The *Arabidopsis thaliana* F-Box Protein FBL17 Is Essential for Progression through the Second Mitosis during Pollen Development

Andi Gusti1, Nicolas Baumberger1, Moritz Nowack2, Stefan Pusch2, Herfried Eisler1, Thomas Potuschak1, Lieven De Veylder3,4, Arp Schnittger1,2, Pascal Genschik1*  
1 Institut de Biologie Moléculaire des Plantes du CNRS, Université de Strasbourg, Strasbourg, France, 2 Unigroupe am Max-Planck-Institut für Züchtungsforschung, Max-Delbrück-Laboratorium, Lehrstuhl für Botanik III, Universität Köln, Köln, Germany, 3 Department of Plant Systems Biology, Flanders Institute for Biotechnology (VIB), Gent, Belgium, 4 Department of Molecular Genetics, Ghent University, Gent, Belgium

Abstract

In fungi and metazoans, the SCF-type Ubiquitin protein ligases (E3s) play a critical role in cell cycle regulation by degrading negative regulators, such as cell cycle-dependent kinase inhibitors (CKIs) at the G1-to-S-phase checkpoint. Here we report that FBL17, an *Arabidopsis thaliana* F-box protein, is involved in cell cycle regulation during male gametogenesis. FBL17 expression is strongly enhanced in plants co-expressing E2Fa and DPa, transcription factors that promote S-phase entry. *FBL17* loss-of-function mutants fail to undergo pollen mitosis II, which generates the two sperm cells in mature *A. thaliana* pollen. Nonetheless, the single sperm cell-like cell in *fbl17* mutants is functional but will exclusively fertilize the egg cell of the gametophyte, giving rise to an embryo that will later abort, most likely due to the lack of functional endosperm. Seed abortion can, however, be overcome by mutations in *FIE*, a component of the Polycomb group complex, overall resembling loss-of-function mutations in the *A. thaliana* cyclin-dependent kinase CDKA;1. Finally we identified ASK11, as an SKP1-like partner protein of FBL17 and discuss a possible mechanism how SCF*FBL17* may regulate cell division during male gametogenesis.

Introduction

Regulation of protein stability through the ubiquitin proteasome system (UPS) is a major mechanism underlying many different cellular and organismal processes, such as cell division, DNA repair, quality control of newly produced proteins, regulation of developmental pathways, important parts of immune defence and in plants, light and phytohormone signal transduction [1–3]. Degradation via the UPS is a two-step process: the target protein is first tagged by covalent attachment of ubiquitin and subsequently degraded by a multicatalytic protease complex called the 26S proteasome. Conjugation of ubiquitin to the protein involves a cascade of three enzymes: E1, E2 and E3. Ubiquitin-activating enzyme (E1) forms a high-energy thioester intermediate, E1-S–Ubi, which is then transferred to one of the several ubiquitin conjugating enzymes (E2s). The transfer of ubiquitin from the E2-S–Ubi to an e-NH2 group of an internal lysine residue in the target protein, requires an ubiquitin protein-ligase (E3). Because E3 enzymes determine the substrate specificity, they are recognized to play the most important role in the ubiquitylation reaction.

Among the different classes of E3s, two of them, the SCF and the Anaphase Promoting Complex/Cyclosome (APC/C), dominate cell cycle regulation, such as DNA replication and cell division, respectively. Whereas the APC/C permits progression and exit from mitosis by inducing proteolysis of different cell cycle regulators including PDS1/SECURIN and CYCLIN B (reviewed in [4]), the budding yeast SCFCDK1 and the mammalian SCFSKP2 (the name of the F-box protein being indicated in uppercase) are required to destroy the cell cycle-dependent kinase inhibitors (CKIs) SIC1 and p27Kip1, respectively (reviewed in [5,6]) and thus promote the entry into S-phase. It is noteworthy that the human SCFSKP5 E3 targets other essential regulators of S-phase progression, including cyclin E [7], E2F1 [8], the RB-like p130 protein [9] and the licensing factor for DNA replication CDT1 [10].

In plants, the role of the APC/C as a mitotic regulator has been established (discussed in [11]), however the proteolytic machinery at the G1/S-phase transition is still poorly characterized. Nevertheless, in the model plant *A. thaliana*, loss-of-function mutants in *CULLIN1* arrest embryogenesis very early at the zygoete stage [12], which is consistent with a role of an SCF E3 in cell cycle control. Moreover, two F-box proteins, similar to the metazoan SKP2, called SKP2A and SKP2B, have been identified in *A. thaliana* [13–15]. SCFSKP2A recuits cyclin-dependent kinase (CDK)-phosphorylated E2Fc, a negative regulator of cell division and its partner DPB, for proteolysis [13,14]. In addition, plant genomes encode also proteins sharing a short amino acid motif...
with the mammalian KIP/CIP-type CKIs [16–18]. *A. thaliana* counts seven such proteins called ICK1/KRP1, ICK2/KRP2, KRP3, KRP4, KRP5, KRP6 and KRP7. Despite poor homology conservation with the metazoan CIP/KIP proteins, plant ICK/KRPs bind to and inhibit several CDK complexes (likely A-type CDK associated to A- or D-type cyclins) (reviewed in [19]). At least two *A. thaliana* ICK/KRPs, ICK1/KRP1 and ICK2/KRP2, are degraded by the 26S proteasome [20,21]. SCF^{SKP2B} may participate in the degradation of *A. thaliana* ICK/KRPs, because overexpression of the F-box protein SKP2B decreases the accumulation of ectopically expressed ICK/KRP1 and suppressed the ICK1/KRP1-dependent serrated leaf phenotype [15]. However, *skp2a skp2b* double mutant plants develop normally and do not stabilize ICK1/KRP1 [15], indicating a residual but sufficient activity of SCF^{SKP2} in this *skp2* mutant background and/or multiple E3s targeting redundantly ICK/KRPs.

Moreover, open siliques from self-pollinated A. thaliana seedlings were genotyped and none of them was found homozygous for the mutation, suggesting that *fbl17-2* is an LRR-type F-box protein, which has no close paralog in *Arabidopsis*. To gain insights into the function of *FBL17*, we searched for F-box protein encoding genes that are expressed in a cell cycle dependent manner. Among the ~700 *A. thaliana* F-box proteins [26], one of them, called *FBL17* (At3g54650) was of particular interest because its expression was found to be 2.5 fold increased in S-phase based on available microarray data [27]. Moreover, this gene was in silico identified as a putative target of E2F transcription factors [28]. *A. thaliana* plants co-expressing ectopically E2Fa with its dimerization partner, DPa, display strongly induced cell proliferation rates [29] and indeed in these plants, we found a 15-fold increase in *FBL17* transcript accumulation (Figure 1A).

**Results**

**FBL17 is an essential gene in Arabidopsis**

To better characterize the role of the SCF in plant cell cycle function, we searched for F-box protein encoding genes that are expressed in a cell cycle dependent manner. Among the ~700 *A. thaliana* F-box proteins [26], one of them, called *FBL17* (At3g54650) was of particular interest because its expression was found to be 2.5 fold increased in S-phase based on available microarray data [27]. Moreover, this gene was in silico identified as a putative target of E2F transcription factors [28]. *A. thaliana* plants co-expressing ectopically E2Fa with its dimerization partner, DPa, display strongly induced cell proliferation rates [29] and indeed in these plants, we found a 15-fold increase in *FBL17* transcript accumulation (Figure 1A).

FBL17 is an LRR-type F-box protein, which has no close paralog in *Arabidopsis*. To gain insights into the function of *FBL17*, we isolated two *A. thaliana* T-DNA insertion mutants, named *fbl17-1* and *fbl17-2*, in which the T-DNA insertions interrupt the 7th exon and 6th intron, respectively (Figure 1B). Ninety-six *fbl17-1* and 102 *fbl17-2* seedlings were genotyped and none of them was found homozygous for the mutation, suggesting that *FBL17* is an essential gene in *Arabidopsis*. However, heterozygous *fbl17-1* and *fbl17-2* plants appeared normal in morphology during sporophytic development. As both lines contained single T-DNA insertions with integral sulphadiazine selection markers, we self-pollinated heterozygous mutant plants and analyzed the segregation of this marker in their progeny. This genetic analysis revealed a segregation ratio close to 1:1 (53% of Sul^{R} plants at most; Table 1), which is consistent with a gametophytic defect. Moreover, open siliques from self-pollinated *fbl17-1* and *fbl17-2*...
heterozygous plants showed a large proportion of aborted seeds (~44%, see Table 2 and Figure 1C–E). Therefore we analyzed the T-DNA transmission of fbl1-1 and fbl1-2 by reciprocal crosses with wild type plants. A severe reduction in the transmission rate was only observed when heterozygous fbl1 mutants were used as a paternal partner (Table 1).

To demonstrate that this phenotype is caused by the fbl1-1 mutation, we engineered a rescue construct (pFBL17:FBL17) consisting of 865-bp of the promoter sequence fused to FBL17 full length genomic sequence. Transgenic lines expressing this construction fully complemented the fbl1-1 seed abortion phenotype (Table 2, Figure 1C–E).

### Table 1. Genetic analysis of fbl17 mutant plants.

| Parental genotype (female x male) | F1 plants genotype (Sulfadiazine resistance) | n  | f(bl17) (%) | TE (%) |
|-----------------------------------|---------------------------------------------|-----|-------------|-------|
| fbl1-1 (selfed)                   | SulfR                                       | 171 | 364         | 47.0  |
| fbl1-2 (selfed)                   | SulfR                                       | 111 | 210         | 52.9  |
| Col-0 x fbl1-1                    | SulfR                                       | 17  | 309         | 5.5   |
| fbl1-1 x Col-0                    | SulfR                                       | 187 | 378         | 49.5  |
| Col-0 x fbl1-2                    | SulfR                                       | 23  | 596         | 3.9   |
| fbl1-2 x Col-0                    | SulfR                                       | 316 | 664         | 47.6  |

Resistance to sulfadiazine (SulfR, sulfadiazine resistant seedlings; SulfS, sulfadiazine sensitive seedlings) was used for the AtFbl17-1 and AtFbl17-2 plants. n = total number. Transmission efficiencies were calculated according to [59]: TE = SulfR/SulfS × 100.

doi:10.1371/journal.pone.0004780.t001

### Table 2. Seed abortion in fbl17 mutants.

| Parental genotype (female x male) | Normal (%) | Aborted (%) | Undeveloped (%) | n     |
|-----------------------------------|------------|-------------|-----------------|-------|
| fbl1-1 (selfed)                   | 53.00      | 44.85       | 2.15            | 466   |
| fbl1-2 (selfed)                   | 56.00      | 43.70       | 0.29            | 341   |
| Col-0 x Col-0                     | 96.35      | 3.99        | 0.26            | 384   |
| fbl1-1 x Col-0                    | 97.10      | 1.76        | 0.53            | 567   |
| Col-0 x fbl1-1                    | 60.35      | 39.33       | 0.32            | 623   |
| fbl1-1 (pFBL17:FBL17) (selfed)    | 96.80      | 2.83        | 0.35            | 283   |

n = total number.

doi:10.1371/journal.pone.0004780.t002

fbl17 single sperm cell shows a large proportion of aborted seeds (~44%, see Table 2 and Figure 1C–E). Therefore we analyzed the T-DNA transmission of fbl1-1 and fbl1-2 by reciprocal crosses with wild type plants. A severe reduction in the transmission rate was only observed when heterozygous fbl1 mutants were used as a paternal partner (Table 1). To demonstrate that this phenotype is caused by the fbl1-1 mutation, we engineered a rescue construct (pFBL17:FBL17) consisting of 865-bp of the promoter sequence fused to FBL17 full length genomic sequence. Transgenic lines expressing this construction fully complemented the fbl1-1 seed abortion phenotype (Table 2, Figure 1C–E).

### Table 1. Genetic analysis of fbl17 mutant plants.

| Parental genotype (female x male) | F1 plants genotype (Sulfadiazine resistance) | n  | f(bl17) (%) | TE (%) |
|-----------------------------------|---------------------------------------------|-----|-------------|-------|
| fbl1-1 (selfed)                   | SulfR                                       | 171 | 364         | 47.0  |
| fbl1-2 (selfed)                   | SulfR                                       | 111 | 210         | 52.9  |
| Col-0 x fbl1-1                    | SulfR                                       | 17  | 309         | 5.5   |
| fbl1-1 x Col-0                    | SulfR                                       | 187 | 378         | 49.5  |
| Col-0 x fbl1-2                    | SulfR                                       | 23  | 596         | 3.9   |
| fbl1-2 x Col-0                    | SulfR                                       | 316 | 664         | 47.6  |

Resistance to sulfadiazine (SulfR, sulfadiazine resistant seedlings; SulfS, sulfadiazine sensitive seedlings) was used for the AtFbl17-1 and AtFbl17-2 plants. n = total number. Transmission efficiencies were calculated according to [59]: TE = SulfR/SulfS × 100.

doi:10.1371/journal.pone.0004780.t001

### Table 2. Seed abortion in fbl17 mutants.

| Parental genotype (female x male) | Normal (%) | Aborted (%) | Undeveloped (%) | n     |
|-----------------------------------|------------|-------------|-----------------|-------|
| fbl1-1 (selfed)                   | 53.00      | 44.85       | 2.15            | 466   |
| fbl1-2 (selfed)                   | 56.00      | 43.70       | 0.29            | 341   |
| Col-0 x Col-0                     | 96.35      | 3.99        | 0.26            | 384   |
| fbl1-1 x Col-0                    | 97.10      | 1.76        | 0.53            | 567   |
| Col-0 x fbl1-1                    | 60.35      | 39.33       | 0.32            | 623   |
| fbl1-1 (pFBL17:FBL17) (selfed)    | 96.80      | 2.83        | 0.35            | 283   |

n = total number.

doi:10.1371/journal.pone.0004780.t002

eXhibited only one generative-like cell (Table 3), indicating a failure to progress through the second mitotic division. To analyze this in more detail, we performed a tetrad analysis by crossing fbl1-1 to quartet mutation qrt1-1 [31], which causes the four products of male meiosis to remain attached (Figure 2C–F). Pollen produced by fbl1-1 qrt1-1 qrt1-2 plants never contained more than two aberrant grains and rarely contained fewer, indicating that fbl1-1 is a highly penetrant male-gametophytic mutation.

To confirm that FBL17 is expressed in mature pollen as suggested by microarray data [32], we generated a GUS promoter-reporter with the FBL17 promoter sequence that we have above shown to be functional, and introduced it into wild type plants. Histochemical staining revealed that FBL17 expression is detectable during the microspore to bi-cellular pollen transition and fades later when pollen reaches the tricellular stage (Figure 2M–U).

To examine whether fbl1-1 mutation affects pollen cell fate, we introduced the fbl1-1 / + background the H3.3 histone variant pHTR10-HTTR10-mRFP marker, which is specifically expressed in the male germ line and accumulates in the nuclei of wild type generative cell and subsequently in sperm cells [33]. Expression of this marker was observed in the generative-like cell nuclei of fbl1-1 mutant (n = 95), indicating that this cell has not lost its gametic fate (Figure 2G–I). Furthermore, the single generative-like cell nuclei of fbl1 mutant pollen is distinctively larger and less dense than the sperm cell nuclei observed in wild type pollen at anthesis (Figure 2D) and its morphology is reminiscent of the generative cell nuclei before the second mitotic division. Accordingly, measurement of DNA content by densitometry of DAPI stained pollen nuclei revealed that fbl1 single generative-like cell nuclei have a slightly higher content than the wild type sperm cell nuclei at anthesis (1.38C vs 1.25C respectively; Figure 2V). Thus, the fbl1 generative-like cell fails to progress into mitosis but has initiated S-phase reminiscent of wild-type sperms before fertilization. This suggested that the single generative-like cell of fbl1 mutant pollen might function as a sperm cell.

fbl17 single sperm cell predominantly fertilizes the egg cell in the embryo sac and causes seed abortion

Overall, our results indicate that fbl1 mutation prevents entry of the generative cell into mitosis, supporting a function of the FBL17 protein in cell cycle regulation in the male germ line. Interestingly, the production of a single sperm cell has recently been described for the loss of function of CDKA1, the central A. thaliana cyclin-dependent kinase [24,25]. In these studies, it was found that the single sperm cell of cdka1 may fertilize preferentially the egg cell in the embryo sac, leading to embryo arrest at the globular stage and fertilization-independent endosperm development.

To further investigate the functionality of the single generative-like cell and the origin of semi-sterility of fbl1 plants, we characterized the embryo sac development of plants fertilized with fbl1 pollen. Siliques of selfed fbl1-1 and fbl1-2 plants showed arrested embryos at the globular stage with undeveloped endosperm (Figure 3). These embryos never developed further and eventually degenerated. When fbl1-1 pollen was used to fertilize wild type plants, the same phenotype was observed (Figure 3A, C, E, F). Hence, fbl1 mutants fully phenocopy the cdka1 mutation.

fbl17 embryos are rescued by fis-class mutants

Malfunction of the FIS-Polycist group complex leads to central cell proliferation even in the absence of fertilization (reviewed in [34–36]). A striking observation was that mutations of genes encoding this complex, including mea, fis2 and fis, could rescue the cdka1 paternally conferred seed abortion phenotype.

---

**PLoS ONE | www.plosone.org**

March 2009 | Volume 4 | Issue 3 | e4780

---

**SCF in Male Gametogenesis**

---

**PloS ONE | www.plosone.org**

3

March 2009 | Volume 4 | Issue 3 | e4780

---

**SCF in Male Gametogenesis**

---
Figure 2. Pollen phenotype of \textit{fbl17} mutants and \textit{FBL17} expression during male gametogenesis. \textit{fbl17} (A) and wild type (B) mature pollen viability test by coloration with Alexander’s stain. The purple staining indicates that the grains are viable. Dehiscent pollen of \textit{qrt1-1} \textsuperscript{-} (C) and \textit{qrt1-1} \textsuperscript{+} \textit{fbl17-1} \textsuperscript{-} (D) stained with DAPI and observed under UV fluorescence. The four pollen grains of the tetrad show two densely stained sperm cell nuclei and one large diffuse vegetative cell nuclei in \textit{qrt-1} mutants, whereas two pollen grains of the tetrad show only a single germ cell nuclei in \textit{qrt1-1,fbl17-1} \textsuperscript{-} double mutants. (E, F) transmitted light picture of C and D. (G–L) Expression of the \textit{HT10} gene in the \textit{fbl17} mutant pollen. Expression of the \textit{HTR10-mRFP} protein under the \textit{HTR10} promoter in \textit{fbl17-1} (H) and wild type (K) pollen, counterstained with DAPI (G, \textit{fbl17-1}; J, wild type). (I, L) transmitted light pictures of G and J. (A, B) bar = 100 μm; (C–L) bar = 10 μm. (M–U) Promoter-GUS analysis of \textit{FBL17} expression in pollen. (M, P, S) DAPI staining is applied to reveal the developmental stage of the pollen grain. (N–U) \textit{X-Gluc} histochemical staining of p\textit{FBL17:GUS} (N, Q, F) and non-transformed Col-0 (O, R, U) pollen grains. Bars = 10 μm. (V) DNA content measurement of wild type germinative cell nuclei at prophase (n = 9; DNA = 2C), wild type sperm cell nuclei at telophase (n = 16), WT sperm cell nuclei at anthesis (n = 111) in comparison to the unique germ-cell like nuclei of \textit{fbl17} pollen (n = 77). Error bars = standard error mean. doi:10.1371/journal.pone.0004780.g002
FBL17 interacts with specific ASKs to form SCF-like complexes

Since FBL17 carries an F-box motif, we next examined whether this protein forms an SCF-type complex. Yeast two hybrid assays were conducted with the different *A. thaliana* SKP1-like proteins, called ASKs [38]. In yeast, several ASKs were able to interact with FBL17 when fused to the GAL4 DNA binding domain while failing to allow yeast growth on selective medium when fused to the activating domain (Figure 4A). In contrast, ASK11 displayed a robust interaction with FBL17 in both fusions. Interestingly, ASK11 is among the members of the *A. thaliana* ASK family that are strongly expressed in pollen [39,40] and constitutes therefore a strong candidate for participating in an SCF<sup>FBL17</sup> complex. It is noteworthy that the most predominant ASK, ASK1, was not found to interact with FBL17 in our yeast-two hybrid experiment. To further test a possible interaction between FBL17 and ASK11, we performed bimolecular fluorescence complementation (BiFC) experiments. As a control we fused to the N- and C-terminal half of YFP to the C-terminus of FBL17 and GUS, respectively. After co-injection of these fusion constructs into *Nicotiana benthamiana* leaf cells no YFP fluorescence could be found (data not shown). In contrast, when split-YFP fusions with FBL17 and ASK11 were co-injected, a strong fluorescence signal was recovered corroborating an interaction between these two components of an SCF-type complex in vivo (Figure 4B-G). Moreover transient expression of YFP fusions revealed that the FBL17 protein is essentially nuclear, whereas ASK11 localizes both to the cytoplasm and the nucleus (Figure 4B-E). Consequently, an interaction between ASK11 and FBL17 in the BiFC assays was restricted to nuclei (Figure 4F,G).

FBL17 interacts with KRP7 in vivo

The FBL17 loss-of-function pollen phenotype is similar, if not identical, to the recently described *edka;1* mutant [25] strongly supporting a cell cycle function of this F-box protein. An interesting hypothesis is that an SCF<sup>FBL17</sup> E3 ligase targets one or more members of the plant cell cycle-dependent kinase inhibitors, called ICK/KRPs [reviewed in [19,41]]. In such a scenario, ICK/KRPs would accumulate in *fbl17* mutant pollen and consequently would inhibit the kinase activity mimicking the *edka;1* mutant phenotype. To test this possibility, we first carried out yeast two-hybrid experiments, with binding and activation domain fusions of both FBL17 and all seven KRPs being tested.

### Table 3. Phenotype of *fbl17* pollen at anther dehiscence.

|         | Normal (%) | Abnormal (%) | n  |
|---------|------------|--------------|----|
| Col-0   | 97         | 3            | 1,355 |
| *fbl17*-1 | 56         | 44           | 1,483 |
| *fbl17*-2 | 55         | 45           | 1,492 |

Pollen from dehiscent anthers of WT and *fbl17*<sup>−/−</sup> plants was stained with DAPI and observed under UV illumination. *n* = total number. Normal pollen all showed two sperm cells. Abnormal pollen showed a single sperm-like cell. [10.1371/journal.pone.0004780.g003]

and allowed the formation of viable seeds with only a diploid endosperm [37]. To test whether *fbl17* fertilized ovules behave in a similar manner, we crossed female *fie*<sup>−/−</sup> mutant with pollen from the heterozygous *fbl17-1* and *fbl17-2* mutants. The F1 generation was then genotyped to assess the transmission of both the *fbl17* and *fie* mutant alleles (Table 4). In contrast with Col-0 fertilized by *fbl17* mutants that only allowed 6–8% of transmission efficiency of the *fbl17* mutant allele, the *fie* mutation raised the transmission efficiency of *fbl17* to 27–32%. At the same time, the transmission efficiency of the *fie* mutant allele through the female gametophyte increased from 8% in crosses with wild-type pollen to 28–32% indicating a mutual rescue of *fbl17* and *fie* similar to what has been previously reported for *fs* mutants and *ckx;1* Taken together, we conclude that the single *fbl17* generative-like cell is able to some degree to function as a sperm cell leading to the production of viable seed when sufficiently developed endosperm is provided.

### Figure 3. Fertilization with *fbl17* mutant pollen leads to embryo developmental arrest and seed abortion.

Embryo development in wild type plants fertilized with *fbl17-1* mutant (A, E) and wild type (B) pollen, 3 days after pollination. Embryo development in wild type plants fertilized with *fbl17-1* mutant (C, F) and wild type (D) pollen, 4 days after pollination. Early fertilization-independent development of the endosperm is visible in *fbl17-1*-fertilized seeds (E) as evidenced by the presence of multiple nuclei (asterisks). Arrested globular embryo in *fbl17-1* mutant seeds with degenerated endosperm (F). Bars: 50 μm. [10.1371/journal.pone.0004780.g003]
observed (not shown). This lack of protein interaction was, however, not unexpected because most SCF substrates require post-translational modifications [42], which may not occur in yeast. To circumvent this problem we performed bimolecular fluorescence complementation (BiFC) experiments with three ICK/KRPs, KRP3, KRP5 and KRP7 that are expressed in pollen [32]. We also used KRP1, which is not expressed in pollen, as a control. No signal could be recovered when split-YFP fusion constructs of KRP1, KRP3, or KRP5 were co-injected with FBL17 into Nicotiana benthamiana leaf cells (data not shown). In contrast, we observed a weak but distinct nuclear interaction when FBL17 and KRP7 were co-injected (Figure 4H–K).

KRP7 protein shows strong sequence similarity to KRP6, which was recently found to be expressed in pollen, as early as meiosis [43]. Interestingly, KRP6 turnover is necessary for cell cycle progression during gametogenesis. Thus we ordered krp6 and krp7 mutants from the stock centers but only krp6 mutants could be obtained. In one T-DNA insertion mutant line, called krp6−1, KRP6 expression was abolished as indicated by qRT-PCR obtained. In one T-DNA insertion mutant line, called krp6−1, KRP6 expression was abolished as indicated by qRT-PCR analysis (not shown). Homozygous krp6−1 mutant plants showed no visible mutant phenotype. Strikingly, the krp6−1/fbl17−1/+ double mutant, showed a partial rescue of fbl17 pollen phenotype in F1 with the ratio of aberrant pollen of 35%, a number significantly lower than the ~48% observed in fbl17 mutant (Table 5). From these experiments, we conclude that KRP6 and most likely KRP7 are targets of an SCF FBL17 during pollen development.

Discussion

FBL17 interacts with ASK11 and other ASKs

SCF-type complexes are involved in many developmental and physiological processes [reviewed in [44]]. However so far, little is known about SCF complexes during gametophyte development. Here we investigated the function of FBL17, an F-box protein that is expressed during pollen development. Because FBL17 carries an N-terminal F-box domain, we first tested whether it associates with ASK proteins. The A. thaliana genome encodes 21 different ASKs [38]. However, the great majority of functionally characterized A. thaliana F-box proteins were shown to associate with ASK1 and ASK2, which exhibit broad expression patterns throughout plant development. Interestingly, ASK1 and ASK2 function during male meiosis [40] and in cell cycle regulation as cell division during embryogenesis is affected in the double ask1 ask2 mutant [45]. Here we provide evidence that FBL17 can interact with several ASKs. In particular we observed the strongest interaction with ASK11, which is also expressed in pollen [39,40] and known to interact with A. thaliana CUL1 [46]. Although we cannot exclude that FBL17 participates in many CUL1-type E3 complexes, our data supports the existence of a novel type of SCF E3 ligase, formed by CUL1, ASK11 and FBL17 regulating male germ line development.

FBL17 is involved in cell cycle function during male gametogenesis

In fungi and metazoans, the SCF-types E3s play a critical role during the cell cycle at the G1-to-S-phase transition by degrading negative regulators such as CKIs. Whether such a mechanism is conserved in plants remained unclear. Consistently with this scenario, A. thaliana cul1 loss-of-function mutants arrest early during embryogenesis at the zygote stage [12] and also exhibit a reduced transmission of the mutation through both male and female gametophytes. However, such a phenotype was not observed in any of the ~700 F-box encoding genes in A. thaliana.

Here, we provide evidence that FBL17 is a key regulator of the cell cycle during gametophyte development. First, FBL17 is expressed at the G1-to-S-phase transition [27] and its expression is strongly enhanced in plants ectopically co-expressing the S-phase transcription factor E2Fa together with its dimerization partner DPs. It is noteworthy that FBL17 promoter sequence carries the canonical E2F binding sites in both A. thaliana and rice [28]. Whether FBL17 is a direct target of E2Fs, however, is not established yet.

Second, the FBL17 loss-of-function mutants arrest germ cell division leading to a single sperm cell phenotype that closely resembles (see also below) the phenotype found in mutants for the major cell cycle regulator; CDKA1.

The paternal effect of fbl17 mutants leads to seed abortion, which is characterised by an arrest of embryo development at the globular stage and a defect in endosperm development. Similarly to the okla1 mutant [25], it is likely that the fbl17 single sperm cell may preferentially fertilize the egg cell, but not the central cell in the embryo sac. In both cases, the uniparental underdeveloped endosperm, which is unable to nourish the embryo, may thus be the cause of seed abortion. Hence, when the arrest of endosperm development is released by the mutation of FIE, a component of the Polycomb group complex, we observed at least a partial rescue of the paternally conferred fbl17 seed abortion phenotype.

FBL17 targets negative regulator(s) of the cell cycle

Based on the pollen phenotype, we propose that FBL17 targets a negative regulator of the cell cycle. Because fbl17 mutants phenocopy CDKA loss-of-function alleles, we speculated that the negative regulator targeted by FBL17 may directly act at the level of CDKA1. Plausible candidate proteins are CKIs, whose

Table 4. Transmission frequencies of fbl17 alleles in a fis-class mutant.

| Parental genotype (female×male) | Genotype of viable progeny (%) | TE% fbl17 | TE% fie | Association factor | n |
|---------------------------------|-------------------------------|----------|--------|--------------------|---|
| Col-0×fbl17-1                   | 6.4                           | 6.5      | NA     | NA                 | 294|
| Col-0×fbl17-2                   | 8.5                           | 8.4      | NA     | NA                 | 142|
| fie×Col-0                       | NA                            | NA       | 8.0    | NA                 | 248|
| fie×fbl17-1                     | 32.3                          | 32.3     | 31.8   | 87.1               | 393|
| fie×fbl17-2                     | 30                            | 26.9     | 28.1   | 84.5               | 438|

N, number of F1 germinated seedlings scored; NA, not applicable; association factor, number of F1 plants carrying both a fis-class mutant (fie) and a fbl17 mutant allele divided by the total number of plants carrying a fis-class mutant allele expressed in per cent.

doi:10.1371/journal.pone.0004780.t004
accumulation during gametogenesis would lead to CDK\(\alpha\)1 inactivation and as a consequence cell cycle arrest. Indeed, we observed a physical interaction between FBL17 and at least one member of the KRP family (e.g., KRP7). Moreover, KRP6 mutation suppressed, at least partially, the fbl17 pollen phenotype. After the completion of this work Kim et al. [47] reported a similar role of FBL17 during male gametogenesis. This study showed that both KRP6 and KRP7 are expressed in the male germ cells after asymmetric division, but disappear in sperm cells. Based on their in vitro degradation assays and the fact that a KRP6-GFP fusion protein remained stable in the fbl17 single germ cell, these authors also proposed that KRP6 and KRP7 are the main target this SCF E3 ligase [47]. Nevertheless, because a krp6 krp7 double loss-of-function mutant is not available, this model still awaits a definitive genetic prove.

An interesting question, which has not yet been fully answered, is at which stage the cell cycle fbl17 mutant arrests? Based on our knowledge of the cell cycle in yeast and animals, a naïve prediction was that fbl17 loss-of-function mutants would arrest at the G1/S transition. However, this was not the case because the mutant pollen had a higher DNA content than wild-type sperm and thus must have entered into S-phase ([47] and our work). In contrast, the DNA content of fbl17 was measured to be lower than 2C and thus, the mutant sperms appear to be not arrested at the entry into mitosis. However, as the fbl17 single germ cell fertilizes the egg cell, even allowing complete embryo development in a fie mutant background, we exclude that an aneuploid situation arose after fertilization and thus, it is likely that DNA replication was completed at the time of fertilization. Thus, we speculate that fbl17 mutants are arrested in the G1 phase and similarly to wild type would be still susceptible to an S-phase trigger late in development. This trigger would then be able to overwrite the FBL17 loss-of-function. It is noteworthy here that accumulation during gametogenesis would lead to CDK\(\alpha\)1 inactivation and as a consequence cell cycle arrest. Indeed, we observed a physical interaction between FBL17 and at least one member of the KRP family (e.g., KRP7). Moreover, KRP6 mutation suppressed, at least partially, the fbl17 pollen phenotype. After the completion of this work Kim et al. [47] reported a similar role of FBL17 during male gametogenesis. This study showed that both KRP6 and KRP7 are expressed in the male germ cells after asymmetric division, but disappear in sperm cells. Based on their in vitro degradation assays and the fact that a KRP6-GFP fusion protein remained stable in the fbl17 single germ cell, these authors also proposed that KRP6 and KRP7 are the main target this SCF E3 ligase [47]. Nevertheless, because a krp6 krp7 double loss-of-function mutant is not available, this model still awaits a definitive genetic prove.

An interesting question, which has not yet been fully answered, is at which stage the cell cycle fbl17 mutant arrests? Based on our knowledge of the cell cycle in yeast and animals, a naïve prediction was that fbl17 loss-of-function mutants would arrest at the G1/S transition. However, this was not the case because the mutant pollen had a higher DNA content than wild-type sperm and thus must have entered into S-phase ([47] and our work). In contrast, the DNA content of fbl17 was measured to be lower than 2C and thus, the mutant sperms appear to be not arrested at the entry into mitosis. However, as the fbl17 single germ cell fertilizes the egg cell, even allowing complete embryo development in a fie mutant background, we exclude that an aneuploid situation arose after fertilization and thus, it is likely that DNA replication was completed at the time of fertilization. Thus, we speculate that fbl17 mutants are arrested in the G1 phase and similarly to wild type would be still susceptible to an S-phase trigger late in development. This trigger would then be able to overwrite the FBL17 loss-of-function. It is noteworthy here that accumulation during gametogenesis would lead to CDK\(\alpha\)1 inactivation and as a consequence cell cycle arrest. Indeed, we observed a physical interaction between FBL17 and at least one member of the KRP family (e.g., KRP7). Moreover, KRP6 mutation suppressed, at least partially, the fbl17 pollen phenotype. After the completion of this work Kim et al. [47] reported a similar role of FBL17 during male gametogenesis. This study showed that both KRP6 and KRP7 are expressed in the male germ cells after asymmetric division, but disappear in sperm cells. Based on their in vitro degradation assays and the fact that a KRP6-GFP fusion protein remained stable in the fbl17 single germ cell, these authors also proposed that KRP6 and KRP7 are the main target this SCF E3 ligase [47]. Nevertheless, because a krp6 krp7 double loss-of-function mutant is not available, this model still awaits a definitive genetic prove.

An interesting question, which has not yet been fully answered, is at which stage the cell cycle fbl17 mutant arrests? Based on our knowledge of the cell cycle in yeast and animals, a naïve prediction was that fbl17 loss-of-function mutants would arrest at the G1/S transition. However, this was not the case because the mutant pollen had a higher DNA content than wild-type sperm and thus must have entered into S-phase ([47] and our work). In contrast, the DNA content of fbl17 was measured to be lower than 2C and thus, the mutant sperms appear to be not arrested at the entry into mitosis. However, as the fbl17 single germ cell fertilizes the egg cell, even allowing complete embryo development in a fie mutant background, we exclude that an aneuploid situation arose after fertilization and thus, it is likely that DNA replication was completed at the time of fertilization. Thus, we speculate that fbl17 mutants are arrested in the G1 phase and similarly to wild type would be still susceptible to an S-phase trigger late in development. This trigger would then be able to overwrite the FBL17 loss-of-function. It is noteworthy here that...
FBL17 expression is abolished in late pollen developmental stages as based on our promoter-reporter lines and [47]. Alternatively, the loss of an SCF FBL17 complex might result in slow, but none the less steady progression into S-phase. This S-phase might then, similar to wild type, be only completed shortly before fertilization and thus, the generative-like-cells of fbl17 mutants would resemble wild-type sperm cells.

Multiple components of UPS regulate A. thaliana male gametogenesis

Recent work has shown that KRP6 is also targeted by two related RING-H2-type E3s, called RHF1a and RHF2a, during pollen development [43]. Contrarily to fbl17, the rhf1a rhf2a double mutant pollen phenotype is partially penetrant and pollen development is arrested at multiple stages. Constitutive overexpression of KRP6 could phenocopy the rhf1a rhf2a double mutant defects. Thus, ICK/KRP degradation is regulated at different levels during pollen development (Figure 5). Although additional experiments are required to determine the exact contribution of each E3 in KRP6/7 turnover, RHF1a and RHF2a do not seem to act redundantly with SCF FBL17 to allow entry into PMII.

With this, KRP turnover in plant cells, at least in the germ line, show similarities with the situation found in mammals where the cell cycle-dependent kinase inhibitor protein p27KIP1 is degraded by two different classes of E3s; the SCF KPC/ and the RING-type KPC1/KPC2 [48]. The KPC E3 mediates polyubiquitylation of p27KIP1 in the cytoplasm in a phosphorylation-independent manner during G1 phase, whereas SCF SKP2 is involved in phosphorylation-dependent p27KIP1 degradation in the nucleus during S and G2 phases [49]. Whether SCF FBL17 recruits phosphorylated ICK/KRPs is unknown. Nevertheless, it has been shown that at least one A. thaliana ICK/KRP is subjected to proteolysis in a phosphorylation-dependent manner [21]. In agreement, putative CDKA;1 phosphorylation sites are present in both KRP6 and KRP7 protein sequences [41].

Not only ubiquitylation, but also deubiquitylation has recently been involved in pollen development [50]. Hence two closely related A. thaliana Ub-specific proteases (UBPs) called UBP3 and UBP4 are important for pollen mitosis II, as their combined disruption also leads to a single sperm cell, which however is unfertile. The target of UBPs/UBP4 is unknown. Interestingly, recent work in mammals identified USP19 as a UBP that regulates the protein level of KPC1, the catalytic subunit of KPC [51]. In their model, USP19 stabilizes KPC1 and thus indirectly promotes degradation of p27KIP1 in the cytoplasm. Whether the plant UBPs/UBP4 have a similar effect on ICK/KRP accumulation during pollen development is worth to investigate.

Materials and Methods

Plant material and growth conditions

A. thaliana plants used in this study were all derived from the Columbia-0 (Col-0) accession. The lines gtt1-1 [31], fbl17-1 (GABI-170E02), fbl17-2 (GABI-436F11), krp6 (SAIL_548B03) and fie (GABI-362D06) were obtained from the NASC stock centre. The position of the T-DNA inserts in the fbl17 lines were verified by DNA sequencing of the flanking regions amplified with primer pairs GABI T-DNA LP/7, GABI T-DNA RP/8 for fbl17-1 and GABI T-DNA LP/9, GABI T-DNA RP/10 for fbl17-2 (see oligo table below the GABI web site: http://www.gabi-kat.de). Those primer pairs were also used for genotyping. The transgenic lines E2Fa/DPa over-expressing line and pHTR10-HTR10-mRFP have been described elsewhere [29,33]. For most experiments, plants were grown on soil under standard greenhouse conditions (22 °C, 16 h light photoperiod). Analysis of the fbl17 alleles transmission efficiency and selection of heterozygous fbl17 plants were performed by growing seedlings on sterile 1 × Murashige and Skoog agar medium supplemented with 10 μg/ml sulfadiazine.

Constructs and transformation

The complementation genomic construct was generated by insertion of a 3747nt-long PCR amplified genomic product from (~)668 bp to the stop codon into the NcoI site of pCBI [52]. PCR amplification of this genomic fragment was done with the primers 11 and 12 (see table below). The construct was transformed into fbl17-1 mutant using Agrobacterium tumefaciens GV3101 with the floral-dip methods [33]. Primers 3 and 4 were used to identify homozygous knock-out mutants rescued by the full genomic construct. The promFBL17:GUS construct was created by PCR amplifying 868nt of the promoter region with primers 3 and 6 and cloning of the fragment in pENTR1A vector between the Not I and Xho I site of pCBI [52]. PCR amplification of this genomic fragment was done with the primers 11 and 12 (see table below). The construct was transformed into fbl17-1 mutant using Agrobacterium tumefaciens GV3101 with the floral-dip methods [33]. Primers 3 and 4 were used to identify homozygous knock-out mutants rescued by the full genomic construct. The promFBL17:GUS construct was created by PCR amplifying 868nt of the promoter region with primers 3 and 6 and cloning of the fragment in pENTR1A vector between the Not I and Xho I sites. The promoter fragment was then mobilized by LR clonase II recombination (Invitrogen) into pMDC162 [54]. For the BiFC constructs FBL17, ASK11, KRPI, 3, 5 and 7 cDNAs were first amplified by PCR without their STOP codons, cloned into pDONR 201 (except FBL17 which was cloned into pDONR 207) by BP clonase recombination (Invitrogen) before to be transferred into the split-YFP destination vector pSYN and pSYC [20] by LR clonase reaction (Invitrogen). 35S:YFP fusion were obtained by remobilizing FBL17, ASK11, and KRPs cDNA from the pDONR vector to the plasmid pEXSG-YFP [55].

Primers

Oligo Sequence (5’→3’)  
1 TGGAGAGTTGAGTCGCAGCG  
2 CCGAGCGCAGAGATGAGGAGAG
Q-PCR
Total RNA for Q-PCR was extracted from 6 days-old E2Fa-DPA of and Col-0 wild-type seedlings grown on sterile 1x Murashige and Skoog agar medium with the Plant RNeasy kit (Qiagen). For krp6-1 mutant analysis, total RNA was extracted from inflorescences of krp6-1 homozygous mutant and Col-0 wild-type plants. 1 μg of total RNA was reverse transcribed with the Superscript II RT kit (Invitrogen) and a mixture of random hexamers and oligo dT (18) according to the manufacturer’s instruction. PCR was performed using oligos 1 and 2 (FBL17 specific) and oligos 13 and 14 (KRP6 specific) in a total volume of 15 μL SYBR Green Master mix (Roche) on a Lightcycler LC480 apparatus (Roche) according to the manufacturer’s instructions. The ACTIN2, AT4G32470 and AT4G26410 genes were used as internal controls for the normalization of the q-PCR.

Microscopy
Developing seeds were prepared from siliques at different days after pollination (DAP) and mounted on microscope slides in a clearing solution of 8:2:1 Chloral hydrate: distilled water: glycerol as described in [56]. Observations were performed on a Zeiss Axioshot using 20x and 40x DIC optic. Pollen viability was assessed by mounting pollen in Alexander’s stain [30] and incubated for 30’ before the observation on a Nikon fluorescent stereo-microscope E800 equipped with a 100x objective. Quantification of DNA content was performed with the software ImageJ [http://rsbweb.nih.gov/ij/] on images taken with constant settings. Background fluorescence values were subtracted to the measure to correct for difference in staining intensity. The obtained measures were normalized against those of generative prophase nuclei from wild type bi-cellular pollen sampled in pre-dehiscent anthers, which are by definition at 2C. Pictures of qr-1/bfl17-1 and qr-1 mutant pollen were obtained on a Zeiss LSM 510 Meta confocal microscope as a Z-stack subsequently flattened into a single image in the ImageJ software. GUS staining of anthers at different stages was performed as described in [57] and the state of pollen development was determined from the pollen DAPI staining taken from the same flower at the same time.

Yeast-two hybrid
The ASK7 to ASK19 cDNAs cloned as fusions to the GAL4 activation domain and GAL4 binding domain, respectively, in Gateway-compatible pGADT7 and pGBT9 (Clontech) yeast two-hybrid vector have been described in [46]. Full length FBL17 cDNA was mobilized from the entry vector pDONR FBL17 to both pGADT7 and pGBK7 (Clontech) with LR clonase II kit (Invitrogen). The yeast strain AH109 [MATa, npk-1, neo2-3, 112, ura3-52, his3-200, gal4, gal80, LYS2::GAL11/AS-GAL11/TATA::HIS3, GAL2::GAL12/TATA::ADE2, URA3::MEV1/AS-MEL1/TATA::MEL1] was transformed with the appropriate combinations of bait and prey vectors. Transformants were selected on synthetic defined (SD)/-Leu/-Trp (SD-LW) medium for 2 days at 30°C. Weak and strong interactions were tested by transferring transformants on SD/-Leu/-Trp/-His (SD-LWH) with 5 μM 3-amino-1,2,4-triazole (3-AT) and SD/-Leu/-Trp/-Ade (-LWA) media, respectively, allowing growth for 3 days at 30°C.

BiFC assay
For infiltration of Nicotiana benthamiana leaves the A. tumefaciens strain GV3101 pMP90RK was used. The A. tumefaciens strains containing the BiFC vectors were infiltrated as described [58]. Infiltration was performed on the abaxial leaf side of two-month-old plants and analyzed 3 to 5 days later.

Acknowledgments
We thank M. Alioua for quantitative RT-PCR analyses and J. Molinier for some plant crossing experiments.

Author Contributions
Conceived and designed the experiments: PG. Performed the experiments: AG NB MN SP HE. Analyzed the data: NB MN TP LDV AS PG. Contributed reagents/materials/analysis tools: AG HE. Wrote the paper: AS PG.

References
1. Cirianchow A, Oriam A, Schwartz Al (2000) Ubiquitin-mediated proteolysis: biological regulation via destruction. Bioessays 22: 442-451.
2. Pickart CM (2001) Mechanisms underlying ubiquitination. Annu Rev Biochem 70: 503-533.
3. Smalle J, Vierstra RD (2004) The ubiquitin 26S proteasome proteolytic pathway. Annu Rev Plant Biol 55: 555-590.
4. Peters JM (2006) The anaphase promoting complex/cyclosome: a machine designed to destroy. Nat Rev Mol Cell Biol 7: 644-656.
5. Dessenais RJ, Frenell JE (2003) Midstarch phosphorylation and the countdown to S phase. Cell 107: 819-822.
6. Pagano M (2004) Control of DNA synthesis and mitosis by the Skp2-p27-Cdk1/2 axis. Mol Cell 14: 414-416.
7. Nakayama K, Nagahama H, Minamishima YA, Matsumoto M, Nakanishi I, et al. (2000) Targeted disruption of Skp2 results in accumulation of cyclin E and p27kip1, polyplody and centrosome overduplication. EMBO J 19: 2069-2081.
8. Marti A, Wirbelauer C, Scheffler M, Krek W (1999) Interaction between ubiquitin-protein ligase SCF and E2F-1 underlies the regulation of E2F-1 degradation. Nat Cell Biol 1: 14-19.
9. Tedesco D, Lukas J, Reed SI (2002) The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCFβTrCP. Genes Dev 16: 2946-2957.
10. Li X, Zhao Q, Liao R, Sun P, Wu X (2003) The SCFβTrCP ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. J Biol Chem 278: 38054-38058.
11. Genschik P, Criqui MC (2007) The UPS: an engine that drives the cell cycle. In: Inze D, ed. Annual Plant Reviews, Blackwell Publishing. pp 87-113.
12. Suen WH, Parmentier Y, Helman J, Lecointre E, Dong A, et al. (2002) Null mutation of AbCell causes arrest in early embryogenesis of Arabidopsis. Mol Biol Cell 13: 1916-1928.
13. del Pozo JC, Boniotti MB, Gutierrez C (2002) Arabidopsis E2F functions in cell division and is degraded by the ubiquitin-SCFβTrCP pathway in response to light. Plant Cell 14: 3057-3071.
14. del Pozo JC, Diaz-Trivino S, Cisneros N, Gutierrez C (2006) The balance between cell division and endoreplication depends on E2F2-DBP, transcription factors regulated by the ubiquitin-SCFβTrCP pathway in Arabidopsis. Plant Cell 18: 2224-2235.
15. Ren H, Santner A, del Pozo JC, Murray JA, Estelle M (2008) Degradation of the cyclin-dependent kinase inhibitor KRP1 is regulated by two different ubiquitin E3 ligases. Plant J 53: 765–716.

16. Wang H, Foske LC, Crosby WL (1997) A plant cyclin-dependent kinase inhibitor gene. Nature 386: 451–452.

17. De Veylder L, Beeckman T, Beemster GT, Krols L, Terras F, et al. (2001) Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. Plant Cell 13: 1655–1668.

18. Vandevoorde K, Raes J, De Veylder L, Rouze P, Rombaerts S, et al. (2002) Genome-wide analysis of core cell cycle genes in Arabidopsis. Plant Cell 14: 903–916.

19. Verkest A, Wein C, Inze D, De Veylder L, Schmitter A (2005) Switching the cell-cycle-Kip-related proteins in plant cell cycle control. Plant Physiol 139: 1099–1106.

20. Jakoby MJ, Wein C, Pusch S, Kuijt SJ, Merkle T, et al. (2006) Analysis of the subcellular localization, function, and proteolytic control of the Arabidopsis cyclin-dependent kinase inhibitor TIC51/KRP37. J Biol Chem 281: 30975–30983.

21. Verkest A, Manes CL, Vercruysse S, Maes S, Van Der Schueren E (2005) The cyclin-dependent kinase inhibitor KRP2 controls the onset of the endoreduplication cycle during Arabidopsis leaf development through inactivation of mitotic CDK1a1 kinase complexes. Plant Cell 17: 1729–1736.

22. Yadegari R, Davies GN (2004) Female gametophyte development. Plant Cell 16 Suppl: S133–141.

23. Ma H (2005) Molecular genetic analyses of microsporogenesis and megagametogenesis in flowering plants. Annu Rev Plant Biol 56: 393–434.

24. Iwakawa H, Shinmyo A, Seke M (2006) Arabidopsis CDKA1, a CDCl homologue, controls proliferation of generative cells in male gametogenesis. Plant Cell 18: 819–831.

25. Nowack MK, Grini PE, Jakoby MJ, Lefos M, Kneze C, et al. (2006) A positive signal from the fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis. Nat Genet 38: 63–67.

26. Gagne JM, Downes BP, Shiu SH, Durski AM, Vierstra RD (2002) The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in flowering plants. Proc Natl Acad Sci U S A 99: 11519–11524.

27. Menges M, Hennig L, Gruissem W, Murray JA (2003) Genome-wide analysis of endoreduplication. Plant Cell 133: 1113–1129.

28. Vandevoorde K, Vliegh K, Fleurquin K, Hennig L, Beemster GT, et al. (2005) Genome-wide identification of potential plant E2F target genes. Plant Physiol 139: 316–328.

29. Verkest A, Beeckman T, Beemster GT, Almeida Engler J, Ormense S, et al. (2002) Control of proliferation, endoreduplication and differentiation by the cyclin-dependent kinase suppressor FUL. Plant J 30: 235–247.

30. Alexander MP (1969) Differential staining of aborted and non-aborted pollen. Stain Technol 44: 117–122.

31. Preuss D, Rhee SY, Davin RW (1994) Tetrad analysis possible in Arabidopsis with cotransformation of the QUARTET (QRT) genes. Science 264: 1458–1460.

32. Pina C, Pinto F, Feijó JA, Becker JD (2005) Functional analysis of the Arabidopsis pollens transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. Plant Physiol 138: 744–756.

33. Ingold M, Hamamura Y, Georgiou G, Higashiyama T, Berger F (2007) Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. Curr Biol 17: 1032–1037.

34. Guittion AE, Berger F (2005) Control of reproduction by Polycomb Group complexes in animals and plants. Int J Dev Biol 49: 707–716.

35. Baroux C, Pina S, Grossniklaus U (2007) Chromatin modification and remodeling during early seed development. Curr Opin Genet Dev 17: 473–479.

36. Kohler C, Villar CB (2008) Programming of gene expression by Polycomb group proteins. Trends Cell Biol 18: 236–243.

37. Nowack MK, Shiroya R, Wissmann N, Dolf A, Ertl E, et al. (2007) Bypassing genomic imprinting allows seed development. Nature 447: 312–315.

38. Farrán R, Ferrando A, Jásik J, Kleinov T, Okrás L, et al. (2001) SKP1–SnRK2 protein kinase interactions mediate proteasomal binding of a plant SCF ubiquitin ligase. EMBO J 20: 2742–2756.

39. Marrocco K, Luzcurial A, Nicolas P, Guérache P (2003) The Arabidopsis SKP1-like genes present a spectrum of expression profiles. Plant Mol Biol 52: 715–727.

40. Zhao D, Han T, Rissevuk E, Crosby WL, Ma H (2003) Conservation and divergence of ASK1 and ASK2 gene functions during male meiosis in Arabidopsis thaliana. Plant Mol Biol 53: 163–173.

41. Wang H, Zhou Y, Torres Acosta JA, Foske LC (2007) CDK inhibitors. In: Inz D, ed. Annual Plant Reviews, Blackwell Publishing. pp 62–86.

42. Petrosw MD, Deshae RJ (2005) Function and regulation of cyclin-RING ubiquitin ligases. Nat Rev Mol Cell Biol 6: 9–20.

43. Liu J, Zhang Y, Qin G, Tong T, Sakaguchi N, et al. (2008) Targeted degradation of the cyclin-dependent kinase inhibitor ICK4/KRP6 by RING-type E3 ligases is essential for mitotic cell cycle progression during Arabidopsis gametogenesis. Plant Cell 20: 1538–1554.

44. Lechner E, Ackard P, Vamiri A, Potuschak T, Genschik P (2006) F-box proteins everywhere. Curr Opin Plant Biol 9: 631–638.

45. Liu F, Ni W, Griffith ME, Huang Z, Chang C, et al. (2004) The ASK1 and ASK2 genes are essential for Arabidopsis early development. Plant Cell 16: 5–20.

46. Marrocco K, Zhou Y, Bury E, Dietzel M, Funk M, et al. (2006) Functional analysis of EDD1, an F-box protein involved in photolyase α-dependent light signal transduction. Plant J 45: 425–436.

47. Kim Hj, Oh SA, Brownfield L, Heng SH, Rya H, et al. (2000) Control of plant germline proliferation by SCFβTrCP degradation of cell cycle inhibitors. Nature 453: 1134–1137.

48. Kamura T, Hara T, Matsumoto M, Ishida N, Okumura F, et al. (2004) Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(Kip1) at G1 phase. Nat Cell Biol 6: 1229–1235.

49. Hengst L (2004) A second RING to destroy p27Kip1. The ubiquitin-specific protease subfamily UBP3/UBP4 is essential for pollen development and transmission in Arabidopsis. Nat Cell Biol 6: 1153–1155.

50. Doelling HJ, Phillips AR, Soyley-Oveigem G, Wise J, Chandler J, et al. (2007) The ubiquitin-specific protease subfamily UBP3/UBP4 is essential for pollen development and transmission in Arabidopsis. Plant Physiol 145: 801–813.

51. Li Y, Adrogue OA, Nepveu A, Nakayama KI, Bedard N, et al. (2006) USP19 Deubiquitinating Enzyme Supports Cell Proliferation by Stabilizing KPC1, a Ubiquitin Ligase for p27Kip1. Mol Cell Biol 29: 547–558.

52. Serravallo O, Perez-Perez JM, Heidstra R, Scheres B (2006) Non-cell-autonomous rescue of anaphase-promoting complex function revealed by mosaic analysis of HOBBIT, an Arabidopsis CDC27 homolog. Proc Natl Acad Sci U S A 103: 13250–13255.

53. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis. Proc Natl Acad Sci U S A 95: 11547–11551.

54. Curtis M, Grossniklaus U (2003) A Gateway TM cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiol 133: 462–469.

55. Dissmeyer N, Nowack MK, Pusch P, Stals H, Inze D, et al. (2007) T-Loop phosphorylation of Arabidopsis CDKA1 is required for its function and can be partially restored by an aspartate residue. Plant Cell 19: 972–985.

56. Grini PE, Jurgens G, Hulskamp M (2002) Embryo and endosperm development is disrupted in the female gametophytic capulet mutants of Arabidopsis. Genetics 162: 1911–1926.

57. Domelly PM, Bonetta D, Tsukaya H, Denger RE, Denger NG (1999) Cell cycling and cell enlargement in developing leaves of Arabidopsis. Dev Biol 215: 407–419.

58. Walter M, Chaban C, Schutte K, Batisic O, Weckermann K, et al. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J 40: 428–438.

59. Howden R, Park SK, Moore JM, Orme J, Grossniklaus U (1998) Selection of T-DNA-transformed male and female gametophytic mutants by segregation distortion in Arabidopsis. Genetics 149: 621–631.