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RESEARCH PAPER

The FRK1 mitogen-activated protein kinase kinase kinase (MAPKKK) from Solanum chacoense is involved in embryo sac and pollen development

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Abstract

The fertilization-related kinase 1 (ScFRK1), a nuclear-localized mitogen-activated protein kinase kinase kinase (MAPKKK) from the wild potato species Solanum chacoense, belongs to a small group of pMEKKs that do not possess an extended N- or C-terminal regulatory domain. Initially selected based on its highly specific expression profile following fertilization, in situ expression analyses revealed that the ScFRK1 gene is also expressed early on during female gametophyte development in the integument and megaspore mother cell and, later, in the synergid and egg cells of the embryo sac. ScFRK1 mRNAs are also detected in pollen mother cells. Transgenic plants with lower or barely detectable levels of ScFRK1 mRNAs lead to the production of small fruits with severely reduced seed set, resulting from a concomitant decline in the number of normal embryo sacs produced. Megagametogenesis and microgametogenesis were affected, as megaspores did not progress beyond the functional megaspore (FG1) stage and the microspore collapsed around the first pollen mitosis. As for other mutants that affect embryo sac development, pollen tube guidance was severely affected in the ScFRK1 transgenic lines. Gametophyte to sporophyte communication was also affected, as observed from a marked change in the transcriptomic profiles of the sporophytic tissues of the ovule. The ScFRK1 MAPKKK is thus involved in a signalling cascade that regulates both male and female gamete development.

Key words: Embryo sac development, gametophyte to sporophyte communication, MAPKKK, megagametogenesis, microgametogenesis, pollen tube guidance, seed and fruit development, Solanaceae.

Introduction

Flowering plants or angiosperms exhibit a two-staged life cycle, alternating between a short-lived haploid gametophyte generation composed of only a few cells, and a temporally predominant diploid sporophytic generation. The haploid generation begins with specialized diploid cells (mother cells) of the sporophyte that undergo meiosis to give rise to haploid spores. These spores undergo cell proliferation and differentiation to produce multicellular haploid gametophytes. The male gametophyte (pollen grain) develops within the stamen of the anther and consists of two sperm cells encased within
a vegetative cell. The female gametophyte (embryo sac or megagametophyte) develops within the carpel of the ovary and, in most cases, leads to the formation of an eight-nucleate, seven-celled *Polygonum*-type embryo sac harbouring one egg cell, two synergid cells, three antipodal cells, and one central cell. The major function of gametophyte generation is thus to produce haploid gametes, the egg and sperm cells, which, upon fusion (one sperm cell fusing with the egg cell and the other with the central cell, giving rise to the embryo and endosperm, respectively), will lead to a new sporophytic generation.

Numerous genetic screens have been carried out to identify genes affecting gametophyte development (Feldmann et al., 1997; Bonhomme et al., 1998; Christensen et al., 1998; Pagnussat et al., 2005; Muralla et al., 2011). One would expect that, considering the developmental complexity involved in gamete production, genes involved in intracellular signalling and cell–cell communication would be readily found among gametophyte essential genes. From the curated data set of *Arabidopsis* genes required for gametophyte function listed in Muralla et al. (2011), of the 173 genes that displayed a gametophyte defective phenotype, only 9 (0.05%) are classified as being involved in signalling pathways, of which three are protein kinases. These include the two-component histidine kinase CKI1 (At1g47430) involved in cytokinin perception that severely affects the female gametophyte but only weakly affects the male gametophyte (Pischke et al., 2002; Hejatko et al., 2003); the FUSED (FU) Ser/Thr kinase, involved in cytokinesis, that severely affects both male and female gametophytes (Oh et al., 2005); and the calmodulin-binding receptor-like cytoplasmic kinase 2 (CRCK2) that severely affects male gametophyte development but only weakly affects the female gametophyte (Boavida et al., 2009).

With >1000 and 1400 members, respectively, the kinase superfamily from *Arabidopsis* and rice represents a very large fraction of the proteome in comparison with other eukaryotes. For example, in *Arabidopsis*, kinases represent 4% of the proteome, compared with ~2% in human, *Caenorhabditis elegans*, *Drosophila melanogaster*, and yeast (Shiu and Bleeker, 2003; Shiu et al., 2004; Champion et al., 2004; Dardick et al., 2007). Nonetheless, only a few kinases have been found in gametophyte defective mutant screens, most probably due to the high level of functional redundancy found in major kinase groups, mainly the receptor kinase family (>600 in *Arabidopsis* and 1200 in rice) and the mitogen-activated protein kinase (MAPK) superfamily (MAPK, MAPKK, MAPKKK, and MAPKKKK) with >100 members in both *Arabidopsis* and rice (Hamel et al., 2006; Rao et al., 2010). An example of such redundancy in the MAPKKK family is observed with the *Arabidopsis* ANP1/2/3 kinases that regulate cell division where the triple mutant is not transmitted through the male and female gametes, although other phenotypes, such as reduction of plant size (*anp2/anp3* double mutants), were also observed (Krysan et al., 2002). The ANP1/2/3 kinases are related to the tobacco NPK1 MAPKKK that is part of a cascade, the NACK–PQR pathway, possibly involved in cellularization/differentiation which occurs during stage FG5 (Nishihama et al., 2002; Soyano et al., 2003; Chevalier et al., 2011).

In this study, the isolation and functional characterization of a new MAPKKK from the pMEKK subfamily in *Solanum chacoense* is described. Down-regulation of this single MAPKKK named ScFRK1 (fertilization-related kinase) severely affects both embryo sac and pollen development and leads to partial parthenocarpic fruit production upon pollination.

### Materials and methods

#### Plant material and plant transformation

All plant material and growth conditions are as described in Gray-Mitsumune et al. (2006). For sense and antisense constructs, the ScFRK1 cDNA was inserted in a modified pBin19 transformation vector with a *Cauliflower mosaic virus* (CaMV) 35S double enhancer promoter (Bussière et al., 2003). Sense and antisense constructs were individually transformed in *Agrobacterium tumefaciens* LBA4404 by electroporation. *Solanum chacoense* plants were transformed by the leaf disc method as previously described (Matton et al., 1997).

#### DNA and RNA analyses

Nucleic acid isolation, blotting, and hybridization are as described in O’Brien et al. (2007). Sequence analysis and phylogeny are as described previously (Gray-Mitsumune et al., 2006). Accession numbers are AY427828, KC768863 (ScFRK1), AY427829 (ScFRK2), and KC768864 (ScFRK3).

#### Protein localization through transient expression

The ScFRK1 coding region was fused in-frame to the N-terminus of green fluorescent protein (GFP) in the 35S-driven Gateway vector pMDC83 (Curtis and Grossniklaus, 2003). A 35S-GFP construct was used as a control. Microparticle bombardment was performed as described previously (Germain et al., 2008).

#### Tissue fixation and electron microscopy observations

For pollen viability estimation through outer structure analysis, fresh pollen was observed with a Hitachi S-3000N variable pressure scanning electron microscope at 30 Pa and 15 kV. For transmission electron microscopy (TEM), samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4, post-fixed in 2% osmium tetroxide (OsO$_4$) in the same buffer, dehydrated in ethanol, and embedded in Spur’s resin. Observations were made on a Hitachi H-7500 microscope. Statistics for pollen defects observed by scanning electron microscopy (SEM) were scored from ≥100 pollen grains per wild-type (WT) or transgenic line.

#### Cytological analysis of microsporogenesis

Meiosis and microspore development were studied by squashing anthers in lacto-acetic orcin (1% orcin), according to Dyer’s method (Dyer, 1963), modified by substituting proponic acid with acetic acid. To monitor starch accumulation inside developing pollen, anthers were also squashed in iodine (I$_2$–KI), according to Eriksson’s method (Eriksson, 1962). Pollen fertility was estimated after staining freshly collected mature pollen with acetocarmine (1%) or iodine. All observations were made with a Zeiss AxiosImager M1 microscope equipped with an AxioCam HRc camera.

#### Tissue fixation and optical microscopy observations

Pistils were fixed in FAA for 24 h at 4 °C (50% ethanol, 1.35% formaldehyde, and 5% glacial acetic acid). Samples were then dehydrated in increasing series of tert-butanol (from 70% to pure
tert-butyl alcohol). Pistils were infiltrated with Paraplast Plus paraffin at 60 °C. Thin sections (10 μm) were prepared from embedded samples and tissue sections were stained in 0.5% Astra Blue and 1% safranine after paraffin removal. Alternatively, thin sections (10 μm) were prepared from embedded samples and tissue sections were stained in 0.05% Toluidine Blue. In situ hybridizations were performed as described previously (O’Brien et al., 2005). For differential interference contrast (DIC) microscopy observations, floral buds were dissected and ovaries were fixed in FAA solution overnight (50% ethanol, 0.5% acetic acid, and 1% formaldehyde). Clearing of ovules was performed with an increasing ratio of ethanol–methylsalcylate solutions (0:100, 75:25, 50:50, 25:75, 100:0) for 1 h each and left overnight in 100% methylsalicylate. After dissection from the placenta, ovules were observed with a Zeiss AxioImager M1 microscope equipped with an AxioCam HRc camera.

cDNA microarrays analysis

DNA microarrays comprised 7741 expressed sequence tags (ESTs) corresponding to 6374 unigenes derived from fertilized ovary cDNA libraries covering embryo development from zygote to late torpedo stages (Germain et al., 2005). Experimental conditions were as described previously (Gray-Mitsumune et al., 2006; Tebbji et al., 2010).

Results

Sequence analysis and cellular localization of the ScFRK1 kinase

Using a subtraction selection screen targeting only genes weakly expressed during fertilization and early embryogenesis, five members from the MAPKKK family were isolated in S. chacoense, a self-incompatible wild potato species (Germain et al., 2005). Three of these, named ScFRK1–ScFRK3, were phylogenetically classified in the pMEKK subfamily of the MAPKKKs (Gray-Mitsumune et al., 2006), although they differed significantly from the majority of the pMEKKs due to their small size, consisting of practically only a kinase domain with little N- or C-terminal putative regulatory domains. In Arabidopsis thaliana, 21 MAPKKKs are classified as pMEKKs (mean size ~675 amino acids), with five of those (AtMAPKKK17–AtMAPKKK21) <400 amino acids in length. Three of these (AtMAPKKK19–AtMAPKKK21) are closely related to the ScFRK1–ScFRK3 family in S. chacoense, although ScFRK3 is closer to the MAPKKK19–MAPKKK21 group (Supplementary Fig. S1A available at JXB online). Amino acid sequence identity within this group ranges from 31% to 75% (46–85% similarity) (Supplementary Fig. S1B). Functional analysis of this family has only been reported for the ScFRK2 kinase, which has been shown to be involved in ovule and pollen development (Gray-Mitsumune et al., 2006; O’Brien et al., 2007), and AtMAPKKK20, involved in the osmotic stress response (Kim et al., 2012). Here the functional analysis of the ScFRK1 kinase is reported.

The ScFRK1 clone codes for an open reading frame of 323 amino acids with an estimated mol. wt of 37kDa. A short C-terminal region of 42 amino acids (position 282–323, Fig. 1A) follows kinase subdomain XI. Analysis of the sequence revealed the presence of a cluster of two short basic amino acid sequences predicted to form a bipartite nucleoplasm-type nuclear localization sequence (NLS) (Brameier et al., 2007) (Fig. 1A). The algorithm also predicted a higher NLS potential for the first one (NLS1). To verify this, the ScFRK1 coding region was fused in-frame to the N-terminus of GFP. A 35S::GFP construct was used as a control. As expected for the GFP alone, expression was detected in both the cytoplasm and the nucleus (Fig. 1B; GFP control). In contrast, fluorescence of the ScFRK1–GFP fusion protein was restricted to the nuclei and co-localized with the 4′,6-diamidino-2-phenylindole (DAPI) signal (Fig. 1B; ScFRK1 full). To determine if the two basic sequences were used as a bipartite NLS or if they acted redundantly, the individual role of each predicted NLS was analysed. As shown in Fig. 1B, a sharp cytoplasmic fluorescence was observed in most of the onion cells bombarded with the ScFRK1Δ1 or ScFRK1Δ1Δ2 constructs. However, ambiguous nucleocytoplasmic localization was obtained in ~45% of bombarded cells with the ScFRK1Δ1 construct. Consistent with the above-mentioned prediction, the deletion of NLS2 did not disrupt the nuclear localization of ScFRK1 to the same extent. Only 25% of the cells bombarded with the Δ2 construct showed cytoplasmic or nucleocytoplasmic fluorescence (data not shown). This suggests that although the two C-terminal NLS in ScFRK1 form a bipartite NLS, NLS1 predominates, consistent with the NLS strength prediction.

Pollination and fertilization trigger a stepwise decrease of ScFRK1 mRNA abundance in ovaries

The ScFRK1 expression pattern was determined by RNA gel blot analysis with various vegetative (roots, stems, and leaves), generative (petals), and reproductive tissues (stamens, pollen, styles, and ovaries). At anthesis, strong ScFRK1 mRNA accumulation was only observed in the ovary (Fig. 2A) and, to a lesser extent, in the style (Fig. 2B). Faint expression

Fig. 1. Characterization of the ScFRK1 bipartite nuclear localization signal. (A) Details of the wild type and modified C-terminal region of ScFRK1 constructs used for protein localization studies. The ScFRK1 coding region was fused in-frame to the N-terminus of GFP. The two short basic amino acid sequences predicted to form the NLS are shown in bold. (B) Visualization of GFP expression (top) and DAPI (nucleus) localization (bottom) in bombarded onion cells expressing the fusion constructs. Scale bar=25 μm.
could also be detected in the leaf (Fig. 2A). Pollination and fertilization had dramatic effects on ScFRK1 accumulation in ovaries. Although pollen tubes only reach the ovules ~36 h after landing on the stigma, ScFRK1 steady-state mRNA levels had already significantly declined 12 h after pollination and were barely detectable after fertilization (Fig. 2A). To determine if this stepwise down-regulation of ScFRK1 steady-state mRNA levels was caused by pollination and fertilization, and was not developmentally regulated, non-pollinated pistils were collected from 3 d before anthesis to 3 d after anthesis. As shown in Fig. 2B, peak accumulation of ScFRK1 mRNAs is observed 1 d prior to anthesis and, without pollination, only slightly declines in ovary thereafter. Even 3 d after anthesis, strong ScFRK1 mRNA accumulation is still observed, confirming the stepwise roles of pollination and fertilization in ScFRK1 mRNA accumulation in ovaries.

ScFRK1 is expressed in both the sporophyte and the gametophyte

In order to determine the spatial expression pattern of the ScFRK1 gene, in situ RNA hybridizations were performed using gynoecia from various developmental stages (Fig. 2C). On the day of anthesis, ScFRK1 mRNA signal was strongly detected in ovules and in the vascular tissue and, to a lesser extent, in the ovary wall (Fig. 2C, i). At medium magnification, the strongest accumulation is detected in the integument of the ovule as well as in the epidermis of the placenta that is in direct continuity with the ovule integument (Fig. 2C, iii). Closer examination of the ScFRK1 expression pattern revealed that the gene is expressed in the synergids and the egg cell of the embryo sac (Fig. 2C, v). The asymmetric staining pattern observed is typical for these cells since the two synergids have their large vacuole located towards the chalazal pole, while the vacuole of the egg cell has the reverse orientation. This concentrates the mRNA signal at the micropylar pole for the synergids and towards the chalazal pole for the egg cell. In young flower buds bearing ovules at the megaspore mother cell (MMC) stage, ScFRK1 mRNA signal was already observed in the single ovule integument (Fig. 2C, vii). Solanum chacoense produces unitegmic-tenuinucellate ovules, a trait that occurs almost universally in the asterid clade (Albach et al., 2001). At this stage, denser staining was consistently observed at the tip of the growing integument as well as in the MMC (Fig. 2C, vii). At the same

expression in the ovule integument and embryo sac of mature ovules at anthesis. (vi and vii) ScFRK1 expression in young ovules at the megaspore mother cell stage isolated from ~2 mm flower buds. (ix and x) Cross-sections of young anthers isolated from ~2 mm flower buds. Scale bars: 200 µm (i, ii); 50 µm (iii, iv, ix, x); and 20 µm (v–viii).
stage, ScFRK1 mRNA signal was also detected in developing anthers, more prominently observable in pollen mother cells (PMCs; Fig. 2C, ix).

ScFRK1 knock-down transgenic lines show reproductive defects

In order to assign a function to the ScFRK1 gene, transgenic plants carrying an ScFRK1 sense or antisense construct were generated. The ScFRK1 cDNA was placed downstream of a double enhancer CaMV35S promoter in a modified pBin19 vector in a sense or antisense orientation (Bussière et al., 2003). Kanamycin-resistant plants were grown to maturity in the greenhouse and cross-pollinated to determine if any abnormal phenotype linked to sexual reproduction, based on the ScFRK1 expression profile, could be observed. Numerous plants showed a marked reduction in fruit size, irrespective of the transgenic population from which they were isolated (sense or antisense lines). The ScFRK1 expression level was monitored in transgenic lines showing a decrease in fruit size by RNA gel blot analyses of ovaries collected on the day of anthesis. All the affected lines showed a reduced accumulation of ScFRK1 mRNAs. Three lines expressing variable levels of ScFRK1, down to almost undetectable levels, were chosen for further analyses (Fig. 3). Lines S27 and S1 were co-suppressed lines retrieved from the ScFRK1 sense overexpression population, while AS13 came from the antisense expressed population. Overall plant growth and vegetative development appeared unaffected in all ScFRK1 transgenic lines. However, the ScFRK1 down-regulated lines exhibited severe defects in seed and fruit development. Fruit volume ranged from 13% (S1) to 35–40% (AS13 and S27) when compared with the WT (Fig. 3A, C) or transgenic plants unaffected in ScFRK1 expression (data not shown). Seed production was also strongly affected, with S1 producing only 2% of the normal seed content of an S. chacoense fruit, while AS13 and S1 produced only 15% of the WT seed count (Fig. 3A). The reduced seed set could thus explain the small fruit size observed.

Down-regulation of ScFRK1 affects embryo sac development

Since ScFRK1 is expressed before and after anthesis and with an expression level influenced by both pollination and fertilization, the reduced seed set observed could result from either aberrant ovule development or post-fertilization seed abortion. To assess this, cleared ovules from all lines were observed prior to pollination. As shown in Table 1, decreasing levels of ScFRK1 mRNA led to a concomitant decrease in the number of normal embryo sacs observed. In the most strongly affected transgenic line, Scfrk1-S1, this led to an almost complete absence of normal embryo sacs, explaining the strongly reduced seed set observed. In order to determine when the defect appeared during female gametophyte development, ovules from different developmental stages were observed in flowers buds of the Scfrk1-S1 line, since almost all of its ovules were affected at anthesis. Table 2 shows the correspondence between flower bud length and developmental stages of the S. chacoense female gametophyte. Observation of cleared ovules revealed that megasporogenesis was unaffected. Ovules from the MMC to the functional megaspore stage could be routinely observed in both WT plants (Fig. 4A, B, E) and the Scfrk1-S1 line (Fig. 4C, D, G). Thus, meiosis of the MMC ultimately producing the functional megaspore appeared normal in the ScFRK1 transgenic line. Afterwards, no cell divisions could be observed in the Scfrk1-S1 line (Fig. 4H) that would correspond to the dyad
stage in the WT shown in Fig. 4F, and later at anthesis in Fig. 4J or K). In the WT plants, the surviving megaspore underwent three successive mitotic divisions to produce an eight-nucleate megagametophyte. In WT ovules at anthesis, the three antipodals have already degenerated and only the central cell with its fused polar nuclei, the synergids, and the egg cell are visible (Fig. 4I). In the transgenic lines, affected embryo sacs showed either a clear lack of organization with a shrunken and filled embryo sac (Fig. 4J) or retained a single cell (Fig. 4K). Thus, down-regulation of the ScFRK1 gene affects megagametogenesis as the functional megaspore never progresses beyond the FG1 stage.

**Pollen development is also affected in ScFRK1 transgenic lines**

Although no ScFRK1 mRNA signal could be detected in mature pollen, a strong signal was observed in cross-sections of flower buds in the anthers (Fig. 2C, ix). The ~2 mm flower buds corresponded to anthers where the sporogenous cells are differentiating into PMCs. Pollen from the Scfrk1-S27 and Scfrk1-S1 lines was used to pollinate a fully compatible S. chacoense genotype. When pollen from the WT (S-alleles S12S14, also used as the host for plant transformation) was used to pollinate this fully compatible genotype (S-alleles S11S13), 100% of the pollinated flowers developed fruits (n=20). When pollen from the Scfrk1-S27 line was used, only one in 20 pollinations led to the production of a fruit, while the use of pollen from the Scfrk1-S1 line did not lead to fruit production (n=20). This suggested that pollen development was also affected in transgenic plants down-regulated in ScFRK1 mRNA levels. Pollen observation in dehiscent anthers revealed that, in transgenic lines, pollen viability was severely affected, as estimated by acetocarmine staining of >1000 pollen grains scored per line (Fig. 5, upper panels).

### Table 2. Correspondence between flower buds length and developmental stages of S. chacoense female gametophyte

| Flower bud length | Female gametophyte development stage |
|-------------------|--------------------------------------|
| 1.0–1.5 mm        | Ovule primordia                      |
| 1.5–2.5 mm        | Megaspore mother cell                |
| 2.5–3.0 mm        | Dyad                                |
| 3.0–4.0 mm        | Tetrads and functional megaspore     |
| 4.0–5.0 mm        | Uninucleated and binucleated embryo sac |
| 5.0–6.0 mm        | Tetraneucleated and octanucleated embryo sac |
| Open flower–anthesis | Mature embryo sac; four nuclei (antipodals have degenerated) |

Abbreviations: FRK, fertilization-related kinase; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; WT, wild type.
WT, where >98% of the pollen grains are stainable, <20% of the Scfrk1-S27 transgenic pollen and <1% of the Scfrk1-S1 transgenic pollen appeared viable. When fresh pollen is observed by SEM under low vacuum conditions, affected lines produced shrivelled and collapsed pollen (Fig. 5, middle panels). TEM of pollen sections revealed that the collapsed or shrivelled pollen grains were devoid of a dense cytoplasm and of organelles, in sharp contrast to WT pollen (Fig. 5, lower panels).

Cytological analysis of microsporogenesis

In order to determine precisely when the pollen started to collapse, a cytological analysis of developing pollen was conducted. Microscopic observations of PMCs both at late prophase II/metaphase II of meiosis (Fig. 6A, B) and at the tetrad stage (Fig. 6C, D) revealed no differences between the WT and the Scfrk1-S1 line. Similarly, the young mononucleate microspores from Scfrk1-S1 and the WT line appeared indistinguishable (Fig. 6E, F), suggesting that the defect occurred at later stages of development. This was indeed the case since at later stages of gametogenesis the two lines started to show substantial differences. Mitosis occurred normally in the microspores of the WT, and was followed by differentiation of the generative and vegetative nuclei (Fig. 6G).

Fig. 6. Comparative cytological analyses of WT and Scfrk1-S1 pollen. Late prophase II/metaphase II showing 12 chromosomes in WT (A) and in transgenics plants (B). Tetrads surrounded by callose from WT plants (C) and transgenics (D). Mononucleate microspores just released from the tetrads of WT plants (E) and transgenics (F). Young binucleate WT pollen stained with lacto-acetic orcein showing generative (dark) and vegetative (pale) nuclei (G), and initiation of starch accumulation, visualized with the iodine test (H). The same developmental stage as G and H in transgenics (I, J). WT pollen grains 3 d before anthesis (K, L). The same developmental stage as K and L in transgenics (M, N). Mature WT (O) and transgenic (P) pollen stained with iodine; by this time, starch hydrolysis has been completed. Note the collapsed pollen grains in the transgenic line surrounding one viable pollen grain (P). Scale bar=20 μm.

In contrast, in line Scfrk1-S1, <20% of the microspores underwent the first pollen mitosis (PMI), but <1% continued their development, leading to differentiation of the generative and vegetative nuclei (Fig. 6I). In S. chacoense, microsporogenesis proceeds similarly to the microsporogenesis reported in both tomato (de Nettancourt and Eriksson, 1968) and S. verrucosum (de Nettancourt and Dijstra, 1969). In these species, starch accumulation begins shortly after PMI, while starch hydrolysis begins 2 d before anthesis and is completed by the time the flower opens. In the present study, the iodine test was used to monitor starch accumulation and hydrolysis in developing pollen. The test revealed that almost all WT young pollen started to accumulate starch just after the pollen mitosis (Fig. 6J), and starch accumulation reached a maximum 3 d before anthesis. At this time, the pollen grains appeared almost black (Fig. 6K), and only the generative nucleus remained visible with the acetocarmine stain (Fig. 6L), the vegetative nucleus being completely hidden by the starch grains. In contrast, in Scfrk1-S1 pollen, starch started to accumulate only in a limited number (<1%) of the pollen grains (Fig. 6M), most probably in those where mitosis had been completed and differentiation of the generative and vegetative nuclei had occurred. Most probably these pollen grains continued their development in a similar way to the WT (Fig 6N, O). At anthesis, almost all WT pollen appeared viable, having completed starch hydrolysis (Fig. 6O). In line Scfrk1-S1, however, >99% of the pollen appeared shrunken, with only very few grains showing a normal appearance (Fig. 6P).

Pollen tube guidance is severely affected in the ScFRK1 transgenic plants

The integrity of the embryo sac, the female gametophyte, has been shown to be a prerequisite for the ability of the ovule to attract pollen tubes. Mutants lacking a mature female gametophyte or affected in the development of its cells are defective in pollen tube guidance (reviewed in Marton and Dresselhaus, 2010; Chevalier et al., 2011;Takeuchi and Higashiyama, 2011). To determine if the ScFRK1 transgenic lines are also affected in pollen tube guidance, a semi in vivo pollen tube guidance system was used. The Scfrk1-S1 line was selected for this analysis as it showed the lowest percentage of functional embryo sacs. WT flowers were hand pollinated with fully compatible pollen and styles were collected 24 h later. The detached styles are then laid on a microscopic slide covered with pollen tube growth medium with ovules placed at ~650 μm from the cut style end, a distance corresponding to the radius of the ovary. Pollen tubes start to emerge ~30 hours after pollination (HAP). Figure 7 shows the result of two different assay systems. First, a single-choice assay was used with ovules from either WT or Scfrk1-S1 plants as shown in Fig. 7A, B, respectively. Attraction was determined from the bulk pattern obtained, with each pollinated style being counted as one assay. An attraction phenotype was scored when there was a clear trend and the majority of the pollen tubes grew toward the ovules as observed in Fig. 7A.
In order to isolate embryo sac-expressed genes and sporophytic genes that would depend on embryo sac gene expression, the Scfrk1-S1 ovule transcriptome was compared with the one from WT ovules using a 7.7K DNA microarray made from ovule-derived ESTs (Tebbji et al., 2010). Analysis of variance (ANOVA) testing, along with a Benjamini and Hochberg multiple testing correction algorithm, was used to select ESTs that showed a statistically significant difference in transcript abundance between the WT and the Scfrk1-S1 ovules. A Welch’s t-test (P<0.05) was initially used to compare the profiles from the Scfrk1-S1 versus control and the control versus control comparisons. They were then further restricted with a ≥1.5 fold variation (1.5 cut-off up or down). Seventy-nine ESTs corresponding to 69 unigenes showed statistically lower transcript abundance between the WT and the Scfrk1-S1 ovules (Supplementary Table S1 online). These genes, identified as down-regulated in Scfrk1-S1 ovules, are thus most probably embryo sac-expressed genes. On the other hand, 118 ESTs (98 unigenes) were transcribed significantly more in Scfrk1-S1 ovules, and may thus be associated with sporophytic adaptation to the absence of female gametophyte (Supplementary Table S2).

Blast2GO was used to analyse functional category enrichment between up- and down-regulated genes, and a Fisher’s exact test was performed to determine which categories were significantly regulated (Supplementary Table S3 at JXB online). Among them, gene ontology (GO) terms related to chromatin remodelling (e.g. DNA packaging, DNA conformation change, histone exchange, nucleosome organization, and chromatin assembly and disassembly), cell cycle control (e.g. cell cycle, interphase), intracellular trafficking, as well as development of reproductive tissues characterized down-regulated genes. On the other hand, up-regulated genes are mostly associated with response to stress (e.g. defence response, systemic acquired resistance, response to cold and to radiation), senescence (ageing, organ senescence), and amino acid metabolism (e.g. serine family amino acid metabolic process).

SignalP 4.1, SecretomeP 1.0, and NetNGlyc 1.0 were also used to predict secretion and glycosylation of proteins encoded by regulated ESTs (Supplementary Fig. S3, Horade et al., 2013). To confirm the results of this quick assay system, the two-sample Kolmogorov–Smirnov test, a non-parametric test comparing empirical distribution functions in two samples, widely used in axon guidance studies, was also used. In this case, growth angles for all distinguishable pollen tubes were calculated from their exit to their end point on a total of ~150 pollen tubes from five semi in vivo single-choice assays (Fig. 7D–F). A mean angle of 2.5° was obtained for the negative control (without ovules), ~0.8° for assays with Scfrk1-S1 ovules, and 16.3° for assays with WT ovules. Attraction was thus observed with WT ovules (P=0.008) but not with Scfrk1-S1 ovules (P=0.285).

Embryo sac-dependent gene expression and gametophytic to sporophytic communication

In order to isolate embryo sac-expressed genes and sporophytic genes that would depend on embryo sac gene expression, the Scfrk1-S1 ovule transcriptome was compared with the one from WT ovules using a 7.7K DNA microarray made from ovule-derived ESTs (Tebbji et al., 2010). Analysis of variance (ANOVA) testing, along with a Benjamini and Hochberg multiple testing correction algorithm, was used to select ESTs that showed a statistically significant difference in transcript abundance between the WT and the Scfrk1-S1 ovules. A Welch’s t-test (P<0.05) was initially used to compare the profiles from the Scfrk1-S1 versus control and the control versus control comparisons. They were then further restricted with a ≥1.5 fold variation (1.5 cut-off up or down). Seventy-nine ESTs corresponding to 69 unigenes showed statistically lower transcript abundance between the WT and the Scfrk1-S1 ovules (Supplementary Table S1 online). These genes, identified as down-regulated in Scfrk1-S1 ovules, are thus most probably embryo sac-expressed genes. On the other hand, 118 ESTs (98 unigenes) were transcribed significantly more in Scfrk1-S1 ovules, and may thus be associated with sporophytic adaptation to the absence of female gametophyte (Supplementary Table S2).

Blast2GO was used to analyse functional category enrichment between up- and down-regulated genes, and a Fisher’s exact test was performed to determine which categories were significantly regulated (Supplementary Table S3 at JXB online). Among them, gene ontology (GO) terms related to chromatin remodelling (e.g. DNA packaging, DNA conformation change, histone exchange, nucleosome organization, and chromatin assembly and disassembly), cell cycle control (e.g. cell cycle, interphase), intracellular trafficking, as well as development of reproductive tissues characterized down-regulated genes. On the other hand, up-regulated genes are mostly associated with response to stress (e.g. defence response, systemic acquired resistance, response to cold and to radiation), senescence (ageing, organ senescence), and amino acid metabolism (e.g. serine family amino acid metabolic process).

SignalP 4.1, SecretomeP 1.0, and NetNGlyc 1.0 were also used to predict secretion and glycosylation of proteins encoded by regulated ESTs (Supplementary Fig. S3,
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Supplementary Table S4 at JXB online). A characteristic of embryo sac genes is their enrichment for small and secreted proteins (Jones-Rhoades et al., 2007). Interestingly, proteins harbouring a signal peptide are significantly more frequent in down- and up-regulated genes than in unregulated genes ($P=5.6e^{-12}$ and $P=3.8e^{-6}$, respectively). In contrast, the proportion of unconditionally secreted proteins decreases significantly in both data sets ($P=0.05$ and $P=0.02$, respectively). Furthermore, 30% of secreted peptides potentially encoded in down and up ESTs correspond to small, cysteine-rich proteins (CRPs) such as defensins, lipid transfer proteins, rapid alkalization factor (RALF) peptides, Papaver S-like proteins, and γ-thionins. Conventionally, up-regulated secreted proteins are significantly less glycosylated ($P=0.03$), which holds true for up-regulated CRPs ($P=4.6e^{-4}$).

Discussion

Among the protein kinases known to affect key aspects of plant reproductive development only a few have been characterized from the MAPK superfamily. These include the Arabidopsis YODA (MAPKKK4), involved in embryo and stoma development (Bergmann et al., 2004; Lukowitz et al., 2004); the S. chacoense FRK2 MAPKKK, involved in ovule (Gray-Mitsumune et al., 2006) and pollen development (O’Brien et al., 2007); the redundant Arabidopsis MAP3K1 (MAPKKK7) and MAP3K2 (MAPKKK6), involved in pollen viability (Chaiwongser et al., 2006); the Arabidopsis MPK6, involved in anther, inﬂorescence as well as in embryo development (Bush and Krysan, 2007); and Arabidopsis MPK3 and MPK6, also involved in defence responses and in ovule development (Wang et al., 2008). Recently, mpk3/mpk6 double mutant pollen tubes were also shown to be defective specifically in the funicular guidance phase (Guan et al., 2014).

In this study, the characterization of ScFRK1, a novel MAPKKK from S. chacoense that affects both male and female gametophyte development, is reported. Although the three FRK genes, ScFRK1, 2, and 3, are expressed in reproductive tissues, they are not genetically redundant, since down-regulation of ScFRK1 is not complemented by the presence of the others. They also share limited amino acid sequence identity, <45% (Supplementary Fig. S1 at JXB online), and are not all predicted to be in the same subcellular compartment (only ScFRK1 and 2 are predicted to be localized in the nucleus). Furthermore, an RNA interference effect on ScFRK2 and 3 is unlikely considering the low nucleotide sequence identity between ScFRK1 and the two other kinases (<38%) and the fact that no stretches of >15 and 12 identical nucleotides are found with ScFRK2 and ScFRK3, respectively. Thus, as expected, ScFRK2 and 3 expression levels were not significantly down-regulated in the Scfrk1-S1 line (Supplementary Fig. S2), confirming that the phenotype observed is due to the down-regulation of the ScFRK1 gene. Furthermore, down-regulation of ScFRK2 showed no observable phenotype, nor any reproductive defects, while overexpression lines led to the conversion of ovules into carpelloid structures (Gray-Mitsumune et al., 2006).

As for ScFRK2, the other member of the family previously examined, ScFRK1 had a complex and peculiar expression pattern. While ScFRK2 expression is weak at anthesis and is fertilization induced, ScFRK1 is strongly expressed at anthesis and is pollination repressed. Since ScFRK1 is expressed early on during pollen and ovule development, with phenotypes observed in down-regulated lines affecting the gametophytes, high expression in the ovary at anthesis and the post-pollination steady-state ScFRK1 mRNA decrease are puzzling and remain to be examined.

In Arabidopsis, the putative orthologues of the Solanum FRK family members are MAPKKK19, 20, and 21. Interestingly, MAPKKK20 was among the genes regulated by the male germline-specific DUO1 MYB transcription factor (Borg et al., 2011). In contrast to ScFRK1 transgenic lines, DUO1 mutants only affect the male gametophyte. DUO1 mutants progress normally through PMI but fail to complete the generative cell cycle (Durbarry et al., 2005; Rotman et al., 2005). In contrast, in line Scfrk1-S1, <20% of the microspores underwent PMI, and <1% continued their development, leading to differentiation of the generative and vegetative nuclei (Fig. 6I). Consistent with this, starch accumulation that normally begins shortly after PMI in solanaceous species (Fig. 6J) was only detected in <1% of the Scfrk1-S1 pollen grains (Fig. 6J), and those were presumably the ones that had progressed through PMI and completed the differentiation of the generative and vegetative nuclei. Thus, in pollen, ScFRK1 most probably does not act in the same pathway or would act upstream of genes such as DUO1.

As ScFRK1 is expressed in both sporophytic and gametophytic tissues, it is puzzling that only the gametophyte is affected in down-regulated transgenic lines. Considering that the ovule sporophytic tissue in ScFRK1 transgenic lines does not show any evidence of developmental defects at maturity, and that in described gametophytic mutants sporophytic tissue develops normally (Bencivenga et al., 2011), this suggests that down-regulation of ScFRK1 mostly affects gametophyte development. Since very weak expression can still be detected in ScFRK1 down-regulated lines, a different threshold effect between the expression observed in the integument and the young ovule at the MMC stage (see Fig. 2, vii for WT expression) could also explain why the sporophyte is not affected. Interestingly, ScFRK1 expression is not equally distributed in the integument, with higher levels at the tip of the integument in young ovules at the MMC stage (Fig. 2, vii) and, in mature ovules, in the cell layers immediately surrounding the embryo sac, the inner epidermis, also called the 'integumentary tape-tum' in unigemtic families such as Solanaceae (Fig. 2ii, v). The inner epidermis has been endowed with numerous features. During the initial stages of gametophyte development, the ultrastructure of the inner epidermis cells has been described as akin to that of meristematic cells. By dividing profusely, its cells were considered to provide the necessary conditions to co-ordinate the intensive growth of the embryo sac. Once fully differentiated, the inner epidermis provides nutrition to the embryo sac (Kapil and Tiwari, 1978).

As expected from Arabidopsis mutants that lack a functional embryo sac (reviewed in Chevalier et al., 2011;
Takeuchi and Higashiyama, 2011; Dresselhaus and Franklin-Tong, 2013), or for plants where the synergid cells had been physically ablated (Higashiyama et al., 2001), pollen tube guidance was severely compromised in the Scfrk1-S1 transgenic line (Fig. 7). Absence of the embryo sac led to the isolation of embryo sac-dependent genes (down-regulated genes; Supplementary Table S1 at JXB online) that could be directly involved in pollen tube guidance or other cell-cell interaction functions. Among these, two RALFs, ScRALF4 and 5, were isolated. These are closely related to Arabidopsis RALF27 and 32 and are quite ubiquitously expressed in S. chacoense (Germain et al., 2005). No functions have yet been ascribed to these RALFs. However, involvement of RALF peptides in plant reproduction has been recently highlighted with the characterization of ScRALF3, involved in sporophyte to gametophyte communication. Although expressed in the sporophytic tissue of the ovule, down-regulation of ScRALF3 expression by RNA interference led to improper embryo sac development through loss of embryo sac nuclei polarization and an increase in asynchronous divisions (Chevalier et al., 2013). Absence of the embryo sac also had an impact on the surrounding sporophytic tissue with the isolation of several up-regulated genes in the ovule, suggesting interactions between the female gametophyte and the maternal sporophyte, as observed previously in Arabidopsis (Johnston et al., 2007). Furthermore, the majority of proteins encoded in up and down ESTs correspond to secreted peptides, 30% of which are small CRPs. These proportions are in line with recent studies in Arabidopsis (Supplementary Figs S4 and S5).

Interestingly, the GO terms associated with down-regulated genes in Scfrk1-S1 (chromatin remodelling, cell cycle control, intracellular trafficking, and development of reproductive tissues) are consistent with the dynamic nature of the development of the embryo sac, with its rounds of mitosis, cellular polarization, and positioning, as well as with the expression of elevated amounts of secreted proteins, such as from the synergid filiform apparatus. Similarly, GO terms associated with up-regulated genes are predominantly linked to stress responses, as if absence of the female gametophyte would be sensed as a scar and elicit a wound or defence response. The characterization of the other members of this MAPK cascade, such as MAPKKK and MAPK, as well as downstream nuclear or cellular targets should reveal essential steps in male and female gametophytes development.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. (A) Section of a pMEKK phylogenetic tree showing the most closely related orthologues of ScFRK1–3 in A. thaliana. (B) Percentage sequence identity and similarity between S. chacoense FRK1, 2, and 3 and A. thaliana MAPKKK19, 20, and 21, based on a ClustalW multiple protein sequence alignment.

Figure S2. Specific down-regulation of ScFRK1 in transgenic plants.

Figure S3. Secretion and glycosylation predictions for proteins up-, down- and not regulated in the Scfrk1 mutant.

Figure S4. Conventional and unconventional secretion predictions for proteins regulated in Scfrk1 and other ovule mutants.

Figure S5. CRP content in proteins regulated in Scfrk1 and other ovule mutants.

Table S1. Information about frk1 down-regulated ESTs.
Table S2. Information about frk1 up-regulated ESTs.
Table S3. GO term enrichment in frk1 up- and down-regulated ESTs compared with unregulated ESTs.
Table S4. Summary of secretion and glycosylation predictions on Scfrk1 up-, down-, and unregulated ESTs.

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