RESEARCH ARTICLE

Live and let die: a REM complex promotes fertilization through synergid cell death in Arabidopsis

Marta Adelina Mendes1,2, Rosalinda Fiorella Guerra1, Beatrice Castelnovo1, Yuriria Silva-Velazquez3, Piero Morandini1, Silvia Manrique1, Nadine Baumann4, Rita Groß-Hardt4,5, Hugh Dickinson3 and Lucia Colombo1,4*

ABSTRACT

Fertilization in flowering plants requires a complex series of coordinated events involving interaction between the male and female gametophyte. We report here molecular data on one of the key events underpinning this process – the death of the receptive synergid cell and the coincident bursting of the pollen tube inside the ovule to release the sperm. We show that two REM transcription factors, VALKYRIE (VAL) and VERDANDI (VDD), both targets of the ovule identity MADS-box complex SEEDSTICK-SEPALLATA3, interact to control the death of the receptive synergid cell. In vdd-1/+ mutants and VAL RNAi lines, we find that GAMETOXYTHETIC FACTOR 2 (GFA2), which is required for synergid degeneration, is downregulated, whereas expression of FERONIA (FER) and MYB98, which are necessary for pollen tube attraction and perception, remain unaffected. We also demonstrate that the vdd-1/+ phenotype can be rescued by expressing VDD or GFA2 in the synergid cells. Taken together, our findings reveal that the death of the receptive synergid cell is essential for maintenance of the following generations, and that a complex comprising VDD and VAL regulates this event.

KEY WORDS: Receptive synergid cell, Pollen tube, Cell death, MADS box, REM, Fertilization

INTRODUCTION

The female gametophyte of flowering plants develops within the ovule and, in many species, including Arabidopsis thaliana, comprises a seven-celled structure containing three antipodal cells, two synergid cells, the egg and a central cell (Wetstein and Russell, 2004). Cell ablation experiments in Torenia fournieri have showed that synergids produce a species-specific signal to attract the pollen tubes (Higashiyama et al., 2006) and at least one viable synergid cell is needed for pollen tube attraction (Higashiyama et al., 2001). In Arabidopsis thaliana, the myb98 mutant has a defective filiform apparatus and consequently fails to attract pollen tubes, confirming a role for the synergid cells in pollen tube attraction (Kasahara et al., 2005). Typically, only one pollen tube penetrates each ovule and delivers two non-motile sperm cells whose release occurs by bursting – an event triggered when the tube interacts with the synergid cell surface (Sandaklie-Nikolova et al., 2007). The receptive synergid cell then degenerates, whereas the other synergid cell remains unaffected.

Hamamura et al. (2011) proposed that it is the loss (through termination of synthesis or degradation) of the pollen tube attractant(s) that results in the inability of the female gametophyte to attract further pollen tubes after a successful fertilization. Fusion of the two sperm cells with the female gametes (egg and central cell) is required to stimulate the degeneration of the second synergid, and the termination of attractant production (Beale et al., 2012; Kasahara et al., 2012). These events have been shown to require both the chromatin-remodeling Polycomb repressive complex 2 (PRC2) (Maruyama et al., 2013) and ethylene signaling (Völz et al., 2013).

Once the pollen tube has interacted with the receptive synergid, its growth becomes arrested. Several gametophytic mutants defective in pollen tube growth arrest have been identified in Arabidopsis thaliana, such as feronia (fer) (Huck et al., 2003), sirène (srm, allelic to fer) (Rotman et al., 2003; Escobar-Restrepo et al., 2007), lorelei (lre) (Capron et al., 2008 and Tsukamoto et al., 2010), scylla (syl) (Rotman et al., 2008), norta (ntr) (Kessler et al., 2010) and abstinence by mutual consent (amc) (Boisson-Dernier et al., 2008).

Synergid cells have recently been reported to coordinate pollen tube reception and burst via a calcium-mediated response requiring the FER signaling pathway (Ngo et al., 2014). The synergid ‘cell-death pathway’ is defective in fer and lre lines, although it is not clear whether the receptive synergid, the non-receptive synergid or even both synergids are affected (Ngo et al., 2014). The first unambiguous female gametophytic mutation identified that showed a defective receptive synergid cell death was gametophytic factor 2 (gfa2) (Christensen et al., 2002). GFA2 is a mitochondrial matrix chaperone protein (Christensen et al., 2002), suggesting that synergid cell death in Arabidopsis requires functional mitochondria, as already proposed by several authors (see review by Scott and Logan, 2008). A similar situation was also reported for animals, where several studies have demonstrated that functional mitochondria are required both for normal cell growth and division, and for programmed cell death. Paradoxically, most tumor cells, which contain active mitochondria, are resistant to apoptosis (Gogvadze et al., 2008; Wang et al., 2013; Yadav and Chandra, 2014).

VERDANDI (VDD), a transcription factor belonging to the reproductive meristem (REM) family (Romanel et al., 2009; Mantegazza et al., 2014), was identified as a target of the ovule identity complex SEEDSTICK-SEPALLATA3 (STK-SEP3) and is required for synergid cell death (Matias-Hernandez et al., 2010). In vdd-1/+ ovules, the synergid cells partially lose their identity. They are still able to attract pollen tubes, but never undergo cell death. We...
show here that a second REM factor, REM11, is also a target of the STK-SEP3 complex and is involved directly in synergid cell death, together with VDD. Since the REM11 mutant exhibited a female phenotype similar to vdd-1/+ , we decided to follow tradition and name it after a Norse goddess, VALKYRIE (VAL) (from Old Norse valkyrja), who chose those who lived and died in battle (Orchad, 2011). We analyzed the role of VDD and VAL in the context of other genes required for pollen tube-synergid interactions, such as MYB98, FER and GFA2. We found the vdd-1/+ phenotype is fully rescued by expression of the VDD coding sequence in synergid cells using the MYB98 promoter, and is partially rescued by expressing GFA2, suggesting that GFA2 lies downstream of VDD. Based on these results, we propose an integrated model in which a VDD-VAL complex regulates key events of the fertilization process, including the death of the receptive synergid cell and the death (by bursting) of the pollen tube after growth arrest.

RESULTS

**VAL is co-expressed with STK and VDD**

Pollen tube reception and synergid cell death are key events in the fertilization process. The female gametophytic phenotype of vdd-1/+ is important to our understanding of these events because 35% of vdd-1/+ ovules remain unfertilized even if a viable pollen tube successfully reaches all of them (Matias-Hernandez et al., 2010). To discover further factors involved in this process, we used Affymetrix microarray data from 2000 experiments in a bioinformatics strategy to identify genes with expression patterns similar to VDD. The Pearson correlation coefficient (PCC) of individual scatterplots was used to determine the extent of similarity in expression profiles (i.e. co-expression level; for more details, see Materials and Methods). The Affymetrix microarray dataset has proved to be consistent and the calculated PCC values are robust indicators (Menges et al., 2008; Berri et al., 2009). Using this dataset, STK and VDD show a high degree of correlation (0.812) and emerge amongst the top correlators (see Tables S1 and S2), a group which also includes SHP1, SHP2 and other genes either known to be involved in the process (e.g. AGO9; Durán-Figueroa and Vielle-Calzada, 2010) or identified in other screens (At5g20520; see Skinner and Gasser, 2009). Nevertheless, VALKYRIE (VAL) – a gene not previously associated with fertilization – scored the highest PCC value of 0.916 in the Lin analysis for STK (cf. VDD value of 0.798) and was therefore selected for further analysis.

**VAL is a direct target of STK-SEP3**

As VAL presented the highest PCC value, even higher than VDD with STK, we performed a sequence analysis of the VAL genomic region searching for putative MADS box binding sites – CArG box sequences. This analysis revealed the presence of two putative CArG box sequences: the first located in the VLD promoter region and the second close to the translation start site (Fig. 1A). We performed a chromatin immunoprecipitation (ChIP) assay using STK and SEP3 native antibodies followed by quantitative real-time PCR (qRT-PCR). We also tested SEP3 because it forms a heterodimer with STK, enabling STK to bind DNA (Mendes et al., 2013). qRT-PCR analysis revealed an enrichment of both CArG box regions in the ChIP assays (Fig. 1B). In the anti-STK experiment, chromatin immunoprecipitated from the stk single mutant was used as a negative control, whereas in the anti-SEP3 study, wild-type (wt) leaves were used as a negative control, because SEP3 is not expressed in leaves. These results indicate that VAL, like VDD, is a direct target of the MADS box domain STK-SEP3 protein complex.

Additionally, in situ hybridization experiments were performed to determine VAL expression pattern during ovule development. VAL is expressed at all stages of ovule development in wt plants (Fig. S1A-F) and high expression was detected in the embryo sac and surrounding layer (Fig. S1E). A higher magnification of the mature ovule demonstrated that a strong signal was present inside the embryo sac in the synergid cell zone (Fig. S1F). Furthermore, the hybridization signal was clearly weaker in stk mutant ovules (Fig. S1G) and no expression was observed in stk shp1 shp2 triple mutant ovules (Fig. S1H). These data confirm that STK, SHP1 and SHP2 redundantly control VAL expression in ovules, as is also the case for VDD (Matias-Hernandez et al., 2010).

**VAL is required for female gametophyte fertility**

As no T-DNA insertion lines are available for VAL we used an RNA interference approach to investigate the function of VAL during ovule development. We obtained 43 transgenic plants containing a p35S::VAL_RNAi (VAL_RNAi) construct. The immature silicules of these lines were analyzed, and different percentages of unfertilized ovules were observed. We then further divided the transgenic plants into three classes based on the number of unfertilized ovules contained in eight immature silicules per plant. The classes were distributed as follows, comparing always with wt (Fig. 2A): class i, 35% unfertilized ovules (28 plants); class ii, 45% unfertilized ovules (10 plants) (Fig. 2B); class iii, 60% unfertilized ovules (5 plants) (Fig. 2C). Although the silicules contained large numbers of unfertilized ovules, the floral organs developed normally (Fig. 2D). Interestingly, further analysis by qRT-PCR showed the number of unfertilized ovules in the transgenic plants to be proportional to the level of downregulation of the VAL transcript (Fig. 2E). VAL_RNAi plants with the most severe phenotype had approximately 60% unfertilized ovules and almost complete silencing of VAL (Fig. 2E).
To determine whether female, male or both gametophytes were affected by downregulation of VAL, we performed reciprocal crosses between VAL_RNAi and wt plants. After fertilizing wt plants with pollen from VAL_RNAi mutant plants (class iii), only 6% (18/300) of the ovules remained unfertilized, which is similar to the control (equivalent wt crosses, 4.5%; 10/250). Using class iii VAL_RNAi plants as female, pollinated with wt pollen, the percentage of unfertilized ovules was 56% (336/600), demonstrating that the reduced fertility phenotype resulted from a female defect.

As in wt plants, the ovules of VAL_RNAi plants reached maturity and contained an embryo sac with seven cells (Fig. S2). As the embryo sac seemed to be formed correctly, we further investigated whether ovule sterility resulted from defects in the specification of female gametophytic cell identity. Embryo sac cell-specific reporter constructs were thus introduced into VAL_RNAi transgenic lines and gene expression was analyzed in F2 generation, in this way plants were homozygous for the marker. EC1::GUS was used as an egg cell identity marker (Sprunck et al., 2012), FIS2::GUS as a central cell marker (Chaudhury et al., 1997) and the promoter of the gene At1g36340 as marker for the antipodal cells (Yu et al., 2005) (Fig. S2). No difference in the GUS marker line expression was detected between VAL_RNAi and wt plants, indicating that their cell fates were unaffected by the presence of the transgene. However, analysis of GUS expression using the synergid-specific cell marker line (ET2634; Groß-Hardt et al., 2007) revealed that 277 of 740 (37.5%) embryo sacs to fail to express the synergid specific marker in VAL transgenic plants. The percentage of unfertilized ovules per silique (37%) in the transgenic plants corresponded to the number of embryo sacs that failed to show GUS expression in the synergids.

Fig. 2. VAL_RNAi plants exhibit a female gametophyte defect. (A) Wild-type siliques showing full seed set. (B,C) Siliques of VAL_RNAi plants with 45% (B) and 60% (C) unfertilized ovules (black asterisks). (D) Class iii VAL_RNAi flower; although the pistil shows 60% unfertilized ovules, all the flower organs are perfectly formed. (E) qRT-PCR in the different VAL_RNAi classes. The number of unfertilized ovules perfectly correlates with downregulation of VAL. (F) pCKX7::GUS in a wt mature ovule. A very strong GUS signal was detected in the synergid region. (G) pCKX7::GUS in VAL_RNAi. Several mature ovules do not present the GUS signal (asterisks). (H) Aniline Blue staining shows that pollen tubes (asterisks) reach all VAL_RNAi mutant ovules 24 h after hand-pollination, even the smaller ovules (unfertilized). (I) Detail of an unfertilized ovule containing a pollen tube. (J) Detail of an unfertilized ovule with two/three pollen tubes (white dots). Scale bars: 50 μm.
(Fig. S2). Kölßmer et al. (2014) proposed that the plant hormone cytokinin plays a role in synergid and egg cell development, because CYTOKININ OXIDASE/DEHYDROGENASE7 (CKX7) is strongly expressed in those cells. A similar construct pCYTOKININ OXIDASE7 (CKX7)::GUS, but with a nuclear localization signal (NLS) signal to confer a stronger and more specific GUS signal, was used to make crosses with vdd-1/+ and VAL_RNAi (class iii) plants. The GUS expression was strong in the wt synergid cells region (Fig. 2F); plants from the F2 generation homozygous for pCKX7::GUS construct showed B-glucuronidase activity in only 72% of vdd-1/+ ovules (n=600) and in 62% (n=500) of VAL_RNAi (class iii) mutant ovules (Fig. 2G).

Synergid cells are responsible for the production of attractant molecules, the guidance cues that ensure each embryo sac (ovule) receives a pollen tube (Higashiyama et al., 2001). We therefore investigated the journey of pollen tubes to the micropyle of mutant ovules. We followed pollen tubes in the transmitting tract using aniline blue staining of the pollen tube walls and found that all ovules of VAL_RNAi plants were targeted by at least one pollen tube, indicating that the pollen tube guidance was not affected in this mutant. Importantly, we noticed the ovules investigated fell into two size classes, large (756 of 1200 ovules investigated) having been fertilized within 24 h of pollination, and small (444 of 1200 ovules), remaining unfertilized, although pollen tubes could be seen adjacent to the receptive synergids (Fig. 2G and detail 2H). Furthermore, a low percentage of the ovules (~7% of 444, compared with 2% of 200 in wt; P=0.008, Fisher’s exact test) were penetrated by more than one pollen tube (Fig. 2I). Our data thus show that VAL_RNAi transgenic lines display a near-identical phenotype to that described for vdd-1/+ mutants, with both lines showing loss of synergid identity while retaining the ability to attract pollen tubes, (Matias-Hernandez et al., 2010).

The VDD-VAL dimer is required for pollen tube bursting
VDD and VAL proteins belong to the same family, whose members have a putative protein-protein binding domain, and a single protein-DNA binding domain (Romanel et al., 2009; Swaminathan et al., 2008; Mantegazza et al., 2014). The similarity of these mutant phenotypes raised the question as to whether these two proteins were able to interact and form a complex. Using a yeast two-hybrid assay (Y2H), we found clear evidence of an interaction between VDD and VAL (Fig. S3). To explore this interaction in vivo, we exploited the bimolecular fluorescence complementation (BiFC) assay to test whether VDD and VAL also interacted in planta, using leaves of N. benthamiana and basing our assays on the live reconstitution of yellow fluorescence protein (YFP), as previously described by Belda-Palazón et al. (2012). The results confirmed that VDD and VAL were able to fully reconstruct the YFP signal, fused either to the C- or N-terminal fragments of the YFP, forming a heterodimer (Fig. 3A). Furthermore, we discovered that VAL is also able to interact with itself, forming a homodimer (Fig. 3B), whereas VDD alone is not able to reconstitute the YFP signal. We further tested VDD and VAL fused with the C- or N-terminus of the YFP, with empty vectors with the YFP C- or N-terminus as negative controls; no reconstitution of YFP was found (Fig. S3).

To further explore the mechanisms underlying the vdd-1/+ and VAL_RNAi phenotypes, we hand-pollinated both mutants with pollen from plants containing the Late Anther Tomato52 (pLAT52):GUS transgene (Tsukamoto et al., 2010). This marker labels the pollen tube cytosol, allowing the investigation of pollen tube growth and, ultimately, the location of its bursting when in contact with the receptive synergid (Fig. 4A). In wt plants, pollen tube burst was detected in 98% of the 300 ovules analyzed. In the case of the mutant ovules, despite the fact that most of them were reached by at least one pollen tube, only some pollen tubes burst, with other tubes entering the ovule without bursting. Only 71.2% of the 300 vdd-1/+ ovules analyzed showed pollen tube burst, while the remaining 28.8% stayed in contact with the receptive synergid without any discharge (Fig. 4D). In VAL_RNAi pistils, 65% of 250 ovules analyzed showed pollen tube discharge, while the remaining 35% failed to show any bursting (Fig. 4G). These results strongly suggest that both VDD and VAL are required for pollen tube bursting.

To confirm these results, we used pollen from plants carrying the HTR10-RFP transgene, which marks the two sperm nuclei, to visualize the location of the sperm cells in the two mutants and to enable gametic fusion to be monitored (Aw et al., 2010; Hamamura et al., 2011). Importantly, in vdd-1/+ (Fig. 4E,F) and VAL_RNAi (Fig. 4H,1) mutant lines, the two sperm cells arrested in the micropylar area, near to the receptive synergid, at a frequency similar to the number of the unfertilized ovules previously observed in these two mutant backgrounds: 30%
(n=500) of vdd-1/+ ovules and 41% (n=200) of VAL_RNAi ovules.

Structural analysis of mutant embryo sacs – receptive synergid degeneration

To investigate in detail the degeneration of the receptive synergid and the integrity of ‘unfertilized’ embryo sacs, we carried out confocal laser scanning (CLS) and transmission electron microscopy (TEM) on vdd-1/+ and VAL_RNAi ovules, from 16 until 48 h after hand-pollination.

As a control for our CLS microscopic analysis, we examined the status of the synergid cells in wt embryo sacs, before and after pollen tube arrival in unpollinated pistils and in hand-pollinated pistils (16 h post pollination). As expected, the two synergids appeared intact prior to pollen tube arrival in the unpollinated pistils (Fig. 5A) and one synergid degenerated after pollen tube arrival (16 h post pollination) (Fig. 5B, detail in C), with the other remaining intact, as described elsewhere.

Both egg and central cells were still clearly visible in vdd-1/+ ovules 48 h after hand-pollination (Fig. 5D), revealing that fertilization had not occurred. Importantly, in 16% of the analyzed ovules (Fig. 5E; 16% of n=150, unfertilized ovules=32%) two intact synergid cells could be seen, albeit in a different confocal plane from the egg and central cells. The two synergids also remained intact after 48 h in VAL_RNAi plants (Fig. 5F; 22% of n=160, unfertilized ovules=42%). Developing endosperm could clearly be seen in ovules within the same ovaries that had been fertilized (Fig. 5G).

TEM analysis was used to investigate finer structural details of the embryo sac cells. In vdd-1/+ and VAL_RNAi lines, those ovules in which the female gametophyte remained unfertilized regularly featured intact egg and central cells (Fig. S4). While these embryo sacs contained two intact synergid cells with classical filiform apparatus (vdd-1/, Fig. 5H,I; VAL_RNAi, Fig. 5J), remains of pollen tubes could be detected (recognizable by a high density of organelles, as described by Sandaklie-Nikolova et al., 2007) in the micropylar region. Although the pollen tube had penetrated the ovule and approached the receptive synergid cell in these ovules, neither the pollen tube nor the receptive synergid had degenerated. Further, the synergids presented entirely normal cytology, with intact membranes and key organelles located correctly.

The molecular network controlling synergid cells and pollen tube interaction

The VAL-VDD heterodimer is required for the death of the receptive synergid and consequentially for pollen tube disintegration, but it is clearly not required for pollen tube attraction, suggesting that two or more independent molecular networks may operate in the synergid. MYB98 has been identified as one of the transcription factors responsible for pollen tube attraction to the synergids and we therefore analyzed crosses between the vdd-1/, and ‘class iii’ VAL_RNAi plants and the marker line pMYB98::GFP. In the F2 from this cross, where the transgene was in a homozygous situation, 15 independent plants were analyzed for each mutant. pMYB98::GFP was found to be expressed in the two mutant backgrounds (vdd-1/: 97% of 1500 ovules; VAL_RNAi: 98% of 800 ovules) as in wt plants (98% of 300 ovules) (Fig. 6A-C), confirming our previous data indicating that pollen tube attraction was not compromised in these mutants.

Fig. 4. Pollen tube burst and sperm cell delivery in vdd-1/+ and VAL_RNAi lines. Pollen tube burst was assessed using LAT52::GUS transgene and sperm cell delivery using HTR10_RFP transgene.
(A) Wild-type ovules, with a clear burst of the pollen tube: a large blue region is seen in the micropylar region. (B) Wild-type ovule showing sperm cell fusion 24 h after pollination. The sperm cells are inside and fusion with the egg and central cell can be observed. (C) Overlay of B with bright-field image. (D) In vdd-1/+ ovule, no pollen tube burst was detected. A blue line is visible in the micropylar region very close to the receptive synergid place. (E) Two sperm cells outside the vdd-1/+ embryo sac. No fusion was detected with the central or egg cell. (F) In more detail, overlay of E with bright-field image. (G) VAL_RNAi ovule, showing no pollen tube burst with pLAT52::GUS marker. (H) VAL_RNAi ovule with the two sperm cells outside the embryo sac. (I) Overlay with the bright-field image. Percentages of all the crosses are summarized in Table 1. Scale bars: 50 μm.
Following attraction to the receptive synergid, growth of the pollen tube is arrested. The receptor like-kinase FERONIA has been proposed to play a key role in this process (Huck et al., 2003) and more recently in the coordination of the Ca²⁺ response during cell death (Ngo et al., 2014). We therefore crossed vdd-1/+ and class iii VAL_RNAi with pFER::FER_GFP, but failed to detect any difference in FER expression compared with wt levels (Fig. S5, vdd-1/+, n=50 and VAL_RNAi, n=50). We also used qRT-PCR to determine FER transcript levels in both mutant backgrounds, but here detected a slight upregulation of FER transcripts (Fig. 6G,H; *P<0.05, t-test). Despite this slight variation, overall, our data are consistent with the absence of the classical fer' pollen tube overgrowth' phenotype in our mutant lines.

The only mutant described so far as having a phenotype similar to vdd-1/+ and VAL_RNAi is GAMETOPHICTIC FACTOR 2 gfa2/+ (Christensen et al., 2002). We therefore crossed vdd-1/+ and ‘class iii’ VAL_RNAi plants with the pGFA2::GUS line and again analyzed the F2 generation. The promoter of GFA2 is active in all flower organs including, mature ovules (Fig. 6D) (Christensen et al., 2002).

In the vdd-1/+ background, GUS was detectable in the ovule (as in wt); however, low levels of GUS activity were detected in VAL_RNAi lines, indicating a strong reduction in pGFA2 promoter activity. Interestingly, GUS signal was not seen in mature ovules, sporophytic and gametophytic tissues, suggesting that GFA2 (Fig. 6F) is also a target of VAL in sporophytic tissues (n=15 plants, 400 ovules). To confirm the downregulation of GFA2 expression in vdd-1/+ heterozygote plants, we used GFA2-specific primers to perform qRT-PCR on vdd-1/+ mature carpels, comparing the data with results from wt and VAL_RNAi (class iii). As shown in Fig. 6G,H, GFA2 expression is slightly downregulated in vdd-1/+ lines, but still significant (t-test, P<0.01), greatly repressed in VAL_RNAi (t-test, P<0.001) compared with wt pistils, implying that GFA2 lies downstream of the VDD-VAL complex, an interpretation that is consistent with the gfa2/+ phenotype.

VDD is expressed throughout the embryo sac and it is unclear whether the vdd-1/+ phenotype is focused solely on the synergid cell, or if other cells in the vdd-1/+ embryo sac are involved. Furthermore, we wished to explore the possibility that a reduction in
GFA2 expression resulted in the vdd phenotype. Since MYB98 is active in the mutant background, we used its promoter to drive expression of both VDD and GFA2 coding sequences. Strikingly, as shown in Fig. 6I the pMYB98::VDD transgene was able to almost fully complement the vdd-1/+ phenotype, reducing unfertilized ovules from 28% to 7% (t-test, P<0.0001, n=10 plants; Fig. 6I). With respect to the role of GFA2, the pMYB98::GFA2 transgene expressed in a vdd-1/+ background partially restored the wt phenotype, reducing the number of unfertilized ovules from 28% to 16% (t-test, P<0.04, n=8 plants). In both circumstances, the percentage of seed abortion was maintained (Fig. 6I). These data demonstrate that unfertilized ovules in vdd-1/+ lines result from defects in the receptive synergid, and that GFA2 lies downstream of VDD in the pathway regulating these events.
DISCUSSION

Synergid cells are essential for successful fertilization: they are responsible for the attraction, reception and the arrest of the pollen tube, and for preventing entry of other pollen tubes (Beale and Johnson, 2013). We present here structural and molecular data demonstrating that the receptive synergid is also responsible for inducing the bursting of the pollen tube. As proposed by Sandaklie-Nikolova et al. (2007), Ngo et al. (2014) and later, Leydon et al. (2015), our results confirm that cell death of the receptive synergid is coordinated with that of the pollen tube. Furthermore, we show that VDD and VAL, two REM transcription factors and direct targets of the MADS domain complex – STK and SEP3—are able to interact in vivo, indicating that cell death in the receptive syngamy requires a complex containing both VDD and VAL, possibly as a heterodimer. Independent disruptions of both VDD (vdd-1/+) and VAL (VAL_RNAi) exhibit a strong female gametophytic defect, stemming from the absence of the degeneration of the receptive synergid and pollen tube. Importantly, our data also strongly suggest that VDD-VAL complex-mediated synergid cell death is required for bursting of the pollen tube.

The VDD-VAL dimer clearly does not affect attraction and perception of the pollen tube because our data confirm not only that expression of synergid-expressed genes known to be involved in these events (e.g. MYB98) are unaffected in our mutant/RNAi lines, but these plants also fail to display the pollen tube overgrowth phenotype characteristic of feronia lines, with FER expression remaining unaffected. However, elements of the FER signaling pathway are certainly involved in modulating and coupling synergid-generated calcium signals. Ngo et al. (2014) have demonstrated that FER collaborates with the GPI-anchored LRE (LORELEI) at the synergid cell surface to receive signals from the pollen during its phase of slow growth at the embryo sac entrance and triggers a synergid calcium signaling cascade, resulting in the coordinated death of the pollen tube and the receptive synergid. To determine the position of the VDD-VAL complex in this signaling pathway it is thus important to determine whether calcium signals are generated in vdd-1/+ and VAL_RNAi lines.

Other mutants known to have an overgrowing pollen tube phenotype such as bre show increased synergid degeneration 48 h after fertilization, suggesting that physical contact with the pollen tube may trigger synergid cell death (Leydon et al., 2015). In our mutants, even 48 h after fertilization, we found both ovules with intact synergids (vdd gametophyte) and ovules containing a developing endosperm (VDD gametophyte), which demonstrates that even if the pollen tube remains in contact with the synergid for a long period, cell death does not occur. This lack of a synergid response suggests that factors other than physical contact may be required for synergid activation. These may include additional ligand-receptor interactions between the pollen tube and synergid, or hormonal pathways within the synergid, involving enzymes such as cytokinin oxidase/dehydrogenase 7 (CKX7), which is responsible for cytokinin degradation and is strikingly downregulated in vdd-1/+ and VAL_RNAi synergids. TEM imaging further supports this persistence of the two synergids, because two perfect synergid cells could be visualized 48 h after pollination, one seemingly in contact a with pollen tube. A low level of ‘polytubey’ (7% of unfertilized ovules) occurs in VAL_RNAi lines, which has also been proposed as evidence of synergid integrity (Beale et al., 2012).

The mitochondrial chaperone GFA2 (Christensen et al., 2002) is required for synergid degeneration. Leydon et al. (2015) recently showed that pLAT52::GUS pollen tubes failed to burst in the gfa2/+ mutant, a phenotype identical to that of our mutant/RNAi lines. Together with our data showing that GFA2 is downregulated in VAL_RNAi and vdd/+ plants, this result reinforces the view that GFA2 lies downstream of VDD and VAL. Further evidence for GFA2 being both essential for synergid cell death, and regulated by VDD (and probably VAL) is provided by our data showing that a construct containing the GFA2 coding region under the control of the MYB98 promoter partially complements the vdd-1/+ mutant phenotype. The data also demonstrate that the vdd-1/+ ‘unfertilized’ ovules phenotype can be complemented using the VDD coding sequence under the control of MYB98 promoter, which is only active in the synergid cells before fertilization, demonstrating that the vdd-1/+ phenotype is synergid specific. In the future, it will be interesting to investigate whether MYB98 is involved in directly or indirectly modulating VDD expression.

Unequivocal evidence is now accumulating that receptive synergid cell death results from a complex exchange of signals between the pollen tube and the synergid. We show here that the receptive synergid controls both pollen tube bursting and its own degeneration and that the VDD-VAL dimer constitutes an essential component of the signaling pathway regulating these events. Based on our findings and those of others, we propose a model (Fig. 7) to explain this crosstalk between female and male gametophytes. The model reflects the independence of diverse pathways and comprises

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**Fig. 7. Model for the control of pollen tube attraction, reception (arrest) and death by the receptive synergid in Arabidopsis thaliana.** The ‘pollen tube attraction module’ constitutes the LUREs produced by the synergids that attract the pollen tubes; the transcription factor MYB98 appears to control pollen tube guidance during this event. The ‘pollen tube reception and pollen tube arrest module’ involves expression of FER, NTA and LRE in the synergid cells, required for pollen tube arrest. The ‘cell death module’ comprises the STK-SEP3 MADS domain complex that directly regulates the expression of VAL and VDD, which encode the VAL-VDD dimer that controls expression of the mitochondrial chaperone GFA2, which is, in turn, required for synergid cell death and consequently for pollen tube cell death (burst). Ca2+ signaling is also involved in the process.
three interacting modules, regulating pollen tube attraction (MYB98, LUREs), pollen tube reception (FER, LRL, NTA) and pollen tube/synergid cell death (VDD, VAL).

Interestingly, in the stk, shp1, shp2 triple mutant with one of the alleles heterozygous (e.g. stk/STK, shp1, shp2) the gametophytic defects as described in this study were never observed. Still, we show here convincing evidence that these MADS domain factors are necessary for VDD and VAL expression in the female gametophyte and for proper synergid function. The most likely explanation is that accumulation of the MADS domain factors in the sporophyte during early stages of ovule development are still available later in the gametophyte cells that derive from the sporophyte to control VDD and VAL expression.

MATERIALS AND METHODS

Plant material and growth conditions

All plants (Arabidopsis thaliana, ecotype Columbia-0, mutants and embryo sac marker lines) were grown at 22°C, under short day (8 h light:16 h dark) or long day (16 h light: 8 h dark) conditions. The Arabidopsis stk and stk, shp1, shp2 mutants were provided by M. Yanofsky (Pinyopich et al., 2003). The transgenic lines: pGFA2::GUS and pMYB98::GFP were provided by Prof. Gary Drews (Kasahara et al., 2005; Christensen et al., 2002); HTR10_RFP was provided by Frederic Berger (Aw et al., 2010); FER::GFP by Prof. Ueli Grossniklaus and LAT52::GUS by Prof. Ravi Palanivelu.

Statistical analysis

Values for Pearson correlation were calculated as described (Toufighi et al., 2005) for the ‘Expression Angler’. For this purpose, a Visual C++ based program was developed (P.M. and L. Mizzi, unpublished) and the correlation values were calculated from data acquired using the ATH1 GeneChip from Affymetrix deposited at the NASC array database (http://affy.arabidopsis.info/link_to_i_plant.shtml). Pearson coefficient calculations were log transformed before calculating the correlation value. This method is explained in Menges et al. (2008) in the section entitled ‘Global expression correlation analysis’.

Table 1. Number and percentage of crosses showing pollen tube burst and sperm cell delivery in vdd_1/+ and VALRNAi lines

<table>
<thead>
<tr>
<th>Cross (female×male)</th>
<th>PT burst</th>
<th>No PT burst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Percentage</td>
</tr>
<tr>
<td>wt×pLAT52::GUS</td>
<td>294</td>
<td>98%</td>
</tr>
<tr>
<td>vdd_1/+×pLAT52::GUS</td>
<td>213</td>
<td>71%</td>
</tr>
<tr>
<td>VAL RNAi×pLAT52::GUS</td>
<td>195</td>
<td>65%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sperm cells in embryo sac</th>
<th>Sperm cells in micropyle</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt×HTR10_RFP</td>
<td>288</td>
</tr>
<tr>
<td>vdd_1/+×HTR10_RFP</td>
<td>350</td>
</tr>
<tr>
<td>VAL RNAi×HTR10_RFP</td>
<td>118</td>
</tr>
</tbody>
</table>

Table 2. Number and percentage of the different crosses with and without GFP/GUS signal in the ovules

<table>
<thead>
<tr>
<th>F2 generation</th>
<th>Signal in the ovules</th>
<th>No signal in the ovules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Percentage</td>
</tr>
<tr>
<td>wt×pMYB98::GFP</td>
<td>294</td>
<td>98%</td>
</tr>
<tr>
<td>vdd_1/+×pMYB98::GFP</td>
<td>1459</td>
<td>97%</td>
</tr>
<tr>
<td>VAL RNAi×pMYB98::GFP</td>
<td>784</td>
<td>98%</td>
</tr>
<tr>
<td>wt×pGFA2::GUS</td>
<td>200</td>
<td>100%</td>
</tr>
<tr>
<td>vdd_1/+×pGFA2::GUS</td>
<td>182</td>
<td>72%</td>
</tr>
<tr>
<td>vdd_1/+×pCKX7::GUS</td>
<td>432</td>
<td>72%</td>
</tr>
<tr>
<td>VAL RNAi×pCKX7::GUS</td>
<td>310</td>
<td>62%</td>
</tr>
</tbody>
</table>

Chi² and quantitative real-time PCR analysis

In the VAL genomic region we found two putative CARG boxes, allowing one mismatch. The first (5′-CTATTAATGG-3′) is located 100 bp before the translation starting site, and the second (5′-CTATTTTGGG-3′), 80 bp after. Primers were designed to test enrichment on these CARG boxes. Primer sequences for first CARG box were: 5′-GGGCCCTTAAGCATACTTG-3′ and 5′-GTATTGTATCTAAGGTTTGCC-3′; and for the second CARG box: 5′-GACACAAAGGATTTTACTCCTG-3′ and 5′-CCAGATCACCAGGATCTACAGG-3′. Real-time PCR assays were performed in order to determine the enrichment of the fragments. The detection was performed in triplicate using the SYBR Green assay (Bio-Rad) and a Bio-Rad C1000 thermal cycler optical system. Chi²-qPCR experiments and relative enrichments were calculated as reported before (Matias-Hernandez et al., 2010).

Generation of VAL RNAi line

To make the p35::VAL RNAi construct, we used the Arabidopsis vector pFGCS941 for dsRNA production obtained from ABRC (stock no. CD3-447). For VAL a 247 bp fragment of VAL cDNA (position 364-611) was amplified by PCR using primers: AtP_2757 (5′-GGGGAAGGATTTTGATCAAAAACGCAGGTATCTCCTGGAACAAATCGGAT-3′) and AtP_2758 (5′-GGGGAACACATTGTTACAAAGAAGCTGGTGATCCATACGCGGATCTC-3′). The fragments were amplified using Phusion High-Fidelity DNA Polymerase (New England BioLabs) and purified using the GeneJET Gel Extraction Kit (Thermo Scientific). The amplified fragments were then cloned into vector pDONR207 (Invitrogen) and subsequently in pFGCS941, using the Gateway system (Invitrogen). Next, Agrobacterium-mediated transformation of Arabidopsis plants was performed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected with 10 ng/ml BASTA herbicide.

Cytological assays

The following gametophytic cell reporter lines were used: for the egg cell, the ECI gene promoter (Sprunk et al., 2012); for synergid cells, the ET2634 marker line (Groß-Hardt et al., 2007) and pMYB98::MYB89-GFP (Kasahara et al., 2005); for the central cell, the promoter of At1g02580 (Chaudhury et al., 1997) and for antipodal cells, the At1g36340 promoter (Yu et al., 2005). All marker lines, except pMYB98::MYB89-GFP, contained GUS as reporter gene.

The CKX7 promoter reporter line fragment primer sequence was used primers 5′-AGTGAGGCAGCGCCCTTTTCTACGGAAACACACAAATTTTT-3′ and 5′-AGTGGATTAAATGTGTGATGTGGTAAAGTCTAACT-3′ and cloned into the pGEM-T vector (Promega). The promoter was excised using Ascl/Pvla and ligated into the binary vector pGoldBar-EC1::NLS_GUS (Völz et al., 2011) in place of the ECI promoter.

The reporter lines were then used as female parental and hand-pollinated with VAL RNAi or vdd-1/+ pollen. Heterozygous plants, from the F1 generation, were self-fertilized and the presence/absence of the VAL RNAi and vdd-1 mutations in the F2 generation were examined by PCR. GUS staining was performed in order to detect the presence of the marker and the mutant expression profile was always used to confirm the correct expression profile. GUS staining on petals of emasculated flowers, was performed as described in Liljegren et al. (2000). After staining, the samples were incubated in a clearing solution containing chloral hydrate:glycerol:water in 8:1:2 proportion; then the petals were dissected and observed using a Zeiss Axioshot D1 microscope equipped with differential interference contrast (DIC) optics. Flowers of VAL RNAi plants were collected at different developmental stages. For ovule development analysis, they were cleared, dissected and analyzed as described previously (Matias-Hernandez et al., 2010). Images were captured with an AxioCam MRc5 camera (Zeiss) using Axiosview software (v.4.1).

In situ hybridization analysis

Arabidopsis flowers were collected at different stages, fixed and embedded in paraffin as described by Huisjes et al. (1992). Plant tissue sections were probed with a 317 bp digoxigenin-labeled VAL antisense RNA probe amplified using primers atp_2759 (5′-ACATCTGGAAAAACTTGGATC-3′) and atp_2760 (5′-GATCATCACCAGGATCTACAG-3′). Hybridization and immunological detection were executed as described previously by...
Coen et al. (1990). Plant tissue sections were also hybridized with VAL digoxigenin-labelled sense probe as a negative control.

**Yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BIFC)**

Full-length VAL and VDD CDS were amplified from Arabidopsis cDNA. Primers including Gateway attB1 and attB2 sites were used to amplify the fragments. Primers, 5′-GGGGAACATGGTGTACAAAAGCCAGCTCGATGACACAAAGTTTCAAATTCTTATTCTTTCCGAC-3′ were used for VDD and 5′-GGGGAACATGGTGTACAAAAGCCAGCTCGATGACACAAAGTTTCAAATTCTTATTCTTTCCGAC-3′ were used for VDD and 5′-GGGGAACATGGTGTACAAAAGCCAGCTCGATGACACAAAGTTTCAAATTCTTATTCTTTCCGAC-3′ were used for VAL and 5′-GGGGAACATGGTGTACAAAAGCCAGCTCGATGACACAAAGTTTCAAATTCTTATTCTTTCCGAC-3′ were used for BIFC through Gateway recombination (Invitrogen). BIFC was executed as described by Belda-Palazón et al. (2012).

For Y2H assays each bait/prey pair was transformed into the yeast strain α-his3, MATa, and each prey with the empty BD vector. The pair (bait/prey) colonies were assessed by genotyping. Presence of unfertilized ovules was assessed under the stereomicroscope.

**Confocal and TEM analysis**

For confocal laser scanning and transmission electron microscopy (TEM), flowers were emasculated and hand pollinated with wt pollen. For confocal laser scanning, 24–48 h after pollination, pistils were fixed as described by Braselton et al. (1996). Samples were then excited using a laser (532 nm) and emission was detected between 570 and 740 nm. A Leica SP5 laser scanning, 24–48 h after pollination, pistils were fixed as described by Sandaklie-Nikolova et al. (2007). H.D. designed the TEM experiment and contributed to writing the manuscript.

**Expression analysis by quantitative real-time RT-PCR**

Quantitative real-time RT-PCR experiments were performed using cDNA obtained from inflorescences. Total RNA was extracted using the Qiagen RNA extraction Kit. Ambion TURBO DNA-free DNase kit was used to eliminate genomic DNA contamination according to the manufacturer’s instructions (http://www.ambion.com/). The lmProm-ITM reverse transcription system (Promega) was used to retro-transcribe the treated RNA. Transcripts were detected using a Sybr Green Assay (iQ SYBR Green Supermix; Bio-Rad) using UBIQUITIN10 (AT4G05320) and ACTIN8 (AT1G49240) as a reference genes. Assays were done in triplicate using a Bio-Rad iCycler iQ Optical System (software v3.0a). The fold changes were calculated by normalizing the amount of mRNA against housekeeping gene fragments.

Diluted aliquots of the reverse-transcribed cDNAs were used as templates in quantitative PCR reactions containing the iQ SYBR Green Supermix (Bio-Rad). The expression of different genes was analyzed, using the following primers: VDD (RT_795 5′-GGGAAGGTGTCATGCGAGTTA-3′; RT_796 5′-CCATCTGTGGTCGATATGTTG-3′), VAL (RT_1019 5′-GAAAAGCGGTATCTGGATGTA-3′; RT_1020 5′-CCTTTGCAAAGATGGCAACCAACA-3′), GFA2 (RT_853 5′-CCCTCTGGTGTTCAGCATTG-3′; RT_854 5′-GGATCTGTGGTGTTCACATTG-3′), VDD (RT_147 5′-CTTCTGGCGAATGCAATTTT-3′; RT_148 5′-CGAGAAGGTCGATGACGACA-3′), and Act8 (RT_861 5′-CAGAGTTATTTGCGAGATTG-3′; RT_862 5′-CTGGAACTGTCCATCTAATG-3′).

**Complementation construct**

To complement the vdd1/+ mutant, we generated a modified version of pBGW7 (Karimi et al., 2002): the 35S promoter was substituted by a 1794 bp fragment of the MYB98 gene promoter (pMYB98 forward primer: 5′-CGGAGATACTGCTGAGAAGT-3′; pMYB98 reverse primer: 5′-GTCCTTTAGCAGTGAAGATG-3′).

VDD and GFA2 coding sequences were amplified with primers including attB1 and attB2 adaptor sites for cloning into pDONR207, and then recombined into pMYB98-pBGW7 which contains attR1 and attR2 Gateway sites. The VDD coding sequence was amplified with primers AtP_3785 (5′-GGGGAACATGGTGTACAAAAGCCAGCTCGATGACACAAAGTTTCAAATTCTTATTCTTTCCGAC-3′) and AtP_3786 (5′-GGGGAACATGGTGTACAAAAGCCAGCTCGATGACACAAAGTTTCAAATTCTTATTCTTTCCGAC-3′) to construct the plasmid pCKX7::GUS marker complementation constructs. N.B. and R.G.H. executed the pCKX7::GUS marker line. H.D. designed the TEM experiment and contributed to writing the manuscript. L.C. designed the experiments and wrote the manuscript.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

M.A.M. designed and performed most of the experiments and wrote the manuscript. R.G.H. designed and employed the RNA interference construct and performed the ChIP experiment. B.C. performed statistical analyses. Y.S.V. performed TEM imaging. P.M. performed the Pearson coefficient analysis. S.M. made the complementation constructs. N.B. and R.G.H. executed the pGEX::GUS marker line. H.D. designed the TEM experiment and contributed to writing the manuscript. L.C. designed the experiments and wrote the manuscript.

**Funding**

M.A.M. was supported by fellowships from the Portuguese National Funding Agency for Science, Research and Technology (Fundaçao para a Ciência e a Tecnologia [SFRH/BDP/99936/2014]), by the European Union Initial Training Network (ITN) SYSFLO 2010–2013, and by a grant from the European Commission, Marie Curie Action: Networks for Initial Training (SYSFLO - Training in Systems Biology Applied to Flowering [FP7-PEOPLE-ITN-2008]). L.C. was supported by Ministero dell’Istruzione, dell’Università e della Ricerca MIUR-PRIN 2012.

**Data availability**

Correlation analysis data for STK and VDD are available on Figshare at: https://dx.doi.org/10.6084/m9.figshare.3464054.v2 (Table S1) and https://dx.doi.org/10.6084/m9.figshare.3464057.v2 (Table S2).

**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.134916.supplemental

**References**


