



JAGGER localization and function are dependent on GPI anchor addition

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Abstract

Key message GPI anchor addition is important for JAGGER localization and in vivo function. Loss of correct GPI anchor addition in JAGGER, negatively affects its localization and function.

Abstract In flowering plants, successful double fertilization requires the correct delivery of two sperm cells to the female gametophyte inside the ovule. The delivery of a single pair of sperm cells is achieved by the entrance of a single pollen tube into one female gametophyte. To prevent polyspermy, *Arabidopsis* ovules avoid the attraction of multiple pollen tubes to one ovule–polytubey block. In *Arabidopsis jagger* mutants, a significant number of ovules attract more than one pollen tube to an ovule due to an impairment in synergid degeneration. JAGGER encodes a putative arabinogalactan protein which is predicted to be anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Here, we show that JAGGER fused to citrine yellow fluorescent protein (JAGGER-cYFP) is functional and localizes mostly to the periphery of ovule integuments and transmitting tract cells. We further investigated the importance of GPI-anchor addition domains for JAGGER localization and function. Different JAGGER proteins with deletions in predicted ω -site regions and GPI attachment signal domain, expected to compromise the addition of the GPI anchor, led to disruption of JAGGER localization in the cell periphery. All JAGGER proteins with disrupted localization were also not able to rescue the polytubey phenotype, pointing to the importance of GPI-anchor addition to in vivo function of the JAGGER protein.

Keywords Arabinogalactan proteins (AGPs) · GPI-anchored proteins (GPI-APs) · *Arabidopsis* · Pollen–pistil interactions · Polytubey

Introduction

Plant reproduction involves a series of cell–cell interactions between the male gametophyte and the female gametophyte and between the male gametophyte and the female sporophyte (Johnson et al. 2019; Lopes et al. 2019). In *Arabidopsis*, the embryo sac (female gametophyte) is surrounded by the ovule maternal tissues, and in addition to the two female gametes (the egg cell and the central cell), it contains five accessory cells: two synergid cells and three antipodal cells (Yadegari and Drews 2004). After the pollen lands on the stigma, it produces a tube, carries the two sperm cells at its tip, and grows past the stigma, style, transmitting tract, before emerging in the ovary. The tube continues to grow in the ovary and when it is attracted to the ovule, it gains access to the embryo sac through one of the synergid cells, which facilitates the double fertilization of the egg cell and the central cell by each of the two sperm cells carried by the pollen

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tube, giving rise to the embryo and the seed endosperm, respectively (Russell 1992).

A functional female gametophyte is essential for the attraction of the pollen tube to the ovule (Shimizu and Okada 2000; Johnson et al. 2019; Lopes et al. 2019). In *in vitro* experiments with *Torenia fournieri*, in which the micropylar end of the female gametophyte protrudes outside the ovule integuments, the pollen tubes were attracted to LURE-type CRP attractant peptides from the synergids (Higashiyama et al. 1998; Zhong et al. 2019; Takeuchi 2021). The two synergid cells located at the micropylar end of the ovule share a specialized secretory structure called the filiform apparatus, which is rich in cell wall, plasma membrane, and secretory vesicles (Huang and Russell 1992). The highly invaginated filiform apparatus vastly increases the plasma membrane surface area that could come in quick and easy contact with the arriving pollen tube and promotes the secretion of small peptides and glycosides involved in the pollen tube attraction, reception, and subsequent release of the sperm cells for double fertilization (Li et al. 2018; Pereira and Coimbra 2019).

The first pollen tube receptor discovered in the synergid filiform apparatus was the receptor-like protein kinase FERONIA/SIRENE (FER/SRN). Unlike in wild type, the *fer/srn* mutation in ovule results in incessant growth of the pollen tube within the embryo sac, and consequently male gametes end up not being released from the pollen tube (Escobar-Restrepo et al. 2007). LORELEI (LRE) is a putative glycosylphosphatidylinositol (GPI)-anchored protein (GPI-AP) that is primarily expressed in the synergids and the *lre* mutant ovule phenocopy all the aberrant behaviours of *fer/srn* mutant ovule (Capron et al. 2008). LRE and FER are believed to be co-receptors and work together at the interface between pollen tube and synergid cells to regulate the rupture of the pollen tube in the synergid cells (Liu et al. 2016). Arabinogalactan proteins (AGPs) are highly glycosylated proteins and are likely involved in several steps of the reproductive process. The AGPs were found to be attractants and/or signalling molecules for pollen tube guidance in *Nicotiana tabacum*, as it displayed a gradient of increasing glycosylation in the extracellular matrix of the transmitting tract, from the stigma to the style, and enabled pollen tube growth and guidance (Cheung et al. 1995; Wu et al. 1995). As evidenced by immunolocalization studies in ovules of different species, AGPs are present in the sporophytic integument cells and in the synergid cells (Costa et al. 2013a, b; Coimbra et al. 2007; Coimbra and Salema 1997; Lopes et al. 2016; Pereira et al. 2016b, 2016a; Hou et al. 2016; Lara-Mondragón and MacAlister 2021). Remarkably, in *T. fournieri*, an AGP-derived disaccharide (AMOR) produced by the ovule sporophytic tissues induced competency of the pollen tube to respond to the LURE-type CRP attractant peptides from the synergids (Mizukami et al. 2016). It has

been suggested that given the importance of Ca^{2+} dynamics for pollen tube growth and the ability of AGPs to bind Ca^{2+} in a pH-dependent manner, AGPs might influence pollen tube growth either by acting as a periplasmic Ca^{2+} reservoir or as a diffusible Ca^{2+} carrier (Lampert et al. 2021). In the ovary of *Arabidopsis*, a group of chimeric AGPs known as early nodulin-like proteins (ENODLs 11–15) are highly expressed in the embryo sac and their loss of function disrupts the bursting of the pollen tubes upon penetration (Hou et al. 2016). The influence of AGPs on pollen tube growth has also been observed in several species using the Yariv reagent that precipitates AGPs (Qin et al. 2007; Lara-Mondragón and MacAlister 2021). Another important AGP is AGP4 or JAGGER, which plays an important role in preventing polytubey (Pereira et al. 2016b). The failure to prevent polytubey in the *jagger* mutant could be due to the continued secretion of pollen tube attractants from the synergid cell that persists after fertilization instead of being rapidly inactivated. Indeed, the persistent synergid in *jagger* mutant remains aberrantly active after fertilization and does not undergo degeneration, resulting in enhanced frequency of polytubey (Pereira et al. 2016b).

Approximately half of the AGP family members in *Arabidopsis*, including JAGGER, are predicted to contain a GPI lipid anchor that directs the proteins to the outer leaflet of the plasma membrane, making them worthy candidates for signal perception and transduction (Seifert and Roberts 2007; Ellis et al. 2010). The functional properties of AGPs probably reside in their variable sugar chains (Moreira et al. 2023). These sugar chains may become easily accessible as signalling molecules through detachment of the AGPs from the cell wall and its secretion to the apoplast by the action of phospholipases (GPI-PLC) (Majewska-Sawka and Nothnagel 2000; Ellis et al. 2010). In eukaryotes, the newly synthesized proprotein chain of GPI-APs typically contain an N-terminal signal peptide (SP) for translocation into the endoplasmic reticulum (ER) during its synthesis, and an extended C-terminal GPI-attachment signal (GAS) region containing several discernible elements: a region of about 11 amino acid residues plus two segments that are critical for addition of the GPI anchor: (i) a cleavable hydrophobic C-terminal tail of 9–24 amino acid residues and (ii) a ω -site region containing four small residues, one of which will be attached to a GPI anchor. The ω -site region and the C-terminal tail are separated by a spacer region of about 6 amino acid residues (Eisenhaber et al. 2003).

To date, the structure has been resolved for only one plant GPI anchor, and it is that of *Pyrus communis* AGP1 (PcAGP1). It was shown to contain a glycan moiety, composed of three α -linked mannose residues and one glucosamine that is linked to the C-terminus of the protein by ethanolamine phosphate, and a lipid moiety of phosphoceramide, composed of phytosphingosine and

tetracosanoic acid and attached to the glycan core by myo-inositol (Oxley and Bacic 1999; Yeats et al. 2018; Desnoyer and Palanivelu 2020). The GPI anchor is synthesized in the ER and is then attached to the ω -site of GPI-APs by the GPI transamidase complex. After GPI anchor attachment to the ω -site of GPI-APs, the glycan and lipid moieties are remodelled. After leaving the ER, GPI-APs are transported by the secretory pathway, through the Golgi complex, to their final destination, the plasma membrane (Ellis et al. 2010; Yeats et al. 2018). Disrupting GPI anchor synthesis or attachment in *Arabidopsis* is often gametophytic or embryo lethal, as assessed by loss-of-function mutant studies of genes that code for specific subunits of the GPI transamidase complex (Bundy et al. 2016; Desnoyer et al. 2020). Indeed, many GPI-APs have important functions in plant reproduction such as AGP4 (JAGGER), AGP6, AGP11, and AGP18, FLA3, ENODL 11–15 and LRE that are important for *Arabidopsis* gametophyte development or fertility (Acosta-García and Vielle-Calzada 2004; Coimbra et al. 2009; Li et al. 2010; Hou et al. 2016; Liu et al. 2016; Pereira et al. 2016a). Other examples include LORELEI-LIKE GPI-anchored proteins 2 and 3, which are involved in maintaining cell wall integrity at the pollen tube tip during its growth through the style and transmitting tract (Ge et al. 2019; Feng et al. 2019), and COBRA-LIKE protein 10 (COBL10), which seems to be important for micropylar guidance of the pollen tube to the synergids (Li et al. 2013).

Despite these critical roles of specific GPI-APs in both gametophytes, the importance of subcellular localization of GPI-APs in the plasma membrane via a GPI anchor is not fully understood and remains to be shown that they are essential for their functions. Indeed, while mutations in the LRE GAS region or the loss of *trans*-acting GPI transamidase resulted in mutant LRE proteins or wild type LRE protein, respectively, not localized in the plasma membrane-rich filiform apparatus of synergid cells, they, however, did not affect LRE protein function in pollen tube reception (Liu et al. 2016). These surprising results showing that GPI anchoring is dispensable for LRE function in the female gametophyte could not still be reconciled with the evolutionary conservation of domains required for GPI anchor attachment in LRE proteins. So, in the present work, we investigated whether loss of GPI anchoring is dispensable in JAGGER, another GPI-AP. For this, we generated JAGGER protein with several deletions that are expected to compromise the addition of the GPI anchor, verified if JAGGER subcellular localization is disrupted in cells of ovules, and examined if these changes reversed the *jagger* mutant phenotype. Our work, besides describing the subcellular localization of an AGP *in planta*, demonstrated that GPI anchor is critical for *in vivo* function of JAGGER.

Material and methods

JAGGER sequence analysis

The *JAGGER-cYFP* DNA constructs were designed as described for LRE in Liu et al. (2016). JAGGER ω -sites were predicted by the BIG-PI plant predictor software (https://mendel.imp.ac.at/gpi/plant_server.html) (Eisenhaber et al. 2003).

Plant material

Arabidopsis thaliana seeds were surface sterilized and plated on half strength Murashige and Skoog medium, containing 2% (w/v) sucrose and the appropriate antibiotics. After stratification at 4 °C for 2–3 days, seeds were placed in a growth chamber and maintained at 20 °C in a 16 h/8 h photoperiod. Seven- to ten-day-old seedlings were transferred to soil and kept in the same growth conditions. The background ecotype of all *Arabidopsis* seeds was Columbia (Col-0). The *jagger1-2* (N412778) mutant line was previously characterized by Pereira et al. (2016b).

JAGGER—cYFP DNA constructs

The *cYFP* (E1403; Tian et al. 2003) reporter gene was fused to wild-type *JAGGER* in the C-terminus and expressed in transgenic *Arabidopsis* plants under the control of *JAGGER* promoter (2273 bp). *cYFP* was used as reporter because it is pH insensitive and capable of fluorescing in the acidic apoplast (Gjetting et al. 2012), which is the likely site of localization of GPI-APs (Schultz et al. 1998). *cYFP* has been used to determine plasma membrane localization of other *Arabidopsis* GPI-anchored surface proteins (Simpson et al. 2009; Liu et al. 2016). The inclusion of a complete GAS sequence downstream of the *cYFP* sequence is expected to make the fusion protein GPI-anchored. Flanking the *cYFP* reporter, two equal JAGGER ω -11 sequences were inserted. Upstream of the *cYFP* is the native ω -11 sequence and the second ω -11 domain was placed between the *cYFP* and the ω -site to prevent any potential hindrance to the GPI anchoring mechanism and the need for regulatory cues. Care was taken to retain the same amino acid sequence of the ω -11 sequence in JAGGER, yet the codons were altered to be consistent with the codon bias in *Arabidopsis*. The JAGGER-*cYFP* construct was structured in this manner, as cleaving the AGP for fusion in the ω -11 keeps the coding sequence unaltered and should not potentially impair the JAGGER and *cYFP* protein folding.

Three DNA fragments were prepared by PCR amplification as follows: (1) a genomic sequence encompassing

JAGGER regulatory region and CDS up to the codon immediately upstream of the predicted ω -site; (2) *cYFP* sequence preceded by a Gly⁹ linker sequence (Chen et al. 2013) corresponding to GRPGGGGA, which was amplified from plasmid DNA; and (3) a codon-optimized ω -11 sequence followed by the predicted ω -site and the 3'-terminal sequence corresponding to the C-terminal end region of *JAGGER*. The primers were designed to allow the fusion of the three fragments by overlap PCR (Table S1). The *JAGGER-cYFP* fusion under the control of the native promoter was cloned into a modified pH7WG (Karimi et al. 2002; Liu et al. 2016). This vector has inherited a unique *AscI* restriction site from *pENTR/D-TOPO* that was used together with *SpeI* restriction site to linearize the vector and clone the *proJAGGER:JAGGER-cYFP* construct using In-Fusion HD Cloning Plus (Clontech). For the mutant constructs used in this study, altered-sequence fragments were introduced by PCR using modified primers (Table S1) and *AscI/SpeI* linearized *pH7WG::ProJAGGER:JAGGER-cYFP* was used as template for in-fusion cloning.

Isolation of insertion lines

pH7WG::ProJAGGER:JAGGER-cYFP and the *ProJAGGER:JAGGER-cYFP* mutant constructs plasmids were transferred to *Agrobacterium tumefaciens* (GV3101 strain) and plants (wild-type and *jagger1-2* mutant line) were then transformed by the floral dip procedure (Clough and Bent 1998). Hygromycin-resistant transformants were selected and verified by PCR with primers flanking the *JAGGER-cYFP* coding sequence (primers described in Table S1) followed by Sanger sequencing of the resultant PCR products.

Confocal imaging

Fluorescent images were captured using a Leica TCS SP8 confocal laser scanning microscope (CLSM). For cYFP imaging, samples were excited with a 514 nm laser line, and emission spectra between 522 and 550 nm were collected. cYFP images were processed with ImageJ software (<http://imagej.nih.gov/ij/>). Pistils from flowers at stage 12 according to Smyth et al. (1990) were dissected under a stereomicroscope (Model C-DSD230, Nikon) using hypodermic needles (0.4 × 20 mm; Braun). The opened carpels and the ovules that remained attached to the septum were kept in water and covered with a coverslip.

Preparation of live plant material for microscopy

For crosses with dehiscent anthers, closed flower buds were emasculated 24–48 h before pollination, at stage 11–12, according to Smyth et al. (1990). Fresh pollen at flower stage

13 was profusely applied to the stigma of the emasculated pistils. Hand-pollinated pistils were collected 2 days after pollination and used for aniline blue staining.

Aniline blue pollen tube staining

Arabidopsis pistils were fixed overnight in 10% (v/v) acetic acid in ethanol at 4 °C, washed three times with water and softened with 8 M NaOH for 16 h, washed three times with water, and incubated overnight in 0.1% (w/v) decolorized aniline blue (DAB) at 4 °C (Kho and Baër 1968). Stained pistils were dissected under a stereomicroscope (Model C-DSD230, Nikon) using hypodermic needles (0.4 × 20 mm; Braun). The opened carpels and the ovules that remained attached to the septum were maintained in a drop of DAB and covered with a coverslip. Observations were made with a Leica DMLB/LB30T at 350–400 nm (UV), and polytubey was scored on a Nikon DS-Ri2 camera using the NIS-Elements BR programme (version 4.60.00).

Statistical analysis

Statistical tests on the data were performed using GraphPad software (www.graphpad.com).

Results

JAGGER-cYFP localizes in the cell periphery of transmitting tissue and ovule integument cells in *Arabidopsis* pistils

The *JAGGER* amino acid sequence contains putative features that define a GPI-AP: the N-terminal signal peptide (SP), the proline-rich yet a less complex ω -11 region, and two domains that are key to the addition of a GPI anchor: the ω -site region and the C-terminal hydrophobic tail (GAS sequence) (Fig. 1). The putative GAS sequence in *JAGGER* has the conserved features of animal GAS sequences (Schultz et al. 1998) and is predicted to span from residue Ala¹¹⁸ through to Leu¹³⁴. *JAGGER* Ser¹¹¹ was the best predicted ω -site residue by BIG-PI plant predictor software (Eisenhaber et al. 2003).

To study subcellular localization of *JAGGER* in the *Arabidopsis* pistil, and to test if the translational fusion constructs can complement the *jagger* mutant phenotype, we fused the citrine YFP (cYFP) reporter gene to the C-terminus of *JAGGER* coding sequence and expressed it from the *JAGGER* promoter. *JAGGER* protein and cYFP amino acid sequences were separated by a semi-flexible linker of 9 amino acids, adapted from the one used in LORELEI-cYFP (Liu et al. 2016) and included in all DNA constructs described in this work.

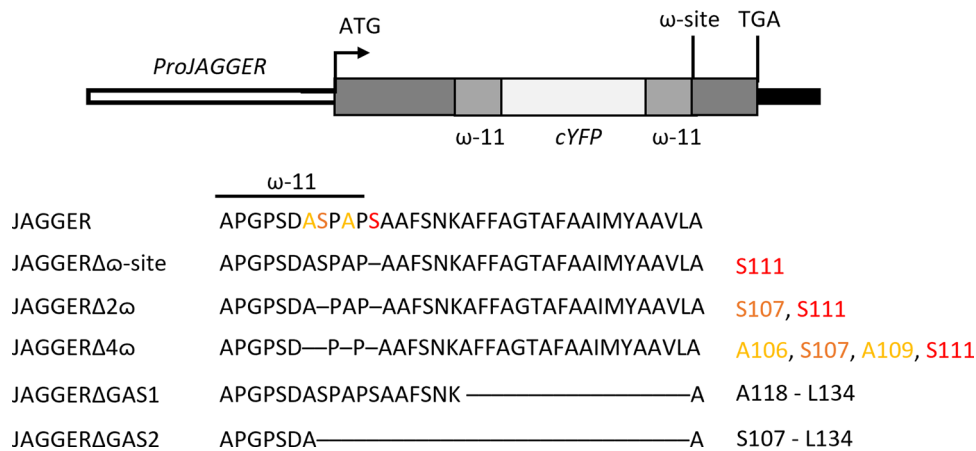


Fig. 1 Diagram of the JAGGER-cYFP protein. Wild-type or altered amino acid sequence on either side of the predicted ω -site amino acid are indicated below the diagram. In JAGGER, the best predicted ω -site and a cryptic ω -site are labelled in red and orange, respectively.

Two additional cryptic ω -sites are labelled yellow. Each dash in the protein sequence represents a deletion of the corresponding amino acid in wild-type JAGGER protein sequence. The corresponding amino acid is referred at right. (Diagram not drawn to scale.)

Confocal laser scanning microscopy (CLSM) images of unpollinated pistils carrying the wild-type JAGGER protein fused to cYFP showed that JAGGER-cYFP fusion proteins were expressed in the sporophytic ovule integument cells and in the transmitting tissue cells (Fig. 2). Ovule integument cells located more closely to the embryo sac region presented a higher level of fluorescence intensity compared to the chalaza region of the ovules (Fig. 2A–D). Also, in these cells, some cYFP fluorescence is detected in puncta in the cell's cytoplasm (Fig. 2E, F). Isolated transmitting tract cells revealed cYFP signal mostly at the cell periphery, with only some punctate fluorescence pattern detected in the cytoplasm (Fig. 2G–I), which did not overlap with the autofluorescent regions of the cell that likely represented the chloroplasts. In both cell types, cYFP signal was detected at the cell periphery, consistent with JAGGER being a putative GPI-AP.

Removal of the GPI anchor attachment signal sequence in JAGGER negatively affects JAGGER-cYFP localization in the cell periphery

The loss of the ω -site in JAGGER-cYFP could prevent it from receiving a GPI anchor and consequently disrupt its localization in the cell periphery. However, in ovule integuments and transmitting tissue cells in which Ser¹¹¹ of JAGGER was deleted (JAGGER-cYFP $\Delta\omega$), predicted by the BIG-PI plant predictor software to have the highest chance of functioning as an ω -site in JAGGER (Eisenhaber et al. 2003), we found that the cYFP fluorescence signal was indistinguishable from that in JAGGER-cYFP (Fig. 3A–D,E–H). To avoid redundant functions caused by cryptic ω -sites in the JAGGER ω -region, we conducted a new analysis using

the BIG-PI plant predictor software (Eisenhaber et al. 2003). This identified Ser¹⁰⁷ as an alternative ω -site to Ser¹¹¹ in JAGGER, when the JAGGER protein sequence lacking Ser¹¹¹ was used as an input at the BIG-PI plant predictor software. When both Ser¹¹¹ and Ser¹⁰⁷ were deleted, JAGGER-cYFP $\Delta 2\omega$ localization in the cell periphery was dramatically affected compared to wild-type JAGGER fused to cYFP (Fig. 3I–L). Concomitant with this decrease in cYFP signal in the cell periphery, there was a noticeable increase in the diffused cYFP signal in the internal regions of the cell (likely the cytoplasm) in both ovule integument cells and transmitting tissue cells of JAGGER-cYFP $\Delta 2\omega$ pistils (Fig. 3I–L). Further, if JAGGER protein sequence lacked both Ser¹¹¹ and Ser¹⁰⁷, the BIG-PI plant predictor software identified Ala¹⁰⁶ and Ala¹⁰⁹ as potential ω -sites. Removing all four predicted ω -sites, Ala¹⁰⁶, Ser¹⁰⁷, Ala¹⁰⁹ and Ser¹¹¹, in the JAGGER protein sequence and using it as an input in the BIG-PI plant predictor software returned no other cryptic ω -sites. The JAGGER-cYFP $\Delta 4\omega$ construct lacking Ala¹⁰⁶, Ser¹⁰⁷, Ala¹⁰⁹ and Ser¹¹¹, showed a subcellular localization similar to that of JAGGER-cYFP $\Delta 2\omega$, with fluorescence signal redistributed from the cell periphery to the internal regions of the cell, but with a strong punctate pattern, both in ovule integuments and transmitting tissue cells (Fig. 3M–P).

The removal of putative ω -sites affected JAGGER-cYFP localization in the cell periphery; hence, we hypothesized that deletion of the GAS signal sequence (Ala¹¹⁸–Leu¹³⁴) should also result in a non-anchored JAGGER-cYFP as predicted by Schultz et al. (1998) and as shown for LRE (Liu et al. 2016). Contrary to these expectations, JAGGER-cYFP Δ GAS^{A118–L134} showed a similar localization in the cell periphery as JAGGER-cYFP, indicating that the absence of

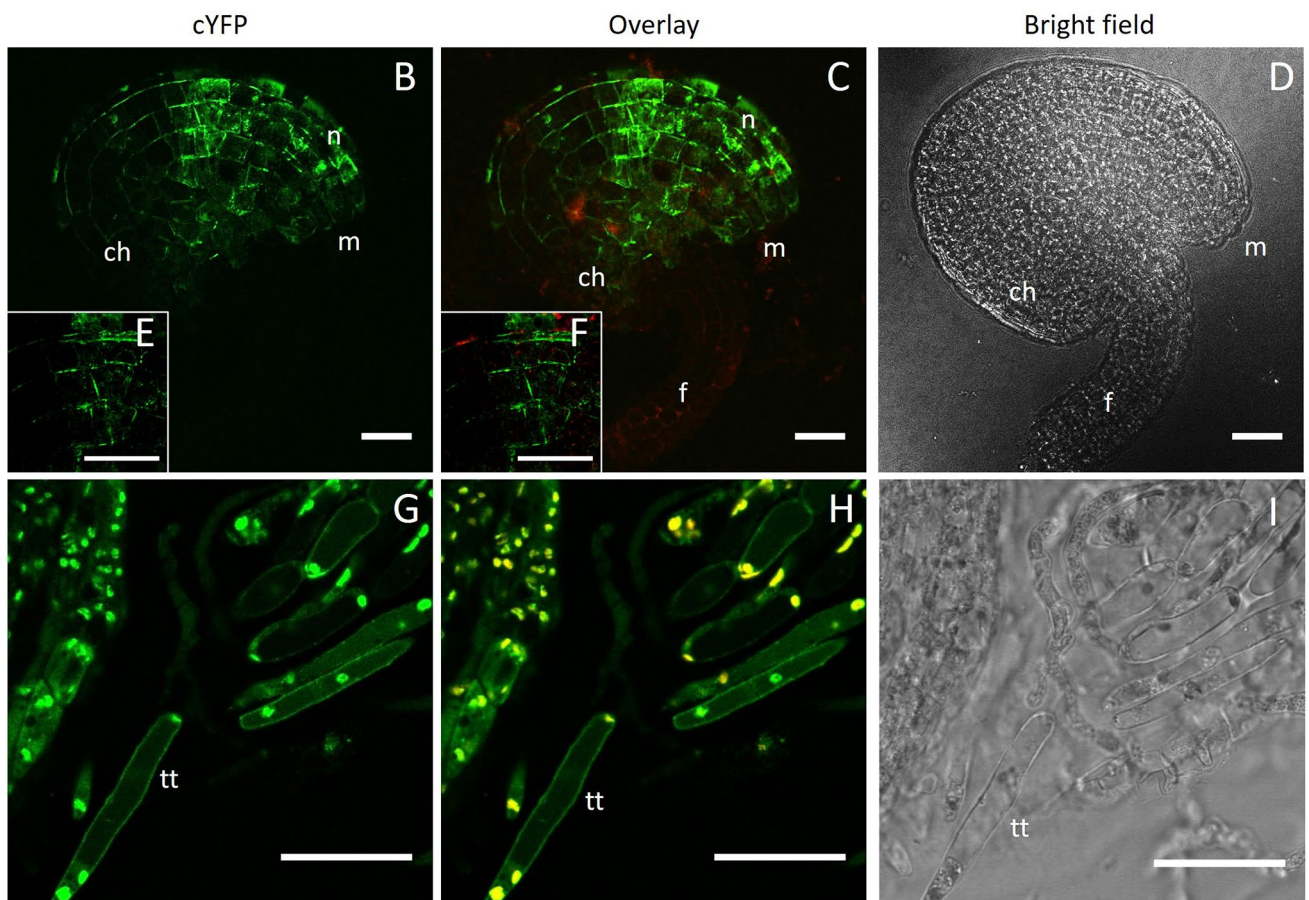
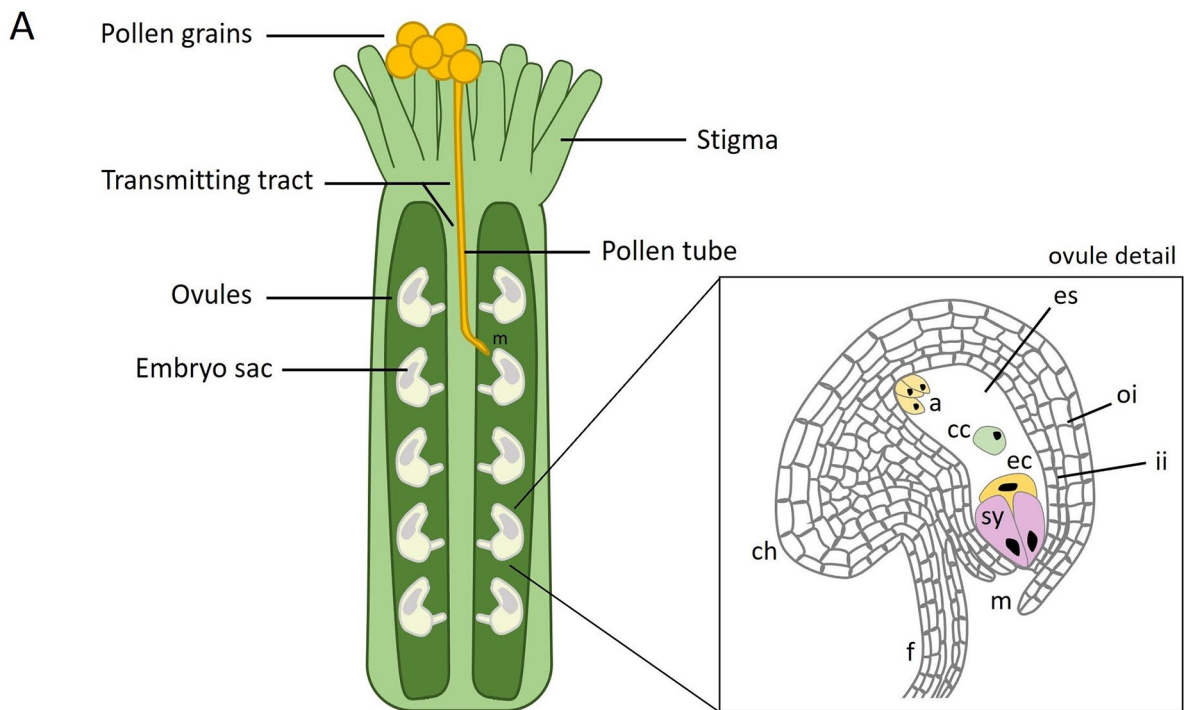


Fig. 2 Confocal laser-scanning images of JAGGER-cYFP localized at ovule outer integument and at the transmitting tissue cells. **A** Schematic representation of the pistil with pollen grains at the stigma surface. A germinated pollen grain is depicted with a pollen tube invading the pistil tissues and growing along the transmitting tract until it reaches an ovule. **B** JAGGER-cYFP localization at the ovule outer integument; **C** cYFP fluorescence image overlayed with chlorophyll autofluorescence; **D** transmitted light image (bright field) corresponding to A and B; **E** cYFP fluorescence of ovule outer integument detail; **F** cYFP fluorescence overlayed with chlorophyll autofluorescence of ovule outer integument detail; **G** JAGGER-cYFP localization at the transmitting tissue cells; **H** cYFP fluorescence image overlayed with chlorophyll autofluorescence; **I** transmitted light image (bright field) corresponding to F and G. ch, chalaza; es, embryo sac; f, funiculus; ii, inner integuments; m, micropyle; oi, outer integuments; tt, transmitting tissue cell. Scale bars: 25 μ m

this short C-terminal tail sequence was not sufficient to disrupt JAGGER protein sorting and localization (Fig. 4E–H). When removing a longer GAS sequence spanning from Ala¹⁰⁹ to Leu¹³⁴ we found that the localization of JAGGER-cYFP Δ GAS^{A109–L134} at the cell periphery was slightly lower than JAGGER-cYFP (Fig. 4I–L) and there was a noticeable increase in the diffuse cYFP signal in the internal regions of the cells in both ovule integuments and transmitting tissue. These results were similar to the ones obtained from JAGGER-cYFP Δ 2 ω and JAGGER-cYFP Δ 4 ω localization studies and are consistent with the model in which JAGGER-cYFP localization in the cell periphery of ovule integument cells and transmitting tissue cells requires a GPI anchor.

The GPI anchor attachment signal sequence in JAGGER is necessary for its function in polytubey prevention

To test if indeed the GPI anchor sequence is essential for JAGGER function in polytubey block, we counted and compared the number of polytubey occurrences in wild-type hand pollinated pistils with that in *jagger*^{-/-} pistils expressing JAGGER-cYFP and different GPI anchor mutants, namely JAGGER-cYFP Δ ω , JAGGER-cYFP Δ 2 ω , JAGGER-cYFP Δ 4 ω , and JAGGER-cYFP Δ 2GAS^{S107–L134} (Fig. 5). Consistent with what was reported previously (Pereira et al. 2016b), polytubey was observed in 1.14% \pm 1.7% of wild-type ovules ($n = 172$). This rate of polytubey was significantly lower than that was detected in *jagger*^{-/-} ovules (14.84% \pm 1.28%; $n = 207$). From the results shown in Fig. 5, we found that in the *jagger*^{-/-} pistils carrying constructs that did not alter JAGGER localization at the cell periphery (JAGGER-cYFP and JAGGER-cYFP Δ ω), there was a complete rescue of the polytubey phenotype, as the polytubey percentages in *jagger*^{-/-} JAGGER-cYFP (2.4% \pm 1.12% of ovules with polytubey occurrence, $n = 185$) and *jagger*^{-/-} JAGGER-cYFP Δ ω -site (1.87% \pm 1.9% of ovules with polytubey occurrence,

$n = 148$) were similar to that observed in wild type. On the contrary, the transgenic plants that showed dramatically altered cYFP localization (*jagger*^{-/-} plants carrying the constructs JAGGER-cYFP Δ 2 ω , JAGGER-cYFP Δ 4 ω , and the JAGGER-cYFP Δ GAS^{A109–L134}) did not fully rescue the polytubey phenotype, as the polytubey percentages in these lines were 8.44% \pm 3.09% ($n = 243$), 4.69% \pm 2.65% ($n = 229$), 11.45% \pm 2.47% ($n = 164$), respectively. Importantly, when we compared the test samples analysed with the *jagger*^{-/-} hand pollinated pistil, they were all found to have significantly different amounts of polytubey occurrences, while when we compared all the samples with wild-type hand pollinated pistils, only the *jagger*^{-/-} pistils carrying the constructs JAGGER-cYFP Δ 2 ω -site, JAGGER-cYFP Δ 4 ω -site and the JAGGER-cYFP Δ GAS^{A109–L134}, presented significant differences. These results showed that the reduction in cell periphery localization of JAGGER is positively correlated with reduction in JAGGER function in preventing polytubey and point to the importance of GPI anchoring in JAGGER function.

Discussion

JAGGER protein expression is consistent with the JAGGER promoter activity and its function in sporophytic tissues

JAGGER protein expression in the ovule integuments and transmitting tissue cells shown in this study is in the same region of the pistils as reported previously for JAGGER promoter fused to a reporter gene (Pereira et al. 2016b). Our protein expression data in pistils are also consistent with a sporophytic role of JAGGER in preventing polytubey (Pereira et al. 2016b). The expression results described in this study are important because the expression of JAGGER protein in reproductive tissues has not been reported before and until now transient expression experiments performed in *Nicotiana tabacum*, *Nicotiana benthamiana* (Martinière et al. 2012; Zavaliev et al. 2016) and *Arabidopsis thaliana* seedlings (Bernat-Silvestre et al. 2020, 2021) used GFP-AGP4 and mCitrine-AGP4 expressed from the constitutive 35S promoter. In this study, we examined the expression of the JAGGER protein in *Arabidopsis* pistil tissues with JAGGER-cYFP expressed from the JAGGER endogenous promoter.

JAGGER is a putative GPI-anchored membrane protein in Arabidopsis pistil tissues

In the present work, we showed that JAGGER is probably a GPI-anchored membrane protein. Until now, experimental evidence supporting JAGGER anchoring by a GPI has been

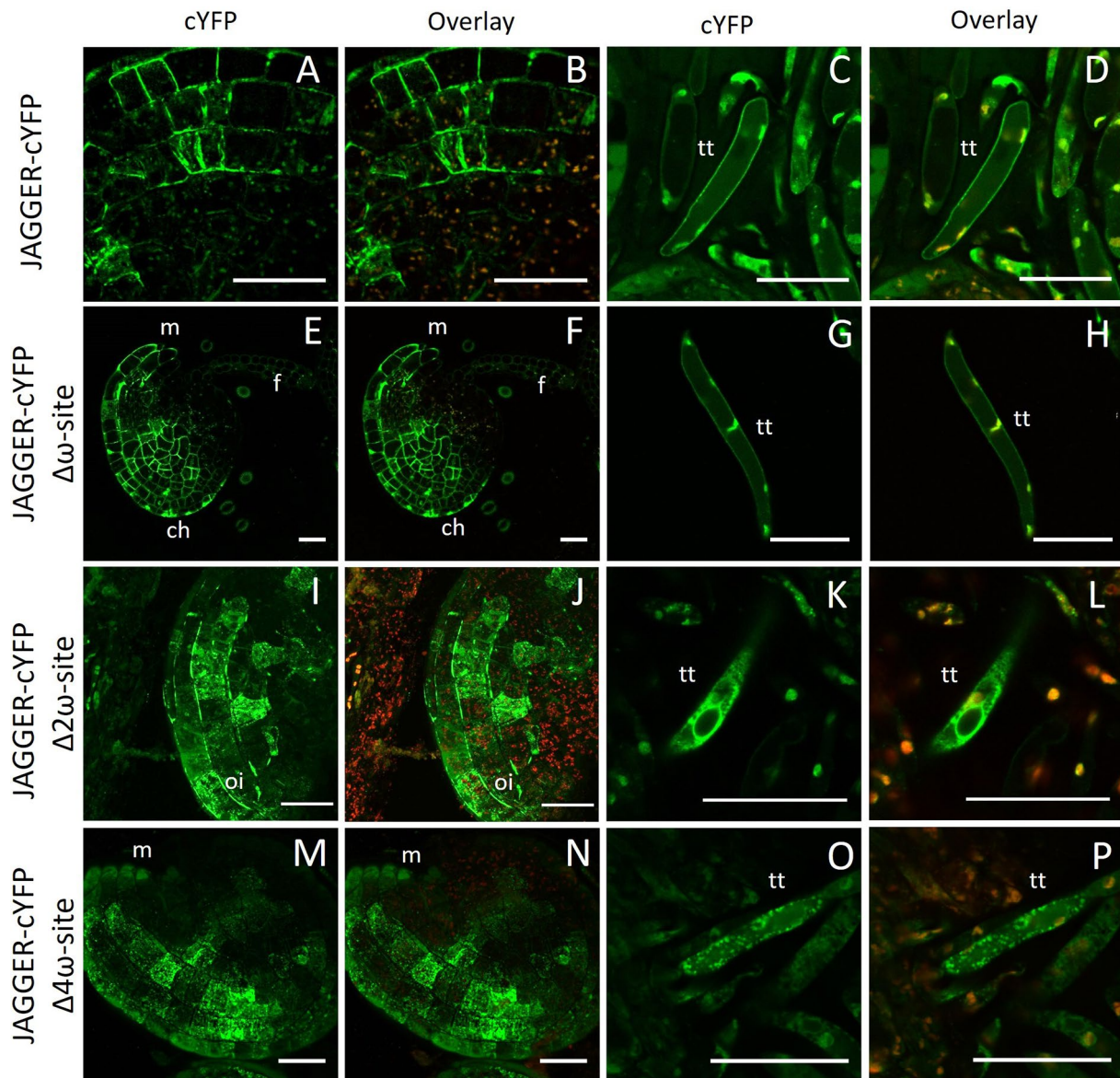


Fig. 3 Confocal laser-scanning images of JAGGER-cYFP and its mutated versions localization in ovule and transmitting tract cells. The predicted ω -site domain is not sufficient to disrupt JAGGER localization at the plasma membrane. **A–D** Localization of JAGGER-cYFP at the ovule outer integuments (**A, B**) and the transmitting tissue cells (**C, D**); **E–H** JAGGER-cYFP $\Delta\omega$ -site localization at the ovule outer integuments (**E, F**) and the transmitting tissue cells

(**G, H**); **I–L** JAGGER-cYFP $\Delta 2\omega$ -site localization at the ovule outer integuments (**I, J**) and the transmitting tissue cells (**K, L**); **M–P** JAGGER-cYFP $\Delta 4\omega$ -site localization at the ovule outer integuments (**M, N**) and the transmitting tissue cells (**O, P**). **A, C, E, G, I, K, M** and **O**, cYFP fluorescence. **B, D, F, H, J, L, N** and **P**, cYFP fluorescence overlaid with chlorophyll autofluorescence. **ch**, chalaza; **f**, funiculus; **m**, micropyle; **tt**, transmitting tissue cell. Scale bars: 25 μ m

reported in biochemical studies (Sherrier et al. 1999). Here, we performed complementation tests with mutant JAGGER proteins showing unexpected subcellular localization patterns. This is important because if it is shown that the biological function of JAGGER depends on its subcellular localization, then it would be consistent with the model that its function is dependent on the presence of a GPI anchor.

The confirmation that JAGGER is a GPI-AP in female tissues involved the preparation of JAGGER fusions with

reporter proteins. The wild-type JAGGER protein fusions allowed us to determine the tissue and subcellular localization of JAGGER in the cells of transmitting tract and ovule integuments. To study the importance of the GPI signal domains (present in the nascent protein) in the subcellular localization and function of JAGGER, we prepared constructs with mutations in the GPI signal domains. Subcellular localization using these proteins showed that JAGGER is a GPI-anchored protein and that its subcellular localization

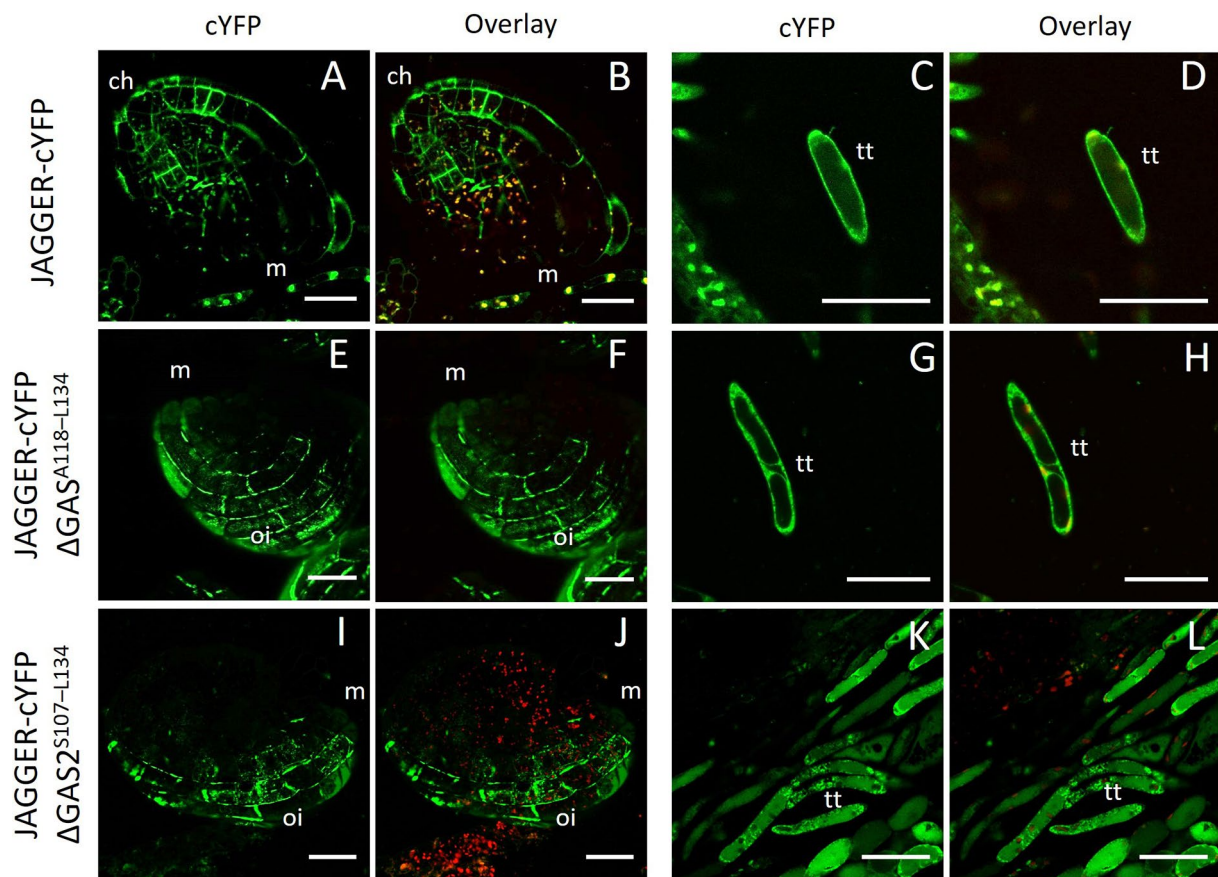


Fig. 4 Confocal laser-scanning images of JAGGER-cYFP and its mutated versions localization in ovule and transmitting tract cells. Predicted GAS A^{118-L134} domain is not sufficient to disrupt JAGGER localization at the cell periphery. **A–D** Localization of JAGGER-cYFP at the ovule outer integuments (**A, B**) and the transmitting tissue cells (**C, D**); **E–H** JAGGER-cYFP Δ GAS^{A118-L134} localization at the ovule outer integuments (**E, F**) and the transmitting tissue cells

(**G, H**); **I–L** JAGGER-cYFP Δ GAS^{S107-L134} localization at the ovule outer integuments (**I, J**) and the transmitting tissue cells (**K, L**). **A, C, E, G, I** and **K**, cYFP fluorescence. **B, D, F, H, J** and **L** cYFP fluorescence overlaid with chlorophyll autofluorescence. ch, chalaza; m, micropyle; oi, outer integuments; tt, transmitting tissue cell. Scale bar 25 μ m

in the cell periphery is dependent of the GPI anchor addition. The cell periphery localization of JAGGER-cYFP is also consistent with its function related to polytubey prevention, as it involves interactions between cells in the integuments of the ovules and pollen tubes.

Another critical finding of our work is that the best predictions of the ω -site using the BIG-PI plant predictor software are not the only ω -site in this GPI-AP (Eisenhaber et al. 2003). We showed that losing the best predicted ω -site (Ser-111) in the JAGGER protein did not significantly alter its subcellular localization. In this way, we have identified another three lower score ω -sites (Ser-107, Ala-106 and Ala-109) as critical for JAGGER localization at the cell surface, suggesting that these residues may play a role in GPI anchor addition. These results are consistent with the results reported by Liu et al. (2016), as the deletion of a single ω -site in LORELEI-cYFP also had an indistinguishable localization from the complete LORELEI-cYFP protein,

with a cryptic ω -site possibly functioning in the absence of the best predicted ω -site in LORELEI (Liu et al. 2016).

Previously, it was observed that GFP and m-Citrine fused to AGP4/JAGGER (GFP-AGP4 and mCit-AG) was exclusively localized to the cell periphery in transient expression experiments (Martinière et al. 2012; Zavaliev et al. 2016; Bernat-Silvestre et al. 2021). In the present work, it was consistently observed JAGGER-cYFP fluorescence in puncta in the cell's cytoplasm. Still, the organelle or subcellular identity of these puncta remains unknown. Similar localization in puncta in the synergid cytoplasm was also observed with LORELEI-cYFP (Liu et al. 2016), a GPI-AP. Also, FLA4-citrine fusion revealed a similar expression in puncta of seedlings root epidermal cells. FLA4 (Fasciclin Arabinogalactan Protein 4) belongs to a subfamily of fasciclin-like AGPs, typically containing fasciclin domains and in most cases a GPI anchor (Johnson et al. 2003). These intracellular FLA4-citrine signal co-localized only partially with markers

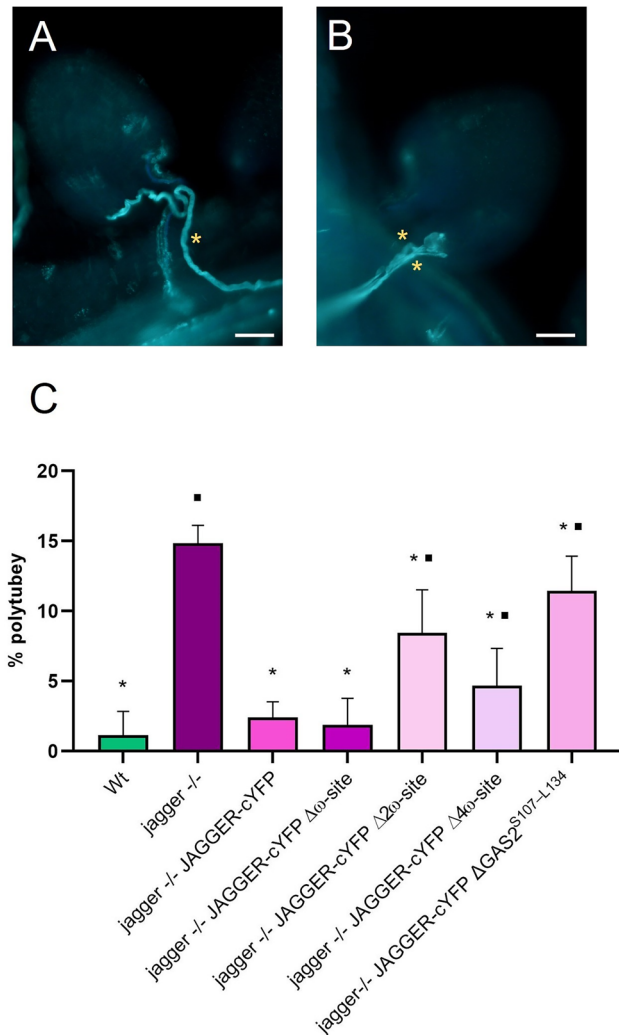


Fig. 5 Decolorized aniline blue staining of pollen tubes growing towards the ovules of hand pollinated pistils from wild type and *jagger-/-* flowers, and the observed frequency of polytubey. **A** ovule receiving one pollen tube (yellow asterisk) from a wild type pistil. **B** Ovule from a *jagger-/-* pistil receiving two pollen tubes (yellow asterisks). **C** Frequency of polytubey observed in ovules from hand pollinated pistils stained with aniline blue in wild type ($n=172$), *jagger-/-* ($n=207$), *jagger-/-* JAGGER-cYFP ($n=185$), *jagger-/-* JAGGER-cYFP $\Delta\omega$ -site ($n=148$), *jagger-/-* JAGGER-cYFP $\Delta 2\omega$ -site ($n=243$), *jagger-/-* JAGGER-cYFP $\Delta 4\omega$ -site ($n=229$) and *jagger-/-* JAGGER-cYFP Δ GAS^{S107-L134} ($n=164$). The frequency of polytubey phenotype was calculated as the number of ovules showing more than one embryo sac entering the embryo sac divided by the total number of ovules observed. Values are expressed as percentages. Error bars denote mean \pm standard deviation, asterisks above bars indicate significant differences between the samples and *jagger-/-* hand pollinated pistils ($P \leq 0.05$), and black squares above bars indicate significant differences between the samples and wild type hand pollinated pistils ($P \leq 0.05$). One-way analysis of variance and the post hoc Tukey test were applied to detect significant differences. Statistical analysis was conducted using GraphPad Prism version 8.0.2 for Windows (www.graphpad.com). Scale bars in A and B, 20 μ m

for late and recycling endosomal compartments (Xue et al. 2017). The authors suggest that there is a certain degree of FLA4 endocytosis, which is afterwards recycled back to the plasma membrane. This has also been proposed for AGP6 and AGP11, two pollen-specific AGPs important for pollen development and pollen tube growth along the pistil tissues, based on transcriptomics and yeast two hybrid assays (Costa et al. 2013a). Interestingly, in transient expression systems, no diffused signal in the cytoplasm has been detected for GFP-AGP4 or Venus-FLA11, another GPI-AP (Bernat-Silvestre et al. 2021). Although we did not find any study of this kind with *Arabidopsis* FLA4, there is one study with FLAs from *Nicotiana benthamiana* which localizes FLA4 in the plasma membrane and the nucleus, in a transient expression experiment (Wu et al. 2020).

The increase in the diffuse cYFP signal in the cytoplasm of both ovule integuments and transmitting tissue cells of JAGGER-cYFP $\Delta 2\omega$ and JAGGER-cYFP $\Delta 4\omega$ lines is most probably due to disruptions in its localization to the cell periphery. This raises the possibility that removal of the two predicted ω -sites led to defects in GPI anchor addition and disrupted the trafficking of JAGGER-cYFP $\Delta 2\omega$ and JAGGER-cYFP $\Delta 4\omega$ to the cell periphery. If that is the case, our results may support the hypothesis that GPI attachment is required for transport of GPI-anchored proteins from the endoplasmic reticulum to the cell periphery (Mao et al. 2003). Similar disruption of localization in the plasma membrane and a concomitant diffused distribution of fluorescence in the cytoplasm happened when the entire GAS sequence in the JAGGER was removed. We verified that JAGGER has a longer GAS sequence than initially predicted, as foreseen to be the case for some GAPs (Martinière et al. 2012). Transgenic plants expressing the GAS^{S107-L134} stretch of amino acid residues fused to GFP was sufficient to drive GFP to the plasma membrane in transient expression experiments performed by Martinière et al. (2012), Bernat-Silvestre et al. (2021). Consistent with these results, we found that the localization of JAGGER-cYFP Δ GAS^{A109-L134} at the cell periphery was markedly lower than JAGGER-cYFP. Most probably, the absence of the GAS region prevents its recognition and the subsequent attachment of the preassembled GPI anchor to the ω -site, supporting the importance of GPI anchor addition for JAGGER localization at the cell surface.

GPI anchor is critical for JAGGER function in polytubey prevention

To test if losing its localization in the plasma membrane via a GPI anchor negatively affected JAGGER function, we performed several hand-pollination experiments with JAGGER-cYFP, JAGGER-cYFP $\Delta\omega$, JAGGER-cYFP $\Delta 2\omega$, JAGGER-cYFP $\Delta 4\omega$, and JAGGER-cYFP $\Delta 2$ GAS constructs introduced in the *jagger-/-* background. Results from these

experiments showed that only those transgenic lines in which the JAGGER localization at the cell periphery were not affected were able to rescue, at least in part, the JAGGER polytubey phenotype. These results led us to conclude that JAGGER localization in the cell periphery through a GPI anchor is critical for its function.

This result obtained for JAGGER function is strikingly different from the conclusions reported for LORELEI protein that lacked a GPI anchor, as none of the GPI anchor addition domain mutants in the LORELEI protein negatively affected LORELEI function in pollen tube reception (Liu et al. 2016). One possibility for this difference is that in the case of LORELEI, the nature of the filiform structure (excessive invagination of the plasma membrane and the increased presence of cell walls) could have masked the negative effects caused by the decreased concentration of mutant proteins in the cell periphery. In other words, the filiform structure in synergid cells could have accumulated enough mutant LORELEI proteins without the GPI anchor to complement the pollen tube reception phenotype. However, in case of cells that express JAGGER, there are no such structural modifications to overcome the loss of JAGGER protein caused by the loss of its GPI anchor.

This result is also quite different from the one obtained for FLA4, which is implicated in many functions, including root elongation and salt stress tolerance (Xue et al. 2017). They demonstrated that FLA4 lacking the GPI anchor addition signal was partially secreted but was not membrane associated. Furthermore, this truncated version of FLA4 was still capable of complementing *sos5-1* and *sos5-2* plants phenotype under salt stress conditions. The author proposed that FLA4 may perform its function in its soluble form, rather than attached to the plasma membrane. In the case of JAGGER, as discussed in Pereira et al. (2016b), we suggest that it may function as a signalling molecule that interacts with receptors from the pollen tubes right before it enters the female gametophyte. In this way, the pollen tubes primed by JAGGER would act as carriers of some sort of signal for persistent synergid degeneration. And this would be in accordance with its localization at the surface of the sporophytic tissues. Nevertheless, our results showed that mutations to GPI anchor addition domains negatively affected JAGGER function, experimentally demonstrating that GPI anchoring is key for in vivo functions of this AGP.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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