RESEARCH PAPER

SUN anchors pollen WIP–WIT complexes at the vegetative nuclear envelope and is necessary for pollen tube targeting and fertility

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Received 19 June 2015; Revised 11 August 2015; Accepted 26 August 2015

Editor: Daphne Goring

Abstract

LINC (linker of nucleoskeleton and cytoskeleton) complexes play an essential role in nuclear migration by connecting the nucleus to the cytoskeleton and/or motor proteins. Plant LINC complexes have recently been identified in Arabidopsis thaliana, with the inner nuclear membrane SUN and outer nuclear membrane WIP proteins comprising the first identified complex. A recent study identified a nuclear movement defect in Arabidopsis pollen vegetative nuclei linked to the outer nuclear envelope WIP and WIT proteins. However, the role that SUN proteins may play in pollen nuclear migration has yet to be addressed. To explore this question, a SUN2 lumenal domain that was targeted to the ER specifically in pollen was over-expressed. It is shown that the ER-targeted SUN2 lumenal domain was able to displace WIP and WIT proteins from the pollen vegetative nuclear envelope. Expression of this dominant-negative transgene led to impaired VN mobility, impaired pollen tube guidance, and defective pollen tube reception. The observed pollen defects are similar to phenotypes observed in a wip1-1 wip2-1 wip3-1 wit1-1 wit2-1 mutant. It is also shown that these defects were dependent on the KASH-binding function of the SUN2 lumenal domain. These data support a model where LINC complexes formed by SUN, WIP, and WIT at the VNE are responsible for VN migration and suggest an important function of SUN, WIP, and WIT in pollen tube guidance and reception.

Key words: Arabidopsis, KASH, nuclear envelope, nuclear migration, plant fertilization, pollen tube ovular guidance, pollen tube reception, SUN, vegetative nucleus.

Introduction

In angiosperms, sperm cells are delivered to ovules by pollen. The pollen vegetative nucleus (VN) and the sperm cells [SCs, or their progenitor, the generative cell (GC)], termed 'male germ unit' (MGU), are usually closely associated and migrate together inside pollen tubes (Dumas et al., 1985; McCue et al., 2011). During migration, the VN precedes (is closer to the growing pollen tube tip than) the GC/SC in many angiosperm species including Arabidopsis thaliana (Heslop-Harrison and Heslop-Harrison, 1989; Lalanne and Twell, 2002; McCue et al., 2011). That the VN and GC/SC migrate as a MGU was proposed to be important for successful fertilization by allowing signal transduction between the VN and SC (Dumas et al., 1985) or by ensuring efficient and simultaneous SC delivery (Russell and Cass, 1981). However, the molecular mechanism for MGU migration has only recently been addressed in Arabidopsis, where VN movement is mediated by WPP domain-interacting proteins (WIPs) and WPP domain-interacting tail anchored proteins (WITs). The Arabidopsis genome encodes three genes for WIPs (WIP1, WIP2,
and WIP3) and two genes for WITs (WIT1 and WIT2) (Xu et al., 2007; Zhao et al., 2008). An Arabidopsis vip1-1 vip2-1 vip3-1 triple null mutant (here abbreviated as vip123) and a wit1-1 wit2-1 double null mutant (here abbreviated as wit12) shows a reversed VN-SC order and frequent loss of the VN during pollen tube growth, suggesting that the VN loses its locomotion and is transported forward by the SCs which still migrate towards the growing pollen tube tip. In addition, vip123 and wit12 exhibit significantly reduced male fertility, resulting from frequent failure of pollen tube reception, exemplified by overgrown pollen tubes inside ovules and polytubey (Zhou and Meier, 2014).

WIPs are outer nuclear membrane Klarsicht/ANC-1/Syne-1 Homology (KASH) proteins. KASH proteins interact with inner nuclear membrane Sad1/UNC-84 (SUN) proteins through the SUN–KASH domain interaction in the nuclear envelope (NE) lumen, forming linkers of the nucleoskeleton and the cytoskeleton (LINC) at the NE (Razafsky and Hodzic, 2009; Starr and Fridolfsson, 2010; Kim et al., 2015). In opisthokonts, LINC complexes play an essential role in nuclear migration by connecting the nucleus to the cytoskeleton and/or motor proteins (Starr and Fridolfsson, 2010; Gundersen and Worman, 2013; Razafsky et al., 2014). In Arabidopsis thaliana, SUN1 and SUN2 interact with WIP1, WIP2, and WIP3, forming NE bridges that anchor WIT1 and WIT2 to the outer nuclear membrane (Zhou et al., 2015a). In roots and leaves, the SUN–WIP–WIT complexes recruit myosin XI-i to the NE, which mediates nuclear elongation and movement (Oda and Fukuda, 2011; Tamura et al., 2013; Zhou et al., 2012, 2015b). Myosin XI-i is dispensable for pollen VN migration (Tamura et al., 2013; Zhou and Meier, 2014), but whether SUN1 and SUN2 are involved in this process is unknown.

In this study, evidence is provided that SUN proteins are involved in VN migration during pollen tube growth. Due to the essential function of SUN proteins in meiotic chromosome movement, synopsis, and recombination, a sun1 sun2 double null mutant has severe pollen developmental defects (Duroc et al., 2014; Murphy et al., 2014; Varas et al., 2015), making it undesirable for this study. Here, the SUN2 luminal domain was expressed under the post-meiotic, pollen-specific Lat52 promoter (Lat52pro) and targeted to the ER, thereby displacing WIPs and WITs (and potentially other known or unknown KASH proteins) from the pollen vegetative nuclear envelope (VNE). It is shown that this largely recapitulates the WIP and WIT deletion phenotypes, specifically when compared with a severe vip1-1 vip2-1 vip3-1 wit1-1 wit2-1 (here abbreviated as wifi) mutant, but that a stronger effect on pollen tube guidance than reported before is caused by this approach. Together, the data presented here make SUN proteins most likely to be the inner NE players in pollen nuclear movement, thus suggesting that the WIP and WIT functions are indeed orchestrated in the context of a plant LINC complex.

Materials and methods

Plant materials

Arabidopsis plants were grown at 25 °C in soil under a 16/8 h light/dark regime or on MS (Caisson laboratories) with 1% sucrose plates under constant light. The Columbia ecotype was used as the wild type (WT) unless indicated otherwise. sun1-KO sun2-KD was reported previously (Zhou et al., 2012) and WIP1pro::GFP-WIP1 vip123, WIT1pro::GFP-WIT1 wit12, and wifi were also reported previously (Zhou and Meier, 2014). sun2-1 was reported previously by Zhou et al. (2015b). Lat52pro::GFP WT was a gift from Dr R Keith Slotkin (McCue et al., 2012).

Constructs

Primers Lat52proinF and Lat52proIR were used to amplify the Lat52pro promoter from a Lat52pro::GFP construct (a gift from Dr R Keith Slotkin). Primers 2SAlbminERF and 2SAlbminERIR were used to amplify the ERS (2SAlbminERF itself served as a template) by PCR. The above two PCR products were mixed and served as templates for overlapping PCR using Lat52proinF and 2SAlbminERIR as primers. The PCR product was then cloned into SacI-and SpeI-digested pK7WGR2 by in-fusion (Clontech). After confirmation by sequencing, the pK7WGRERS2 vector was obtained. Primers 35SERSinF and 35SERSinR were used to amplify ERS from the pK7WGRERS2 vector and the PCR product was cloned in to SpeI-digested pK7WGF2 by in-fusion (Clontech). After confirmation by sequencing, the pK7WGFERS2 vector was obtained. All primer sequences are listed in Supplementary Table S2 at JXB online.

SUN2Lm was amplified by PCR from a SUN2 pENTR D/TOPO clone using SUN2LmF and SUN2LmR as primers. SUN2Lm PCR product was cloned into pENTR D/TOPO (Life Technologies). After confirmation by sequencing, SUN2Lm was moved to pK7WGRERS2 and to pK7WGFERS2 by LR reactions (Life Technologies) to obtain Lat52pro::ERS-RFP-SUN2Lm and Cauliflower Mosaic Virus 35S Promoter (35S)-driven ERS-GFP-SUN2Lm, respectively. SUN2dMut was amplified by PCR from a SUN2dMut pENTR D/TOPO clone using SUN2LmF and SUN2LmR as primers. SUN2Lm PCR product was cloned into pENTR D/TOPO (Life Technologies). After confirmation by sequencing, SUN2dMutLm was moved to pK7WGRERS2 and to pK7WGFERS2 by LR reactions (Life Technologies) to obtain Lat52pro::ERS-RFP-SUN2dMutLm and Cauliflower 35S-driven ERS-GFP-SUN2dMutLm, respectively.

Generation of transgenic plants

Binary constructs were transformed to Agrobacterium strain ABI by triparental mating (Wise et al., 2006). The ER-mCherry marker Agrobacterium strain (Nelson et al., 2007) was obtained from the Arabidopsis Biological Resource Center. Transgenic Arabidopsis lines were obtained by the Agrobacterium-mediated floral dip method (Clough and Bent, 1998).

Hoechst 33342 staining

For Hoechst 33342 staining, a solution containing 4% paraformaldehyde, 18% sucrose, and 4 µM Hoechst 33342 was used to stain pollen tubes for at least 20 min. Pollen tubes were viewed under a Nikon C90i microscope. The UV-2E/C filter cube (Nikon) was used for imaging the Hoechst 33342-stained VN and SN. Images were taken by a Nikon DS-Qi1Mc digital camera.

In vitro pollen germination and Alexander staining

Pollens grains from the stamens of fully opened flowers were germinated on a pollen germination medium containing 18% sucrose, 0.01% boric acid, 1 mM CaCl2, 1 mM Ca(NO3)2, 1 mM MgSO4, and 0.5% agar. Several wild-type stigmas were placed adjacent to pollen grains to stimulate pollen germination (Qin et al., 2009, 2011). For the pollen competition assay, the pollen grains were directly germinated on stigmas. Alexander pollen staining was performed as described previously by Alexander (1969).
Ovule imaging and pollen tube aniline blue staining

A magnifier was used to identify opening flowers with protruding unpollinated stigmas. These flowers were colored and the ovaries from these flowers were collected 3 d later. For imaging ovules, ovaries were dissected and ovules were mounted in 80 mM sorbitol for microscopy (Leydon et al., 2013). For aniline blue staining, ovaries were fixed in a solution containing acetic acid:ethanol (1:3 v/v) for 2 h, washed in a 70%, 50%, 30%, 0% ethanol gradient for 10 min each time, and softened in 8 M NaOH overnight. Aniline blue solution containing 0.1% (w/v) aniline blue and 108 mM K$_2$PO$_4$ (pH 11) was used to stain the softened ovaries overnight. Stained ovaries were dissected and ovules were imaged using a Nikon C90i microscope equipped with a UV-2A filter cube (Nikon). Images of pistils with stained pollen tubes were collected using a Nikon C90i confocal microscope. The aniline blue dye was excited by a 403 nm laser and the emission above 470 nm was collected as aniline blue signal.

Co-immunoprecipitation experiments

Nicotiana benthamiana leaves were collected, ground to powder in liquid nitrogen, and Co-IP experiments were performed at 4 °C. One milliliter NP-40 buffer (50 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 3 mM DTT, 1 mM PMSF, and 1% protease inhibitor cocktail [Sigma-Aldrich]) was used to extract 500 μl of plant tissue. One-tenth of the protein extracts was used as the input sample and the rest were used for IP using protein A-Sepharose beads (GE Healthcare) pre-coated with a rabbit anti-GFP antibody (catalogue number ab290, Abcam Cambridge). After washing three times in NP-40 buffer, the immunoprecipitates and the input samples were separated by 8% SDS-PAGE, transferred to PVDF membranes (Bio-Rad), and detected with a mouse anti-GFP (1:2000, catalogue number 632569, Clontech) or a mouse anti-Myc (1:1000, catalogue number ab290, Abcam Cambridge). After washing three times in 50 mM NaCl, 0.5% NP-40, 1% Triton X-100 buffer, the immunoprecipitates and the input samples were separated by 8% SDS-PAGE, transferred to PVDF membranes (Bio-Rad), and detected with a mouse anti-GFP (1:2000, catalogue number 632569, Clontech) or a mouse anti-Myc (1:1000, catalogue number M5546, Sigma-Aldrich) antibody. The input/IP ratio was 1/9.

Results

Single SUN null alleles cause no major fertility or VN movement defects

Since the wip123 and wit12 null mutants have a severe reduction in seed set, seed production was analysed in the sun1-knockout sun2-knockdown (sun1-KO sun2-KD) mutant that recapitulates the wip123 and wit12 nuclear shape phenotypes in root hairs, trichomes, and root epidermal cells (Oda and Fukuda, 2011; Zhou et al., 2012). Compared with the WT, sun1-KO sun2-KD has a small reduction of seeds per silique (12%) (Fig. 1A). This is far less severe than the 33% seed loss of the wip123 mutant or the 50% seed loss of the wit12 mutant (Zhou and Meier, 2014). To determine whether sun1-KO sun2-KD has impaired VN movement, its pollen nuclear order was examined 5 h after pollen germination as described previously by Zhou and Meier (2014). Unlike the strong effect seen for wip123 and wit12, there is no apparent difference between sun1-KO sun2-KD and the WT (Fig. 1B) indicating that the VN of sun1-KO sun2-KD migrates normally in pollen tubes. This suggests either that SUN1 and SUN2 are not involved in the role of WIP and WIT in pollen nuclear migration or that the remaining amount of SUN2 in the sun1-KO sun2-KD is sufficient for this function. To dissect if SUN2 is the main WIP and WIT anchor in pollen tubes, a SUN2 null mutant in the Ws-4 ecotype, sun2-1, was acquired (Zhou et al., 2015b). Figure 1A shows that sun2-1 has no deficiency in seed production when compared with the Ws-4 WT. Together, these data suggest either that SUN1 and SUN2 are not involved in the role of WIP and WIT in pollen nuclear migration or that they act redundantly and—unlike in vegetative tissue—the remaining SUN2 in sun1-KO sun2-KD pollen is sufficient for this function.

A mistargeted SUN2 lumenal domain depletes WIP1 and WIT1 from the pollen VNE

To address the requirement of SUN1/SUN2 for VN migration, a dominant-negative approach was therefore used as described previously by Crisp et al. (2006). The KASH-binding SUN domain of SUN2 was over-expressed in the ER lumen with the aim of outcompeting native SUN–KASH interactions at the NE. Specifically, a Golgi retrieval signal, HDEL, was fused to the C-terminus of the SUN2 lumenal domain (SUN2Lm). This construct was then N-terminally tagged with a fluorescent protein (FP) with an ER targeting signal (ERS), resulting in the ERS-FP-SUN2Lm construct

![Fig. 1. SUN1 and SUN2 function redundantly in seed production.](https://example.com/fig1.png)
(Fig. 2A). As a negative control, the NE luminal domain of a SUN2 mutant carrying the H434A and Y438F point mutations (SUN2dMutLm) was used to make the ERS-FP-SUN2dMutLm construct (Fig. 2A). The H434A and Y438F point mutations impair the KASH binding ability of SUN2 (Zhou et al., 2014).

Both ERS-GFP-SUN2Lm and ERS-GFP-SUN2dMutLm co-localized with an ER-mCherry marker in Nicotiana benthamiana leaves, confirming their presence at the ER (see Supplementary Fig. S1 at JXB online). Next, ERS-GFP-SUN2Lm or ERS-GFP-SUN2dMutLm was co-expressed with Myc-WIP1, Myc-WIP2, or Myc-WIP3 in N. benthamiana leaves to test for protein–protein interactions. An anti-GFP antibody was used to immunoprecipitate (IP) protein complexes from the cell extracts. As shown in Fig. 2B, Myc-WIP1, Myc-WIP2, and Myc-WIP3 interact with ERS-GFP-SUN2Lm, but not with ERS-GFP-SUN2dMutLm.

To test whether expression of ERS-FP-SUN2Lm de-localizes WIPs and WITs from the VNE, an RFP version of this construct was generated and expressed in Arabidopsis under the post-meiotic, pollen-vegetative-cell-specific Lat52 promoter (Lat52pro) (Twell, 1992; Twell et al., 1990). Lat52pro-driven ERS-RFP-SUN2Lm (Lat52pro:ERS-RFP-SUN2Lm) was transformed into WIP1pro:GFP-WIP1-rescued wip123 line 3 and WIT1pro:GFP-WIT1-rescued wit12 line 1, respectively, which have no pollen nuclear migration or seed production defects (Zhou and Meier, 2014). Figure 3A shows the resulting pollen grains segregating for ERS-RFP-SUN2Lm while expressing GFP-WIP1 or GFP-WIT1. The GFP fusion proteins were delocalized from the NE in pollen grains expressing ERS-RFP-SUN2Lm, but in pollen grains lacking ERS-RFP-SUN2Lm GFP-WIP1 and GFP-WIT1 strongly labelled the VNE (Fig. 3A; see Supplementary Fig. S2 at JXB online). The analogous experiment was carried out using ERS-RFP-SUN2dMutLm driven by Lat52pro (Lat52pro:ERS-RFP-SUN2dMutLm). As shown in Fig. 3B, ERS-RFP-SUN2dMutLm did not affect the localization of GFP-WIP1 or GFP-WIT1, although it was expressed at a similar level to ERS-RFP-SUN2Lm as suggested by the RFP intensity.

Lat52pro::ERS-RFP-SUN2Lm is likely to deplete other KASH proteins from the VNE. Additional KASH protein families have been identified, but only the SINE1/2 KASH family is also conserved across land plants (Zhou et al., 2014). Other plant KASH families are present only in subgroups of plant species (Zhou et al., 2014) and, therefore, they are less likely to play a role in the widely conserved process of pollen tube ovular guidance. Among Arabidopsis SINE1 and SINE2, SINE1 appears not to be expressed in pollen, but SINE2 is present at the SC NE and somewhat more weakly at the VNE (see Supplementary Fig. S3 at JXB online). However, despite the VNE localization of SINE2, sine2 null mutants do not have a fertility defect (data not shown). Hence, it was reasoned that, in the context of known plant KASH proteins, the effect of Lat52pro:ERS-RFP-SUN2Lm is specific to WIP proteins.

ERS-RFP-SUN2Lm causes severe seed loss in sun1-KO sun2-KD

If the remaining amount of SUN2 in sun1-KO sun2-KD is functioning in WIP and WIT VNE anchoring, then
ERS-RFP-SUN2Lm in this background should compete with this interaction. Thus, sun1-KO sun2-KD was transformed with Lat52pro::ERS-RFP-SUN2Lm and Lat52pro::ERS-RFP-SUN2dMutLm, respectively, and homozygous transgenic lines were selected with similar expression levels.

Figure 4 shows the seed set of two independent transgenic lines, each compared to sun1-KO sun2-KD. While Lat52pro::ERS-RFP-SUN2Lm lines had drastically reduced seed set, Lat52pro::ERS-RFP-SUN2dMutLm lines had seed set very similar to the sun1-KO sun2-KD background (Fig. 4A). Reciprocal crosses between Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD line 3 and WT confirmed that the seed loss phenotype was derived from the male (Fig. 4B). It was then assessed whether expressing ERS-RFP-SUN2Lm or ERS-RFP-SUN2dMutLm in sun1-KO sun2-KD would affect pollen morphology or pollen tube growth. Supplementary Fig. S4 at JXB online shows that no visible difference was seen compared with the WT.

To determine the position and order of VN and GC, pollen tubes were examined 5h after in vitro pollen germination and stained with Hoechst to visualize nuclei (see Materials and methods). Figure 5 shows that, in the majority of Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD pollen tubes, the SN preceded the VN, very similar to the phenotype previously reported for wip123, wit12, and wifi (Zhou and Meier, 2014).
Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD plants exhibited no change in pollen nuclear order when compared with untransformed sun1-KO sun2-KD. Plants expressing ERS-RFP-SUN2Lm have ovular guidance and reception defects.

To address whether the seed loss phenotype observed in Fig. 4 reflects defects in pollen tube reception, as described for WIP and WIT mutants, fertilized ovules were examined in Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD or Lat52pro::ERS-RFP-SUN2dMutLm sun1-KO sun2-KD plants. When ovaries of Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD were observed 3 d after the flowers opened, there was a small number of big ovules and a large number of small ovules (see Supplementary Fig. S5A at JXB online). The number of big ovules per ovary was similar to the seed number per silique, suggesting that the ovules that remain small at this stage do not develop into seeds (see Supplementary Fig. S5B at JXB online). Aniline blue staining showed that the big ovules were targeted by at least one pollen tube, suggesting that they were fertilized and developed into seeds. In small ovules, the central cell nucleus and the egg cell were visible (see Supplementary Fig. S5C at JXB online), suggesting that they were unfertilized. Among the total ovules examined, approximately one half (43% for line 1 and 52% for line 3) were without visible pollen tubes (Fig. 6; see Supplementary Table S1 at JXB online).

Ovaries of sun1-KO sun2-KD plants transformed with Lat52pro::ERS-RFP-SUN2Lm had randomly distributed big fertilized ovules (see Supplementary Fig. S5A at JXB online), and their pollen tubes were able to reach the receptacle end of a pistil (see Supplementary Fig. S6 at JXB online), suggesting that there is no defect in pollen tube growth and that, instead, the pollen tubes expressing ERS-RFP-SUN2Lm might have an ovular guidance defect (Higashiyama and Takeuchi, 2015). In addition, one-quarter of the ovules of Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD examined bore overgrown pollen tubes (28% for line 1 and 25% for line 3, Fig. 6), a phenotype typical for a pollen tube reception defect. Both pollen tube defects were not observed in WT, sun1-KO sun2-KD, or Lat52pro::ERS-RFP-SUN2dMutLm sun1-KO sun2-KD (Fig. 6).

It has been shown previously that the pollen tubes of wip123 and wit12 have a high frequency of overgrowth in ovules and a minor defect in ovule targeting (Zhou and Meier, 2014). Since ERS-RFP-SUN2Lm can displace both WIP1 and WIT1 from the NE, the fate of wifi pollen tubes was characterized as a comparison. Wifi pollen tubes are also able to reach the
receptacle end of a pistil (see Supplementary Fig. S6 at JXB online). As shown in Fig. 6, and in Supplementary Table S1 at JXB online, approximately 14% of wifi ovules examined were without pollen tubes, which is less severe than the effect seen with Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD. By contrast, approximately 36% of the wifi ovules examined had overgrown pollen tubes, which is more severe than that found for Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD. Similar to wip123 and wit12 mutants, wifi also has a pronounced polytubey phenotype, which was not observed in the other mutants and transgenic lines including Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD, suggesting that there are quantitative differences between the mutants examined.

Discussion

Function of SUN–WIP–WIT complexes

In vegetative tissues, SUNs, WIPs, and WITs form a complex at the NE that regulates nuclear shape and nuclear movement. Previously, it has been shown that WIPs and WITs at the pollen VNE are essential for VN migration (Zhou and Meier, 2014). In this study, ERS-RFP-SUN2Lm was specifically expressed in post-meiotic pollen grains and showed that it displaced GFP-WIP1 and GFP-WIT1 from the pollen VNE in the presence of both native SUN1 and SUN2 (Fig. 3), suggesting that ERS-RFP-SUN2Lm is able to outcompete KASH interactions with both SUN1 and SUN2. This is in line with our previous report that both SUN1 and SUN2 interact with WIP1, WIP2, and WIP3 (Zhou et al., 2012). Expressing ERS-RFP-SUN2Lm in pollen grains of sun1-KO sun2-KD impaired the VN movement and caused severe seed loss, both of which were not observed in sun1-KO sun2-KD or Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD. Furthermore, no seed loss was observed in sun2-1, supporting our preferred model where SUN1 and SUN2 redundantly anchor WIP and WIT at the VNE, and the SUN–WIP–WIT complex is responsible for VN migration during pollen tube growth. This suggests that WIP and WIT perform their role in male fertility in the context of a LINC complex and thus adds further evidence that the function of LINC complexes in nuclear migration is conserved in eukaryotes.

Function of SUN, WIP, and WIT in pollen tube ovular guidance and reception

sun1-KO sun2-KD pollen grains expressing ERS-RFP-SUN2Lm have no defects in pollen viability and pollen tube growth. However, these pollen tubes have severe ovular guidance and reception defects, and similar phenotypes, yet with different severity, were observed in wifi (Fig. 6; see Supplementary Table S1 at JXB online). These severe phenotypes were not observed in Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD (Fig. 6; see Supplementary Table S1 at JXB online). Unlike wifi, Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD did not show an obvious polytubey phenotype. This can be explained by its strong ovular guidance defect, because of which not enough pollen tubes can target ovules to cause the polytubey phenotype. These data indicate that the SUN–WIP–WIT LINC complexes are involved in pollen tube ovular guidance and reception.

Pollen tube ovular guidance and reception involve signals from both female and male parts (Leydon et al., 2014; for a recent review see Higashiyama and Takeuchi, 2015). Growing through pistil tissues, pollen tubes receive signals from female sporophytic tissues and undergo an activation or differentiation process which makes them responsive to attraction signals secreted from ovules (Leydon et al., 2014; Higashiyama and Takeuchi, 2015). Known Arabidopsis genes expressed in pollen tubes that mediate ovular guidance include the ER-localized potassium transporters CHX21 and CHX23 (Lu et al., 2011), the ER protein POD1 (Li
membrane-anchored receptor-like cytoplasmic kinases LIP1 and LIP2 (Liu et al., 2013), glycosylphosphatidylinositol-anchored protein COBL10 (Li et al., 2013), glycosylphosphatidylinositol-biosynthesis-related proteins SETH1, SETH2, and APTGT (Lalanne et al., 2004; Dai et al., 2014), F-actin severing proteins MAP18 and MDP25 (Zhu et al., 2013; Qin et al., 2014), glutathione transferase GSTU26 (Lin et al., 2014), xylanogucan endotransglycosylase/hydrolase XTH19 (Lin et al., 2014), and mitogen-activated protein kinases MPK3 and MPK6 (Guan et al., 2014). Three pollen-specific transcription factors MYB97, MYB101, and MYB120 have been reported to be essential for pollen tube reception (Leydon et al., 2013; Liang et al., 2013). Therefore, it is tempting to speculate that a transcriptionally-active VN at the growing pollen tube tip is required for locally expressing the male factors responsible for pollen tube guidance and reception during the late stages of pollen tube growth. As shown by our previous study, disrupting SUN–WIP–WIT LINC complexes can lead to a loss of the VN during pollen tube growth (Zhou and Meier, 2014), which would, by this model, lead to the pollen tube guidance and reception defects described here.

It was noted that Lat52pro::ERS-RFP-SUN2dMutLm sun1-KO sun2KD has a stronger ovular guidance defect than wifi. One possible explanation is that unidentified KASH proteins or other SUN-domain interacting proteins are involved in this specific aspect of male fertility and that they, too, are depleted in this mutant. Alternatively, the delocalized WIP and WIT proteins in pollen might cause this strong ovular guidance defect through unknown mechanisms.

ERS-RFP-SUN2Lm as a tool to study the function of SUN proteins

ERS-RFP-SUN2Lm has been successfully used here to out-compete SUN1 and SUN2 for binding KASH proteins in mature pollen and pollen tubes and the function of SUN proteins in these two cell types has been revealed. SUN1 and SUN2 are expressed in various tissues (Graumann et al., 2010; Oda and Fukuda, 2011) and probably play multiple roles in plant development. ERS-RFP-SUN2Lm can now be used as a tool to dissect the function of SUN proteins in a specific cell type or at a certain developmental stage without the interference of unrelated developmental phenotypes of a sun double null mutant.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Fig. S1. ERS-GFP-SUN2Lm and ERS-GFP-SUN2dMutLm localize to the ER in N. benthamiana.

Supplementary Fig. S2. ERS-RFP-SUN2Lm delocalizes WIP1 and WIT1 from the NE in pollen grains.

Supplementary Fig. S3. Expression and localization pattern of SINE1 and SINE2 in WT pollen.

Supplementary Fig. S4. Both Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD and Lat52pro::ERS-RFP-SUN2dMutLm sun1-KO sun2-KD transgenic Arabidopsis plants produce healthy pollen grains.

Supplementary Fig. S5. Fertilization defects in ovaries of Lat52pro::ERS-RFP-SUN2Lm sun1-KO sun2-KD.

Supplementary Fig. S6. Pollen tube growth in pistils revealed by aniline blue staining.

Supplementary Table S1. Pollen tube fate in ovaries 3 d after flower opening.

Supplementary Table S2. Primers used for cloning.

Acknowledgements

We thank Ms Anna Griffis and Ms Alecia Wagner for many helpful discussions and for critical reading of the manuscript and Dr David Evans for critical reading of the manuscript. We thank the National Science Foundation for financial support (NSF-MCB 1243844).

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