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The SERK1 receptor-like kinase regulates organ separation in *Arabidopsis* flowers

Michael W. Lewis[1], **Michelle E. Leslie**[2], **Emilee H. Fulcher**[1], **Lalitree Darnielle**[1], **Patrick Healy**[1], **Ji-Young Youn**[1], and **Sarah J. Liljegren**[1,2,*]

Michael W. Lewis: mwlewis@unc.edu; Michelle E. Leslie: meleslie@gmail.com; Emilee H. Fulcher: emileefulcher@gmail.com; Lalitree Darnielle: lalitree@gmail.com; Patrick Healy: hpatrick@email.unc.edu; Ji-Young Youn: jy.youn@utoronto.ca

[1] Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280, USA

[2] Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC 27599-3280, USA

Summary

Through a sensitized screen for novel components of pathways regulating organ separation in *Arabidopsis* flowers, we have found that the leucine-rich repeat receptor-like kinase SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1) acts as a negative regulator of abscission. Mutations in *SERK1* dominantly rescue abscission in flowers without functional NEVERSHED (NEV), an ADP-ribosylation factor GTPase-activating protein required for floral organ shedding. We previously reported that the organization of the Golgi apparatus and location of the *trans*-Golgi network (TGN) are altered in *nev* mutant flowers. Disruption of *SERK1* restores Golgi structure and the close association of the TGN in *nev* flowers, suggesting that defects in these organelles may be responsible for the block in abscission. We have also found that the abscission zones of *nev serk1* flowers are enlarged compared to wild-type. A similar phenotype was previously observed in plants constitutively expressing a putative ligand required for organ separation, INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), suggesting that signaling through IDA and its proposed receptors, HAESA and HAESA-LIKE2, may be deregulated in *nev serk1* abscission zone cells. Our studies indicate that in addition to its previously characterized roles in stamen development and brassinosteroid perception, SERK1 plays a unique role in modulating the loss of cell adhesion that occurs during organ abscission.

Keywords

receptor-like kinase; membrane trafficking; abscission; ARF-GAP; NEVERSHED; flower development

Introduction

A defining feature of multicellular organisms is their ability to regulate the adhesive properties of individual cells and cell layers. In plants, programmed changes in cellular adhesion allow the abscission of leaves, flowers, and fruit, and dispersal of pollen and seeds. Understanding the signaling pathways that regulate the loss of cell adhesion in model plants offers tremendous potential to develop novel approaches to control abscission in plants of agronomic value (Ferrandiz et al., 2000; Liljegren et al., 2004; Østergaard et al., 2005). In recent years, the *Arabidopsis* flower has become a valuable model system for investigating

Correspondence to: Sarah J. Liljegren, (liljegren@unc.edu; phone 919 962-3216; fax 919 962-1625).

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several distinct cell separation processes including organ abscission, fruit opening, and pollen release (Aalen et al., 2006; Lewis et al., 2006).

Arabidopsis flowers shed their outer organs—the sepals, petals and stamens—after fertilization of the internal gynoecium. The detachment sites are a few differentiated cell layers at the base of each organ known as abscission zones (AZs). Once AZ cells are specified, a series of steps must be successfully coordinated for organ separation to occur and to protect the plant body remaining behind. The dissolution of cell walls must be initiated and controlled, after which the remaining AZ cells form a scar-like epidermis (Patterson, 2001).

Recent studies have defined a signaling pathway required for the initiation of organ separation in *Arabidopsis* (Cho et al., 2008; Stenvik et al., 2008; Butenko et al., 2009). Two redundant, leucine-rich repeat receptor-like kinases (LRR-RLKs) HAESA (HAE) and HAESA-LIKE2 (HSL2) are likely activated by the small peptide INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) (Jinn et al., 2000; Butenko et al., 2003; Stenvik et al., 2006; Cho et al., 2008; Stenvik et al., 2008). HAE and HSL2 trigger cell separation by transmitting signals through Mitogen-Activated Protein Kinase Kinase 4 (MKK4)/MKK5 and Mitogen-Activated Protein Kinase 6 (MPK6)/MPK3 (Cho et al., 2008). Despite the discovery of signaling molecules that promote abscission, the mechanisms that prevent premature organ separation are largely unknown. Two identified inhibitors are the MADS domain transcription factors AGL15 and AGL18; ectopic expression of either is sufficient to delay both floral senescence and organ abscission (Fernandez et al., 2000; Fang and Fernandez, 2002; Adamczyk et al., 2007).

More than 200 LRR-RLKs have been identified in *Arabidopsis* (Shiu and Bleecker, 2001). While the functions of most are still unknown, characterized family members regulate diverse growth and developmental processes including plant immunity, hormone signaling, meristem maintenance and stomatal patterning (reviewed in Diévart and Clark, 2004; Afzal et al., 2008; Aker and de Vries, 2008; Butenko et al., 2009). While many LRR-RLKs potentially bind unique ligands, others, such as the members of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family, are thought to act instead as multi-functional regulators of LRR-RLK pathways (Torii, 2004; Albrecht et al., 2008). SERK1 belongs to the LRR-RLK subfamily II; each of the 14 members of this subfamily has 5 LRRs and most exhibit broad temporal and spatial expression profiles (Shiu and Bleecker, 2001; Schmid et al., 2005). BRI1 ASSOCIATED KINASE1 (BAK1, SERK3) functions in BR perception and pathogen response (Li et al., 2002; Chinchilla et al., 2007). BAK1-LIKE1 (BKK1, SERK4) also participates in BR signaling and, together with BAK1, is proposed to regulate a BR-independent cell death pathway (He et al., 2007). Three other members of subfamily II—NSP-INTERACTING KINASE1 (NIK1), NIK2 and NIK3—act in a defense signaling pathway targeted by plant viruses (Fontes et al., 2004; Carvalho et al., 2008). Specialization within the SERK family has been attributed to differences in protein activity rather than in expression profiles, with functional specificity likely governed through interactions with higher order protein complexes (Albrecht et al., 2008).

Recently, we demonstrated that the NEVERSHED (NEV) ADP-ribosylation factor GTPase-activating protein (ARF GAP) is required for floral organ abscission (Liljegren et al., 2009). Loss of NEV activity causes defects in membrane trafficking at the time of organ separation including the appearance of circularized multilamellar structures, which may consist of chimeric fusions of the Golgi apparatus and *trans*-Golgi network (TGN). Here we show that mutations in *SERK1* restore floral organ shedding, Golgi structure and TGN location in *nev* mutant flowers. Our studies suggest that the SERK1 receptor-like kinase functions as an inhibitor of organ abscission.

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Results

Mutations in *SERK1* dominantly restore organ shedding in *nev* flowers

To identify novel genes that regulate floral organ abscission, a suppressor screen of *nev-3* mutant plants was carried out. The *nev-3* mutation replaces an invariant arginine near the zinc finger of the NEV ARF GAP domain with a lysine; this residue is essential for ARF GAP activity in all proteins tested (Luo et al., 2007; Liljegren et al., 2009). Of eight independent mutants found to rescue organ shedding in *nev* flowers, three behave recessively and five act dominantly (Table S1; Fig. 1c; Fig. S1a–d,f).

Two of the dominant mutations were mapped to the lower arm of chromosome 1. By sequencing candidate genes in the 168 kb interval, unique point mutations were discovered in four of the five dominant mutants within the *SERK1* gene (At1g71830) (Fig. 1k; Table S1). We confirmed that interactions between *SERK1* and *NEV* are not allele-specific: the *serk1-4* and *serk1-5* mutations dominantly suppress the *nev-3* mutation as well as a mutation (*nev-6*) predicted to encode a truncated protein missing the ARF GAP domain (Table S2; Fig. 1c; Fig. S1f; data not shown).

SERK1 encodes a 625-amino acid protein with five leucine-rich repeats in its extracellular domain, a single-pass transmembrane domain and a cytoplasmic kinase domain (Fig. 1k) (Hecht et al., 2001). The extracellular domain includes a signal peptide, and the LRRs are flanked by a leucine zipper and a Serine-Proline-Proline motif (Fig. 1k) (Hecht et al., 2001). Previously, SERK1 has been demonstrated to act redundantly with the SERK2 LRR-RLK in directing differentiation of the tapetal cell layer during stamen development (Albrecht et al., 2005;Colcombet et al., 2005), and to work together with the BRI1 LRR-RLK to promote brassinosteroid (BR)-mediated signaling controlling hypocotyl growth (Karlova et al., 2006;Albrecht et al., 2008).

Each of the four *serk1* alleles identified in the *nev* suppressor screen introduce missense mutations within the extracellular or transmembrane domain of the SERK1 protein whereas the previously characterized mutant alleles contain T-DNA insertions in the kinase domain (Fig. 1k) (Albrecht et al., 2005;Colcombet et al., 2005;Albrecht et al., 2008). *serk1-4* affects a glycine residue in the second LRR which is invariant among the members of LRR-RLK subgroup II; this allele was used as a representative allele in most of our subsequent studies. *serk1-7* introduces a serine in place of a glycine between the predicted signal peptide cleavage site and the leucine zipper. The *serk1-5* allele changes the 3′ splice acceptor site of intron 3 from an AG to an AA; defective splicing of exon 4 is predicted to cause production of a 577 amino acid protein with an in-frame deletion of the second and third LRRs. *serk1-6* introduces an aspartic acid residue into the hydrophobic transmembrane domain.

The previously reported *serk1* mutants behave as a recessive, loss-of-function alleles in the context of anther development and/or hypocotyl growth, and the truncated serk1-1 protein was demonstrated to be kinase-dead *in vitro* (Albrecht et al., 2005; Colcombet et al., 2005; Albrecht et al., 2008). To determine if a partial loss of SERK1 kinase activity is sufficient to rescue shedding in *nev* flowers, we generated *nev* plants carrying either one or two mutant copies of the *serk1-1* allele. As with the *serk1* alleles identified in our screen, organ shedding was dominantly rescued in *nev serk1-1/+* flowers (Fig. 1d; Fig. S1e). This result suggests that mutations in *SERK1* restore abscission in a haploinsufficient, rather than dominant-negative, fashion.

Organ abscission was observed to occur at the same developmental stage in wild-type and *nev serk1* mutant flowers. After shedding, sections of *nev serk1* flowers (Fig. 1i) show that many of the remaining cells of the floral AZs have expanded as in wild-type (Fig. 1g),

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whereas the AZ cells of *nev* flowers have remained small and the organs are still attached (Fig. 1h). Like the mutant alleles identified previously (Albrecht et al., 2005;Colcombet et al., 2005;Albrecht et al., 2008), we did not detect any phenotypic differences between the *serk1* single mutants reported here and wild-type plants (Fig. 1e,j; Fig. S1h–k).

Since *SERK1* and *SERK2* function redundantly during anther and pollen development (Albrecht et al., 2005; Colcombet et al., 2005), we tested whether a mutation in *SERK2* might also suppress the abscission defects of *nev* flowers. We found that the previously characterized *serk2-1* allele (Colcombet et al., 2005) does not restore shedding in *nev* flowers (Fig. 1f). This result suggests that SERK1 modulates both organ separation and BR signaling independently of SERK2 (Albrecht et al., 2008).

SERK1 is expressed in floral buds and most vegetative tissues examined with the highest expression in the vasculature of roots, hypocotyls and inflorescence stems (Albrechet et al., 2005; Kwaaitaal et al., 2005; Kwaaitaal et al., 2007). Like *SERK1*, *NEV* expression is not restricted to specific cell types and functional redundancy within the ARF GAP family is predicted to mask broader roles for NEV in promoting plant growth and development (Liljegren et al., 2009). In addition to organ abscission defects, *nev* inflorescences produce shorter fruit than wild-type (Fig. S2) (Liljegren et al., 2009). Mutations in *SERK1* do not suppress this aspect of the *nev* mutant phenotype; *nev* and *nev serk1* fruit are both significantly shorter than either wild-type or *serk1* fruit (Fig. S2). This result suggests that NEV likely functions with factors other than SERK1 to promote fruit growth.

Disruption of the Golgi structure in *nev* flowers is stage-dependent

We previously described a unique set of membrane trafficking defects in *nev* flowers compared to wild-type at the time of organ shedding (floral stage 16) (Liljegren et al., 2009). Instead of the flat Golgi cisternae characteristic of wild-type cells (Fig. 2a), cup-shaped multilamellar structures (CMS) are predominantly present in *nev* AZ cells (Fig. 2b,k). The tubular-vesicular networks of the TGN, which are closely associated with Golgi stacks in wild-type cells (Fig. 2a), are not readily apparent near the CMS in *nev* cells (Fig. 2b,d). Furthermore, vesicle clusters known as paramural bodies (PMBs) are frequently found between the plasma membrane and cell wall of *nev* AZ cells (Fig. 2h) (Liljegren et al., 2009).

Using transmission electron microscopy (TEM), we examined whether disruption of the Golgi is also evident in *nev* AZ regions at later stages of flower development. We found that a significant amount of CMS are also present in older *nev* flowers, along with multilamellar structures that closely resemble the flat Golgi stacks of wild-type cells (Fig. 2a–d). While nearly all (97%) multilamellar structures in *nev* cells are cup-shaped at the timepoint when organ shedding would normally occur (stage 16) (Liljegren et al., 2009), in older *nev* flowers, 36% (early stage 17) and 29% (mid-stage 17) of the multilamellar structures present are cup-shaped (Fig. 2k). These results suggest that the severity of the Golgi structural defect in *nev* flowers depends on the stage of development, and correlates with the surge of membrane trafficking required for cell separation.

Mutations in *SERK1* restore Golgi structure in *nev* flowers

To determine whether any of the trafficking defects present in *nev* flowers might be rescued by mutations in *SERK1*, we examined cells in the sepal AZ regions of *nev serk1* flowers immediately after shedding (early stage 17). Whereas a mixture of CMS and linear Golgi stacks were observed in *nev* cells at this stage (Fig. 2b–d,k), Golgi with a wild-type appearance were primarily observed in *nev serk1* double mutant cells (Fig. 2e,k). As in wild-type, only flat stacks of Golgi cisternae were present in *serk1* cells (Fig. 2a,f,k). In *nev* cells

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(early stage 17), TGN were usually visible near the flat stacks of Golgi present (Fig. 2c,d), but were not readily observed near CMS (Fig. 2b,d). As in wild-type and *serk1* cells, TGN were observed closely associated with the Golgi cisternae of *nev serk1* cells (Fig 2a,e,f). These results suggest that disruption of *SERK1* activity is able to restore the structure of the Golgi and location of the TGN in *nev* cells.

We also examined whether the accumulation of PMBs in *nev* cells was influenced by mutations in *SERK1*. For this study, PMBs were defined as clusters of ten or more vesicles located between the plasma membrane and the cell wall (Fig. 2h). In *nev* sepal AZ regions (early stage 17), PMBs were observed in 75% of the cells examined, compared to 10% of wild-type and *serk1* cells (Fig. 2g,h,j,l). Significantly more PMBs (51%) were also observed in *nev serk1* cells compared to wild-type (Fig. 2g,i,l). While the decrease in PMB accumulation between *nev* and *nev serk1* cells is significant (Fig 2l), our analysis of *nev serk1* flowers suggests that the structural changes of the Golgi cisternae and altered location of the TGN are likely the primary membrane trafficking defects associated with blocked abscission in *nev* flowers.

The abscission zones of *nev serk1* flowers are enlarged

Although disruption of *SERK1* activity rescues organ shedding in *nev* flowers, the AZ scars of older *nev serk1* flowers can have a rough appearance compared to those of wild-type (Fig. 1a,c,d; Fig. S1a–f). To determine the basis for these differences, wild-type, *serk1* and *nev serk1* flowers were fixed for scanning electron microscopy (SEM) during or immediately after shedding. Whereas no differences between *serk1-4* and wild-type AZ cells were detected (Fig. 3a,e), the AZs of *nev serk1-4* appeared to slightly larger than wild-type and those of *nev serk1-6* flowers to be significantly enlarged (Fig. 3b–d). To quantify this change, the average height of the floral AZ region, from the base of the sepal AZ to the top of the stamen AZ was determined for wild-type and *nev serk1-6* flowers for the first flower undergoing organ shedding (stage 16; position 1) to older flowers with well-developed AZ scars (mid-stage 17; position 7). At all positions tested, *nev serk1-6* flowers were found to contain significantly larger floral AZs compared to wild-type (Fig. 3i). Similar measurements of the height of the sepal AZ were conducted for wild-type, *serk1-4* and *nev serk1-4* flowers; *nev serk1-4* flowers were also found to contain significantly larger sepal AZs compared to wild-type (Fig. 3j). Since the petals and sepals of *nev* flowers remain firmly attached (Fig. 1b,h; Fig. 3f), an equivalent measurement of *nev* sepal AZ cells was not possible. However, we have observed that the stamens sometimes slough off in older *nev* flowers, and expansion of the remaining stamen AZ cells appears uneven and somewhat disorganized (Fig. 3f).

To explore whether the enlarged AZs of *nev serk1-4* flowers could be due to increased cell division or cell expansion, we also examined the number of sepal AZ cells within a defined area for wild-type and mutant flowers (Fig. 3k). *nev serk1-4* sepal AZs were found to contain significantly more cells compared to wild-type at each floral position tested (Fig. 3k). We also observed that the relative difference in cell number between *nev serk1-4* and wild-type flowers remained constant at the positions tested, suggesting that after organ separation, the growth rate of *nev serk1-4* AZ cells mirrors that of wild-type (Fig. 3k). Taken together, our results suggest that the enlargement of *nev serk1-4 and nev serk1-6* AZs occurs prior to or during organ abscission.

Flowers ectopically expressing the putative signaling ligand IDA develop massive bands of AZ tissue at their bases due to increased AZ cell expansion and the presence of additional AZ cells (Stenvik et al., 2006; Cho et al., 2008). We saw similar results in plants engineered to express a chimeric *IDA-GFP/GUS* fusion protein under the control of the viral 35S promoter (Fig. 3g,h; Fig. S1g). After organ detachment, the sepal, petal, and stamen AZs of

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35S::IDA-GFP/GUS flowers (early stage 17) already appear enlarged, show poorly defined boundaries and have begun to engulf the nectaries (Fig. 3g). To determine whether the increase in AZ size at this stage is due to ectopic cell expansion or the presence of additional cells, we measured the height and checked the cell density of the *35S::IDA-GFP/GUS* floral AZ regions compared to wild-type (Fig. 3i,k). Although the density of cells in *35S::IDA-GFP/GUS* AZs at this stage resembles that of wild-type (Fig. 3k), the size of the *35S::IDA-GFP/GUS* AZs was significantly larger (Fig. 3i). These results suggest that either increased cell division or recruitment of neighboring cells is responsible for the initial AZ enlargement of flowers misexpressing IDA.

In older *35S::IDA-GFP/GUS* flowers (mid-stage 17), the contribution of ectopic cell expansion to the collars of AZ tissue that form at their bases is readily apparent (Fig. 3h). As *35S::IDA-GFP/GUS* flowers continue to age, the cells in the AZ region appear to swell until they burst, leaving rough, broken tissue behind (Fig. S1g). We also observed large bands of rough tissue in the AZ regions of older *nev* flowers carrying one or two copies of the *serk1-5* or *serk1-6* mutant alleles (Fig. S1b,c,f; Fig. 3n,o). Since the AZ regions of *nev serk1-4* and *nev serk1-7* flowers present a more smooth appearance (Fig. S1a,d), yet all five *serk1* alleles tested dominantly rescue organ separation in *nev* flowers, this phenotypic distinction may point to differences in the activity levels of the respective serk1 mutant proteins.

Flowers constitutively expressing IDA have been shown to contain detectable arabinogalactin proteins in their enlarged AZs after organ separation (Stenvik et al., 2006). To determine whether *nev serk1* flowers might also share this characteristic, we stained wild-type and *nev serk1-6* flowers (stage 17) with the Yariv reagent (β-GlcY) Yariv et al., 1962; Yariv et al., 1967). As in *35S::IDA* flowers, a red precipitate indicative of arabinogalactan protein was observed in *nev serk1-6* flowers (Fig. 4m) but not readily observed in wild-type (Fig. 4l), or in flowers treated with a control reagent (α-GlcY) (Fig. 4n,o). Taken together, the strong resemblence of *nev serk1-6* flowers to flowers ectopically expressing IDA suggests the intriguing possibility that deregulated signaling of the IDA/HAE pathway may be responsible for the rescue of abscission in *nev serk1* flowers and the significant enlargement of the *nev serk1* AZs.

Mutations in *SERK1* do not rescue organ shedding in the *ida* or *hae hsl2* abscission mutants

Since the redundant LRR-RLKs, HAE and HSL2 (Cho et al., 2008; Stenvik et al., 2008), and their proposed ligand IDA (Butenko et al., 2003), appear to promote organ separation at the same stage as NEV (Liljegren et al., 2009), we tested for genetic interactions between *SERK1*, *IDA*, and *HAE/HSL2*. We found that as in the *ida* single and *hae hsl2* double mutants (Fig. 4a,c), organ abscission is also blocked in the *ida serk1-4* double and *hae hsl2 serk1-4* triple mutants (Fig. 4b,d). To further examine the relationship between *SERK1*, *NEV* and *IDA*, we generated the *nev ida* double and *nev ida serk1-4* triple mutant. We discovered that mutations in *SERK1* also rescue abscission in *nev ida* flowers (Fig. 4e,f), and that the abscission zones of *nev ida serk1-4* flowers (Fig. 4g) do not show the well-defined boundaries typical of wild-type abscission zones (Fig. 4h). These results are consistent with *SERK1* regulating abscission upstream of *HAE/HSL2,* and/or functioning in a parallel or downstream pathway to modulate organ separation.

Discussion

Here we report a novel role for the SERK1 LRR-RLK as a negative regulator of floral organ abscission. We were able to uncover SERK1's inhibitory role in organ separation in the context of plants with defective NEV, an ARF GAP which likely facilitates the movement of key cargo molecules required for cell separation (Liljegren et al., 2009). As described below,

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our studies suggest that SERK1 acts to inhibit organ abscission and restrict the size of floral AZs by modulating the localization or activity of signaling components that promote abscission.

Insights into the potential function of SERK1 in modulating organ separation are provided by two well-characterized LRR-RLK signaling pathways, both regulated by BAK1 (SERK3). Signaling in response to the BR hormone begins when the extracellular domain of the BRI1 LRR-RLK binds BR (Li and Chory, 1997; Wang et al., 2001; Kinoshita et al., 2005). After release of an inhibitory protein, the affinity of BRI1 for its co-receptor, BAK1, is increased and is followed by oligomerization of the receptors and transphosphorylation (Li et al., 2002; Wang et al., 2005a; Wang et al., 2005b). BRI1 cycles between the plasma membrane and endomembrane system in a ligand-independent manner; formation of the BRI1-BAK1 complex may promote endosomal sorting of these receptors for enhanced signaling (Russinova et al., 2004; Karlova et al., 2006; Geldner et al., 2007; Geldner and Robatzek, 2008). BAK1 also functions as a co-receptor for the FLS2 LRR-RLK in regulating the plant immune response (Chinchilla et al., 2007). Interaction of BAK1 with the flagellin peptide-bound FLS2 receptor is required for endocytosis of FLS2 (Robatzek et al., 2006; Chinchilla et al., 2007; Heese et al., 2007). SERK1 has previously been shown to undergo endocytosis from the plasma membrane and to interact with proteins such as KINASE-ASSOCIATED PROTEIN PHOSPHATASE that likely regulate its movement, recycling and degradation (Stone et al., 1994; Shah et al., 2001; Shah et al., 2002; Rienties et al., 2005; Aker et al., 2006; Karlova et al., 2006; Aker and de Vries, 2008; Hink et al., 2008). Like BAK1, SERK1 and other members of the SERK family may enable internalization and/or sorting of ligand-binding LRR-RLKs that function in divergent signaling pathways.

Two attractive LRR-RLK candidates that may be regulated by SERK1 are HAE and HSL2, which are proposed to bind the secreted peptide IDA and activate abscission via a MAPK signaling cascade (Cho et al., 2008; Stenvik et al., 2008). Our genetic analysis suggests that *NEV* and *SERK1* function upstream of *HAE/HSL2* and/or in a parallel signaling pathway regulating cell separation (Fig. 4). These results are consistent with the possibility of direct regulation of HAE/HSL2 by SERK1. Rather than acting as a co-receptor for ligand-bound HAE/HSL2, SERK1 may directly interact in a complex with HAE/HSL2 to promote endocytosis and/or internal sorting of these putative receptors prior to ligand binding, and thereby limit their ability to activate abscission signaling. Our previous studies indicated that NEV is localized in the TGN/early endosome and putative recycling endosomal compartments (Liljegren et al., 2009). In *nev* flowers, the trafficking of receptor complexes including SERK1 may be affected, such that recycling of the HAE/HSL2 receptors back to the plasma membrane is compromised. Partial loss of SERK1 activity may be sufficient to restore abscission in *nev* flowers by increasing the amount of activated HAE/HSL2 receptor complexes at the plasma membrane or in internal endosomal compartments, thereby bypassing *nev*-mediated defects in membrane trafficking.

At the time of organ separation, the sepal AZs of *nev serk1* flowers are larger and appear to contain more cells than those of wild-type, suggesting a role for SERK1 in regulating AZ size (Fig. 3). This phenotype is exaggerated in flowers ectopically expressing IDA, and these flowers also show premature organ abscission and ectopic AZ cell expansion (Fig. 3g,h) (Stenvik et al., 2006;Cho et al., 2008). As highlighted in Figure 5, we propose that SERK1 may spatially restrict the domain of cell separation during abscission to ensure that loss of cell adhesion does not occur in cells beyond the AZs. Disruption of SERK1 activity in *nev* flowers may lead to deregulated signaling of the IDA/HAE pathway in AZ cells. This in turn could trigger the increased secretion and spread of cell wall-modifying and -degrading enzymes to cells adjacent to the AZ regions giving *nev serk1* flowers the

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appearance of larger AZ scars with an increased number of cells (Fig. 5). In plants ectopically expressing *IDA*, early and prolonged signaling of HAE/HSL2, accompanied by increased ectopic cell wall loosening, could account for the uncontrolled AZ cell expansion and premature organ abscission observed (Fig. 3h) (Stenvik et al., 2006;Cho et al., 2008).

Alternatively, the larger AZs of *nev serk1* flowers may indicate that SERK1 regulates cell division or fate during a late stage of AZ differentiation. During anther development, SERK1 and SERK2 are redundantly required for formation of the tapetal cell layer and proper maturation of the microspores, which become the pollen grains (Albrecht et al, 2005; Colcombet et al., 2005). In the male-sterile *serk1 serk2* double mutant, the tapetum is absent and extra sporogenous cells are found, suggesting that SERK1/2 activities influence the balance between these two cell fates. Mutations in the genes that encode the EXCESS MICROSPOROCYTES1 (EMS1, also known as EXTRA SPOROGENOUS CELLS) LRR-RLK and its putative secreted ligand TAPETUM DETERMINANT1 (TPD1) also result in an increased number of sporogenous cells at the expense of the tapetal cell layer (Canales et a., 2002; Zhao et al., 2002; Yang et al., 2003; Yang et al., 2005). SERK1/2 are considered as potential co-receptors of EMS1 or of an unknown LRR-RLK regulating the intertwined fates of these cell types (Albrecht et al, 2005; Colcombet et al., 2005). Although very little is currently known about the process of floral organ AZ differentiation in *Arabidopsis,* in some plant species, cells in the separation or neighboring layers undergo an additional round(s) of cell division immediately prior to or after abscission (Sexton and Roberts, 1982; van Doorn and Stead, 1997). While this has not been demonstrated to occur in wild-type *Arabidopsis* flowers (Patterson, 2001), ectopic cell division or a cell-fate transformation could also be responsible for the enlarged AZs of *nev serk1* and *35S::IDA* flowers.

Abscission is likely to be particularly sensitive to perturbations in membrane trafficking as it relies on secretion of a suite of hydrolytic enzymes that extensively modify cell walls as well as dissolve the pectin-rich middle lamellae between the walls of AZ cells (Sexton and Roberts, 1982; Gonzalez-Carranza et al., 1998). Strict control of these enzymes is essential to ensure that loss of cell adhesion only occurs at the appropriate time and in the designated regions. We previously discovered that the Golgi structure is disrupted, the location of the TGN is altered, and increased numbers of paramural vesicles are present in *nev* floral AZ cells at the time when organ shedding would normally occur (Liljegren et al., 2009). Further analysis reported here shows that the Golgi structural defect shows a temporal fluctuation in *nev* AZ cells, and is significantly alleviated in older flowers (Fig. 2). These results dovetail with early ultrastructural studies of leaf AZ cells that showed significant increases in the ER and Golgi present after abscission was induced (Sexton and Hall, 1974; Sexton et al., 1977). It is intriguing that disruption of SERK1 activity not only rescues abscission in *nev* flowers but also restores the structure of the Golgi and TGN location (Fig. 2). These results suggest the possibility that a backlog of cargo molecules may directly contribute to the disruption of the Golgi structure observed in *nev* flowers. Alternatively, these results may indicate that SERK1 plays a broader role in regulating endosomal traffic, rather than simply facilitating endocytosis and/or sorting of ligand-binding LRR-RLKs.

Our research illustrates the power of sensitized forward genetic screens to identify novel factors that regulate signaling and membrane traffic (Rojo and Denecke, 2008). Like innovative screens designed to detect mutants with defects in vacuolar trafficking (Li et al., 2006; Shimada et al., 2006; Sanmartin et al., 2007; Sohn et al., 2007), we were able to successfully use a discrete developmental phenotype as a readout for restoration of membrane trafficking and secretion in specific flower cells. The rescue by partial loss of SERK1 suggests that *nev* mutant flowers represent an unbuffered background where moderate changes in the activity of RLK(s) regulating organ abscission are sufficient to allow the programmed loss of cell adhesion to occur. SERK1 was known to function

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redundantly in distinct signaling pathways required for anther development and BR perception (Albrecht et al., 2005, Colcombet et al., 2005; Karlova et al., 2006; Albrecht et al., 2008), but a role in regulating organ abscission was unsuspected. The productivity of our screen (Table S1) (Leslie et al., 2010; C. Burr and S. Liljegren, unpublished results) suggests that we will be able to identify additional components of pathway(s) that modulate the timing and extent of cell separation in *Arabidopsis* flowers.

Experimental Procedures

Plants

Mutant alleles of *SERK1* were obtained through ethyl methanesulfonate screens of *nev-3* mutant plants as previously described (Liljegren et al., 2000). The *serk1-4*, *serk1-6*, and *serk1-7* alleles contain nucleotide substitutions within codons 107, 242, and 30, which change a glycine to an aspartic acid, an aspartic acid, and a serine, respectively. The *serk1-5* allele contains a G to A nucleotide substitution at the splice acceptor site of the 3rd intron. All alleles identified are of the Ler ecotype. Primers and restriction enzymes used for genotyping these alleles are listed in Table S3. The *serk1-1*, *serk2-1*, *ida-2*, *hae-1*, and *hsl2-1* mutant alleles and *35S::IDA-GFP/GUS* transgenic plants were described previously (Albrecht et al., 2005; Colcombet et al., 2005; Cho et al., 2008; Stenvik et al., 2008; Leslie et al., 2010).

Mapping

To map dominant mutations that restore organ shedding in *nev* flowers, the *serk1-4 nev-3* and *serk1-5 nev-3* double mutants were crossed to *nev-6* (Col) and *nev-3* (stock backcrossed to Col three times), respectively. 118 *nev serk1* and 244 *nev serk1/+* F2 plants were identified by segregation analysis of the F3 progeny. Using DNA isolated from these plants and PCR-based markers including several designed from Monsanto Ler polymorphisms (http://www.Arabidopsis.org/Cereon/), both mutations were mapped to chromosome 1, between positions 26940342 and 27108102. The coding regions of 5 of 42 predicted genes in this 168 kb region were sequenced from *nev serk1* genomic DNA.

Microscopy

Flower images were captured using a Leica MZ FLIII dissecting microscope and a MicroPublisher 3.3 RTV digital camera (Qimaging, Surrey, British Columbia, Canada). For transmission electron microscopy, flowers were analyzed as described (Liljegren et al., 2009). Images of Toluidine Blue stained sections were captured using a Nikon Eclipse 80i microscope with a Nikon Ds-Fi 7 color camera and Nikon elements BR2.3 software (Nikon Americas Inc, Melville, NY). For scanning electron microscopy, flowers were fixed in 2% glutaraldehyde in 0.05M sodium phosphate buffer overnight, treated for 30 min with 2% osmium tetroxide, and dehydrated through an ethanol series. Samples were dried using a Samdri-795 critical point dryer (Tousimis Research Corporation, Rockville, MD), and coated in gold-palladium using a Hummer X sputtering system (Anatech, Alexandria, VA). Images were captured using a Zeiss Supra 25 scanning electron microscope with SmartSEM acquisition and imaging software (Zeiss, Peabody, MA). Image brightness and contrast were adjusted with Photoshop CS4 (Adobe, Mountain View, CA); the color balance of the images shown in Fig. 1a–f was adjusted with Photoshop. AZ measurements were performed using NIH imageJ software. To determine sepal AZ cell density, all cells which fell completely or partially within an 1800 μm^2 area between the medial nectaries were counted. Yariv staining was conducted as described previously (Stenvik et al., 2006).

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Supplementary Material

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Acknowledgments

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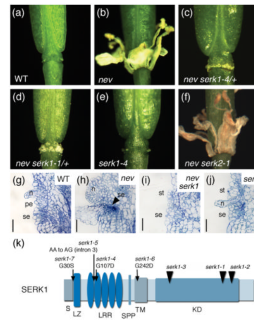

Figure 1. Mutations in *SERK1* dominantly rescue organ shedding in *nev* flowers

(a) Wild-type flower after organ separation (stage 17). The sepals, petals and stamens have detached, leaving behind the maturing fruit.

(b) Floral organ shedding is blocked in *nev* mutant flowers.

(c) Organ separation is dominantly restored in *nev* flowers carrying one mutant copy of the *serk1-4* allele.

(d) The previously characterized, kinase-dead *serk1-1* allele (Albrecht et al., 2005; Colcombet et al., 2005) dominantly rescues organ shedding in *nev* flowers.

(e) *serk1-4* flowers shed their organs normally.

(f) A mutation in *SERK2* does not rescue abscission in *nev* flowers.

(g–j) Longitudinal sections of wild-type and mutant flowers (stage 17) stained with Toluidine Blue. In *nev serk1-4* flowers (i), the remaining AZ cells show a similar amount of cell expansion as those of wild-type (g) and *serk1-4* (j) flowers. The sepal (se), petal (pe) and stamen (st) AZs and floral nectaries (n) are indicated; in *nev* flowers (h) the AZs are still intact. Scale bars: 50 μm.

(k) Diagram of the SERK1 protein. The locations of four novel point mutations and three previously described T-DNA mutants are indicated by arrows and arrowheads, respectively. The *serk1-4* mutation affects the second LRR within exon 4 and the *serk1-5* mutation alters the 3′ splice site of intron 3. The *serk1-6* mutation falls within the transmembrane domain (TM) and the *serk1-7* mutation affects the leucine zipper (LZ) near the N-terminus. The previously identified T-DNA mutations, *serk1-1, serk1-2* and *serk1-3,* affect the kinase domain (KD). The locations of the SERK1 signal peptide (S) and Serine-Proline-Proline (SPP) motif are also indicated.

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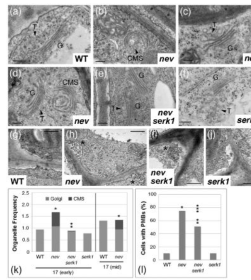

Figure 2. Mutations in *SERK1* restore Golgi structure in *nev* flowers

Transmission electron micrographs of cells in wild-type and mutant sepal AZ regions (first stage 17 flower).

(a) In wild-type cells, the Golgi (G) are composed of several flat cisternae closely associated with the tubular-vesicular structures of the *trans*-Golgi network (TGN) (T, arrowhead).

(b–d) A mixture of cup-shaped multilamellar structures (CMS, arrowhead) (b,d) and Golgi with a wild-type appearance (c,d) are found in *nev* cells at this developmental stage. TGN are usually observed nearby the linearized Golgi (c) but are not readily apparent near the CMS (b,d) in *nev* cells.

(e) Golgi consisting of flat cisternae are primarily found in *nev serk1* cells, and are typically associated with structures resembling the TGN.

(f) The structure of the Golgi and location of the TGN are not affected in *serk1* cells.

(g,h) Clusters of vesicles termed paramural bodies (PMBs, see asterisks in [h]) are infrequently seen between the plasma membrane and cell wall of wild-type cells (g), and are frequently observed in *nev* cells (h).

(i) Accumulation of PMBs still occurs in *nev serk1* double mutant cells.

(j) As in wild-type, PMBs are infrequently observed in *serk1* cells.

(k) Frequency of Golgi and CMS per cell in sections of wild-type and mutant AZ regions. A temporal reduction is seen in the proportion of CMS to linear Golgi in *nev* cells (stage 17), although a significant number of CMS are found in *nev* cells at all stages tested compared to wild-type (Fisher's exact test, **P*<0.0001). In *nev serk1* double mutant cells (early stage 17), primarily Golgi with flattened cisternae are observed compared to *nev* cells (***P*<0.0001). For each type of tissue analyzed, *n* (cells) ≥17. Data from two independent experiments are presented, as indicated by separate sections in the graph.

(l) Quantification of PMBs present in wild-type and mutant cells (early stage 17). Compared to wild-type cells, significantly more *nev* (Fisher's exact test, **P*<0.001) and *nev serk1* (***P*<0.002) cells in sepal AZ regions display one or more PMBs. A significant decrease was observed in the number of *nev serk1* cells with PMBs compared to *nev* cells (****P*<0.03). For each type of tissue analyzed, *n* (cells) ≥40.

Scale bars: 0.2 μm (a–f), 0.5 μm (g–j).

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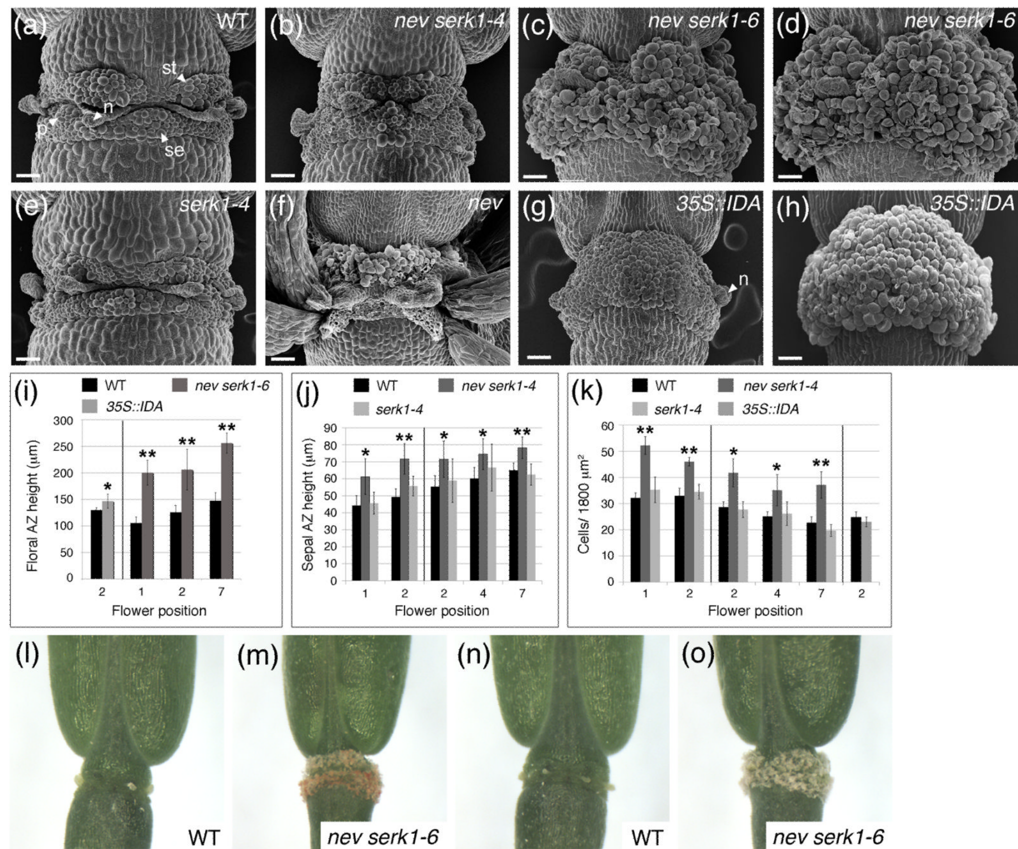

Figure 3. *nev serk1* flowers have enlarged abscission zones

(a–h) Scanning electron micrographs of wild-type, *serk1-4* and *nev serk1-4* flowers (position seven, mid-stage 17), and of *nev serk1-6* and *35S::IDA-GFP/GUS* flowers (position two, early stage 17; mid-stage 17).

(a) After organ separation, the remaining AZ cells of wild-type flowers expand and form smooth, protective scars at the detachment sites. The sepal (se), petal (pe) and stamen (st) AZs can be easily distinguished from each other and the floral nectaries (n).

(b) The floral AZs of *nev serk1-4* flowers are slightly larger and contain more cells than those of wild-type.

(c–d) *nev serk1-6* flowers contain significantly enlarged AZs which merge and form visible rings of tissue at their bases.

(e) The floral AZs of *serk1* flowers appear like those of wild-type.

(f) Although the sepal and petals remain firmly attached, the stamens of *nev* flowers sometimes slough away in older flowers. Cells remaining in these *nev* stamen AZs appear expanded and somewhat disorganized. The medial sepal and stamens of the *nev* flower shown were removed for clarity.

(g) The enlarged AZs of *35S::IDA-GFP/GUS* flowers have merged, enveloping the floral nectaries.

(h) Due to ectopic cell expansion in the floral AZ regions, massive collars of tissue encircle the bases of older *35S::IDA-GFP/GUS* flowers.

(i) After organ abscission, *nev serk1-6* (position one, stage 16; positions two and seven, stage 17) and *35S::IDA-GFP/GUS* (position two, stage 17) flowers show significantly larger floral AZs compared to wild-type (Independent groups *t*-test, $*P<0.002$, $**P<0.0001$). The total height of the floral AZ regions, from the top of the stamen AZ to the bottom of the sepal AZ, was measured since the boundaries between the sepal, petal and stamen AZs

become unclear. For each genotype, *n* (flowers) ≥5. Wild-type flowers used as controls for analysis of *nev serk1-6* and *35S::IDA-GFP/GUS* flowers were of the Ler and Columbia ecotypes, respectively.

(j) The height of *nev serk1-4* sepal AZs is significantly increased compared to wild-type at every position measured (Independent groups *t*-test, *$P<0.005$, **$P<0.001$). For each genotype and position, *n* (flowers) ≥5. Data from independent experiments are shown in separate sections of the graph.

(k) The sepal AZs of *nev serk1-4* flowers contain more cells than those of wild-type or *serk1-4* flowers. Significant differences in cell density between *nev serk1-4* flowers and wild-type are apparent from the time of organ abscission (position one, stage 16) (Independent groups *t*-test, *$P<0.001$, **$P<0.0001$). Soon after organ shedding (position 2, stage 17), *35S::IDA-GFP/GUS* sepal AZs are enlarged (e,h) but show a similar cell density compared to wild-type (Columbia). For each genotype and position, *n* (flowers) ≥4. Data from independent experiments are shown in separate sections of the graph.

(l–o) Wild-type and *nev serk1-6* flowers (stage 17) stained with the Yariv reagent (β-GlcY) or with a negative control (α-GlcY). A red precipitate appears in the AZs of *nev serk1* flowers (m) stained with β-GlcY, indicating the presence of arabinogalactan proteins. Wild-type flowers stained with β-GlcY (l), or flowers stained with α-GlcY (n,o), did not show a detectable precipitate. Scale bars: 50 μm.

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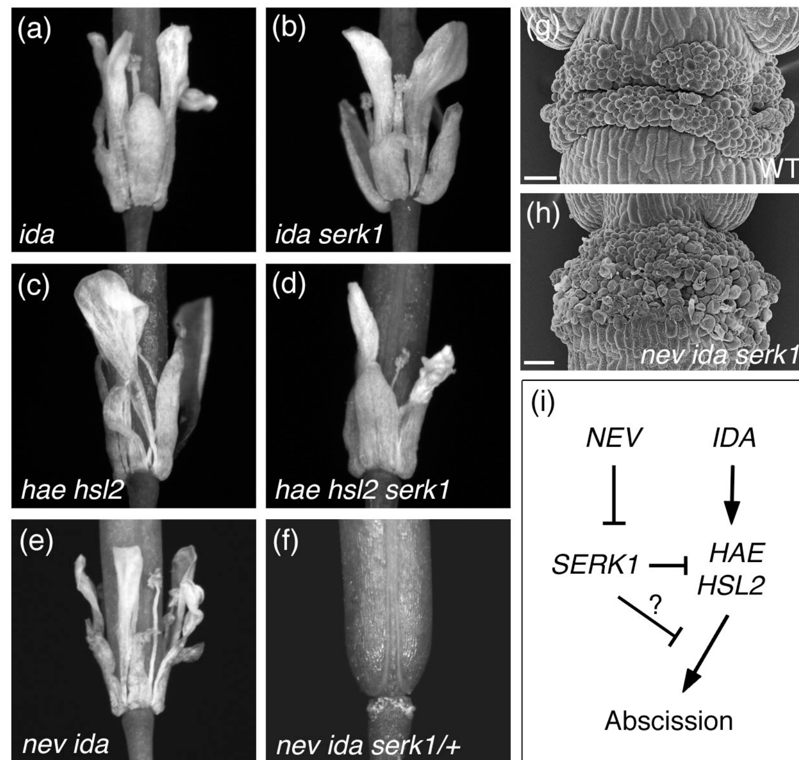

Figure 4. Genetic analysis of pathways regulating floral organ abscission

Organ abscission is blocked by mutations in genes encoding the putative signaling molecule IDA (a) or its proposed receptors, the redundant HAE and HSL2 LRR-RLKs (c). Mutations in *SERK1* do not rescue organ shedding in either *ida* (b) or *hae hsl2* (d) flowers. As in the *ida* and *nev* single mutants, abscission is blocked in the *nev ida* double mutant (e). Mutations in *SERK1* dominantly restore shedding in *nev ida* flowers (f). *nev ida serk1* triple mutant flowers (h) show disorganized abscission zones compared to the well-defined boundaries of those in wild-type flowers (g). As a membrane trafficking regulator, NEV may promote organ separation by potentially controlling the localization and activity of SERK1 and other receptor-like kinases (i). SERK1 may inhibit organ separation by acting upstream and/or downstream of HAE/HSL2. Scale bars: 50 µm.

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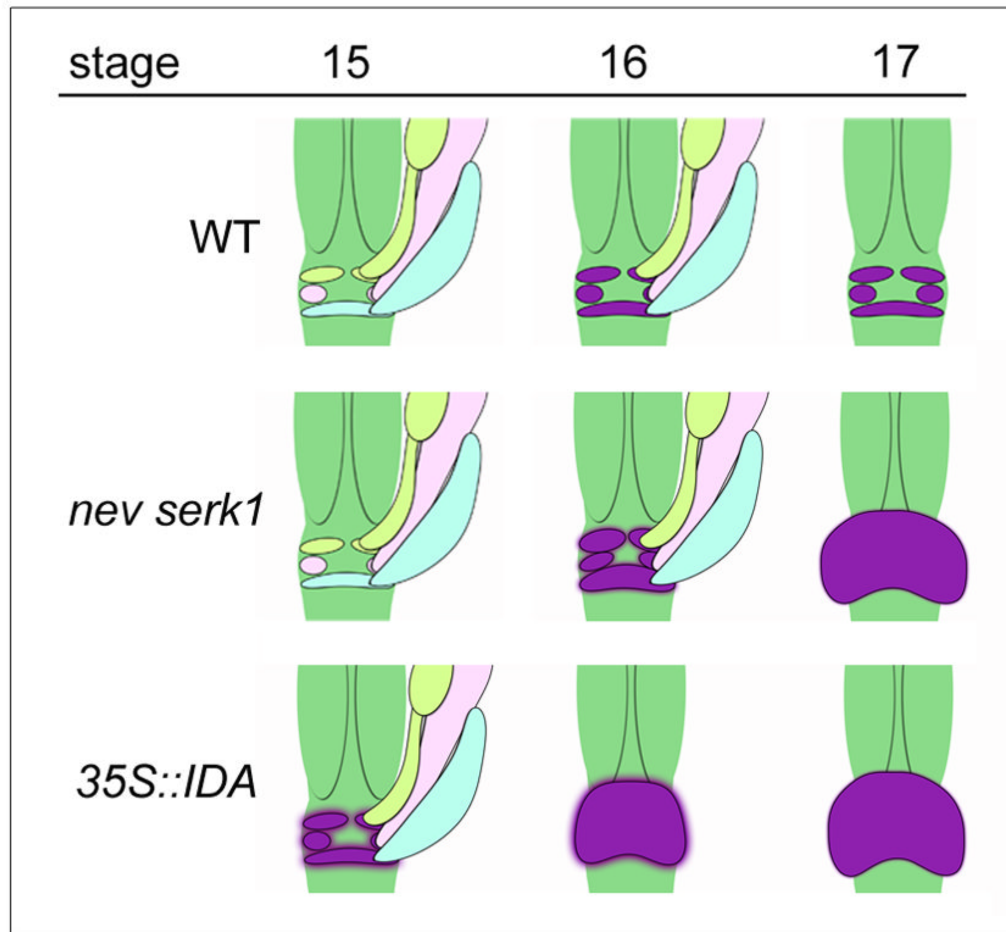

Figure 5. SERK1 may spatially restrict AZ signaling during organ abscission

In wild-type flowers (stage 16), floral organ shedding occurs when signaling activated by IDA and HAE/HSL2 leads to loss of cell adhesion within the floral AZs. After the floral organs have detached (stage 17), the remaining AZ cells expand and form protective scars at the sites of separation. Mutations in *SERK1* not only restore abscission in *nev* flowers (stage 16), but lead to an enlargement of the floral AZs. Ectopic expression of IDA causes premature abscission (stage 15) and pronounced AZ enlargement (stage 17) (Stenvik et al., 2006). Our studies suggest the possibility that deregulated signaling of IDA/HAE is responsible for the rescued organ separation and larger AZs of *nev serk1* flowers. Deregulated activation of abscission could cause the release of hydrolytic enzymes beyond the original AZ boundaries and ectopic loss of cell adhesion in the neighboring cells. In wild-type flowers, SERK1 and other factors may modulate the activity of HAE/HSL2, and thereby control the timing and regions of cell separation. Activated AZs are indicated in purple.