Organ abscission is a sophisticated cell-separation process that allows plants to sculpt their shape in response to developmental cues, environmental stress or pathogen attack (for review see refs. 1-3). Over the past decade, the Arabidopsis flower has become a model system to investigate the developmental programming required to release its sepals, petals and stamens (Fig. 1A).4-6 Abscission zones form at organ boundaries with the floral receptacle. After pollination, secretion of cell wall modifying enzymes leads to cell separation within these zones and detachment of the floral organs.

Genetic dissection of organ abscission has revealed several components of a signaling pathway required for the cell separation phase of organ abscission. The redundant HAESA (HAE) and HAESA-LIKE2 (HSL2) receptor-like kinases switch on a mitogen-activated protein (MAP) kinase cascade.7,8 Reduced transcription of cell wall modifying enzymes is found in hae hsl2 mutant abscission zones.9 Genetic analysis points to INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) as the peptide ligand for HAE and HSL2.8,10,11 Flowers with ectopic expression of IDA shed their organs prematurely and contain enlarged, disorganized abscission zones.12 Loss of HAE and HSL2 blocks abscission in 35S::IDA flowers; treatment of hae hsl2 mutant flowers with a synthetic IDA peptide is not sufficient to rescue organ shedding.8,11 NEVERSHEd (NEV/AGD5), an ADP-ribosylation factor GTPase-activating protein (ARF-GAP), is also required during the cell separation phase of abscission (1B).13 ARF-GAPs are thought to regulate intracellular traffic by linking cargo recruitment with vesicle formation.14 Trafficking defects identified in nev mutant flowers include altered organization of the Golgi, mislocation of the Golgi-associated trans-Golgi network (TGN), and the presence of numerous extracellular vesicles in the apoplastic space.15 NEV co-localizes with endomembrane markers of the TGN/early endosome and recycling endosomes.13,15,16 A recent study has demonstrated that NEV physically interacts in vivo with the clathrin heavy chain and labels a subset of purified clathrin-coated vesicles.16 In vegetative tissue and seeds, NEV acts cooperatively with MODIFIED TRANSPORT TO THE VACUOLE1 (MTV1), an EPSIN N-TERMINAL HOMOLOGY protein to regulate cargo movement from the TGN to the pre-vacuolar compartment.16 Trafficking of vacuolar cargo is strongly affected in nev mtv1 double mutants, which are severely dwarfed compared with wild-type plants.10 During abscission, NEV may potentially regulate exocytosis of cell wall-modifying enzymes, endocytosis of cell wall materials, cycling of signaling components between the cell surface and endosomes, and/or transport of specific cargo to the pre-vacuolar compartment.

Three receptor-like kinases—EVERSHEd (EVR), SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1) and CAST AWAY (CST)—limit the extent of cell separation in abscission zones by potentially altering HAE/HSL2 localization or activity. The NEVERSHEd (NEV) ADP-riboylation factor GTPase-activating protein facilitates the intracellular movement of molecules required for organ abscission and fruit growth. Here we report further analysis of the relationship between NEV-mediated intracellular traffic, EVR activity and IDA-HAE/HSL2 signaling during flower development. Our results support a model in which cell separation is mediated by HAE/HSL2 signaling downstream of NEV and EVR. We discuss the possibility that conserved circuits control organ abscission and modulate fruit growth.
serk1 and nev cst mutant flowers.\(^{17-19}\) Furthermore, like 35S::IDA flowers, nev evr flowers shed their organs prematurely.\(^{17}\) Loss of EVR, SERK1 or CST restores the organization of the Golgi and placement of the TGN in nev flowers.\(^{17-19}\) Bimolecular fluorescence complementation assays in Arabidopsis leaf protoplasts suggest that CST, a receptor-like cytoplasmic kinase, interacts at the plasma membrane with both EVR and HAE.\(^{19}\) As enlarged abscission zones with indistinct boundaries are a common feature of nev evr, nev serk1, nev cst and 35S::IDA flowers, it has been proposed that ectopic signaling of the IDA-HAE/HSL2 signaling pathway is responsible for the rescue of organ abscission in these flowers.\(^{5,20}\) In wild-type flowers, CST and EVR may repress cell separation by altering the localization or activity of the HAE/HSL2 receptors.\(^{5,19}\)

In support of these hypotheses, we have shown that loss of EVR, SERK1 or CST function does not restore organ shedding in either ida or hae hsl2 mutant flowers.\(^{17-19}\) However, despite similarities between the nev evr, nev serk1 and 35S::IDA phenotypes, loss of IDA activity is not sufficient to block organ shedding in either nev evr (1D) or nev serk1 flowers.\(^{18,21}\) These results suggest that the rescue of cell separation in nev evr and nev serk1 flowers is IDA-independent, and that ectopic activation of the HAE/HSL2 signaling pathway may occur either at the level of the HAE/HSL2 receptors or further downstream.

In addition to its role in modulating abscission, the EVR/ SUPPRESSOR OF BIR1 receptor-like kinase functions in plant defense responses to bacterial and fungal pathogens.\(^{22-24}\) EVR acts downstream of and may be negatively regulated by BAK1-INTERACTING RECEPTOR-LIKE KINASE1 (BIR1).\(^{22}\) Loss of BIR1 results in ectopic activation of cell death, which is rescued by mutations in EVR.\(^{22}\) The tomato ortholog of EVR, SSOBIR1, interacts with the Cf-4 and Ve1 receptor-like proteins, which do not contain cytoplasmic domains.\(^{23}\) When the Cf-4 or Ve1 receptors recognize their fungal effectors, SSOBIR1 is required as a co-receptor to transmit the signal activating the immune response.\(^{23}\) Similarly, interaction between EVR and RECEPTOR-LIKE PROTEIN30 is required to activate defense signaling against a fungal pathogen of Arabidopsis.\(^{24}\)

Disruption of NEV-mediated intracellular traffic significantly affects fruit growth as well as organ abscission (Figs. 1A, B, and 2).\(^{13}\) The regulatory regions of NEV, EVR and HAE have previously been shown to direct expression of the β-Glucuronidase (GUS) reporter gene in the fruit style.\(^{13,16,17}\) Furthermore, the regulatory regions of several of the MAP kinases shown to act downstream of HAE and HSL2 during organ abscission, also direct expression of GUS in the style.\(^{8}\) Whereas mutations in EVR suppress the organ shedding defects of nev flowers, they enhance the reduced growth of nev fruit (Figs. 1B, C, and 2).\(^{17}\) Fruit length is also reduced in the evr single mutant (Fig. 2).\(^{17}\)

To test whether HAE/HSL2 activity is required for the rescue of organ abscission and/or reduced fruit growth observed in nev evr flowers (1C), we generated nev evr hae triple (1E) and nev evr hae hsl2 quadruple (1F) mutants. Mutants were generated using the nev-3 (Ler), evr-2 (Ler), hae-2 (Col) and hsl2-1 (Col) alleles and confirmed by genotyping as previously described in Liljegren et al.\(^{13}\) Leslie et al.\(^{15}\) and Cho et al.\(^{8}\) Since fruit size is significantly reduced in the Ler ecotype due to a mutation in the ERECTA (ER) receptor-like kinase gene,\(^{26}\) all mutant plants analyzed were also er homozygous.

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**Figure 1.** Loss of HAE and HSL2 activity blocks organ abscission in nev evr flowers. (A) Floral organ abscission occurs soon after pollination in wild-type (Ler) flowers. (B) Mutations in NEV prevent organ abscission by blocking intracellular traffic. (C-E) Mutations in EVR restore organ shedding in nev evr mutant flowers (C), even if IDA (D) or HAE (E) activity is also compromised. (F) Loss of HAE and HSL2 signaling prevents organ abscission in nev evr hae hsl2 flowers. Scale bars: 1 cm. Plants were grown at 21 °C with 50% humidity and a 16-h light:8-h dark cycle.
When HAE activity alone is compromised, shedding is still rescued in nev evr flowers (1E). In contrast, when the activities of HAE and HSL2 are both lost, organ abscission in nev evr flowers is disrupted (1F). These results indicate that rescue of organ abscission in nev evr flowers is dependent on HAE and HSL2 activity.

Our results are consistent with models in which NEV-mediated regulation of intracellular traffic during organ abscission converges with the IDA-HAE/HSL2 signaling pathway at the level of the HAE/HSL2 receptors (Fig. 3). NEV may indirectly regulate HAE/HSL2 localization or activity by targeting the EVR receptor-like kinase for degradation (3A). Alternatively, NEV may promote cell separation by directly facilitating transport or recycling of HAE/HSL2 to the cell surface (3B). In both scenarios, loss of EVR activity may rescue organ abscission in nev flowers by increasing the pool of activated HAE/HSL2 receptors at the cell surface (3A,B).5,17

Recent analysis of the genetic interactions between NEV and IDA suggests that they assume independent as well as overlapping functions in regulating organ abscission.11 Two lines of evidence make a persuasive case for independent roles. Breakstrength assays, which allow comparison of the force required to remove wild-type and mutant petals over time, reveal similar, yet distinct V-shaped profiles in the nev-3 and ida-1 single mutants.21 In both mutants, petal breakstrength decreases after floral pollination, as is observed in wild type, then gradually recovers to the initial level. In nev-3 ida-1 double mutant flowers, petal breakstrength remains high over time, reflecting a more complete block in cell wall loosening than in either single mutant alone.21 Genetic analysis of an ida suppressor mutant is also consistent with NEV functioning independently, at least in part, of IDA-HAE/HSL2 signaling. Disruption of the BREVIPEDICELLUS (BP) gene, which encodes a KNOTTED1-like homeodomain transcription factor, restores organ shedding in ida and hae hsl2 flowers but does not suppress the abscission defects of nev flowers.21,22 Further insights into the relationship of BP with regard to the convergence of the NEV, EVR and HAE/HSL2 signaling pathways may be possible by examining the nev evr bp triple and nev evr bp hae hsl2 quintuple mutants. While NEV likely performs HAE/HSL2-independent roles in cell wall loosening and/or cell expansion, its function in cell separation appears to be HAE/HSL2-dependent (Fig. 3).

nev evr fruit (late stage 17; 7.4 ± 0.5 mm) are 56% the length of wild-type fruit (13.2 ± 0.5 mm) (1A,C; Fig. 2). Loss of HAE activity alone does not affect their size, as nev evr hae fruit (7.3 ± 0.5 mm) are also 56% the length of wild-type fruit (13.1 ± 0.6 mm) (1A,C,E; Fig. 2). In contrast, loss of both HAE and HSL2 activity partially restores fruit growth in nev evr plants (1C,F; Fig. 2). nev evr hae hsl2 fruit (10.2 ± 0.7 mm) are 78% the length of wild-type, 94% of evr (10.9 ± 0.5 mm), 103% of nev (9.9 ± 0.5 mm) and 140% of nev hae fruit (Fig. 2). To determine whether this effect on fruit growth is also mediated by loss of IDA activity, we analyzed the nev-3 evr-2 ida-2 triple mutant21 (1D). Although nev evr ida fruit (8.3 ± 0.6 mm) are slightly longer than nev evr fruit, this difference is not statistically significant (1C,D; Fig. 2). These results suggest that the reduced size of nev evr fruit is partially dependent on HAE and HSL2 activity but largely independent of IDA activity.

Our results raise the intriguing possibility that some of the molecular circuitry that controls organ abscission also modulates fruit development. Images of 35S::IDA and 35S::IDA hae hsl2 fruit are consistent with potential involvement of the IDA-HAE/HSL2 ligand-receptor module in regulating fruit growth. In independent studies9,11 35S::IDA fruit are depicted as smaller than wild-type fruit. Loss of HAE/HSL2 activity in 35S::IDA flowers not only blocks abscission, but also appears to substantially restore fruit growth.9,11

NEV and EVR appear to have an additive relationship in promoting fruit growth. The length of nev evr and nev evr fruit is reduced by 24, 17 and 44%, respectively, compared with wild-type fruit (Fig. 2). The partial rescue of growth in nev evr...
The model in which EVR promotes fruit growth by inhibiting HAE/HSL2 activity or localization (3C).

Since *nev evr* and 35S::IDA flowers shed their outer floral organs prematurely,12,17 a contributing factor to the reduced fruit growth observed could be that their pistils are exposed to less pollen than those of wild-type flowers. By preventing abscission, loss of HAE and HSL2 activity in *nev evr* and 35S::IDA flowers could indirectly promote fertilization and fruit growth. Analysis of the timing of abscission in *nev evr* flowers showed that organ shedding begins one day earlier (stage 15) than in wild-type flowers (stage 16).17 However, this developmental time point (stage 15) corresponds with elongation of the gynoecium above the stamens, and occurs after pollen dehiscence (stage 13) and ovule fertilization (stage 14).28,29 Furthermore, fruit growth is significantly reduced in both the *nev* and *evr* single mutants (1B; Fig. 2). Within the fruit, EVR or NEV may positively impact fertility and growth by negatively regulating HAE and HSL2 in the style (3C,D). Dissection of the features and tissue specificity of the mutant phenotypes should yield fresh insights into the respective contributions of NEV-mediated intracellular traffic and EVR receptor-like kinase signaling to fruit development.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank John Walker for kindly providing the hae-2 allele and Charles McCrory for technical assistance. This research was supported by National Science Foundation grants to Liljegren (IOS-1239311) and the Mississippi EPSCoR program (EPS-0903787).

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**Figure 3. Models of the intracellular traffic and receptor-like kinase signaling that control floral organ abscission and promote fruit growth.** (A, B) The HAE and HSL2 receptor-like kinases redundantly regulate organ abscission via activation of a MAP kinase cascade. IDA, a secreted peptide, is the proposed ligand of the HAE and HSL2 receptors. Through its interaction with the CST receptor-like kinase (not shown), EVR may modulate the timing and extent of cell separation in organ abscission zones by regulating the localization and/or activity of HAE and HSL2. By mediating intracellular traffic, NEV may promote organ abscission by either removing EVR from the cell surface to allow unimpeled HAE/HSL2 signaling (A) or by directly facilitating delivery of HAE/HSL2 to the cell surface (B). As the rescue of organ abscission in *nev evr* flowers is dependent on HAE/HSL2 activity, the NEV, EVR, and IDA-HAE/HSL2 pathways appear to converge during the cell separation phase of abscission. However, NEV likely directs some abscession-related processes, such as cell wall loosening, independently of IDA-HAE/HSL2 (dotted arrow). (C, D) NEV and EVR appear to positively regulate fruit elongation through independent pathways. Since loss of HAE/HSL2 signaling partially restores the growth of *nev evr* fruit, either EVR (C) or NEV (D) is proposed to inhibit HAE/HSL2 signaling in the style.

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