MEMBRANE TRAFFICKING AND RECEPTOR-KINASE SIGNALING EVENTS MODULATE CELL ADHESION IN *ARABIDOPSIS*

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ABSTRACT

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Membrane trafficking and receptor-kinase signaling events modulate cell adhesion in *Arabidopsis*
(Under the direction of Sarah Liljegren)

Plants shed entire organs to facilitate remodeling of their architecture and reproductive success. Organ shedding, or abscission, is a highly specialized process that involves the differentiation of abscission zones (AZs) wherein given the proper cues, cell separation is activated. In a screen for mutations that block the shedding of *Arabidopsis* floral organs, an ADP-ribosylation factor-GTPase-activating protein (ARF-GAP) was identified and named NEVERSHED (NEV). Using a NEV-specific antibody and a set of plant endomembrane markers, I found that NEV localizes to the *trans*-Golgi network and putative recycling endosome. Interestingly, mutations in *NEV* cause defects in the structure of the Golgi apparatus and extensive accumulation of vesicles adjacent to the AZ cell walls. Through a secondary screen for mutations that restore organ shedding in *nev* flowers, I identified a leucine-rich repeat receptor-like kinase (LRR-RLK), EVERSHED (EVR), that functions as an inhibitor of abscission. EVR belongs to LRR-RLK subfamily XI, which also includes the HAESA and HAESA-LIKE2 RLKs that are redundantly required for abscission. Defects in the Golgi structure in *nev* AZ cells are rescued by a mutation in *EVR*, suggesting that like NEV, EVR may regulate membrane trafficking during abscission. In addition to *EVR*, we identified the *SERK1* LRR-RLK and *CAST AWAY* receptor-like cytoplasmic kinase as inhibitors of abscission through

suppressor analysis of *nev*. Using the bimolecular fluorescence complementation assay in *Arabidopsis* leaf protoplasts, we have detected interactions between EVR, CAST AWAY and HAESA. I present here a working model that integrates the roles of membrane trafficking and cell signaling during the abscission process.

This work is dedicated to my husband, J. Mark Leslie, without whose caring support it would not have been possible.

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LIST OF ABBREVIATIONS

ARF-GAP	ADP-ribosylation factor GTPase-activating protein
AZ	abscission zone
BAK1	BRI1 ASSOCIATED KINASE 1
BFA	brefeldinA
BRI1	BRASSINOSTEROID INSENSITIVE 1
CIP	calf intestinal alkaline phosphatase
CST	CASTAWAY
EVR	EVERSHED
ER	ERECTA
GFP	green fluorescent protein
GUS	β-glucuronidase
LRR	leucine-rich repeat
LRR-RLK	leucine-rich repeat receptor-like kinase
FLS2	FLAGELLIN-SENSITIVE 2
HAE	HAESA
HSL2	HAESA-LIKE 2
IDA	INFLORESCENCE DEFICIENT IN ABSCISSION
KD	kinase domain
NEV	NEVERSHED
PEPR1	PEP1 RECEPTOR 1
PM	plasma membrane
PMB	paramural body

RLK	receptor-like kinase
SEM	scanning electron micrograph
SERK1	SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE 1
SOBIR1	SUPPRESSOR OF BIR1 1
TEM	transmission electron micrograph
TGN	*trans*-Golgi network
WT	wildtype
YFP	yellow fluorescent protein

CHAPTER 1

INTRODUCTION

The first section of this introduction, "Overview of Plant Organ Abscission", was originally published as the book chapter "Organ Abscission" (Leslie et al., 2007) in Plant Cell Separation and Adhesion (ed J. Roberts and Z. Gonzalez-Carranza). Research in the field of organ abscission has been productive, and the succeeding section "The regulation of floral organ abscission" introduces modulators of cell separation that have been identified in the past three years in the model plant *Arabidopsis thaliana*.

SECTION I. OVERVIEW OF PLANT ORGAN ABSCISSION

At certain stages in the life cycle of many multicellular organisms, targeted separation of cells at predetermined sites enables the shedding of entire organs. Plants undergo abscission of many diverse types of organs, such as leaves, flowers, fruit, seeds, bark, and branches (Addicott, 1982). While less common, separation events in animals that involve the loss of entire organs include antler shedding in deer, skin shedding in snakes, and tail or arm autotomy in lizards and starfish (Rolf and Enderle, 1999; Alibardi, 2000; Wilkie, 2001; Alibardi, 2005). Each of these events occurs within specific tissue layers or autotomy planes, with some requiring dissolution of cell adhesion similar to organ abscission in plants.

The agronomical benefits of understanding and manipulating organ abscission in plants are well known. Since the advent of agriculture, humans have selected for crop varieties with desirable abscission traits. According to the "Teosinte Hypothesis", the selection of four key traits within the past 6000 to 10,000 years was required for the domestication of maize from the primitive teosinte grass (Doebley, 2004). Seed dispersal from teosinte ears is facilitated by the initial separation of eight fruitcases from one another. Each fruitcase then disperses its seed individually. A significant feature of domesticated maize is that ears remain intact until harvest; thus it is hypothesized that a mutation(s) disrupting abscission layer formation between teosinte fruitcases was one of the first traits selected for by our farming ancestors (Doebley, 2004). Much more recently, the spontaneously arising *jointless* mutation was found to block fruit abscission in tomato (Butler, 1936). Since its discovery, this trait has been introgressed into a variety of tomato cultivars, providing a modern example of human-directed evolution of crop species. Today, we aim to increase our understanding of abscission as a unique developmental process as well as an agronomically important event.

Organ shedding in plants facilitates several key processes, including **(1) Reproduction:** Cell separation events in plants are orchestrated to drop ripened fruit, disperse seeds from opened fruit, release spores from fern leaf sori, scatter leaves or branches capable of vegetative propagation, and abscise leaves that obstruct pollination. **(2) Recycling:** Many organs are shed after they have served their biological function. For example, senescent leaves fall from deciduous trees and floral organs are released after fertilization of the flower. These organs are large mineral sinks; thus shedding of leaves and flowers allows minerals to be redirected to other organs. During the

photoperiod-induced senescence of aspen (*Populus tremula*) leaves, many nutrients, such as nitrogen and phosphorous, are redirected out of the leaves prior to their abscission (Keskitalo et al., 2005). **(3) Remodeling:** Entire branches are often shed to allow for directed growth of a plant. **(4) Defense:** As a guard against the spreading of disease, pathogen infected leaves, branches, and bark can be shed. Organ shedding can also produce protective scars or spines that deter pathogen attack. **(5) Competition:** Frequently, a plant will generate more than enough floral buds to be fertilized. Once sufficient fertilization has occurred, a portion of the remaining buds or developing fruit will be dropped to ensure optimized growth of a subset. In addition, leaves from certain plants contain protective chemicals that can inhibit the growth of other plants in the soil where the "poisonous" leaves drop and decompose.

Organ abscission events may be diverse in function, yet all events require the proper formation of an abscission zone (AZ) at the base of the organ to be shed. The AZ is morphologically distinct from neighboring cells and is often visible to the naked eye (Addicott, 1982). At the microscopic level, AZ cells are smaller than surrounding cells, and more cytoplasmically dense. The size of the AZ varies from as few as 1-2 rows of cells in the model plant *Arabidopsis thaliana* to as many as 50 rows in *Sambucus nigra* (Taylor et al., 1994; Bleecker and Patterson, 1997). Diverse environmental cues such as drought, nutrient deficiency, and pathogen attack can trigger the initiation of abscission at these sites (Addicott, 1982; Taylor and Whitelaw, 2001). Developmentally, the timing of abscission is coordinated with fertilization, fruit maturation, and senescence depending on the particular organ to be shed.

Studies of AZ morphology together with physiological, genetic, and biochemical experiments have led to a general model of the organ abscission process across plant species (Figure 1.1). First, cells at the future site of detachment must receive and respond to differentiation signals. Differentiation of the AZ is dependent upon the proper developmental patterning of the organ itself. Second, activation of the AZ, allowing the initiation of cell separation, is triggered by developmental and hormonal cues. Third, the organ is abscised when the middle lamella between AZ cells loses integrity due to the activity of cell wall modifying and hydrolytic enzymes. Finally, protective scarring is evident at the site of organ detachment on the plant body. Scarring is associated with expansion of the AZ cells that remain behind—it remains to be determined whether cell expansion may itself play a role in the separation process.

In this chapter we discuss how genetic analysis in model plants has contributed to our current understanding of the organ abscission process as outlined above (Figure 1.2; Table 1.1). The introduction of *Arabidopsis* as a model organism has been particularly useful for the isolation of genes involved in floral organ abscission and seed dispersal (Figure 1.2A and B). With the advances in genome analysis in *Lycopersicon esculentum* (tomato), the classic *jointless* mutants have recently been molecularly characterized, suggesting that tomato will continue to be an ideal plant to study the abscission of fruit (Figure 1.2C). In addition, the recent isolation and characterization of mutations that affect seed abscission in *Pisum sativum* (pea) and flower, leaf and cotyledon abscission in the Australian crop plant, *Lupinus angustifolius*, point toward the use of these plants in future genetic experiments (Figure 1.2D). Since the abscission process appears to be quite similar across species, genes that are essential for the abscission of particular organs

in *Arabidopsis*, tomato, pea, and *L. angustifolius* likely control abscission of these organs in other species as well.

A. SETTING THE STAGE: PATTERNING AND DIFFERENTIATION

Differentiation of an organ AZ is thought to be dependent on the proper patterning of plant organs from meristematic tissue. So far, the characterization of mutants in *Arabidopsis*, tomato, and *L. angustifolius* that affect both organ patterning and AZ differentiation has provided evidence to support this hypothesis (Mao et al., 2000; Clements and Atkins, 2001; Pinypopich et al., 2003; Hepworth et al., 2005; Norberg et al., 2005). Analysis of a pea mutant in which differentiation of the seed AZ is affected, apparently without altering development of the seed stalk (von Stackelberg et al., 2003), suggests that these two processes can be uncoupled and that proper organ patterning precedes AZ formation.

BLADE ON PETIOLE1 and *2* establish lateral organ polarity

In *Arabidopsis* flowers, the sepals, petals and stamens are shed after pollination at distinct AZs located at the sites of organ attachment to the flower pedicel (see Figure 1.2A). Recent studies suggest that the redundant BLADE-ON-PETIOLE1 (BOP1) and BOP2 proteins may contribute to the patterning of *Arabidopsis* floral organs prior to abscission (Hepworth et al., 2005; Norberg et al., 2005). Single loss-of-function alleles of *BOP1* or *BOP2* do not show any phenotypic differences compared to wild type;

however, floral organ shedding is completely blocked in *bop1 bop2* double mutant flowers. In addition to abscission defects, *bop1 bop2* floral organs and leaves display alterations in organ patterning. In *bop* flowers, the abaxial sepal is replaced by one or two sepal/petal mosaic organs, and the first and second whorl organs cluster around the adaxial side of the flower. In *bop1 bop2* leaves, blade tissue arises aberrantly on the petiole, resulting in multi-lobed leaves. Although the proximal patterning of these lateral organs is disrupted, the distal regions appear to be unaffected by loss of *BOP* activity. *bop1 bop2* patterning defects also include ectopic outgrowths at the bases of leaves, cotyledons, sepals and petals.

BOP1 and *BOP2* encode proteins with a BTB/POZ domain and four ankyrin repeats (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). The BTB/POZ domain, named for the *Drosophila* Broad-Complex, Tramtrak and Bric-a-brac transcriptional regulators and multiple poxvirus zinc-finger proteins in which it was first identified, can facilitate homodimerization as well as interactions with other proteins that may or may not contain a BTB/POZ domain (Stogios et al., 2005). Ankyrin repeats are also predicted to mediate protein-protein interactions. Interestingly, the semi-dominant *bop1-1* allele appears to encode a dominant negative BOP1 isoform with aberrant protein interactions (Ha et al., 2003; Ha et al., 2004). Although abscission defects have not been reported for *bop1-1* plants, similarities in patterning defects between *bop1 bop2* and *bop1-1* plants suggest that the *bop1-1* mutant protein may interfere with the activity of a BOP1-BOP2 heterodimer or perhaps with the activity of shared target proteins (Ha et al., 2004). Of the other 75 BTB/POZ domain-containing proteins in *Arabidopsis*, BOP1 and BOP2 are most closely related to NONEXPRESSOR OF PR GENES1 (NPR1) (Cao et

al., 1997; Ha et al., 2004; Stogios et al., 2005). NPR1, which lacks a canonical DNA-binding domain, mediates transcriptional changes during systemic acquired resistance through interactions with members of the TGA family of bZIP transcription factors (reviewed in Dong, 2004). Thus, it is likely that BOP1 and BOP2 interact with or activate unique transcription factors to promote floral organ patterning and abscission. Indeed, BOP interactions with the TGA transcription factor PERIANTHIA likely control the formation of four sepals in the first whorl rather than five (Hepworth et al., 2005).

Expression analyses indicate that *BOP1* and *BOP2* are expressed in similar patterns during leaf and flower development (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). During normal vegetative growth, *BOP1* and *BOP2* are expressed in the proximal half of rosette leaves. During reproductive development, *BOP* expression is seen throughout stage 1 and 2 floral primordia. After stage 3, *BOP* expression becomes restricted to the bases of developing sepals, petals, stamens and carpels. In mature flowers, the *BOP* genes are strongly expressed at the bases of sepals, petals and stamens, in domains overlapping the floral organ AZs. This expression profile and the *bop* mutant phenotype described above suggest that BOP1 and BOP2 might function during the organ patterning required for AZ differentiation or during a later stage of the abscission process.

Early BOP activity at the bases of developing lateral organs may specify a proximal fate by restricting the expression domains of two sets of transcription factors (Ha et al., 2004; Norberg et al., 2005). In wild type plants, BOP activity excludes expression of the shoot meristem *KNOX* transcription factors *BREVIPEDICELLUS* (*BP)*, *KNAT2* and *KNAT6* from developing leaves while limiting expression of the C_2H_2 zinc

finger transcription factor *JAGGED (JGD)* to a distal domain (Ha et al., 2003; Dinneny et al., 2004; Ohno et al., 2004; Norberg et al., 2005). When BOP function is compromised, expression of *BP, KNAT2*, *KNAT6*, and *JAG* expands into the proximal region of developing leaves (Ha et al., 2003; Norberg et al., 2005). Since the roles of many organ polarity factors are conserved between leaves and floral organs (reviewed in Bowman et al., 2002), it is likely that BOP function is similarly conserved. Indeed, *JAG* expression was found to expand into the proximal domains of *bop* mutant floral organs as in leaves, although shedding is not restored in a *jag bop1 bop2* triple mutant (Nordberg et al., 2005). Another C_2H_2 zinc finger transcription factor, *JAGGED-LIKE* (*JGL*), is thought to act redundantly with *JAG* (Dinneny et al., 2004; Ohno et al., 2004; Norberg et al., 2005), and represents another potential target of BOP regulation. Additional molecular and genetic analyses are now required to determine whether this model of BOP-dependent establishment of proximal-distal polarity also applies to floral organs, and can explain the abscission defects of *bop* flowers. It is not yet known whether *BP*, *KNAT2*, *KNAT6,* and *JGL* are ectopically expressed in the proximal regions of *bop* floral organs. Characterization of higher order mutant combinations such as the *jag jgl bop1 bop2* quadruple and *bp knat2 knat6 bop1 bop2* quintuple mutants should reveal whether ectopic expression of these two sets of transcription factors prevents the establishment of floral organ boundaries in *bop* mutant flowers, and thus interferes with AZ development. An alternative, but not exclusive, possibility is that BOP1 and BOP2 may independently act to establish a proximal fate by regulating a third set of candidate target genes with proximal-specific expression patterns in lateral organs, such as *LATERAL ORGAN BOUNDARIES (LOB)* and *LATERAL ORGAN JUNCTIONS (LOJ)* (Shuai et al., 2002;

Prasad et al., 2005). Although *lob* and *loj* single mutants do not display any defects in floral organ shedding, both of the affected genes are predicted to act redundantly. Analysis of *LOB* and *LOJ* expression in *bop* mutant floral organs may reveal yet another link between polarity establishment, pattern formation and organ differentiation, all of which must be properly coordinated for organ abscission to occur.

SEEDSTICK **promotes proper development of the seed stalk**

In dry dehiscent fruit, like that of *Arabidopsis*, two sequential cell separation events are required for seed dispersal (see Figure 6.1B). First, opening of the fruit along the margins of its walls, or valves, exposes the seed (fruit dehiscence is discussed in greater detail in Chapter Ten). Second, cell separation within seed AZs releases the seed from the fruit replum (Figure 1.2B). Seeds are attached to the replum by stalks called funiculi. The seed AZ, a thin layer of distinctly small cells of the funiculus, immediately adjacent to the developing seed, differentiates after fertilization (Pinypopich et al., 2003). A MADS domain transcription factor, *SEEDSTICK* (*STK*), is now known to be required for seed abscission. Characterization of the *stk* mutant has revealed that the entire funiculus is enlarged due to cellular overgrowth and overproliferation. Thus, *STK* may be indirectly required for proper AZ differentiation through its regulation of funiculus development (Pinypopich et al., 2003). Identification of the transcriptional targets of *STK* may lead to more specific regulators of AZ differentiation. In contrast to the *stk* mutant, differentiation of the seed AZ appears to be specifically blocked in the *development funiculus* (*def*) mutation in pea (von Stackelberg et al., 2003). Molecular

characterization of the *def* locus should reveal another key factor controlling seed abscission.

JOINTLESS is essential for differentiation of the pedicel abscission zone

Tomato is an important model plant to study differentiation of the flower pedicel AZ. Unlike plants such as *Arabidopsis* that only release flower parts, entire tomato flowers and fruit are shed by separation at a single point on the floral pedicel (see Figure 1.2C). Cells in the pedicel AZ are anatomically distinct, and form a visible 'joint'. In *jointless* (*j*), a spontaneous mutation discovered in a tomato crop variety, the pedicel AZ fails to differentiate (Butler, 1936). This mutation has proven to be agronomically desirable and is widely cultivated since the resulting "stemless" tomato fruit are more easily harvested and transported. In addition to preventing abscission, the *j* mutation affects the transition between vegetative and reproductive growth. In wild type plants, inflorescence meristems arise from vegetative or sympodial meristems then transition into floral meristems, whereas *j* inflorescence meristems often revert back to sympodial meristems after initiating the growth of only a few flowers (Szymkowiak and Irish, 1999). Recently, it has been proposed that the lack of AZ differentiation in *j* pedicels may be an indirect effect of disrupted inflorescence development (Szymkowiak and Irish, 2005).

J was found to correspond to *LeMADS,* a MADS domain transcription factor (Mao et al., 2000). Analysis of the *j* locus revealed a 939 base-pair (bp) deletion including the 5' UTR and the first 33 bp of the MADS-box region; a TAPIR (tomato anionic peroxidase inverted repeat) transposable element was likely responsible for this

deletion (Mao et al., 2000; Mao et al., 2001). Although the *Arabidopsis* MADS box family includes two genes closely related to *J—SHORT VEGETATIVE PHASE* (*SVP*/*AGL22*) and *AGAMOUS-LIKE24 (AGL24*)—neither *SVP* nor *AGL24* have known roles in abscission (Hartmann et al., 2000; Yu et al., 2004). *AGL24* is thought to promote an inflorescence meristem fate and *SVP* to act in the autonomous flowering time pathway repressing the transition from vegetative to reproductive growth.

To investigate how J affects both meristem identity and the differentiation of multiple tissue layers into a properly formed pedicel AZ, Szymkowiak and Irish (1999) generated chimeric tomato plants with L1, L2 and L3 meristem layers of wildtype (*J*) and mutant (*j*) origins. Their work demonstrated that a *J* signal from the L3 tissue is sufficient to maintain inflorescence meristem identity, and specify pedicel AZ formation, in plants with overlaying L1 and L2 tissues of *j* origin. This result suggests that the *J* signal can spread radially out of the L3 tissue layer to coordinate formation of the AZ. In addition, the authors found that the *J* signal cannot spread laterally within a tissue layer. When *J* and *j* cells are juxtaposed in the L3 layer, wild type and mutant sectors are seen in the L1 and L2 layers of the tomato pedicel corresponding to the underlying genotype. Only those tissues overlying wild type *J* cells differentiate into AZ pedicel tissue. This work clearly indicates that J-mediated cell-cell signaling is required for inflorescence patterning and AZ differentiation, and raises the fundamental question of how the J signal is communicated. Further studies are also necessary to explore the relationship between meristem identity and AZ differentiation, particularly since *J* is expressed at high levels in inflorescence and flower meristems but has not been detected in the pedicel itself (Szymkowiak and Irish, 2005).

jointless-2 maps near a predicted transcriptional regulator

A second non-allelic mutation in tomato, *jointless-2* (*j-2*), also prevents differentiation of the pedicel AZ. Extensive linkage mapping and sequencing has established that the *j-2* mutation is located within the centromeric region of chromosome 12 (Budiman et al., 2004; Yang et al., 2005). Yang et al (2005) have recently identified a candidate gene, *ToCPL1*, which is closely related to *Arabidopsis C-terminal phosphatase-like gene 3 (AtCPL3)*. *AtCPL3* has been shown to regulate gene expression in response to plant stress, and play a role in growth and development (Koiwa et al., 2002). Confirmation that *J-2* corresponds to *ToCPL1* is currently waiting on complementation of *j-2* mutant plants (Yang et al., 2005).

The predicted ToCPL1 and AtCPL3 proteins contain a conserved catalytic domain characteristic of the CPL family and a BRCA1 carboxy-terminal (BRCT) domain. FCP1, a closely related yeast carboxy-terminal domain (CTD) phosphatase, associates with and dephosphorylates the CTD of RNA polymerase II (Pol II) through the interaction of its BRCT domain with the RAP74 subunit of the TFIIF complex (Archambault et al., 1998). TFIIF is part of the Pol II complex during transcription elongation; dephosphorylation of Pol II by CTD phosphatases serves as a release and recycling signal for the complex (Cho et al., 1999). If the proposed identity of *J-2* is confirmed to be that of *ToCPL1*, it may be required to repress transcription of a specific suite of genes for pedicel AZ development to occur. Alternatively, a region of homology between AtCPL3 and the yeast CAPPING ENZYME SUPPRESSOR protein suggest a link to mRNA capping (Schwer and Shuman, 1996; Koiwa et al., 2002). Identification of

the *in vivo* substrate(s) of ToCPL1 should further define its function during pedicel AZ development. In the future, chimeric and expression studies to investigate whether J-2 acts in a cell or non-cell autonomous manner, as well as genetic analysis of the interactions between *J* and *J-2*, should be particularly informative.

B. COMMUNICATION INVOLVED IN CELL SEPARATION

Around the turn of the 20th century, scientists were intrigued by the defoliating effect of illuminating gas upon nearby shade trees. Ethylene, a component of this coal-manufactured gas, was soon shown to be the "poisoning" agent as well as a potent inducer of the "triple response" in dark-grown (etiolated) seedlings (Neljubow, 1901; Doubt, 1917). When etiolated seedlings are exposed to ethylene, hypocotyls and roots fail to elongate, hypocotyls thicken, and the apical hook shows exaggerated curving—a phenotype reminiscent of the stunted and twisted plants growing near street lamps in 19th century cities (Neljubow, 1901). Since then, ethylene, the simplest of plant hormones, has been shown to affect a multitude of plant processes including germination, fruit ripening, leaf and flower senescence, pathogen defense, stress response, and abscission (Abeles et al., 1992). In support of an *in vivo* role for ethylene in developmental events, disruption of the endogenous biosynthesis of ethylene has been found to delay abscission, ripening, and senescence (Ecker and Theologis, 1994).

The role of small, diffusible hormones such as ethylene in regulating the timing of abscission has been studied in depth. Early experiments with bean and cotton leaf

explants, in which controlled amounts of hormone were applied to the stem of the cut leaf, support a model in which ethylene promotes and auxin delays cell separation at the AZ (reviewed in Sexton and Roberts, 1982; Brown, 1997). Following the differentiation of organ AZ cells, there are two distinct phases of hormone sensitivity prior to abscission. During the first phase, a constant flux of auxin into the AZ is thought to inhibit cell separation. Exogenous ethylene application during this time is insufficient to promote abscission, suggesting that AZ cells are largely insensitive to ethylene. Just prior to abscission, during the second phase, hormone sensitivities are reversed—AZ cells become competent to respond to ethylene whereas auxin can no longer inhibit cell separation. Continual exposure to ethylene is required to accelerate cell separation (Sexton and Roberts, 1982; Taylor and Whitelaw, 2001). Studies such as these point out the importance of ethylene in controlling the timing and speed of abscission; however, no experiment has convincingly shown that ethylene perception and signaling are required for abscission (reviewed in Patterson, 2001). The recent identification of several ethylene-independent genes required for the proper initiation of abscission supports a model in which ethylene-dependent and -independent pathways work to promote abscission (Butenko et al., 2003; Patterson and Bleecker, 2004). Thus, the goal of this section is to describe the genes from each of these pathways that are known be involved in the abscission process.

(a) Involvement of the ethylene signaling pathway

Screens for *Arabidopsis* seedlings that are defective in the "triple response" have been extremely successful in identifying genes of the primary ethylene signaling pathway (reviewed in Alonso and Stepanova, 2004; Guo and Ecker, 2004). A subset of the genes required for ethylene sensitivity, including *ETHYLENE RECEPTOR1* (*ETR1)*, *ETHYLENE INSENSITIVE2* (*EIN2*), and members of the *EIN3* transcription factor family, are implicated in the proper timing of floral organ abscission (Bleecker and Patterson, 1997; Tieman et al., 2001; Patterson and Bleecker, 2004). In *Arabidopsis*, turgid floral organs are normally shed just following pollination. Although signs of senescence are evident in wild type floral organs prior to abscission (Fang and Fernandez, 2002), dominant gain-of-function and recessive loss-of-function mutations in *ETR1* and *EIN2*, respectively, block floral organs from shedding until after sepals and petals have begun to wither (Bleecker and Patterson, 1997; Patterson, 2001; Patterson and Bleecker, 2004). AZ formation is normal in these plants, suggesting that ETR1 and EIN2 play specific roles in the initiation of abscission rather than earlier stages (Patterson and Bleecker, 2004). Tomato homologs of ethylene signaling genes are also implicated in cell separation; *LeETR3* and the *EIN3-like* genes, *LeEIL1*, *2*, and *3*, are all required for proper coordination of abscission within the timeline of development. It is probable that most, if not all, components of the primary ethylene signaling pathway will likewise be shown to affect abscission.

***ETHYLENE RECEPTOR1* links ethylene perception with abscission initiation**

ETR1 was the first gene from the ethylene signaling pathway to be directly implicated in *Arabidopsis* floral organ abscission. Despite the normal appearance of adult *etr1* plants, they are insensitive to ethylene-induced senescence, and flowering and abscission are delayed (Bleecker et al., 1988; Chao et al., 1997; Bleecker and Patterson, 1997). *ETR1* is broadly expressed in *Arabidopsis*, with highest transcript levels in flowers, particularly the anthers and developing carpels (Hua et al., 1998). The *etr1* allele was mapped to a region that encodes a protein with domains similar to both the receiver and sensor proteins of the "two-component" histidine kinase receptors in bacteria (Chang et al., 1993). Since then, ETR1 has been localized to the endoplasmic reticulum (ER) membrane, where the receptor is predicted to bind ethylene at its N-terminus and transmit a signal to the nucleus through a MAPK-like cascade (Chen et al 2002; reviewed in Guo and Ecker, 2004). The *Arabidopsis* ethylene receptors have been assigned to two subfamilies based upon sequence similarity: (1) *ETR1* and *ERS1* and (2) *ETR2, EIN4,* and *ERS2*. In screens for ethylene-insensitivity, only dominant gain-of-function mutations have been isolated for these receptor genes, suggesting that genetic redundancy masks any single mutant loss-of-function phenotypes (Hua and Meyerowitz, 1998; Hua et al., 1998). In support of this hypothesis, higher order combinations of loss-of-function ethylene receptor mutants result in stronger ethylene responses than those observed for wild type or any single loss-of-function allele (Hua and Meyerowitz, 1998; Cancel and Larson, 2002). Both the triple *etr1 etr2 ein4* and quadruple *etr1 etr2 ein4 ers2* loss-of-function mutants display a constitutive response in the absence of ethylene (Hua and Meyerowitz, 1998). The effects of higher order combinations of either gain- or

loss-of-function mutations upon the timing of floral organ shedding have not been investigated.

The *NEVER-RIPE* ethylene receptor promotes abscission in tomato

The semi-dominant, ethylene-insensitive *Never-ripe* (*Nr*) mutant was isolated in tomato half a century ago, and found to affect the ethylene receptor *LeETR3* (Rick and Butler, 1956; Wilkinson et al., 1995; Lashbrook et al., 1998b). Tomato cultivars with the *Nr* mutation are deficient in fruit ripening, senescence, and abscission at the floral pedicel (Rick and Butler, 1956; Lanahan et al., 1994). Whereas 87.5% of unfertilized, wild type flowers of the Pearson tomato cultivar abscise after 20 days on the plant, less than 4% of unfertilized Pearson *Nr* flowers abscise after the same amount of time. Furthermore, ethylene exposure does little to accelerate this process in flower explants (Lanahan et al., 1994). Antisense inhibition of the *Nr* allele has confirmed that *Nr* plays an inhibitory role in ethylene signal transduction, akin to its *Arabidopsis* homologs (Hackett et al., 2000). The ethylene receptor family in tomato has four additional genes, *LeETR1*, *2*, *4*, and *5; LeETR4* appears to be partially redundant with *Nr* in regulating fruit ripening (Tieman et al., 2000).

***Never-ripe2* and *Green-ripe* may represent mutations in a novel ethylene signaling gene**

Two partially ethylene-insensitive, dominant mutations, *Never-ripe 2* (*Nr-2*) and *Green-ripe* (*Gr*), confer defects in tomato fruit ripening (Barry et al., 2005). Both mutants also show a subtle delay in floral organ shedding as compared to the slight delay

observed in the *Nr* mutant. Like *Nr*, *Nr-2* and *Gr* also affect abscission at the floral pedicel; wild type tomato explants abscise almost all flowers after 48 hours of ethylene treatment whereas nearly isogenic *Nr, Nr-2* and *Gr* explants abscise only 60%, 80%, and 95% of flowers, respectively, after 72 hours of ethylene treatment. The roots of *Nr-2* and *Gr* etiolated seedlings exhibit weak ethylene insensitivity, although hypocotyl elongation is not affected, suggesting that the affected gene(s) contributes to a specific subset of ethylene responses involved in fruit ripening, flower senescence, abscission, and root elongation (Barry et al., 2005). *Nr-2* and *Gr* were both mapped to the same 2-centiMorgan region of chromosome 1, and are predicted to disrupt a novel gene in the ethylene signaling pathway.

ETHYLENE-INSENSITIVE2, a transmembrane protein of unknown function, is required for the proper timing of abscission

To date, *EIN2* is the only gene functioning downstream of the ethylene receptors that has been reported to affect the timing of *Arabidopsis* floral organ abscission (Patterson and Bleecker, 2004). *ein2* mutants exhibit a strong ethylene-insensitive phenotype, as etiolated seedlings are deficient in the "triple response", and flower senescence and organ abscission is not accelerated by treatment with ethylene (Guzmán and Ecker, 1990; Chao et al., 1997; Patterson and Bleecker, 2004). *EIN2* was found to encode a protein with two functional domains: an N-terminal cluster of twelve predicted transmembrane helices with homology to the Nramp class of metal ion transporters and a hydrophilic C-terminus that may facilitate protein-protein interactions (Alonso et al., 1999). Membrane bound EIN2 is predicted to function in transmission of the ethylene

signal from the ER-bound receptor(s) to transcription factors in the nucleus; however, the specific subcellular localization of EIN2 has not yet been determined (Alonso et al., 1999). Delayed flower senescence has also been reported for antisense lines of a EIN2 homolog in Petunia (Shibuya et al., 2004). Future studies in *Arabidopsis* should increase our understanding of the *EIN2*-mediated ethylene response and abscission.

***EIN3-LIKE1*, *2* and *3* may induce the transcription of abscission-related genes**

While a delay in floral organ shedding has not yet been reported for the *Arabidopsis ein3* mutant, genes from the tomato *EIN3* family are known to be involved in the ethylene-mediated initiation of abscission (Tieman et al., 2001). Knocking down the expression of all three tomato *EIN3-like* genes—*LeEIL1*, *2* and *3*—by non-specific antisense RNA (*LeEIL-AS)* causes plants to become ethylene-insensitive. Treatment of wild type tomato plants with ethylene induces premature abscission of flowers prior to fruit development; however, when *LeEIL-AS* plants with the most severe reduction in *LeEIL* expression are treated with ethylene, flower abscission is delayed until after fruit development begins (Tieman et al., 2001). Recently, it was found that overexpression of *LeEIL1-GFP* can partially compensate for the ethylene-insensitivity of *Nr* plants (Chen et al., 2004). This result suggests that *LeEIL1* acts downstream of *NR* (*LeETR3*) and that the primary ethylene signaling pathway is conserved from tomato to *Arabidopsis.* Like the *Arabidopsis* EIN3 family of transcription factors, the predicted LeEIL proteins contain nuclear localization signals that are most likely responsible for movement of LeEIL1-GFP chimeric protein to the nucleus (Chen et al., 2004). Since *LeEIL1*, *2* and *3* appear to be constitutively expressed in seedlings, leaves, flower buds and fruit (Tieman

et al., 2001), interacting proteins may be required to impart specificity to LeEIL activity during abscission.

(b) Ethylene Independent Avenues to Abscission

INFLORESCENCE DEFICIENT IN ABSCISSION encodes a putative signaling ligand essential for abscission

Characterization of an *Arabidopsis* T-DNA mutant led to the discovery of the first gene required for floral organ shedding*, INFLORESCENCE DEFICIENT IN ABSCISSION* (*IDA*; Butenko et al., 2003). Although *ida* mutant flowers develop and senesce normally, the sepals, petals and stamens remain attached indefinitely. In contrast to the ethylene-insensitive mutants, *ida* plants exhibit a wild type response to ethylene during all stages of development tested except floral organ shedding. Whereas exogenous application of ethylene accelerates the senescence and abscission of wild type flowers, *ida* flowers treated with ethylene senesce more rapidly but do not shed their organs (Butenko et al., 2003).

IDA encodes the founding member of a new class of putative signaling ligands, which include a predicted signal peptide at the N-terminus, and a conserved 12 amino acid sequence of unknown function at the C-terminus. Although receptors have yet to be identified for IDA or any of the IDA-like ligands identified in *Arabidopsis* and other plants, it has been shown that a chimeric IDA-GFP fusion protein transiently expressed in onion epidermal cells localizes to the extracellular space and/or cell wall, supporting its proposed function as a secreted ligand (Butenko et al., 2003).

IDA is thought to act after differentiation of the floral AZs, during the stage of active cell separation. An *IDA* promoter::ß-glucuronidase (GUS) reporter gene is strongly expressed in floral AZs, first appearing at stage 15 just prior to organ abscission at stage 16 (Butenko et al., 2003). Measurements of the force required to remove petals from sequentially older flowers of the *ida* mutant show an initial decrease, as in wild type, followed by a striking increase in later *ida* flowers (Butenko et al., 2003). Scanning electron microscopy (SEM) of *ida* and wildtype flowers after petals are manually removed mirror these breakstrength data. Initially, the petal AZ cells of *ida* and wild type flowers have the same broken appearance, however, at the time of abscission, wild type cells expand and become fully rounded whereas *ida* cells are less rounded (Butenko et al., 2003). At later timepoints, after abscission has occurred in wild type flowers, *ida* AZ cells again exhibit a broken appearance, as if the connections between them had been repaired. Taken together, these results suggest that IDA signaling enables cell separation within the AZ, either by promoting secretion of hydrolytic enzymes or by inhibiting repair of the middle lamella and cell walls (Butenko et al., 2003).

HAESA, a receptor-like kinase, may perceive an abscission signal

HAESA (HAE) belongs to the leucine-rich repeat (LRR) class of receptor-like protein kinases (RLKs). Proteins within this class are highly divergent in sequence; however, all are predicted to contain an extracellular ligand-binding domain, a transmembrane hydrophobic domain, and a cytoplasmic serine-threonine protein kinase domain (Shiu and Bleecker, 2001). Appropriate ligand binding is thought to trigger autophosphorylation of the RLK and/or phosphorylation of intracellular proteins,

affecting a downstream signaling pathway(s). Approximately 200 LRR-RLKs are annotated within the *Arabidopsis* genome (Tarutani et al., 2004); HAE (previously known as RLK5) was among the first for which biochemical studies were conducted and is localized to the plasma membrane (Horn et al., 1994; Stone et al., 1994; Jinn et al., 2000). When *HAE* expression was knocked down in transgenic plants by antisense suppression, floral organ abscission is blocked (Jinn et al., 2000). Since *HAE* antisense RNA (*HAE-AS*) may have also knocked down the expression of closely-related RLKs, it will be necessary to analyze single loss-of-function mutants of *HAE* to confirm its function in abscission. As in the *ida* mutant, the floral organs of *HAE-AS* plants senesce normally yet completely fail to abscise (Jinn et al., 2000). Considering the similarities between these phenotypes, it will be intriguing to determine whether these genes function in the same pathway, perhaps with HAE functioning as a receptor for IDA (Butenko et al., 2003).

The expression profile of *HAE* is consistent with its predicted role in floral organ abscission. Jinn et al. (2000) used antisense RNA *in situ* hybridization and a *HAE::GUS* reporter construct to show that *HAE* is first expressed at the bases of the floral organs at stage 14, when flowers are undergoing pollination. Since the expression pattern of *HAE* is not affected by disrupting the ethylene signaling pathway, *HAE* is predicted to function in an ethylene-independent pathway to abscission (Jinn et al., 2000). Expression of *HAE* is also detected in leaves at the base of the petioles, where the leaf attaches to the plant stem. Since *Arabidopsis* rosette leaves are not shed, this may represent an evolutionarily conserved expression pattern (Jinn et al., 2000).

Two additional *Arabidopsis* LRR-RLKs, RLK902 and RLK1, which share 75% amino acid identity overall, may also be involved in floral organ shedding (Tarutani et al., 2004). RNA blot analysis of these genes shows the highest levels of expression in floral tissue, and the promoters of *RLK902* and *RLK1* drive GUS reporter expression in floral AZs. However, single *rlk902* and *rlk1* mutants, as well as the double *rlk902 rlk1* mutant, do not display a detectable phenotype (Tarutani et al., 2004). Since LRR-RLKs have been shown to heterodimerize with other receptor-like proteins (Jeong et al., 1999), it will be interesting to determine whether RLK902 and/or RLK1 interact with HAE. Further characterization of LRR-RLK family members, and the identification of potential HAE-interacting proteins (Jinn et al., 2000), should be particularly informative in dissecting the complex signaling pathways that promote abscission.

DELAYED ABSCISSION **mutations uncouple the initiation of abscission from ethylene signaling**

Recently, a set of three new *Arabidopsis* mutants—*dab1, dab2* and *dab3*—was isolated in a screen for T-DNA lines with delayed floral organ abscission (Patterson and Bleecker, 2004). Each of the *dab* mutants exhibits a normal response to ethylene, suggesting that the initiation of abscission can be uncoupled from ethylene perception. In comparison to the ethylene-insensitive mutants *etr1* and *ein2*, a longer delay in shedding is seen for all three *dab* mutants (Patterson and Bleecker, 2004). Although the sepal, petal and stamen AZs of *dab* flowers appear morphologically normal by light microscopy, SEM has revealed that *dab2* AZ cells elongate irregularly (Patterson and Bleecker, 2004). Since the role of cell expansion during abscission is currently unclear, it

should be particularly interesting to investigate this aspect of the *dab2* phenotype. Slight variations between the *dab1, dab2* and *dab3* phenotypic profiles, as detected by SEM and petal breakstrength calculations, suggest that these mutations disrupt genes with unique functions in cell separation (Patterson and Bleecker, 2004). Thus, mapping and further characterization of *DAB1*, *2* and *3* are expected to enhance our understanding of abscission initiation and the connections between ethylene-dependent and -independent pathways.

ACTIN-RELATED PROTEINS may globally regulate transcription during senescence and abscission

In a recent study by Kandasamy et al. (2005b), *Arabidopsis* ACTIN-RELATED PROTEIN7 (ARP7) was found to be required for embryogenesis and adult plant development. Complete loss of ARP7 causes embryo lethality, with development arresting just prior to the torpedo stage. When *ARP7* expression was knocked down using RNA interference (RNAi), transgenic plants with the strongest viable phenotype exhibit stunted organs, reduced fertility, and defects in flower opening, anther dehiscence and fruit growth. Another striking characteristic of the *ARP7* RNAi plants is a delay in floral organ shedding such that 12 or more open flowers on an inflorescence retain their sepals and petals as compared to 4 or 5 in wild type (Kandasamy et al., 2005b). Senescence of the floral organs appears to be correspondingly delayed. Based on sections of *ARP7* RNAi and wild type flowers just before anthesis, AZ differentiation occurs normally. *ARP7* RNAi plants are partially competent to respond to ethylene; etiolated seedlings from a strong RNAi line show similar morphological changes as wild type seedlings in

the ethylene triple-response assay, yet ethylene exposure does not expedite floral organ shedding in *ARP7* RNAi adult plants as in wild type. Therefore, the ARP7 abscission pathway may be independent of or downstream of ethylene perception in flowers (Kandasamy et al., 2005b).

Arabidopsis ARP7 belongs to a phylogenetic class of ARPs unique to plants (McKinney et al., 2002). Other eukaryotic ARPs have been implicated in polymerization of conventional actin filaments, dynein motor function and chromatin remodeling (Schafer and Schroer, 1999; Goodson and Hawse, 2002). Of the eight classes of ARPs that are evolutionarily conserved from humans to budding yeast (*Saccharomyces cerevisiae*), four are localized to the cytoplasm and function in actin polymerization or dynein motor function, and the other four are localized to the nucleus, in association with high molecular weight chromatin remodeling complexes or heterochromatin (Goodson and Hawse, 2002). ARP7 also associates with a high molecular weight complex (Kandasamy et al., 2005b), and was previously shown to be localized to the nucleus during cytokinesis, interphase, and prophase (Kandasamy et al., 2003); therefore, it is predicted to play a role in chromatin remodeling during transcription.

Arabidopsis ARP4, with homologs in both humans and yeast, has also been implicated in floral organ abscission. When *ARP4* expression is knocked down using RNAi, a pleiotropic phenotype results that partially overlaps with that of *ARP7* RNAi plants (Kandasamy et al., 2005a). Paradoxically, *ARP4* RNAi plants with the strongest viable phenotype flower early yet senesce and abscise late (Kandasamy et al., 2005a). Thus, the observed delay in floral organ shedding may be an indirect result of an impaired developmental clock in *ARP4* RNAi plants. Detailed characterization of AZ

development in these plants should more clearly define the stage of abscission affected. Like ARP7, ARP4 is localized to the nucleus, and is predicted to function as part of a chromatin-remodeling complex to modulate transcription as has been demonstrated for its yeast and human homologs (Kandasamy et al., 2005a). Both proteins are expressed ubiquitously in all cell types, and ARP4 protein expression was not found to be affected in *ARP7* RNAi plants (Kandasamy et al., 2003; Kandasamy et al., 2005b). Identification of ARP4 and ARP7 interacting proteins as well as their transcriptional targets should reveal more clues about global regulation of genes involved in senescence and abscission.

Transcriptional activity of *AGAMOUS-LIKE 15* inhibits senescence and abscission

Arabidopsis AGAMOUS-LIKE 15 (AGL15) is a MADS domain transcription factor that appears to promote a juvenile state and/or inhibit senescence-related events, such as abscission, in the adult plant (Fernandez et al., 2000; Harding et al., 2003). Transgenic plants that constitutively express *AGL15* under the control of the viral *35S* promoter show delays in several developmental processes, including embryonic development, flowering time, fruit maturation, floral organ senescence and abscission (Fernandez et al., 2000; Harding et al., 2003). *35S::AGL15* plants are ethylene-sensitive: treatment of adult plants with exogenous ethylene promotes floral organ abscission as in wild type. Since development of the floral organ AZ occurs normally in these plants, AGL15 appears to inhibit floral organ shedding after AZ differentiation (Fernandez et al., 2000).

Consistent with the proposed role for AGL15 in inhibiting senescence-related events, the *AGL15* promoter drives reporter gene expression in many developing tissues,

including the vegetative shoot apex and meristem, leaf and stipule primordia, young leaves, and floral buds stage 4 through 13—but not in senescing or abscising floral organs. As leaves and floral organs mature, reporter activity declines and becomes concentrated at the base of the organs prior to disappearing completely (Fernandez et al., 2000). To determine the floral stage at which ectopic *AGL15* expression can cause developmental delays, Fang and Fernandez (2002) made use of the glucocorticoid-inducible expression system (Aoyama and Chua, 1997). These experiments demonstrate that AGL15 must be active at the time of flower opening (stage 13) to delay senescence and abscission (Fang and Fernandez, 2002).

Several questions remain concerning the role of AGL15 in flower development. First, genetic redundancy has so far proved an obstacle to loss-of-function analysis of *AGL15*. Single *agl15* mutants or plants carrying double mutant combinations of *AGL15* and *AGL18*, the MADS-box gene most closely related to *AGL15*, do not display any detectable defects in embryonic or adult development (Lehti-Shiu et al., 2005). Genetic analysis of additional related MADS-box genes may be necessary to elucidate *AGL15* function. Second, although several *DOWNSTREAM TARGET OF AGL15* (*DTA1-4*) genes have been identified based on the DNA-binding properties of AGL15 (Wang et al., 2002; Tang and Perry, 2003), to identify the genes regulated by AGL15 that affect the timing of senescence and floral organ shedding, genetic screens for suppressors of the *35S::AGL15* phenotype would be useful. Finally, it is not yet clear whether ectopic expression of *AGL15* delays floral organ abscission through a cell autonomous or cell non-autonomous pathway. When using the AZ-specific bean chitinase promoter to direct *AGL15* expression instead of the *35S* promoter, Fang and Fernandez (2002) observed no

delay in floral organ abscission. These results can be interpreted in one of two ways: either AGL15 inhibits abscission through a cell non-autonomous pathway, or this particular promoter drives *AGL15* expression too late in the developmental process to affect abscission. Earlier AZ-specific promoters should be identified and tested to resolve this question.

(c) Integrative pathways that promote ethylene biosynthesis and abscission

AUXIN RESPONSE FACTORS regulate the timing of abscission

For over half a century, abscission has been known to be affected by auxin levels based on experiments carried out with bean leaf AZ explants (Addicott, 1982). Until recently, however, auxin-related mutants that affect organ abscission had not been identified. Genetic analysis of four members of the *Arabidopsis AUXIN RESPONSE FACTOR* (*ARF*) family—*ARF2*, *ARF1*, *NONPHOTOTROPIC HYPOCOTYL4* (*NPH4*)/*ARF7*, and *ARF19*—has now revealed that these transcriptional regulators function with partial redundancy to promote senescence and floral organ shedding (Ellis et al., 2005; Okushima et al., 2005b). *arf2* single mutant flowers show a delay in the onset of both senescence and floral organ abscission. Although the single *arf1*, *nph4*, and *arf19* single mutants do not demonstrate any shedding defects, *arf1 arf2* double mutants and *arf2 nph4 arf19* triple mutants show an enhanced delay in abscission compared to *arf2* single mutants (Ellis et al., 2005). ARF proteins contain an N-terminal DNA binding domain specific to auxin response elements, and a regulatory middle region that either stimulates or represses transcription (Ulmasov et al., 1999; Tiwari et al., 2003). Since ARF2 and ARF1 have been shown to repress transcription while NPH4 and ARF19

stimulate it (Ulmasov et al., 1997; Ulmasov et al., 1999; Tiwari et al., 2003), the mechanism of how these transcriptional regulators function alone or in complexes to regulate senescence and organ shedding is not yet clear. One possible scenario is that their individual activities could be affected by changes in auxin gradients across floral organ AZs, since such gradients likely affect leaf senescence and abscission (Addicott, 1982; Okushima et al., 2005b).

A key role of ARF2 may be to promote ethylene biosynthesis prior to senescence and floral organ shedding. Three members of the 1-aminocyclopropane-1-carboxylate (ACC) synthase family—*ACS2, ACS6* and *ACS8*—show decreased transcript levels in *arf2* flowers (Okushima et al., 2005b). Expression of each of these *ACS* family members is normally observed in flowers after pollination and can be induced in seedlings by auxin treatment. However, ARF2 must clearly play additional roles in regulating the onset of senescence and abscission, since *ein2 arf2* double mutant flowers show an additive delay. An additional point raised by these results is whether *ACS2*, *ACS6*, and *ACS8* regulation by ARF2 is mediated by AUX/IAA signaling (Ellis et al., 2005, Yamagami et al., 2003).

G-protein signaling promotes ethylene biosynthesis and abscission of citrus leaves

The integration of G-protein signaling with the ethylene biosynthetic pathway of the 'Valencia' orange tree (*Citrus sinensis* L. Osbeck) is unprecedented. In a study based on the application of 2-chloroethylphosphonic acid (ethephon), of which ethylene is a byproduct, to stimulate both fruit and leaf abscission, Yuan and colleagues (2005) found that ethephon specifically increases steady state RNA levels of the ethylene biosynthesis genes *ACS1* and *ACC Oxidase* (*ACO*) in fruit and leaf AZs. Increased expression of

ACS1 and *ACO* corresponds with increased ethylene evolution. Two G-protein coupled α_{2A} – adreno-receptor agonists, guanfacine and clonidine, were found to inhibit ethephon-induced *ACS1* and *ACO* RNA expression in the AZ of the leaf but not that of the fruit (Yuan et al., 2005). These studies implicate G-protein signaling in the regulation of ethylene biosynthesis related to abscission, and demonstrate the feasibility of specifically controlling fruit abscission. In the future, it will be of interest to identify the G-protein coupled receptor(s) and pathway(s) that are the target of the guanfacine and clonidine agonists.

C. DISSOLUTION OF THE CELL WALL – CELL SEPARATION

The late stages of abscission are marked by AZ-specific loosening of the primary cell walls and dissolution of the middle lamella, a pectin-rich connective layer between cells (Osborne, 1989). Cellulose microfibrils, hemicelluloses, and pectins are highly cross-linked within the cell wall; therefore, cell separation necessitates the activity of a variety of cell wall modifying and hydrolytic enzymes. Expression studies in multiple plant species underscore the importance of two major types of hydrolytic enzymes for abscission: the endo-beta-1,4-glucanase (EGase or cellulase) and the polygalacturonase (PG) families. EGase family enzymes are thought to loosen cell walls by releasing xyloglucan, one of the most abundant hemicellulose species, from the cellulose microfibrils (Cosgrove, 2005). PG family enzymes, which hydrolyze polygalacturons of the pectin class, are most likely required for dissolution of the middle lamella. Other

proteins that have been implicated in AZ cell wall modification include the cell wall loosening family of expansins (Belfield et al., 2005; Sampredo and Cosgrove, 2005) and pathogenesis-related (PR) chitinases (reviewed in Roberts et al., 2002).

(d) Hydrolytic enzymes

Endo-beta-1,4-Glucanases

EGase class enzymes are encoded by a multigene family, with evidence of gene duplication and divergence of function. In the *Arabidopsis* genome alone, 25 EGases are annotated with numerous examples in which *Arabidopsis* paralogs are predicted to be more similar to each other than to homologous genes from other species. This phylogenetic arrangement suggests a history of gene duplications (Libertini et al., 2004). The EGase family is divided into three subfamilies, of which the alpha- and beta-EGases are implicated in cell elongation, ripening and abscission. gamma-EGases, on the other hand, are predicted to play roles in cell elongation and cellulose biosynthesis at the plasma membrane (Libertini et al, 2004). The first AZ-specific cell wall degrading enzyme to be characterized and cloned was bean abscission-specific cellulase (BAC) from *Phaseolus vulgaris* (Lewis and Varner, 1970; Tucker et al., 1988; Koehler et al., 1996). *BAC* promoter-driven expression of a reporter gene (*pBAC::GUS*) is a commonly used marker associated with abscission across species (Fernandez et al., 2000). In addition, *BAC* sequence has been successfully used to isolate related EGase genes expressed within the AZs of *Glycine max* (soybean; Kemmerer and Tucker, 1994), *S. nigra* (Taylor et al., 1994) and tomato (Lashbrook et al., 1994).

Whereas a single EGase is known to be expressed in the bean leaf AZ, multiple EGases have been detected in the AZ tissue of *S. nigra* leaves, *Capsicum annuum* (pepper) leaves and flowers, *Prunus persica* (peach) leaves, and tomato flowers, suggesting cooperativity and/or redundancy of function (Lashbrook et al., 1994; Taylor et al., 1994; Ferrarese et al., 1995; del Campillo and Bennett, 1996; Traniotti et al., 1997; Traniotti et al., 1998). EGase expression patterns in tomato and peach suggest overlapping functions in fruit maturation and abscission. In tomato, the EGase genes *Cel1*, *Cel2, Cel5* and *Cel6* are expressed to varying extents within pedicel AZs, while *Cel1* and *Cel2* are also expressed in developing fruit (Lashbrook et al., 1994; del Campillo and Bennett, 1996). Knocking down expression of either *Cel1* or *Cel2* with antisense RNA impairs abscission, as measured by decreased frequency of abscission in *Cel1-AS* flower explants and increased force required to remove fruit from *Cel2-AS* plants. However, neither fruit ripening nor softening are affected in *Cel1-AS* and *Cel2-AS* plants (Lashbrook et al.. 1998a; Brummell et al., 1999). These results suggest that Cel1 and Cel2 activity is redundant for fruit maturation, and cooperative for abscission. Future analysis of genetic interactions among mutant EGase alleles should tell us more about their cooperative and/or redundant functions.

Interestingly, the spontaneously arising *abs1* mutant of *L. angustifolius,* a grain used for livestock feed in Australia, is deficient in the abscission of leaves, flowers and cotyledons (Clements and Atkins, 2001; see Figure 1.2D). This deficiency corresponds with a lack of EGase expression in ethylene-treated *abs1* AZ cells (Henderson et al., 2001). Otherwise, *abs1* mutants appear like wild type, with no detectable differences in senescence, root-cap border cell separation, or the triple response in ethylene-treated

seedlings. Although AZ differentiation appears to be unaffected, imaging of *abs1* cotyledon AZs by transmission electron microscopy reveals a lack of middle lamella dissolution in comparison to wild type (Clements and Atkins, 2001). Although Henderson and colleagues (2001) did not detect any changes in the expression and/or enzyme activity of PGase, beta-galactosidase, beta-glucosidase, and beta-1,3-glucanhydrolase, more sensitive assays might detect differences in enzymatic activity. Alternatively, an unidentified class of enzymes may facilitate cell wall dissolution in *L. angustifolius*. In contrast to tomato, in which multiple EGases are expected to play a role in cell wall dissolution within the pedicel AZ, *abs1* may represent a single genetic lesion that disrupts the expression of an EGase necessary for all types of *L. angustifolius* abscission. Another possibility is that *abs1* mutants lack a crucial regulator of multiple cell wall modifying and hydrolytic enzymes. Mapping of the *abs1* mutation is sure to increase our understanding of the mechanism and requirements of cell wall dissolution during abscission.

Polygalacturonases

Plants are well equipped with enzymes for the modification of pectin in the primary cell wall and middle lamella. In *Arabidopsis*, approximately 150 proteins are predicted to have pectin-modifying activity, of which 52 belong to the PG family of enzymes (The *Arabidopsis* Genome Initiative, 2000). PGs are implicated in the hydrolysis of the polygalacturon class of pectins during many developmental processes, including fruit ripening, abscission, dehiscence, pollen tube growth and pollen grain maturation (reviewed in Hadfield and Bennett, 1998). Based upon the phylogenetic

relationship of 43 PGs from multiple plant species, the enzymes were divided into five clades (A-E) with unique intron-exon structures (Torki et al., 2000). All PGs contain a signal peptide for secretion of the enzyme and four functional domains (I-IV). Domains I-III are thought to be required for catalytic activity, and domain IV may facilitate interaction with the polygalacturon substrate (reviewed in Torki et al., 2000). PGs of clade B are unique in that they contain an additional N-terminal prosequence that may act as a novel protein-sorting signal (Dal Degan et al., 2001). Although initial expression studies and sequence analysis of the first PGs isolated suggested that all PGs acting during organ abscission belong to clade A (Torki et al., 2000), recent studies of a few *Brassica napus* and *Arabidopsis* PGs belonging to clade B (see below) suggest that this simple classification is not absolute.

PG family genes were originally implicated in abscission by expression studies in a variety of plants, including tomato, peach, and *S. nigra* (Tucker et al., 1984; Bonghi et al., 1992; Taylor et al., 1993). The first AZ-specific PG mRNA to be isolated was *TAPG1* in tomato (Kalaitzis et al., 1995), suggesting that PG expression is more specific to the abscission process than the more broadly expressed EGase tomato genes. Using *TAPG1* as a probe of a tomato leaf AZ cDNA library, three additional PG sequences were identified: *TAPG2*, *TAPG4*, and *TAPG5* (Kalaitzis et al., 1997; Hong and Tucker, 2000). In general, tomato abscission PG genes are more related to each other (76-95% identity) than to tomato fruit PG genes (38-41% identity) (Kalaitzis et al., 1997). All four tomato PGs are expressed in the AZs of the leaf and of the flower pedicel, but not in stems, petioles or fruit (Kalaitzis et al., 1997; Hong and Tucker, 2000); and RNase protection assays indicate that expression is 7-fold higher in flower AZs than leaf AZs (Kalaitzis et

al., 1997). PG expression is also detected within the AZ of the corolla floral organs at the base of the flower (Hong et al., 2000). Furthermore, promoter-GUS fusion constructs for *TAPG1* and *TAPG4* indicate that *TAPG4* is expressed earlier than *TAPG1*, suggesting that the enzymes function at different stages of cell wall dissolution (Hong et al., 2000).

Whereas numerous PG transcripts have been isolated from the AZ tissue of tomato, the small number of cells within *Arabidopsis* separation zones makes RNA and protein isolation from these sites difficult. To circumvent this problem, González-Carranza and colleagues (2002) turned to a closely related species, *B. napus* (oilseed rape), which has a larger AZ. A PG cDNA isolated from *B. napus* leaf AZs was used as a probe to identify corresponding genomic clones in *B. napus* (*PGAZBRAN*) and *Arabidopsis* (*PGAZAT*). *PGAZAT* expression was analyzed using *PGAZAT::GUS* and *PGAZAT::GFP* promoter fusion constructs. PGAZAT::GUS expression is first detected in a ring pattern at the anther bases (González-Carranza et al., 2002), similar to that seen for *TAPG1* and *TAPG4* promoter-driven GUS expression in tomato pedicel AZs (Hong et al., 2000). Expression spreads throughout the floral AZ prior to separation, with ring patterns at the sites of petal abscission also becoming apparent. Ethylene exposure accelerates this process, so that expression of the reporter correlates with the accelerated timing of abscission. Reporter expression was not detected in the seed AZ or the dehiscence zones (DZ) of anthers and fruit, suggesting that PGAZAT functions primarily in floral organ abscission (González-Carranza et al., 2002).

As in tomato, functional redundancy is expected among PGs during *Arabidopsis* and *B. napus* abscission. Another *Arabidopsis* PG, ADPG1, shares 69% amino acid identity with PGAZAT, suggesting that these two PGs of clade B might have overlapping

activities. While *ADPG1* expression has only been reported for fruit DZs, expression of a *B. napus* ortholog, *RDPG1*, is also detected in floral AZs by RT-PCR (Sander et al., 2001). In addition, a partial *RDPG1* promoter drives reporter expression in *Arabidopsis* at several sites of cell separation, including fruit and anther DZs and floral AZs. Thus, functional analysis of both *PGAZAT* and *ADPG1* in *Arabidopsis* may be required to dissect their respective roles in abscission.

(e) Expansins

***EXPANSINS* are positioned to affect cell wall loosening during abscission**

In addition to the hydrolytic enzymes necessary for cell wall dissolution, a family of enzymes that facilitate cell wall extension, known as expansins, have recently been implicated in abscission. Using an *in vitro* biochemical assay, Belfield et al. (2005) observed that similar levels of expansin activity are present in *Sambucus nigra* (elder) leaflet AZ and non-AZ tissue prior to ethylene treatment; however, following ethylene treatment, expansin activity increases 7-fold exclusively in AZ cells (Belfield et al., 2005). Two expansins, *SniEXP2* and *SniEXP4*, were found to be enriched in a cDNA library constructed from ethylene-treated leaflet AZ cells, and RNA blot analysis showed that transcription of both genes is induced in AZ cells 12-24 hours after ethylene exposure. RT-PCR amplification of two additional expansins, *SniEXP1* and *SniEXP3,* suggested that they are enriched in leaflet AZ cells as well (Belfield et al., 2005).

Expansins are a multigene family, with 38 predicted *Arabidopsis* members (Li et al., 2003). Proteins of this family have two characteristic domains: (1) a predicted active site with homology to the family-45 EGases, and (2) a putative polysaccharide binding

region with homology to a grass pollen allergen. Expansins are thought to weaken the hydrogen bonds between cell wall polysaccharides, particularly cellulose microfibrils and xyloglucans, easing the cell wall tension that impedes growth. Although cellulose and xyloglucans are also targeted by EGases, *in vitro* biochemical experiments have not shown that expansins are capable of hydrolyzing cell wall material (Cosgrove et al., 2002; Li et al., 2003). The localization of expansins is consistent with their role in cell wall expansion. Immunogold labeling of cucumber and maize epidermal cells with an antibody for cucumber EXP1 shows that expansins are dispersed throughout all layers of the cell wall and are occasionally found in golgi-derived vesicles, suggesting that these enzymes are targeted to the cell wall through the secretory system (Cosgrove et al., 2002).

Historically, expansins have been shown to function in events that require extension of the cell wall, such as polarized growth and general cell expansion—not in events that require softening and breakdown of the cell wall, like fruit ripening and abscission. Although functional analysis of the *Arabidopsis* expansin family has not yet revealed any single mutants with defects in floral organ shedding, the likelihood of genetic redundancy will require construction of higher order mutants to fully address their potential role in cell separation (reviewed in Cosgrove et al., 2002 and Li et al., 2003). In addition to the induction of expansin activity in ethylene-treated *S. nigra* leaflet AZs, a few other observations in *Arabidopsis* flowers suggest that certain expansins might be involved in the abscission process. First, it has been shown that changes in the expression of *AtEXP10*, which is normally found at the bases of flower pedicels, affects the degree of pedicel breakage when flowers are forcibly removed from the inflorescence

stem. Overexpression of *AtEXP10* in transgenic plants appeared to increase the frequency of complete breakage at this apparently vestigial AZ site compared to wild type, whereas antisense *AtEXP10* expression reduced the frequency of complete breakage (Cho and Cosgrove, 2000). Second, SEM of floral organ AZs over time has revealed that proximal AZ cells begin to expand and become rounded at the time of abscission (Patterson and Bleecker, 1997; Butenko et al., 2003). An intriguing possibility is that expansion of these AZ cells, facilitated by expansin activity, might be a prerequisite for abscission, perhaps by affecting the ability of cells within the AZ to adhere to one another (Figure 1.1).

D. DISCUSSION AND FUTURE DIRECTIONS

The organ abscission studies described in this chapter represent only a few enticing episodes of a much more intricate story yet to be told. Since abscission mutants are currently categorized based on our superficial understanding of the cell separation process, as we gain a deeper knowledge of the complex genetic interactions that underlie the morphological changes that take place, the stages of organ abscission are certain to come into a clearer focus (Figure 1.1). In the future, sensitized genetic screens and protein interaction experiments should dramatically expand the cast of abscission players, and begin to reveal the structure and intersections of the pathways in which they act. Additional insights into the functional mechanisms of those proteins we already know to be involved in organ abscission are likely to be provided by subcellular localization approaches such as fluorescent-tagging and immunofluorescence analysis. For instance,

the nuclear localization of ARP4 and ARP7 lends strong support to their predicted functions in chromatin remodeling and global regulation of gene networks involved in senescence and abscission. Research in model plants with sequenced genomes also facilitates several powerful approaches including: (1) map-based cloning of abscission mutants, (2) systematic analysis of gene families in which genetic redundancy is expected, (3) cross-species comparisons to identify candidate genes in agronomically important species, (4) microarray studies of AZ-specific gene expression using laser capture microdissection, and (5) ChIP-chip analysis to identify downstream targets of transcription factors that regulate abscission. Together with more traditional genetic screens and reverse genetic strategies, these techniques are certain to uncover new tales of genetic interactions required for organ abscission over the next decade.

In addition to investigating the shared characteristics of cell separation across plant species, a parallel goal of abscission research is the ability to manipulate the shedding of specific organs in important crop plants. Characterization of new mutants such as *abs2*, which is non-allelic with *abs1* and completely blocks cotyledon abscission yet only delays flower and leaf shedding in *L. angustifolius* (Clements and Atkins, 2001), should increase our understanding of the regulatory differences between various types of organ abscission. Since ethylene promotes the abscission of all lateral organs in 'Valencia' orange trees, research is ongoing to identify compounds that affect the timing of mature fruit abscission without inducing citrus trees to drop their flowers, immature fruit or leaves. One such compound, 5-chloro-3-methyl-4-nitro-1*H*-pyrazole (CMNP), marketed as "Release" 30 years ago, specifically induces the abscission of mature fruit. When applied to citrus peel, CMNP appears to promote membrane breakdown and the

activation of lipid signaling pathways (Alferez et al., 2005). This study suggests that lipid signaling is yet another component of particular pathways controlling plant separation, and nicely illustrates the feedback potential between the knowledge gained from applied and basic research of organ abscission.

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SECTION II. THE REGULATION OF FLORAL ORGAN ABSCISSION

While the abscission process has captured the interest of botanists for hundreds of years, at the outset of this dissertation, research had yet to describe a genetic pathway(s) for the regulation of organ shedding. The model plant *Arabidopsis thaliana*, with a sequenced genome and a high-density map of DNA polymorphisms, is an excellent system for studying the genetics of abscission. In fact, the study of cell separation during *Arabidopsis* floral organ shedding has been extremely fruitful in the identification of novel genetic factors for the regulation of plant cell adhesion. These factors include a redundant pair of polygalacturanases, a putative F-box protein, as well as novel members of a signaling pathway essential for cell wall dissolution (González-Carranza et al 2007b; Cho et al., 2008; Ogawa et al., 2009). Further study of the BLADE-ON-PETIOLE 1 (BOP1) and BOP2 BTB/POZ domain proteins shows that these factors are redundantly required for AZ differentiation (McKim et al., 2008).

PGAZAT and QUARTET 2 are required for AZ cell wall dissolution

Multiple genes encoding polygalacturanase (PG) pectin-modifying enzymes are expressed within plant AZs, yet none had been found to be essential for organ shedding. (reviewed in Leslie et al., 2007). As predicted by its expression within *Arabidopsis* floral AZs, loss of *ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURANASE 2* (*ADPG2*; previously named *PGAZAT*) delays floral organ abscission (González-Carranza et al., 2002; González-Carranza et al., 2007a; Ogawa et al., 2009). Additional PGs likely function with *PGAZAT*; however, the most closely related *ADPG1* is predominantly expressed in anther dehiscence zones and mature fruit, and *adgp1* plants shed their organs

in a similar time course to wildtype (González-Carranza et al., 2007a; Ogawa et al., 2009). A third member of this PG family is encoded by *QUARTET* 2 (*QRT2*). *QRT2* is expressed within floral AZs, and plants deficient in *QRT2* exhibit delayed shedding (Ogawa et al., 2009). Double loss-of-function *adpg2 qrt2* plants show a greater delay in shedding in comparison to either single mutant (Ogawa et al., 2009), demonstrating that an additive relationship exists between at least two PG enzymes during *Arabidopsis* floral organ abscission.

The putative F-box protein, HAWAIIAN SKIRT, promotes sepal abscission

In a forward genetic screen for novel floral phenotypes in *Arabidopsis*, a loss-of-function mutation in the previously uncharacterized *HAWAIIAN SKIRT* (*HWS*) was found to block floral organ shedding—senesced organs cling to the base of the developing fruit (González-Carranza et al., 2007b). HWS encodes a putative F-box protein that may target proteins for degradation in the 26S proteosome. Scanning electron micrographs of developing *hws* floral buds reveal that sepal fusion, rather than a delay in cell separation within AZs, ultimately blocks organ shedding. However, cell separation is delayed within *hws* sepal AZs (González-Carranza et al., 2007b), and identifying HWS targets may reveal novel abscission factors and/or enhance our understanding of those currently known.

An essential signaling pathway for *Arabidopsis* floral organ shedding

Recent work has elucidated a signaling pathway for abscission, of which all factors are essential for floral organ shedding. This pathway includes the putative

signaling ligand INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), a set of receptor-like kinases (RLKs) and a downstream MAP kinase cascade (Cho et al., 2008; Stenvik et al., 2008). Previously, the leucine-rich repeat receptor-like kinase (LRR-RLK) HAESA (HAE) was implicated in the abscission process by antisense suppression of *HAE* and potentially related RLKs (Jinn et al., 2000). It is now known that HAE and the closely related LRR-RLK, HAESA-LIKE 2 (HSL2), are redundantly required for *Arabidopsis* floral organ abscission (Cho et al., 2008; Stenvik et al., 2008). Similarity in phenotype between *ida* and *hae hsl2* mutant plants—both block cell separation without affecting earlier AZ differentation—as well as overlapping expression patterns, have led the authors to speculate that IDA is the signaling ligand for the HAE/HSL2 RLKs (Jinn et al., 2000; Butenko et al., 2003; Cho et al., 2008; Stenvik et al., 2008). As would be predicted for a receptor-ligand interaction, the HAE/HSL2 RLKs function downstream of the IDA signaling ligand (Cho et al., 2008; Stenvik et al., 2008). *IDA* misexpression causes ectopic expansion of wildtype floral AZs, occasionally leading to premature organ abscission (Stenvik et al., 2006; Cho et al., 2008; Leslie et al., 2010), whereas the floral organs of plants deficient in *HAE* and *HSL2* remain attached to the plant indefinitely, regardless of *IDA* misexpression (Cho et al., 2008; Stenvik et al., 2008). Further downstream of *HAE/HSL2*, two sets of MAP kinases, *MPK3*/*MPK6* and *MKK4*/*MKK5*, are required for abscission (Cho et al., 2008). Decreased MPK6 activity was reported for *hae hsl2* non-shedding AZs, while constitutively active MKK4 or MKK5 is sufficient to rescue shedding in *ida* or *hae hsl2* flowers (Cho et al., 2008). While transcriptional targets are yet to be found for the proposed signaling pathway, the identification of

downstream MAP kinase factors allows for a quantifiable read-out of the activation or repression of HAE/HSL2 signaling.

In this work, we uncover a novel link between membrane trafficking and abscission signaling. We have identified the NEVERSHED (NEV) ADP-ribosylation factor GTPase-activating protein (ARF-GAP), which is essential for cell wall dissolution (Chapter 2; Liljegren et al., 2009), and the EVERSHED (EVR) LRR-RLK that inhibits abscission downstream of *NEV* (Chapter 3; Leslie et al., 2010). We provide genetic and physical evidence that is consistent with an interaction between the newly identified NEV-EVR pathway and HAE/HSL2 signaling (Chapter 4).

REFERENCES

Abeles, F. B., Morgan, P. W., and Saltveit, M. E. (1992) *Ethylene in Plant Biology*, 2nd edn, Academic Press, San Diego, CA.

Addicott, F. T. (1982) *Abscission*, University of California Press, Berkeley, CA.

Alferez, F., Singh, S., Umbach, A. L., Hockema, B. and Burns, J. K. (2005) Citrus abscission and *Arabidopsis* plant decline in response to 5-chloro-3-methyl-4-nitro-1*H*-pyrazole are mediated by lipid signaling, *Plant, Cell and Environment,* **28**, 1436-1449.

Alibardi, L. (2000) Ultrastructural localization of alpha-keratins in the regenerating epidermis of the lizard Podarcis muralis during formation of the shedding layer, *Tissue and Cell,* **32**(2), 153-162.

Alibardi, L. (2005) Differentiation of snake epidermis, with emphasis on the shedding layer, *Journal of Morphology,* **264**(2), 178-190.

Alonso, J. M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker, J. R. (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*, *Science,* **284**(5423), 2148-2152.

Alonso, J. M. and Stepanova, A. N. (2004) The ethylene signaling pathway, *Science,* **306**(5701), 1513-1515.

Aoyama, T. and Chua, N. H. (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants, *Plant Journal,* **11**(3), 605-612.

Archambault J., Pan, G., Dahmus, G. K., Cartier, M., Marshall, N., Zhang, S., Dahmus, M. E. and Greenblatt, J. (1998) FCP1, the RAP74-interacting subunit of a human protein phosphatase that dephosphorylates the carboxyl-terminal domain of RNA polymerase IIO, *Journal of Biological Chemistry*, **273**(42), 27593-27601.

Barry, C. S., McQuinn, R. P., Thompson, A. J., Seymour, G. B., Grierson, D. and Giovannoni, J. J. (2005) Ethylene insensitivity conferred by the *Green-ripe* and *Never-ripe 2* ripening mutants of tomato, *Plant Physiology,* **138**(1), 267-275.

Belfield, E. J., Ruperti, B., Roberts, J. A. and McQueen-Mason, S. (2005) Changes in expansin activity and gene expression during ethylene-promoted leaflet abscission in *Sambucus nigra*, *Journal of Experimental Botany,* **56**(413), 817-823.

Bleecker, A. B., Estelle, M. A., Somerville, C. and Kende, H. (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*, *Science,* **241**(4869), 1086-1089.

Bleecker, A. B. and Patterson, S. E. (1997) Last exit: senescence, abscission, and meristem arrest in *Arabidopsis*, *Plant Cell,* **9**(7), 1169-1179.

Bonghi, C., Rascio, N., Ramina, A. and Casadoro, G. (1992) Cellulase and polygalacturonase involvement in the abscission of leaf and fruit explants of peach, *Plant Molecular Biology,* **20**(5), 839-848.

Bowman, J. L., Eshed, Y. and Baum, S. F. (2002) Establishment of polarity in angiosperm lateral organs, *Trends in Genetics,* **18**(3), 134-141.

Brown, K. M. (1997) Ethylene and abscission, *Physiologia Plantarum,* **100**, 567-576.

Brummell, D. A., Hall, B. D. and Bennett, A. B. (1999) Antisense suppression of tomato endo-1,4-beta-glucanase Cel2 mRNA accumulation increases the force required to break fruit abscission zones but does not affect fruit softening, *Plant Molecular Biology,* **40**(4), 615-622.

Budiman, M. A., Chang, S.-B., Lee, S., Yang, T. J., Zhang, H.-B., de Jong, H. and Wing, R. A. (2004) Localization of *jointless-2* gene in the centromeric region of tomato chromosome 12 based on high resolution genetic and physical mapping, *Theoretical and Applied Genetics,* **108**(2), 190-196.

Butenko, M. A., Patterson, S. E., Grini, P. E., Stenvik, G. E., Amundsen, S. S., Mandal, A. and Aalen, R. B. (2003) *INFLORESCENCE DEFICIENT IN ABSCISSION* controls floral organ abscission in *Arabidopsis* and identifies a novel family of putative ligands in plants, *Plant Cell*, **15**(10), 2296-2307.

Butler, L. (1936) Inherited characters in the tomato. II. Jointless pedicel, *The Journal of Heredity,* **37**, 25-26.

Cancel, J. D. and Larsen, P. B. (2002) Loss-of-function mutations in the ethylene receptor ETR1 cause enhanced sensitivity and exaggerated response to ethylene in Arabidopsis, *Plant Physiology,* **129**(4), 1557-1567.

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S. and Dong, X. (1997) The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats, *Cell,* **88**(1), 57-63.

Chang, C., Kwok, S. F., Bleecker, A. B. and Meyerowitz, E. M. (1993) *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators, *Science,* **262**(5133), 539-544.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W. and Ecker, J. R. (1997) Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins, *Cell,* **89**(7), 1133-1144.

Chen, G., Alexander, L. and Grierson, D. (2004) Constitutive expression of EIL-like transcription factor partially restores ripening in the ethylene-insensitive *Nr* tomato mutant, *Journal of Experimental Botany,* **55**(402), 1491-1497.

Chen, Y.-F., Randlett, M. D., Findell, J. L. and Schaller, G. E. (2002) Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of *Arabidopsis*, *Journal of Biological Chemistry*, **277**(22), 19861-19866.

Cho, H. T. and Cosgrove, D. J. (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in *Arabidopsis thaliana*, *Proceedings of the National Academy of Sciences of the United States of America,* **97**(17), 9783-9788.

Cho, S., Jang, S., Chae, S., Chung, K. M., Moon, Y.-H., An, G. and Jang, S. K. (1999) Analysis of the C-terminal region of *Arabidopsis thaliana* APETALA1 as a transcriptional activation domain, *Plant Molecular Biology,* **40**(3), 419-429.

Cho, S. K., Larue, C. T., Chevalier, D., Wang, H., Jinn, T-L., Zhang, S. and Walker, J. C. (2008). Regulation of floral organ abscission in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **105**, 15629-15634.

Clements, J. and Atkins, C. (2001) Characterization of a non-abscission mutant in *Lupinus angustifolius*. I. Genetic and structural aspects, *American Journal of Botany,* **88**(1), 31-42.

Cosgrove, D. J. (2005) Growth of the plant cell wall, *Nature Reviews. Molecular Cell Biology,* **6**(11), 850-861.

Cosgrove, D. J., Li, L. C., Cho, H.-T., Hoffmann-Benning, S., Moore, R. C. and Blecker, D. (2002) The growing world of expansins, *Plant and Cell Physiology,* **43**(12), 1436-1444.

Dal Degan, F., Child, R., Svendsen, I. and Ulvskov, P. (2001) The cleavable N-terminal domain of plant endopolygalacturonases from clade B may be involved in a regulated secretion mechanism, *The Journal of Biological Chemistry,* **276**(38), 35297-35304.

del Campillo, E. and Bennett, A. B. (1996) Pedicel breakstrength and cellulase gene expression during tomato flower abscission, *Plant Physiology,* **111**(3), 813-820.

Dinneny, J. R., Yadegari, R., Fischer, R., Yanofsky, M. F. and Weigel, D. (2004) The role of *JAGGED* in shaping lateral organs, *Development*, **131**(5), 1101-1110.

Doebley, J. (2004) The genetics of maize evolution, *Annual Review of Genetics,* **38**37-59.

Dong, X. (2004) NPR1, all things considered, *Current Opinion in Plant Biology,* **7**(5),

547-552.

Doubt, S. L. (1917) The response of plants to illuminating gas, *Botanical Gazette,* **63**(3), 209-224.

Ecker, J. R. and Theologis, A. (1994) Ethylene: a unique plant signaling molecule, in *Arabidopsis* (eds E. M. Meyerowitz and C. R. Somerville), Cold Spring Harbor Press, Plainview, NY pp. 485-521.

Ellis, C. M., Nagpal, P., Young, J. C., Hagen, G., Guilfoyle, T. J. and Reed, J. W. (2005) *AUXIN RESPONSE FACTOR1* and *AUXIN RESPONSE FACTOR2* regulate senescence and floral organ abscission in *Arabidopsis thaliana*, *Development***,** **132**(20), 4563-4574.

Fang, S.-C. and Fernandez, D. E. (2002) Effect of regulated overexpression of the MADS domain factor AGL15 on flower senescence and fruit maturation, *Plant Physiology,* **130**(1), 78-89.

Fernandez, D. E., Heck, G. R., Perry, S. E., Patterson, S. E., Bleecker, A. B. and Fang, S.-C. (2000) The embryo MADS domain factor AGL15 acts postembryonically. Inhibition of perianth senescence and abscission via constitutive expression, *Plant Cell* **12**(2), 183-98.

Ferrarese, L., Trainotti, L., Moretto, P., Polverino De Laureto, P., Rascio, N. and Casadoro, G. (1995) Differential ethylene-inducible expression of cellulase in pepper plants, *Plant Molecular Biology,* **29**(4), 735-747.

González-Carranza, Z. H., Elliott, K. A. and Roberts, J. A. (2007a) Expression of polygalacturonases and evidence to support their role during cell separation processes in Arabidopsis thaliana. *J. Exp. Bot.* **58**, 3719-3730.

González-Carranza, Z. H., Rompa, U., Peters, J. L., Bhatt, A. M., Wagstaff, C., Stead, A. D. and Roberts, J. A. (2007b) Hawaiian skirt: an F-box gene that regulates organ fusion and growth in Arabidopsis. *Plant Physiol.* **144**, 1370-1382.

González-Carranza, Z. H., Whitelaw, C. A., Swarup, R. and Roberts, J. A. (2002) Temporal and spatial expression of a polygalacturonase during leaf and flower abscission in oilseed rape and Arabidopsis, *Plant Physiology,* **128**(2), 534-543.

Goodson, H. V. and Hawse, W. F. (2002) Molecular evolution of the actin family, *Journal of Cell Science,* **115**(Pt 13), 2619-2622.

Guo, H. and Ecker, J. R. (2004) The ethylene signaling pathway: new insights, *Current Opinion in Plant Biology,* **7**(1), 40-49.

Guzmán, P. and Ecker, J. R. (1990) Exploiting the triple response of *Arabidopsis* to

identify ethylene-related mutants, *Plant Cell,* **2**(6), 513-523.

Ha, C. M., Jun, J. H., Nam, H. G. and Fletcher, J. C. (2004) *BLADE-ON-PETIOLE1* encodes a BTB/POZ domain protein required for leaf morphogenesis in *Arabidopsis thaliana*, *Plant and Cell Physiology*, **45**(10), 1361-1370.

Ha, C. M., Kim, G. T., Kim, B. C., Jun, J. H., Soh, M. S., Ueno, Y., Machida, Y., Tsukaya, H. and Nam, H. G. (2003) The *BLADE-ON-PETIOLE1* gene controls leaf pattern formation through modulation of meristematic activity in *Arabidopsis, Development*, **130**(1), 161-172.

Hackett, R. M., Ho, C.-W., Lin, Z., Foote, H. C. C., Fray, R. G. and Grierson, D. (2000) Antisense inhibition of the *Nr* gene restores normal ripening to the tomato *Never-ripe* mutant, consistent with the ethylene receptor-inhibition model, *Plant Physiology,* **124**(3), 1079-1086.

Hadfield, K. A. and Bennett, A. B. (1998) Polygalacturonases: many genes in search of a function, *Plant Physiology,* **117**(2), 337-343.

Harding, E. W., Tang, W., Nichols, K. W., Fernandez, D. E. and Perry, S. E. (2003) Expression and maintenance of embryogenic potential is enhanced through constitutive expression of *AGAMOUS-Like 15*, *Plant Physiology,* **133**(2), 653-663.

Hartmann, U., Hohmann, S., Nettesheim, K., Wisman, E., Saedler, H. and Huijser, P. (2000) Molecular cloning of *SVP*: a negative regulator of the floral transition in *Arabidopsis*, *Plant Journal,* **21**(4), 351-360.

Henderson, J., Lyne, L. and Osborne, D. J. (2001) Failed expression of an *endo*-beta-1,4-glucanhydrolase (cellulase) in a non-abscinding mutant of *Lupinus angustifolius* cv Danja, *Phytochemistry,* **58**(7), 1025-1034.

Hepworth, S. R., Zhang, Y., McKim, S., Li, X. and Haughn, G. W. (2005) *BLADE-ON-PETIOLE1* dependent signaling controls leaf and floral patterning in *Arabidopsis*, *Plant Cell*, **17**(5), 1434-48.

Hong, S.-B., Sexton, R. and Tucker, M. L. (2000) Analysis of gene promoters for two tomato polygalacturonases expressed in abscission zones and the stigma, *Plant Physiology,* **123**(3), 869-881.

Hong, S.-B. and Tucker, M. L. (2000) Molecular characterization of a tomato polygalacturonase gene abundantly expressed in the upper third of pistils from opened and unopened flowers, *Plant Cell Reports,* **19**(7), 680-683.

Horn, M. A. and Walker, J. C. (1994) Biochemical properties of the autophosphorylation of RLK5, a receptor-like protein kinase from Arabidopsis thaliana, *Biochimica et*

Biophysica Acta, **1208**(1), 65-74.

Hua, J. and Meyerowitz, E. M. (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*, *Cell,* **94**(2), 261-271.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q. G., Bleecker, A. B., Ecker, J. R. and Meyerowitz, E. M. (1998) *EIN4* and *ERS2* are members of the putative ethylene receptor gene family in Arabidopsis, *Plant Cell,* **10**(8), 1321-1332.

Jeong, S., Trotochaud, A. E. and Clark, S. E. (1999) The Arabidopsis *CLAVATA2* gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase, *Plant Cell,* **11**(10), 1925-1934.

Jinn, T.-L., Stone, J. M. and Walker, J. C. (2000) *HAESA*, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission, *Genes and Development*, **14**(1), 108-17.

Kalaitzis, P., Koehler, S. M. and Tucker, M. L. (1995) Cloning of a tomato polygalacturonase expressed in abscission, *Plant Molecular Biology,* **28**(4), 647-656.

Kalaitzis, P., Solomos, T. and Tucker, M. L. (1997) Three different polygalacturonases are expressed in tomato leaf and flower abscission, each with a different temporal expression pattern, *Plant Physiology,* **113**(4), 1303-1308.

Kandasamy, M. K., Deal, R. B., McKinney, E. C. and Meagher, R. B. (2005a) Silencing the nuclear actin-related protein AtARP4 in *Arabidopsis* has multiple effects on plant development, including early flowering and delayed floral senescence, *Plant Journal*, **41**(6), 845-858.

Kandasamy, M. K., McKinney, E. C., Deal, R. B. and Meagher, R. B. (2005b) Arabidopsis ARP7 is an essential actin–related protein required for normal embryogenesis, plant architecture and floral organ abscission, *Plant Physiology*, **138**(4), 2019-2032.

Kandasamy, M. K., McKinney, E. C. and Meagher, R. B. (2003) Cell cycle-dependent association of *Arabidopsis* actin-related proteins AtARP4 and AtARP7 with the nucleus, *Plant Journal,* **33**(5), 939-948.

Kemmerer, E. C. and Tucker, M. L. (1994) Comparative study of cellulases associated with adventitious root initiation, apical buds, and leaf, flower, and pod abscission zones in soybean, *Plant Physiology,* **104**(2), 557-562.

Keskitalo, J., Bergquist, G., Gardestrom, P. and Jansson, S. (2005) A cellular timetable of autumn senescence, *Plant Physiology*, **139**(4), 1635-48.

Koehler, S. M., Matters, G. L., Nath, P., Kemmerer, E. C. and Tucker, M. L. (1996) The gene promoter for a bean abscission cellulase is ethylene-induced in transgenic tomato and shows high sequence conservation with a soybean abscission cellulase, *Plant Molecular Biology,* **31**(3), 595-606.

Koiwa, H., Barb, A. W., Xiong, L., Li, F., McCully, M. G., Lee, B.-H., Sokolchik, I., Zhu, J., Gong, Z., Reddy, M., Sharkhuu, A., Manabe, Y., Yokoi, S., Zhu, J. K., Bressan, R. A., Hasegawa, P. M. (2002) C-terminal domain phosphatase-like family members (AtCPLs) differentially regulate *Arabidopsis thaliana* abiotic stress signaling, growth, and development, *Proceedings of the National Academy of Sciences of the United States of America*, **99**(16), 10893-10898.

Lanahan, M. B., Yen, H.-C., Giovannoni, J. J. and Klee, H. J. (1994) The *Never ripe* mutation blocks ethylene perception in tomato, *Plant Cell,* **6**(4), 521-530.

Lashbrook, C. C., Gonzalez-Bosch, C. and Bennett, A. B. (1994) Two divergent endo-beta-1,4-glucanase genes exhibit overlapping expression in ripening fruit and abscising flowers, *Plant Cell,* **6**(10), 1485-1493.

Lashbrook, C. C., Giovannoni, J. J., Hall, B. D., Fischer, R. L. and Bennett, A. B. (1998a) Transgenic analysis of tomato endo-beta-1,4-glucanase gene function. Role of *cel1* in floral abscission, *The Plant Journal,* **13**(3), 303-310.

Lashbrook, C. C., Tieman, D. M. and Klee, H. J. (1998b) Differential regulation of the tomato *ETR* gene family throughout plant development, *Plant Journal,* **15**(2), 243-252.

Lehti-Shiu, M. D., Adamczyk, B. J. and Fernandez, D. E. (2005) Expression of MADS-box genes during the embryonic phase in *Arabidopsis*, *Plant Molecular Biology,* **58**(1), 89-107.

Leslie, M. E, Lewis, M. W. and Liljegren, S. J. (2007). Organ Abscission. In *Plant Cell Separation and Adhesion* (ed. J. Roberts and Z. Gonzalez-Carranza), pp. 106-136. Oxford: Blackwell Publishing.

Leslie, M. E., Lewis, M. W., Youn, J-Y., Daniels, M. J. and Liljegren, S. J. (2010) The EVERSHED receptor-like kinase modulates floral organ shedding in Arabidopsis. *Development* **137**, 467-476.

Lewis, L. N. and Varner, J. E. (1970) Synthesis of cellulase during abscission of *Phaseolus vulgaris* leaf explants, *Plant Physiology,* **46**(2), 194-199.

Li, Y., Jones, L. and McQueen-Mason, S. (2003) Expansins and cell growth, *Current Opinion in Plant Biology,* **6**(6), 603-610.

Libertini, E., Li, Y. and McQueen-Mason, S. J. (2004) Phylogenetic analysis of the plant

endo-beta-1,4-glucanase gene family, *Journal of Molecular Evolution,* **58**(5), 506-515.

Liljegren, S. J., Leslie, M. E., Darnielle, L., Lewis, M. W., Taylor, S. M., Luo, R., Geldner, N., Chory, J., Randazzo, P. A., Yanofsky, M. F. and Ecker, J. R. (2009). Regulation of membrane trafficking and organ separation by the NEVERSHED ARF-GAP protein. *Development* **136**, 1909-1918.

Mao, L., Begum, D., Chuang, H. W., Budiman, M. A., Szymkowiak, E. J., Irish, E. E. and Wing, R. A. (2000) *JOINTLESS* is a MADS-box gene controlling tomato flower abscission zone development, *Nature*, **406**(6798), 910-913.

Mao, L., Begum, D., Goff, S. A. and Wing, R. A. (2001) Sequence and analysis of the tomato *JOINTLESS* locus, *Plant Physiology,* **126**(3), 1331-1340.

McKim S. M., Stenvik, G-E., Butenko, M. A., Kristiansen, W., Cho, S. K., Hepworth, S. R., Aalen, R. B. and Haughn, G. W. (2008). The *BLADE-ON-PETIOLE* genes are essential for abscission zone formation in *Arabidopsis*. *Development* **135**, 1537-1546.

McKinney, E. C., Kandasamy, M. K. and Meagher, R. B. (2002) Arabidopsis contains ancient classes of differentially expressed actin-related protein genes, *Plant Physiology,* **128**(3), 997-1007.

Neljubow, D. N. (1901) Über die horizontale nutation der stengel von *Pisum sativum* und einiger anderen pflanzen. *Beitrage und Botanik Zentralblatt*, **10**, 128-139.

Norberg, M., Holmlund, M. and Nilsson, O. (2005) The *BLADE ON PETIOLE* genes act redundantly to control growth and development of lateral organs, *Development*, **132**(9), 2203-2213.

Ogawa, M., Kay, P., Wilson, S. and Swain, S. M. (2009) ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and QUARTET2 are Polygalacturonases required for cell separation during reproductive development in Arabidopsis. *Plant Cell* **21**, 216-233.

Ohno, C. K., Reddy, G. V., Heisler, M. G. B. and Meyerowitz, E. M. (2004) The *Arabidopsis JAGGED* gene encodes a zinc finger protein that promotes leaf tissue development, *Development*, **131**(5), 1111-1122.

Okushima, Y., Mitina, I., Quach, H. L. and Theologis, A. (2005a) AUXIN RESPONSE FACTOR 2 (ARF2): a pleiotropic developmental regulator, *Plant Journal*, **43**(1), 29-46.

Okushima, Y., Overvoorde, P. J., Arima, K., Alonso, J. M., Chan, A., Chang, C., Ecker, J. R., Hughes, B., Lui, A., Nguyen, D., Onodera, C., Quach, H., Smith, A., Yu, G.

and Theologis, A. (2005b) Functional genomic analysis of the *AUXIN RESPONSE FACTOR* gene family members in *Arabidopsis thaliana*: unique and overlapping functions of *ARF7* and *ARF19*, *Plant Cell,* **17**(2), 444-463.

Osborne, D. J. (1989) Abscission, *Critical Reviews in Plant Sciences*, **8**(2), 103-129.

Patterson, S. E. (2001) Cutting loose. Abscission and dehiscence in Arabidopsis, *Plant Physiology,* **126**(2), 494-500.

Patterson, S. E. and Bleecker, A. B. (2004) Ethylene-dependent and independent processes associated with floral organ abscission in *Arabidopsis*. *Plant Physiology* **134**(1), 194-203.

Pautot, V., Dockx, J., Hamant, O., Kronenberger, J., Grandjean, O., Jublot, D. and Traas, J. (2001) KNAT2: Evidence for a Link between Knotted-like Genes and Carpel Development, *Plant Cell,* **13**(8), 1719-1734.

Pinyopich, A., Ditta, G. S., Savidge, B., Liljegren, S. J., Baumann, E., Wisman, E. and Yanofsky, M. F. (2003) Assessing the redundancy of MADS-box genes during carpel and ovule development, *Nature*, **424**(6944), 85-88.

Prasad, A. M., Sivanandan, C., Resminath, R., Thakare, D. R., Bhat, S. R. and Srinivasan (2005) Cloning and characterization of a pentatricopeptide protein encoding gene (*LOJ*) that is specifically expressed in lateral organ junctions in *Arabidopsis thaliana*, *Gene,* **353**(1), 67-79.

Rick, C. M., and Butler, L. (1956) Phytogenetics of the tomato, *Advances in Genetics,* **8**, 267-382.

Roberts, J. A., Elliott, K. A. and González-Carranza, Z. H. (2002) Abscission, dehiscence, and other cell separation processes, *Annual Review of Plant Biology,* **53**, 131-158.

Rolf, H. J. and Enderle, A. (1999) Hard fallow deer antler: a living bone till antler casting?, *Anatomical Record,* **255**(1), 69-77.

Sampredro, J. and Cosgrove, D. J. (2005) The expansin superfamily, *Genome Biology*, **6**(12), 242.

Sander, L., Child, R., Ulvskov, P., Albrechtsen M. and Borkhardt, B. (2001) Analysis of a dehiscence zone endo-polygalacturonase in oilseed rape (*Brassica napus*) and *Arabidopsis thaliana*: evidence for roles in cell separation in dehiscence and abscission zones, and in stylar tissues during pollen tube growth, *Plant Molecular Biology*, **46**(4), 469-479.

Schafer, D. A. and Schroer, T. A. (1999) Actin-related proteins, *Annual Review of Cell*

and Developmental Biology, **15**, 341-363.

Schwer, B. and Shuman, S. (1996) Multicopy suppressors of temperature-sensitive mutations of yeast mRNA capping enzyme, *Gene Expression,* **5**(6), 331-344.

Sexton, R. and Roberts, J. A. (1982) Cell Biology of Abscission, *Annual Review of Plant Physiology,* **33,** 133-162.

Shibuya, K., Barry, K. G., Ciardi, J. A., Loucas, H. M., Underwood, B. A., Nourizadeh, S., Ecker, J. R., Klee, H. J. and Clark, D. G. (2004) The central role of PhEIN2 in ethylene responses throughout plant development in petunia, *Plant Physiology,* **136**(2), 2900-2912.

Shiu, S. H. and Bleecker, A. B. (2001) Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases, *Proceedings of the National Academy of Sciences of the United States of America,* **98**(19), 10763-10768.

Shuai, B., Reynaga-Peña, C. G. and Springer, P. S. (2002) The *LATERAL ORGAN BOUNDARIES* gene defines a novel, plant-specific gene family, *Plant Physiology*, **129**(2), 747-761.

Stenvik, G-E., Butenko, M. A., Urbanowicz, B. R., Rose, J. K. and Aalen, R. B. (2006). Overexpression of *INFLORESCENCE DEFICIENT IN ABSCISSION* activates cell separation in vestigial abscission zones in *Arabidopsis*. *Plant Cell* **18**, 1467-1476.

Stenvik, G-E., Tandstad, N. M., Guo, Y., Shi, C-L., Kristiansen, W., Holmgren, A., Clark, S. E., Aalen, R. B. and Butenko, M. A. (2008). The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION is sufficient to induce abscission in *Arabidopsis* through the receptor-like kinases HAESA and HAESA-LIKE2. *Plant Cell* **20**, 1805-1817.

Stogios, P. J., Downs, G. S., Jauhal, J. J., Nandra, S. K. and Prive, G. G. (2005) Sequence and structural analysis of BTB domain proteins, *Genome Biology,* **6**(10), R82.

Stone, J. M., Collinge, M. A., Smith, R. D., Horn, M. A. and Walker, J. C. (1994) Interaction of a protein phosphatase with an *Arabidopsis* serine-threonine receptor kinase, *Science*, **266**(5186), 793-795.

Szymkowiak, E. J. and Irish, E. E. (1999) Interactions between *jointless* and wild-type tomato tissues during development of the pedicel abscission zone and the inflorescence meristem, *Plant Cell,* **11**(2), 159-175.

Szymkowiak, E. J. and Irish, E. E. (2005) *JOINTLESS* suppresses sympodial identity in inflorescence meristems of tomato, *Planta,* in press.

Tang, W. and Perry, S. E. (2003) Binding site selection for the plant MADS domain protein AGL15: an *in vitro* and *in vivo* study, *Journal of Biological Chemistry,* **278**(30), 28154-28159.

Tarutani, Y., Morimoto, T., Sasaki, A., Yasuda, M., Nakashita, H., Yoshida, S., Yamaguchi, I. and Suzuki, Y. (2004) Molecular characterization of two highly homologous receptor-like kinase genes, *RLK902* and *RKL1*, in *Arabidopsis thaliana*, *Bioscience, Biotechnology, and Biochemistry,* **68**(9), 1935-1941.

Taylor, J. E., Coupe, S. A., Picton, S. and Roberts, J. A. (1994) Characterization and accumulation pattern of a messenger-RNA encoding an abscission-related beta-1,4-glucanase from leaflets of *Sambucus nigra*, *Plant Molecular Biology,* **24**(6), 961-964.

Taylor, J. E., Webb, S. T. J., Coupe, S. A., Tucker, G. A. and Roberts, J. A. (1993) Changes in polygalacturonase activity and solubility of polyuronides during ethylene-stimulated leaf abscission in *Sambucus nigra*, *Journal of Experimental Botany,* **258,** 93-98.

Taylor, J. E. and Whitelaw, C. A. (2001) Signals in abscission, *New Phytologist,* **151**, 323-339.

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*, *Nature,* **408**(6814), 796-815.

Tieman, D. M., Ciardi, J. A., Taylor, M. G. and Klee, H. J. (2001) Members of the tomato LeEIL (EIN3-like) gene family are functionally redundant and regulate ethylene responses throughout plant development, *Plant Journal,* **26**(1), 47-58.

Tieman, D. M., Taylor, M. G., Ciardi, J. A. and Klee, H. J. (2000) The tomato ethylene receptors NR and LeETR4 are negative regulators of ethylene response and exhibit functional compensation within a multigene family, *Proceedings of the National Academy of Sciences of the United States of America,* **97**(10), 5663-5668.

Tiwari, S. B., Hagen, G. and Guilfoyle, T. (2003) The roles of auxin response factor domains in auxin-responsive transcription, *Plant Cell,* **15**(2), 533-543.

Torki, M., Mandaron, P., Mache, R. and Falconet, D. (2000) Characterization of a ubiquitous expressed gene family encoding polygalacturonase in Arabidopsis thaliana, *Gene,* **242**(1-2), 427-436.

Trainotti, L., Ferrarese, L. and Casadoro, G. (1998) Characterization of cCel3, a member of the pepper endo-beta-1,4-glucanase multigene family, *Hereditas,* **128**(2), 121-126.

Trainotti, L., Spolaore, S., Ferrarese, L. and Casadoro, G. (1997) Characterization of

ppEG1, a member of a multigene family which encodes endo-beta-1,4-glucanase in xch, *Plant Molecular Biology,* **34**(5), 791-802.

Tucker, M. L., Sexton, R., del Campillo, E. and Lewis, L. N. (1988) Bean abscission cellulase, *Plant Physiology,* **88**(4), 1257-1262.

Tucker, G. A., Schindler, C. B. and Roberts, J. A. (1984) Flower abscission in mutant tomato plants, *Planta,* **160,** 164-167.

Ulmasov, T., Hagen, G. and Guilfoyle, T. J. (1997) ARF1, a transcription factor that binds to auxin response elements, *Science,* **276**(5320), 1865-1868.

Ulmasov, T., Hagen, G. and Guilfoyle, T. J. (1999) Activation and repression of transcription by auxin-response factors, *Proceedings of the National Academy of Sciences of the United States of America,* **96**(10), 5844-5849.

von Stackelberg, M., Lindemann, S., Menke, M., Riesselmann, S. and Jacobsen, H.-J. (2003) Identification of AFLP and STS markers closely linked to the *def* locus in pea, *Theoretical and Applied Genetics*, **106**(7), 1293-1299.

Wang, H., Tang, W., Zhu, C. and Perry, S. E. (2002) A chromatin immunoprecipitation (ChIP) approach to isolate genes regulated by AGL15, a MADS domain protein that preferentially accumulates in embryos, *Plant Journal,* **32**(5), 831-843.

Wilkie, I. C. (2001) Autotomy as a prelude to regeneration in echinoderms, *Microscopy Research and Technique*, **55**(6), 369-396.

Wilkinson, J. Q., Lanahan, M. B., Yen, H. C., Giovannoni, J. J., and Klee, H. J. (1995) An ethylene-inducible component of signal transduction encoded by *Never-ripe*, *Science*, **270**(5243), 1807-1809.

Yamagami T., Tsuchisaka A., Yamada K., Haddon W. R., Harden L. A. and Theologis A. (2003) Biochemical diversity among the 1-amino-cyclopropane-1-carboxylate synthase isozymes encoded by the *Arabidopsis* gene family, *Journal of Biological Chemistry*, **278**(49)**,** 49102-49112.

Yang, T.-J., Lee, S., Chang, S.-B., Yu, Y., de Jong, H. and Wing, R. A. (2005) In-depth sequence analysis of the tomato chromosome 12 centromeric region: identification of a large CAA block and characterization of pericentromere retrotransposons, *Chromosoma*, **114**(2), 103-117.

Yu, H., Ito, T., Wellmer, F. and Meyerowitz, E. M. (2004) Repression of AGAMOUS-LIKE 24 is a crucial step in promoting flower development, *Nature Genetics* **36**(2), 157-161.

Yuan, R., Wu, Z., Kostenyuk, I. A., Burns, J. K. (2005) G-protein-coupled alpha2A-adrenoreceptor agonists differentially alter citrus leaf and fruit abscission by affecting expression of ACC synthase and ACC oxidase, *Journal of Experimental Botany*, **56**(417), 1867-1875.

CHAPTER 2

REGULATION OF MEMBRANE TRAFFICKING AND ORGAN SEPARATION BY THE NEVERSHED ARF GAP PROTEIN

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SUMMARY

Cell separation, or abscission, is a highly specialized process in plants that facilitates remodeling of their architecture and reproductive success. Because few genes are known to be essential for organ abscission, we conducted a screen for mutations that alter floral organ shedding in *Arabidopsis*. Nine recessive mutations that block shedding were found to disrupt the function of an ADP-ribosylation factor-GTPase-activating protein (ARF-GAP) we have named NEVERSHED (NEV). As predicted by its homology to the yeast Age2 ARF-GAP and transcriptional profile, NEV influences other aspects of plant development including fruit growth. Co-localization experiments carried out with NEV-specific antiserum and a set of plant endomembrane markers revealed that NEV localizes to the *trans*-Golgi network and endosomes in *Arabidopsis* root epidermal cells. Interestingly, transmission electron micrographs of abscission zone regions from wild-type and *nev* flowers reveal defects in the structure of the Golgi apparatus and extensive accumulation of vesicles adjacent to the cell walls. Our results suggest that NEV ARF-GAP activity at the *trans*-Golgi network and distinct endosomal compartments is required for the proper trafficking of cargo molecules required for cell separation.

INTRODUCTION

A distinctive feature of plants is their remarkable ability to release entire organs such as leaves, flowers, fruit and seeds by modifying cellular adhesion. Organ separation occurs through the trafficking and secretion of enzymes that locally alter cell walls and dissolve the pectin-rich middle lamella between cells in an abscission zone. The *Arabidopsis* flower has become a model system to investigate several independently-regulated separation events, including stamen dehiscence, floral organ separation, fruit opening, and seed abscission (Aalen et al., 2006; Lewis et al., 2006; Roberts et al., 2002). Through organ abscission, the three outer whorls of floral organs—the sepals, petals, and stamens—are shed after the stamens release their pollen and fertilization occurs (Fig. 2.1A,B). The abscission zones consist of a few cell layers at the base of each of these organs adjacent to the floral stem.

Genetic analysis in *Arabidopsis* is revealing an expanding set of factors that are required for or affect the timing of organ separation. Integral components include a pair of closely-related, redundant leucine-rich repeat receptor-like kinases, HAESA (HAE) and HAESA-LIKE2 (HSL2), which are likely activated by the IDA and IDA-LIKE secreted peptides, and which in turn switch on a conserved mitogen-activated protein kinase signal transduction pathway essential for activating the separation process (Butenko et al., 2003, Cho et al., 2008, Jinn et al., 2000, Stenvik et al., 2008). Two of the polygalacturonase enzymes that contribute to the cell wall remodeling required for floral organ shedding have been identified (González-Carranza et al., 2007; Ogawa et al., 2009). While factors that directly influence abscission zone differentiation have remained elusive, a pair of redundant transcription factors, BLADE-ON-PETIOLE1 (BOP1) and

BOP2, affects the patterning of leaf and floral organ proximal zones, a prerequisite for abscission zone specification (Hepworth et al., 2005, McKim et al., 2008, Norberg et al., 2005). The timing of organ abscission is influenced by signaling via the plant hormones ethylene and auxin (Ellis et al., 2005; Okushima et al., 2005; Patterson and Bleecker, 2004; Patterson et al., 2007) and by transcriptional regulation and chromatin-remodeling that may globally affect both senescence and abscission (Fernandez et al., 2000, Kandasamy et al., 2005a, Kandasamy et al., 2005b).

In contrast to animal cells that undergo frequent changes in adhesion to facilitate cell migration and the development of a multilayered body plan, plant cells rarely separate from one another. When separation does occur, unique changes in membrane trafficking can be expected to play an essential role in modifying the highly cross-linked cell walls. While numerous *Arabidopsis* mutants that alter membrane trafficking have been found to affect cell expansion and polarized growth (Lycett, 2008; Yang, 2008), none have yet been reported to affect cell separation.

Key regulators of membrane trafficking in yeast, animal and plant cells include members of two families that influence the activity and localization of the small ADP-ribosylation factor (ARF) G-proteins during vesicle formation and cargo recruitment (D'Souza-Schorey and Chavrier, 2007; Inoue and Randazzo, 2007; Nielsen et al., 2008). ARF-guanine exchange factors (ARF-GEFs) increase levels of the active GTP-bound ARF protein, which is tethered to the membrane by a myristoylated tail. By stimulating GTP hydrolysis, ARF-GTPase-activating proteins (ARF-GAPs) regenerate the inactive, soluble GDP-bound ARF protein. In addition, interactions of ARF-GAPs with lipids, coat

proteins and cargo receptors promote efficient loading of cargo into vesicles and remodeling of the actin cytoskeleton (Inoue and Randazzo, 2007).

Functional characterization of *Arabidopsis* ARF-GAP and ARF-GEF families is revealing roles for these trafficking regulators during specific phases of plant development. The ARF-GAP VASCULAR NETWORK DEFECTIVE3/SCARFACE (VAN3/SCF) and three closely-related homologs—ARF-GAP DOMAIN1 (AGD1), AGD2, and AGD4—together control vein patterning in developing leaves, potentially by affecting auxin signaling and transport (Koizumi et al., 2005, Sieburth et al., 2006). AGD1 also plays a key role in the orientation of root hair growth by influencing cytoskeletal organization (Yoo et al., 2008). ROOT AND POLLEN ARF-GAP (RPA) affects polarized tip growth of root hairs and of pollen tubes (Song et al., 2006). The ARF-GEF GNOM plays a crucial role during embryo development by regulating endosomal cycling of the PIN1 auxin efflux carrier and thereby controlling auxin transport (Geldner et al., 2003; Steinmann et al., 1999). Together with its homolog GNOM-LIKE1 (GNL1), GNOM also functions during gametophytic development and root growth (Richter et al., 2007). With the *Arabidopsis* genome predicted to encode fifteen ARF-GAPs and eight ARF-GEFs potentially regulating twenty-one ARF-GTPases (Vernoud et al., 2003), much work remains to uncover the unique and overlapping functions of each member of these families in membrane trafficking and their impact on plant development.

Here, we report that the ARF-GAP protein NEVERSHED (NEV) is required for floral organ abscission. Our results suggest that mutations in *NEV* alter the organization

of the Golgi apparatus, and block the movement of molecules required for cell separation, providing a link between membrane trafficking and cell separation in plants.

MATERIALS AND METHODS

Plants

Mutant alleles of *NEV* were obtained through EMS screens as described (Liljegren et al., 2000). The *nev-1*, *nev-3*, and *nev-9* alleles contain nucleotide substitutions within codons 51, 59, and 34, which change a cysteine to a tyrosine, an arginine to a lysine, and a cysteine to a tyrosine, respectively. The *nev-2*, *nev-4*, and *nev-5* alleles contain nucleotide substitutions within codons 198, 260, and 122, which change a glutamine, a tryptophan, and a tryptophan to stop codons, causing production of truncated proteins of 197, 259, and 121 amino acids, respectively. The *nev-8* allele contains a nucleotide substitution at the splice acceptor site of the seventh intron. The *nev-6* (SALK_075680) and *nev-7* (SALK_079928) alleles contain T-DNA insertions in the first intron. With the exception of *nev-6* and *nev-7*, all alleles identified are of the Ler ecotype. The *nev-3* mutant was backcrossed to L*er* three times prior to phenotypic analyses; homozygous mutants can be detected by a BspCNI site introduced by the dCAPs oligo 5'-CTGCATGCAATGTTCTGGGATTCTCA-3'.

Mapping

To map *NEV*, *nev-1* and *nev-2* mutants were crossed to wildtype (Col). Using DNA isolated from 515 *nev* F2 plants and PCR-based markers including several designed

from Monsanto Ler polymorphisms (http://www.Arabidopsis.org/Cereon/index.html), both mutations were mapped to chromosome 5 between CER436970 and CER465330. The coding regions of 8 of 13 predicted genes in this region were sequenced from *nev* genomic DNA.

Generation of NEV-specific antiserum and immunodetection

NEV cDNA (GenBank accession number FJ794601) was PCR amplified with 5'-AGATATTGAAGAGCGCTGAAGGCG-3' and 5'-TCCGTTTAAGTCTTTTTCAGAGAGAGAG-3' using Col RNA as template. After cloning the cDNA into pCR2.1 (Life Technologies, Carlsbad, CA), a fragment encoding the unique carboxy-terminus corresponding to amino acids 266-483 was amplified with 5'-CACCGCTGGAAGTGGTCAAACG-3' and 5'-TCAATGTTTTGTGAACATTCCATCC-3' and inserted into pENTR/D-TOPO (Life Technologies, Carlsbad, CA). Recombination of this construct with pDEST17 (Life Technologies, Carlsbad, CA) was used to express truncated, 6XHis-tagged NEV protein in *Escherichia coli*. Recombinant protein purified by Ni^{2+} affinity chromatography (HisTrap: GE Healthcare, Chalfont St. Giles, UK) was used to immunize rabbits (Covance Research Products, Denver, PA).

Protein extracts were prepared by grinding floral tissue, incubating samples with extraction buffer (50 mM TrisHCl [pH 8.5], 2% SDS) at 60°C for 20 minutes, pelleting by centrifugation at 16K *g* for 5 minutes, and resuspending the supernatant in loading buffer (5 mM EDTA [pH 8.5], 10% glycerol, 50 mM mercaptoethanol, 0.05%

bromophenol blue). Proteins were separated by SDS-PAGE and analyzed by western blotting. Anti-NEV antibody was used at 1:20K concentration.

Microscopy

Wild-type flowers were analyzed by scanning electron microscopy as described (Liljegren et al., 2000). For transmission electron microscopy, flowers were fixed in 2% paraformaldehyde/1% glutaraldehyde in 0.05M sodium phosphate buffer overnight at 4^0C. After fixation, samples were stained with 1% osmium tetroxide, dehydrated through an ethanol series and embedded in Spurr's resin. Sections (60-70 nm) were cut with a diamond knife, mounted on copper mesh grids, stained with 4% uranyl acetate followed by Reynolds lead citrate, and examined with a LEO EM-910 transmission electron microscope (LEO Electron Microscopy, Thornwood, NY) at 80 kV with images recorded using a Gatan Orius SC1000 CCD camera (Gatan, Pleasanton, CA). Sections (1 μm) from the above samples were stained with 1% toluidine blue for light microscopy. To determine the extent of paramural body accumulation, individual cells were examined for the presence of plasma membrane-associated clusters of ten or more vesicles. Plasma membrane perimeters were determined with NIH imageJ software.

For immunofluorescence assays, seedlings were grown vertically on MS media plates for 5-10 days then prepared as described (Lauber et al., 1997) with minor modifications. Primary antibodies were used at the following concentrations: anti-NEV, 1:2500; chicken anti-GFP, 1:500 (Abcam, Cambridge, MA). Fluorochrome-conjugated secondary antibodies were used at a 1:500 concentration: Alexa Fluor 488 anti-rabbit F(ab')2 and Alexa Fluor 647 anti-rabbit (Molecular Probes, Eugene, OR); anti-chicken

Cy2 (Abcam, Cambridge, MA). Confocal laser scanning microscopy was performed with a Zeiss LSM-410 or 510 (Carl Zeiss, Thornwood, NY). Image brightness and contrast were adjusted with Photoshop 7.0 (Adobe, Mountain View, CA). For BFA treatment, 5-10 day old seedlings were incubated in MS liquid media with or without 100 μM BFA for 50-60 minutes, fixed in 4% formaldehyde and prepared for immunofluorescence detection as described above.

ARF-GTPase-activating assay

The *NEV* coding region was amplified with 5'-CACCATGAACGAGAAAGCCAACGTC-3' and 5'-TCAATGTTTTGTGAACATTCCATCC-3' and inserted into pENTR/D-TOPO (Life Technologies, Carlsbad, CA). Recombination of this construct with pDEST17 was used to express full-length 6XHis-tagged NEV protein in *E. coli.* Recombinant NEV protein was purified as described above. Nonmyristoylated Arf1 was prepared as described (Randazzo et al., 1992) for use in the GTPase-activating assay performed as described using recombinant AGAP1 as a positive control (Che et al., 2006). Briefly, [α32P]GTP was exchanged for GDP bound to Arf1. [α32P]GTP•Arf1 was incubated with the indicated concentration of NEV for 3 minutes at 30°C. The reaction was terminated by dilution and shifting to 4°C. Arf1 was separated from free nucleotide by filtering on nitrocellulose. Nucleotide was released from Arf1 with formic acid and the relative level of GDP and GTP was determined by quantifying P32 following thin layer chromatographic separation of the nucleotides. The data are presented as the percentage of GTP converted to GDP. No conversion was observed in the absence of a GAP.

Generation of transgenic lines

A 1.8 kb genomic region of NEV, from 908 nucleotides upstream of the predicted translational start site into exon two, was amplified with 5'-GAGCCAGCGAGAGACCACTTCTC-3' and 5'-CTCTAGAAGAATCTGCAGATAAGTAGCAC-3' using Col DNA as template. This fragment was cloned into pCR2.1, excised as XbaI fragment, and cloned into pDW137 (Blázquez et al., 1997) to create a translational fusion of NEV to the ß-glucuronidase (GUS) reporter. GUS expression was analyzed as described (Blázquez et al., 1997) with minor modifications.

The transgenic marker lines YFP-VTI12, YFP-RabA1e, YFP-RabA1g, YFP-RabF2a, YFP-RabD2a, YFP-NIP1;1, and YFP-NPSN12 used in co-localization studies are part of the "Wave" marker collection (Geldner et al., 2009). The VHA-a1-RFP and NAG-GFP markers were described previously (von der Fecht-Bartenbach et al., 2007; Grebe et al., 2003).

RESULTS

Mutations in *NEV* prevent floral organ shedding

To identify novel loci required for organ abscission, we carried out mutant screens of *Arabidopsis* plants as flowers matured. Six mutants that retain their floral organs indefinitely represent a unique complementation group we have named *nevershed*

(*nev*) (Fig. 2.1C). A seventh *nev* mutant allele was kindly provided by Pataradawn Pinyopich (UCSD).

As each of the identified mutant alleles blocks floral organ abscission, we characterized *nev-3* as a representative allele. In examining sections of *nev* flowers at a range of developmental stages compared to wild type (Fig. 2.1F,G; data not shown), differences in abscission zone morphology were first detected when organ separation normally occurs in wild-type flowers (stage 16). At this point, abscission zone cells expand and become vacuolated in wild type (Fig. 2.1F), whereas in *nev* flowers the organs stay attached and cells within the sepal and petal abscission zones remain small and densely cytoplasmic (Fig. 2.1G). Since abscission zones are thought to be specified in the proximal regions of the floral organs as they differentiate, enhancer trap lines with early expression profiles at the bases of wild-type floral organs (Campisi et al., 1999) were examined in *nev* flowers (Fig. 2.S1). No temporal or spatial differences were detected for four different markers in *nev* flowers compared to wild type. Taken together, these results suggest that NEV acts during the separation phase of organ abscission, rather than during the initial patterning and differentiation of abscission zone cells.

To determine whether NEV function is specific to abscission, we examined whether other aspects of plant development are affected by mutations in *NEV*. While floral organ separation is tightly linked to the initiation of organ senescence (Fang and Fernandez, 2002), the aerial (cauline) leaves of wild-type *Arabidopsis* plants can be shed well after senescence has occurred (Fig. 2.1D). Detachment of these leaves is blocked in *nev* mutants (Fig. 2.1E). Fruit (silique) growth is also significantly affected by mutations

in *NEV* (Fig. 2.S2A,B). These results indicate that NEV plays a role in multiple developmental processes.

NEV encodes an ADP-ribosylation factor GTPase-activating protein

Using standard mapping procedures, the *nev-1* and *nev-2* mutations were located within a 39 kb interval on the lower arm of chromosome 5, including the BACs MDK4 and GA469. Within one of thirteen predicted genes in this interval, At5g54310, a single nucleotide change was identified in the *nev-2* mutant that would introduce a stop codon into the corresponding transcript (Fig. 2.2A). This gene includes 11 exons and encodes a predicted 483-amino acid protein with a zinc finger ($CX_2CX_{16}CX_2C$) characteristic of ARF-GAP proteins located near its N-terminus (Fig. 2.2A). Three of the nine *nev* alleles introduce missense mutations within the highly conserved 115-amino acid ARF-GAP domain, with two, *nev-1* and *nev-9*, altering critical cysteine residues in the zinc finger motif to tyrosines, and a third, *nev-3*, changing an invariant arginine in close proximity to the zinc finger to a lysine (Fig. 2.2B). This particular arginine has been demonstrated to be essential for ARF-GAP activity for all ARF-GAPs examined (Luo et al., 2007) and structural analysis of the PAPß/ARF1 complex suggests that it may act as an "arginine finger" directly contacting the active site of the ARF substrate during GTP hydrolysis (Mandiyan et al., 1999). Four additional alleles are predicted to affect the C-terminal region of the protein by introducing premature stop codons (*nev-2*, *nev-4*, *nev-5*) or affecting splicing (*nev-8*) (Fig. 2.2A). Two alleles of *NEV* identified in the Salk T-DNA mutant collection (Alonso et al., 2003), *nev-6* and *nev-7*, contain insertions in the first intron and are predicted to encode a truncated protein without an ARF-GAP domain (Fig.

2.2A). Since plants carrying either of these null alleles are phenotypically indistinguishable from the other *nev* mutants, all of the identified mutations appear to completely disrupt the function of *NEV* and are considered to be strong loss-of-function alleles.

Comparison of NEV with other eukaryotic ARF-GAPs has revealed that NEV shares 49% amino acid identity within its ARF-GAP domain with yeast ArfGAP effector2 (Age2), 50% identity with Age2-like ARF-GAPs in worms and mice, and 73% identity with another Age2-like ARF-GAP in *Arabidopsis*, AGD15 (Fig. 2.2B). In contrast to NEV, Age2 activity has been found to be entirely redundant with that of another yeast ARF-GAP, Growth cold sensitive1 (Gcs1) (Poon et al., 2001). The yeast *age2 gcs1* double mutant disrupts the morphology of the Golgi, membrane trafficking from the *trans*-Golgi network to the endosome, vacuole, and plasma membrane, and is lethal. In mammals, two Age2-related ARF-GAPs, SMAP1 and SMAP2, regulate clathrin-mediated endocytosis from the plasma membrane and retrograde traffic from the early endosome to the *trans*-Golgi network, respectively (Natsume et al., 2006; Tanabe et al., 2005). Outside of the ARF-GAP domain, the NEV protein is poorly conserved with Age2 and Age2-like ARF-GAPs, and the conserved clathrin-binding domains of SMAP1 and SMAP2 are not present in the C-terminal regions of either NEV or Age2.

Homology searches also revealed predicted proteins with strong sequence similarity to NEV in diverse species of flowering plants, such as *Oryza sativa* (rice). A candidate rice ortholog shares 90% amino acid identity within its ARF-GAP domain and 48% amino acid identity overall with NEV; several conserved regions outside of the ARF-GAP domain are apparent (Figs 2.2B, 2.S3).

To determine whether NEV is able to function as an ARF-GAP, we tested its ability to promote GTP hydrolysis of the mammalian ARF1 G-protein substrate *in vitro* (Fig. 2.2C). Recombinant full-length NEV protein was expressed in *E. coli*, purified, and used in an ARF-GAP assay, along with a mammalian ARF-GAP, AGAP1, as a positive control. NEV promoted GTP hydrolysis of ARF1 in a concentration-dependent manner with enzymatic activity levels similar to those of other *Arabidopsis* ARF-GAPs acting on heterologous ARF substrates (Koizumi et al., 2005).

NEV is expressed broadly during development

Global analysis of *Arabidopsis* gene expression profiles revealed that *NEV* is expressed ubiquitously during development in floral, leaf, stem, and root tissue (Fig. 2.S4; Schmid et al., 2005). To determine whether expression of *NEV* might be enriched in certain cell types within these tissues, transgenic plants were generated that express a translational fusion of NEV, including 908 nucleotides upstream of the predicted translational start site through part of the second exon, to the complete coding region of ß-Glucuronidase (GUS). Of 19 T1 plants analyzed, 9 showed broad expression patterns of GUS in the inflorescence stem, abscission zones, stigma, floral and/or leaf vasculature (Fig. 2.2D,E), 8 showed expression profiles in a subset of these tissues, and 2 showed no apparent expression. These results together with our phenotypic analysis suggest that *NEV* likely functions broadly during plant development.

NEV localizes to the *trans*-Golgi network and endosomes

ARF-GAPs modulate membrane trafficking from multiple sites, including the Golgi, *trans*-Golgi network, and recycling endosome (Inoue et al., 2008; Min et al., 2008; Poon et al., 1999; Poon et al., 2001). Analysis of NEV using WoLF PSORT (Horton et al., 2007) showed a possible monopartite nuclear localization signal between amino acids 14 and 17 (RHRK) and no other known sorting motifs. To determine the subcellular site of NEV action, we examined its localization using NEV polyclonal antiserum raised against its unique C-terminal region. This antiserum recognized a ~55 kDa protein in wild-type flower extracts, consistent with the predicted size of NEV (53 kDa) (Fig. 2.3A). Because this protein was not recognized in extracts from *nev-2* flowers containing truncated nev protein, the antiserum appears to be specific for NEV (Figs 2.2A, 2.3A). Immunolocalization and confocal laser-scanning microscopy revealed that the NEV antiserum labels punctate spots in wild-type root epidermal cells (Fig. 2.3B). As expected, the signal profile observed in wild-type roots is not consistent with nuclear localization and a substantial reduction of signal is observed in *nev-2* roots (Fig. 2.3B,C).

We next used a set of markers for specific plant endomembrane compartments (Table 2.S1) to explore the precise localization of NEV (Figs 2.3, 2.S5). Because Age2 localizes to and regulates traffic from the *trans*-Golgi network (TGN) in yeast (Poon et al., 2001), we first examined the co-localization of NEV with markers of the TGN and Golgi (Fig. 2.3). While NEV localization is distinct from the Golgi marker NAG-GFP (Fig. 2.3D-F) (Grebe et al., 2003), the punctate spots labeled by NEV antiserum co-localize with spots of a similar size and structure labeled by the TGN/early endosome (EE) marker YFP-VTI12 (arrows, Fig. 2.3G-I) (Sanderfoot et al 2001). To further confirm that this marker labels the TGN/EE, co-localization of YFP-VTI12 with the

TGN/EE marker VHA-a1-RFP was shown (Fig. 2.S6) (von der Fecht-Bartenbach et al., 2007). In plants, accumulating evidence exists for a unified TGN/EE compartment (Dettmer et al., 2006; Müller et al., 2007; Robinson et al., 2008). These results suggest that NEV is localized at the TGN/EE.

As we observed occasional sites where NEV fluorescence does not overlap with the YFP-VTI12 marker (arrowheads, Fig. 2.3G-I), a wider range of endomembrane markers was tested for co-localization with NEV. We discovered that NEV also co-localizes with two novel endosomal markers, YFP-RabA1e and YFP-RabA1g (arrows, Fig. 2.3J-L; data not shown) proposed to label recycling endosomes (Geldner et al., 2009). The endosomal compartment labeled by these markers is distinct from the TGN/EE and shows a high sensitivity to BFA (Geldner et al., 2009). Rab11, the closest mammalian homolog of the expanded RabA class in *Arabidopsis*, regulates trafficking through the recycling endosome; other characterized RabA family members in *Arabidopsis* and tomato are thought to localize at the TGN/EE (Chow et al., 2008; Nielsen et al., 2008; Rehman et al., 2008). NEV did not co-localize with the YFP-RabF2A marker of the late endosome/pre-vacuolar complex (Fig. 2.S5A) (Lee et al., 2004; Ueda et al., 2004) or the YFP-NIP1;1 marker of ER network (Fig. 2.S5B) (Geldner et al., 2009). Vesicles labeled by NEV occasionally overlapped with YFP-NPSN12, a marker of the plasma membrane (Fig. 2.S5C) (Zheng et al., 2002). NEV also partially localized with YFP-RabD2a, a marker associated with the Golgi network and endosome (Fig. 2.S5D) (Zheng et al., 2005). Taken together, these results are consistent with roles for NEV in regulating trafficking at the TGN/EE and a distinct endosomal compartment which may correspond to recycling endosomes.

To further explore NEV localization, we used Brefeldin A (BFA), a fungal toxin that interferes with the activity of a subset of BFA-sensitive ARF-GEFs, inhibiting ARF-mediated vesicular transport from the target membrane (Peyroche et al., 1999). In *Arabidopsis* roots, BFA inhibits recycling of vesicles back to the plasma membrane without blocking endocytosis or ER-Golgi traffic, resulting in the accumulation of the TGN/EE and rapidly cycling proteins such as PIN1 into BFA-bodies (Geldner et al., 2003; Grebe et al., 2003; Richter et al., 2007; Robinson et al., 2008; Teh and Moore, 2007). Golgi, on the other hand, tend to cluster around the BFA-bodies while remaining functionally intact. When we treated primary roots with 100 μM BFA prior to immunodetection of NEV, NEV localizes to structures resembling BFA-bodies rather than the punctate spots characteristic of untreated cells (Figs 2.3N, 2.S7). In the presence of BFA, NEV localization within the BFA-compartment overlaps with but is more disperse than that of the endosomal marker YFP-RabA1e found at the core of the BFA-compartment (arrows, Fig. 2.3N-O). These results suggest that NEV is not only localized to the TGN/EE and distinct endosomes, but also to additional BFA-resistant membranes.

Mutations in *NEV* disrupt Golgi structure, alter location of the *trans*-Golgi network, and cause accumulation of paramural vesicles

Since *nev* mutations affect a member of a protein family known to regulate membrane trafficking, we examined whether subcellular defects could be detected in *nev* mutant cells. Transmission electron microscopy was performed on *nev* flowers compared to wild type at the time of organ shedding (stage 16). We discovered that cells in the region of *nev* sepal abscission zones predominantly exhibited circularized or cup-shaped

multilamellar structures (Figs 2.4B, 2.5D, 2.S8D-F) rather than the flat stacks of Golgi cisternae typical of wild type (Figs 2.4A, 2.5D, 2.S8A-C). We also often observed the TGN closely associated with the *trans*-face of the Golgi in wild-type cells (Figs 2.4B, 2.S8A-C), but did not readily observe these tubular-vesicular networks near the circularized structures in *nev* abscission zone cells (Figs 2.4B, 2.S8D-F). In older *nev* flowers (stage 17), a mixture of Golgi with a wild-type appearance and circularized structures were observed in sepal abscission zones (data not shown). These unusual compartments were also observed in the two other regions of *nev* flowers (stage 16) examined—fruit walls and floral stems (pedicels), although vesicular structures resembling the TGN were more apparent in the fruit cells examined (Fig. 2.5C,D). Taken together, these results suggest that defects in NEV-regulated trafficking can severely affect the structure of the Golgi apparatus and alter the location of the TGN.

Extensive accumulation of vesicles between the plasma membrane and cell wall was also observed in the sepal abscission zone regions of *nev* flowers (Fig. 2.4D). Vesicle clusters associated with pockets of the plasma membrane have been termed paramural bodies (PMBs) by researchers who have also observed them in the embryos of the *Arabidopsis vps9a* Rab-GEF mutant and in barley leaf cells upon fungal infection (An et al., 2007; Goh et al., 2007). While paramural vesicles and bodies are present in wild-type cells (Fig. 2.4C,E), far more PMBs containing numerous vesicles were found in *nev* flowers (Fig. 2.4D,F) (0.002 +/- 0.01 PMB/µm plasma membrane in wild-type cells [n=20] compared to 0.082 +/- 0.066 PMB/µm plasma membrane in *nev* cells [n=28]). Some PMBs in *nev* flowers have the appearance of a recent fusion of a multivesicular body to the plasma membrane (Fig. 2.4F). Additional PMBs were also observed in the

fruit walls and pedicels of *nev* flowers (Figs 2.5F,H; 2.S9B,D) compared to wild type (Figs 2.5A,C; 2.S9A,C). These results suggest that NEV may play a role in PMB turnover or biogenesis.

DISCUSSION

We report here the identification and characterization of NEV, an *Arabidopsis* Age2-like ARF-GAP that is required for floral organ abscission. Our studies suggest that NEV plays an essential role in membrane trafficking during the separation stage of organ abscission by facilitating the movement of key cargo molecules through the TGN/EE and other distinct endosomes. While mutations in *NEV* do not appear to affect the early differentiation of abscission zone cells, they alter their fate. Instead of undergoing cell separation and expansion, cells in the abscission zone regions of *nev* flowers remain small and cytoplasmic (Figs 2.1, 2.4). Disruption of membrane trafficking in *nev* flowers could affect the localization of signaling molecules required to initiate the separation process, the subsequent secretion of cell wall modifying enzymes, or the endocytosis of cell wall materials. Key molecules in the signaling network thought to activate the separation phase of organ abscission include the IDA and IDA-LIKE peptides, and their proposed receptors, the transmembrane HAE and HSL2 leucine-rich repeat receptor-like kinases (Butenko et al., 2003, Cho et al., 2008, Jinn et al., 2000, Stenvik et al., 2008). Cell wall modifying and hydrolytic enzymes—polygalacturonases, cellulases, expansins, and others—are secreted by abscission zone cells to loosen their cell walls and and dissolve the pectin-rich middle lamellae. Identifying which cargo molecules are

incorrectly trafficked in *nev* flowers will be central to understanding further the role of NEV in abscission.

We discovered two unusual subcellular defects in the cells of *nev* mutant flowers: circularized multilamellar structures and extensive paramural bodies. Either or both of these defects may be responsible for blocking organ abscission. Because of their resemblence to the Golgi cisternae, and the corresponding lack of Golgi with a wild-type morphology and closely associated TGN (Figs 2.4, 2.5, 2.S8), we believe the multilamellar structures are Golgi-derived and may represent TGN-Golgi fusions. It is noteworthy that localization of NEV at the TGN and its role in maintaining normal Golgi morphology further support a conserved function with its closest yeast ArfGAP relative, Age2. Although the *age2* single mutant is unaffected, the *age2 gcs1* double mutant accumulates atypical membrane-bound structures due to a fragmentation of the *trans*-Golgi network (Poon et al., 2001). The appearance of these structures is clearly distinct from the circularized structures we see in *nev* flowers, which is not surprising as wild-type *Saccharomyces cerevisiae* Golgi are composed of a single disk in contrast to the multilamellar organization of the plant Golgi system (Preuss et al., 1992). In the future, we plan to test the potential chimeric nature of the aberrant multilamellar structures we have observed.

Interestingly, the multilamellar structures that we see in *nev* flower cells (Figs 2.4, 2.S8) are similar in appearance to Golgi-derived structures that form when the activity of *Arabidopsis* vacuolar H^+-ATPase (V-ATPase) complexes are reduced (Brüx et al., 2008; Dettmer et al., 2005; Dettmer et al., 2006; Neubert et al., 2008). The eukaryotic V-ATPase complex is a multi-subunit proton transporter that is critical for acidification of

intracellular organelles and vesicles, thereby affecting secretory and endocytic trafficking (Dettmer et al., 2006; Marshansky and Futai, 2008). The characteristic changes in Golgi organization that are seen in cases of reduced V-ATPase activity, bending of the stacks and swelling at the ends of the cisternae, are thought to be due to defects in vesicle trafficking (Dettmer et al., 2006). Disruption of V-ATPase activity has severe effects on development, as illustrated by the block in pollen development associated with hypomorphic mutations in the TGN/early endosome-localized VHA-a1 subunit (Dettmer et al., 2005; Dettmer et al., 2006). V-ATPases are known to be required for the recruitment of proteins involved in vesicle budding, including ARF G-proteins, ARF-GEFs, and coat components (Aniento et al., 1996; Hurtado-Lorenzo et al., 2006; Maranda et al., 2001; Zeumen et al., 1992). Since ARF-GAPs modulate vesicle formation by promoting GTP hydrolysis of ARF G-proteins and interacting with coat components, the similarity between the multilamellar structures found in *nev* and V-ATPase mutants suggests the possibility that TGN-localized NEV and V-ATPase complexes may jointly regulate the recruitment and activity of the same ARF G-protein(s).

The hyperaccumulation of paramural vesicles in *nev* flowers, fruit and pedicels (Figs 2.4D; 2.S8B,D) is a surprising discovery, and would appear to represent a role for NEV in membrane trafficking that is not conserved with yeast Age2. NEV and the Rab-GEF VPS9a are the first reported *Arabidopsis* proteins with potential roles in PMB biogenesis or turnover (Goh et al., 2007). The functions and origin of paramural bodies and vesicles in plants are unknown, although their appearance has been associated with cell-plate formation, secondary cell wall thickening, and exposure to pathogens (An et al., 2007). One possibility, previously offered by Goh et al., (2007) is that PMBs could

represent a novel form of endocytosis, by which cell wall materials are taken up into vesicles and transported into the cell for recycling or degradation. Whereas this model is congruent with the need for extensive cell wall modifications during plant cell separation events, the currently favored model is that paramural vesicles in plants may be analogous to the exosomes produced by other higher eukaryotes (An et al., 2007). In animals, exosomes are formed in multivesicular bodies by the reverse budding of membrane into the lumen of late endosomal compartments, and secreted by fusion of the multivesicular body with the plasma membrane rather than with a lytic compartment (van Niel et al., 2006). Exosomes can carry transmembrane signaling molecules and are known to play roles in immunity, development, and diseases such as cancer; a host cell's multivesicular body pathway can also be hi-jacked by viruses to release viral particles within exosomes (Gould et al., 2003; van Niel et al., 2006; Lakkaraju and Rodriguez-Boulan, 2008). Based on the current understanding of multivesicular body biogenesis, interlumenal vesicles destined for exocytosis versus degradation are indistinguishable in size yet differ in membrane composition (Trajkovic et al., 2008). Analysis of the orientation, content and membrane composition of *nev* and wild-type paramural vesicles will provide further insights into the origin and intended destination of these structures as well as their potential role in cell separation.

The expression profile of *NEV* and its homology to Age2 suggest that it likely plays a more fundamental role in development than is indicated by the *nev* single mutant phenotype (Figs 2.1, 2.S2). Since Age2 activity is redundant with that of the Gcs1 ArfGAP in controlling traffic in yeast cells from the *trans*-Golgi network to the endosome, plasma membrane and vacuole (Poon et al., 2001), further functional analysis

of the *Arabidopsis* Age2-like and Gcs1-like family members NEV, AGD15, AGD6, and AGD7 may reveal broad, overlapping requirements for these proteins in plant growth and development.

If NEV is indeed regulating membrane trafficking in multiple plant cell types, why do mutations in *NEV* have such a profound effect in abscission zone cells? One possibility is that because these cells rely on a tremendous surge of vesicle trafficking to carry out the extensive cell wall remodeling necessary for cell separation within a short time frame, even a minor delay in traffic could affect this process. More than thirty years ago, ultrastructural studies of leaf abscission zone cells revealed evidence of the importance of vesicle trafficking and protein synthesis in cell separation, with dramatic increases in the area of cytoplasm occupied by the ER and Golgi bodies observed upon induction of abscission (Sexton and Hall, 1974; Sexton et al., 1977). Furthermore, the response of abscission zone cells to *nev*-mediated trafficking defects could be exacerbated by a feedback loop pushing increased vesicle traffic through the system in response to the lack of cell separation. The potential of increased traffic in *nev* mutant flowers suggest that it may represent a sensitized background to screen for mutations in the signaling components NEV may regulate.

To date, analysis of distinct cell separation events during plant development has not uncovered any common regulatory factors (Lewis et al., 2006). Leaf and floral organ abscission may prove to be an exception. Floral organs and leaves have long been considered to be derived from the same basal structure, and many of the same regulatory networks act to establish their early pattern and polarity (Bowman et al., 2002; Smyth, 2005). It is intriguing that neither floral organ shedding nor aerial leaf detachment occurs

in *Arabidopsis* plants carrying mutations in *NEV*. Because *Arabidopsis* leaf detachment is not considered a classical form of abscission, future studies to test whether candidate orthologs of *NEV* control the shedding of both types of lateral organs in other angiosperms will be informative.

Throughout the history of crop domestication, breeders have selected varieties of grains with reduced shattering characteristics and adopted plants such as the 'jointless' tomato mutant that lack fruit abscission (Butler, 1936; Doebley, 2004; Li et al., 2006). Chemicals that control abscission are currently sprayed on fruit trees to prevent preharvest fruit drop, and used to extend the lifetime of cut flowers and potted plants. Alternative strategies that target specific cell separation events and allow fine-tuning of the timing of separation are expected to arise from increased knowledge of the molecular basis of domestication-related traits and the mechanisms that model plants use to orchestrate this remarkable process.

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REFERENCES

Aalen, R. B., Butenko, M. A., Stenvik, G. E., Tandstad, N. M. and Patterson, S. E. (2006). Genetic control of floral abscission. In *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues Vol. I* (ed. J.A. Teixeira da Silva), pp. 101-108. Isleworth, UK: Global Science Books.

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**, 653-657.

An, Q., van Bel, A. J. E. and Hückelhoven, R. (2007). Do plant cells secrete exosomes derived from multivesicular bodies? *Plant Signaling & Behavior* **2**, 4-7.

Aniento, F., Gu, F., Parton, R. G. and Gruenberg, J. (1996). An endosomal beta COP is involved in the pH-dependent formation of transport vesicles destined for late endosomes. *J. Cell Biol*. **133**, 29-41.

Blázquez, M. A., Soowal, L. N., Lee, I. and Weigel, D. (1997). *LEAFY* expression and flower initiation in *Arabidopsis*. *Development* **124**, 3835–3844.

Bleecker, A. B., Estelle, M. A., Somerville, C. and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in Arabidopsis thaliana. *Science* **241**, 1086-1089.

Bowman, J. L., Eshed, Y. and Baum, S. F. (2002). Establishment of polarity in angiosperm lateral organs. *Trends in Genetics* **18**, 134-141.

Brüx, A., Liu, T.-Y., Krebs, M., Stierhof, Y.-H., Lohmann, J. U., Miersch, O., Wasternack, C. and Schumacher, K. (2008). Reduced V-ATPase activity in the trans-Golgi network causes oxylipin-dependent hypocotyl growth inhibition in *Arabidopsis*. *Plant Cell* **20**, 1088-1100.

Butenko, M. A., Patterson, S. E., Grini, P. E., Stenvik, G. E., Amundsen, S. S., Mandal, A. and Aalen, R. B. (2003). *INFLORESCENCE DEFICIENT IN ABSCISSION* controls floral organ abscission in Arabidopsis and identifies a novel family of putative ligands in plants. *Plant Cell* **15**, 2296-2307.

Butler, L. (1936). Inherited characters of tomato. II. Jointless pedicel. *The Journal of Heredity* **37**, 25-26.

Campisi, L., Yang, Y., Yi, Y., Helig, E., Herman, B., Cassista, A. J., Allen, D. W., Xiang, H. and Jack, T. (1999). Generation of enhancer trap lines in *Arabidopsis* and characterization of expression patterns in the inflorescence. *Plant J.* **17**, 699-707.

Che, M. M., Nie, Z. and Randazzo, P. A. (2006). Assays and properties of the Arf GAPs AGAP1, ASAP1 and Arf GAP1. *Methods Enzymol.* **404**, 147-163.

Cho, S. K., Larue, C. T., Chevalier, D., Wang, H., Jinn, T.-L., Zhang, S. and Walker, J. C. (2008). Regulation of floral organ abscission in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* **105**, 15629-15634.

Chow, C. M., Neto, H., Foucart, C. and Moore, I. (2008). Rab-A2 and Rab-A3 GTPases define a trans-golgi endosomal membrane domain in Arabidopsis that contributes substantially to the cell plate. *Plant Cell* **20**, 101-123.

Dettmer, J., Schubert, D., Calvo-Weimar, O., York-Dieter, S., Schmidt, R. and Schumacher, K. (2005). Essential role of the V-ATPase in male gametophyte development. *Plant J.* **41**, 117-124.

Dettmer, J., Hong-Hermesdorf, A., Stierhof, Y.-H. and Schumacher, K. (2006). Vacuolar H+-ATPase activity is required for endocytic and secretory trafficking in *Arabidopsis*. *Plant Cell* **18**, 715-730.

Doebley, J. (2004). The genetics of maize evolution. *Annual Review of Genetics* **38**, 37-59.

D'Souza-Schorey, C. and Chavrier, P. (2006). ARF proteins: roles in membrane traffic and beyond. *Nature Reviews Molecular Cell Biology* **7**, 347-358.

Ellis, C. M., Nagpal, P., Young, J. C., Hagen, G., Guilfoyle, T. J. and Reed, J. W. (2005). AUXIN RESPONSE FACTOR1 and AUXIN RESPONSE FACTOR2 regulate senescence and floral organ abscission in *Arabidopsis thaliana*. *Development* **132**, 4563-4574.

Eshed, Y., Baum, S. F. and Bowman, J. L. (1999). Distinct mechanisms promote polarity establishment in carpels of *Arabidopsis*. *Cell* **99,** 199-209.

Fang, S.-C. and Fernandez, D. E. (2002). Effect of regulated overexpression of the MADS domain factor AGL15 on flower senescence and fruit maturation. *Plant Physiol.* **130**, 78-89.

Fernandez, D. E., Heck, G. R., Perry, S. E., Patterson, S. E., Bleecker, A. B. and Fang, S.-C. (2000). The embryo MADS domain factor AGL15 acts postembryonically. Inhibition of perianth senescence and abscission via constitutive expression. *Plant Cell* **12**, 183-98.

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A. and Jurgens, G. (2003). The *Arabidopsi*s GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-mediated plant growth. *Cell* **112**, 219-30.

Geldner, N., Dénervaud-Tendon, V., Hyman, D. L., Mayer, U., Stierhof, Y-D., and Chory, J. (2009). Rapid, combinatorial analysis of membrane compartments in intact plants with a multi-color marker set. *Plant J.*, accepted article. doi:10.1111/j.1365-313X.2009.03851.x.

Goh, T., Uchida, W., Arakawa, S., Ito, E., Dainobu, T., Ebine, K., Takeuchi, M., Sato, K., Ueda, T. and Nakano, A. (2007). VPS9a, the common activator for two distinct types of Rab5 GTPases, is essential for the development of *Arabidopsis thaliana*. *Plant Cell* **19**, 3504-3515.

González-Carranza, Z. H., Elliott, K. A. and Roberts, J. A. (2007). Expression of polygalacturonases and evidence to support their role during cell separation processes in Arabidopsis thaliana. *J. Exp. Bot.* **58**, 3719-30.

Gould, S. J., Booth, A. M. and Hildreth, J. E. K. (2003). The Trojan exosome hypothesis. *PNAS* **100**, 10592-10597.

Grebe, M. Xu, J., Mobius, W., Ueda, T., Nakano, A., Geuze, H. J., Rook, M. B. and Scheres, B. (2003). Arabidopsis sterol endocytosis involves actin-mediated trafficking via ARA6-positive early endosomes. *Curr. Biol.* **13**, 1378-1387.

Hepworth, S. R., Zhang, Y., McKim, S., Li, X. and Haughn, G. W. (2005). *BLADE-ON-PETIOLE1* dependent signaling controls leaf and floral patterning in *Arabidopsis*. *Plant Cell* **17**, 1434-1448.

Horton, P., Park, K-J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C. J. and Nakai, K. (2007). WoLF PSORT: protein localization predictor. *Nucleic Acids Research* 35, W585-587.

Hurtado-Lorenzo, A., Skinner, M., El Annan, J., Futai, M., Sun-Wada, G. H., Bourgoin, S., Casanova, J., Wildeman, A., Bechoua, S., Ausiello, D. A. et al. (2006). V-ATPase interacts with ARNO and Arf6 in early endosomes and regulates the protein degradative pathway. *Nat. Cell Biol*. **8**, 124-136.

Inoue, H. and Randazzo, P. A. (2007). Arf GAPs and their interacting proteins. *Traffic* **8**, 1465-1475.

Inoue, H., Ha, V. L., Prekeris, R. and Randazzo, P. A. (2008). Arf GTPase-activating protein ASAP1 interacts with Rab11 effector FIP3 and regulates pericentrosomal localization of transferrin receptor-positive recycling endosome. *Mol. Biol. Cell* **19**, 4224-4237.

Jinn, T. L., Stone, J. M. and Walker, J. C. (2000). *HAESA*, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. *Genes & Dev.* **14**, 108-17.

Kandasamy, M. K., Deal, R. B., McKinney, E. C. and Meagher, R. B. (2005a). Silencing the nuclear actin-related protein AtARP4 in *Arabidopsis* has multiple effects on plant development, including early flowering and delayed floral senescence. *Plant J.* **41**, 845-858.

Kandasamy, M. K., McKinney, E. C., Deal, R. B. and Meagher, R. B. (2005b). Arabidopsis ARP7 is an essential actin–related protein required for normal embryogenesis, plant architecture and floral organ abscission. *Plant Physiol.* **138**, 1-14.

Keller, S., Sanderson, M. P., Stoeck, A., and Altevogt, P. (2006). Exosomes: From biogenesis and secretion to biological function. *Immunology Letters* **107**, 102-108.

Koizumi, K., Naramoto, S., Sawa, S., Yahara, N., Ueda, T., Nakano, A., Sugiyama, M. and Fukuda, H. (2005). VAN3 ARF-GAP-mediated vesicle transport is involved in leaf vascular network formation. *Development* **132**, 1699-711.

Lakkaraju, A. and Rodriguez-Boulan, E. (2008). Itinerant exosomes: emerging roles in cell and tissue polarity. *Trends in Cell Biology* **18**, 199-209.

Lauber, M. H., Waizenegger, I., Steinmann, T., Schwarz, H., Mayer, U., Hwang, I., Lukowitz, W. and Jurgens, G. (1997). The Arabidopsis KNOLLE protein is a cytokinesis-specific syntaxin. *J. Cell Biol.* **139**, 1485–1493.

Lee, G. J., Sohn, E. J., Lee, M. H. and Hwang, I. (2004). The arabidopsis rab5 homologs rha1 and ara7 localize to the prevacuolar compartment. *Plant Cell Physiol.* **45**, 1211-20.

Lewis, M. W., Leslie, M. E. and Liljegren, S. J. (2006). Plant cell separation: 50 ways to leave your mother. *Current Opinion in Plant Biology* **9**, 59-65.

Li, C., Zhou, A. and Sang, T. (2006). Rice domestication by reduced shattering. *Science* **311**, 1936-1939.

Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L. and Yanofsky, M. F. (2000). *SHATTERPROOF* MADS-box genes control seed dispersal in *Arabidopsis*. *Nature* **404**, 766-70.

Luo, R., Ahvazi, B., Amariei, D., Shroder, D., Burrola, B., Losert, W., and Randazzo, P. A. (2007). Kinetic analysis of GTP hydrolysis catalysed by the Arf1-GTP-ASAP1 complex. *Biochem J.* **402**, 439-47.

Lycett, G. (2008). The role of Rab GTPases in cell wall metabolism. *J. Exp. Bot.* **59**, 4061-74.

Mandiyan, V., Andreev, J., Schlessinger, J. and Hubbard, S. R. (1999). Crystal structure

of the ARF-GAP domain and ankyrin repeats of PYK2-associated protein ß. *Embo J*. **18**, 6890-6898.

Maranda, B., Brown, D., Bourgoin, S., Casanova, J. E., Vinay, P., Ausiello, D. A. and Marshansky, V. (2001). Intra-endosomal pH-sensitive recruitment of the Arf-nucleotide exchange factor ARNO and Arf6 from cytoplasm to proximal tubule endosomes. *J. Biol. Chem.* **276**, 18540-18550.

Marshansky, V. and Futai, M. (2008). The V-type H+-ATPase in vesicular trafficking: targeting, regulation and function. *Curr. Opin. Cell Biol.* **20**, 415-26.

McKim, S. M., Stenvik, G. E., Butenko, M. A., Kristiansen, W., Cho, S. K., Hepworth, S. R., Aalen, R. B. and Haughn, G. W. (2008). The BLADE-ON-PETIOLE genes are essential for abscission zone formation in Arabidopsis. *Development* **135**, 1537-1546.

Min, M. K., Kim, S. J., Miao, Y., Shin, J., Jiang, L. and Hwang, I. (2008). Overexpression of Arabidopsis AGD7 causes relocation of Golgi-localized proteins to the endoplasmic reticulum and inhibits protein trafficking to plant cells. *Plant Physiol*. **143**, 1601-1614.

Müller, J., Mettbach, U., Menzel, D. and Sˇamaj, J. (2007). Molecular dissection of endosomal compartments in plants. *Plant Physiol*. **145**, 293-304.

Natsume, W., Tanabe, K., Kon, S., Yoshida, N., Watanabe, T, et al. (2006). SMAP2, a novel ARF GTPase-activating protein, interacts with clathrin and clathrin assembly protein and functions on the AP1-positive early endosome/*trans*-Golgi network. *Mol. Biol. Cell*. **17**, 2592-603.

Neubert, C., Graham, L. A., Black-Maier, E. W., Coonrod, E. M., Liu, T. Y., Stierhof, Y. D., Seidel, T., Stevens, T. H. and Schumacher, K. (2008). Arabidopsis has two functional orthologs of the yeast V-ATPase assembly factor Vma21p. *Traffic* **9**, 1618-28.

Nie, Z. and Randazzo, P. A. (2006). Arf GAPs and membrane traffic. *J. Cell Sci.* **119**, 1203-1211.

Nielsen, E., Cheung, A. Y. and Ueda, T. (2008). The regulatory RAB and ARF GTPases for vesicular trafficking. *Plant Physiol.* **147**, 1516-1526.

Norberg, M., Holmlund, M. and Nilsson, O. (2005). The *BLADE ON PETIOLE* genes act redundantly to control growth and development of lateral organs. *Development* **132**, 2203-2213.

Ogawa, M., Kay, P., Wilson, S. and Swain, S. M. (2009). ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and

QUARTET2 are polygalacturonases required for cell separation during reproductive development in *Arabidopsis*. *Plant Cell* **21**, 216-233.

Okushima, Y., Mitina, I., Quach, H. L. and Theologis, A. (2005). AUXIN RESPONSE FACTOR2 (ARF2): a pleiotropic developmental regulator. *Plant J.* **43**, 29-46.

Patterson, S. E. and Bleecker, A. B. (2004). Ethylene-dependent and independent processes associated with floral organ abscission in Arabidopsis. *Plant Physiol.* **134**, 194-203.

Patterson, S., Butenko, M. and Kim, J. (2007). Ethylene responses in abscission and other processes of cell separation in Arabidopsis. In *Advances in Plant Ethylene Research* (eds. A. Ramina, C. Chang, J. Giovannoni, H. Klee, P. Perata, and E. Woltering), pp. 271-278. Netherlands: Springer.

Peyroche, A., Antonny, B., Robineau, S., Acker, J., Cherfils, J. and Jackson, C. L. (1999). Brefeldin A acts to stabilize an abortive ARF-GDP-Sec7 domain protein complex: involvement of specific residues of the Sec7 domain. *Molecular Cell* **3**, 275-285.

Poon, P. P., Cassel, D., Spang, A., Rotman, M., Pick, E., Singer, R. A. and Johnston, G. C. (1999). Retrograde transport from the yeast Golgi is mediated by two ARF GAP proteins with overlapping function. *EMBO J.* **18**, 555-564.

Poon, P. P., Nothwehr, S. F., Singer, R. A. and Johnston, G. C. (2001). The Gcs1 and Age2 ArfGAP proteins provide overlapping essential function for transport from the yeast *trans*-Golgi network. *J. Cell Biol.* **155**, 1239-1250.

Preuss, D., Mulholland, J., Franzusoff, A., Segev, N. and Botstein, D. (1992). Characterization of the Saccharomyces Golgi complex through the cell cycle by immunoelectron microscopy. *Mol. Biol. Cell* **3**, 789-803.

Randazzo, P. A., Weiss, O. and Kahn, R. A. (1992). Preparation of recombinant ADP-ribosylation factor. *Methods Enzymol.* **219**, 362-369.

Rehman, R. U., Stigliano, E., Lycett, G. W., Sticher, L., Sbano, F., Faraco, M., Dalessandro, G., and Di Sansebastiano, G. (2008). Tomato Rab11a characterization evidenced a difference between SYP121-dependent and SYP122-dependent exocytosis. *Plant Cell Phys.* **49**, 751-766.

Richter, S., Geldner, N., Schrader, J., Wolters, H., York-Dieter, S., Rios, G., Konca, C., Robinson, D. G. and Jürgens, G. (2007). Functional diversification of closely related ARF-GEFs in protein secretion and recycling. *Nature* **448**, 488-492.

Roberts, J. A., Elliott, K. A. and González-Carranza, Z. H. (2002). Abscission, dehiscence, and other cell separation processes. *Annual Review of Plant Biology* **53**, 131-158.

Robinson, D. G., Jiang, L. and Schumacher, K. (2008). The endosomal system of plants: Charting new and familiar territories. *Plant Physiol*. **147**, 1482-1492.

Rutherford, S. and Moore, I. (2002). The *Arabidopsis* Rab GTPase family: another enigma variation. *Curr. Opin. Plant Biol*. **5**, 518-528.

Sanderfoot, A. A., Kovaleva, V., Bassham, D. C. and Raikhel, N. V. (2001). Interactions between syntaxins identify at least five SNARE complexes within the Golgi/prevacuolar system of the arabidopsis cell. *Mol Biol Cell.* **12**, 3733-43.

Sato, Y., Hong, H. N., Yanai, N. and Obinata, M. (1998). Involvement of stromal membrane-associated protein (SMAP-1) in erythropoietic microenvironment. *J. Biol. Chem.* **124**, 209-216.

Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., Vingron, M., Schölkopf, B., Weigel, D. and Lohmann, J. U. (2005). A gene expression map of *Arabidopsis thaliana* development. *Nature Genetics* **37**, 501-506.

Sexton, R. and Hall, J. L. (1974). Fine structure and cytochemistry of the abscission zone cells of *Phaseolus* leaves. I. Ultrastructural changes occuring during abscission. *Ann. Bot.* **38**, 849-54.

Sexton, R., Jamieson, G. G. and Allan, M. H. (1977). An ultrastructural study of abscission zone cells with special reference to the mechanism of enzyme secretion. *Protoplasma* **91,** 369-87.

Sieburth, L. E., Muday, G. K., King, E. J., Benton, G., Kim, S., Metcalf, K. E., Meyers, L., Seamen, E. and Van Norman, J. M. (2006). SCARFACE encodes an ARF-GAP that is required for normal auxin efflux and vein patterning in Arabidopsis. *Plant Cell* **18**, 1396-411.

Smyth, D. R. (2005). Morphogenesis of flowers—our evolving view. *Plant Cell* **17**, 330-341.

Song, X. F., Yang, C. Y., Liu, J. and Yang, W. C. (2006). RPA, a class II ARFGAP protein, activates ARF1 and U5 and plays a role in root hair development in Arabidopsis. *Plant Physiol.* **141**, 966-76.

Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C. L., Paris, S., Galweiler, L., Palme, K. and Jurgens, G. (1999). Coordinated polar localization of the auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* **286**, 316-318.

Stenvik, G-E., Tandstad, N. M., Guo, Y., Shi, C-L., Kristiansen, W., Holmgren, A., Clark, S. E., Aalen, R. B. and Butenko, M. A. (2008). The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION is sufficient to induce

abscission in *Arabidopsis* through the receptor-like kinases HAESA and HAESA-LIKE2. *Plant Cell* **20**, 1805-1817.

Tanabe, K., Torii, T., Natsume, W., Braesch-Andersen, S., Watanabe, T. and Satake, M. (2005). A novel GTPase-activating protein for ARF6 directly interacts with clathrin and regulates clathrin-dependent endocytosis. *Mol. Biol. Cell* **16**, 1617-28.

Teh, O. and Moore, I. (2007). An ARF GEF acting at the Golgi and in selective endocytosis in polarized plant cells. *Nature* **448**, 493-496.

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brügger, B. and Simons, M. (2008). Ceramide triggers budding of exosome vesicles into multivesciular endosomes. *Science* **319**, 1244-1247.

Ueda, T., Uemura, T., Sato, M. H. and Nakano, A. (2004). Functional differentiation of endosomes in *Arabidopsis* cells. *Plant J*. **40**, 783-789.

Uemura, T., Ueda, T., Ohniwa, R. L., Nakano, A., Takeyasu, K. and Sato, M. H. (2004). Systematic analysis of SNARE molecules in *Arabidopsis*: Dissection of the post-Golgi network in plant cells. *Cell Struct. Funct*. **29**, 49-65.

van Niel, G., Porto-Carreiro, I., Simoes, S., Raposo, G. (2006). Exosomes: a common pathway for a specialized function. *J. Biochem*. **140**, 13-21.

Vernoud, V., Horton, A. C., Yang, Z. and Nielsen, E. (2003). Analysis of the small GTPase gene superfamily of Arabidopsis. *Plant Physiol.* **131**, 1191-208.

Vida, T. A. and Emr, S. D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* **128**, 779-792.

von der Fecht-Bartenbach, J., Bogner, M., Krebs, M., Stierhof, Y-D., Schumacher, K., and Ludewig, U. (2007). Function of the anion transporter At*CLC*-d in the *trans*-Golgi network. *Plant J.* **50**, 466-474.

Woollard, A. A. and Moore, I. (2008). The functions of rab GTPases in plant membrane traffic. *Curr. Opin. Plant Biol.* **10**, 1-10.

Yang, Z. (2008). Cell polarity signaling in Arabidopsis. *Annu Rev Cell Dev Biol* **24**, 551-575.

Yoo, C-M., Wen, J., Motes, C. M., Sparks, J. A. and Blancaflor, E. B. (2008). A class one ADP-ribosylation factor GTPase-activating protein is critical for maintaining directional root hair growth in *Arabidopsis thaliana*. *Plant Physiol*. **147**, 1659-1674.

Zeuzem, S., Feick, P., Zimmermann, P., Haase, W., Kahn, R. A. and Schulz, I. (1992). Intravesicular acidification correlates with binding of ADP-ribosylation factor to microsomal membranes. *PNAS* **89**, 6619-6623.

Zhang, C.-J., Cavenagh, M. M. and Kahn, R. A. (1998). A family of Arf effectors defined as suppressors of the loss of Arf function in the yeast Saccharomyces cerevisiae. *J. Biol. Chem.* **273**, 19792-19796.

Zheng, H., Bednarek, S. Y., Sanderfoot, A. A., Alonso, J., Ecker, J. R. and Raikhel, N. V. (2002). NPSN11 is a cell plate-associated SNARE protein that interacts with the syntaxin KNOLLE. *Plant Physiol.* **129**, 530-9.

Zheng, H., Camacho, L., Wee, E., Batoko, H., Legen, J., Leaver, C. J., Malho, R., Hussey, P. J. and Moore, I. (2005). A rab-E GTPase mutant acts downstream of the rab-D subclass in biosynthetic membrane traffic to the plasma membrane in tobacco leaf epidermis. *Plant Cell* **17**, 2020-2036.

CHAPTER 3

THE EVERSHED RECEPTOR-LIKE KINASE MODULATES FLORAL ORGAN SHEDDING IN ARABIDOPSIS

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SUMMARY

Plant cell signaling triggers the abscission of entire organs, such as fruit, leaves and flowers. Previously, we characterized an ADP-ribosylation factor GTPase-activating protein, NEVERSHED (NEV), that regulates membrane trafficking and is essential for floral organ shedding in *Arabidopsis*. Through a screen for mutations that restore organ separation in *nev* flowers, we have identified a leucine-rich repeat receptor-like kinase, EVERSHED (EVR), that functions as an inhibitor of abscission. Defects in the Golgi structure and location of the trans-Golgi network in *nev* abscission zone cells are rescued by a mutation in *EVR*, suggesting that EVR may regulate membrane trafficking during abscission. In addition to shedding their floral organs prematurely, *nev evr* flowers show enlarged abscission zones. A similar phenotype was reported for plants ectopically expressing INFLORESCENCE DEFICIENT IN ABSCISSION, a predicted signaling ligand for the HAESA/HAESA-like2 receptor-like kinases, indicating that this signaling pathway may be constitutively active in *nev evr* flowers. We present a model in which EVR may modulate the timing and region of abscission by promoting the internalization of other receptor-like kinases from the plasma membrane.

INTRODUCTION

Plants have evolved expansive groups of transmembrane receptor-like kinases (RLKs) that allow complex sampling of environmental conditions and developmental status. With over 400 RLKs annotated in the *Arabidopsis* genome, signaling complexity is enhanced by genetic redundancy (Shiu and Bleecker, 2001). Allowing for even further complexity, RLKs containing extracellular leucine-rich repeats (LRRs) can oligomerize and participate in multiple receptor complexes (Dievárt and Clark, 2003). For example, by interacting with the ligand-binding LRR-RLKs BRASSINOSTEROID INSENSITIVE1 (BRI1) and FLAGELLIN SENSITIVE2 (FLS2), BRI1-associated kinase1 (BAK1) functions in two discrete signaling complexes that regulate cell growth and pathogen defense, respectively (Li et al., 2002; Chinchilla et al., 2007).

Although RLKs are expected to play critical roles in plant development, deduction of these roles has often required phenotypic analysis of multiple loss-of-function mutants (Shpak et al., 2004; DeYoung et al., 2006; Morillo and Tax, 2006; Xu et al., 2008). A recent breakthrough is the discovery that a pair of LRR-RLKs, HAESA (HAE) and HAESA-like2 (HSL2), are redundantly required for floral organ abscission (Jinn et al., 2000; Cho et al., 2008; Stenvik et al., 2008). Whereas the outer whorls of the *Arabidopsis* flower, containing the sepals, petals and stamens, are shed following fertilization in wild type, floral organs remain attached indefinitely in *hae hsl2* flowers. As shown for other LRR-RLKs (Li et al., 2002; Kinoshita et al., 2005; Karlova et al., 2006; Chinchilla et al., 2007), HAE and HSL2 likely function as members of larger receptor complexes that are activated through interactions with an extracellular ligand. A small, secreted peptide, INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), is

required for abscission and is likely the ligand for HAE/HSL2 (Butenko et al., 2003; Cho et al., 2008; Stenvik et al., 2008; Butenko et al., 2009).

With the identification of these and other mutations that block or delay floral organ shedding (reviewed in Leslie et al., 2007; González-Carranza et al., 2007; McKim et al., 2008; Ogawa et al., 2009; Liljegren et al., 2009), the *Arabidopsis* flower has become a model for studying abscission. In plants, abscission events allow the shedding of leaves, flowers, fruit and seeds, and can facilitate growth, reproduction, and defense against pathogens. As with most developmental events, proper timing and spacing are critical during organ separation (Roberts and González-Carranza, 2007). In order for shedding to occur at the correct time, cell wall modifying enzymes must be secreted from cells within abscission zones (AZs) at the base of each organ to be shed (Roberts et al., 2002). If these enzymes are released too soon, premature organ loss could have an irreparable effect on reproduction (e.g. shedding of stamens before pollination). Conversely, a delay or block in abscission can also have an adverse effect on plant growth and reproduction (e.g. lack of seed dispersal) (Pinyopich et al., 2003). Of equal importance is the spatial regulation of abscission—the release of cell wall modifying enzymes must be restricted to discrete AZs to prevent general loss of tissue integrity or the shedding of neighboring organs. For example, misexpression of IDA leads to deregulated expansion of floral AZ cells and can cause premature organ shedding, as well as ectopic loss of developing fruit (Stenvik et al., 2006; Cho et al., 2008).

Membrane trafficking is emerging as an additional means of regulating plant transmembrane RLKs and likely modulates signaling during complex developmental events (Geldner and Robatzek, 2008). We recently reported that NEVERSHED (NEV),

an ADP-ribosylation factor GTPase-activating protein, is required for proper membrane trafficking and abscission (Liljegren et al., 2009). Mutations in *NEV* lead to altered organization of the Golgi and associated trans-Golgi network, accumulation of vesicles in paramural bodies, impaired fruit growth, and a block in organ separation. We hypothesized that NEV, which is localized to the trans-Golgi network and other distinct endosomal compartments, is required for the proper trafficking of key abscission factors.

Here we report that mutations in *EVERSHED* (*EVR*), which encodes an LRR-RLK, rescue organ shedding in *nev* flowers. The sepals, petals, and stamens of *nev evr* flowers are shed prematurely, and ectopic AZ cell expansion occurs. Loss of EVR kinase activity also restores normal Golgi morphology in *nev* AZ cells. Our results suggest that the EVR RLK functions as an inhibitor of abscission.

MATERIALS AND METHODS

Plants

The *evr-1* and *evr-2* alleles were obtained through ethyl methanesulfonate screens of *nev-3* (Ler) plants as described (Liljegren et al., 2000). The *evr-3* (*sobir1-12;* SALK_050715) and *evr-4* (*srrlk-2*; SALK_009453) alleles (Col) contain T-DNA insertions within codons 516 and 600 (Alonso et al., 2003; Gao et al., 2009; Katiyar-Agarwal et al., 2007). The *At1g17750* (SALK_098161) allele contains an insertion within codon 137. Genotyping primers and restriction enzymes are listed in Table S1. Mutant alleles described previously include *nev-2*, *nev-3*, *nev-6*, *ida-2*, *hae-1*, *hsl2-1*, and *pepr1-1*

(SALK_014538) (Yamaguchi et al., 2006; Cho et al., 2008; Stenvik et al., 2008; Liljegren et al., 2009).

A 759 bp region of *EVR*, from 750 nucleotides upstream of the predicted translational start site into the third codon, was PCR amplified from Col DNA with 5'-GATCAAGCTTTCACTATGGAACCCACAG-3' and 5'-GATCTCTAGAACAGCCATTTTAATTAGAG-3. This fragment was cloned into pCR2.1 (Life Technologies, Carlsbad, CA), excised with HindIII/XbaI, and cloned into pDW137 (Blázquez et al., 1997) to create a translational fusion of the *EVR* promoter to β-glucuronidase (GUS). A 1689 bp region of *HAE* (Jinn et al., 2000) was amplified from Col DNA with 5'-GTTTCTGTTGCATGTCAGGATTAGC-3' and 5'-GGATCCAGCATTTTTTTGGAAAAGGAATCG-3', cloned into pCR2.1, excised with XbaI/BamHI, and cloned into pDW137. 11 of 12 *HAE::GUS* T1 lines showed AZ expression. GUS expression was analyzed as described (Blázquez et al., 1997).

A 2670 bp region of *EVR*, including the predicted promoter and open reading frame, was amplified from Col DNA with 5'-CACCTCACTATGGAACCCACAGCG-3' and 5'-GTGCTTGATCTGGGACAACATGGTC-3', cloned into pENTR/D-TOPO (Life Technologies, Carlsbad, CA) and recombined into pGWB40 to add a YFP C-terminal tag (Nakagawa et al., 2007). 64 *EVR::EVR-YFP* T1 lines were generated.

To generate *35S::IDA-GFP/GUS* plants, a tandem repeat of the 35S promoter was excised as an EcoRI/HindIII fragment from pBIN-JIT (Ferrandiz et al., 2000), and cloned in pBluescriptIISK(+) (Stratagene, La Jolla, CA). The *IDA* cDNA was amplified with 5'-AAGCTTGACCCTTCATTCATTTACTC-3' and 5'-GTCGACTGAGGAAGAGAGTTAACAAAAGAG-3' from Col DNA, cloned into

pCR2.1, excised with HindIII/SalI, and cloned into the 35S/pBluescript construct. The *35S::IDA* fragment was excised with SacI/SalI, and replaced the *35S::CLV3* fragment in the pBGF-0 vector (Chytilova et al., 1999; Rojo et al., 2002) to create a translational fusion of IDA to GFP/GUS. 12 of 68 T1 lines showed the described phenotype.

Mapping

To determine the identity of *EVR*, the *nev-3 evr-1* mutant was crossed to *nev-6* (Col). Using 631 F2 plants and PCR-based markers including several designed from Ler polymorphisms (http://www.Arabidopsis.org/Cereon/), the *evr-1* mutation was mapped to a 122 kb interval on chromosome 2, between the CER100665 and CER103194 markers.

Kinase Activity

EVR kinase domains were amplified with 5'-CACCAGAGGATCAGAAAAACCACCAGG-3' and 5'-CTAGTGCTTGATCTGGGACAACATG-3' from Col and *evr-2* DNA, to create KD^{WT} and KD^{E407K}, respectively. Fragments were cloned into pENTR/D-TOPO. Site-directed mutagenesis (Stratagene, La Jolla, CA) with 5'-GGAAGATCATAGCTGTGGAGAAAGTGATCCAACCG-3' and 5'-CGGTTGGATCACTTTCTCCACAGCTATGATCTTCC-3' was used to generate $EVR\text{-}KD^{K377E}$. Recombination with pDEST17 (Life Technologies, Carlsbad, CA) generated N-terminal 6X-His-tagged EVR kinase domains for expression in *E. coli*. Recombinant proteins were purified by Co^{2+} affinity chromatography (Clontech Laboratories, Mountain View, CA).

To generate EVR-specific antiserum, a C-terminal peptide, CTLDDPKQRPNSKDVRTMLSQIKH, was synthesized and used to immunize chickens (Aves Labs, Tigard, Oregon). Antisera were used at the following dilutions: anti-EVR (1:10000), anti-phosphoserine (Sigma-Aldrich; 1:2000), anti-phosphotyrosine (Sigma-Aldrich; 1:2000), anti-phosphothreonine (Zymed/Invitrogen; 1:800). HRP-conjugated goat anti-chicken (Abcam, Cambridge, MA) and chicken anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) secondary antibodies were used at a 1:10000 dilution.

Microscopy

For scanning electron microscopy, flowers were fixed in 2% glutaraldehyde in 0.05M sodium phosphate buffer, treated 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. For transmission electron microscopy, flowers were analyzed as described (Liljegren et al., 2009). Confocal laser scanning microscopy of leaf petiole (stem), root, and cotyledon epidermal cells was performed with an LSM-510 (Carl Zeiss, Thornwood, NY). Image brightness and contrast were adjusted with Photoshop CS4 (Adobe, Mountain View, CA).

RESULTS

Mutations in *EVR* restore floral organ shedding in *nev* plants

Wild-type floral organs are shed after fertilization, whereas floral organs senesce and remain attached indefinitely in *nevershed* (*nev*) flowers (Fig. 3.1A,B). To identify novel regulators of abscission, we carried out a suppressor screen for mutations that restore floral organ shedding in *nev-3* plants (M. Lewis, E. Glaub, and S. Liljegren, unpublished results). Two recessive mutations identified in this screen form a complementation group that we have named *evershed* (*evr*) (Figs 3.1C, 3.S1A). The interaction between *NEV* and *EVR* was subsequently observed in multiple allele combinations (Fig. 3.S1C-F). Since the phenotypes of *nev evr-1 and nev evr-2* flowers are indistinguishable, analysis was primarily performed using the *evr-2* allele.

Mutations in *NEV* block abscission during the separation phase; the early patterning and differentiation of *nev* abscission zone (AZ) cells are unaffected (Liljegren et al., 2009). The secondary loss of *EVR* restores cell separation within *nev* AZs, such that all of the outer floral organs are shed from *nev evr* flowers (Figs 3.1C, 3.S1A). Since the densely cytoplasmic cells of *nev* sepal and petal AZs fail to become vacuolated and expand as in wild type (Fig. 3.1E,F; Liljegren et al., 2009), we examined longitudinal sections of *nev evr* flowers at the time of shedding to see if these defects were rescued by mutations in *EVR*. We found that cell vacuolation and expansion are not only rescued, but that *nev evr* AZ cells expand to a greater extent than those of wild type (Fig. 3.1E-G).

To determine whether mutations in *EVR* affect organ separation in otherwise wild-type flowers, we isolated the *evr-1* and *evr-2* single mutants. Floral organ shedding

and AZ expansion appear to be unaffected by loss of *EVR* alone compared to wild type (Figs 3.1A,D-E,H; 3.S1B).

Organ abscission occurs prematurely in *nev evr* flowers

By examining the progression through flower development, we discovered that *nev evr* floral organs are shed prematurely (Fig. 3.2A,C,E,G). In wild-type flowers, buds open and pollen grains are released by the anthers (stage 13); fertilization of the ovules occurs shortly thereafter (stage 14) (Müller, 1961; Smyth et al., 1990). After the fruit begins to elongate (stage 15), the outer floral organs start to senesce and undergo abscission (stage 16) (Fang and Fernandez, 2002). To compare these developmental stages in wild-type and mutant flowers, the youngest open bud (stage 13) at the inflorescence apex was defined as the first floral position, the next older flower produced as the second position, and so on (Patterson, 2001). The percentage of flowers at each floral stage (13-17) was assessed for positions one to ten. Whereas all floral organs were shed (stage 17) from wild-type inflorescences by position 8, shedding was complete in *nev evr* inflorescences by position 6 (Fig. 3.2A,C). In *nev evr* flowers, abscission occurs before the petals wither, at a developmental timepoint resembling stage 15 (Fig. 3.2C, stage 15*) rather than stage 16. Similar results were obtained with petal breakstrength assays (Lease et al., 2006) of wild-type, *nev* and *nev evr* flowers. Whereas a similar force was required to remove petals from *nev* flowers at each position, the force required to remove *nev evr* petals dropped to zero by position 4, one position earlier than for wild type (Fig. 3.S2). These results suggest that *EVR* prevents premature organ abscission during the transition between fertilization and early fruit development.

nev and *nev evr* mutants show other growth defects, including reduced stature (Fig. 3.S3A) and decreased fruit growth (Fig. 3.2F,G) (Liljegren et al., 2009). To confirm that premature shedding of *nev evr* floral organs was not due to a general defect in the timing of floral meristem formation, we also tracked individual wild-type and mutant flowers over the course of 5 days, from bud opening (day 0; stage 13) to the completion of abscission (stage 17). Whereas wild-type floral organs began shedding 3 days post-bud opening, *nev evr* floral organs began shedding one day earlier (Fig 3.S3B). These results suggest that *nev evr* floral organs abscise prematurely with respect to both floral age and stage of development.

Mutations in *EVR* alone do not substantially affect the timing of abscission (Figs 3.2D,H; 3.S3B). However, *evr* fruit are significantly shorter (79%) than wild type (Fig. 3.2E,H), and *nev evr* fruit are shorter (88%) than *nev* fruit (Fig. 3.2F,G).

AZ regions in *nev evr* flowers are larger and show increased cell expansion

We found that expansion of *nev evr* AZ cells is increased in comparison to wild type at the time of shedding (Fig. 3.1E,G). To further characterize this phenotype, we took scanning electron micrographs (SEMs) of wild-type, *evr* and *nev evr* flowers after organ separation was complete (youngest stage 17). In wild-type flowers, discrete AZs are found at the base of the developing fruit (Fig. 3.3A). Loss of *EVR* alone does not appear to affect AZ development in comparison to wild type (Fig. 3.3B). In contrast, *nev evr* AZs are less defined and appear to expand into the neighboring fruit and stem tissues (Fig. 3.3C). In a comparison of sepal AZ height in stage 17 SEMs, we found that *nev evr* sepal AZs are 2-fold taller than either wild-type or *evr* sepal AZs (Fig. 3.3E). In addition,

the stem-like base of the fruit known as the gynophore is hidden due to expansion of the stamen AZs in mature *nev evr* flowers (Fig. 3.3C, arrow). Cells within *nev evr* sepal AZs are also significantly larger in comparison to wild-type and *evr* cells (cells/10^3 μm^2 measurement, Fig. 3.3F), indicating that cell expansion contributes in part to the increase in AZ size. In older flowers, the cell expansion is even more striking—discrete AZs are less recognizable and the floral nectaries become enveloped in the expanding tissue (Fig. 3.3G). Later on, the expanded *nev evr* AZ cells burst, leaving behind a visible collar of rough tissue around the base of the fruit (Fig. 3.1C).

A similar phenotype was reported for flowers misexpressing the putative signaling ligand INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) (Stenvik et al., 2006; Cho et al., 2008). We generated plants that constitutively express an IDA-GFP/GUS translational fusion under the control of the viral *35S* promoter. We also observed enlargement of the floral AZs and overexpansion of individual AZ cells (Fig. 3.3D-F,H). Similar to what we observed in *nev evr* flowers, premature abscission of the sepals, petals and stamens was reported for *35S::IDA* flowers (Stenvik et al., 2006). These results suggest that regulation of AZ size and cell expansion by *NEV*, *EVR*, and *IDA* is important for the proper timing of organ shedding in *Arabidopsis*.

EVR encodes a plasma membrane-localized LRR-RLK with dual specificity

The *nev evr* phenotype suggests an important role for EVR in abscission. To clarify its mechanism of action, we identified single nucleotide changes for the *evr-1* and *evr-2* alleles within At2g31880, 1 of 34 predicted genes in the mapping interval. This intronless gene encodes a 641 amino acid protein of the leucine-rich repeat receptor-like

kinase (LRR-RLK) family (Fig. 3.4A). 11 additional alleles of *EVR* were identified in a screen for mutations that rescue seedling lethality in *bak1-interacting receptor-like kinase1 (bir1*) mutants (Gao et al., 2009). Loss of *BIR1* is predicted to cause constitutive activation of a SUPPRESSOR of BIR1 (SOBIR1/EVR)-mediated signaling pathway for disease resistance (Gao et al., 2009). Also encoded within the *EVR* open-reading frame is a heterogeneous cluster of small RNAs that are expressed upon pathogen infection, and for which *EVR* has also been named *small RNA-generating RLK* (Katiyar-Agarwal et al., 2007) (Fig. 3.S4A).

Based on the sequence of its kinase domain, EVR has been assigned to the 28-member LRR-RLK subfamily XI (Shiu and Bleecker, 2001). This subfamily includes the HAESA (HAE) and HAESA-like2 (HSL2) RLKs known to redundantly promote organ shedding (Jinn et al, 2000; Cho et al., 2008; Stenvik et al., 2008), the CLATAVA1 and BARELY ANY MERISTEM RLKs that control stem cell differentiation in shoot and flower meristems (Clark et al., 1997; DeYoung et al., 2006; DeYoung and Clark, 2008), the HAIKU RLK that regulates seed size (Luo et al., 2005) and the PEP1 RECEPTOR1 (PEPR1) that is involved in pathogen response (Yamaguchi et al., 2006). Although most members of subfamily XI have extensive sets of 19-32 LRRs in their extracellular domains (Shiu and Bleecker, 2001), EVR has only 5 predicted LRRs (Fig. 3.4A). However, in the context of the entire LRR-RLK family, EVR is not unique; the majority of *Arabidopsis* LRR-RLKs have fewer than ten LRRs (Shiu and Bleecker, 2001).

The *evr-1* and *evr-2* mutations are both predicted to be loss-of-function alleles that are unlikely to affect transcriptional regulation of *EVR* or the alternatively encoded small RNAs. The *evr-1* allele introduces a stop codon upstream of the first LRR,

suggesting that it is a null allele (Fig. 3.4A). The *evr-2* allele changes a conserved glutamic acid to a lysine within subdomain III of the kinase domain (Fig. 3.4A,B). This subdomain is involved in ATP binding and catalysis; a loss-of-function mutation in the *HAR1* LRR-RLK gene of *Lotus japonicus* introduces an identical missense mutation (Nishimura et al., 2002; Diévart and Clark, 2003). Two additional mutant alleles (Alonso et al., 2003), *evr-3* and *evr-4*, contain T-DNA insertions within subdomains VII and X of the kinase domain, respectively (Figs 3.4A, 3.S1H-I). These mutations also restore organ separation and cause premature abscission in *nev* flowers (Figs 3.S1G,J-L, 3.S2; data not shown).

Although up to 20% of *Arabidopsis* RLKs are predicted to be kinase-dead (Castells and Casacuberta, 2007), isolation of the *evr-2* mutation suggests that EVR kinase activity is required to regulate abscission (Fig. 3.4A-B). To facilitate analysis of EVR kinase activity, we generated EVR-specific antiserum using a 24-aa peptide corresponding to its unique C-terminus. The EVR antibody recognizes the EVR-KDs of wild type (WT), a kinase-dead mutant (K377E) (Horn and Walker, 1994), and the *evr-2* mutant (E407K) expressed as N-terminal 6X-His-tagged fusion proteins in *E.coli* (Fig. 3.4B-C). Whereas the purified KD^{K377E} and KD^{E407K} proteins migrate as single bands of ~40 kDa near the predicted size of 39kDa, the purified KD^{WT} migrates as two distinct bands of ~40 and 46 kDa, suggesting that the wild-type protein is phosphorylated (Fig. 3.4C).

To test whether EVR is a functional serine/threonine kinase, we used phosphoserine and phosphothreonine antisera to detect phosphorylated residues on the recombinant KDs. Both antisera recognized the upper KD^{WT} band but not the lower,

presumably unphosphorylated, KD^{WT} band. Neither KD^{K377E} nor KD^{E407K} appear to have kinase activity since the antisera do not recognize these mutant proteins (Fig. 3.4C). Since some LRR-RLKs have also been demonstrated to be dual-specificity kinases (Mu et al., 1994; Oh et al., 2009), we also tested the ability the recombinant EVR-KDs to undergo autophosphorylation at tyrosine residues. We found that phosphotyrosine antiserum exhibits the same recognition pattern as that of the phosphoserine and phosphothreonine antisera (Fig. 3.4C), and that treatment with calf intestinal alkaline phosphatase is able to completely dephosphorylate the tyrosine residues of EVR-KD^{WT}. (Fig. 3.S4B). These results suggest that EVR is a dual-specificity kinase that autophophorylates on serine, threonine and tyrosine residues in vitro.

EVR, like other transmembrane receptor-like kinases, is predicted to localize to the plasma membrane (Alexandersson et al., 2004). To visualize EVR localization in vivo, we generated transgenic plants expressing *EVR-YFP* under control of the *EVR* promoter (Fig. 3.S4A). Using confocal laser scanning microscopy, 6 of 6 independent transgenic lines showed localization of the EVR-YFP chimeric protein to the plasma membrane of epidermal cells (Fig. 3.4D). To confirm these results, we treated *pEVR::EVR-YFP* seedlings with the lipophilic dye FM4-64 (Vida and Emr, 1995), and found that EVR-YFP colocalizes with FM4-64 at the plasma membrane (Fig. 3.S4C).

EVR is expressed in floral organ AZs prior to cell separation

RT-PCR and global expression experiments indicate that *EVR* transcripts are present in multiple tissues, with increasing expression in older flowers (Fig. 3.S5A-B) (Schmid et al., 2005). To determine whether *EVR* is expressed at the right time and place

to modulate organ separation, we generated transgenic plants that express β-Glucuronidase (GUS) under the control of the predicted *EVR* promoter (Fig. 3.S4A). Of 83 T1 plants examined, 58 showed GUS expression in floral organ AZs, typically just prior to organ shedding (stage 15) with strengthened expression in older flowers (stages 16 and early 17) (Fig. 3.5A,C). This profile is similar to the reported expression patterns of *HAE::GUS* and *HSL2::GUS* (Fig. 3.5A-D) (Jinn et al., 2000; Cho et al., 2008), and is consistent with a role for EVR in modulating the timing of organ shedding.

Two independent *EVR::GUS* lines with single transgene insertions were characterized in the T2 generation. In addition to expression in floral organ AZs, each line showed GUS expression within the style of the developing fruit (stage 15; Fig. 3.5E), at the bases of the cauline leaves (Fig. 3.5G), and in the stems of the first rosette leaves (Fig. 3.S5C). Since *evr* mutants show defects in fruit growth (Fig. 3.2H), and global transcriptional analysis suggests that *EVR* has a broader expression profile (Fig. 3.S5B) (Schmid et al., 2005), our *EVR::GUS* markers may not reflect the complete expression profile of *EVR*. The *HAE* promoter was also observed to direct GUS expression in the style (Fig. 3.5F), at the cauline leaf bases (Fig. 3.5H), and at the junction between the floral and inflorescence stems (Fig. 3.5B, arrow). These observations suggest that EVR and HAE may co-regulate the development of a set of plant tissues.

Mutations in *EVR* restore the structure of the Golgi and location of the TGN in *nev* AZ cells

Since mutations in *EVR* rescue abscission in *nev* flowers, we examined AZ cells at the time of organ shedding to determine whether the membrane trafficking defects

observed in *nev* flowers were also rescued. Loss of *NEV* leads to a distinctive bending of the Golgi cisternae, disruption of the trans-Golgi network (TGN) and the accumulation of clusters of vesicles known as paramural bodies (PMBs) between the plasma membrane and cell wall of AZ cells (Liljegren et al., 2009). Unlike *nev* cells, *nev evr* cells contain flat stacks of Golgi cisternae with a wild-type appearance and closely associated TGN (Fig. 3.6A-C,E). The Golgi stacks and TGN of *evr* cells resemble those of wild type (Fig. 3.6D-E). As with *nev* cells, PMBs with more than 30 vesicles were observed in *evr* cells, but not in wild-type or *nev evr* cells (Figs 3.6F-J, S6). Since the defects in Golgi morphology and TGN location correlate with non-shedding floral organs, these may represent the primary cellular changes associated with and potentially responsible for the block of organ shedding in *nev* flowers. Furthermore, the restoration of Golgi/TGN structure in *nev evr* flowers and the defects in PMB formation in *evr* flowers suggest the possibility that like NEV, EVR may regulate membrane trafficking at the transition to floral organ shedding.

Mutations in *EVR*-like genes do not restore abscission in *nev* flowers

To potentially identify additional regulators of abscission, we evaluated whether mutations in two *EVR*-related genes also rescue *nev*-mediated defects. EVR shares 42 and 45% amino acid identity within the kinase domains of the PEPR1 and At1g17750 LRR-RLKs, respectively (Fig. 3.4B). PEPR1 was previously found to be a receptor for the PEP1 peptide involved in the innate immune response of plants to pathogen attack (Yamaguchi et al., 2006), and At1g17750 was shown to be transcriptionally induced by the fungal protein, Nep1 (Necrosis and ethylene-inducing peptide1; Qutob et al., 2006).

EVR is also expressed in response to viral and bacterial infection (Whitham et al., 2003; Katiyar-Agarwal et al., 2007; Ascencio-Ibanez et al., 2008). Despite the similarities within the kinase domain and overlapping expression patterns (Fig. 3.S5B), predicted loss-of-function mutations in *PEPR1* and At1g17750 did not rescue organ shedding in *nev* flowers (Fig. 3.7). These results suggest that these closely-related kinases do not regulate organ separation, or that their roles are hidden by additional genetic redundancy.

Mutations in *EVR* do not rescue shedding in *ida* or *hae hsl2* mutant flowers

Since mutations in *NEV*, *IDA*, and the redundant genes, *HAE* and *HSL2*, all appear to block floral organ shedding at a similar point during the cell separation stage (Butenko et al., 2003; Cho et al., 2008; Stenvik et al., 2008; Liljegren et al., 2009), we sought to use mutations in *EVR* to test the genetic relationship between these genes. We found that loss of *EVR* does not rescue shedding in either *ida* or *hae hsl2* flowers (Fig. 3.8A-D). These results suggest that *NEV* and *EVR* could act upstream of *IDA* and *HAE/HSL2* or in a parallel pathway that converges at the point of *HAE/HSL2* function or further downstream (Fig. 3.8E).

DISCUSSION

Here we report the characterization of EVR, an *Arabidopsis* LRR-RLK that modulates floral organ abscission and promotes fruit development. Our studies suggest

that EVR functions to inhibit organ separation by regulating signaling that affects the timing of AZ activity, AZ size and cell expansion.

We were able to detect a role for EVR in organ separation through a sensitized genetic screen of *nev* flowers. *NEV* encodes an ADP-ribosylation factor GTPase-activating protein that likely regulates membrane trafficking during multiple aspects of plant development (Liljegren et al., 2009). At the time of abscission, loss of NEV activity may disrupt the movement of signaling molecules critical for activating this process, thereby blocking release of the hydrolytic enzymes essential for AZ cell wall modifications associated with cell separation. As described below, we propose that secondary loss of the EVR LRR-RLK may bypass the requirement for NEV-mediated trafficking during abscission by affecting the localization and/or activity of such a signaling complex.

We have discovered that EVR acts as both a temporal and spatial regulator of abscission. *EVR* regulatory regions first direct *GUS* expression in floral AZs during the transition from fertilization to fruit growth (stage 15) (Fig. 3.5A,C). In wild-type flowers of the Landsberg *erecta* ecotype, organ separation (stage 16) occurs about three days after the buds open and the stamens release their pollen (Fig. 3.S3B). Loss of *EVR* causes premature abscission of floral organs in *nev* flowers (stage 15*) two positions earlier than in wild-type inflorescences (Fig. 3.2C), and about two days after bud opening (Fig. 3.S3B). These results indicate that younger flowers are competent to respond to signals to shed their organs, as was previously observed for flowers treated with ethylene or misexpressing IDA (Butenko et al., 2003; Stenvik et al., 2006). More importantly, our results suggest that EVR is one of the factors that acts to inhibit this response. Although

mutations in *EVR* alone do not affect the timing of abscission, multiple levels of genetic redundancy likely exist to ensure that organ separation does not occur prematurely.

Mutations in *EVR* appear to alter the spatial restriction of AZ signaling. Whereas smooth, scar-like surfaces form at the sites of organ detachment in wild-type flowers (Fig. 3.3A), increased expansion of individual AZ cells leads to visible collars of rough, broken tissue at the bases of *nev evr* fruit (Figs 3.1C, 3.3F,G). AZ regions are also enlarged in *nev evr* flowers compared to wild type (Fig. 3.3E). These AZ phenotypes strongly resemble those of plants constitutively expressing *IDA* (Fig. 3.3E-H; Stenvik et al., 2006; Cho et al., 2008), suggesting that excess levels of the IDA ligand or an activated HAE/HSL2 receptor complex may be present in *nev evr* flowers. An intriguing possibility is that ectopic AZ signaling causes cell wall loosening and expansion of cells both within and neighboring the original AZs of *nev evr* flowers. While the relationship between AZ cell expansion and organ separation in wild-type *Arabidopsis* flowers is unclear (Patterson, 2001), cell expansion may physically enable the shedding of floral organs during the cell wall loosening process. In this capacity, the contribution of AZ cell expansion to organ abscission may be analogous to that of lignified fruit cells to *Arabidopsis* pod dehiscence (Liljegren et al., 2004).

With five LRRs in its extracellular domain, EVR may inhibit organ separation by regulating the behavior of ligand-binding LRR-RLKs. One of the best characterized interactions between non-ligand and ligand-binding LRR-RLKs is that of BAK1/SOMATIC EMBRYOGENESIS RECEPTOR KINASE3 and FLAGELLIN-SENSITIVE2 (FLS2) during pathogen-triggered immunity (Geldner and Robatzek, 2008; Boller and Felix, 2009). Recognition of the flagellin-derived flg22 peptide by FLS2

triggers interaction with BAK1 (Chinchilla et al., 2007; Heese et al., 2007). As a co-receptor for FLS2, BAK1 promotes both flg22-induced signaling and internalization of FLS2 from the plasma membrane (Chinchilla et al., 2007). While EVR may function similarly to BAK1—by mediating the membrane trafficking of a ligand-binding receptor—we predict that it plays a unique role by acting as an inhibitor of its LRR-RLK partner prior to ligand-binding, rather than as a co-receptor after ligand-binding.

A model for EVR function during the transition from fertilization to floral organ shedding is shown in Figure 3.9. In wild-type AZ cells, plasma membrane-localized EVR may interact with and inhibit the activity of ligand-binding LRR-RLK(s) such as HAE and HSL2. In contrast to BAK1, for which FLS2 binding is enhanced by the presence of ligand, EVR may promote the internalization of an inactive receptor complex, thereby limiting the pool of available receptors and delaying abscission signaling. Increased availability of the HAE/HSL2 receptors or of the putative ligand, IDA, may trigger cell separation in older flowers. Loss of EVR function alone would not alter the timing of floral organ abscission due to the activity of a redundant LRR-RLK(s). This RLK would also be predicted to promote internalization of inactive receptor complexes in wild-type cells. Loss of NEV function in the TGN and other endosomal compartments could alter the trafficking of receptor complexes containing EVR and other inhibitory RLKs, such that the components required to activate cell separation are not recycled back to the plasma membrane. Secondary loss of EVR function could bypass the requirement for NEV in floral organ shedding, potentially resulting in a constitutive signal for cell separation that is no longer internalized or targeted for degradation. Increased signaling

may be responsible for the premature organ shedding, enlarged AZs and deregulated cell expansion that we observe in *nev evr* flowers.

In addition to restoring abscission, we found that a mutation in *EVR* rescues the structural defects in the Golgi and TGN that are present in *nev* flowers. Defects in NEV-mediated membrane trafficking may leave the integrity of the Golgi/TGN vulnerable to organizational stress due to the high volume of traffic blocked during organ abscission. If mutations in *EVR* restore abscission signaling and thereby alleviate the backlog of traffic in *nev* AZ cells, they may be sufficient to indirectly restore Golgi/TGN structure. Alternatively, EVR/SOBIR1 may function in a pathway(s) that directly regulates Golgi dynamics during periods of cellular stress, such as abscission and pathogen attack. Perturbations in Golgi structure have been associated with cell death-associated kinase signaling and GTPase activity (Landry et al., 2009), and AZ tissues that undergo periodically high volumes of membrane trafficking may require specific signaling pathways to maintain the integrity of membrane-bound organelles. Future experiments investigating the role of the EVR RLK in regulating membrane trafficking during abscission are likely to advance our understanding of the signaling complexities that control plant growth and development.

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REFERENCES

Aker, J. and de Vries S. C. (2008). Plasma membrane receptor complexes. *Plant Physiol*. **147**, 1560-1564.

Alexandersson, E., Saalbach, G., Larsson, C. and Kjellbom, P. (2004). *Arabidopsis* plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. *Plant Cell Physiol.* **45**, 1543-1556.

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R. et al**.** (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**, 653-657.

Ascencio-Ibáñez, J. T., Sozzani, R., Lee, T-J., Chu, T-M., Wolfinger, R. D., Cella, R. and Hanley-Bowdoin, L. (2008). Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. *Plant Physiol.* **148**, 436-454.

Belkhadir, Y. and Chory, J. (2006). Brassinosteroid signaling: a paradigm for steroid hormone signaling from the cell surface. *Science* **314**, 1410-1411.

Blázquez, M. A., Soowal, L. N., Lee, I. and Weigel, D. (1997). *LEAFY* expression and flower initiation in *Arabidopsis*. *Development* **124**, 3835–3844.

Boller, T. and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol*. **60**, 379-406.

Butenko, M. A., Patterson, S. E., Grini, P. E., Stenvik, G. E., Amundsen, S. S., Mandal, A. and Aalen, R. B. (2003). *INFLORESCENCE DEFICIENT IN ABSCISSION* controls floral organ abscission in Arabidopsis and identifies a novel family of putative ligands in plants. *Plant Cell* **15**, 2296-2307.

Butenko, M. A., Vie, A. K., Brembu, T., Aalen, R. B. and Bones, A. M. (2009). Plant peptides in signaling: looking for new partners. *Trends Plant Sci*. **14**, 255-263.

Castells, E. and Casacuberta, J. M. (2007). Signalling through kinase-defective domains: the prevalence of atypical receptor-like kinases in plants. *J. Exp. Bot*. **58**, 3503-3511.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J. D., Felix, G. and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* **448**, 497-500.

Cho, S. K., Larue, C. T., Chevalier, D., Wang, H., Jinn, T-L., Zhang, S. and Walker, J. C. (2008). Regulation of floral organ abscission in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **105**, 15629-15634.

Chytilova, E., Macas, J. and Galbraith, D. W. (1999). Green fluorescent protein targeted to the nucleus, a transgenic phenotype useful for studies in plant biology. *Ann. Bot.* **83**, 645-654.

Clark, S. E., Williams, R. W. and Meyerowitz, E. M. (1997). The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* **89**, 575-585.

DeYoung, B. J., Bickle, K. L., Schrage, K. J., Muskett, P., Patel, K. and Clark, S. E. (2006). The CLAVATA1-related BAM1, BAM2 and BAM3 receptor kinase-like proteins are required for meristem function in Arabidopsis. *Plant J.* **45**, 1-16.

DeYoung, B. J. and Clark, S. E. (2008). BAM receptors regulate stem cell specification and organ development through complex interactions with CLAVATA signaling. *Genetics* **180**, 895-904.

Diévart, A. and Clark, S. E. (2003). Using mutant alleles to determine the structure and function of leucine-rich repeat receptor-like kinases. *Curr. Opin. Plant Biol.* **6**, 507-516.

Fang, S.-C. and Fernandez, D. E. (2002). Effect of regulated overexpression of the MADS domain factor AGL15 on flower senescence and fruit maturation. *Plant Physiol.* **130**, 78-89.

Ferrándiz, C., Liljegren, S. J. and Yanofsky, M. F. (2000). Negative regulation of the *SHATTERPROOF* genes by FRUITFULL during *Arabidopsis* fruit development. *Science* **289**, 436-438.

Gao, M., Wang, X., Wang, D., Xu, F., Ding, X., Zhang, Z., Bi, D., Chen, Y. T., Chen, S., Li, X. and Zhang, Y. (2009) Regulation of cell death and innate immunity by two receptor-like kinases in *Arabidopsis*. *Cell Host Microbe*. **6**, 34-44.

Geldner, N., Hyman, D. L., Wang, X., Schumacher, K. and Chory, J. (2007). Endosomal signaling of plant steroid receptor kinase BRI1. *Genes Dev*. **21**, 1598-1602.

Geldner, N. and Robatzek, S. (2008). Plant receptors go endosomal: a moving view on signal transduction. *Plant Physiol*. **147**, 1565-1574.

González-Carranza, Z. H., Rompa, U., Peters, J. L., Bhatt, A. M., Wagstaff, C., Stead, A. D. and Roberts J. A. (2007). Hawaiian skirt: an F-box gene that regulates organ fusion and growth in Arabidopsis. *Plant Physiol.* **144**, 1370-1382.

Heese, A., Hann, D. R., Gimenez-Ibanez, S., Jones, A. M., He, K., Li, J., Schroeder, J. I., Peck, S. C. and Rathjen, J. P. (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc. Natl. Acad. Sci. USA* **104**, 12217-12222.

Horn, M. A. and Walker J. C. (1994). Biochemical properties of the auto-phosphorylation of RLK5, a receptor-like protein kinase from *Arabidopsis thaliana. Biochim. Biophys. Acta* **1208**, 65-74.

Jinn, T. L., Stone, J. M. and Walker, J. C. (2000). *HAESA*, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. *Genes Dev.* **14**, 108-117.

Karlova, R., Boeren, S., Russinova, E., Aker, J., Vervoort, J. and de Vries, S. C. (2006). The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 protein complex includes BRASSINOSTEROID-INSENSITIVE 1. *Plant Cell* **18**, 626-638.

Katiyar-Agarwal, S., Gao, S., Vivian-Smith, A. and Jin, H. (2007) A novel class of bacteria-induced small RNAs in Arabidopsis. *Genes Dev*. **21**, 3123-3134.

Kinoshita, T., Caño-Delgado, A., Seto, H., Hiranuma, S., Fujioka, S., Yoshida, S. and Chory, J. (2005). Binding of brassinosteroids to the extracellular domain of plant receptor kinase BRI1. *Nature* **433**,167-171.

Landry, M., Sicotte, A., Champagne, C. and Lavoie, J. N. (2009). Regulation of cell death by recycling endosomes and Golgi membrane dynamics via a pathway involving Src-family kinases, Cdc42 and Rab11a. *Mol Biol. Cell* **20**, 4091-4106.

Lease, K. A., Cho, S. K. and Walker, J. C. (2006). A petal breakstrength meter for Arabidopsis abscission studies. *Plant Methods* **2**, 2.

Leslie, M. E., Lewis, M. L. and Liljegren, S. J. (2007). Organ Abscission. In *Plant Cell Separation and Adhesion* (ed. J. Roberts and Z. Gonzalez-Carranza), pp. 106-136. Oxford, United Kingdom: Blackwell Publishing.

Li, J., Wen, J., Lease, K. A., Doke, J. T., Tax, F. E. and Walker, J. C. (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* **110**, 213-222.

Liljegren, S. J., Ditta, G. S., Eshed, Y., Savidge, B., Bowman, J. L. and Yanofsky, M. F. (2000). *SHATTERPROOF* MADS-box genes control seed dispersal in *Arabidopsis*. *Nature* **404**, 766-770.

Liljegren, S. J., Leslie, M. E., Darnielle, L., Lewis, M. W., Taylor, S. M., Luo, R., Geldner, N., Chory, J., Randazzo, P. A., Yanofsky, M. F. and Ecker, J. R. (2009).

Regulation of membrane trafficking and organ separation by the NEVERSHED ARF-GAP protein. *Development* **136**, 1909-1918.

Liljegren, S. J., Roeder, A. H., Kempin, S. A., Gremski, K., Østergaard, L., Guimil, S., Reyes, D. K. and Yanofsky, M. F. (2004) Control of fruit patterning in Arabidopsis by INDEHISCENT. *Cell* **161**, 843-853.

Luo, M., Dennis, E. S., Berger, F., Peacock, W. J. and Chaudhury, A. (2005). MINISEED3 (MIN3), a WRKY family gene, and HAIKU2 (IKU2), a leucine-rich repeat (LRR) KINASE gene, are regulators of seed size in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **102**, 17531-17536.

McKim S. M., Stenvik, G-E., Butenko, M. A., Kristiansen, W., Cho, S. K., Hepworth, S. R., Aalen, R. B. and Haughn, G. W. (2008). The *BLADE-ON-PETIOLE* genes are essential for abscission zone formation in *Arabidopsis*. *Development* **135**, 1537-1546.

Morillo, S. A. and Tax, F. E. (2006). Functional analysis of receptor-like kinases in monocots and dicots. *Curr. Opin. Plant Biol*. **9**, 460-469.

Mu, J-H., Lee, H-S. and Kao, T-H. (1994). Characterization of a pollen-expressed receptor-like kinase gene of *Petunia inflata* and the activity of its encoded kinase. *Plant Cell* **6**, 709–721.

Müller, A. (1961). Zur Charakterisierung der Blüten und Infloreszenzen von *Arabidopsis thaliana* (L.) Heynh. *Kulturpflanze* **9**, 364-393.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K, Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T. and Kimura, T. (2007). Development of Series of Gateway Binary Vectors, pGWBs, for realizing Efficient Construction of Fusion Genes for Plant Transformation. *J. Biosci. Bioeng.* **104**, 34-41.

Nam, K. H. and Li, J. (2002). BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell* **110**, 203-212.

Nishimura, R., Hayashi, M., Wu, G-J., Kouchi, H., Imaizumi-Anraku, H., Murakami, Y., Kawasaki, S., Akao, S., Ohmori, M., Nagasawa, M. et al. (2002). HAR1 mediates systemic regulation of symbiotic organ development. *Nature* **420**, 426-429.

Ogawa, M., Kay, P., Wilson, S. and Swain, S. M. (2009). ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and QUARTET2 are polygalacturonases required for cell separation during reproductive development in *Arabidopsis*. *Plant Cell* **21**, 216-233.

Oh, M-H., Wang, X., Kota, U., Goshe, M. B., Clouse, S. D. and Huber, S. C. (2009). Tyrosine phosphorylation of the BRI1 receptor kinase emerges as a component of

brassinosteroid signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **106**, 658-663.

Patterson, S. E. (2001). Cutting loose. Abscission and dehiscence in Arabidopsis. *Plant Physiol*. **126**, 494-500.

Pinyopich, A., Ditta, G. S., Savidge, B., Liljegren, S. J., Baumann, E., Wisman, E. and Yanofsky, M. F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature* **424**, 85-88.

Qutob, D., Kemmerling, B., Brunner, F., Kufner, I., Engelhardt, S., Gust, A. A., Luberacki, B., Seitz, H. U., Stahl, D., Rauhut, T., et al. (2006). Phytotoxicity and innate immune responses induced by Nep1-like proteins. *Plant Cell* **18**, 3721-3744.

Roberts, J. A., Elliott, K. A. and González, Z. H. (2002). Abscission, dehiscence, and other cell separation processes. *Annu. Rev. Plant Biol.* **53**, 131-58.

Roberts, J. A. and González-Carranza, Z. H. (2007). Abscission. In *Handbook of Plant Science, Volume 1* (ed. K. Roberts), pp. 512-519. Chichester, West Sussex, United Kingdom: John Wiley & Sons Ltd.

Rojo, E., Sharma, V. K., Kovaleva, V., Raikhel, N. V. and Fletcher, J. C. (2002). CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem cell signaling pathway. *Plant Cell* **14**, 969-977.

Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., Vingron, M., Schölkopf, B., Weigel, D. and Lohmann, J. U. (2005). A gene expression map of *Arabidopsis thaliana* development. *Nature Genetics* **37**, 501-506.

Shpak, E. D., Berthiaume, C. T., Hill, E. J. and Torii, K. U. (2004). Synergistic interaction of three ERECTA-family receptor-like kinases controls *Arabidopsis* organ growth and flower development by promoting cell proliferation. *Development* **131**, 1491-1501.

Shiu, S. H. and Bleecker, A. B. (2001). Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. *Proc. Natl. Acad. Sci. USA* **98,** 10763-10768.

Smyth, D. R., Bowman, J. L. and Meyerowitz, E. M. (1990). Early flower development in *Arabidopsis*. *Plant Cell* **2**, 755-767.

Stenvik, G-E., Butenko, M. A., Urbanowicz, B. R., Rose, J. K. and Aalen, R. B. (2006). Overexpression of *INFLORESCENCE DEFICIENT IN ABSCISSION* activates cell separation in vestigial abscission zones in *Arabidopsis*. *Plant Cell* **18**, 1467-1476.

Stenvik, G-E., Tandstad, N. M., Guo, Y., Shi, C-L., Kristiansen, W., Holmgren, A., Clark, S. E., Aalen, R. B. and Butenko, M. A. (2008). The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION is sufficient to induce abscission in *Arabidopsis* through the receptor-like kinases HAESA and HAESA-LIKE2. *Plant Cell* **20**, 1805-1817.

Swarup, G., Cohen, S. and Garbers, D. L. (1981). Selective dephosphorylation of proteins containing phosphotyrosine by alkaline phosphatases. *J. Biol. Chem.* **15**, 8197-201.

Vida, T. A. and Emr, S. D. (1995). A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol*. **128**, 779-792.

Wang, X., Goshe, M. B., Soderblom, E. J., Phinney, B. S., Kuchar, J. A., Li, J., Asami, T. Yoshida, S., Huber, S. C. and Clouse, S. D. (2005a). Identification and functional analysis of in vivo phosphorylayion sites of the Arabidopsis BRASSINOSTEROID-INSENSIVE1 receptor kinase. *Plant Cell* **17**, 1685-1703.

Whitham, S. A., Quan, S., Chang, H-S., Cooper, B., Estes, B., Zhu, T., Wang, X. and Hou, Y-M. (2003). Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. *Plant J.* **33***,* 271-283.

Xu, S-L., Rahman, A., Baskin, T. I. and Kieber, J. J. (2008). Two leucine-rich repeat receptor kinases mediate signaling, linking cell wall biosynthesis and ACC synthase in *Arabidopsis*. *Plant Cell* **20**, 3065-3079.

Yamaguchi, Y., Pearce, G. and Ryan, C. A. (2006). The cell surface leucine-rich repeat receptor for *At*Pep1, an endogenous peptide elicitor in *Arabidopsis*, is functional in transgenic tobacco cells. *Proc. Natl. Acad. Sci. USA* **103**, 10104-10109.

CHAPTER 4

DISCUSSION:

MODELS FOR TRAFFICKING AND SIGNALING EVENTS THAT AFFECT CELL ADHESION IN *ARABIDOPSIS*

SUMMARY

We originally identified the NEVERSHED (NEV) ARF-GAP as an essential factor for both abscission and proper membrane trafficking in *Arabidopsis* flowers. Here we show that NEV also acts to keep abscission zone cells from undergoing aberrant expansion, and even inhibits abscission in certain genetic backgrounds. These results suggest that NEV is required for regulating the trafficking of both positive and negative factors for floral organ abscission. With the identification of novel signaling factors that regulate *Arabidopsis* floral organ shedding, we have developed a working model for the integration of membrane trafficking and cell signaling during the cell separation process. Based on other well studied signaling pathways in *Arabidopsis*, we hypothesize that the EVERSHED (EVR) and SERK1 leucine-rich repeat receptor-like kinases (LRR-RLKs), along with the CAST AWAY (CST) receptor-like cytoplasmic kinase, interact with and negatively regulate signaling through the HAESA (HAE) and HAESA-like2 (HSL2) LRR-RLKs, which are redundantly required for abscission. Using the bimolecular fluorescence complementation assay in *Arabidopsis* leaf protoplasts, we have detected evidence for interactions between EVR, CST and HAE. Further experiments will be aimed at confirming these interactions in vitro and in planta, testing for interactions with SERK1, and determining whether NEV activity potentially affects these observed interactions.

INTRODUCTION

While multiple plant receptor-like kinases (RLKs) have been implicated in development and environmental responses, there are over 600 RLKs encoded within the *Arabidopsis* genome and the factors that determine receptor specificity and signaling activity are largely unknown (Shiu and Bleecker, 2001; Chinchilla et al., 2009; Tör et al., 2009). In a genetic screen for mutations that restore floral organ shedding in the *nevershed* (*nev*) mutant background, we identified mutations within genes encoding two leucine-rich repeat (LRR) RLKs, EVERSHED (EVR) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1), and a receptor-like cytoplasmic kinase (RLCK), CASTAWAY (CST) (Leslie et al., 2010; Lewis et al., 2010; Burr et al., in prep). Having identified a group of RLK/RLCKs that negatively regulate cell separation during *Arabidopsis* floral organ shedding, we aim to discern the genetic and physical interactions between EVR, SERK1 and CST and the previously identified LRR-RLKs, HAESA (HAE) and HAESA-LIKE2 (HSL2), which are redundantly required for abscission (Cho et al., 2008; Stenvik et al., 2008). Loss of *EVR*, *SERK1*, or *CST* does not rescue abscission in the *hae hsl2* mutant background, suggesting that EVR, SERK1 and CST function upstream of HAE/HSL2 or in a separate pathway (Leslie et al., 2010; Lewis et al., 2010; Burr et al., in prep). Since *EVR*, *CST*, *HAE* and *HSL2* are expressed within AZs at the transition to floral organ shedding (Jinn et al., 2000; Cho et al., 2008; Leslie et al., 2010; Burr et al., in prep), and *SERK1* has a broad expression pattern throughout development (Albrechet et al., 2005; Kwaaitaal et al., 2005; Kwaaitaal et al., 2007), it is possible that EVR, SERK1 and/or CST physically interact with and regulate signaling downstream of the HAE/HSL2 RLKs. The predicted signaling ligand

for the HAE/HSL2 RLKs, which is encoded by *INFLORESCENCE DEFICIENT IN ABSCISSION* (*IDA*), is also expressed within AZs at the time of organ shedding and likely activates HAE/HSL2 (Butenko et al., 2003; Cho et al., 2008; Stenvik et al., 2008).

Existing models for signaling from plant LRR-RLKs are remarkably diverse (Abrash and Bergmann, 2010; Kim and Wang, 2010; Lu et al., 2010; Zhu et al., 2010; Chinchilla et al., 2009; DeYoung and Clark, 2008); however, most interacting RLKs are predicted to enhance the signaling of a ligand-bound LRR-RLK(s). For example, the SERK family LRR-RLK, BRI1-ASSOCIATED KINASE 1 (BAK1/SERK3), participates in RLK signaling pathways for brassinosteroid (BR) perception and plant innate immunity, interacting with and positively regulating downstream signaling from the ligand-binding BRASSINOSTERIOD INSENSITIVE RECEPTOR-LIKE KINASE 1 (BRI1) and FLAGELLIN SENSING 2 (FLS2) LRR-RLKs, respectively (Chinchilla et al., 2007; Heese et al., 2007; Li et al., 2002; Nam and Li, 2002). Current models of BR signaling involve the sequential binding of BR to BRI1, autophosphorylation of BRI1, and interaction with the BAK1 co-receptor. Transphosphorylation within a BRI1-BAK1 heterotetrameric complex may lead to full activation of the signaling complex (reviewed in Chinchilla et al., 2009). Of note, SERK1, which we identified in our *nev* suppressor screen, also interacts with and positively regulates BRI1 (Lewis et al., 2010; Karlova et al., 2006), although the mechanism is less well understood.

Although RLCKs account for 118 of 610 annotated RLKs (Shiu and Bleecker, 2001), little is known about the function of these kinases within signaling complexes, and so far only one RLCK, BOTRYTIS-INDUCED KINASE 1 (BIK1), has been shown to interact with LRR-RLKs, specifically the ligand-binding FLS2 and its co-receptor BAK1

(Lu et al., 2010). In brief, FLS2 binding of the flagellin-derived peptide, flg22, triggers an FLS2-BAK1 interaction and downstream signaling for pathogen immunity (Chinchilla et al., 2007; Heese et al., 2007). BIK1, which independently associates with FLS2 and BAK1 in the absence of ligand, is rapidly phosphorylated by BAK1 upon flg22 treatment (Lu et al., 2010). According to the model of Lu et al. (2010), phosphorylated BIK1 subsequently transphosphorylates FLS2 and BAKI, leading to an activated FLS2-BAK1-BIK1 complex. Unlike the predicted role for the CST RLCK in the inhibition of abscission signaling (Burr et al., in prep), the BIK1 RLCK promotes pathogen immune responses downstream of FLS2 (Lu et al., 2010).

The regulation of signaling by receptor-mediated endocytosis is now gaining attention in the field of plant biology (reviewed in Geldner and Robatzek, 2008). While both BRI1 and BAK1 undergo constitutive endocytosis, the BRI1-BAK1 complex is preferentially internalized and likely recycled back to the PM (Russinova et al., 2004; Geldner et al., 2007). FLS2 is rapidly internalized following flg22-binding, and the receptor may signal from endosomal compartments prior to degradation (Robatzek et al., 2006; Geldner and Robatzek, 2008). In a well-studied animal model of receptor-mediated endocytosis, inactive EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) is predominantly localized to the plasma membrane yet undergoes constitutive recycling like BRI1 and BAK1. Ligand-activated EGFR forms heterocomplexes that are rapidly internalized and targeted for degradation. As predicted for activated FLS2, activated EGFR signals from endosomal locations (reviewed in Sorkin and Goh, 2008).

Here we show that the HAE, EVR and CST RLKs interact within *Arabidopsis* leaf protoplasts and propose a model in which interactions may occur in a step-wise

manner to down-regulate abscission signaling. We have also uncovered evidence that during the abscission process, the NEV ARF-GAP likely regulating the trafficking of factors that both promote and inhibit the cell separation process within floral AZs. We speculate that NEV activity is required to recycle internalized RLKs back to the PM.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana crosses were performed with wild-type (L*er* ecotype) and previously described mutant alleles: *nev-3 evr-1* and *nev-3 evr-2* (L*er* ecotype; Leslie et al., 2010), and *ida-2* (Col-0 ecotype; Cho et al., 2008). For protoplast experiments, wild-type *Arabidopsis* (Col-0 ecotype) seeds were germinated on 1X Murashige and Skoog (MS) salts supplemented with 0.5% sucrose and 0.8% agar. Seven-day-old seedlings were transferred to soil and grown in a 22°C chamber with a 12-hour photoperiod for 3-4 additional weeks.

Plasmid construction

The coding regions for *CST*, *ER*, and *HAE* were PCR amplified from the respective cDNA containing vectors, *U18406* (ABRC, The Ohio State U.), *pKUT161* (Torii et al., 1996) and *pBS-HAE* (kindly provided by J. Walker, UM-Columbia), and cloned into pENTR/D-TOPO (Life Technologies, Carlsbad, CA). Using methods previously described (Leslie et al., 2010), the 5'-gccccttcaccatggctgcttgtatttcgttc-3' and 5'-gaacgaaatacaagcagccatggtgaaggggggc-3' primers were used to generate *pENTR::CST*^{G2A}. The *EVR* coding region was amplified from genomic Col-0 DNA and

cloned into pENTR/D-TOPO. Recombination with pHBT-gw-GFP, pUC-gw-SPYNE, and pUC-gw-SPYCE destination vectors (Punwani et al., 2010) using Gateway technology (Life Technologies, Carlsbad, CA), was used to generate C-terminal fusions of each RLK to GFP, YFPn and YFPc, respectively.

Protoplast isolation and transfection

Protoplasts were isolated and transformed according to the method previously described (Yoo et al. 2007) with minor modifications. Mesophyll cells were exposed to digestive enzyme solution (1.5% Cellulase R-10 and 0.4% Macerozyme R-10; Yakult Pharmaceutical, Tokyo, Japan) for 1 hour after removing the epidermal cell layer by the Tape-*Arabidopsis* Sandwich method (Wu et al., 2009). For each transfection reaction, 2-3x10^4 protoplasts were incubated with 10-20 μg of plasmid DNA in a 20% polyethylene glycol (PEG; Sigma, St. Louis, MO) solution for 10-15 minutes. Cells were washed and incubated overnight in the dark to allow for gene expression. Protoplasts were imaged 12-16 hours after transfection.

Microscopy

Confocal laser scanning microscopy of leaf protoplasts was performed with a Zeiss LSM7 Duo (Carl Zeiss, Thornwood, NY). The following excitation (ex) lines and emission (em) ranges were used: GFP (ex 488, em 498-532), chlorophyll autofluorescence with GFP imaging (ex 560, em 572-716), YFP (ex 512, em 516-577), chlorophyll autofluorescence with YFP imaging (ex 512, em 603-732. Image brightness and contrast were adjusted with Photoshop CS4 (Adobe, Mountain View, CA). Flowers

were analyzed by scanning electron microscopy as previously described (Leslie et al., 2010). The *nev-3 evr-1* and *nev-3 evr-1/+* floral samples were not treated with OsO_4 prior to the ethanol series deyhydration; thus cells were more fragile and many broke during the drying process.

RESULTS

Loss of *NEV* causes ectopic abscission zone expansion

The NEVERSHED (NEV) ADP-ribosylation factor GTPase activating protein (ARF-GAP) is required for floral organ shedding and proper membrane trafficking in wildtype *Arabidopsis* flowers (Liljegren et al., 2009). The *Arabidopsis* flower is composed of four concentric whorls—the sepals, petals, and stamens, which are all shed after fertilization is complete—and the fused carpels, which remain attached to the stem and form the ripening fruit. We have previously observed that loss of *NEV* leads to ectopic cell expansion within the stamen abscission zones (AZs), which may account for the occasional shedding of stamens (Lewis et al., 2010). While the role of AZ cell expansion in *Arabidospis* floral organ abscission is unclear (Patterson 2001), the expansion of small, cytoplasmically dense AZ cells is correlated with abscission in many plant species (Webster, 1970; Wright and Osborne, 1974; Sexton and Redshaw, 1980; Polito and Stallman, 1981; Mackenzie 1988; Clements and Atkins, 2001).

We had previously discovered that loss of either one of the EVERSHED (EVR) or SERK1 leucine-rich repeat receptor-like kinases (LRR-RLKs), or the previously uncharacterized CASTAWAY (CST) receptor-like cytoplasmic kinase (RLCK), rescues

shedding in *nev* flowers and leads to increased expansion of sepal, petal and stamen AZ cells in comparison to wildtype (Leslie et al., 2010; Lewis et al., 2010; Burr et al., in prep). Furthermore, *nev evr* floral organs are shed prematurely in comparison to those in wildtype (Leslie et al., 2010). Plants carrying single loss-of-function mutations in *EVR*, *SERK1* or *CST* have no discernable phenotype—the AZ cells expand to the same extent as in wildtype and early organ shedding has not been observed. These results suggest that the additional loss of *NEV* may be the trigger for ectopic cell expansion in all AZ regions. In back-crossing *nev evr* plants to wildtype (L*er*), I have found that recovery of a single wildtype copy of *NEV* is sufficient to suppress the AZ cell expansion phenotype of *nev evr* flowers (Fig. 4.1A,B). While *nev-3 evr-1* AZs are enlarged, and individual cells expand to such a great extent that AZ boundaries are blurred (Fig. 4.1A), *nev-3/+ evr-1* AZ regions appear as in wildtype (Fig. 4.1B; see also Fig. 3.3A). This result suggests that the NEV ARF-GAP functions to both trigger AZ cell separation and prevent aberrant AZ cell expansion, and that there is a delicate balance between these two events.

Loss of *NEV* and *EVR* rescues shedding of *ida* mutant flowers

We have previously found that loss of a single negative regulator of abscission—*EVR*, *SERK1* or *CST*—is not sufficient to rescue organ shedding in *INFLORESCENCE DEFICIENT IN ABSCISSION* (*ida*) mutant flowers (Leslie et al., 2010; Lewis et al., 2010; Burr et al., in prep). As seen for *nev* flowers, AZ differentiation occurs as in wildtype, but AZ cell separation is blocked in *ida* flowers (Butenko et al., 2003; Liljegren et al., 2009). These results suggest a pathway for abscission in which EVR, SERK1 and CST either function upstream of the predicted signaling peptide IDA, or in a separate

pathway functioning at the same developmental point as IDA (see Fig 3.8E). Since NEV function during abscission is complex—loss of *NEV* inhibits floral organ shedding while at the same time promotes AZ cell expansion—I tested the effect of a *nev* mutation upon the abscission of *ida evr* floral organs (Fig 4.2A). Whereas *nev ida* floral organs remain attached to the plant (Lewis et al., 2010), loss of *NEV* does rescue abscission of *ida evr* floral organs (Fig 4.2B). Scanning electron micrographs of *nev ida evr* floral AZs show increased expansion of individual cells and spreading of AZ regions (Fig 4.2D), as previously shown for *nev evr*, *nev serk1*, and *nev cst* flowers (Leslie et al., 2010; Lewis et al., 2010; Burr et al., in prep). Similar results were found when introducing a *nev* mutation into *ida serk1* flowers—*nev ida serk1* floral organs abscise (Lewis et al., 2010). These unanticipated results suggest that NEV ARF-GAP activity functions during abscission to regulate the trafficking of both positive and negative factors for cell separation.

The CST receptor-like cytoplasmic kinase localizes to the plasma membrane and internalized structures

The HAE, EVR and SERK1 RLKs, which contain hydrophobic transmembrane domains, are all shown to be associated with the PM or closely associated membrane structures (Jinn et al., 2000; Shah et al. 2001; Alexandersson et al., 2004; Leslie et al., 2010). CST, which lacks a transmembrane domain, may associate with the membrane via myristoylation of its N-terminus, as previously shown for *Brassica* MLPK (Murase et al., 2004; Kakita et al., 2007). To visualize CST protein within the cell, we generated a *CST-GFP* construct driven by the constitutive viral 35S promoter that could be

transfected into *Arabidopsis* leaf protoplasts. Previous attempts to visualize CST-YFP under the control of its native promoter *in vivo* were unsuccessful due to the limited expression profile of *CST* in roots and leaves (C. Burr and M. Leslie, unpublished results). CST-GFP transformed protoplasts exhibit fluorescent localization of the protein to the PM and internalized, punctate structures (Figs 4.3A,B, 4.4E,F). Mutation of the putative myristoylation site (G2A) causes a partial redistribution of CST^{G2A}-GFP to the cytoplasm, as observed for transfection of the GFP tag alone (Fig. 4.3C-F). To further examine the localization of HAE and EVR, which have not previously been localized in *Arabidopsis* protoplasts, we also generated *35S::HAE-GFP* and *35S::EVR-GFP* constructs for transfection. Whereas CST-GFP shows clear localization to the PM, HAE-GFP and EVR-GFP were observed at the PM and at what appears to be the ER network (Fig 4.4A,B,E,F,I,J). It was previously reported that SERK1-YFP localizes to the PM, endosomal compartments and the ER network in *Arabidopsis* leaf protoplasts, and that this distribution varied with respect to time after protoplast transfection (Aker et al., 2006). Occasional nuclear localization was also observed for HAE-GFP and EVR-GFP, suggesting that the GFP tags may be cleaved from some of the fusion proteins (data not shown).

The HAE, CST and EVR receptor-like kinases interact in *Arabidopsis* protoplasts

As a method to detect interactions between RLKs that modulate abscission, we are using the bimolecular fluorescence complementation (BiFC) assay in *Arabidopsis* protoplasts (Walter et al., 2004; Yoo et al., 2007). This approach has been successfully used to detect interactions between membrane-bound RLKs, including the SERK family

RLKs (SERK1, SERK2, BAK1 and BKK1) with BAK1-INTERACTING RECEPTOR-LIKE KINASE1 (BIR1) (Gao et al., 2009). To facilitate this analysis, we have cloned full-length versions of HAE, CST, and EVR with C-terminal translational fusions to either the N-terminal half of YFP (YFPn) or the C-terminal half of YFP (YFPc). When transformed into *Arabidopsis* leaf protoplasts, these fusion proteins are expressed under the control of the constitutive *35S CaMV* promoter. When visualizing protoplasts approximately 12 hours after plasmid transfection, the presence of YFP fluorescence indicates a likely interaction between the two target proteins (ie. SERK1 and BIR1), bringing the N- and C-terminal halves of YFP into close enough proximity to reconstitute the fluorescent protein.

Multiple RLKs have been shown to form homodimers (Russinova et al., 2004; Hink et al., 2008; Zhu et al., 2010); thus we first tested for the self-interaction of HAE, CST and EVR in protoplasts. When co-transfected, HAE-YFPn and HAE-YFPc appear to interact in a similar pattern to what we observe for HAE-GFP transfection alone—localization to the PM and internal structures (Fig 4.4A-D). The CST RLCK can also self-interact since CST-YFPn/CST-YFPc co-transfection results in a similar pattern of fluorescence to CST-GFP (Fig. 4.4E-H). We were unable to detect any interactions between EVR-YFPn and EVR-YFPc (Fig. 4.4I-K), which could reflect an inability of the EVR RLK to homodimerize, an insufficient flexibility of the proteins for YFP reconstitution, or an artifact of protein overexpression (Bhat et al., 2006; Kerpolla 2009). While SERK1 and BRI1 were both found to homodimerize in cowpea protoplasts, BAK1 did not, suggesting that not all LRR-RLKs can self-interact (Hink et al., 2008).

We next began testing our hypothesis that EVR and CST may inhibit abscission signaling by forming receptor hetero-complexes with HAE. First, we found that HAE and CST can interact in *Arabidopsis* leaf protoplasts. Upon co-transfection of either *CST-YFPn*/*HAE-YFPc* or *HAE-YFPn*/*CST-YFPc*, we detected reconstituted YFP at the PM (Fig. 4.5A, data not shown). Unlike CST-GFP that is uniformly localized to the PM (Fig. 4.4E,F), the HAE-CST interaction is restricted to subdomains of the PM, or perhaps closely associated vesicles (Fig. 4.5A-C). We also detected interactions between EVR-YFPn and HAE-YFPc, occasionally at sites near the PM, but primarily within the cell (arrows, Fig. 4.5D,E). Co-transfection of *EVR-YFPn* and *CST-YFPc* resulted in interactions at the PM alone (Fig. 4.5F,G). These results support a model in which the CST and EVR RLKs may inhibit signaling that promotes abscission, by physically interacting with HAE at either the PM or internal membrane structures.

As a control for non-specific interactions, we tested for interactions between the ERECTA (ER) LRR-RLK (subfamily XIII) and HAE, CST and EVR. We were unable to detect reconstituted YFP for co-transfection of *ER-YFPn* with *HAE-YFPc*, *CST-YFPc* or *EVR-YFPc* (Fig. 4.5H-J). Another standard control we will be conducting is to confirm expression of the fusion proteins by SDS-PAGE and Western blotting. While the observed interactions between EVR-YFPn and both CST-YFPc and HAE-YFPc are experimentally reproducible, results with EVR-YFPc have been too variable to determine the validity of interactions (data not shown). Since we also failed to detect EVR-YFPn/EVR-YFPc interactions, the *EVR-YFPc* construct may not be expressed correctly, or the EVR-YFPc fusion protein could be non-functional.

DISCUSSION

Modifications to the genetic pathway(s) for abscission

Our current understanding of the genetic pathway(s) for abscission takes into account the following new observations: 1) *NEV* acts to both positively and negatively regulate organ shedding and 2) organ shedding and AZ cell expansion can occur independently of *IDA*. Since mutations in any one of the receptor-like kinases encoded by *EVR*, *SERK1* or *CST* rescues abscission in the *nev* mutant background, these kinases likely function downstream of *NEV* to negatively regulate organ separation. Therefore, *NEV* appears to promote abscission through inhibition of these three identified kinases. Disruption of NEV activity in the *ida evr* and *ida serk1* backgrounds rescues organ separation—uncovering a role for *NEV* as an inhibitor of abscission. Since abscission occurs in the absence of *IDA* (Fig. 4.2B), pathways including *NEV, EVR* and *SERK1* likely regulate organ shedding at the point of signaling through the redundant RLKs, HAE and HSL2, or further downstream (Fig. 4.2E).

While the phenotypic similarities of misexpressing IDA or disrupting NEV-mediated trafficking suggest a convergent genetic pathway (Fig. 4.2E), we have yet to obtain conclusive genetic evidence. To date, we have been unable to test the genetic relationship between *NEV* and *HAE*/*HSL2* due to *nev hae* synthetic lethality during gametophytic development (data not shown). We now suspect that this lethality may be linked to a potential chromosomal arrangement caused by the *hae-1* T-DNA insertional mutation, and will continue our genetic analysis using an alternative T-DNA allele, *hae-2.* Alternatively, we can look further downstream and test for genetic interactions

between the predicted *NEV* pathway and the two sets of MAP kinases, *MPK3*/*MPK6* and *MKK4*/*MKK5*, required for abscission downstream of *HAE*/*HSL2* (Cho et al., 2008). Decreased MPK6 activity was reported for *hae hsl2* non-shedding AZs, while constitutively active MKK4 or MKK5 is sufficient to rescue shedding in *ida* or *hae hsl2* flowers (Cho et al., 2008). Similar results in a *nev* mutant background would be compelling evidence to conclude that pathways including *NEV*, *IDA*, and *HAE*/*HSL2* converge, and would further support our hypothesis that NEV ARF-GAP activity is required for the trafficking of signaling factors during abscission.

While abscission is non-essential in *Arabidopsis* flowers—non-abscised sepals, petals and stamens do not have an apparent effect upon the maturation or growth of *hae hsl2* or *ida* mutant fruit—premature shedding of floral organs could negatively affect fertilization. Thus, it is not surprising that we have discovered multiple negative regulators of abscission—the EVR, SERK1 and CST RLKs. Plants carrying a loss-of-function mutation in a single negative regulator have no phenotype, suggesting that these RLKs may function redundantly in an otherwise wildtype background. As a test for genetic redundancy between *EVR*, *SERK1* and *CST*, we are generating *evr serk1 cst* triple-mutant plants—for which we may observe premature floral organ shedding. EVR, SERK1 and closely related RLKs, which have similar domain structure to the SERK family LRR-RLK, BAK1, may jointly regulate the internalization of receptor complexes; and CST belongs to a large family of RLCKs, of which other membrane-bound kinases could function redundantly in receptor recruitment.

Model for receptor signaling and endocytosis

Our RLK interaction studies suggest a stepwise model for receptor complex formation and endocytosis during abscission signaling (Fig. 4.6). First, CST may sequester the EVR RLK at the PM. While CST-GFP is primarily localized to the PM (Figs 4.3A,B, 4.4E,F), EVR-GFP is distributed between the PM and unknown internal compartments having the appearance of the ER network (Fig. 4.4I,J). CST-EVR complexes are uniformly localized to the PM (Fig. 4.5F), suggesting that a direct interaction between CST and EVR could limit receptor mobility away from the PM, thereby enabling subsequent interactions to occur. Second, CST-containing complexes may interact with the HAE RLK. In contrast to the uniform localization of CST-GFP at the PM (Fig 4.3A,B), CST-HAE interactions are stabilized within subdomains of the PM or perhaps closely associated vesicles (Fig. 4.5A-C). Receptor aggregation, which could be triggered by RLK interactions between complexes, may be important for packaging into endocytic vesicles (Zappel and Panstruga, 2008). In the third step of the model, a direct interaction between EVR and HAE may facilitate internalization and trafficking of receptor complexes. While CST-EVR and CST-HAE interactions are primarily associated with the PM (Fig. 4.5A-C,F), the EVR-HAE interaction occurs primarily within the cell, at unknown locations (Fig. 4.5E). Since CST and EVR act as negative regulators of cell separation, the stepwise aggregation and internalization of HAE RLKs may function to dampen signaling or even target the complex for degradation. Interestingly, the *Brassica* S-RECEPTOR KINASE (SRK) that perceives an extracellular ligand for self-incompatibility and interacts with the M-LOCUS PROTEIN KINASE (MLPK) RLCK, predominantly localizes to intracellular membrane structures enriched in a negative regulator of SRK signaling (Kakita et al., 2007; Ivanov and Gaude, 2009). A

similar mechanism may function to down-regulate HAE/HSL2 signaling throughout abscission.

Aside from our proposed models of EVR, SERK1 and CST inhibiting HAE/HSL2 signaling during abscission, no other LRR-RLKs have yet been reported to inhibit signaling of interacting RLKs, and only one genetic study suggests an inhibitory role for a receptor-like protein (RLP) in LRR-RLK signaling. Briefly, signaling downstream of the ERECTA (ER) family LRR-RLKs inhibits stomatal production in multiple tissues (Shpak et al., 2005). This epidermal patterning is governed in part by the LRR-RLP, TOO MANY MOUTHS (TMM), which lacks an intracellular kinase domain. Loss of *TMM* leads to excess stomata on leaves but the elimination of stomata from stems (Geisler et al., 1998; Shpak et al., 2005; Bhave et al., 2009). Genetic pathway analysis supports a model in which the TMM RLP inhibits signaling from the ER family RLKs activated by the putative signaling ligand CHALLAH (CHAL), yet promotes signaling from the ER family RLKs activated by the EPIDERMAL PATTERNING FACTOR 1 (EPF1) or EPF2 peptides (Abrash and Bergmann, 2010). Like TMM, the inhibitory roles of EVR, SERK1 and CST could be ligand-dependent, such that in the absence of the IDA ligand (or possibly when presented with a different, unknown ligand), interactions with HAE/HSL2 block downstream signaling events.

NEV, as a global regulator of membrane trafficking, may be required for the trafficking of both positive and negative regulators of abscission, including HAE, HSL2, CST, EVR and SERK1. From its localization at the *trans*-Golgi network/early endosome and the putative recycling endosome (Liljegren et al., 2009), NEV may function to traffic receptors within the early endosomal system and ultimately recycle them back to the PM

(Fig. 4.6). Loss of *NEV* could lead to the hyperaccumulation of inactivated receptors within endosomal compartments, while a secondary loss of *EVR*, *SERK1*, or *CST*, may help to stabilize the HAE/HSL2 RLKs at the PM. At the proper timing for abscission in wild-type flowers, our model predicts that IDA ligand-binding stabilizes HAE/HSL2, perhaps leading to phosphorylation, conformational change and activation of downstream signaling events required for the loss of cell adhesion (Fig. 4.6). In the absence of IDA, IDA-LIKE (IDL) peptides expressed within the floral AZs could activate hyper-stabilized HAE/HSL2 RLKs in *nev ida evr* triple mutant flowers. Several *IDL* promoters are active specifically within AZs, and overexpression of all five *IDA-LIKE* genes has been shown to cause AZ cell expansion to varying degrees (Stenvik et al., 2008).

According to our proposed model for receptor function during abscission signaling (Fig. 4.6), the primary function of CST may be to sequester RLKs at the PM and facilitate interactions between intracellular kinase domains, while EVR may act as a trigger for RLK internalization, perhaps sensing the presence/absence of extracellular ligand binding. Both CST and EVR kinase activity are required for inhibition of abscission (Leslie et al., 2010; Burr et al., in prep), thus it will be interesting to test the effect of kinase-dead variants on receptor interactions and localization in the protoplast system. In addition, the removal or disruption of extracellular LRRs can be used to test whether EVR or HAE intracellular kinase domains are sufficient for receptor interactions or localization. Recent work on FLS2-mediated signaling shows that interactions between FLS2, BAK1 and the newly identified BIK1 RLCK occur independently of kinase activity (Lu et al., 2010; Schulze et al., 2010). Future experiments will also investigate interactions with the SERK1 RLK, which like EVR, contains 5 extracellular

LRRs that likely promote protein-protein interactions (Hecht et al., 2001). We have identified several mutations within the extracellular and kinase domains of SERK1 that affect cell separation (Lewis et al., 2010), and will test the effect of these mutations upon any observed interactions. By monitoring the phosphorylation status of full-length tagged RLKs extracted from protoplasts, or of truncated kinase domains *in vitro*, we can also test for transphosphorylation of identified, interacting RLKs.

Based on the genetic characterization of abscission, RLK interactions and/or localization might be regulated by NEV ARF-GAP and/or IDA ligand activity. The transient transfection system in *Arabidopsis* protoplasts allows us the flexibility to rapidly test the effects of native copy mutant alleles (i.e. generating and transfecting *nev-3* protoplasts) or exogenous application of molecules (i.e. a synthetic 20aa IDA EPIP peptide) (Stenvik et al., 2008). In preliminary experiments, loss of *NEV* does not appear to affect interactions between HAE and CST at the PM or in closely, associated vesicles (data not shown). Since NEV is likely redundant with one or more ARF-GAPs in most cell types, it may be necessary to isolate protoplasts deprived of *NEV* and a closely-related ARF-GAP. While abscission occurs independently of *IDA* in certain genetic backgrounds (i.e. *nev ida evr*), the application of IDA peptide to wild-type protoplasts may affect interactions between RLKs and/or RLK localization. In pathogen defense signaling, exposure to the bacterial elicitor peptide flg22 causes rapid activation, internalization and degradation of the FLS2 LRR-RLK, which binds flg22 (reviewed in Boller and Felix, 2009). However, internalization of other RLKs such as BRI1 has been shown to be ligand-independent (Geldner et al., 2007). According to our model (Fig. 4.6), IDA may stabilize HAE/HSL2 at the PM and thereby prolong the activation of

downstream signaling events. Using a multicolor BiFC vector system, in which various interactions between the N- and C-terminal fragments of the CFP and VENUS fluorophores are detected by distinct emission spectra, binding preferences of target proteins can be analyzed under varying conditions (i.e. presence or absence of IDA ligand) (Hu and Kerppola, 2003; Gehl et al., 2009). As when FLS2 binds flg22, IDA peptide binding may also trigger intracellular phosphorylation of RLKs and disassociation of an RLCK (Lu et al., 2010). The phosphorylation status of the HAE, EVR, SERK1 and CST RLKs can be monitored following IDA peptide treatment, perhaps elucidating a chain of phosphorylation events as for BRI1-BAK1 and FLS2-BAK1-BIK1 signaling (Lu et al., 2010; Wang et al., 2008).

Future experiments will move beyond the *in vivo* protoplast system and *in vitro* transphosphorylation assays to test the observed interactions *in planta*. While a transient protoplast expression system is ideal for rapid identification of protein interactions, results should be confirmed by experiments in cells with intact cell walls, in developmentally relevant tissues, and preferably with native protein expression levels. Overexpression of protein targets, as with the *35S CaMV* promoter, could lead to the identification of false-positive interactions, or even inhibit certain protein-protein interactions (Bhat et al., 2006; Kerpolla 2009). The identification of particular amino acids that are necessary for interaction, yet do not affect protein localization or stability, lends validity to BiFC results (Bhat et al., 2006; Kerpolla 2009). One particular limitation of the BiFC assay, with regard to studying rapid signaling events, is the stabilization of transient protein interactions—in one instance, reconstituted YFP exhibited a half-life of greater than 24 hours (reviewed in Kerpolla 2009). Confirmation

of our results requires transgenic expression of full-length epitope-tagged RLKs, which will allow for the isolation of protein complexes from AZ cells, and could be used for the immuno-localization of RLKs within AZ cells by transmission electron microscopy. By introducing tagged RLKs into the *nev* mutant backgrounds, we can more directly test the effect of NEV ARF-GAP activity upon RLK interactions and localization during abscission. To further understand the role of NEV, it would be particularly interesting to observe RLK localization with respect to the hyperaccumulation of vesicles in *nev* AZ cells. If NEV is required for the proper trafficking of abscission factors, RLKs may aggregate in aberrant locations in *nev* cells. Secondary loss of a negative regulator (i.e. *EVR*, *SERK1*, *CST*), may rescue the localization of HAE/HSL2 RLKs. With the identification of both signaling and membrane trafficking factors converging on a single pathway, our ongoing studies are uniquely positioned to make a significant contribution to the understanding of intracellular signaling during *Arabidopsis* development.

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REFERENCES

Abrash, E. B. and Bergmann, D. C. (2010) Regional specification of stomatal production by the putative ligand CHALLAH. *Development* **137**, 447-455.

Aker, J., Borst, J. W., Karlova, R. and de Vries, S. C. (2006) The Arabidopsis thaliana AAA protein CDC48A interacts in vivo with the somatic embryogenesis receptor-like kinase 1 receptor at the plasma membrane. *J. Struct. Biol.* **156**, 62-71.

Albrecht, C., Russinova, E., Hecht, V., Baaijens, E and de Vries, S. (2005) The Arabidopsis thaliana SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES1 and 2 control male sporogenesis. *Plant Cell* **17**, 3337-3349.

Alexandersson, E., Saalbach, G., Larsson, C. and Kjellbom, P. (2004). *Arabidopsis* plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. *Plant Cell Physiol.* **45**, 1543-1556.

Bhat, R. A., Lahaye, T. and Panstruga, R. (2006) The visible touch: in planta visualization of protein-protein interactions by fluorophore-based methods. *Plant Methods* **2**, 12.

Bhave, N. S., Veley, K. M., Nadeau, J. A., Lucas, J. R., Bhave, S. L. and Sack, F. D. (2009) TOO MANY MOUTHS promotes cell fate progression in stomatal development of Arabidopsis stems. *Planta* **229**, 357-367.

Boller, T. and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol*. **60**, 379-406.

Burr, C. A., Leslie, M. E., Wright, C. E., and Liljegren, S. J. CAST AWAY, a membrane-associated receptor-like kinase, inhibits floral organ shedding in *Arabidopsis* (in preparation).

Butenko, M. A., Patterson, S. E., Grini, P. E., Stenvik, G. E., Amundsen, S. S., Mandal, A. and Aalen, R. B. (2003). *INFLORESCENCE DEFICIENT IN ABSCISSION* controls floral organ abscission in Arabidopsis and identifies a novel family of putative ligands in plants. *Plant Cell* **15**, 2296-2307.

Chinchilla, D., Shan, L., He, P., de Vries, S. and Kemmerling, B. (2009) One for all; receptor-associated kinase BAK1. *Trends Plant Sci.* **14**, 535-541.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J. D., Felix, G. and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* **448**, 497-500.

Cho, S. K., Larue, C. T., Chevalier, D., Wang, H., Jinn, T-L., Zhang, S. and Walker, J. C. (2008). Regulation of floral organ abscission in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **105**, 15629-15634.

Clements, J. and Atkins, C. (2001) Characterization of a non-abscission mutant in Lupinus angustifolius. I. Genetic and structural aspects. *American J. Bot.* **88**, 31-42.

DeYoung, B. J. and Clark, S. E. (2008). BAM receptors regulate stem cell specification and organ development through complex interactions with CLAVATA signaling. *Genetics* **180**, 895-904.

Gao, M., Wang, X., Wang, D., Xu, F., Ding, X., Zhang, Z., Bi, D., Chen, Y. T., Chen, S., Li, X. and Zhang, Y. (2009) Regulation of cell death and innate immunity by two receptor-like kinases in *Arabidopsis*. *Cell Host Microbe*. **6**, 34-44.

Gehl, C., Waadt, R., Kudla, J., Mendel, R. R. and Hänsch, R. (2009) New GATEWAY vectors for high throughput analyses of protein-protein interactions by bimolecular fluorescence complementation. *Mol. Plant* **2**, 1051-1058.

Geisler, M., Yang, M. and Sack, F. D. (1998) Divergent regulation of stomatal initiation and patterning in organ and suborgan regions of the Arabidopsis mutants too many mouths and four lips. *Planta* **205**, 522-530.

Geldner, N., Hyman, D. L., Wang, X., Schumacher, K. and Chory, J. (2007). Endosomal signaling of plant steroid receptor kinase BRI1. *Genes Dev*. **21**, 1598-1602.

Geldner, N. and Robatzek, S. (2008). Plant receptors go endosomal: a moving view on signal transduction. *Plant Physiol*. **147**, 1565-1574.

Hecht, V., Vielle-Calzada, J. P., Hartog, M. V., Schmidt, E. D., Boutilier, K., Grossniklaus, U. and de Vries, S. C. (2001) The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiol* **127**, 803-816.

Heese, A., Hann, D. R., Gimenez-Ibanez, S., Jones, A. M., He, K., Li, J., Schroeder, J. I., Peck, S. C. and Rathjen, J. P. (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc. Natl. Acad. Sci. USA* **104**, 12217-12222.

Hink, M. A., Shah, K., Russinova, E., de Vries, S. C. and Visser, A. J. (2008) Fluorescence fluctuation analysis of Arabidopsis thaliana somatic embryogenesis receptor-like kinase and brassinosteroid insensitive 1 receptor oligomerization. *Biophys. J.* **94**, 1052-1062.

Hu, C. D. and Kerppola, T. K. (2003) Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat Biotechnol*. **21**, 539-545.

Ivanov, R. and Gaude, T. (2009) Endocytosis and endosomal regulation of the S-receptor kinase during the self-incompatibility response in Brassica oleracea. *Plant Cell* **21**, 2107-2117.

Jinn, T. L., Stone, J. M. and Walker, J. C. (2000). *HAESA*, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. *Genes Dev.* **14**, 108-117.

Kakita, M., Murase, K., Iwano, M., Matsumoto, T., Watanabe, M., Shiba, H., Isogai, A. and Takayama, S. (2007) Two distinct forms of M-locus protein kinase localize to the plasma membrane and interact directly with S-locus receptor kinase to t ransduce self-incompatibility signaling in Brassica rapa. *Plant Cell* **19**, 3961-3973.

Karlova, R., Boeren, S., Russinova, E., Aker, J., Vervoort, J. and de Vries, S. C. (2006). The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 protein complex includes BRASSINOSTEROID-INSENSITIVE 1. *Plant Cell* **18**, 626-638.

Kerpolla, T. K. (2009) Visualization of molecular interactions using bimolecular fluorescence complementation analysis: characteristics of protein fragment complementation. *Chem. Soc. Rev.* **38**, 2876-2886.

Kim, T. W. and Wang, Z. Y. (2010) Brassinosteroid signal transduction from receptor kinases to transcription factors. *Ann. Rev. Plant Bio.,* Kim:2010p1537; 10.1146/annurev.arplant.043008.092057

Kwaaitaal, M. A. and de Vries, S. C. (2007) The SERK1 gene is expressed in procambium and immature vascular cells. *J. Exp. Bot.* **58**, 2887-2896.

Kwaaitaal, M. A., de Vries, S. C. and Russinova, E. (2005) Arabidopsis thaliana Somatic Embryogenesis Receptor Kinase 1 protein is present in sporophytic and gametophytic cells and undergoes endocytosis. *Protoplasma* **226**, 55-65.

Leslie, M. E., Lewis, M. L. and Liljegren, S. J. (2007). Organ Abscission. In *Plant Cell Separation and Adhesion* (ed. J. Roberts and Z. Gonzalez-Carranza), pp. 106-136. Oxford, United Kingdom: Blackwell Publishing.

Leslie, M. E., Lewis, M. W., Youn, J-Y., Daniels, M. J. and Liljegren, S. J. (2010) The EVERSHED receptor-like kinase modulates floral organ shedding in Arabidopsis. *Development* **137**, 467-476.

Lewis, M. W., Leslie, M. E., Fulcher, E. H., Darnielle, L., Healy, P., Youn, J-Y. and Liljegren, S. J. (2010) The SERK1 receptor-like kinase regulates organ separation in Arabidopsis flowers. *Plant J.*, Lewis:2010p1430; 10.1111/j.1365-313X.2010.04194.x

Li, J., Wen, J., Lease, K. A., Doke, J. T., Tax, F. E. and Walker, J. C. (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* **110**, 213-222.

Liljegren, S. J., Leslie, M. E., Darnielle, L., Lewis, M. W., Taylor, S. M., Luo, R., Geldner, N., Chory, J., Randazzo, P. A., Yanofsky, M. F. and Ecker, J. R. (2009). Regulation of membrane trafficking and organ separation by the NEVERSHED ARF-GAP protein. *Development* **136**, 1909-1918.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L. and He, P. (2010) A receptor-like cytoplasmic kinase, BIK1, associates with flagellin receptor complex to initiate plant innate immunity. *Proc. Natl. Acad. Sci. USA* **107**, 496-501.

Mackenzie, K. A. D. (1988) The anatomy of fruit abscission in loganberries. *Ann. Bot.* **62**, 249-263.

Murase, K., Shiba, H., Iwano, M., Che, F. S., Watanabe, M., Isogai, A. and Takayama S. (2004) A membrane-anchored protein kinase involved in Brassica self-incompatibility signaling. *Science* **303**, 1516-1519.

Nam, K. H. and Li, J. (2002). BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell* **110**, 203-212.

Patterson, S. E. (2001). Cutting loose. Abscission and dehiscence in Arabidopsis. *Plant Physiol*. **126**, 494-500.

Polito, V. S. and Stallman, V. (1981) Localized cell growth in ethephon-treated olive leaf abscission zone. *Scientia Horticulturae* **15**, 341-347.

Punwani, J. A., Hutchinson, C. E., Eric Schaller, G. and Kieber, J. J. (2010) The subcellular distribution of the Arabidopsis histidine phosphotransfer proteins is independent of cytokinin signaling. *Plant J.*, Punwani:2010p584; 10.1111/j.1365-313X.2010.04165.x

Robatzek, S., Chinchilla, D. and Boller, T. (2006) Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. *Genes Dev.* **20**, 537-542.

Russinova, E., Borst, J. W., Kwaaitaal, M., Caño-Delgado, A., Yin, Y., Chory, J. and de Vries, S. C. (2004) Heterodimerization and endocytosis of Arabidopsis brassinosteroid receptors BRI1 and AtSERK3 (BAK1). *Plant Cell* **16**, 3216-3229.

Schulze, B., Mentzel, T., Jehle, A., Mueller, K., Beeler, S., Boller, T., Felix, G. and Chinchilla, D. (2010) *J. Biol. Chem.* Schulze:2010p293; 10.1074/jbc.M109.096842

Sexton, R. and Redshaw, A. J. (1980) The role of cell expansion in the abscission of *Impatiens sultani* leaves. *Ann. Bot.* **48**, 745-756.

Shah, K., Gadella, T. W., van Erp, H., Hecht, V. and de Vries, S. C. (2001) Subcellular localization and oligomerization of the Arabidopsis thaliana somatic embryogenesis recpetor kinase 1 protein. *J. Mol Biol.* **309**, 641-655.

Shiu, S. H. and Bleecker, A. B. (2001). Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. *Proc. Natl. Acad. Sci. USA* **98,** 10763-10768.

Shpak, E. D., McAbee, J. M., Pillitteri, L. J. and Torii, K. U. (2005) Stomatal patterning and differentiation by synergistic interactions of receptor kinases. *Science* **309**, 290-293.

Sorkin, A and Goh, L. K. (2008). Endocytosis and intracellular trafficking of ErbBs. *Exp. Cell Res.* **314**, 3093-3106.

Stenvik, G-E., Tandstad, N. M., Guo, Y., Shi, C-L., Kristiansen, W., Holmgren, A., Clark, S. E., Aalen, R. B. and Butenko, M. A. (2008). The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION is sufficient to induce abscission in *Arabidopsis* through the receptor-like kinases HAESA and HAESA-LIKE2. *Plant Cell* **20**, 1805-1817.

Tör, M., Lotze, M. T., and Holton, N. (2009) Receptor-mediated signalling in plants: molecular patterns and programmes. *J. Exp. Bot.* **60**, 3645-54.

Torii, K. U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R., Whittier, R. F. and Komeda, Y. (1996) The Arabidopsis ERECTA gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. *Plant Cell* **8**, 735-746.

Walter, M., Chaban, C., Schütze, K., Batistic, O., Wechermann, K., Näke, C., Blazevic, D., Grefen, C., Shumacher, K., Oecking, C., Harter, K. and Kudla, J. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J.* **40**, 428-438.

Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M. B., Huber, S. C. and Clouse, S. D. (2008) Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling. *Dev. Cell* **15**, 220-235.

Webster, B. D. (1970). A morphogenetic study of leaf abscission in Phaseolus. *American J. Bot.* **57**, 443-451.

Wright, M and Osborne, D. J. (1974) Abscission in *Phaseolus vulgaris*: The positional differentiation and ethylene-induced expansion growth of specialised cells. *Planta* **120**, 163-170.

Wu, F. H., Shen, S. C., Lee, L. Y., Lee, S. H., Chan, M. T. and Lin, C. S. (2009) Tape-Arabidopsis-Sandwich – a simpler Arabidopsis protoplast isolation method. *Plant Methods* **5**, 16.

Yoo, S. D., Cho, Y. H. and Sheen, J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc*, **2**, 1564-1572.

Zappel, N. F. and Panstruga, R. (2008) Heterogeneity and lateral compartmentalization of plant plasma membranes. *Curr. Opin. Plant Biol.* **6**, 632-40.

Zhu, Y., Wang, Y., Li, R., Song, S., Wang, Q., Huang, S., Jin, J. B., Liu, C. M. and Lin, J. (2010) Analysis of interactions among the CLAVATA3 receptors reveals a direct interaction between CLAVATA2 and CORYNE in Arabidopsis. *Plant J.* **61**, 223-233.

Table 1.1 Genes affecting organ abscission in model plants

Gene	Plant	Mutant phenotype	Function	References
Organ patterning and AZ differentiation				
BLADE ON PETIOLE1 (*BOP1*) and *BOP2*	*At*	*bop1 bop2* double LOF[a]: floral organ position disrupted, abscission blocked	Establishment of proximo-distal polarity in lateral organs	Norberg *et al.*, 2005; Hepworth *et al.*, 2005; Ha *et al.*, 2004
SEEDSTICK (*STK*)	*At*	LOF: funicular overgrowth blocks seed release from mature fruits	Funicular patterning, seed AZ differentiation	Pinyopich *et al.*, 2003
development funiculus (*def*)	*Ps*	Recessive: seed abscission blocked	Unknown	von Stackelberg *et al.*, 2003
JOINTLESS (*J*)	*Le*	LOF: pedicel AZ fails to form, fruit abscission blocked	MADS domain transcription factor	Mao *et al.*, 2000; Syzmkowiak and Irish, 1999, 2005
JOINTLESS-2 (*J-2*)	*Le*	Recessive: pedicel AZ fails to form, fruit abscission blocked	Transcriptional regulator**	Budiman *et al.*, 2004; Yang *et al.*, 2005
Modified stipules (*Mstips*)*	*La*	Recessive: defects in AZ differentiation; delayed senescence	Unknown	Clements and Atkins, 2001
Abscission initiation – ethylene signaling and biosynthesis				
ETHYLENE RECEPTOR1 (*ETR1*)	*At*	Dominant GOF[b]: ethylene insensitivity, delayed abscission	Ethylene perception	Bleecker *et al.*, 1988; Bleecker and Patterson, 1997; Hua *et al.*, 1998
NEVER-RIPE (*LeETR3*)	*Le*	Dominant GOF: ethylene insensitivity, deficient in fruit ripening, and delayed senescence and floral abscission	Ethylene perception	Lanahan *et al.*, 1994; Lashbrook *et al.*, 1998
ETHYLENE INSENSITIVE2 (*EIN2*)	*At*	LOF: ethylene insensitive, delayed abscission	Ethylene signal transduction	Patterson and Bleecker, 2004; Chao *et al.*, 1997; Zimmerman *et al.*, 2004
EIN3-LIKE1 (*EIL1*), *EIL2*, and *EIL3*	*Le*	Nonspecific RNAi: delayed flower abscission	Predicted transcription factors for ethylene signal transduction	Tiemen *et al.*, 2001; Chen *et al.*, 2004
Green-ripe and *Never-ripe 2**	*Le*	Dominant: ethylene-insensitive, deficient in fruit ripening, delayed flower abscission and senescence	Unknown function in ethylene signal transduction	Barry *et al.*, 2005
AUXIN RESPONSE FACTOR2 (*ARF2*), *ARF1*, *ARF7*, *ARF19*	*At*	*arf2* LOF: delayed abscission and senescence; *arf1 arf2* double LOF or *arf2 arf7 arf19* triple LOF: enhanced delay in abscission	Predicted transcriptional repressors and activators; Role in ethylene biosynthesis	Okushima *et al.*, 2005b; Ellis *et al.*, 2005

(*Continued*)

Table 1.1 Genes affecting organ abscission in model plants (*Continued*)

Gene	Plant	Mutant phenotype	Function	References
Abscission initiation – ethylene independent pathway(s)				
delayed abscission1 (*dab1*)*	*At*	Recessive: delayed abscission, ethylene-sensitive	Unknown	Patterson and Bleecker, 2004
*dab2**	*At*	Dominant: delayed abscission, ethylene-sensitive, irregular AZ cell expansion	Unknown	Patterson and Bleecker, 2004
*dab3**	*At*	Recessive: delayed abscission, ethylene-sensitive	Unknown	Patterson and Bleecker, 2004
INFLORESCENCE DEFICIENT IN ABSCISSION (*IDA*)	*At*	LOF: abscission blocked	Predicted signaling ligand	Butenko *et al.*, 2003
HAESA	*At*	AS[c]: abscission blocked	Leucine-rich receptor-like kinase	Jinn *et al.*, 2000
AGAMOUS-LIKE15 (*AGL15*)	*At*	OX[d]: prolonged embryonic development, delayed flowering time, maturation, senescence and abscission, ethylene sensitive; LOF: no phenotype	MADS domain transcription factor	Fernandez *et al.*, 2000; Harding *et al.*, 2003; Lehti-Shiu *et al.*, 2005
ACTIN RELATED PROTEIN7 (*ARP7*)	*At*	LOF: embryonic lethality; RNAi: delayed abscission	Predicted role in chromatin remodeling	Kandasamy *et al.*, 2003, 2005b
Abscission initiation – unknown pathways				
ARP4	*At*	RNAi: early flower maturation, delayed flower senescence and abscission	Predicted role in chromatin remodeling	Kandasamy *et al.*, 2003, 2005a
Delayed abscission (*abs2*)*	*La*	Recessive: cotyledon abscission blocked; reduced frequency of leaflet and flower abscission	Unknown	Clements and Atkins, 2001
Cell wall hydrolysis				
*abs1**	*La*	Recessive: abscission of all organ types blocked, ethylene-sensitive	Predicted role in cell wall dissolution	Clements and Atkins, 2001; Henderson *et al.*, 2001
Cel1, Cel2	*Le*	*Cel1* AS: decreased frequency of fruit abscission. *Cel2* AS: increased force required to remove fruit	Cell wall hydrolysis	Lashbrook *et al.*, 1998; Brummell *et al.*, 1999

[a] Loss-of-function.
[b] Gain-of-function.
[c] Antisense RNA.
[d] Overexpression.
At = *Arabidopsis thaliana*; *La* = *Lupinus angustifolius*; *Le* = *Lycopersicon esculentum* (tomato); *Ps* = *Pisum sativum* (pea). *Gene not yet cloned; **putative gene.

Table 2.S1 Markers used in NEV co-localization experiments

Gene	Marker	AGI locus	Description	Localization	Reference(s)*
NIP1;1	Wave 6y	At4g19030	aquaporin	Endoplasmic reticulum	Geldner et al., 2009
RabF2a (Rha1)	Wave 7y	At5g45130	Rab5 family GTPase	Pre-vacuolar compartment/late endosome	Lee et al., 2004; Ueda et al., 2004
VTI12	Wave 13y	At1g26670	v-SNARE	Trans-Golgi network/early endosome	Sanderfoot et al., 2001; Uemura et al., 2004
RabD2a	Wave 29y	At1g02130	Rab GTPase	Golgi-associated/endosomal	Zheng et al., 2005
RabA1e	Wave 34y	At4g18430	Rab GTPase	Endosomal/recycling endosome	Geldner et al., 2009
RabA1g	Wave 129y	At3g15060	Rab GTPase	Endosomal/recycling endosome	Geldner et al., 2009
NPSN12	Wave 131y	At1g48240	syntaxin	Plasma membrane	Zheng et al., 2002; Uemura et al., 2004
CGL1 (NAG)	NAG-EGFP	At4g38240	N-acetyl glucosaminyl transferase I	Golgi	Grebe et al., 2003

*For assessment of localization patterns.

Reference

Uemura, T., Ueda, T., Ohniwa, R. L., Nakano, A., Takeyasu, K. and Sato, M. H. (2004). Systematic analysis of SNARE molecules in *Arabidopsis*: Dissection of the post-Golgi network in plant cells. *Cell Struct. Funct.* **29**, 49-65.

Table 3.S1 Genotyping mutant alleles

Allele	Enzyme	PCR product (bp)	Digest products (bp)		Oligonucleotides
evr-1	*Dde*I	220	evr-1	150, 70	5′-TCTTCAGGTCATCGAAACAGAGCTCG-3′
			L*er*	220	5′-TAAGTTCCTTGAGCTCCGACAACATTCCG-3′
evr-2	*Hpy*188I	430	evr-2	220, 180, 30	5′-GCAATAAGAGGATCAGAAAAACCACCAGG-3′
			L*er*	220, 150, 60	5′-CAGCTTGTACGTCAGTCAATATATCTTG-3′
evr-3		530 WT			5′-GCTTGAGTACCTTCACATGGATCATAAC-3′
					5′-AGATAGAAGCAAACAATACATATTGAAACAC-3′
		600 T-DNA			5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′
					5′-AGATAGAAGCAAACAATACATATTGAAACAC-3′
evr-4		530 WT			5′-GCTTGAGTACCTTCACATGGATCATAAC-3′
					5′-AGATAGAAGCAAACAATACATATTGAAACAC-3′
		400 T-DNA			5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′
					5′-AGATAGAAGCAAACAATACATATTGAAACAC-3′
pepr1-1		525 WT			5′-TCTATCGACCCACATAATTTCCGTTTC-3′
					5′-CATACTCAGCTCCACAAGCTCCTTAGC-3′
		350 T-DNA			5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′
					5′-CATACTCAGCTCCACAAGCTCCTTAGC-3′
evl1-1		600 WT			5′-GGAATCTTGGGTTACTCGAAATTACTC-3′
					5′-GTTCCCAAGCAACTCTGGAATGGTAC-3′
		300 T-DNA			5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′
					5′-GTTCCCAAGCAACTCTGGAATGGTAC-3′

FIGURES

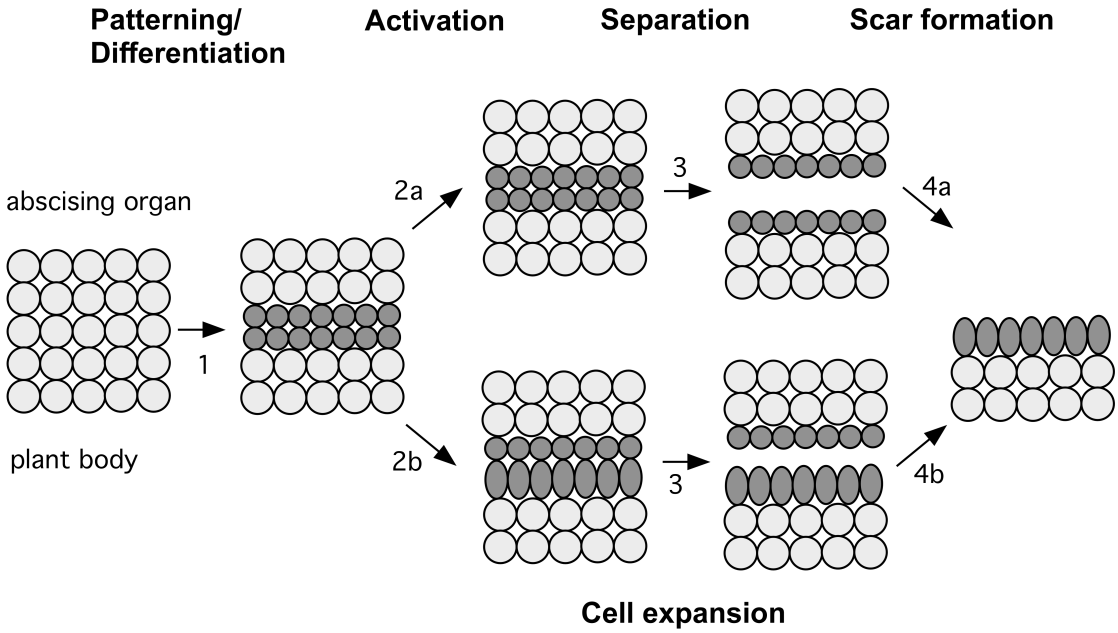

Figure 1.1 Model of the organ abscission process. Morphological changes during organ abscission are conserved across species and between different types of organs that are shed. Cells at the future site of detachment undergo patterning and differentiation within the context of the organ (1) to form distinct layers of small, cytoplasmically dense abscission zone (AZ) cells (shown in dark gray). Next, the AZ is activated for cell separation (2) through the interplay of unknown developmental and hormonal signals. The organ is shed (3) when cell wall modifying enzymes are secreted within the AZ layers, causing dissolution of cell adhesion. Following abscission, a protective scar forms (4) at the site of organ detachment to protect the plant from pathogen attack. It is not yet clear what role cell expansion plays in the abscission process. Cell expansion of a subset of AZ cells during the activation stage (2b) may promote subsequent cell separation; alternatively, cell expansion of the remaining AZ cells on the plant body after abscission (4a) may be solely involved in scar formation.

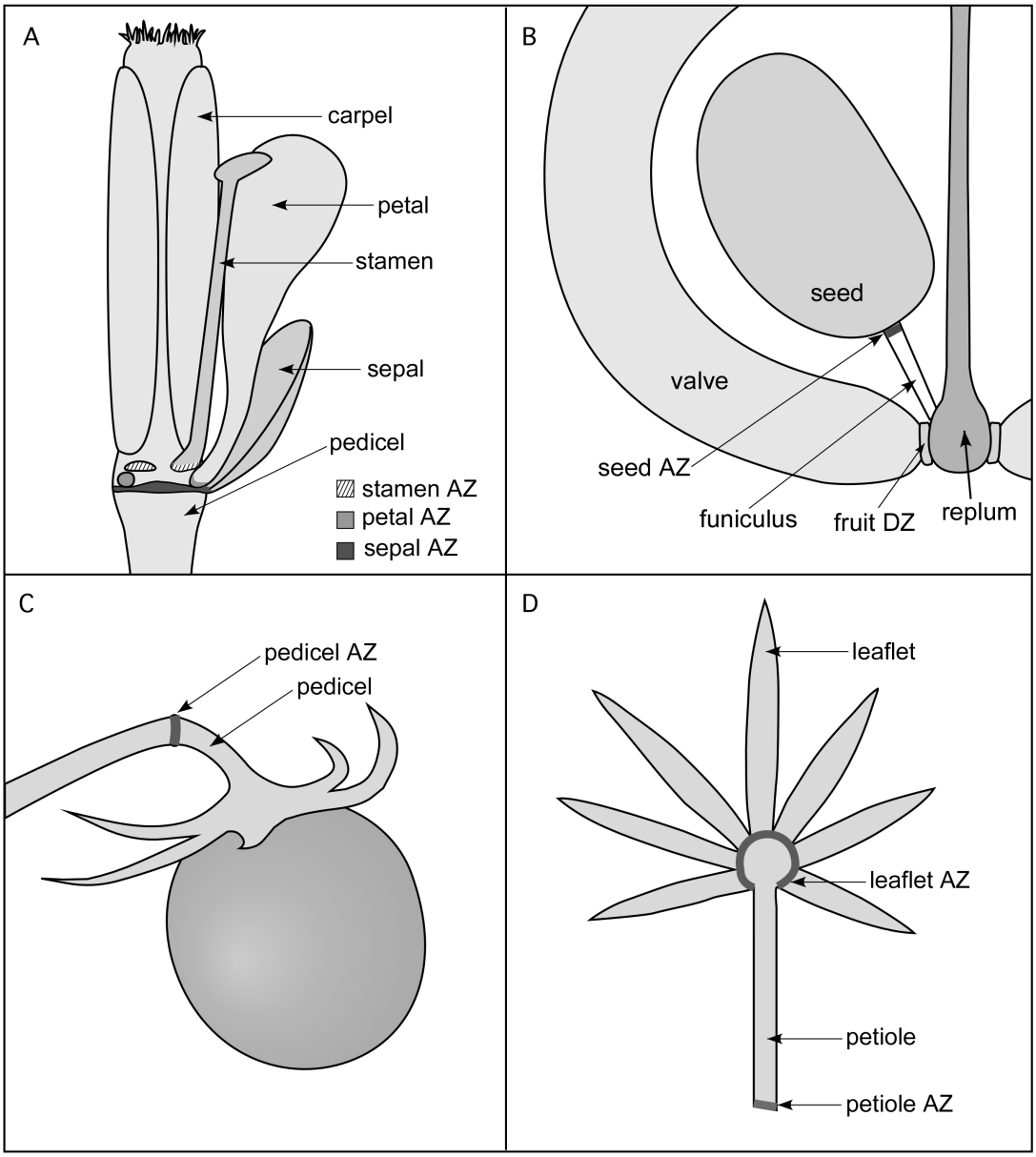

Figure 1.2 Sites of organ abscission in selected model plants. Mutations have been isolated in several model plants that block the abscission of specific organs. Depicted here are the *Arabidopsis thaliana* flower (A) and fruit (B), tomato (*Lycopersicon esculentum*) fruit (C), and *Lupinus angustifolius* compound leaf (D). In *Arabidopsis* flowers (A), the sepals, petals, and stamens are shed at abscission zones (AZs) located at the base of each organ. Within the dry, dehiscent *Arabidopsis* fruit (B), an AZ forms at the site of seed attachment to the funiculus (seed stalk). Seed abscission occurs after the fruit opens at the dehiscence zones (DZ) along its valves, or walls. In tomato (C), abscission of either unfertilized flowers or ripened fruit occurs at the pedicel AZ. The entire *L. angustifolius* leaf (D) can be shed by cell separation at the petiole AZ. Alternatively, leaflet AZs allow the shedding of individual leaflets.

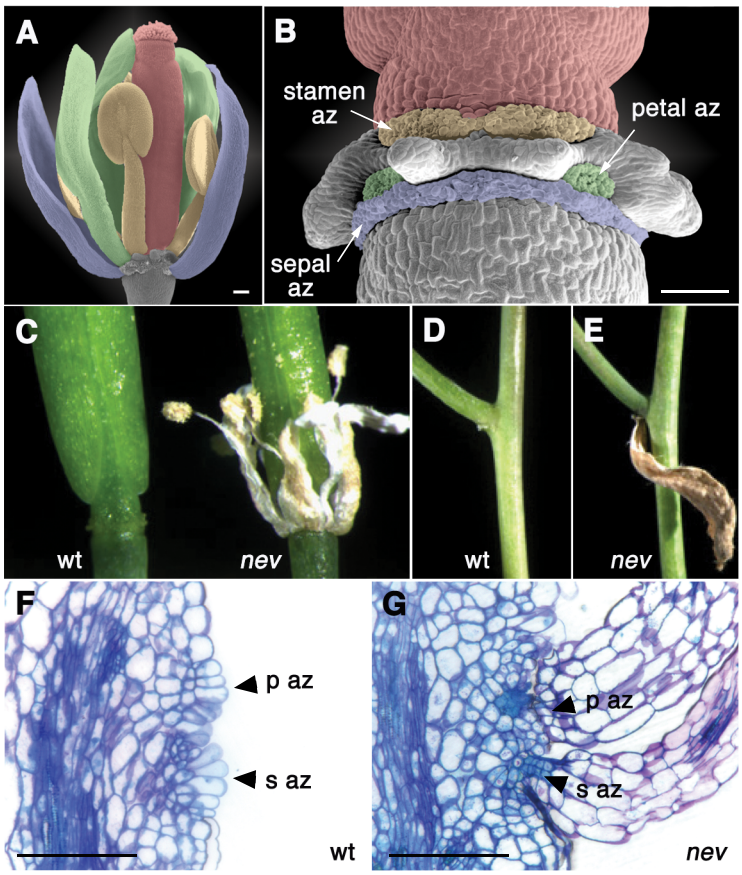

Figure 2.1 Mutations in *NEV* prevent organ separation.
(**A**) Scanning electron micrograph (SEM) of a wild-type *Arabidopsis* flower before organ separation (stage 13). The sepals, petals, stamens, and gynoecium are colorized in purple, green, yellow, and red, respectively. A few organs were removed for clarity.
(**B**) SEM of a wild-type flower after organ separation (stage 17). The remaining abscission zone (az) cells of each organ are colorized as in (A).
(**C**) Floral organs remain attached in *nev* flowers (stage 17) compared to wild type.
(**D,E**) Cauline leaves in *nev* plants (E) fail to detach after senescence compared to wild type (D).
(**F,G**) Longitudinal sections of wild-type (E) and *nev* (F) flowers at the time of shedding (stage 16) stained with toluidine blue. Adjacent petal (p az) and sepal (s az) abscission zones at the base of each flower are indicated. In wild-type flowers, the remaining abscission zone cells have expanded, which does not occur in *nev* flowers in the absence of organ separation. Scale bars: 100 µm.

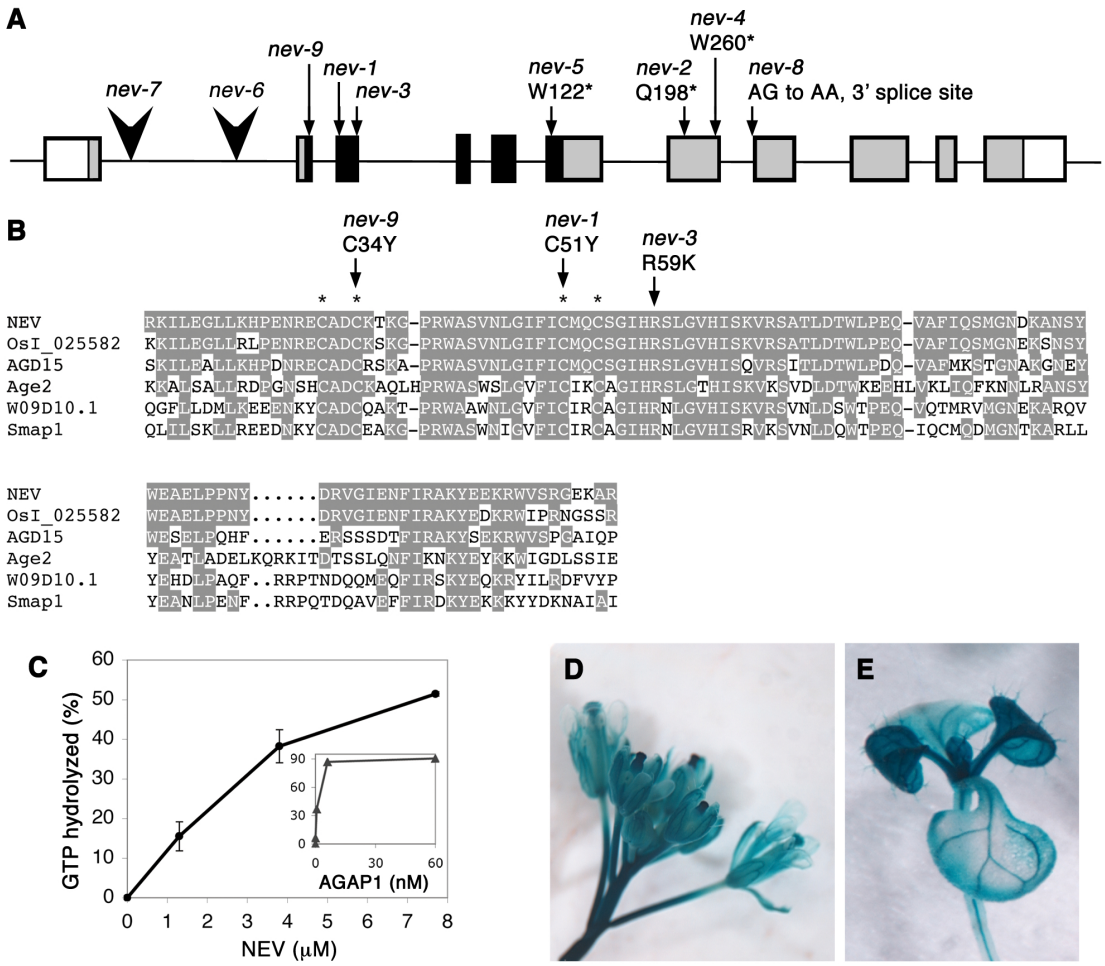

Figure 2.2 *NEV* encodes an ADP-ribosylation factor GTPase activating protein (ARF GAP).

(**A**) Diagram of the At5g54310 locus, showing the sites and sequence alterations of characterized *nev* mutations. Exons are shown as boxes, and the translated regions corresponding to the ARF-GAP domain and the rest of the corresponding protein are indicated in black and gray, respectively. Point mutations are marked by arrows, and T-DNA insertions by arrowheads.

(**B**) Sequence alignment of the ARF-GAP domain from NEV and related proteins from plants, yeast, worm and mouse. Amino acids conserved between NEV and other proteins are shaded, and the four cysteine residues that constitute the zinc finger are indicated with asterisks. The sites of *nev* missense mutations that affect two of these cysteines and a critical arginine residue are marked by arrows above the alignment. Characterized ARF-GAP proteins in the alignment include yeast Age2 (Zhang et al 1998) and mouse Smap-1 (Sato et al 1998). Uncharacterized proteins with closely-related ARF-GAP regions include OsI_025582, AGD15 (Vernoud et al 2003), and W09D10.1, predicted from *Oryza sativa*, *Arabidopsis*, and *Caenorhabditis elegans* sequences, respectively. *(continued on next page)*

Figure 2.2 *(continued)*

(**C**) NEV promotes GTP hydrolysis of mammalian ARF1. The ARF-GAP activity of recombinant, full-length NEV protein was measured using AGAP1, a mammalian ARF-GAP, as a positive control (inset graph). The percentage of GTP bound to Arf1 that was converted to GDP is presented. The data are the summary of two experiments.
(**D-E**) *NEV* regulatory regions direct broad expression of ß-glucuronidase in flowers and shoot inflorescence stems (D), and in developing leaves and vascular strands (E).

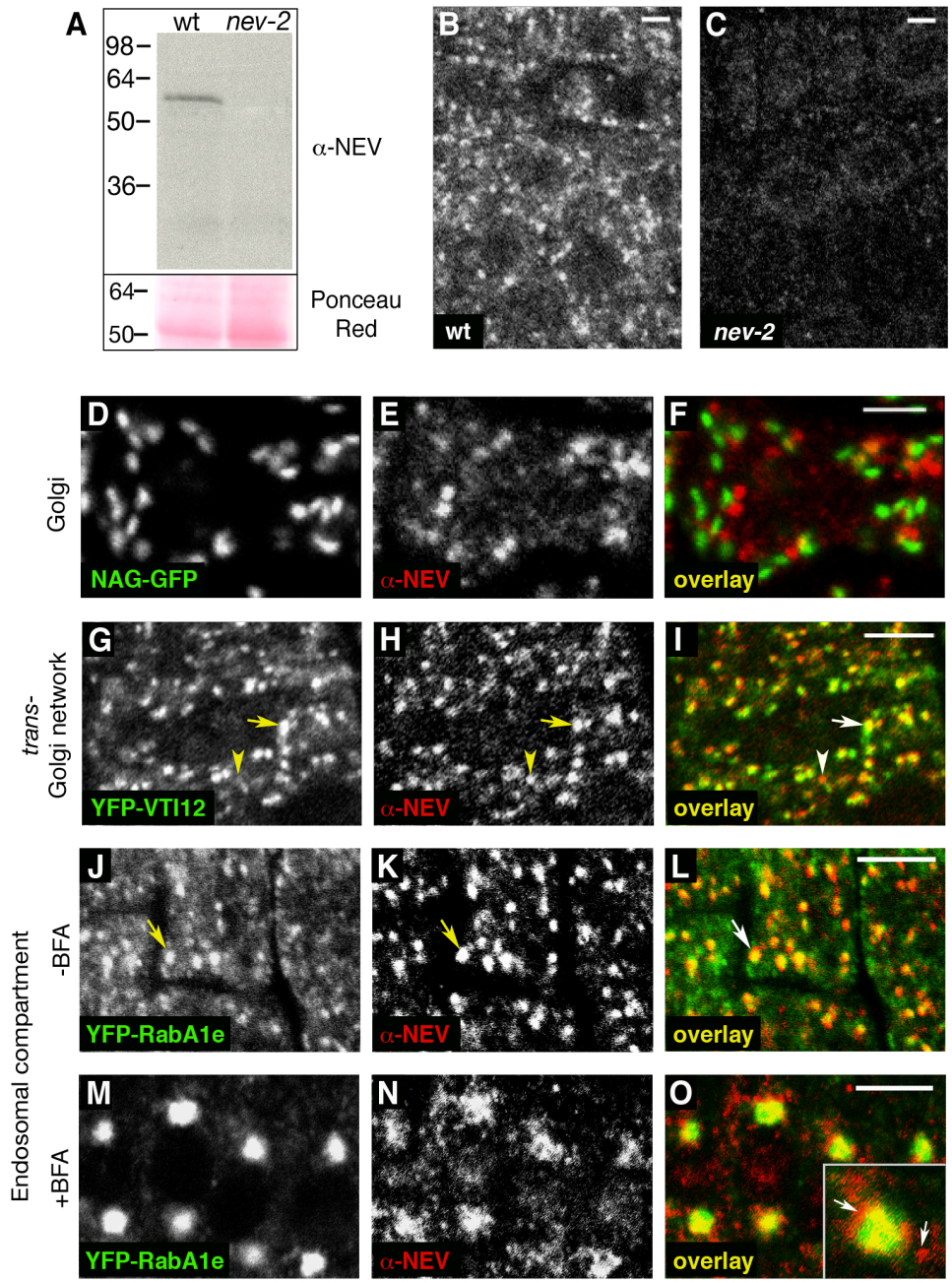

Figure 2.3 NEV localizes to the *trans*-Golgi network and endosomes.
(**A**) NEV antiserum recognizes a ~55-kDa protein in wild-type flower extracts. Since the *nev-2* allele encodes a truncated protein lacking the C-terminal recognition sequence of the NEV antiserum, no protein is detected in *nev-2* flower extracts. Ponceau Red staining was used as a protein loading control.
(B-O) Immunofluorescent localization of NEV and endomembrane markers in primary root epidermal cells of wild-type (B), *nev* (C) and transgenic marker (D-O) plants. In (M-O), the primary roots of YFP-RabA1e plants were incubated for 1 hour with 100µM BFA prior to fixation and immunofluorescent staining.
(continued on next page)

Figure 2.3 *(continued)*
(**B,C**) NEV antiserum detects NEV protein in punctate structures in wildtype cells (B). Background fluorescence is shown for *nev-2* cells (C).
(**D-I**) NEV localization is distinct from the Golgi (D-F), and shows more precise localization with YFP-VTI12, a marker of the *trans*-Golgi network (arrows, G-I). Occasional spots labeled by NEV (arrowheads, G-I) do not co-localize with VTI12.
(**J-O**) NEV also co-localizes with YFP-RabA1e, a novel endosomal marker proposed to localize to the recycling endosome (arrows, J-L). BFA treatment of the primary root causes both NEV and YFP-RabA1e to localize to BFA-bodies (M-O). Whereas the localization of YFP-RabA1e is confined to the core of the BFA-bodies, NEV is also found at the periphery (arrows, M-O).
Individual channels: YFP markers, green; NEV, red. Scale bars: 5 μm.

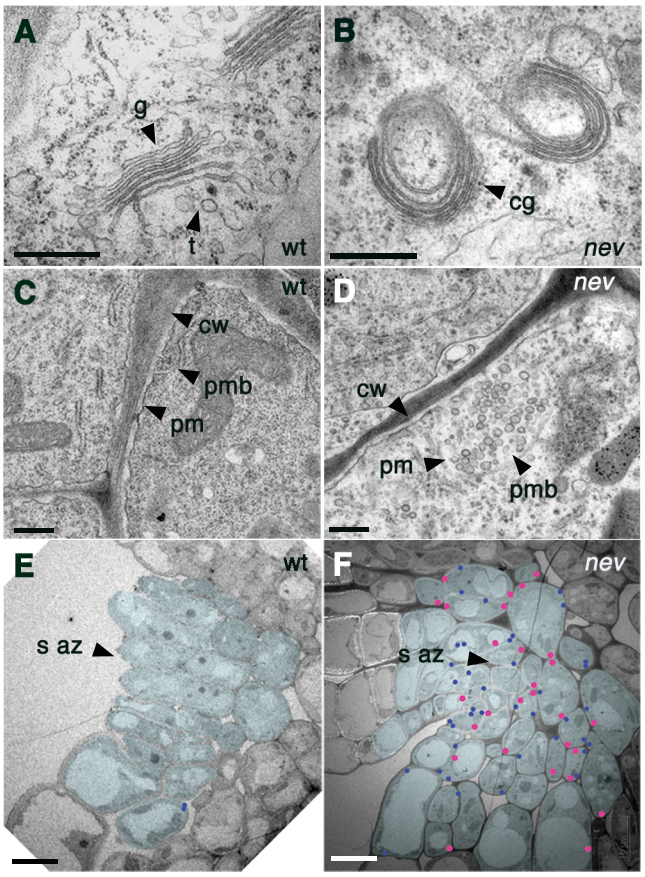

Figure 2.4 Mutations in *NEV* alter Golgi structure, location of the *trans*-Golgi network, and cause accumulation of paramural vesicles.
(A-F) Transmission electron micrographs of cells at the base of wild-type and *nev* sepals at the time of shedding (stage 16).
(**A,B**) Cup-shaped and circularized multilamellar structures (cg) are predominantly found in *nev* cells (B) instead of the typically flat Golgi cisternae (g) of wild-type cells (A). While the *trans*-Golgi network (t) is frequently observed (84%, n=19) near the Golgi (0.041 +/- 0.018 µm, n=16) in wild-type cells (A), these tubular-vesicular compartments are not clearly identifiable near the circularized structures of *nev* cells (B).
(**C,D**) While paramural vesicles are infrequently observed between the plasma membrane (pm) and cell wall (cw) of wild-type cells (C), numerous vesicles are frequently observed in large paramural bodies (pmb) in *nev* cells (D).
(**E-F**) Distribution of paramural vesicles in cells at the base of wild-type (E) and *nev* (F) sepals. Abscission zone regions are indicated by arrowheads, analyzed cells are colorized light blue, and paramural bodies with more than 30 vesicles or 10-30 vesicles are indicated by pink and blue circles, respectively. Scale bars: 0.5 µm in A-D; 10 µm in E,F.

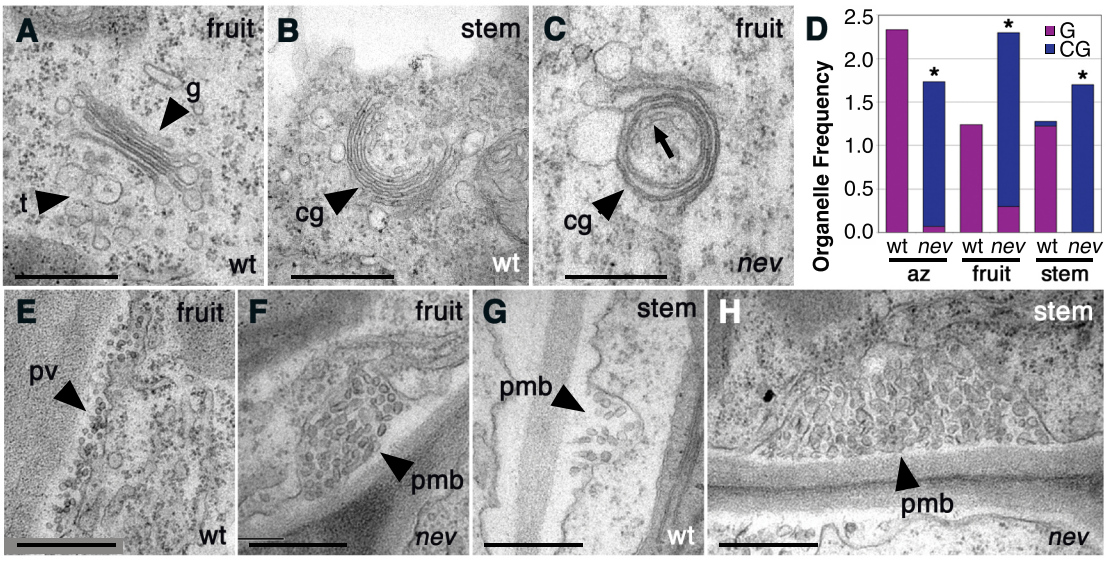

Figure 2.5 The fruit and stems of *nev* flowers also show membrane trafficking defects.

(A-C,E-H) Transmission electron micrographs of cells in the fruit wall and pedicel (stem) of wild-type and *nev* flowers (stage 16).

(**A-C**) Circularized multilamellar structures (cg) are enriched in *nev* fruit and stem cells (C,D) in comparison to the linear Golgi cisternae (g) of wild-type cells (A). Rarely, Golgi cisternae with a curved appearance were observed in wild-type cells (B,D). Vesicular structures resembling the *trans*-Golgi network (t) of wild-type cells (A) are often observed in the vicinity or inside the multilamellar structures of *nev* cells (C, arrow) (73%, n=15).

(**D**) Frequency of Golgi with a wild-type appearance (G, purple) and circularized multilamellar structures (CG, blue) per cell in sections of *nev* and wild-type sepal AZ regions, fruit walls, and pedicels. For each type of tissue analyzed, n (cells) ≥ 15. Statistical differences between *nev* and wild-type tissues are indicated by asterisks (Fisher's Exact Test $P < 0.0001$).

(**E-H**) Large paramural bodies (pmb) are found in *nev* fruit (F) and stem (H) cells. Paramural vesicles (pv) and paramural bodies were also observed in wild-type fruit (E) and stem (G) cells. Scale bars: 0.5 µm in A-C, E-H.

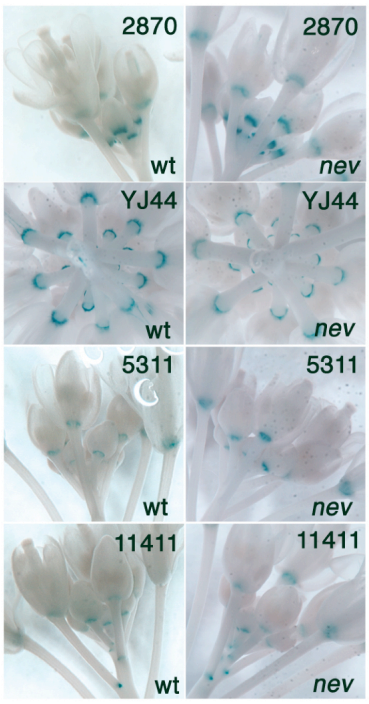

Figure 2.S1 Early markers of floral organ boundaries are not affected in *nev* flowers.
ß-Glucuronidase expression of the 2870, YJ44, 5311, and 11411 markers in *Arabidopsis* inflorescences. The temporal and spatial patterns of each of these markers of the proximal regions of the outer differentiating floral organs is not affected in *nev* mutant flowers compared to wildtype. The YJ44 enhancer trap line was kindly provided by Yuval Eshed and John Bowman and was generated by *Agrobacterium*-mediated transformation with the plasmid pOCA-28-15-991 (Eshed et al., 1999). The 2870, 5311, 11411 enhancer trap lines were kindly provided by Tom Jack and are from the Jack collection described previously (Campisi et al., 1999).

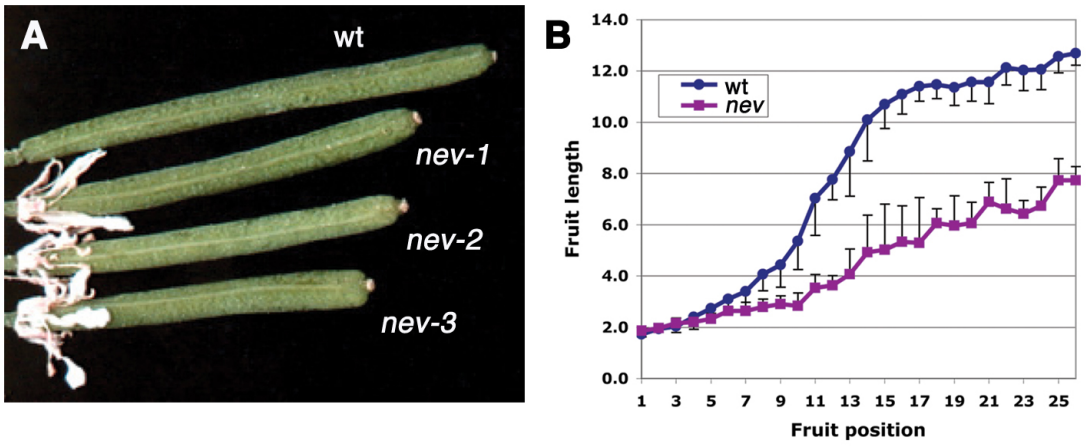

Figure 2.S2 Mutations in *NEV* affect fruit growth.
(**A**) Mature *nev* fruit (late stage 17) are significantly shorter than wild type. The average length of *nev-3* fruit (7.7 ± 0.5 mm; n=50) was 61% the length of wild-type fruit (12.7 ± 0.5 mm; n=50).
(**B**) Measurement of wild-type and *nev-3* fruit length throughout development, from the youngest open flower (position 1; stage 13/14) to mature fruit (position 25; late stage 17) on primary inflorescences. A significant decrease in length is apparent in *nev* fruit (position 8) just prior to the initiation of a rapid growth phase in wild-type fruit (position 9). For each position on an inflorescence, n = 6.

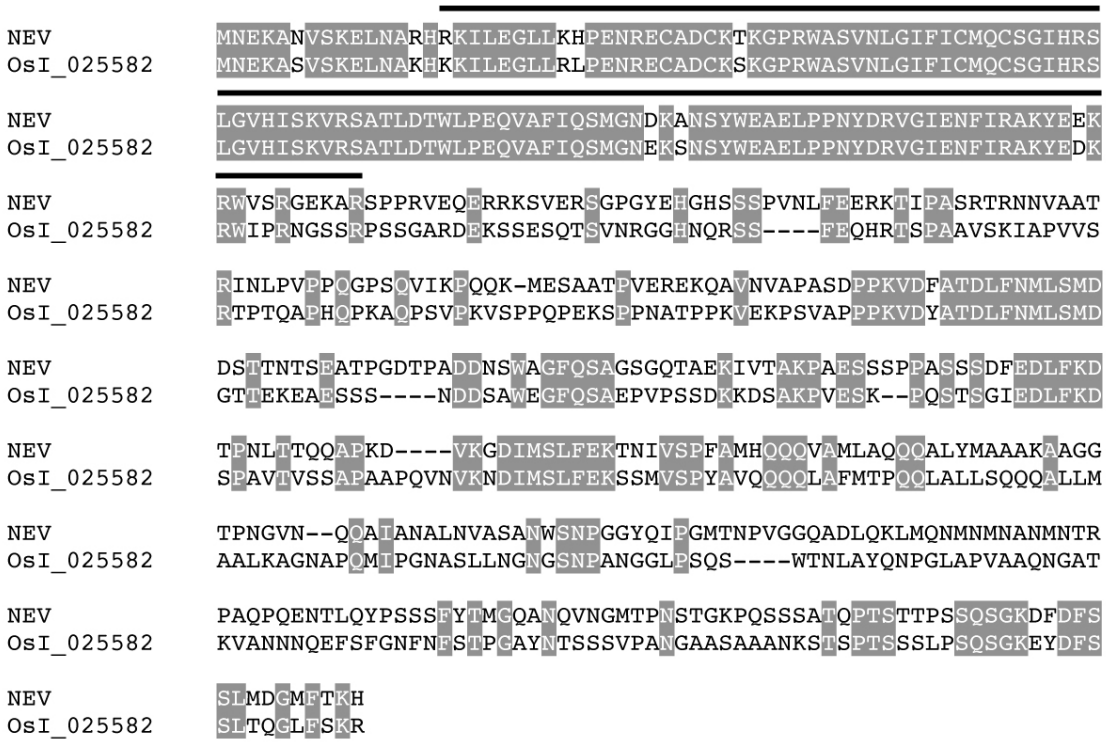

Figure 2.S3 Identification of a candidate NEV ortholog in rice.
Sequence alignment of NEV and a related protein from rice (*Oryza sativa*). Amino acids conserved between NEV and other proteins are shaded, and a line above the sequences indicates the region corresponding to the ARF-GAP domain.

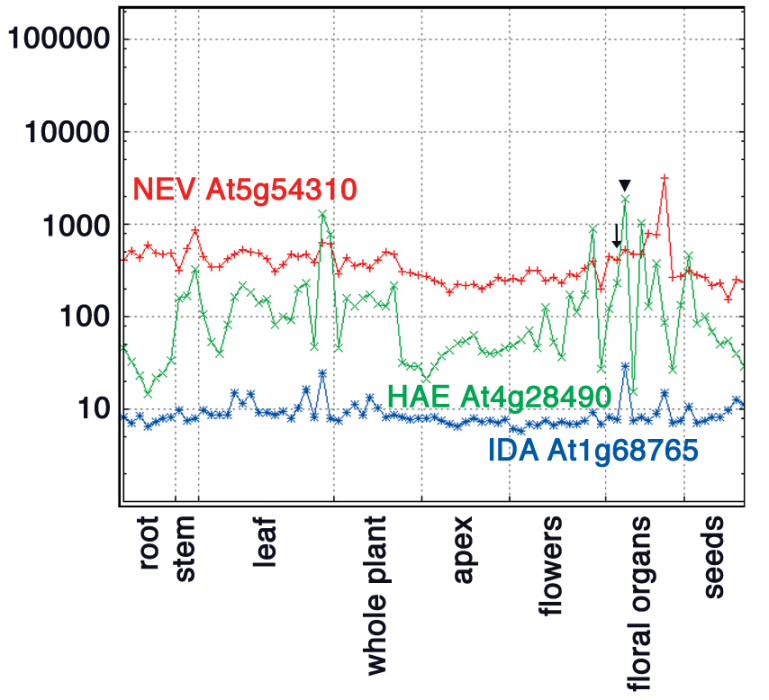

Figure 2.S4 Developmental expression profile of *NEV*.
NEV is expressed ubiquitously in contrast to the dynamic expression profiles of *HAE* and *IDA*. Substantially higher levels of *HAE* and *IDA* transcripts are apparent in sepals just before abscission (stage 15, arrowhead) compared to sepals in unfertilized flowers (stage 12, arrow), whereas levels of *NEV* increase only slightly. (AtGenExpress data: Schmid et al., 2005; http://jsp.weigelworld.org/resources).

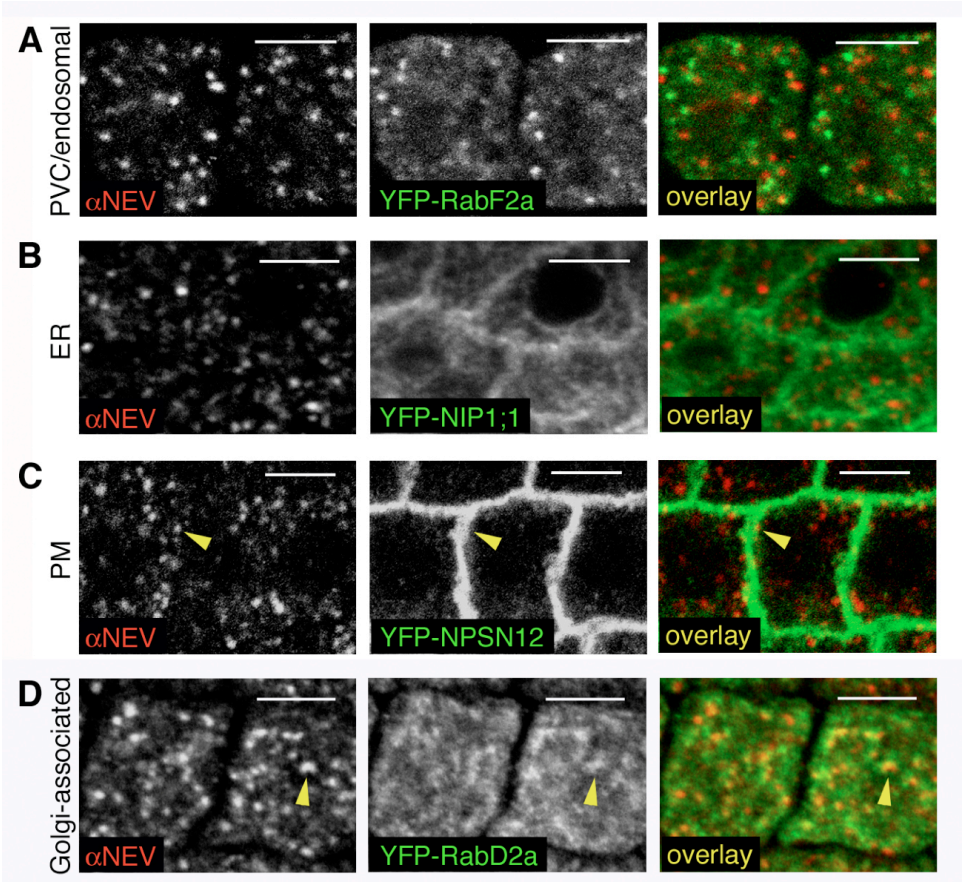

Figure 2.S5 NEV colocalizes with a subset of endosomal compartments.
(**A-D**) Immunofluorescent localization of NEV and endomembrane markers in primary root epidermal cells of transgenic marker plants. Compartments tested for co-localization included the pre-vacuolar compartment (PVC)/late endosome (YFP-RabF2a), the Endoplasmic reticulum (ER; YFP-NIP1;1), and the plasma membrane (PM) (YFP-NPSN12). NEV staining is distinct from both the PVC/late endosome (A) and the ER (B); however, frequent association is seen between NEV and the PM marker NPSN12 (arrows, C). Partial overlap is also seen between NEV and YFP-RabD2a, a marker that is predicted to associate with Golgi and endosomal compartments (arrows, D). Scale bars: 5 μm.

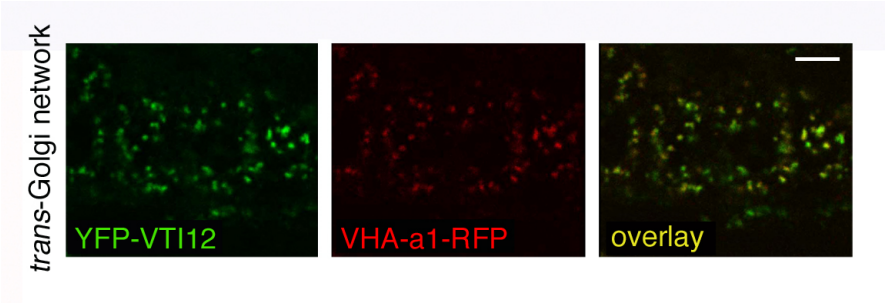

Figure 2.S6 VTI12 colocalizes with VHA-a1 at the *trans*-Golgi network. Immunofluorescent detection of the YFP-VTI12 and VHA-a1-RFP *trans*-Golgi/early endosome markers in primary root epidermal cells of transgenic plants carrying both markers. Nearly complete association is seen between these markers. Scale bar: 5 μm.

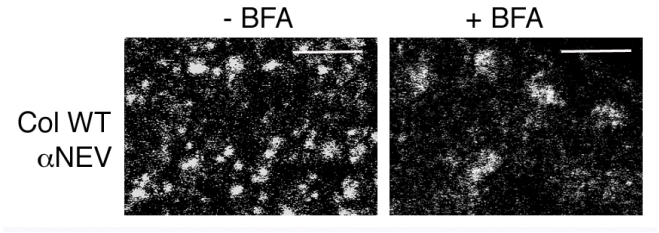

Figure 2.S7 Immunofluorescent detection of NEV in wild-type primary root epidermal cells treated with (+BFA, 100 μM) or without BFA (-BFA) suggests that NEV is associated with endosomes undergoing BFA-sensitive sorting. NEV antigen condenses into structures resembling BFA-bodies derived from TGN/early endosomal membranes. Scale bars: 5 μm.

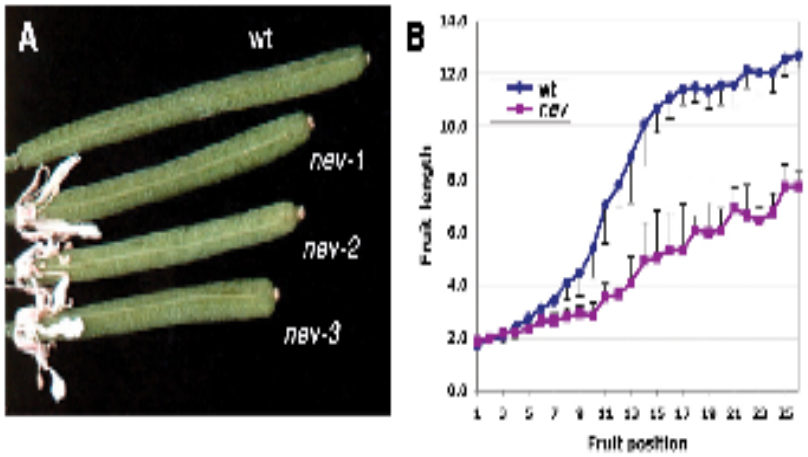

Figure 2.S8 Mutations in *NEV* alter the location of the *trans*-Golgi network.
(**A-F**) Transmission electron micrographs of cells at the base of wild-type and *nev* sepals at the time of shedding (stage 16). Whereas vesicular-tubular clusters of the *trans*-Golgi network (t, TGN) are frequently observed (84%, n=19) near Golgi stacks (g) in wild-type cells (A-C), these compartments are not readily apparent near the circularized multilamellar structures (cg) characteristic of *nev* cells (D-F). Occasionally, structures that could be TGN vesicles (arrow) are found within these circularized structures (F; 12%, n=17). Scale bars: 0.5 µm.

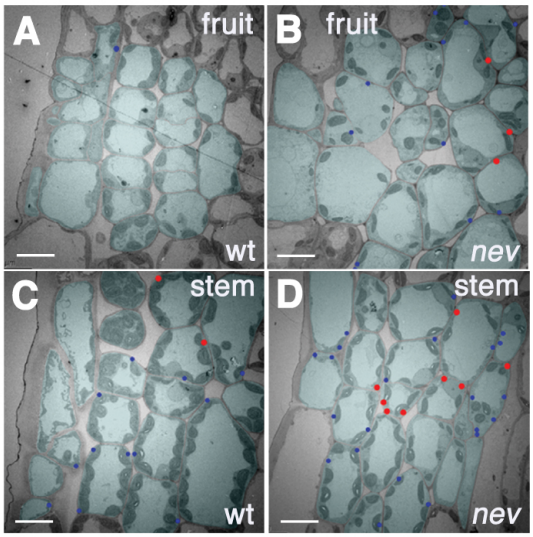

Figure 2.S9 The fruit and stems of *nev* flowers also show membrane trafficking defects.
(**A-D**) Low magnification transmission electron micrographs of cells in the fruit wall and pedicel (stem) of wild-type and *nev* flowers (stage 16). Paramural vesicles are found at a higher frequency in *nev* cells (B,D) compared to wild-type (A,C). Analyzed cells are colorized in aquamarine, and sites with more than 30 vesicles or 10-30 vesicles are indicated by red and blue circles, respectively. Scale bars: 10 µm in A-D.

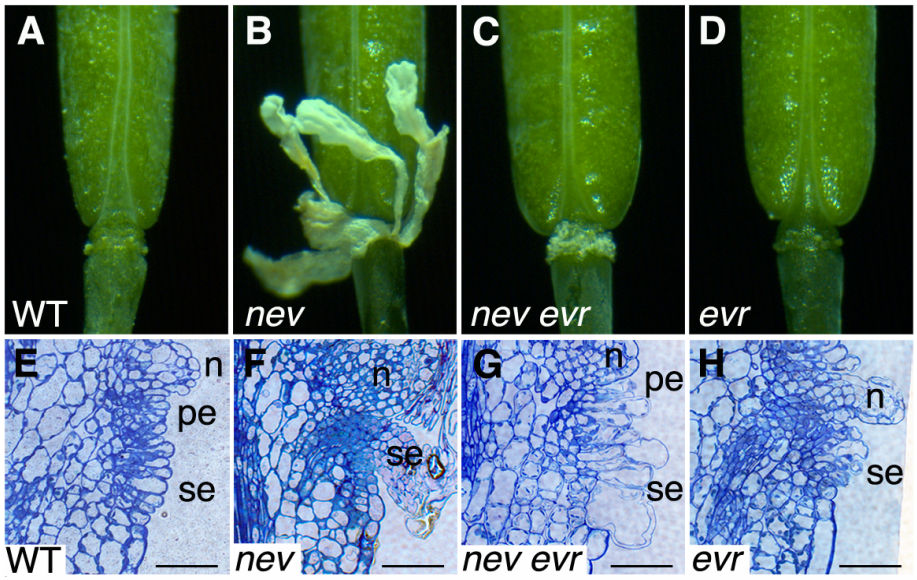

Figure 3.1 Mutations in *EVR* rescue organ separation in *nev* flowers.
(**A**) Wild-type flower after abscission (stage 17).
(**B**) *nev* mutant flowers (stage 17) retain their floral organs indefinitely.
(**C**) Organ shedding is rescued in *nev evr* flowers (stage 17).
(**D**) *evr* flower after abscission (stage 17).
(**E-H**) Longitudinal sections of wild-type (E), *nev* (F), *nev evr* (G), and *evr* (H) flowers (stage 16) stained with Toluidine Blue. In *nev evr* flowers (G), the remaining abscission zone (AZ) cells expand to greater extent than those of wild type (E). The sepal (se) and petal (pe) AZ regions are indicated, as are the nectaries (n). Scale bars: 50 μm.

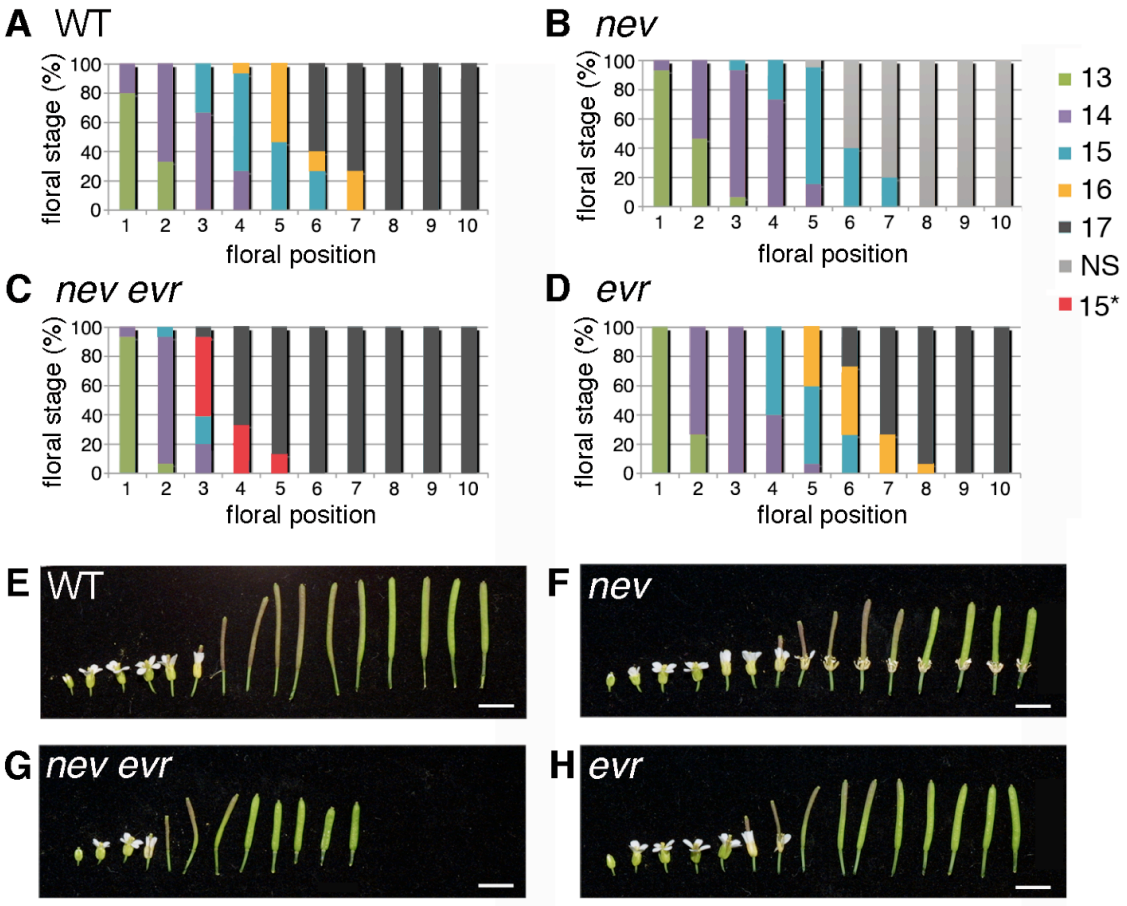

Figure 3.2 Abscission occurs prematurely in *nev evr* flowers.
Progression of flower development in wild-type, *nev*, *nev evr*, and *evr* plants from the first open flower to maturing fruit.
(**A-D**) From the first open flower (position 1), stages were assessed for up to 10 flowers per inflorescence (*n*=15 inflorescences per genotype). For each position, the percentage of flowers at each stage is shown. In wild-type flowers (A), organ separation (stage 16) is first observed at position 5.7 ± 1.0. By position 8, all flowers have shed their organs (stage 17). *nev* flowers (B) retain their floral organs and are labeled as NS (non-shedding, stage 16 on). In *nev evr* flowers (C), organ separation (stage 15*) is first observed at position 3.4 ± 0.5 and is complete by position 6. In *evr* flowers (D), organ separation (stage 16) is first observed at position 5.9 ± 0.8 and is complete by position 9.
(**E-H**) *evr-2* fruit (stage 17) (8.7±0.3 mm; *n*=11) are 79% the length of wild type (11.0±0.5 mm; *n*=11). *nev-3 evr-2* fruit (6.4±0.3 mm; *n*=13) are 88% the length of *nev-3* fruit (7.3±0.5 mm; *n*=15). Scale bars: 5 mm.

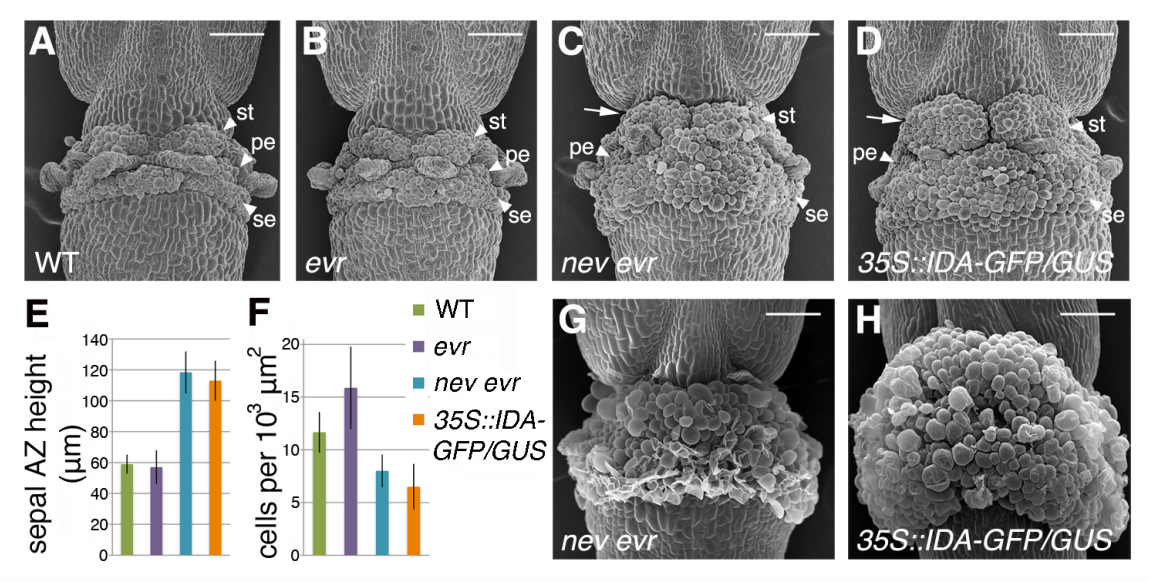

Figure 3.3 Ectopic AZ cell expansion occurs in *nev evr* flowers.
(**A-D**) Scanning electron micrographs (SEMs) of flowers immediately after organ separation (first stage 17 flower). Due to increased cell expansion and the presence of additional cells, *nev evr* flowers (C), like flowers constitutively expressing *IDA* (D), develop larger AZs than wild-type (A) and *evr* (B) flowers that cover the stem-like gynophore of the fruit (arrow).
(**E-F**) Quantification of AZ size (E) and cell expansion (F) in wild-type and mutant flowers ($n \geq 4$, second stage 17 flower). In *nev evr* and *35S::IDA-GFP/GUS* flowers, the average heights of the sepal AZs are 2-fold greater than that of wild-type or *evr* flowers (E). A decrease in the number of sepal AZ cells in a defined area was observed for *nev evr* and *35S::IDA-GFP/GUS* flowers compared to wild-type and *evr* flowers, suggesting that cell expansion contributes in part to the increase in AZ size (F).
(**G-H**) The expanding AZs of older *nev evr* (G) and *35S::IDA-GFP/GUS* (Col) (H) flowers (stage 17) envelop the nectaries and form visible collars of tissue at the fruit bases. The sepal (se), petal (pe) and stamen (st) AZs cannot be distinguished.
Scale bars: 100 µm.

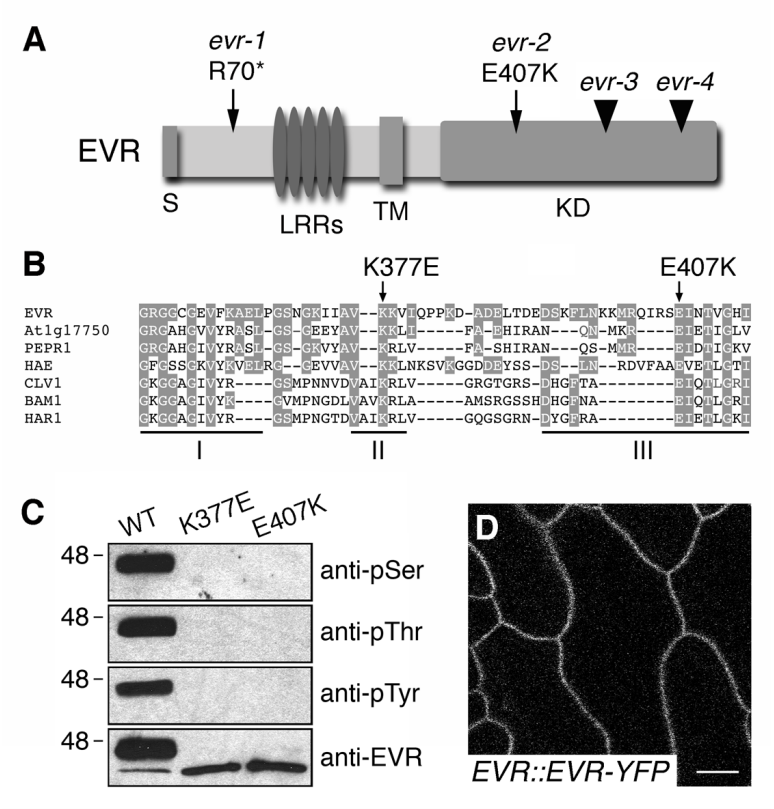

Figure 3.4 *EVR* encodes a plasma membrane-localized LRR-RLK.
(**A**) Diagram of the EVR protein, with the sites of the identified *evr* mutations indicated. The regions corresponding to the signal peptide (S), leucine-rich repeats (LRRs), transmembrane domain (TM), and kinase domain (KD) are indicated. Point mutations are marked by arrows, and T-DNA insertions by arrowheads.
(**B**) Sequence alignment of kinase subdomains I-III from EVR and related LRR-RLKs from *Arabidopsis* and *Lotus japonicus*. Amino acids conserved between EVR and other proteins are shaded. The sites of the conserved glutamic acid in subdomain III affected by the *evr-2* mutation and an invariant lysine in subdomain II required for kinase activity are marked by arrows above the alignment.
(**C**) The recombinant EVR KD autophosphorylates at serine, threonine, and tyrosine residues in vitro. Mutations in subdomains II (K377E) and III (E407K) of the kinase domain block the kinase activity of EVR. EVR antiserum recognizes ~46 and ~40 kDa phosphorylated and unphosphorylated proteins, respectively.
(**D**) EVR localizes to the plasma membrane. Fluorescent localization of EVR-YFP marker in epidermal cells of wild-type leaf petioles (stems). Scale bar: 10 μm.

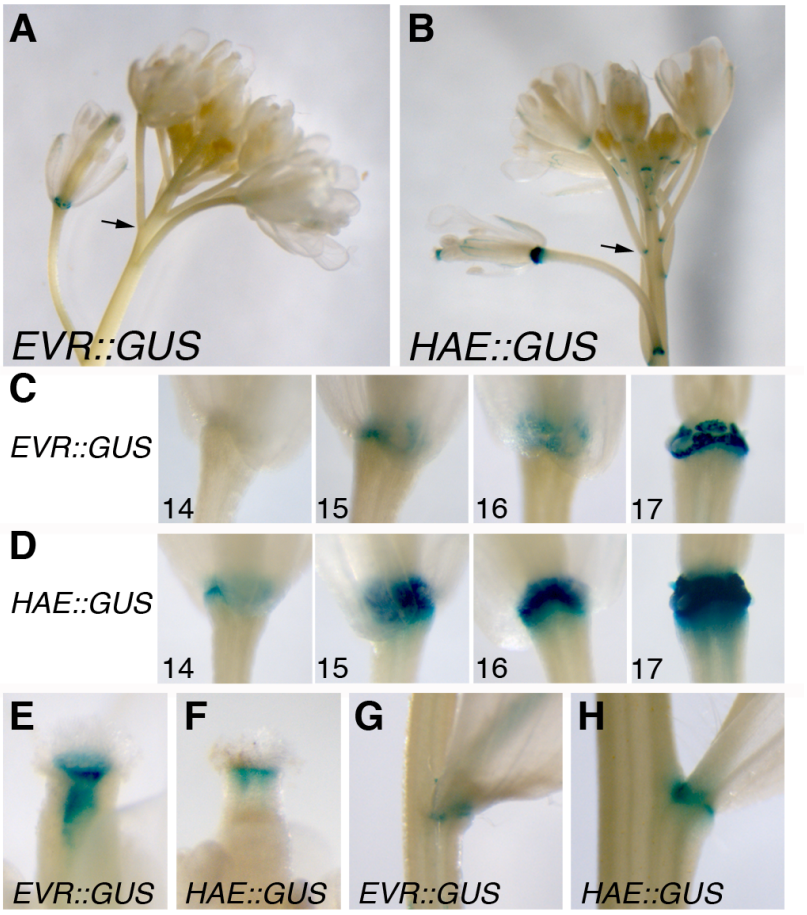

Figure 3.5 *EVR* is expressed in organ AZs.
(**A-H**) The regulatory regions of *EVR* and *HAE* direct expression of β-glucuronidase (GUS) in AZs (A-D), internal tissues of the floral style (E,F) and at the junction between the cauline leaves and the inflorescence stem (G,H). The *HAE::GUS* marker is also expressed at the bases of the floral pedicels (B, arrow). Within floral AZs (C), the *EVR* promoter directs GUS expression prior to organ separation (stage 15), during abscission (stage 16), and as the remaining cells form protective scar tissue (stage 17). GUS expression directed by the *HAE* promoter shows a similar, yet stronger profile in AZs with an earlier stage of initiation (Jinn et al., 2000).

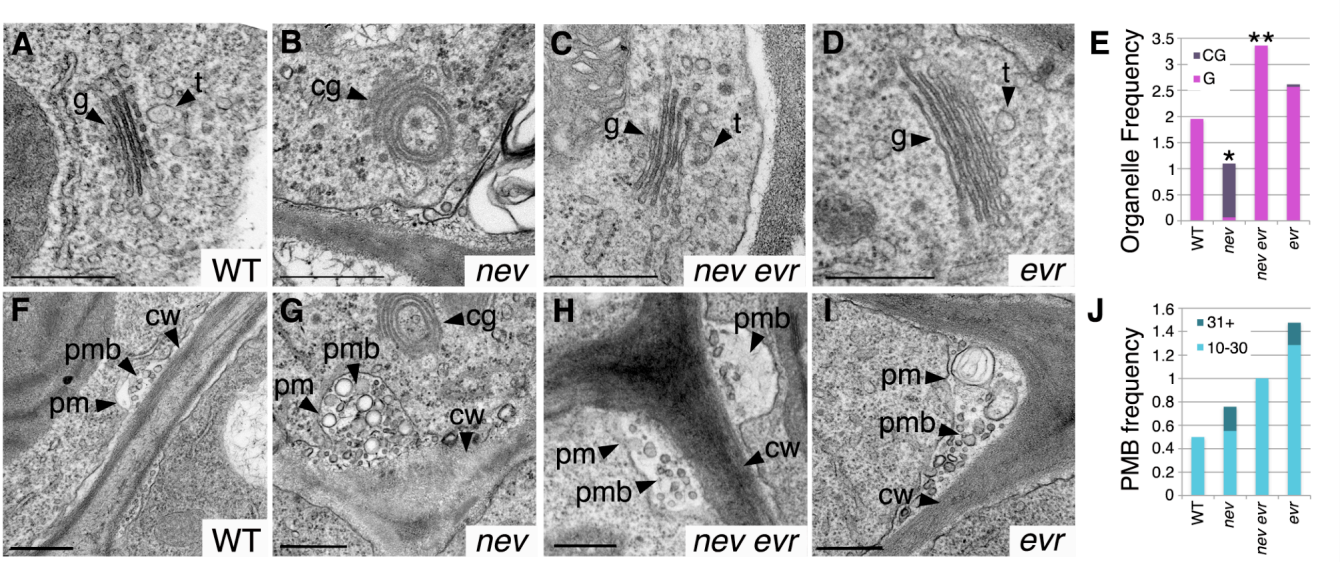

Figure 3.6 Mutations in *EVR* restore Golgi structure and location of the TGN in *nev* flowers.

Transmission electron micrographs and analysis of cells in sepal AZ regions at the time of organ separation for wild-type (stage 16), *nev* (stage 16 non-shedding), *nev evr* (stage 15*) and *evr* (stage 16) flowers.

(**A-D**) Instead of the flat stacks of Golgi cisternae characteristic of wild type (A), circularized multilamellar structures are observed in *nev* cells (B). Golgi with a wild-type appearance are found in *nev evr* (C) and *evr* cells (D). We frequently observed vesicular-tubular structures characteristic of the trans-Golgi network (TGN) (81%, *n*=16) closely associated with the Golgi cisternae (34±21 nm, *n*=13) in wild-type cells (a), whereas the TGN (15%, *n*=13) was less often observed near the circularized multilamellar structures (40±25 nm, *n*=2) of *nev* cells (B). The location of the TGN was restored in *nev evr* cells (83% associated with Golgi, *n*=24; 37±12 nm, *n*=20) and was unaffected by loss of *EVR* alone (76% associated with Golgi, *n*=21; 38±25 nm, *n*=15).

(**E**) Frequency of flat Golgi cisternae (G, purple) and circularized multilamellar structures (CG, blue) per cell in sections of wild-type and mutant sepal AZ regions. For each genotype, *n* (cells)≥11. Statistical differences between *nev* and wild-type, and between *nev evr* and *nev* tissues are indicated by single and double asterisks, respectively (Fisher's exact test, $P<0.0001$). A statistical difference was not detected between *evr* and wild-type tissues.

(**F-I**) Paramural bodies (PMBs) were observed in the cells of wild-type (F), *nev* (G), *nev evr* (H) and *evr* (I) flowers. Whereas PMBs were observed in cells from each genotype, PMBs with greater than 30 vesicles were only observed in *nev* and *evr* cells.

(**J**) Frequency of PMBs (10-30 and 31+ vesicles) per cell in sections of wild-type and mutant sepal AZ regions. For each genotype, *n* (cells)≥11. Statistical differences in PMB accumulation were not detected.

cg, circularized multilamellar structures; cw, cell wall; g, Golgi cisternae; pm, plasma membrane; pmb, paramural body; t, trans-Golgi network. Scale bars: 0.5 µm.

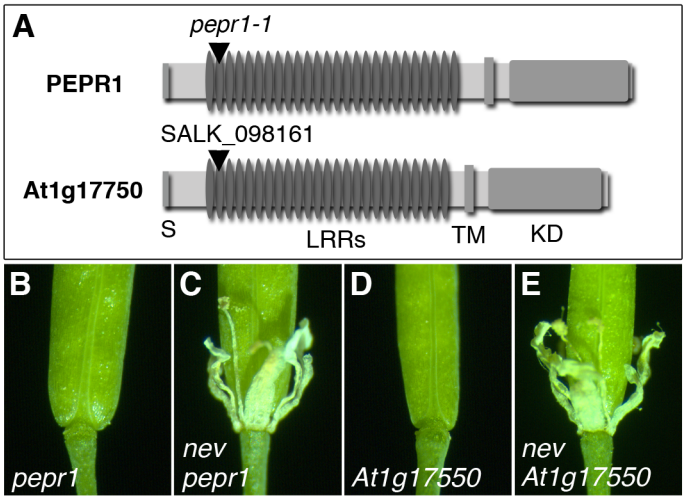

Figure 3.7 Mutations in *PEPR1* or At1g17750 do not rescue shedding in *nev* flowers.
(**A**) Diagrams of the predicted PEPR1 and At1g17750 proteins, with the sites of the T-DNA mutations indicated by arrowheads. The regions corresponding to the signal peptide (S), leucine-rich repeats (LRRs), transmembrane domain (TM), and kinase domain (KD) are indicated.
(**B-E**) Floral organ shedding occurs normally in *pepr1* (B) and *At1g17750* (D) mutant flowers (stage 17), and is blocked in *nev pepr1* (C) and *nev At1g17750* (E) flowers (stage 17).

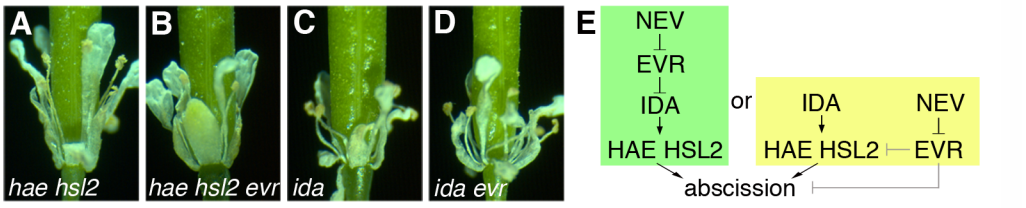

Figure 3.8 Mutations in *EVR* do not restore abscission in *ida* or *hae hsl2* mutant flowers.
(**A-D**) Organ separation is blocked in *ida* (A) and *hae hsl2* (C) mutant flowers (stage 17), and is not rescued in *ida evr* (B) or *hae hsl2 evr* (D) flowers (stage 17).
(**E**) Alternate pathways for the relationships between NEV, EVR, IDA, and HAE/HSL2. In both, EVR is predicted to act as a negative regulator of abscission and to function downstream of NEV. In the simplest model, NEV and EVR act upstream of both IDA and HAE/HSL2. Alternatively, NEV and EVR may function in a converging pathway, in which EVR acts upon HAE/HSL2 or further downstream.

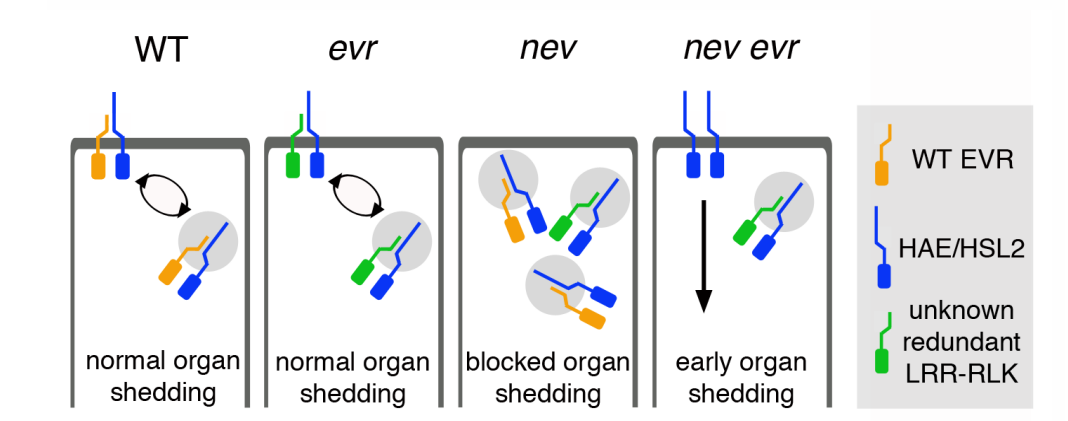

Figure 3.9 A model for EVR function during the transition to floral organ separation.
In wild-type AZ cells before organ shedding, EVR may inhibit cell separation by interacting with ligand-binding LRR-RLKs, such as HAE/HSL2, that trigger cell wall loosening and separation. This interaction could promote the internalization and recycling of inactive receptor complexes through the endosomal system. Continued signaling from a ligand-activated HAE/HSL2 complex would lead to organ shedding in older flowers. The EVR RLK may act redundantly with another LRR-RLK(s), such that loss of *EVR* alone would not alter the timing of abscission. Disrupting NEV activity may alter the trafficking of receptor complexes containing EVR or EVR-like RLKs, thereby blocking a signal required for cell separation. Mutations in *EVR* could bypass the requirement for *NEV* in floral organ shedding, resulting in constitutive signaling of the HAE/HSL2 LRR-RLKs, premature activation of organ separation, enlargement of AZ regions, and deregulated AZ cell expansion.

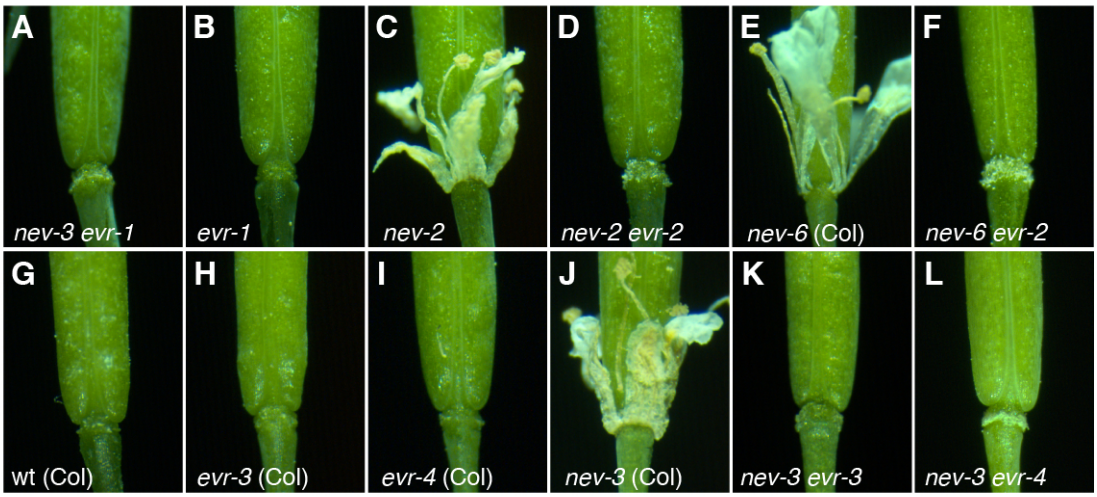

Figure 3.S1 Multiple *evr* alleles rescue shedding in *nev* flowers.
Several mutant alleles of *EVR* rescue organ shedding in *nev-3* flowers, including the *evr-1* allele isolated from the suppressor screen (A), and the *evr-3* and *evr-4* T-DNA insertion alleles (J-L). The *evr-2* mutation also rescues organ separation in *nev-2* (C-D) and *nev-6* (E-F) flowers (Liljegren et al., 2009), demonstrating that interactions between *NEV* and *EVR* are not allele-specific. As described for *nev-3 evr-2* flowers (Fig. 1C), all *nev evr* double mutant flowers show collars of broken AZ cells at their bases (A,D,F,K,L). The morphology of AZ cells appears to be unaffected in each of the *evr* single mutants (B,G-I). Flowers (stage 17) of Ler (A-D), Col (E,G-L) and mixed Ler/Col (F) ecotypes are shown.

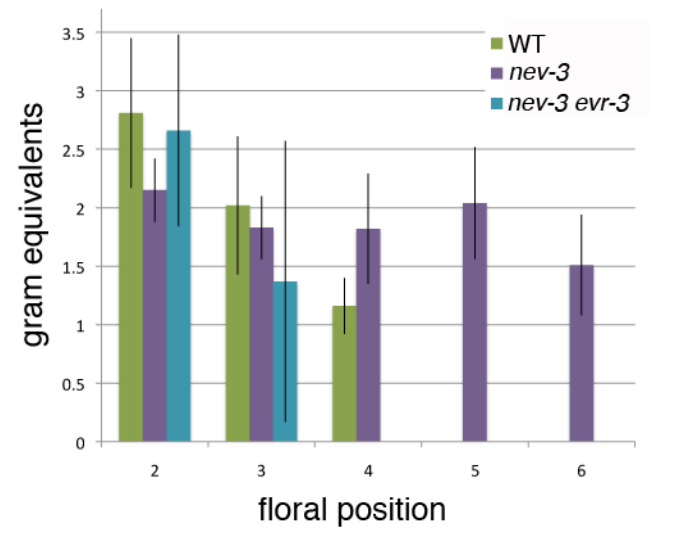

Figure 3.S2 Loss of *EVR* decreases the force required to remove petals from *nev* flowers.
The force required to remove petals from wild-type, *nev-3* and *nev-3 evr-3* flowers (Col ecotype) was measured using a breakstrength meter constructed as described (Lease et al., 2006). Assays were conducted using the second open flower on an inflorescence through the sixth open flower (positions 2-6). If petals were already absent or shed during experimental manipulation, the force was recorded as zero. The breakstrength of wild-type petals ($n \geq 7$) gradually decreased from positions 2 to 4, and all organs were shed by position 5. For *nev* petals ($n \geq 4$), a significant decrease in breakstrength was not observed at the positions tested. The breakstrength of *nev evr* petals ($n \geq 5$) sharply decreased from positions 2 to 3, and all floral organs were shed by position 4.

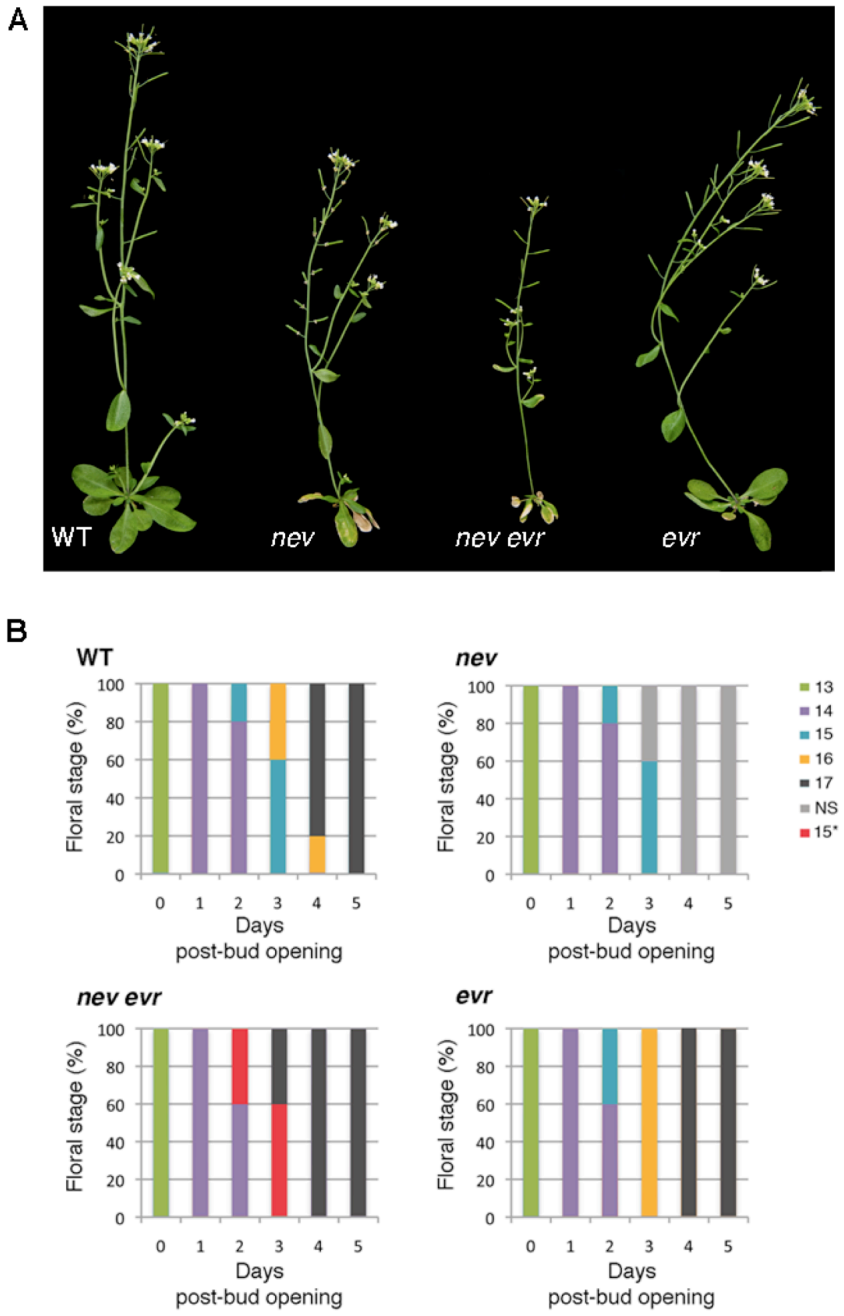

Figure 3.S3 *nev evr* mutants show reduced growth and premature organ abscission.
(**A**) Mutations in *NEV* cause a general decrease in plant size compared to that of wildtype, and *nev evr* plants are typically smaller than *nev* plants of the same age. Other than reduced fruit growth (Fig. 2H), loss of *EVR* alone does not appear to affect plant size. *(continued on next page)*

Figure 3.S3 *(continued)*
(**B**) To track the shedding of floral organs with respect to time, we tagged individual flowers from wild-type and mutant plants at the time of bud opening (stage 13; *n*=5 per genotype). For each day, the percentage of flowers at each stage is shown. While wild-type and *evr* floral organs begin to shed on day 3, we observed *nev evr* floral organ shedding from stage 15* flowers beginning on day 2.

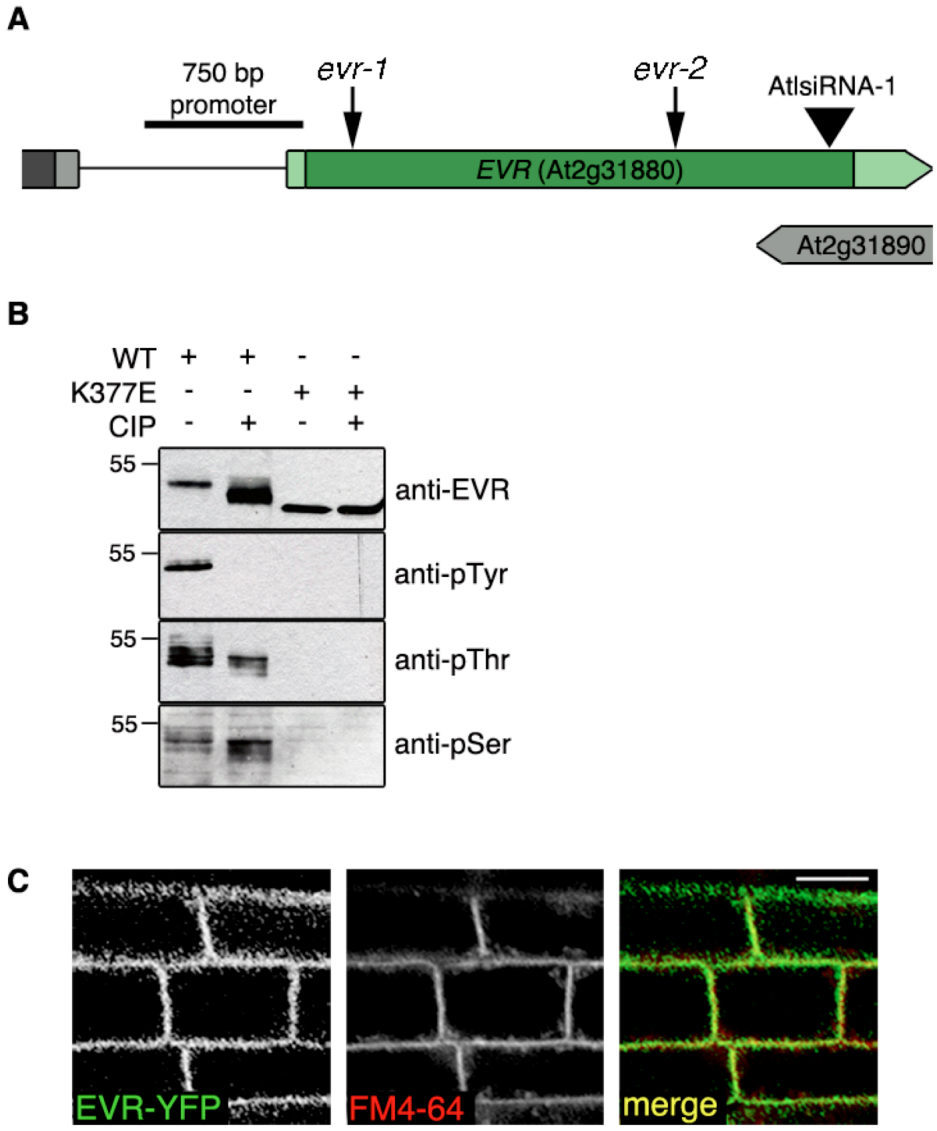

Figure 3.S4 Recombinant EVR can be dephosphorylated at tyrosine residues in vitro.

(**A**) Gene diagram of *EVR* (At2g31880) and its neighboring genes. The protein coding regions are shaded dark gray or green, and the 5' and 3' UTRs are shaded light gray or green. The region selected for the *EVR* promoter begins 750 bp upstream of the *EVR* translational start site. The location of the small RNA cluster (AtsliRNA-1) encoded within the *EVR*/At2g31890 overlapping region is indicated by the arrowhead (Katiyar-Agarwal et al., 2007). *(continued on next page)*

Figure 3.S4 *(continued)*
(**B**) Wild-type (WT) and kinase-dead (K377E) EVR kinase domains (KDs) were treated with calf intestinal alkaline phosphatase (CIP; New England Biolabs; Ipswich, MA) for 2 hours at 37°C to determine the extent of protein migration that is due to phosphorylation. CIP-treated EVR KD^{WT} was observed to migrate at a position intermediate to that of the mock-treated KD^{WT} and KD^{K377E}, suggesting that the protein was not completely dephosphorylated. Duplicate blots were probed with antibodies specific for phosphorylated tyrosine, threonine and serine residues. While phosphate groups appear to be completely removed from EVR tyrosine residues by CIP treatment, dephosphorylation of both threonine and serine residues appears to be incomplete. Since CIP is known to preferentially dephosphorylate tyrosine residues (Swarup et al., 1981), these results are not unexpected. Each of the phospho-antibodies recognizes both the CIP- and mock-treated EVR KD^{WT}.
(**C**) EVR colocalizes with the lipophilic dye FM4-64 at the plasma membrane of epidermal root cells. The primary roots of *pEVR::EVR-YFP* T2 plants were stained with 10 μM FM4-64 (Invitrogen) for 5 minutes on ice, washed in MS liquid media, and imaged within 15 minutes. Imaging parameters were as follows: EVR-YFP (excitation, 488; emission, BP516-548) and FM4-64 (excitation, 543; emission, BP601-655). Scale bar: 10 μm.

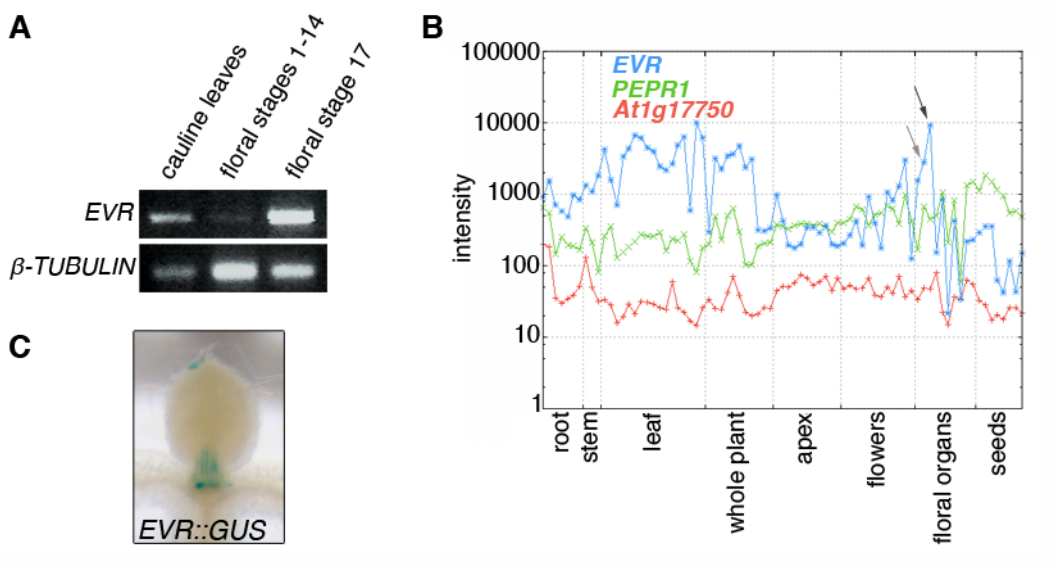

Figure 3.S5 *EVR* shows a broad expression pattern during development.
(**A**) RT-PCR analysis of *EVR* expression. *EVR* is expressed in mature cauline leaves and in flowers after the fruit begins to elongate and all floral organs are shed (stage 17) but only weakly expressed in flowers prior to fruit elongation and shedding (stages 1-14). *β-TUBULIN* RT-PCR was used as a loading control. RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). The reverse transcriptase reaction was performed on DNase-treated RNA using the Omniscript RT Kit (Qiagen, Valencia, CA). *EVR* was amplified with 5'-GAGTGATTCTTGGGATTCTGGTGATTG-3' and 5'-AGATAGAAGCAAACAATACATATTGAAACAC-3' for 30 cycles. *β-TUBULIN* was amplified with 5'-AGAGGTTGACGAGCAGATGA-3' and 5'-ACCAATGAAAGTAGACGCCA-3' for 35 cycles, using an annealing temperature of 55°C.
(**B**) Global expression profiles of *EVR*, *PEPR1*, and *At1g17750* (Schmid et al., 2005). Stage 12 sepals (light gray arrow); stage 15 sepals (dark gray arrow).
(**C**) The regulatory region of *EVR* directs expression of β-glucuronidase (GUS) in the pedicels of the first true leaves of 5-day old seedlings.

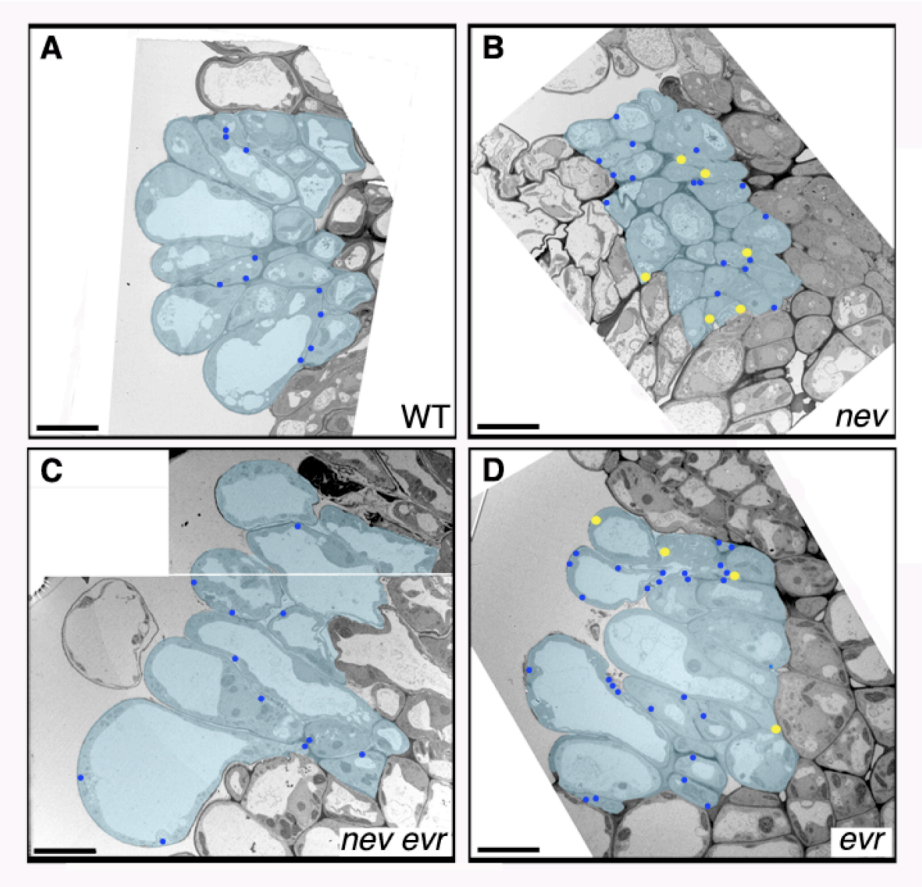

Figure 3.S6 PMB accumulation in *nev*, *nev evr*, and *evr* AZs.
(**A-D**) Transmission electron micrographs of longitudinal sections through wild-type (stage 16), *nev* (stage 16 non-shedding), *nev evr* (stage 15*) and *evr* (stage 16) flowers. Small PMBs (10-30 vesicles, blue dots) accumulate in sepal AZ cells of wild-type, *nev*, *nev evr* and *evr* flowers, whereas large PMBs (31 or more vesicles, yellow dots) were only observed in *nev* and *evr* AZ cells. Scale bars: 10 μm.

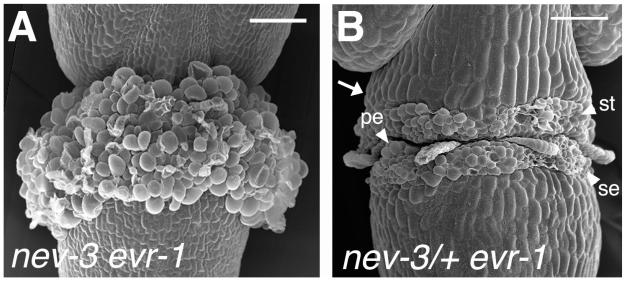

Figure 4.1 Disruption of NEV activity is required for ectopic expansion of AZ cells.
Scanning electron micrographs (SEMs) of flowers after organ separation (floral stage 17). *nev-3 evr-1* flowers, like *nev-3 evr-2* flowers, develop larger AZs than wild-type (A) (Leslie et al., 2010). Recovery of a single wildtype copy of *NEV* rescues AZ morphology, such that sepal, petal and stamen AZ regions can be identified (B), and the AZ regions no longer cover the stem-like gynophore of the fruit (B, arrow). Scale bars, 100 μm.

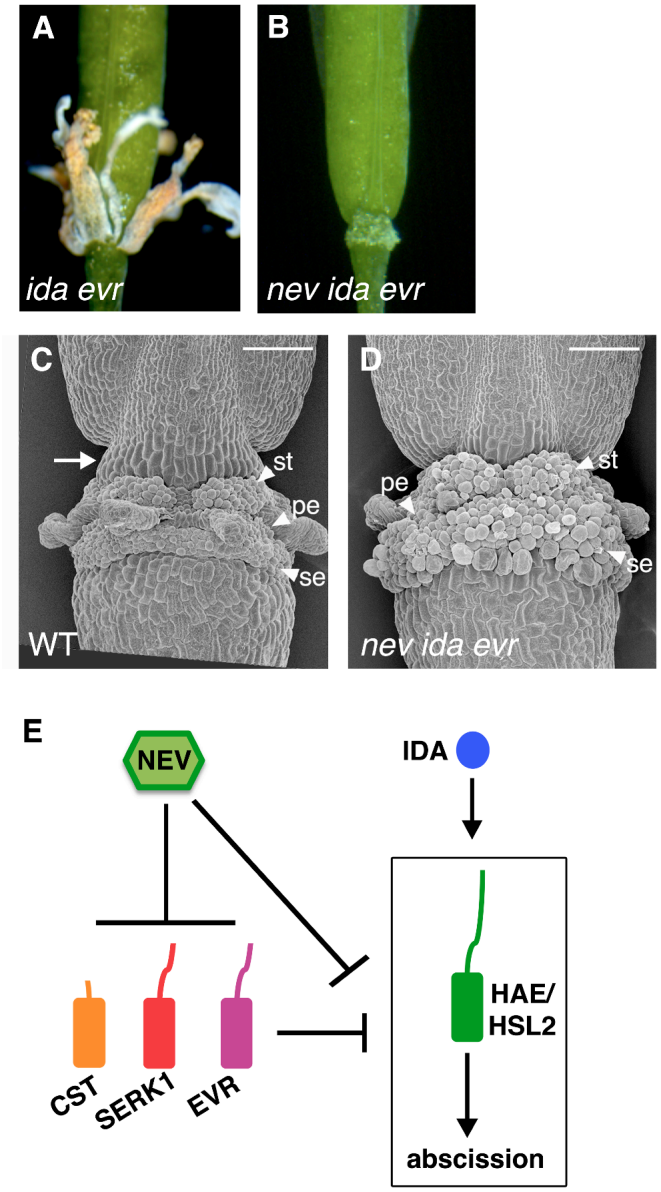

Figure 4.2 AZ cell separation and ectopic expansion can occur independently of *IDA*.
(A) *ida evr* plants retain their floral organs indefinitely (floral stage 17).
(B) Organ shedding is rescued in *nev ida evr* flowers (floral stage 17).
(C-D) SEMs of flowers immediately after organ separation (first stage 17 flower). *nev ida evr* flowers (D), like *nev evr* flowers and those constitutively expressing *IDA* (Leslie et al., 2010) develop larger AZs than wildtype (C). The stem-like gynophore of the fruit that is easily seen in wildtype (C, arrow) is covered by the enlarged *nev ida evr* AZs (D). Scale bars, 100 µm. (*continued on next page*)

Figure 4.2 (*continued*)
(E) Predicted pathway for the relationship between *NEV*, *IDA*, *EVR*, *SERK1*, *CST* and *HAE/HSL2*. As previously shown, *EVR*, *SERK1* and *CST* are predicted to act as negative regulators of organ shedding and function downstream of *NEV*. Mutations in *NEV* block abscission in wildtype flowers (Liljegren et al., 2009) yet rescue abscission of *ida evr* and *ida serk1* floral organs (B) (Lewis et al., 2010), suggesting that NEV ARF-GAP activity plays dual roles in promoting and inhibiting *Arabidopsis* floral organ shedding. Since the *nev evr* and *nev serk1* mutant combinations are epistatic to *ida*, *NEV*, *EVR*, and *SERK1* likely function in a genetic pathway that converges downstream of *IDA*, either acting upon *HAE*/*HSL2* or further downstream (boxed area). Similarities in phenotypes between *nev evr*, *nev serk1* and *nev cst* flowers suggest that *CST* functions at the same point in the pathway for abscission as *EVR* and *SERK1* (Burr et al., in prep). We have also found that mutations in *NEV* promote cell expansion within *ida evr*, *ida serk1*, *evr*, *serk1*, and *cst* flowers (D) (Fig 5.1) (Leslie et al., 2010; Lewis et al., 2010; Burr et al., in prep), which is further evidence that NEV-mediated membrane trafficking can inhibit cell separation.

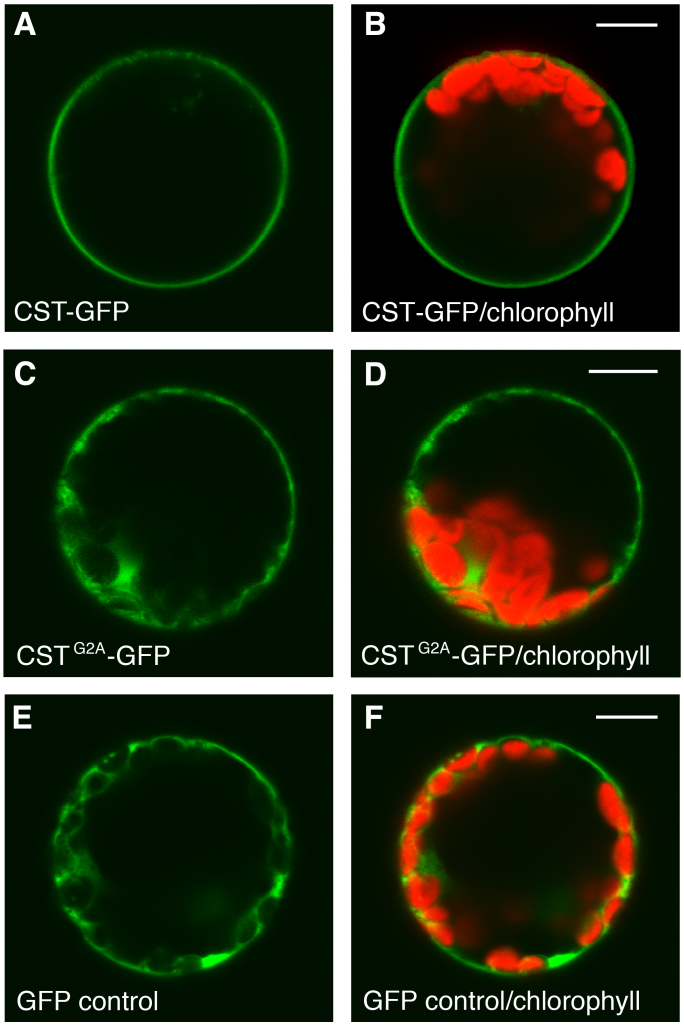

Figure 4.3 CST is localized to the plasma membrane.
Arabidopsis leaf protoplasts were transfected with plasmids containing *35S::CST-GFP* (A-B), *35S::CST^{G2A}-GFP* (C-D), or *35S::GFP* alone (E-F). GFP signal is shown in green (left panels) with an overlay of chlorophyll autofluorescence (right panels). CST-GFP localizes to the plasma membrane (A-B), while CST^{G2A}-GFP is distributed broadly throughout the cells in a pattern similar to that of the GFP tag alone (C-F). Scale bars, 10 μm.

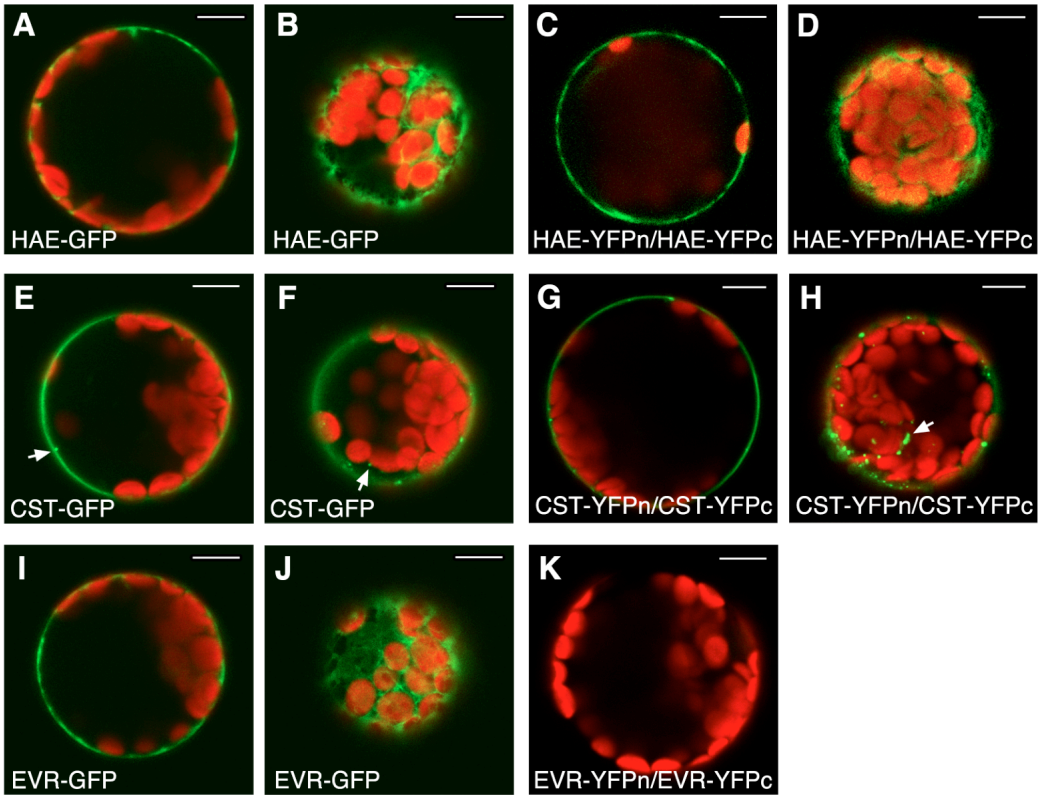

Figure 4.4 HAE and CST form homodimers within *Arabidopsis* leaf protoplasts.
Image overlays of GFP or reconstituted YFP (green) and chlorophyll autofluorescence (red). Two optical sections are shown for each protoplast, except for *35S::EVR-YFPn*/*35S::EVR-YFPc* co-transfection, for which only one optical section is shown (K).
(A-B) HAE-GFP localizes to the plasma membrane and internalized structures that resemble the endoplasmic reticulum network.
(C-D) Co-transfection of *35S::HAE-YFPn* and *35S::HAE-YFPc* results in reconstituted YFP signal from HAE-HAE homodimers in a similar pattern to that of HAE-GFP alone (A-B).
(E-F) CST-GFP localizes to the plasma membrane and to punctate, internalized structures (arrows).
(G-H) Co-transfection of *35S::CST-YFPn* and *35S::CST-YFPc* results in reconstituted YFP signal similar to that of CST-GFP alone (E-F). CST-CST homodimers at visible at the plasma membrane (G) and at internalized, punctate structures (H, arrow).
(I-J) Like HAE-GFP (A-B), EVR-GFP localizes to the plasma membrane and internalized structures that resemble ER network (I-J).
(K) Reconstituted YFP is not detected in protoplasts co-transfected with *35S::EVR-YFPn* and *35S::EVR-YFPc*, suggesting that EVR does not homodimerize.
Scale bars, 10 μm.

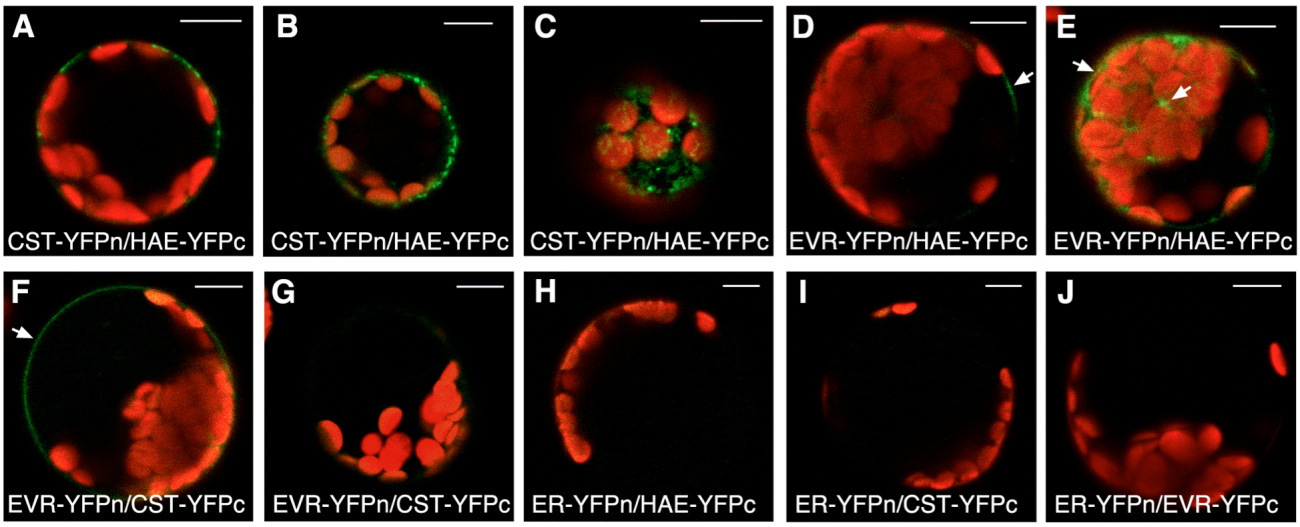

Figure 4.5 HAE, CST and EVR heterodimers are detected within distinct regions of *Arabidopsis* leaf protoplasts.
Image overlays of reconstituted YFP (green) and chlorophyll autofluorescence (red).
(A-C) Co-transfection of *35S::CST-YFPn* and *35S::HAE-YFPc* results in reconstituted YFP within subdomains of the plasma membrane. Three optical sections of a single protoplast are shown. While CST-GFP localizes to punctate, internalized sturctures (Fig. 5.4E,F, arrows), CST-HAE heterodimers are not visible within internalized structures at similar optical sections (A-B). Rather, punctate patterns of CST-HAE appear to be associated with the plasma membrane underlying the chloroplasts (C).
(D-E) Co-transfection of *35S::EVR-YFPn* and *35S::HAE-YFPc* results in reconstituted YFP at occasional subdomains of the plasma membrane (D, arrow), and at internal locations resembling the ER network (E, arrows).
(F-G) Co-transfection of *35S::EVR-YFPn* and *35S::CST-YFPc* results in uniform reconstitution of YFP at the plasma membrane (F). EVR-CST heterodimers appear to be sequestered at the plasma membrane (F) and are not internalized like EVR-HAE heterodimers (E, G).
(**H-J)** The ERECTA (ER) LRR-RLK does not heterodimerize with HAE, CST or EVR. Reconstitued YFP is not detected for co-transfection of *35S::ER-YFPn* with either *35S::HAE-YFPc* (H), *35S::CST-YFPc* (I), or *35S::EVR-YFPc* (J).
Scale bars, 10 µm.

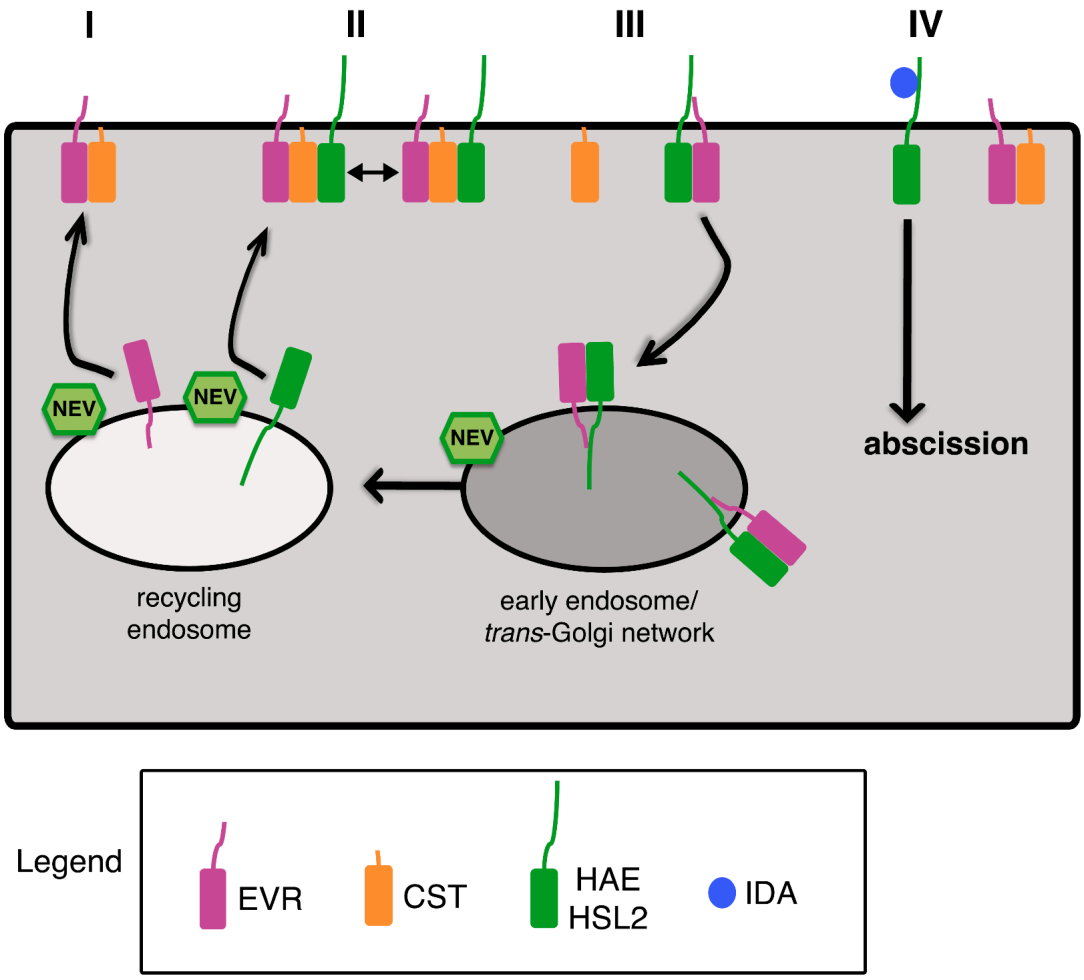

Figure 4.6 A model for signaling and membrane trafficking during the transition to floral organ shedding.
Step-wise oligomerization and internalization of the EVR, CST and HAE/HSL2 RLKs may function to dampen abscission signaling prior to IDA ligand binding. Our protein interaction studies in protoplasts support a model in which the membrane-associated CST sequesters EVR at the plasma membrane (I). CST may then interact with HAE/HSL2 at the plasma membrane, placing EVR and HAE/HSL2 in close proximity (II). Interactions between EVR and HAE/HSL2 (II) may trigger internalization of a receptor complex (III). The NEV ARF-GAP, which localizes to the early endosome/*trans*-Golgi network and recycling endosome (Liljegren et al., 2009), may be required for the trafficking of RLKs through the endomembrane system and/or recycling of RLKs back to the plasma membrane. At the proper timing for abscission, HAE/HSL2 signaling may be activated by IDA ligand binding (IV).