MEMBRANE TRAFFICKING AND RECEPTOR KINASE SIGNALING DURING PLANT DEVELOPMENT

Christian Anne Burr

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Genetics and Molecular Biology.

Chapel Hill
2011

Approved by:

Sarah Liljegren

Mara Duncan

Bob Goldstein

Shawn Ahmed

Jason Reed

ABSTRACT

CHRISTIAN ANNE BURR: Membrane Trafficking and Receptor Kinase Signaling during Plant Development
(Under the direction of Sarah Liljegren)

Abscission is a programmed cell separation process that allows plants to shed their organs. In *Arabidopsis* flowers, each outer organ has a set of differentiated abscission zone cells at its base, which allow the organs to detach after pollination. Previous studies have shown that the NEVERSHED (NEV) ADP-ribosylation factor-GTPase-activating protein regulates membrane trafficking and is required for organ abscission. A *nev* suppressor screen has revealed that mutations in *CAST AWAY* (*CST*), a receptor-like cytoplasmic kinase, are capable of rescuing abscission. Localization of CST to the plasma membrane is supported by *N*-myristoylation and the kinase activity of CST is required for its activity. Mutations in *CST* were found to rescue the trafficking defects of *nev* mutant flowers: in *nev cst* flowers the organization of the Golgi apparatus and location of the *trans*-Golgi network are restored, and the accumulation of extracellular vesicles between the plasma membrane and cell wall is significantly reduced. Organ abscission is known to be triggered via activation of the HAESA (HAE) and HAESA-LIKE2 (HSL2) receptor-like kinases. In addition to CST, two other receptor-like kinases, EVERSHED (EVR) and SOMATIC EMBRYOGENESIS DEFICIENT IN ABSCISSION, are known to inhibit organ abscission. Interaction studies of CST,

HAE, and EVR using the bimolecular fluorescence complementation assay suggest that CST may directly and indirectly modulate cell separation by regulating the localization and/or activity of HAE/HSL2 and EVR.

Functional analysis of *NEV* and three other ARF-GAP genes has revealed that *NEV* and *ARF-GAP DOMAIN6* (*AGD6*) redundantly control plant growth and reproduction. *nev agd6* double mutants are very small compared to wild-type plants; those that survive long enough to flower do not produce seeds. Analysis of leaf cell size suggests that reduced cell expansion is at least partially responsible for the growth defects of *nev agd6* plants. We have also found that the root meristems of *nev agd6* mutants are smaller than those of wild-type, and that growth of the primary roots is significantly reduced. Our studies suggest that NEV- and AGD6-mediated membrane trafficking regulates the localization of cell signaling molecules involved in establishing or maintaining meristem size.

ACKNOWLEDGEMENTS

Without the support of my co-workers, friends and family this thesis would not have been possible. First I need to thank the various members of the Liljegren lab over the years. There were two graduate students that overlapped with my time in the Liljegren Lab, Michelle Leslie and Mike Lewis. I need to thank Mike for all his help as I learned how to handle the plants. Michelle and I became close friends through the years that we worked together, and she has been invaluable in her advice. She has taught me so much and helped me grow as a scientist. There have been a few undergraduate students that I have had the opportunity to work with, but the most important of them is Iris Chen. Iris has been incredibly helpful over the years, she has been given whole projects to work on and proven herself as a scientist through these. Without her help and support in the lab, I would not have been able to accomplish all that I have. I especially need to thank Sarah Liljegren; when I started working in her lab I was a little lost, and over the years she has given me direction and motivation. She has always been kind and allowed me the space to make my own mistakes and learn from them. Without her, I would lack the confidence and skill as I scientist that I have today. There are many other graduate students and post-docs in Coker Hall that I have gone to for help over the years, including Blaire Steinwand, Tracy Hargiss, Mindy Roberts, Cris Argueso, Shouling Xu and Smadar Harpaz-Saad. Additionally there are many faculty members who

have given me very important advice and support, including Joe Kieber, Jason Reed, Mara Duncan, Bob Goldstein, Shawn Ahmed, Mark Peifer and Bob Duronio. The members of microscopy suite here at UNC: Tony Purdue, Vicky Madden and Steve Ray. Their expertise willingness to teach has made my life so much easier. Lastly I need to thank my family for the support they've given me my entire life. My parents, Suzanne and Julian Burr, have always been so proud of me and excited for the career I've chosen and have given nothing less than their fullest support. Nathan and Lauren, my siblings, they keep me laughing. No matter happens, they are always there for me and I'll never need to be anything more than their sister.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

LIST OF ABBREVIATIONS

AGD6	ARF-GAP DOMAIN6
AGE2	ARF-GAP effector 2
ARF	ADP-ribosylation factor
ARF-GAP	ADP-ribosylation factor GTPase-activating protein
ARF-GEF	ADP-ribosylation factor GTPase-exchange factor
AZ	abscission zone
BAK1	BRI1 ASSOCIATED KINASE 1
BFA	Brefeldin A
CST	CASTAWAY
EVR	EVERSHED
ER	ERECTA
GCS1	GROWTH-COLD SENSITIVE1
GFP	green fluorescent protein
GN	GNOM
GNL1	GNOMLIKE 1
GUS	β-glucuronidase
HAE	HAESA
HSL2	HAESA-LIKE 2
IDA	INFLORESCENCE DEFICIENT IN ABSCISSION
KD	kinase domain
LRR	leucine-rich repeat
LRR-RLK	leucine-rich repeat receptor-like kinase

MLPK	M-LOCUS PROTEIN KINASE
NEV	NEVERSHED
PIN	PIN-FORMED
PID	PINOID
PMB	paramural body
RLK	receptor-like kinase
RLCK	receptor-like cytoplasmic kinase
SEM	scanning electron micrograph
SERK1	SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE 1
SSP	SHORT SUSPENSOR
TEM	transmission electron micrograph
TGN	*trans*-Golgi network
WT	wildtype
YFP	yellow fluorescent protein

CHAPTER 1

INTRODUCTION

For my dissertation, I have carried out research on two aspects of plant development. The first project began with mapping *cast away* (*cst*), a suppressor of the abscission mutant *nevershed (nev).* My studies characterizing the role of CST, a membrane-associated receptor-like kinase, in inhibiting abscission have led to a model of how receptor kinase signaling and membrane trafficking may intersect in regulating this process. The second project was to examine the broader role of the NEV ARF GAP in regulating plant development, and led to my discovery that NEV acts redundantly with ARF-GAP DOMAIN6 (AGD6). In this chapter I will review the roles of ARF-related membrane trafficking regulators in plants, cell signaling pathways controlled by receptor-like kinases and the developmental process of abscission. I will also discuss how membrane trafficking contributes to cell signaling regulation using examples of the GNOM/PIN interations and how membrane trafficking regulate signaling through Delta and Notch.

SECTION I. MEMBRANE TRAFFICKING IN PLANTS

Membrane and secreted proteins are produced the endoplasmic reticulum (ER) then trafficked through the Golgi to the organelles or the plasma membrane. The proteins enter the Golgi on the cis side and exit on the trans side into the

trans-Golgi network (TGN). From the TGN, vesicles can enter the endosomal system in the recycling endosome (RE). They could also stay in the biosynthetic pathway and move to either the prevacuolar compartment (PVC) or multivesicular bodies (MVBs). From the PVC the proteins can be trafficked to the lytic vacuole or the protein storage vacuole. Protein traffic can also bypass the Golgi completely and move straight from the ER to the protein storage vacuole (Otegui and Spitzer, 2008).

Endocytosis brings proteins into the cell from the surface. Materials are endocytosed into the plant cell through vesicles coated with proteins, in many cases clathrin. The earliest of these vesicles, formed by the invagination and budding off of part of the plasma membrane first join with the early endosome, which is the same compartment as the *trans*-Golgi network in plants (Richter et al., 2009). From this location, plasma membrane proteins can be moved either back to the plasma membrane via the recycling endosomes or move to the multivesicular bodies for degradation in the vacuole. The ARF-GEF GNOM plays a role in recycling the proteins back to the cell membrane (Otegui and Spitzer, 2008). From the late endosome, proteins may enter the biosynthetic pathway through the TGN, or move to the lytic or protein storage vacuole.

In other eukaryotes such as yeast and mammals, the recognition of cargo and the budding of vesicles are controlled by small GTPase ARF proteins. The activity of the ARF proteins managed by ARF-GEFs and ARF-GAPs, which cycle these proteins from active to inactive through GTP hydrolysis. ARF-GAPs are responsible for switching an ARF protein from active to inactive through GTP hydrolysis, while

ARF-GEFs activate ARF proteins through addition of a GTP (Randazzo and Hirsch, 2004).

There are three types of ARF-GEF proteins in eukaryotes: small, medium and large. All ARF-GEFs contain a Sec7 domain, which is responsible for binding ARF proteins and exchanging GTP for GDP. All three types are present in humans, while yeast only have medium and large ARF-GEFs (Anders and Jurgens, 2008). In *Arabidopsis* there are eight ARF-GEFs, all large. Large ARF-GEFs are split into two groups, the GBF1/Gea/GNOM (GGG) group, represented by GNOM (GN) and GNOM-like 1 and 2 in *Arabidopsis,* and the BIG group, represented by BIG1-5 in *Arabidopsis* (Anders and Jurgens, 2008).

Brefeldin A (BFA) is a fungal toxin that inhibits endosomal recycling by blocking the active sites of susceptible ARF-GEFs, and has become a useful tool for understanding the function of many ARF-GEFs in vesicle trafficking (Peyroche et al., 1996). In *Arabidopsis,* cells treated with BFA most notably causes the formation of BFA bodies, which are aggregates of the *trans*-Golgi network and endosomal compartments. From these bodies normal trafficking to the plasma membrane is seen, but trafficking to the lysosomes is inhibited (Lippincott-Schwartz et al., 1991).

GNOM/EMB30 (GN) is an ARF-GEF that localizes to endosomal compartments (Geldner et al., 2003). Seedlings mutant for *GN* show a variety of different phenotypes as a consequence of being unable to establish apical/basal patterning (Mayer et al., 1991; Shevell et al., 1994). Establishment of an apical/basal axis is based on the polar localization of PIN1, which is responsible for the flow of auxin in the plant (reviewed in Kracek et al., 2009). In the early embryo

the PIN proteins become polarly localized to specific membranes, this creates a network of PIN proteins that create an auxin gradient responsible for regulating much of plant development (Blilou et al., 2005). GN is responsible for the correct localization of the PIN proteins (Steinmann et al., 1999). GN is sensitive to BFA, which has served as an invaluable tool for understanding GN trafficking (Steinmann et al., 1999).

A set of partial loss-of-function alleles of *gn* have allowed a more detailed look into how GN regulates development post-embryonically (Geldner et al., 2004). Plants carrying the strongest of these weak alleles show disruption of the vasculature, no lateral roots, smaller primary roots with smaller meristems that eventually collapse and a disturbed auxin gradient. The collapse of the meristems is most likely a product of a lack of auxin, as it can be rescued by ectopic auxin treatment (Geldner et al., 2004). These partial loss-of-function alleles show that *GN* is necessary, not just in embryogenesis, but throughout plant development.

Another ARF-GEF GNOM-like 1 (GNL1) localizes the Golgi, where it regulates COPI-coated vesicle formation and plays an important role in trafficking between the endoplasmic reticulum (ER) and Golgi and in endocytosis (Richter et al., 2007; Teh and Moore, 2007). GNL1 plays a role in trafficking both PIN1 and PIN2 proteins (Richter et al., 2007; Teh and Moore, 2007). GNL1 also plays an important role in protein secretion. GNL1 is BFA resistant and required for the creation of COPI-coated vesicles and maintenance of the Golgi stacks (Richter et al., 2007; Teh and Moore, 2007). *gnl1* plants are significantly shorter than wild type. Although GN normally has no known role in ER-Golgi trafficking, it can still

compensate for *gn1* in ER-Golgi trafficking, indicating that GN could functionally replace GNL1. This redundancy was shown further by the fact that *gn gnl1* plants showed gametophytic lethality (Richter et al., 2007). This redundancy was further proven through a promoter swap test where *GN* behind the *GNL1* promoter was able to rescue both *gn* and *gnl1*, but *GNL1* behind the *GN* promoter was only able to rescue *GNL1.* This indicates that GN has the ability to control traffic in both the endosomal routes and the ER-Golgi route, whereas GN is specific to only the ER-Golgi route (Richter et al., 2007).

In contrast to GN and GNL1, GNL2 does not have broad developmental responsibilities but is essential for pollen germination and pollen tube growth (Jia et al., 2009). It is possible that GNL1 may also play a role in pollen germination and pollen tube growth, as *gnl1* heterozygous plants show slightly reduced transmission of the mutated *gnl1* allele (Richter et al., 2007). Vesicle trafficking is critical for pollen tube growth, as it establishes the polarity of growth and controls the export of building materials to the extending tip of the pollen tube (Zhang and McCormick, 2010).

In *Arabidopsis* there are three classes of ARF-GAPs containing a total of fifteen proteins (Vernoud et al., 2003). Class I are ARF-GAP proteins with ankyrin domains, PH domains, a BAR domain and an ARF-GAP domain, class II contain only an ARF-GAP domain and class III contain an ARF-GAP domain and a C2 domain (Vernoud et al., 2003). Class I ARF-GAPs are the most like those found in animal ARF-GAPs where they are mostly multi-domain proteins. There are four known ARF-GAPs that have single mutant phenotypes in plants: NEVERSHED

(NEV), SCARFACE (SFC)/VAN3 , ROOT AND POLLEN ARF-GAP (RPA) and AGD1 (Koizumi et al., 2005; Sieburth et al., 2006; Song et al., 2006; Yoo et al., 2008; Liljegren et al., 2009).

NEV is a class II ARF-GAP, which is a class of plant ARF-GAPS that contains only the ARF-GAP domain. Plants carrying a mutation in *NEV* do not shed their floral organs, and are slightly smaller in stature than wild type plants (Liljegren et al., 2009; unpublished data). *nev* plants show two trafficking defects, the first trafficking defect involves the Golgi and the *trans*-Golgi network (TGN) and has been seen in the abscission zones, fruit and stems of the plant. The Golgi takes on a distinctive circular shape and the TGN appear to be fused to the Golgi (Liljegren et al., 2009). The second trafficking defect is the build up of vesicles in the apoplast, or in the layer of space between the plasma membrane and the cell wall, which was seen in the sepal abscission zones. NEV was found to co-localize with markers of the TGN and the recycling endosome, which was a novel location for ARF-GAPs in plants (Liljegren et al., 2009). NEV colocalizes with ARF1 at the TGN and interacts with ARF1 *in vitro*, although findings indicate that NEV interacts with other ARF proteins and may be promiscuous (Stefano et al., 2010).

This distinctive shape and fusion of the Golgi and TGN seen in *nev* mutant plants has also been seen when the proton pump that regulates the acidity of the TGN is disrupted. V-ATPases are proton pumps, in *Arabidopsis* they are found throughout the endosomal system (Dettmer et al., 2005). V-ATPase-a1 encodes a subunit of the V-ATPase proton pump. Plants carrying mutations of *v-atpase-a1* and plants treated with concanamycin A, which inhibits V-ATPase (Dettmer et al., 2005;

Viotti et al., 2010) show the same Golgi/TGN phenotype seen in *nev* mutant plants. In *Arabidopsis* these proteins are necessary for development past the gametophytic stage, however weak knockdowns of V-ATPases show a defect in cell elongation leading to smaller plants (Schumacher et al., 1999; Dettmer et al., 2005; Dettmer et al., 2006).

Another class II ARF-GAP that plays a role in plant development is ROOT AND POLLEN ARF-GAP (RPA), *RPA* is expressed only in the roots and pollen of the plants and shows colocalization with Golgi markers in root hairs. The mutation of *RPA* causes a retardation of pollen tube growth and root hairs are shorter and thicker than wild type (Song et al., 2006). The change in pollen tube growth rate means it may be possible that RPA plays an important role in male gametophytic competition. Song et al. (2006) found through *in vitro* tests that ARF1 and, the ARF-like protein, U5 were putative RPA substrates.

Several class I ARF-GAPs that control the patterning of leaf vasculature. A plant carrying a mutation in *SCARFACE (SFC)/VAN3* shows the formation of vascular islands instead of the connected vascular structure seen in wild type plants. This phenotype is exacerbated in higher order mutant combinations with *agd1, agd2* and *agd4* (Sieburth et al., 2006). SFC localizes to the *trans*-Golgi network (Koizumi et al., 2005). Auxin flow, regulated by a complex pattern of PIN protein localization, plays a large role in vascular patterning (Sachs, 1981; Sachs, 1989). In the *SFC* single mutant roots PIN1 localized in a much smaller area of cells than in wild type, and occasionally was seen to localize to the wrong side of the cell. These results

suggest that SFC is involved in the proper localization of PIN1. (Koizumi et al., 2005; Sieburth et al., 2006).

In addition to playing a role in vascular patterning *AGD1* is also important in root hair formation (Yoo et al., 2008). In the *agd1* single mutant instead of growing straight out from the root, root hairs grow in a wavy pattern. This phenotype appears to be the result of an inability to maintain growth direction, possibly caused by the disorganization of the actin and microtubule cytoskeleton (Yoo et al., 2008). Treatment of roots with BFA, a chemical capable of inhibiting ARF-GEF activity, causes the root hairs to grow normally. It is possible that the loss of AGD1 causes a build up of an active state ARF protein, but this is relieved when treated with BFA, as the ARF-GEF is no longer able to activate the ARF, relieving the build up (Yoo et al., 2008). Currently the AGD1 localization pattern is unknown, but colocalization studies have shown that it is not located at the Golgi cisternae.

ADP-ribosylation factors (ARF) are small GTPase proteins that play a large role in regulating membrane trafficking; their activity is controlled by ARF-GAPs and ARF-GEFs (D'Souza-Schorey and Chavrier, 2006). In *Arabidopsis* there are twelve predicted ARF proteins, and many of these show high levels of redundancy (Hwang and Robinson, 2009). ARFs 1-6 show a great deal of similarity to the ARF proteins in mamallian class 1. ARFs 7-9 show enough difference from mammalian ARFs that they are grouped into two plant classes. ARF7 and 8 are members of plant class A, and ARF9 makes up plant class B (Jurgens and Geldner, 2002).

The high levels of redundancy and overlapping expression patterns of class 1 ARF proteins in make it difficult to genetically determine the functions of ARF1-6.

ARF1 has been shown to play an important role trafficking from the ER to the Golgi and in recruiting coat complexes at the Golgi (Lee et al., 2002; Takeuchi et al., 2002; Matheson et al., 2007). In order to further study the function of ARF1 one group used inducible promoters and mutant versions of GDP and GTP locked ARF1 (Xu and Scheres, 2005). By expressing the mutant ARF1 proteins in specific tissues it was possible to begin to see the processes ARF1 plays a role in regulating. These mutant versions of ARF1 showed changes in Golgi trafficking, endocytic traffic, and disturbed the localization of PIN2 (Xu and Scheres, 2005). ARF1 was shown to play a large role in maintaining cell polarity, and when ARF1 mutants were expressed in adult plant tissue that tissue showed an inability to maintain correct cell polarity (Xu and Scheres, 2005).

I have been working to better understand the role that ARF-GAPs play in plant development. By following the example of Age2/Gcs1 redundancy seen in yeast, we have found one Age2-like ARF-GAP, NEV, and one Gcs1-like ARF-GAP, AGD6, are redundantly required for plant development. Parts of this thesis will explore the work that I have done to characterize the developmental defects in the *nev agd6* mutants, and I will also discuss the plans for further understanding the overlapping developmental role these two proteins play.

SECTION II. RECEPTOR-LIKE KINASE SIGNALING IN PLANTS

Receptor-like kinases (RLKs) are a large gene family in *Arabidopsis* with over 600 members that are broken into two groups. One group of these proteins contains a receptor-like domain, a transmembrane domain and a kinase domain. This

configuration could mean that these proteins are responsible for receiving signals from out side the cell (Shiu and Bleeker, 2003). The second, smaller group of these proteins does not contain a receptor-like domain, these are called the receptor-like cytoplasmic kinases (RLCKs). There are about 125 RLCKs known in the *Arabidopsis* genome, but few have been studied in depth (Shiu and Bleeker, 2003). RLKs and RLCKs play a broad role in the life of a plant, for example they are instrumental in many aspects of plant development, from the production of viable seed to broad growth patterns, and they play a large role in plant pathogen defense.

SHORT SUSPENSOR (SSP) is a member of the RLCK II family (Shiu and Bleecker, 2001) that is involved in zygote development (Bayer et al., 2009). In *Arabidopsis* the zygote developing into the proembryo is caused by an asymmetric cell division that yields a proembryo made up of an apical and a basal cell. The apical cell will go onto become the embryo, while the basal cell becomes the suspensor (Grossniklaus, 2009). In plants mutant for *SSP* the zygote has suspensors with various mutant phenotypes, anything from the complete absence of a suspensor to shorter suspensor with extraneous cell layers (Bayer et al., 2009). The mutant allele of *SSP* has to be inherited from the father to have this effect. It is the first paternally acting locus found in *Arabidopsis* and it appears to be instrumental in the resource allocation process where it works downstream of the mitogen-activated protein kinase kinase (MAPKK) YODA (Bayer et al., 2009). The amino acid sequence of SSP predicts it will be myristoylated, and as predicted the protein is plasma membrane localized and localization is disrupted when the myristoylation site is mutated (Bayer et al., 2009).

Although *Arabidopsis* does not exhibit self-incompatibility, it appears to be a trait that was relatively recently lost, as *Arabidopsis* has most of the genes required for self-incompatibility in other plant species. A self-incompatible *Arabidopsis* was recently created as a model by transferring the genes for just two of the proteins required for self incompatibility into wild type *Arabidopsis* (Rea et al., 2010). *Brassica* is an example of a species that is self-incompatible and mutation of the *MLPK* gene disrupts the self-compatibility. MLPK is the *Brassica* homologue of the *Arabidopsis* class VII RLCK, APK1b (Murase et al., 2004). The predicted *N*-myristoylation of MLPK may cause the plasma membrane localization of the protein, as it is disrupted when the myristoylation site is mutated (Murase et al., 2004). S RECEPTOR KINASE (SRK), a transmembrane receptor like kinase, is activating by the presence of self-incompatible pollen and recruits MLPK, the phosphorylation of these proteins leads to downstream signaling cascades, which prevent fertilization (Murase et al., 2004). Through alternative splicing *MLPK* has two distinct isoforms, one membrane bound through *N*-myristoylation and one that localizes to the membrane through an N-terminal hydrophobic domain. Both of these isoforms have been shown to interact directly with SRK (Kakita et al., 2007).

Overexpression of *CONSTITUTIVE DIFFERENTIAL GROWTH 1* (*CDG1)* dominantly causes a variety of phenotypes, most noticeably dwarfism, a severe epinasty of the leaves, and the stems and fruit grow in spirals (Muto et al., 2004). *CDG1* encodes a class VII RLCK that is expressed throughout the entire plant at a low level. Overexpression of *CDG1* may cause the constitutive activation of the brassinosteroid (BR) signaling pathway. However, *CDG1* is not essential in this

pathway as loss of function mutations of *CDG1* showed no phenotype, perhaps indicating some redundancy between class VII RLCKs (Muto et al., 2004).

The BR signaling pathway plays a large role in plant development. The BSK proteins are members of RLCK subfamily XII that play a role in BR signaling downstream of the BR receptor (Tang et al., 2008). The RLCKs in subfamily XII contain a *N*-myristoylation domain, a kinase domain and a C-terminal tetratricopeptide repeat (TPR) domain, which have been shown to mediate protein-protein interactions (Shiu et al., 2004; Smith, 2004). BSK1 and BSK3 interact with and are phosphorylated by BRI1 (Tang et al., 2008). BSK3 is the only BSK with a mutant phenotype, *bsk3* plants are weakly insensitive to BR. Overexpression of BSK3 can suppress mutations in *BRI1*, but not *BIN2*, this suggests that BSK3 is downstream of BRI1 and upstream of BIN2 (Tang et al., 2008). BRs signal through the LRR-RLK BRI1 by interacting with the protein's extracellular domain (Vert et al., 2005)

Initial work on the class VII RLCK PBS1 showed that it interacted with the nucleotide-binding site leucine-rich repeat (NBS-LRR) protein RPS5 and was activated through cleavage by AvrPphB, a bacterial effector. Activation of PBS1 triggers phosphorylation of RPS5 and sets off a downstream signaling cascade (Shao et al., 2003). Plants that are mutant for *PBS1* show increased susceptibility to the plant pathogen *P. syringae* (Swiderski et al., 2001). Nine additional proteins from RLCK subfamily VII were found to undergo similar cleavage events in the presense of AvrPphB (Zhang et al., 2010). Of these proteins three were upregulated in the presence of flagellin, BOTRYTIS INDUCED KINASE 1 (BIK1) and the PBS1-

like protein, PBL1 and PBL2 (Zhang et al., 2010). These three proteins have been found to initiate PAMP triggered immunity (PTI), while cleavage of PBS1 activates effector-triggered immunity (ETI).

BIK1 is a membrane associated RLCK that interacts with the LRR-RLKs FLS2 and BAK1 (Veronese et al., 2006; Lu et al., 2010). *bik1* plants are smaller and more susceptible to *Botrytis* infection, they have longer root hairs and a shorter primary roots (Veronese et al., 2006). In the model for BIK1 signalling in the presence of flagellin FLS2 interacts with and activates BAK1. The activated BAK1 phosphorylates BIK1, which in turn phosphorylates both FLS2 and BAK1. The now active BIK1 may be released from the protein complex and may activate downstream signaling (Lu et al., 2010).

Two RLCK subfamily XIb proteins, RBK1 and RBK2, interact with a group of Rho small-GTPases, called Rops, which play a role in plant growth and response to plant pathogens (Molendijk et al., 2008). RBK1 localizes to the cytoplasm in tobacco leaf protoplasts and to endomembranes in *Arabidopsis* leaf protoplasts. Increased expression of *RBK1* was seen about six hours after infection with *P. infestans*. Rop interacts with the kinase domain of RBK1.

This dissertation describes work on a class VII RLCK that I've named CAST AWAY (CST). Mutations in *CST* were found through a screen of *nev* that also identified the RLKs *EVR* and *SERK1* (Leslie et al., 2010; Lewis et al., 2010). In this thesis I will explain the work done to understand *cst* and how it suppresses the *nev* mutation. I will also talk about the work we have been doing to lead to an

understanding of how the RLKs implicated in abscission work together to lead to floral organ shedding.

SECTION III. REGULATION OF SIGNALING BY MEMBRANE TRAFFICKING

Membrane trafficking is essential for signaling and various modes of regulation through trafficking are possible. I will discuss two well studied examples of how membrane trafficking can directly influence the localization and activity of cell signaling factors. In plants, GNOM (GN) has been found to effect the polar localization of the auxin-efflux carriers the PIN-FORMED (PINs) proteins, and is therefore critical for establishing the flow of auxin during root development (reviewed in Richter et al., 2010). In animals, the Notch/Delta pathway is conserved from *C. elegans* to humans. Notch/Delta signaling regulates cell fate determination such as, neural differentiation and stem cell maintenance in vertebrates and flies (reviewed in Bray, 2006). Defects in this pathway can lead to developmental defects and cancer (reviewed in Kopan and Ilagen, 2009).

One of the most well studied trafficking pathway in *Arabidopsis* is how the ARF-GEF GNOM regulates polar PIN localization. PIN proteins are regulators of polar auxin efflux and are responsible for moving auxin properly in order to maintain proper root growth and meristem maintenance. This is accomplished through the localization of the different PIN proteins to specific membranes (Kleine-Vehn et al., 2009). The ability to maintain the polarity of the PIN proteins is extremely important in plant development. *gnom* mutants that unable to properly localize PIN proteins do not form an apical/basal axis and the seedlings develop incorrectly (Mayer et al.,

1991; Shevell et al., 1994). The PIN proteins are maintained at these specific membranes through the relationship between GN and the serine/threonine kinase PINOID (PID) (Kleine-Vehn et al., 2009). The phosphorylation of PIN1 proteins by PID dictates where PIN localizes: unphosphorlyated proteins go through the GN dependent pathway and are basally localized, and phosphorylated PIN proteins are managed independently of GN and are apically localized (Kleine-Vehn et al., 2009). GN localizes to the early endosome and from there continually cycles the unphosphorylated population of PIN1 proteins to the basal side of the cell. BrefeldinA (BFA) acts as an inhibitor of GN by binding to the ARF-GEF active site causing it to be unable to trigger the activation of the ARF proteins, leading to the loss of membrane trafficking. Roots treated with BFA show a loss of polar PIN1 localization, as GN is no longer able to activate the trafficking that would maintain their basal localization (Geldner et al., 2003).

In a broader context, the role of trafficking in regulating signaling ligands is epitomized by Delta, a transmembrane ligand that is regulated by the Notch receptor (Bray, 2006). The Delta/Notch pathway is conserved throughout many organisms, including *Drosophila*, *C. elegans* and humans, but it is not found in plants. Delta is very tightly regulated and its activity is dependant on its placement in the cell as well as the organism's phase of development (La Bras et al., 2010). In the canonical pathway binding of the Delta ligand triggers Notch cleavage events and the eventual trafficking of Notch to the nucleus. In order for the proper interactions to take place the Delta ligand is endocytosed, is reactivated and then is trafficked through the cell to where it is able to interact with Notch (La Bras et al., 2010). One example of this

is a two step pathway that has been shown cultured mammalian cells. The first step uses ubiquitination of Delta ligand as a signal for the endocytotic machinery to take up the ligand and then recycle to a specific lipid domain of the plasma membrane. Once there it will interact with Notch, the interaction with Notch triggers another round of endocytosis. This endocytosis of Delta while it is still interacting with Notch creates a pulling force that triggers the cleavage of Notch (Heuss et al., 2008). Another example of this occurs in the *Drosophila* sensory organ lineage. Here inactive Delta needs to be activated and trafficked to the apical actin rich area of the cell. In order to accomplish this inactive, ubiquitinated Delta undergoes endocytosis, where the ubiquitin molecule is removed and then Delta is recycled to the proper actin rich area of the cell (Wang and Struhl, 2004). NEV may play a similar role in the constant cycling of the signaling regulators of abscission, recycling them to the proper membranes until abscission initiation.

SECTION IV. ORGAN ABSCISSION

Organ shedding, or abscission, is a process that allows plants to drop their organs. This process is important in the life cycle of plants, as losing organs that are no longer necessary helps to conserve energy and the shedding of fruit is important for reproduction and dispersal in many species. Being able to manipulate abscission is of interest to industrial agriculture because manipulating abscission can help to increase yield and make harvesting many fruit easier. For example, the *jointless* variety of tomatoes is popular because the fruit develop without an abscission zone in the stem that attaches the fruit to the plant. Instead of falling off the plant when

ripened, the fruit stays attached until it is harvested making it easier to harvest with mechanical harvesters. (Butler, 1936). The more we understand about the abscission process the more opportunities there will be for manipulating abscission in crop plants and for this reason understanding the genetics of abscission is an important goal.

Arabidopsis is an advantageous model organism for studying organ abscission. The plant undergoes abscission of the floral organs in a very defined manner and the disruption of the stages of the abscission process is easily observed. Each set of floral organs has a distinct set of abscission zones at their base. These are sets of differentiated cells that allow the organ to detach. The differentiation of abscission zones is first step in abscission, and in mutants where the differentiation doesn't take place abscission cannot occur. Abscission is then initiated through a complex interaction of hormone and molecular signaling. In the final phase of abscission, the activity of cell wall modifying enzymes enables the break down of the connection between the plant abscission zones and the floral organs causing the floral organs to be shed.

Floral organ shedding is carefully timed to not interfere with seed production and fruit viability. Flower growth and transition into fruit is very carefully regulated and the timing of each developmental phase has been called a stage. The stages that are the most important to the process of abscission are stages 13 through 17. At stage 13 the buds begin to open and the very tops of the petals are visible. Fertilization takes place at stage 14, when the anthers, male flower parts, are above and shed pollen onto the stigma, the female flower part. This is followed by stage 15

when the stigma begins to extend beyond the anthers. The petals, sepals and stamens may begin to take on a withered appearance as they start senescing at stage 16. At stage 17, abscission has occurred and the outer organs have left the plants (Smyth et al., 1990).

Abscission is accompanied by a rise in auxin levels in the abscission zone, and ethylene has been shown to hasten abscission (Sexton and Roberts, 1982; Taylor and Whitelaw, 2001). Ethylene is a plant hormone that plays a large role in plant development; it has been implicated in developmental processes as far ranging as seed germination and senescence. The triple response is the classically studied response to ethylene by seedlings that have etiolated when grown in the dark. The triple response is an exaggerated apical hook, and a shorter, thicker hypocotyl (Neljubow, 1901).

In order to better understand the effect of ethylene on *Arabidopsis*, screens have been carried out to look for genes that may inhibit the triple response. Mutants for two of these genes *ETHYLENE RECEPTOR 1 (ETR1)* and *ETHYLENE INSENSITIVE 2 (EIN2)* have shown abscission phenotypes (Bleeker et al., 1988; Patterson and Bleecker, 2004). The mutations in *ETR1* are dominant and plants carrying them have a few small delays in development, including delays in senescence and abscission (Patterson and Bleecker, 2004). ETR1 is highly similar to histidine protein kinase receptors and has four homologs (Cheng et al., 1993). Although ETR1 appears to be more dominant, these five proteins appear to play redundant roles (Hua and Meyerowitz, 1998). Recently, ETR1 has been show to associate with a transmembrane protein, REVERSION-TO-ETHYLENE

SENESITIVITY 1 (RTE1) (Dong et al., 2010). Both ETR1 and RTE1 localize to the ER and Golgi, and may activate a MAPK cascade to send a signal to the nucleus (Chen et al., 2002; Dong et al., 2008). Plants with mutant copies of *RTE1* display a hypersensitivity to ethylene, and RTE1 may be responsible for the signaling state of ETR1 (Resnick et al., 2008). Plants with mutations in *EIN2* show delayed abscission phenotypes (Patterson and Bleecker, 2004). *EIN2* encodes a transmembrane protein with a C-terminal end that could engage in protein-protein interactions (Alonso et al., 1999). EIN2 also localizes to the ER and physically interacts with ETR1 (Bisson et al., 2009). This may be the first step in abscission signaling that activates transcription factors in the nucleus (Alonso et al., 1999).

Differentiation

In order for abscission to occur correctly the abscission zones must differentiate properly. Plants with mutations in the *BLADE-ON-PETIOLE 1* (*BOP1*) and *BLADE-ON-PETIOLE 2* (BOP2) genes fail to differentiate their abscission zones leading to failure to abscise (Hepworth et al., 2005). BOP1 and BOP2 are two closely related protein in the NPR1 (NON-EXPRESSOR OF PR1) family (Hepworth et al., 2005). NPR1 play a role in disease resistance, however, *bop1 bop2* plants show no changes is resistance. *BOP1* and *BOP2* have overlapping areas of expression and are redundantly required for abscission zone differentiation and leaf patterning (Hepworth et al., 2005; McKim et al., 2008). BOP1 and BOP2 localize to the cytoplasm and nucleus where they interact with and may regulate the activity of the transcription factor, PAN, which plays a role in leaf and floral patterning

(Hepworth et al., 2005). Overexpression of the signaling ligand, *INFLORESENCSE DEFICIENT IN ABSCISSION (IDA)*, normally causes premature organ abscission, and extreme cell expansion at the abscission zones (Butenko et al., 2003; Stenvik et al., 2006). *35S::IDA bop1 bop2* plants do not abscise and do not show increased cell expansion, suggesting a differentiated abscission zone is necessary for IDA activity (McKim et al., 2008). In *bop1 bop2* plants the undifferentiated abscission zone is unable to respond to the signal that would normally lead to abscission.

Abscission Initiation

After the abscission zone differentiates, abscission regulators are required to ensure proper temporal regulation of abscission. One of these regulators is the EPIP (extended PIP) domain protein IDA (Stenvik et al., 2008). *IDA* is expressed in the abscission zones of the flower and when mutated does not shed its floral organs, but when IDA is overexpressed it results in early abscission and increased cell expansion (Stenvik et al., 2006). IDA is secreted from the cell and is the putative signaling peptide for a pair of redundant RLKs, HAE and HSL2 (Stenvik et al., 2006; Stenvik et al., 2008; Cho et al., 2008). IDA is part of a family of five IDA-like (IDL) proteins, although not all of the genes share the same expression pattern as *IDA*, when overexpressed they all show phenotype analogous to *IDA* overexpression.

A pair of RLKs, HAE and HSL2, are the putative IDA receptors and are redundantly required for abscission (Stenvik et al., 2008; Cho et al., 2008). HAE is downstream of IDA and is able to suppress the *35S::IDA* phenotype. Activation of HAE and HSL2 triggers a downstream MAPK signaling cascade (Cho et al., 2008). This signaling cascade includes MPK3, MPK6, MKK4 and MKK5. Its been found

that when any of these genes are knocked down the plants show a non-shedding phenotype, and MPK6 shows decreased levels of phosphorylation in *hae hsl2* and *ida* plants (Cho et al., 2008). In addition, ectopically expressing MKK4 restores a normal shedding phenotype in *hae hsl2* and *ida* plants. Thus, MAPKs and MAPKKs are part of the downstream signaling cascade that regulates abscission (Cho et al., 2008).

The mutation of the ARF-GAP *NEVERSHED (NEV)* also results in plants that fail to abscise their floral organs. *NEV* mutants display trafficking defects throughout the plant. The Golgi circularizes and fuses with the TGN, and vesicles build up between the plasma membrane and the cell wall. These defects may result in the loss of required trafficking of signaling molecules necessary for abscission, like HAE and HSL2, or the enzymes required for breaking down the middle lamellae (Liljegren et al., 2009). In order to gain a deeper understanding of how NEV mediated trafficking affects abscission a screen of *nev* mutant plants was performed. Three proteins have been isolated from this screen so far, two LRR-RLKs and an RLCK. These proteins may be signaling molecules that require NEV for proper trafficking.

The two LRR-RLKs are EVERSHED (EVR) and SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE 1 (SERK1). Mutations in *EVR* recessively suppress *nev*, while mutations in *SERK1* are dominant suppressors (Leslie et al., 2010; Lewis et al., 2010). In the double mutant *nev evr*, floral organ shed about two positions earlier than wild type, but this was not seen in *nev serk1* mutants (Leslie et al., 2010; Lewis et al., 2010). In both of the double mutants Golgi and TGN structure was restored to wild type, but the increase in extracellular

vesicles was not relieved. The relief of the Golgi/TGN defect indicates that the loss of the proper Golgi and TGN structure correlates with the loss of organ shedding in *nev* mutants and the build up of vesicles between the plasma membrane and cell wall is not responsible (Leslie et al., 2010; Lewis et al., 2010). The third gene isolated from the *nev* screen was *CAST AWAY (CST)*, a RLCK.

Transcriptional Regulation of Abscission

The MADS domain family of proteins is a large family conserved through all eukaryotes. In plants they play a variety of roles and are especially important in floral development, with roles as varying from assisting in the development of floral meristems to playing a role in fruit dehiscence, which allows seed to shed (Rounsley et al., 1995; Liljegren et al., 2000). *AGL15* and *AGL18* are closely related MADS domain proteins expressed in many tissues throughout plant development, with some amount of overlap (Fernandez et al., 2000; Adamczyk et al., 2007). Overexpression of *AGL15* and *AGL18* produces plants with many developmental defects, especially pertaining to fruit development and including the failure to senesce or abscise their floral organs. The plants overexpressing *AGL15* could be induced to abscise their floral organs through exogenous application of ethylene, but this work has not been shown for *AGL18* overexpressors (Fernandez et al., 2000; Adamczyk et al., 2007). Plants mutant for either or both *AGL15* or *AGL18* show some changes in plant development but no changes in floral organ senescence or abscission (Adamczyk et al., 2007). Its possible that overexpression of these

proteins keeps the floral organs from senescing and that leads to the failure of signals vital for the initiation of abscission from arising (Fernandez et al., 2000).

The *AUXIN RESPONSE FACTOR (ARF)* gene family is a group of transcription factors that controls the way plants respond to the plant hormone auxin. Two of these *ARF1* and *ARF2*, have been implicated in the regulation of senescence and floral organ abscission, in mutants for either of these genes floral organ abscission is delayed (Ellis et al., 2005). Interestingly, the floral organs of *arf2* and *arf1 arf2* plants do not begin to wilt as would normally be seen at stage 16, instead they remain turgid until the time of their abscission (Ellis et al., 2005). These ARF proteins may interpret the auxin gradient that plays a role in abscission, and from that the plant may establish when abscission should occur. Another possibility is because of delayed senescence conveyed by the *arf2* mutation abscission signaling never begins.

After the abscission zone is differentiated and signaling has initiated abscission a suite of enzymes designed to break down cell walls and the middle lamella, a layer of pectin that connects the cell walls, needs to be trafficked to the correct areas of the cell. The middle lamella and the cell walls are complex and made of many highly crosslinked materials (Osborne, 1989). The complexity of the cell walls requires a few different kinds of enzymes, important examples are endoglucanases, polygalacturonases and expansins. The expression of these enzymes may be controlled by transcription factors necessary for abscission.

Overexpression of the transcription factor *AtDOF4.7* results in loss of floral organ shedding (Wei et al., 2010). This phenotype indicates that AtDOF4.7 may be

responsible for transcriptional repression of genes responsible for the correct temporal initiation of abscission. *AtDOF4.7* is highly expressed in abscission zones, and the protein localizes to the nucleus (Wei et al., 2010). AtDOF4.7 has been shown to bind at the suspected promoter region of *PGAZAT* (polygalacturonase), and *PGAZAT* is down regulated in plants that overexpress *AtDOF4.7*. These results suggest that AtDOF4.7 regulates the expression of *PGAZAT* to help ensure the proper timing of abscission (Wei et al., 2010).

The transcription factor AtZFP2 interacts with AtDOF4.7 in *Arabidopsis* protoplasts, and may form a protein complex to regulate transcription (Wei et al., 2010). AtZFP2 is a zinc finger protein that shows high levels of expression in abscission zones (Cai and Lashbrook, 2008). Plants that moderately overexpress *AtZFP2* show delayed abscission and floral organs that do not senesce, while plants with high levels of overexpression do not open and therefore cannot self-fertilize. These plants can therefore not self-fertilize and therefore do not abscise, however, when the flowers are hand-fertilized the fruits still fail to abscise or senesce their floral organs (Cai and Lashbrook, 2008).

ARP7 and ARP4 are *Arabidopsis* actin-related proteins, these proteins are present throughout eukaryotes and are similar to actin, but are highly divergent in functions (Kandasamy et al., 2005a; Kandasamy et al., 2005b). These are both nuclear proteins that may be involved in transcriptional regulation through chromatin remodeling (Kandasamy et al., 2003; Kandasamy et al., 2005a; Kandasamy et al., 2005b). While *ARP4* mutants show mostly stamen and pollen phenotypes, the *ARP7* mutants showed more severe phenotypes and arrested at the torpedo stage

of embryo development. RNAi knockdown either gene resulted in plants that do not undergo floral organ abscission or senescence, indicating that they are involved in regulation the expression of genes required for abscission (Kandasamy et al., 2005a; Kandasamy et al., 2005b).

REFERENCES

Adamczyk BJ, Lehti-Shiu MD and Fernandez DE: The MADS domain factors AGL15 and AGL18 act redundantly as repressors of the floral transition in *Arabidopsis*. *Plant J*. 2007, 50: 1007-1019.

Alonso JM, Hirayama T, Roman G, Nourizadeh S and Ecker JR: EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science*. 1999, 284: 2148-2152.

Anders N and Jurgens G: Large ARF guanine nucleotide exchange factors in membrane trafficking. *Cell Mol Life Sci*. 2008, 65: 3433-3445.

Bayer M, Nawy T, Giglione C, Galli M, Meinbel T and Lukowitz W: Paternal control of embryonic patterning in Arabidopsis thaliana. *Science.* 2009, 323: 1485-1488.

Bisson MM, Bleckmann A, Allekotte S and Groth G: EIN2, the central regulator of ethylene signaling, is localized at the ER membrane where it interacts with the ethylene receptor ETR1. *Biochem J*. 2009, 424: 1-6.

Bleecker AB, Estelle MA, Somerville C and Kende H: Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science*. 1988, 241: 1086-1089.

Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aida M, Palme K and Schere B: The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature*. 2005, 433: 39-44.

Bray SJ: Notch signaling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol*. 2006, 7: 678-689.

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

Butenko MA, Patterson SE, Grini PE, Stenvik GE, Amundsen SS, Mandal A and Aalen RB: Inflorescence deficient in abscission controls floral organ abscission in *Arabidopsis* and identifies a novel family of putative ligands in plants. *Plant Cell*. 2003, 15: 2296-2307.

Cai S and Lashbrook CC: Stamen abscission zone transcriptome profiling reveals new candidates for abscission control: enhanced retention of floral organs in transgenic plants overexpressing *Arabidopsis* ZINC FINGER PROTEIN2. *Plant Physiol*. 2008, 146: 1305-1321.

Chang C, Kwok SF, Bleecker AB and Meyerowitz EM: *Arabidopsis* ethylene response gene ETR1: similarity of product to two-component regulators. *Science*. 1993, 262: 539-544.

Chen YF, Randlett MD, Findell JL and Schaller GE: Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of *Arabidopsis*. *J Biol Chem*. 2002, 277: 19861-19866.

Cho SK, Larue CT, Chevalier D, Wang H, Jinn TL, Zhang S and Walker JC: Regulations of floral organ abscission in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA*. 2008, 105: 15629-15634.

Dettmer J, Schubert D, Calvo-Weimar O, Stierhof YD, Schmidt R and Schumacher K: Essential role of the V-ATPase in male gametophyte development. *Plant J*. 2005, 41: 117-124.

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

Dong CH, Rivarola M, Resnick JS, Maggin BD and Chang C: Subcellular co-localization of *Arabidopsis* RTE1 and ETR1 supports a regulatory role for RTE1 in ETR1 ethylene signaling. *Plant J*. 2008, 53: 275-286.

Dong CH, Jang M, Scharein B, Malach A, Rivarola M, Liesch J, Groth G, Hwang I and Chang C: Molecular association of the *Arabidopsis* ETR1 ethylene receptor and a regulator of ethylene signaling, RTE1. *J Biol Chem*. 2010, 285: 40706-40713.

D'Souza-Schorey C and Chavrier P: ARF proteins: roles in membrane traffic and beyond. *Nat Rev Mol Cell Biol*. 2006, 7: 347-358.

Ellis CM, Nagpal P, Young JC, Hagen F, Guilfoyle TJ and Reed JW: AUXIN RESPONSE FACTOR 1 and AUXIN RESPONSE FACTOR 2 regulate senescence and floral organ abscission in *Arabidopsis thaliana*. *Development*. 2005, 132: 4563-4574.

Fernandez DE, Heck GR, Perry SE, Patterson SE, Bleecker AM and Fang SC: The embryo MADS domain factor AGL15 acts postembryonically: Inhibition of perianth senescence and abscission via constitutive expression. *Plant Cell.* 2000, 12: 183-198.

Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A and Jurgens G: The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell*. 2003, 112: 219-230.

Geldner N: The plant endosomal system—its structure and role in signal transduction and plant development. *Planta*. 2004, 219: 547-560.

Geldner N, Richter S, Vieten A, Marquardt S, Torres-Ruiz RA, Mayer U and Jurgens G: Partial loss-of-function alleles reveal a role for GNOM in auxin transport-related, post-embryonic development of *Arabidopsis*. *Development*. 2004, 131: 389-400.

Gonzalez-Carranza Zh, Rompa U, Peters JL, Bhatt AM, Wagstaff C, Stead AD and Roberts JA: Hawaiian skirt: an F-box gene that regulates organ fusion and growth in *Arabidopsis*. *Plant Physiol*. 2007, 144: 1370-1382.

Grossniklaus U: Paternal patterning cue. *Science*. 2009, 323: 1439-1440.

Hepworth SR, Zhang Y, McKim S, Li X and Haughn GW: BLADE-ON-PETIOLE-dependent signaling controls leaf and floral patterning in *Arabidopsis. Plant Cell*. 2005, 17: 1434-1448.

Heuss SF, Ndiaye-Lobry D, Six EM, Israel A and Logeat F: The intracellular region of Notch ligands Dll1 and Dll3 regulates their trafficking and signaling activity. *Proc Natl Acad Sci USA*. 2008, 105: 11212-11217.

Hua J and Meyerowitz EM: Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell*. 1998, 94: 261-271.

Hwang I and Robinson DG: Transport vesicle formation in plant cells. *Current Opinion in Plant Biology.* 2009, 12: 660-669.

Jia DJ, Cao X, Wang W, Tan XY, Zhang XQ, Chen LQ and Ye D: GNOM-LIKE 2, encoding an adenosine diphosphate-ribosylation factor-guanine nucleotide exchange factor protein homologous to GNOM and GNL1, is essential for pollen germination in Arabidopsis. *J Integr Plant Biol*. 2009, 51: 762-773.

Jurgens G and Geldner N: Protein secretion in plants: from the *trans*-Golgi network to the outer space. *Traffic*. 2002, 3: 605-613.

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

Kandasamy MK, McKinney EC and Meagher RB: Cell cycle-dependent association of *Arabidopsis* actin-related proteins AtARP4 and AtARP7 with the nucleus. *Plant J.* 2003, 33: 939-948.

Kandasamy MK, Deal RB, McKinney EC and Meagher RB: Silencing the nuclear actin-related protein AtARP4 in *Arabidopsis* has multiple effects on plant development, including early flowering and delayed floral senescence. *Plant J.* 2005a, 41: 845-858.

Kandasamy MK, McKinney EC, Deal RB and Meagher RB: *Arabidopsis* ARP7 is an essential actin-related protein required for normal embryogenesis, plant architecture, and floral organ abscission. *Plant Physiol*. 2005b, 2019-2032.

Kleine-Vehn J, Huang F, Naramoto S, Zhang J, Michniewicz M, Offringa R and Friml J: PIN auxin efflux carrier polarity is regulated by PINOID kinase mediated recruitment in GNOM-independent trafficking in Arabidopsis. *Plant Cell*. 2009, 21: 3839-3849.

Koizumi K, Naramoto S, Sawa S, Yahara N, Ueda T, Nakano A, Sugiyama M and Fukada H: VAN3 ARF-GAP-mediated vesicle transport is involved in leaf vascular network formation. *Development*. 2005, 132: 1699-1711.

Kopan R and Ilagan MX: The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*. 2009, 137: 216-233.

Krecek P, Skupa P, Libus J, Naramoto S, Tejos R, Friml J and Zazimalova E: The PIN-FORMED (PIN) protein family of auxin transporters. *Genome Biol*. 2009, 10: 249.

Le Bras S, Loyer N and Le Borgne R: The Multiple Facets of Ubiquitination in the Regulation of Notch Signaling Pathway. *Traffic*. 2010, 12: 149-161.

Lee MH, Min MK, Lee YJ, Jin JB, Shin DH, Kim DH, Lee KH and Hwang I: ADP ribosylation factor 1 of *Arabidopsis* plays a critical role in intracellular trafficking and maintenance of endoplasmic reticulum morphology in *Arabidopsis*. *Plant Physiol*. 2002, 129: 1507-1520.

Leslie ME, Lewis MW, You JY, Daniels MJ and Liljegren SJ: The EVERSHED receptor-like kinase modulates floral organ shedding in *Arabidopsis*. *Development*. 2010, 137: 467-476.

Lewis MW, Leslie ME, Fulcher EH, Darnielle L, Healy P, Youn JY and Liljegren SJ: The SERK1 receptor-like kinase regulates organ separation in *Arabidopsis* flowers. *Plant J*. 2010, 62: 817-828.

Liljegren SJ, Ditta GS, Eshed Y, Savidge B, Bowman JL and Yanofsky MF: SHATTERPROOF MADS-box genes control seed dispersal in *Arabidopsis. Nature*. 2000, 404: 766-770.

Liljegren SJ, Leslie ME, Darnielle L, Lewis MW, Taylor SM, Luo R, Geldner N, Chory J, Randazzo PA, Yanofsky MF and Ecker JR: Regulation of membrane trafficking and organ separation by the NEVERSHED ARF GAP protein. *Development.* 2009, 136: 1909-1918.

Lippincott-Schwartz J, Yuan LC, Bonifacino JS and Klausner RD: Rapid redistribution of Golgi proteins in the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell*. 1989, 56: 801-813.

Lippincott-Schwartz J, Yuan L, Tipper C, Amherdt M, Orci L and Klausner RD: Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. *Cell*. 1991, 67: 601-616.

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

Matheson LA, Hanton SL, Rossi M, Latjinhouwers M, Stefano G, Renna L and Brandizzi F: Multiple roles of ADP-ribosylation factor 1 in plant cells include spatially regulated recruitment of coatamer and elements of the Golgi matrix. *Plant Physiol*. 2007, 143: 1615-1627.

Mayer U, Ruiz R, Berleth T, Miseera S and Jurgens G: Mutations affecting body organization in the Arabidopsis embryo. *Nature*. 1991, 353: 402-407.

McKim SM, Stenvik GE, Butenko MA, Kristiansen W, Cho SK, Hepworth SR, Aalen RB and Haughn GW: The BLADE-ON-PETIOLE genes are essential for abscission zone formation in *Arabidopsis*. *Development*. 2008, 135: 1537-1546.

Molendijk AJ, Ruperti B, Singh MK, Dovzhenko A, Ditengou FA, Milia M, Westphal L, Rosahl S, Soellick TR, Uhrig J, Weirgarten L, Huber M and Palme K: A cysteine-rich receptor-like kinase NCRK and a pathogen induced protein kinase RBK1 are Rop GTPase interactors. *Plant J*. 2008, 53: 909-923.

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

Muto H, Yabe N, Asami T, Hasunuma K and Yamamoto KT: Overexpression of constitutive differential growth 1 gene, which encodes a RLCKVII subfamily protein kinase, causes abnormal differential and elongation growth after organ differentiation in Arabidopsis. *Plant Phys*. 2004, 136: 3124-3133.

Neljubow DN: ber die horizontale nutation der Stengel von *Pisum sativum* und einiger anderen pflanzen. *Beitrage und Botanik Zentrablatt*. 1901, 10: 128-139.

Osborne DJ: Abscission. *Critical Reviews in Plant Sciences*. 1989, 8: 103-129.

Otegui MS and Spitzer C: Endosomal functions in plants. *Traffic*. 2008, 9: 1589-1598.

Patterson SE and Bleecker AB: Ethylene-dependent and independent processes associated with floral organ abscission in Arabidopsis. *Plant Physiol*. 2004, 134: 194-203.

Peyroche A, Paris S and Jackson CL: Nucleotide exchange on ARF mediated by yeast Gea1 protein. *Nature*. 1996, 384: 479-481.

Randazzo PA and Hirsch DS: Arf GAPs: multifunctional proteins that regulate membrane traffic and actin remodeling. *Cell Signal.* 2004, 16: 401-413.

Rea AC, Liu P and Nasrallah JB: A transgenic self-incompatible Arabidopsis thaliana model for evolutionary and mechanistic studies of crucifer self-incompatibility. *Journal of Experimental Botany*. 2010, 61: 1897-1906.

Resnick JS, Rivarola M and Chang C: Involvement of RTE1 in conformational changes promoting ETR1 ethylene receptor signaling in *Arabidopsis*. *PlantJ*. 2008, 56: 423-431.

Richter S, Geldner N, Schrader J, Wolters H, Stierhof YD, Rios G, Koncz C, Robinson DG and Jurgens G: Functional diversification of closely related ARF-GEFs in protein secretion and recycling. *Nature*. 2007, 448: 488-492.

Richter S, Anders N, Wolters H, Beckmann H, Thomann A, Heinrich R, Schrader J, Singh MK, Geldner N, Mayer U and Jurgens G: Role of the GNOM gene in *Arabidopsis* apical-basal patterning—From mutant phenotype to cellular mechanism of protein action. *Eur J Cell Biol*. 2010, 89: 138-144.

Richter S, Voss U and Jurgens G: Post-Golgi traffic in plants. Traffic. 2009. 10: 819-828.

Rounsley SD, Ditta GS and Yanofsky MF: Diverse roles for MADS box genes in *Arabidopsis* development. *Plant Cell*. 1995, 7: 1259-1269.

Schumacher K, Vafeados D, McCarthy M, Sze H, Wilkins T and Chory J: The *Arabidopsis det3* mutant reveals a central role for the vacuolar H^+-ATPase in

plant growth and development. *Genes & Development*. 1999, 13: 3259-3270.

Sexton R and Roberts JA: Cell biology of abscission. *Annual Review of Plant Physiology*. 1982, 33: 133-162.

Sieburth LE, Muday GK, King EJ, Kenton G, Kim S, Metcalf KE, Meyers L, Seamen E and Van Norman JM: SCARFACE encodes an ARF-GAP that is required for normal auxin efflux and vein patterning in Arabidopsis. *Plant Cell*. 2006, 18: 1396-1411.

Shao F, Golstein C, Ade J, Stoutemyer M, Dixon JE and Innes RW: Cleavage of Arabidopsis PBS1 by a bacterial type III effector. *Science*. 2003, 301: 1230-1233.

Shevell DE, Leu WM, Gillmor CS, Xia G, Feldmann KA and Chua NH: EMB30 is essential for normal cell division, and cell adhesion in Arabidopsis and encodes a protein that has similarity to Sec7. *Cell*. 1994, 77: 1051-1062.

Shiu SH and Bleecker AB: Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. *Proc Natl Acad Sci USA*. 2001, 98: 10763-10768.

Shiu SH and Bleeker AB: Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in Arabidopsis. *Plant Phys.* 2003, 132: 530-543.

Shiu SH, Karlowski WM, Pan R, Tzeng YH, Mayer KF and Li WH: Comparative= analysis of the receptor-like kinase family in Arabidopsis and rice. *Plant Cell*. 2004, 16: 1220-1234.

Smith DF: Tetratricopeptide repeat cochaperones in steroid receptor complexes. *Cell Stress Chaperones*. 2004, 9: 109-121.

Smyth DR, Bowman JL and Meyerowitz EM: Early flower development in Arabidopsis. *Plant Cell*. 1990, 2: 755-767.

Song XF, Yang CY, Liu J and Yang WC: RPA, a class II ARF-GAP protein, activates ARF1 and U5 and plays a role in root hair development in Arabidopsis. *Plant Physiol*. 2006, 141: 966-976.

Stefano G, Renna L, Rossi M, Azzarello E, Pollastri S, Brandizzi F, Baluska F and Mancuso S: AGD5 is a GTPase-activating protein at the trans-Golgi network. *Plant J*. 2010, 64: 790-799.

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

Stenvik GE, Butenko MA, Urbanowicz BR, Rose JK and Aalen RB: Overexpression of INFLORESCENCE DEFICIENT IN ABSCISSION activates cell separation in vesitgal abscission zones in *Arabidopsis*. *Plant Cell.* 2006: 1467-1476.

Stenvik GE, Tandstad NM, Guo Y, Shi CL, Kristiansen W, Holmgren A, Clark SE, Aalen RB and Butenko MA: 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.* 2008. 20: 1805-1817.

Strompen G, Dettmer J, Stierhof YD, Schumacher K, Jurgens G and Mayer U: *Arabidopsis* vacuolar H-ATPase subunit E isoform 1 is required for Golgi organization and vacuole function in embyrogenesis. *Plant J*. 2005: 41: 125-132.

Swiderski MR and Innes RW: The Arabidopsis PBS1 resistance gene encodes a member of a novel protein kinase subfamily. *Plant J*. 2001: 101-112.

Takeuchi M, Ueda T, Yahara N and Nakano A: Arf1 GTPase plays roles in the protein traffic between the endoplasmic reticulum and the Golgi apparatus in tobacco and *Arabidopsis* cultured cells. *Plant J*. 2001, 31: 499-515.

Tang W, Kim TW, Oses-Prieto JA, Sun Y, Deng Z, Zhu S, Wang R, Burlingame AL and Wang ZY: BSKs mediate signal transduction from the receptor BRI1 in Arabidopsis. *Science*. 2008, 321: 557-560.

Taylor JE and Whitelaw CA: Signals in abscission. *New Phytologist.* 2001, 151: 323-339.

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

Vernoud V, Horton AC, Yang Z and Nielsen E: Analysis of the small GTPase gene superfamily of Arabidopsis. *Plant Physiol*. 2003, 131: 1191-1208.

Veronese P, Nakagami H, Bluhm B, Abuqamar S, Chen X, Salmeron J, Dietrich RA, Hirt H and Mengiste T: The membrane-anchored BOTRYTIS INDUCED KINASE1 plays distinct roles in Arabidopsis resistance to necrotrophic and biotrophic pathogens. *Plant Cell*. 2006, 18: 257-273.

Vert G, Nemhauser JL, Geldner N, Hong F and Chory J: Molecular mechanisms of steroid hormone signaling in plants. *Annu Rev Cell Dev Biol*. 2005, 21: 177-201.

Viotti C, Bubeck J, Stierhof Y, Krebs M, Langhans M, van den Berg W, van Dongen W, Richter S, Geldner N and Takano J: Endocytic and secretory traffic in Arabidopsis merge in the *trans*-Golgi network/early endosome, and independent and highly dynamic organelle. *The Plant Cell*. 2010, 22: 1344-1357.

Wang W and Struhl G: *Drosophila* Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. *Development*. 2004, 131: 5367-5380.

Wei PC, Tan F, Gao XQ, Zhang XQ, Wang GQ, Xu H, Li LJ, Chen J and Wang XC: Overexpression of AtDOF4.7, an *Arabidopsis* DOF family transcription factor, induces floral organ abscission deficiency in *Arabidopsis*. *Plant Physiol*. 2010, 153: 1031-1045.

Xu J and Scheres B: Dissection of *Arabidopsis* ADP-RIBOSYLATION FACTOR 1 function in epidermal cell polarity. *Plant Cell*. 2005, 17: 525-536.

Yoo CM, Wen J, Motes CM, Sparks JA and Blancaflor EB: A class I ADP ribosylation factor GTPase-activating protein is critical for maintaining directional root hair growth in Arabidopsis. *Plant Physiol*. 2008, 147: 1659 1674.

Zhang J, Li W, Xiang T, Liu Z, Laluk K, Ding X, Zou Y, Gao M, Zhang X, Chen S, Mengiste T, Zhang Y and Zhou JM: Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a Pseudomona syringae effector. *Cell Host Microbe.* 2010, 7: 290-301.

Zhang Y and McCormick S: The regulation of vesicle trafficking by small GTPases and phosphlipids during pollen tube growth. *Sexual Plant Reproduction*. 2010, 23:87-93.

CHAPTER 2

CAST AWAY, A MEMBRANE-ASSOCIATED RECEPTOR-LIKE KINASE, INHIBITS ORGAN ABSCISSION IN ARABIDOPSIS

This chapter was submitted for publication as:

Christian A. Burr, Michelle E. Leslie, Sara K. Orlowski, Catherine E. Wright, Mark J. Daniels, Iris Chen and Sarah J. Liljegren. (2011) CAST AWAY, a membrane-associated receptor-like kinase, inhibits organ abscission in Arabidopsis.

PREFACE

This chapter has been accepted for publication with revisions in *Plant Physiology*. I am co-first author on this paper with Michelle Leslie. Michelle Leslie, Sara Orlowski, Catherine Wright and I carried out the CST interaction experiments (Figures 2.7, 2.9 and 2.10), Michelle Leslie and Catherine Wright generated the CST and CST^{G2A} localization data (Figure 2.5), Mark Daniels purified the 6XHis-tagged CST, CST^{K124E} and CST^{G157R} proteins for the kinase assay (Figure 2.4D), and Iris Chen assisted with abscission zone measurements (Figure 2.2H) and with preparation of Figure 2.8. The figures and tables were re-numbered for this chapter.

ABSTRACT

Receptor-like kinase-mediated cell signaling pathways play fundamental roles in many aspects of plant growth and development. A pair of *Arabidopsis thaliana* leucine-rich repeat receptor-like kinases (LRR-RLKs), HAESA (HAE) and HAESA-LIKE2 (HSL2), have been shown to activate the cell separation process that leads to organ abscission. Another pair of LRR-RLKs, EVERSHED (EVR) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1), act as inhibitors of abscission, potentially by modulating HAE/HSL2 activity. Cycling of these RLKs to and from the cell surface may be regulated by NEVERSHED (NEV), a membrane trafficking regulator that is essential for organ abscission. We report here the characterization of CAST AWAY (CST), a receptor-like cytoplasmic kinase that acts as a spatial inhibitor of cell separation. Disruption of *CST* suppresses the abscission defects of *nev* mutant flowers, and restores the discrete identity of the *trans*-Golgi

network in *nev* abscission zones. After organ shedding, enlarged abscission zones with obscured boundaries are found in *nev cst* flowers. We show that CST is a dual-specificity kinase in vitro, and that myristoylation at its N-terminus promotes association with the plasma membrane. Using the bimolecular fluorescence complementation assay, we have detected interactions of CST with HAE and EVR at the plasma membrane of Arabidopsis protoplasts and hypothesize that CST negatively regulates cell separation signaling directly and indirectly. A model integrating the potential roles of receptor-like kinase signaling and membrane trafficking during organ separation is presented.

INTRODUCTION

Signaling by transmembrane receptor-like kinases (RLKs) underlies diverse aspects of plant growth and development. Surprisingly, a substantial number of plant RLKs do not contain either extracellular or transmembrane domains. Although receptor-like cytoplasmic kinases (RLCKs) account for at least 125 of the 610 annotated RLKs in Arabidopsis, much remains to be learned about their functions within cell signaling complexes (Shiu and Bleecker, 2001; Shiu et al., 2004; Goring and Walker, 2004; Jurca et al., 2008). Several of the 46 RLCKs assigned to the class VII subfamily have been found to function in pathogen response and developmental signaling pathways (Swiderski and Innes, 2001; Shao et al., 2003; Murase et al., 2004; Muto et al., 2004; Veronese et al., 2006; Ade et al., 2007; Lu et al., 2010; Zhang et al., 2010).

Functional studies of class VII RLCKs have identified four different modes of action; RLCKs can act as co-receptors of RLKs, in signal relays, as repressors, and as activators of signaling. The M-Locus Protein Kinase (MLPK) RLCK functions as a co-receptor of a ligand-binding RLK to transduce signaling. During the self-incompatibility response in *Brassica rapa* flowers, MLPK has been found to interact with the ligand-activated S-Locus Receptor Kinase and is essential for the cell signaling leading to rejection of self-pollen (Murase et al., 2004; Kakita et al., 2007). The BOTRYTIS-INDUCED KINASE 1 (BIK1) RLCK functions in a signaling relay with an activated ligand-binding RLK and its co-receptor. BIK1 was shown to interact with two leucine-rich repeat receptor-like kinases (LRR-RLKs), the ligand-binding FLAGELLIN-SENSITIVE 2 (FLS2) and

its co-receptor BRI1-ASSOCIATED KINASE 1 (BAK1) (Veronese et al., 2006; Lu et al., 2010). FLS2 binding of the bacterial flagellin-derived peptide, flg22, triggers interaction of FLS2 and BAK1, and downstream signaling for pathogen-associated molecular patterns (PAMP)-triggered 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 and promotion of pathogen immune responses downstream of FLS2.

The AvrPphB Susceptible 1 (PBS1) RLCK acts as a repressor. PBS1 is cleaved by an effector of the pathogen *Pseudomonas syringae,* activating the nucleotide binding site-leucine rich repeat protein, RPS5, which triggers programmed cell death (Warren et al., 1999; Swiderski and Innes, 2001; Shao et al., 2003; Ade et al., 2007). A recent study has revealed that like PBS1, BIK1 and several PBS1-like (PBL) RLCKs are also substrates of the bacterial AvrPphB protease effector (Zhang et al., 2010). This discovery and other work suggests that a bacterial effector can suppress PAMP-triggered immunity in plants by cleaving RLCKs known (BIK1) or proposed (PBL1, PBL2) to positively interact with RLKs that bind PAMPs, including FLS2, CHITIN ELICITOR RECEPTOR KINASE1, and the EF-Tu RECEPTOR (Zipfel et a., 2006; Miya et al., 2007; Zhang et al., 2010).

In Arabidopsis, organ abscission is controlled by the competing activities of several LRR-RLKs. The HAESA (HAE) and HAESA-LIKE2 (HSL2) LRR-RLKs redundantly activate a MAPK signaling cascade that leads to cell separation and release of the outer floral organs (Jinn et al., 2000; Cho et al., 2008). The predicted signaling ligand for HAE/HSL2 is INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), a small, secreted peptide (Butenko et al., 2003; Cho et al., 2008; Stenvik et al., 2008). Two inhibitors of organ separation that may directly regulate HAE/HSL2 are the EVERSHED (EVR) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1) LRR-RLKs (Leslie et al., 2010; Lewis et al., 2010). Mutations in *EVR* or *SERK1* were found to restore abscission in plants defective for *NEVERSHED* (*NEV*), an ADP-ribosylation factor GTPase-activating protein. NEV has been proposed to regulate the movement of proteins essential for activating cell separation (Liljegren et al., 2009).

Here we show that CAST AWAY (CST), a membrane-associated class VII RLCK, acts as a spatial inhibitor of abscission zone (AZ) signaling. We have found that CST interacts at the plasma membrane with EVR and HAE, LRR-RLKs that inhibit and promote organ separation, respectively. Our studies of CST suggest a distinct mode of RLCK action in which an RLCK and RLK partner may act in a step-wise fashion to inhibit the activity or alter the location of a ligand-binding receptor-like kinase.

RESULTS

Organ separation is restored in *nev cst* flowers

To identify novel regulators of organ abscission, a genetic screen was conducted for mutations that restored abscission in *nev-3* mutant flowers (Lewis et al., 2010). A recessive mutation identified in this screen, *cast away* (*cst-1*), was found to rescue organ separation in *nev* flowers (Fig. 2.1A-C). A second mutant allele of *CST* from the SAIL T-DNA collection (*cst-2*; SAIL_296_A06; Sessions et al., 2002) dominantly rescues organ abscission in *nev-3* flowers (Fig. 2.1D). Flowers with mutations in *CST* alone have a wild-type appearance and organ shedding occurs normally (Fig. 2.1E,F).

Since NEV, IDA, HAE and HSL2 each regulate the cell separation stage of organ separation (Butenko et al., 2003; Cho et al., 2008; Stenvik et al., 2008; Liljegren et al., 2009), we tested whether disruption of CST activity might also suppress the *ida* and *hae hsl2* mutant phenotypes. We found that mutations in *CST* do not rescue the shedding defects of *ida* or *hae hsl2* flowers (Fig. 2.1G-J). These results suggest that *CST* acts upstream of IDA and HAE/HSL2, or in a parallel pathway that converges at HAE/HSL2 activity or further downstream.

The organ AZs of *nev cst* flowers are enlarged and disorganized.

Although organ separation occurs in *nev-3 cst-1, nev-3 cst-2/+* and *nev-3 cst-2* flowers, the AZ regions have a visibly rough appearance compared to the smooth surfaces of the organ detachment sites in wild-type flowers (Fig. 2.1A,C,D). To further characterize this phenotype, we examined longitudinal

sections and scanning electron micrographs of *nev cst* flowers at the time of shedding compared to wild-type and *cst* single mutant flowers (Fig. 2.2A,C-G). While the remaining AZ cells of wild-type expand to form discrete scars (Fig. 2.2A,E), cells in the AZ regions of *nev cst* flowers have a disordered appearance and show increased, uneven cell expansion (Fig. 2.2C,F). After organ shedding, *nev cst* AZ regions were found to be significantly enlarged compared to wild-type (Fig. 2.2H) and the boundaries between individual organ detachment sites and with the floral stem are notably obscured (Fig. 2.2E-G). These results suggest that CST acts as a spatial inhibitor of signaling that modulates AZ cell adhesion and expansion.

Disruption of CST activity suppresses the subcellular defects of *nev* flowers

Our studies have previously shown that mutations in *NEV* are associated with a unique set of trafficking defects in flowers undergoing organ separation (Liljegren et al., 2009). To determine whether disruption of CST activity suppresses these subcellular changes as well as restoring organ separation in *nev cst* mutant flowers, we carried out transmission electron microscopy of wild-type and mutant flowers shortly after organ abscission (stage 17) (Fig. 2.2A-D). Whereas the structure and organization of the Golgi cisternae and *trans*-Golgi network are altered in *nev* mutant cells (Fig 3B,E) compared to wild-type (Fig. 2.3A,E), we found that these organelles are unaffected in *nev cst* (Fig. 2.3C,E) and *cst* cells (Fig. 2.3D,E). We also discovered that the hyperaccumulation of

extracellular vesicles in *nev* cells (Fig. 2.3G,J) compared to wild-type (Fig. 2.3F,J) and *cst* (Fig. 2.3I,J) cells, is significantly reduced in *nev cst* (Fig. 2.3H,J) cells. These results suggest that specific mutations in *CST* are sufficient to alleviate the disruption of vesicular traffic in *nev* cells that blocks organ separation.

CST encodes a receptor-like cytoplasmic kinase with dual specificity

The *cst-1* mutation was mapped to chromosome 4 and found to affect At4g35600, a gene encoding a predicted receptor-like cytoplasmic kinase (RLCK) of the class VII subfamily (Fig. 2.4A). This mutation introduces an amino acid substitution immediately after subdomain IV of the kinase domain, which is involved in binding ATP (Fig. 2.4B) (Hanks, 2003). Although residues in this region are not highly conserved among eukaryotic protein kinases (Hanks and Hunter, 1995), the affected glycine is invariant in the kinase domains of all 46 predicted class VII RLCKs in Arabidopsis (Fig. 2.4B, data not shown). The *cst-2* mutant allele contains a T-DNA insertion upstream of the kinase domain, and is predicted to cause production of a truncated protein (Fig. 2.4A).

To test CST kinase activity, full-length proteins of wild-type (WT), a traditional kinase-dead mutant (K124E) (Horn and Walker, 1994), and the *cst-1* mutant (G157R) were expressed as N-terminal 6XHis-tagged fusion proteins in *E. coli*. Whereas a His antibody recognizes purified, presumably phosphorylated CST^{WT} protein migrating as a single band of ~55 kDa, it recognizes purified CST^{K124E} and CST^{G157R} proteins migrating as single bands of ~49 kDa in

agreement with the predicted size of the tagged protein (49 kDa) (Fig. 2.4D). Phosphoserine, phosphothreonine, and phosphotyrosine antisera were used to detect phosphorylated residues on the recombinant proteins. Each of these antisera recognized the CST^{WT} protein and neither of the mutant proteins (Fig. 2.4D). These results suggest that CST is a dual-specificity kinase that autophosphorylates serine, threonine and tyrosine residues *in vitro*, and that its kinase activity is abolished by the *cst-1* mutation.

Analysis of allele-specific interactions between *NEV* and *CST*

We have observed allele-specific differences in the number of copies of *cst-1* and *cst-2* required to restore organ shedding in *nev-3* flowers. While a single *cst-2* allele is sufficient to dominantly rescue abscission in *nev-3* flowers (Fig. 2.1B,D), both copies of the *cst-1* mutant allele must be present to restore organ shedding in this background (Fig. 2.1B,C).

To determine whether we could uncover additional allele-specific interactions between *CST* and *NEV*, the *cst-1* and *cst-2* alleles were crossed to the *nev-2* and *nev-6* mutant alleles. While we have not detected significant phenotypic differences between the *nev-2*, *nev-3* and *nev-6* mutants in a Landsberg *erecta* background, the molecular nature of the respective mutations is quite different. The *nev-3* mutation introduces an amino acid substitution in the ARF GAP domain at an invariant arginine (R59K) which is known to be essential for ARF GAP enzymatic activity (Luo et al., 2007; Liljegren et al., 2009). The *nev-2* mutation introduces a stop codon downstream of the ARF GAP domain

(Q198*) and is predicted to cause production of a truncated protein with an ARF GAP domain. The *nev-6* allele contains a T-DNA insertion in the first intron, and is expected to produce a truncated protein without an ARF GAP domain.

While the *cst-1* mutation recessively rescues organ shedding in *nev-2* and *nev-6* flowers (Fig. 2.1M,N; data not shown), and *cst-2* dominantly rescues shedding in *nev-6* flowers (data not shown), the *cst-2* allele was unable to restore abscission in *nev-2* flowers even if both mutant copies of *CST* were present (Fig. 2.1O). These results are partially consistent with an allele-specific compensatory mutation, in which the suppressor mutation restores a physical interaction between the affected components (Michels, 2002). However, if the truncated cst-2 mutant protein were to interact with the nev-3 mutant protein in such a way that its ARF GAP activity was restored, one would expect that the *cst-2* mutant allele should also not be able to rescue abscission in a nev mutant protein missing the ARF GAP domain.

To address whether the dominant rescue of organ shedding in *nev* flowers by the *cst-2* allele is due to either a dominant-negative interaction or haploinsufficiency, a wild-type copy of the *CST* cDNA driven by its predicted 1.4 kb promoter was introduced into *nev-3 cst-2* homozygous mutant plants. We observed that for two independent T1 lines with T2 kanamycin-resistance segregation ratios characteristic of a single insertional locus, presence of the *CST::CST* transgene was sufficient to block organ abscission in *nev cst* flowers, restoring the *nev* mutant phenotype (Fig. 2.1K,L; Table S2). This result may suggest that haploinsufficiency of *CST* rescues floral organ shedding in *nev-3*

cst-2/+ flowers. However, since multiple T-DNA insertions can be present at a single locus (Jorgensen et al., 1987), these results do not rule out a dominant-negative interaction involving truncated CST protein produced from the *cst-2* allele. Multiple copies of wild-type *CST* could efficiently dilute the dominant-negative effect of a single locus producing truncated CST protein.

Localization of CST to the plasma membrane is supported by N-terminal myristoylation

CST is predicted to associate with membranes in part via myristoylation of its N-terminus (Fig. 2.4C), as previously shown for the PBS1, MLPK and BIK1 class VII RLCKs (Warren et al., 1999; Boisson et al., 2003; Murase et al., 2004; Veronese et al., 2006; Kakita et al., 2007). Palmitoylation of N-terminal cysteine residues is also predicted to allow reversible membrane association for CST and many other class VII RLCKs (Fig. 2.4C; Sorek et al., 2009; Zhang et al., 2010). To visualize CST protein within Arabidopsis cells, we generated a *CST-GFP* fusion construct driven by the constitutive viral 35S promoter that could be transfected into mesophyll protoplasts. Attempts to visualize CST-YFP under the control of its native promoter *in vivo* were unsuccessful, likely due to the limited expression of *CST* in roots and leaves (Fig 6F,G,J; data not shown). CST-GFP transformed protoplasts exhibit fluorescent localization of the protein to the plasma membrane and some internal, punctate structures (Fig. 2.5A,A'). Mutation of the predicted myristoylation site (G2A; Fig. 2.4C) causes a partial redistribution of CST^{G2A}-GFP to the cytoplasm (Fig. 2.5B), as independently

observed in tobacco epidermal cells (Stael et al., 2011), and observed for transfection of the GFP tag alone (Fig. 2.5C). These results indicate that myristoylation of CST supports but is not solely required for its localization at the plasma membrane.

The EVR, SERK1 and HAE LRR-RLKs, which contain transmembrane domains, have been previously shown to be associated with the plasma membrane or closely associated membrane structures (Jinn et al., 2000; Shah et al. 2001; Alexandersson et al., 2004; Leslie et al., 2010). To compare the localization profile of CST with HAE and EVR in protoplasts, we also generated *35S::HAE-GFP* and *35S::EVR-GFP* transfection constructs. HAE-GFP and EVR-GFP were observed at the plasma membrane and in structures with the appearance of the ER network (Fig. 2.9A,A',C,C'). SERK1-YFP was previously reported to localize to endosomal compartments and the ER network as well as the plasma membrane in Arabidopsis leaf protoplasts; this distribution was found to vary with respect to time after transfection (Aker et al., 2006). The distinct localization profile of CST compared to those of EVR, SERK1 and HAE may indicate that the mechanism of CST inhibition of abscission differs from that of EVR and SERK1.

CST is expressed in organ AZs, lateral roots, and developing guard cells

To determine the expression pattern directed by *CST* regulatory regions, a construct with a translational fusion of the predicted 1.4 kb promoter region to the β-glucuronidase (GUS) reporter was generated (Fig. 2.6A). Of 35 *CST::CST-*

GUS transgenic lines analyzed, five showed GUS expression in floral organ AZs. Expression of GUS first appears prior to organ shedding (stage 15) in subepidermal cells within the floral pedicel underlying the organ attachment sites (Fig. 2.6B,C). During and after organ shedding (stage 16-17), individual epidermal cells in the AZ regions show GUS expression in a dynamic fashion, and increased GUS expression is observed in subepidermal cells of the gynophore (fruit stem) and pedicel (Fig. 2.6B,D). Expression of GUS in the AZ regions decreases as the fruit matures (mid-stage 17). GUS expression is also observed within the gynoecium, in the style of the developing fruit, and in the axils of the floral stems (Fig. 2.6E,H,I).

Several *CST::CST-GUS* transgenic lines also showed expression in vegetative tissues. In seedlings, GUS expression is found within the lateral roots (Fig. 2.6F,J) and in the guard cells and large pores (hydathodes) at leaf edges (Fig. 2.6G). These results suggest that expression of *CST* is restricted to specific cell types and tissues, and that CST may function during other phases of plant development.

CST interacts with the HAE and EVR receptor-like kinases at the plasma membrane

To test for interactions between CST and other RLKs that modulate abscission, we used the bimolecular fluorescence complementation (BiFC) assay in Arabidopsis mesophyll protoplasts (Walter et al., 2004; Yoo et al., 2007). This approach has been successfully used to detect interactions between membrane-

bound LRR-RLKs, such as the SERK family members (SERK1, SERK2, BAK1 and BAK1-LIKE1) and BAK1-INTERACTING RECEPTOR-LIKE KINASE1 (BIR1) (Russinova et al., 2004; Albrecht et al., 2005; Karlova et al., 2006; Gao et al., 2009). To facilitate this analysis, full-length versions of CST, HAE, and EVR with C-terminal translational fusions to either the N-terminal half of YFP (YFPn) or the C-terminal half of YFP (YFPc) were generated. When transfected into protoplasts, these fusion proteins are expressed under the control of the constitutive 35S promoter. The presence of YFP fluorescence in protoplasts visualized about 16 hours after plasmid transfection indicates a likely interaction between the two target proteins, as the N- and C-terminal halves of YFP were brought into close enough proximity to reconstitute the fluorescent protein.

To allow for detection of protoplasts successfully transfected with plasmid DNA, as well as for the calculation of transfection efficiency, we co-transfected all protoplasts with a previously described CFP-tagged mitochondrial marker (CD3-986; Nelson et al., 2007). Only those experiments with a protoplast transfection efficiency of greater than or equal to 50% were used for BiFC analysis.

Since multiple RLKs are known to form homodimers (Russinova et al., 2004; Hink et al., 2008; Zhu et al., 2010), we first tested for interaction of CST with itself. CST was found to self-interact at the plasma membrane as co-transfection of *CST-YFPn/CST-YFPc* resulted in a similar pattern of fluorescence as CST-GFP (Figs. 5A; 7A). When co-transfected, *HAE-YFPn/HAE-YFPc* and *EVR-YFPn/EVR-YFPc* also appear to interact in a similar pattern to what we observe for HAE-GFP and EVR-GFP transfections alone—localization to the

plasma membrane and internal structures (Fig. 2.9A,A',C,C'). While the SERK1 and BRI1 LRR-RLKs 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 tested the hypothesis that CST may inhibit abscission signaling by forming receptor heterocomplexes with HAE. Upon co-transfection of either *CST-YFPn*/*HAE-YFPc* or *HAE-YFPn*/*CST-YFPc*, reconstituted YFP was detected at the plasma membrane (Fig. 2.7B; Fig. 2.10A). Interestingly, unlike the uniform localization of CST-GFP (Fig. 2.5A), the CST-HAE interaction appears to be restricted to subdomains of the plasma membrane (Fig. 2.7B; Fig. 2.10A; arrowheads). Co-transfection of *CST-YFPn*/*EVR-YFPc* or *EVR-YFPn*/*CST-YFPc* also revealed interactions of CST and EVR at the plasma membrane in a uniform pattern (Fig. 2.7C; Fig. 2.10B).

As a control for non-specific interactions between RLCKs and YFP alone, co-transfection of *CST-YFPn* with *YFPc* (Walter et al., 2004) alone was performed (Fig. 2.7D). As depicted, no fluorescence was observed in the majority of protoplasts that were successfully transfected with the CFP-tagged mitochondrial expression control, indicating that CST and the C-terminus of YFP do not interact.

As a control for non-specific interactions between RLCKs and LRR-RLKs, we tested for interactions between the PBS1 class VII RLCK and the HAE and EVR LRR-RLKs. Co-transfection of *PBS1-YFPn/PBS1-YFPc* resulted in uniform plasma membrane fluorescence (Fig. 2.7E) indicating that PBS1 is also able to

homodimerize. However, co-transfection with *PBS1-YFPn/HAE-YFPc* (Fig. 2.7F), *PBS1-YFPn/EVR-YFPc* (Fig. 2.7G), *HAE-YFPn/PBS1-YFPc* (Fig. 2.10C), or *EVR-YFPn/PBS1-YFPc* (Fig. 2.10D) did not show evidence of interactions between PBS1 and either HAE or EVR at the plasma membrane.

Taken together, these results suggest that CST inhibits signaling that promotes abscission both directly and indirectly by physically interacting with HAE and EVR at the cell surface.

DISCUSSION

We report here the identification of CST, a membrane-associated RLCK that functions as an inhibitor of organ abscission. Like the EVR and SERK1 LRR-RLKs (Leslie et al., 2010; Lewis et al., 2010), CST appears to restrict the extent of cell separation signaling, such that only cells within designated domains at the base of each outer organ undergo cell loosening, separation and expansion. In *nev cst* flowers, the boundaries between the individual organ attachment sites and the border with the floral pedicel become notably obscured after organ abscission (Fig. 2.2F). Enlarged AZs that form visible collars of rough tissue are also found in *nev evr*, *nev serk1* and *35S::IDA* flowers instead of the smooth, discrete AZ scars of wild type (Stenvik et al., 2006; Leslie et al., 2010; Lewis et al., 2010). The remarkable similarity of these phenotypes suggests that the rescue of organ abscission, AZ enlargement and AZ boundary blurring in *nev cst* flowers may be due to ectopic, prolonged signaling of HAE and HSL2, the putative receptors of the IDA peptide.

We have found that the *cst-2* mutation restores the structure of the Golgi and location of the TGN in *nev* flowers, and significantly reduces the hyperaccumulation of extracellular vesicles (Fig. 2.3). Golgi-derived structures like those in *nev* mutant flowers have also been seen in pollen mutants affecting the a1 subunit (VHA-a1) of the Arabidopsis vacuolar H^+-ATPase proton transporter or when cells are treated with a specific V-ATPase inhibitor, concanamycin A (Dettmer et al., 2005; Liljegren et al., 2009). Recent studies have shown that the concanamycin A-induced structures are labeled with VHA-a1 and SYP61, markers of the TGN/early endosome (Viotti et al., 2010). These results suggest that the circularized structures in *nev* flowers are also chimeric fusions of the Golgi cisternae and the TGN, and that traffic through the TGN/early endosome is affected by mutations in *NEV*. Disruption of CST, EVR or SERK1 activity appears to restore the identity and independence of the TGN from the Golgi in *nev* flowers (Leslie et al., 2010; Lewis et al., 2010). Defects in membrane trafficking are thought to underlie the inappropriate fusion of the TGN and the Golgi, as V-ATPase-mediated endomembrane acidification is required to recruit ARF G-proteins, ARF-GEFs and coat components involved in vesicle budding (Zeuzem et al., 1992; Aniento et al., 1996; Maranda et al., 2001; Hurtado-Lorenzo et al., 2006; Viotti et al., 2010). As an ARF-GAP, NEV may regulate the activity and recruitment of the same TGN-localized ARF G-protein(s) and adaptor molecules as the V-ATPase complex. The significant rescue of extracellular vesicle defects in *nev cst* flowers, which was not observed in *nev serk1* or *nev evr* flowers (Leslie et al., 2010; Lewis et al., 2010), suggests the possibility that

CST may interact more directly with NEV and in a different manner than EVR and SERK1 to inhibit abscission. Alternatively, if EVR and SERK1 act redundantly, suppression of extracellular vesicle accumulation may also be observed in *nev serk1 evr* flowers.

Functional studies of class VII RLCKs have revealed that several act in plant defense cell signaling pathways; CST is one of the first RLCKs found to regulate a developmental process (Table I). The *mlpk* allele discovered in the Yellow Sarson variety of *Brassica rapa* was found to allow pollen self-compatibility in this species; MLPK and the ligand-activated S-Locus receptor kinase are proposed to form a heteromeric complex in stigmatic cells that mediates signaling leading to the rejection of self pollen (Murase et al., 2004; Kakita et al., 2007). As with the *cst* mutant, redundancy appears to mask the phenotype of the Arabidopsis *constitutive differential growth1* (*cdg1*) single mutant (Muto et al., 2004). CDG1 is predicted to play a role in brassinosteroid signal transduction based on the phenotype of a dominant, activation-tagged line with stunted growth, epinastic leaves and twisted stems; *cdg1* loss-of-function alleles were identified as intragenic suppressors of the gain-of-function phenotype (Muto et al., 2004). Since *CST* regulatory regions direct GUS expression in the lateral roots, gynoecium, and developing guard cells, CST may function in other aspects of plant development.

Although the genetic nature of the *cst-2* allele has not been resolved, we speculate that it is likely acting as a dominant negative mutation. If the truncated mutant protein is acting as a non-functional kinase and is capable of

homodimerizing with wild-type CST protein and/or heterodimerizing with the HAE and EVR LRR-RLKs, it has the potential to interfere with the activity and regulation of its partners. Dominant negative LRR-RLK mutants are associated with particular missense mutations in the extracellular LRR and kinase domains of CLAVATA1 (Diévart et al., 2003; Diévart and Clark, 2003) and with truncation of the entire kinase domain in some LRR-RLKs such as ERECTA (ER) (Shpak et al., 2003). While most class VII RLCK mutations either occur within the kinase domain or are expected to truncate part of the kinase domain, the *cst-2* mutation affects a codon positioned upstream of the kinase domain (Fig. 2.4A; Table I). It is intriguing that *cst-2* dominantly suppresses the *nev-3* and *nev-6* mutants, but not the *nev-2* mutant (Fig. 2.1D,O; data not shown). Allele-specific interactions for genes with opposing functions in a biological process suggest that the CST and NEV proteins may function in a single complex, and that the ARF GAP domain may regulate this interaction. We plan to explore these possibilities in future genetic and biochemical studies.

Integrated model for RLK regulation of organ separation

Our studies suggest a model in which CST and EVR inhibit cell separation signaling by acting in a stepwise manner to mediate HAE/HSL2 receptor complex formation and internalization (Fig. 2.8) (Leslie et al., 2010). First, CST may sequester the EVR RLK at the plasma membrane. While CST-GFP is primarily localized to the plasma membrane (Fig. 2.5A,A'), EVR-GFP is distributed between the plasma membrane and unknown internal compartments having the

appearance of the ER network (Fig. 2.9C,C'). CST-EVR complexes are uniformly localized to the plasma membrane (Fig. 2.7C; Fig. 2.10B), suggesting that a direct interaction between CST and EVR could limit EVR movement from the plasma membrane, and enable subsequent interactions of EVR with HAE. Second, CST-containing complexes may interact with the HAE RLK. In contrast to the uniform localization of CST-GFP at the plasma membrane (Fig 5A), CST-HAE interactions are stabilized within subdomains of the plasma membrane or perhaps closely associated vesicles (Fig. 2.7B; Fig. 2.10A). Receptor aggregation may be important for packaging into endocytic vesicles (Zappel and Panstruga, 2008). Third, we speculate that interactions of EVR and/or SERK1 with HAE may facilitate internalization and trafficking of HAE-containing receptor complexes (Leslie et al., 2010; Lewis et al., 2010). Since CST, EVR and SERK1 act as negative regulators of cell separation, the stepwise aggregation and internalization of HAE and HSL2 may function to attenuate signaling or target the receptors for degradation.

NEV, as a global regulator of membrane trafficking, may be required for the trafficking of both positive (HAE, HSL2) and negative (EVR, SERK1) regulators of abscission (Fig. 2.8). From its locations at the *trans*-Golgi network/early endosome and the recycling endosome (Liljegren et al., 2009), NEV may function to traffic receptors within the early endosomal system and ultimately recycle them back to the plasma membrane (Fig. 2.8). Loss of *NEV* could lead to the hyperaccumulation of inactivated receptors within endosomal compartments, while a secondary loss of *CST*, *EVR* or *SERK1* may stabilize the

HAE/HSL2 RLKs at the plasma membrane. At the proper timing for abscission in wild-type flowers, our model predicts that IDA ligand-binding stabilizes HAE/HSL2, leading to activation of downstream signaling events required for the loss of cell adhesion (Fig. 2.8). As for activation of the FLS2 receptor by the bacterial elicitor flg22, which coincides with disassociation of the BIK1 RLCK (Lu et al., 2010), IDA peptide binding may trigger intracellular phosphorylation of HAE/HSL2 and disassociation of CST, EVR, and/or SERK1.

While a transient protoplast expression system is ideal for rapid identification of interacting proteins, the interactions of CST with EVR and HAE must be confirmed by future experiments in floral tissues. By introducing epitope-tagged RLKs into the *nev*, *nev cst* and *nev evr* mutants, the effect of NEV ARF-GAP activity upon RLK localization can also be tested. If NEV is required for the proper trafficking of RLKs that regulate AZ cell signaling, HAE and EVR may aggregate in aberrant locations in *nev* cells, either internally in endosomes or externally in the observed extracellular vesicles. Secondary loss of either the CST or EVR negative regulators may restore localization of the HAE/HSL2 RLKs to the plasma membrane. Since the primary function of CST may be to sequester RLKs at the plasma membrane and facilitate interactions between their intracellular kinase domains, future experiments will also investigate whether CST interacts with SERK1, and whether EVR and/or SERK1 interact with HAE.

There is a growing body of evidence that class VII RLCKs can mediate cell signaling by forming heteromeric complexes with RLKs. MLPK acts as a co-receptor for the S-Locus receptor kinase during the self-incompatibility reponse

(Murase et al., 2004; Kakita et al., 2007). BIK1 has been found to interact with the FLS2 and BAK1 LRR-RLKs to enable the PAMP-triggered immune response (Lu et al., 2010). Proposed interactions of the PBL1-like RLCKs with the EFR LRR-RLK and CERK1, an RLK with chitin oligosaccharide-recognizing LysM motifs in its extracellular domain, may promote PAMP-triggered immunity (Zhang et al., 2010). Our work highlights the potential significance of a sequential relay of receptor complex interactions and the regulated trafficking of receptor complexes to and from the cell surface as key mechanisms to modulate RLK-mediated cell signaling during plant growth and development.

MATERIALS AND METHODS

Plants and growth conditions

The *cst-1* allele was identified through an ethyl methanesulfonate screen of *nev-3* mutant plants (Landsberg *erecta* ecotype) as described (Lewis et al., 2010). *cst-1* is caused by a glycine to arginine substitution at amino acid 157. *cst-2* (SAIL_296_A06; Columbia ecotype) contains a T-DNA insertion within codon 79, (Sessions et al., 2002). The primers and restriction enzyme for genotyping these alleles are described in Table 2.3. Mutant alleles described previously include *hae-1, hsl2-1, ida-2, nev-2, nev-3,* and *nev-6* (Cho et al., 2008; Stenvik et al., 2008; Liljegren et al., 2009).

For protoplast experiments, wild-type *Arabidopsis thaliana* (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.

A 1.4 kb region of *CST* through the translational start site, was PCR amplified from Col DNA using 5'-CACCAAGAAGAGAGGAACTCTTGTA-3' and 5'-CCACTCTGCAAATCCTTGAACA-3', cloned into pENTR/D-TOPO (Life Technologies, Carlsbad, CA) and recombined into pGWB3 (Nakagawa et al., 2007) to create a translational fusion of CST to β-glucuronidase (GUS). Of 35 *CST::CST-GUS* transgenic lines analyzed, five showed expression in floral AZs and 23 showed the described expression pattern in seedlings.

Mapping

The *cst-1* mutation was mapped by crossing *nev-3 cst-1* (Ler) and *nev-6* (Col) flowers. Using DNA isolated from 518 *nev cst* F2 plants and PCR-based markers including several designed from Ler polymorphisms (http://www.Arabidopsis.org/Cereon/), *cst-1* was located to the F8D20 BAC on chromosome 4. The coding regions of 11 of 15 genes in the 49 kb interval were sequenced from *nev cst* plants.

Kinase Activity

The CST open reading frame was amplified using 5'-CACCATGGGTGCTTGTATT-3' and 5'-TTATTTTTCTACTGATCCAAACCGT-3' from Col DNA, to create CST^{WT}. This fragment was cloned into pENTR/D-TOPO and site-directed mutagenesis (Stratagene, La Jolla, CA) with 5'-

GGTTCTGGTATGATCGTTGCCATCGAGAGATTGAATTCTGAGAGTGTTC-3' and 5'-GAACACTCTCAGAATTCAATCTCTCGATGGCAACGATCATACCAGAACC-3', or 5'-CACCGAAATCTGGTGAAGTTATTGAGATACTGTCGTGAAGACAAAGAGC-3' and 5'-GCTCTTTGTCTTCACGACAGTATCTCAATAACTTCACCAGATTTCGGTG-3' were used to create CST^{K124E} and CST^{G157R}, respectively. Recombination with pDEST17 (Life Technologies, Carlsbad, CA) was used to generate 6X-His-tagged CST. Recombinant proteins were expressed in E. coli and purified by Co^{2+} affinity chromatography (Clontech Laboratories, Mountain View, CA). Antisera were used at the following dilutions: anti-polyHis-HRP (Sigma-Aldrich; 1:50,000), anti-phosphoserine (Sigma-Aldrich; 1:2000), anti-phosphotyrosine (Sigma-Aldrich; 1:2000), anti-phosphothreonine (Zymed/Invitrogen; 1:800). HRP-conjugated chicken anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) secondary antibodies were used at a 1:10,000 dilution.

Protein interaction and localization assays

The coding regions for *CST*, *HAE* and *PBS1* were PCR amplified from the U18406 (ABRC), pBS-HAE (kindly provided by John Walker, University of Missouri), and U12315 (ABRC) cDNA constructs, and cloned into pENTR/D-TOPO (Life Technologies, Carlsbad, CA). The *EVR* coding region was amplified from genomic (Col-0) DNA and cloned into pENTR/D-TOPO. Using site-directed

mutagenesis, 5'-GCCCCCTTCACCATGGCTGCTTGTATTTCGTTC-3' and 5'-GAACGAAATACAAGCAGCCATGGTGAAGGGGGC-3' were used to generate *pENTR::CST^{G2A}.* 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. The pUC-SPYCE plasmid was used as a YFPc control (Walter et al., 2004).

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. For BiFC assays, protoplasts were co-transfected with the experimental constructs and a mitochondrial CFP marker (ABRC CD3-986; Nelson et al., 2007) to track transfected cells and measure transfection efficiency. Cells were washed and incubated overnight in the dark to allow for gene expression. Protoplasts were imaged 16-20 hours after transfection. Only those transfection experiments for which 50% or greater of the protoplasts showed expression of the co-transfected mitochondrial CFP marker were used for BiFC analysis.

Microscopy

Flowers were fixed and processed for scanning and transmission electron microscopy as described (Liljegren et al., 2009; Leslie et al., 2010). Confocal laser scanning microscopy (CLSM) of root epidermal cells was performed with an LSM-510 (Carl Zeiss, Thornwood, NY). CLSM 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).

Acknowledgments

We thank J. Walker, S. Patterson, M. Duncan, B. Goldstein, S. Ahmed, M. Peifer and J. Reed for helpful discussions; S. Hasty, P. Healy, and H. Kizer for technical assistance; J. Punwani and J. Kieber for protoplast transfection vectors and reagents; T. Perdue, S. Ray and V. Madden for microscopy assistance; J. Walker and ABRC for DNA and seed stocks; Monsanto for access to Ler polymorphisms; and Syngenta for access to the SAIL T-DNA collection.

TABLES

Mutant	Cause	Effect	Reference
pbs1-1	fast neutron	chromosomal inversion	Warren et al., 1999
pbs1-2	EMS	G252R (kinase subdomain VIII)	Swiderski and Innes, 2001
mlpk	natural variation	G194R (kinase subdomain VIa)	Murase et al., 2004
cdg1-1	EMS	W184* (before kinase subdomain VIa)	Muto et al., 2004
cdg1-2	EMS	splicing disruption (after kinase subdomain V)	Muto et al, 2004
bik1	T-DNA insertion	truncation in kinase subdomain IX	Veronese et al., 2006
pbl1	T-DNA insertion	truncation in kinase subdomain VIa	Zhang et al., 2010
pbl2	T-DNA insertion	Insertion in second exon (At1g14370 SALK_149140)	Zhang et al., 2010

Table 2.1. Mutations in class VII RLCKs.

Mutations previously identified in the PBS1, MLPK, CDG1 and BIK1 receptor-like cytoplasmic kinases (RLCKs).

Line	T2 plants	Segregation ratio	Expected kanamycin resistant/ sensitive	Observed kanamycin resistant/ sensitive	χ^2 test (*P*)	Observed phenotype* (shedding/ nonshedding)
1a-1	192	3:1	144:48	148:44	0.5	1:86
4a-1	53	3:1	40:13	40:13	0.975	0:39

*phenotype scored for kanamycin resistant plants

Table 2.2. Complementation of *nev cst* mutant phenotype.

Segregation analysis of *CST::CST* transgene complementation of the *nev cst* mutant.

Allele	Enzyme	PCR product (bp)	Digest products (bp)		Oligos
cst-1	BfuI	1024	*cst-1* Ler	1024 518, 506	5'- CAAGAAACGACTTCTACAGAAT-3' 5'- CATATGTGCCCATGATCC-3'
cst-2		825 WT			5'- CCACTAGCTCTTGTCTGAA-3' 5'- GTTATCATAGGAGAAAACATCATCTG-3'
		521 T-DNA			5'- CCACTAGCTCTTGTCTGAA-3' 5'- TTTCATAACCAATCTCGATACAC-3'

Table 2.3. Genotyping the *cst-1* and *cst-2* mutant alleles.

Information to identify the *cst-1* and *cst-2* mutant alleles is provided.

FIGURES

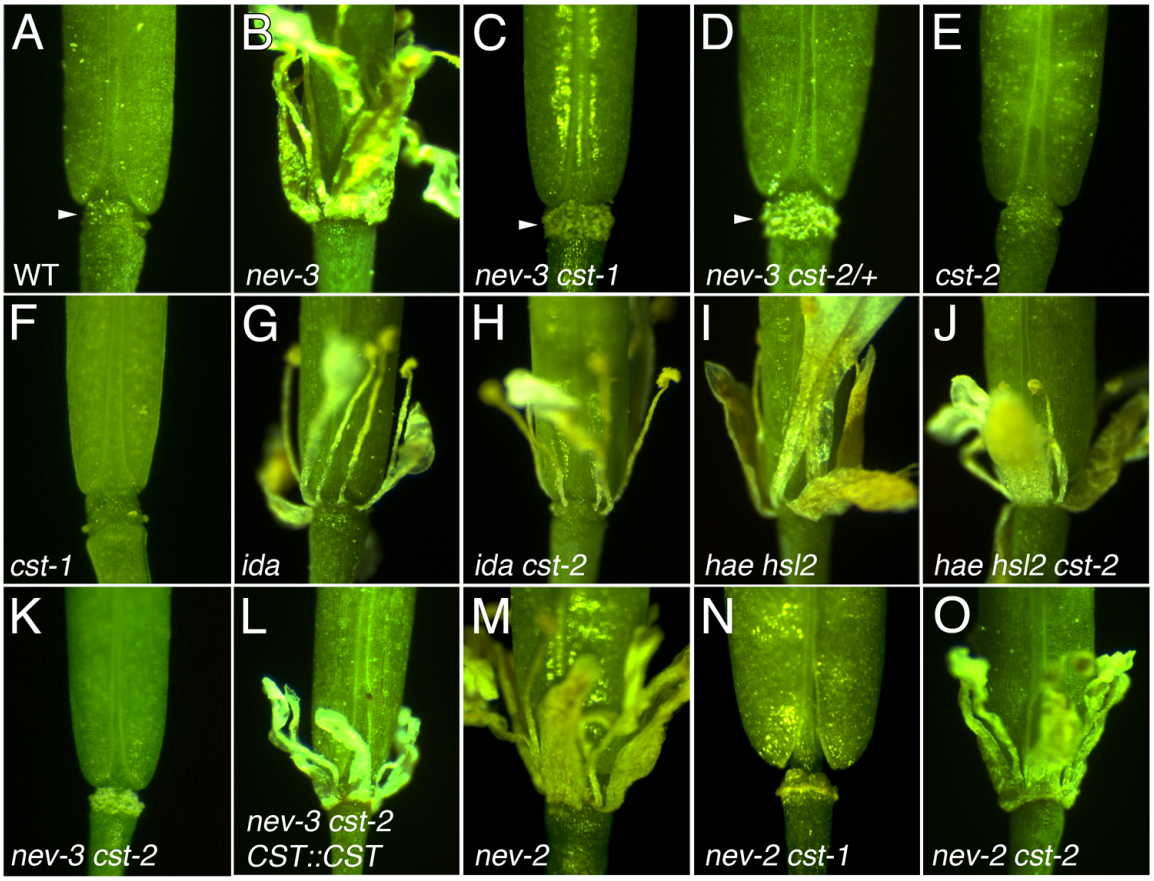

Figure 2.1. Loss of *CAST AWAY* rescues floral organ shedding in *nevershed* plants.

A-D, Sepals, petals and stamens are shed from wild-type flowers by floral stage 17 (A), and remain attached in *nev-3* flowers (B). Organ separation is recessively restored in *nev-3 cst-1* plants (C), while the *cst-2* allele acts dominantly to restore floral organ shedding in the *nev-3* background (D). The abscission zone (AZ) regions of *nev cst* flowers (C,D; see arrows) are enlarged and visibly rougher than those of wild-type flowers (A, arrow). **E-F,** In the *cst-2* (E) and *cst-1* (F) single mutants, the organ AZs appear like those of wild-type flowers and shedding occurs normally. **G-J,** The abscission defects of the *ida* (G) and *hae hsl2* (I) mutants are not rescued by the *cst-2* allele (H,J). **K-L,** The *nev-3 cst-2* mutant phenotype (K) can be complemented by a *CST::CST* transgene (L). Presence of the *CST* transgene blocks organ abscission, restoring the *nev* mutant phenotype. **M-O,** The abscission defects of *nev-2* flowers (M) are rescued by *cst-1* (N) but not by *cst-2* (O) mutant alleles.

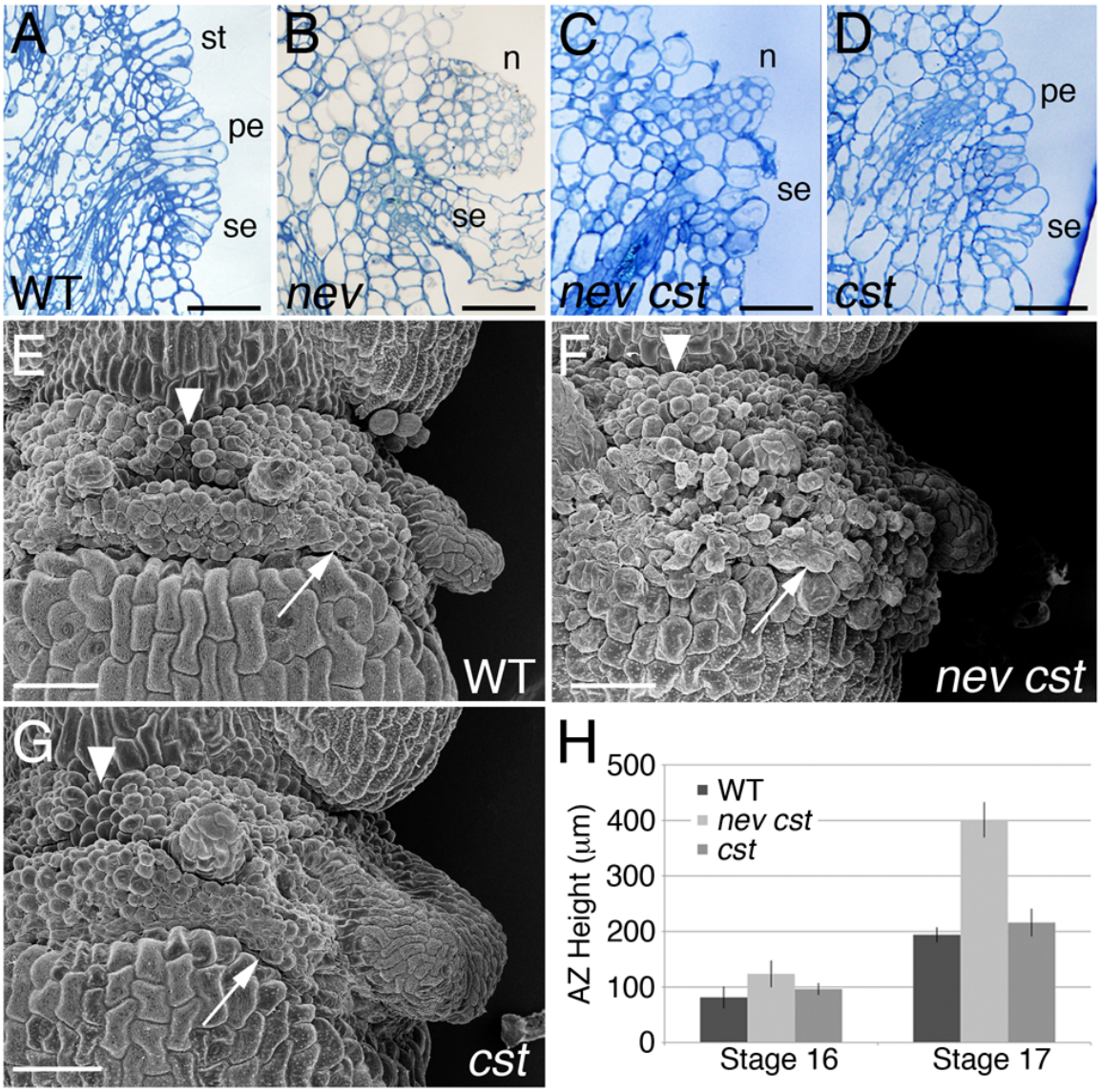

Figure 2.2. *nev cst* abscission zones are disorganized and enlarged.

A-D, Longitudinal sections of flowers (stage 17) stained with Toluidine Blue. The remaining AZ cells of wild-type (A) and *cst-2* (D) flowers show coordinated cell expansion, while the floral organs remain attached in *nev* flowers (B). Although organ abscission is rescued in *nev cst-2* flowers (C), the AZ cells have a disordered appearance. The petal (pe), sepal (se), and stamen (st) AZs and (n) nectaries are indicated. Scale bars, 50 μm. **E-G,** Scanning electron micrographs of flowers after organ separation (stage 17). Distinct AZs are apparent in wild type (E) and *cst* (G) flowers, whereas the AZ regions of *nev cst* flowers have formed an enlarged, disorganized band of cells at the fruit base (F). In *nev cst* flowers, the junction between the medial stamen AZs is no longer visible (E-G, arrowheads), and the border between the sepal AZ and floral stem is not clearly defined (E-G, arrows). Scale bars, 500 μm. **H,** Quantification of AZ size in wild-

type and mutant flowers. The distance between the lower border of the sepal AZ and upper border of the stamen AZ was measured in stage 16 and the first stage 17 flowers ($n \geq 4$ per genotype). *nev cst* flowers contain significantly enlarged AZs after organ shedding compared to wild-type and *cst* flowers.

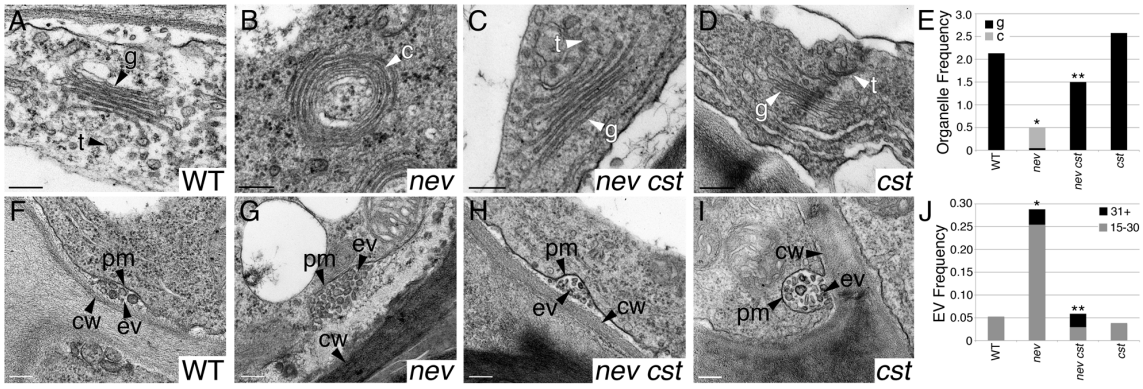

Figure 2.3. Mutations in *CST* rescue the subcellular defects of *nev* mutant flowers.

Transmission electron micrographs of cells in the sepal AZs of wild-type and mutant flowers (stage 17). **A-D,** The linear stacks of Golgi cisternae and associated *trans*-Golgi network seen in wild-type (A) and *cst* (D) flowers are replaced by circularized multilamellar structures in *nev* flowers (B). In *nev cst* flowers (D), the discrete structures of the Golgi and *trans*-Golgi network are restored. **E,** Frequency of Golgi cisternae (g, black) and circularized structures (c, gray) per cell in sections of wild-type and mutant sepal AZs. Statistical differences between *nev* and wild-type, and between *nev cst* and *nev* tissues are indicated by single and double asterisks, respectively (Fisher's exact test, P=0). A statistical difference was not detected between *cst* and wild-type tissues. $n \geq 26$ cells per genotype. **F-I,** Extracellular vesicles are frequently observed in the apoplastic space of *nev* AZ cells (G). In wild type (F), *nev cst* (H) and *cst* (I) AZs the appearance of extracellular vesicles is significantly reduced. **J,** Frequency of extracellular vesicles (clusters of 15-30, gray; clusters of 31+, black) in wild-type and mutant sepal AZ cells. Statistical differences between *nev* and wild-type, and between *nev cst* and *nev* tissues are indicated by single (Fisher's exact test: $P<0.012$) and double ($P<0.02$) asterisks, respectively. A statistical difference was not detected between *cst* and wild-type tissues. $n \geq 26$ cells per genotype. c, circularized structures; cw, cell wall; ev, extracellular vesicles; g, Golgi cisternae; pm, plasma membrane; t, *trans*-Golgi network. Scale bars, 200 nm.

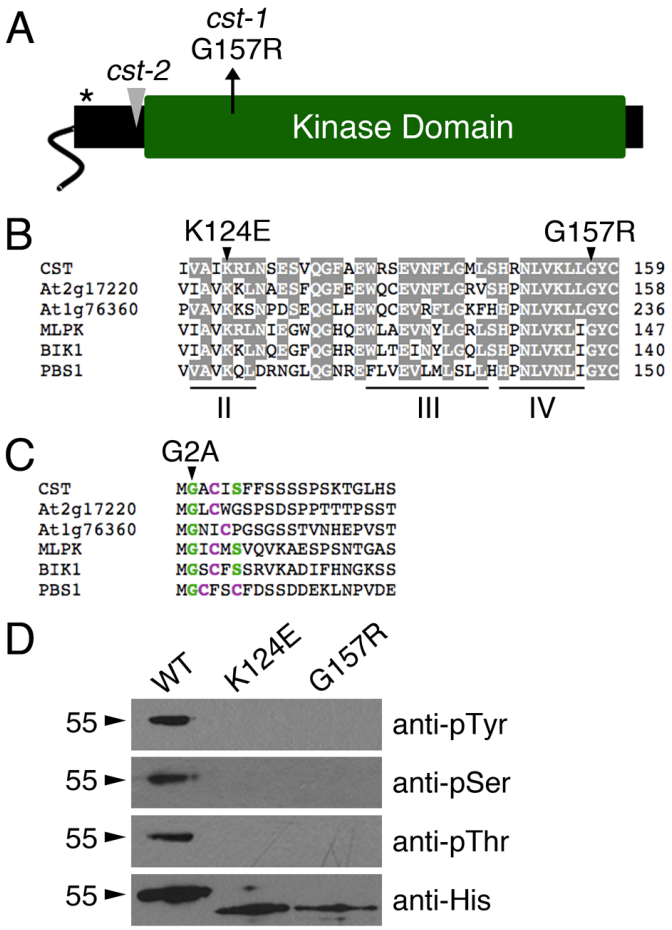

Figure 2.4. *CST* encodes a dual-specificity RLCK.

A, *CST* encodes a receptor-like cytoplasmic kinase (RLCK) with a predicted myristoylation site at its N-terminus (asterisk). The *cst-1* point mutation affects a conserved residue within the kinase domain, and the *cst-2* allele contains a T-DNA insertion upstream of the kinase domain. **B,** Sequence alignment of kinase subdomains II-IV from CST and other class VII RLCKs from Arabidopsis and *Brassica rapa*. The *cst-1* mutation (G157R) alters an amino acid that is invariant among class VII RLCKs. Conserved amino acids between CST and other proteins are shaded. **C,** Sequence alignment of the N-terminus of CST and other related RLCKs. Residues predicted to undergo myristoylation are highlighted in green, and expected palmitoylation sites are highlighted in purple. **D,** Recombinant CST autophosphorylates on serine, threonine and tyrosine residues. Mutations in subdomain II (K124E) and next to subdomain IV (G157R) interfere with the kinase activity of CST. Antiserum that recognizes the 6XHis-tag labels ~55 and ~49 kDa phosphorylated and unphosphorylated CST fusion proteins, respectively.

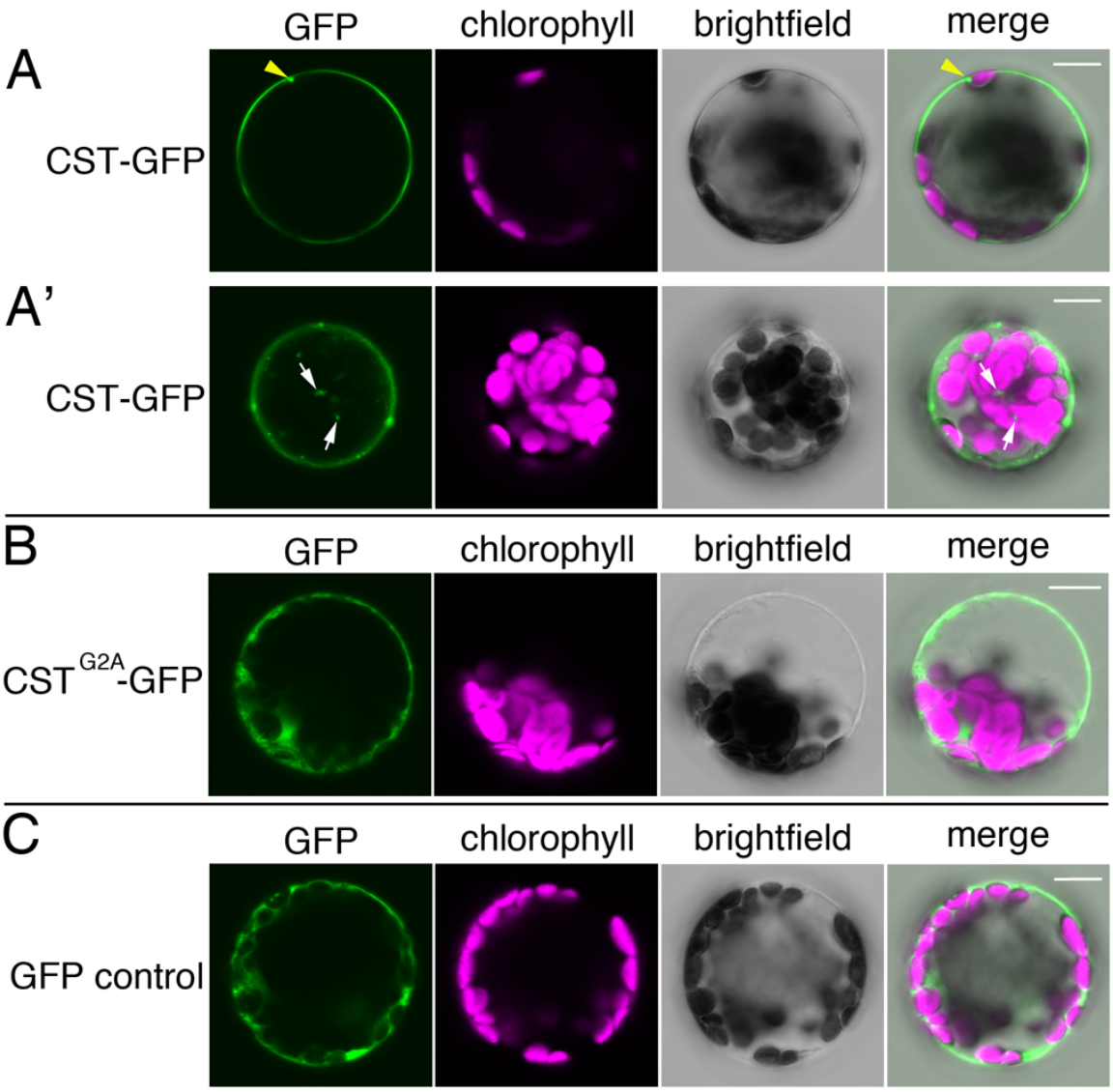

Figure 2.5. CST localizes to the plasma membrane.

Transfected Arabidopsis leaf protoplasts were imaged using confocal microscopy. GFP fluorescence (green), chlorophyll autoflorescence (magenta), brightfield and merged images are shown for each protoplast. **A-A'**, CST (CST-GFP) localizes to the plasma membrane (A) and in internal speckles (A', arrows). Spots of increased CST aggregation associated with the plasma membrane are observed (A, arrowhead). Images of the same protoplast at different focal planes are shown. **B,** Mutation of the predicted myristoylation site (CST^{G2A}-GFP) results in a broad localization of the mutant protein throughout the cytoplasm. Localization of the mutant protein is still observed at or near the plasma membrane. **C,** GFP (-GFP) is localized in a similarly broad pattern throughout the cytoplasm and near the plasma membrane.
Scale bars, 10 μm.

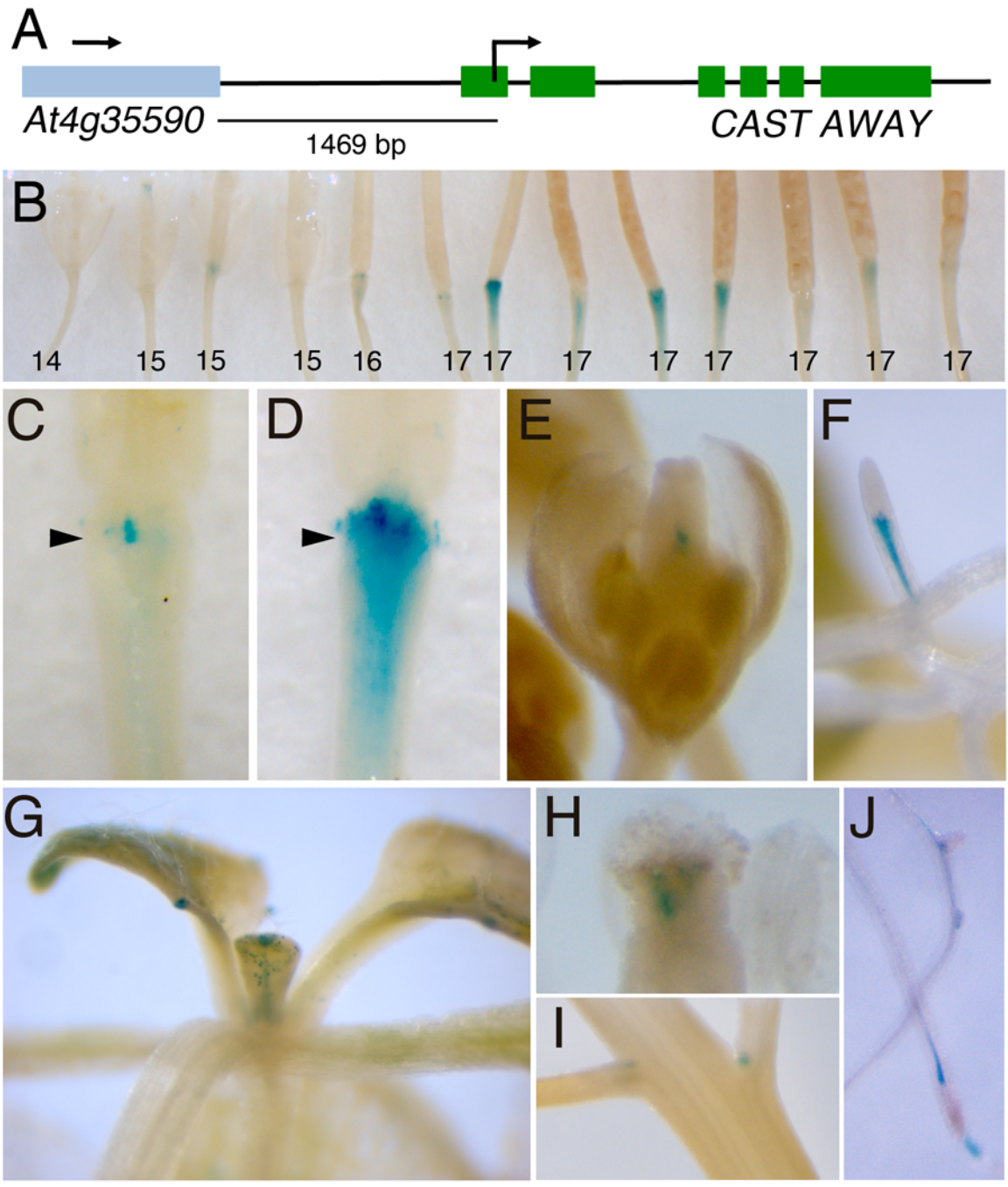

Figure 2.6. *CST* is expressed in floral organ AZs and other specific tissues.

A, A translational fusion of the *CST* regulatory region to the β-glucuronidase (GUS) reporter was created. **B,** Expression of GUS is first detected in stage 15 flowers prior to organ shedding (stage 16) and diminishes by mid-stage 17. Flowers at consecutive positions along the inflorescence are shown with the floral stages indicated below. **C-D,** During and after organ abscission, a dynamic pattern of GUS expression is seen in epidermal AZ cells. The early patches of GUS expression in subepidermal cells of the floral pedicel (C) expand into a

broad, diffuse domain within the gynophore and pedicel of older flowers (D). Arrowheads indicate the position of the sepal AZs. **E-J,** GUS expression was also detected in seedling roots (F, J) and leaves (G), the axils of floral stems (I), and in developing (E) and mature (H) gynoecia.

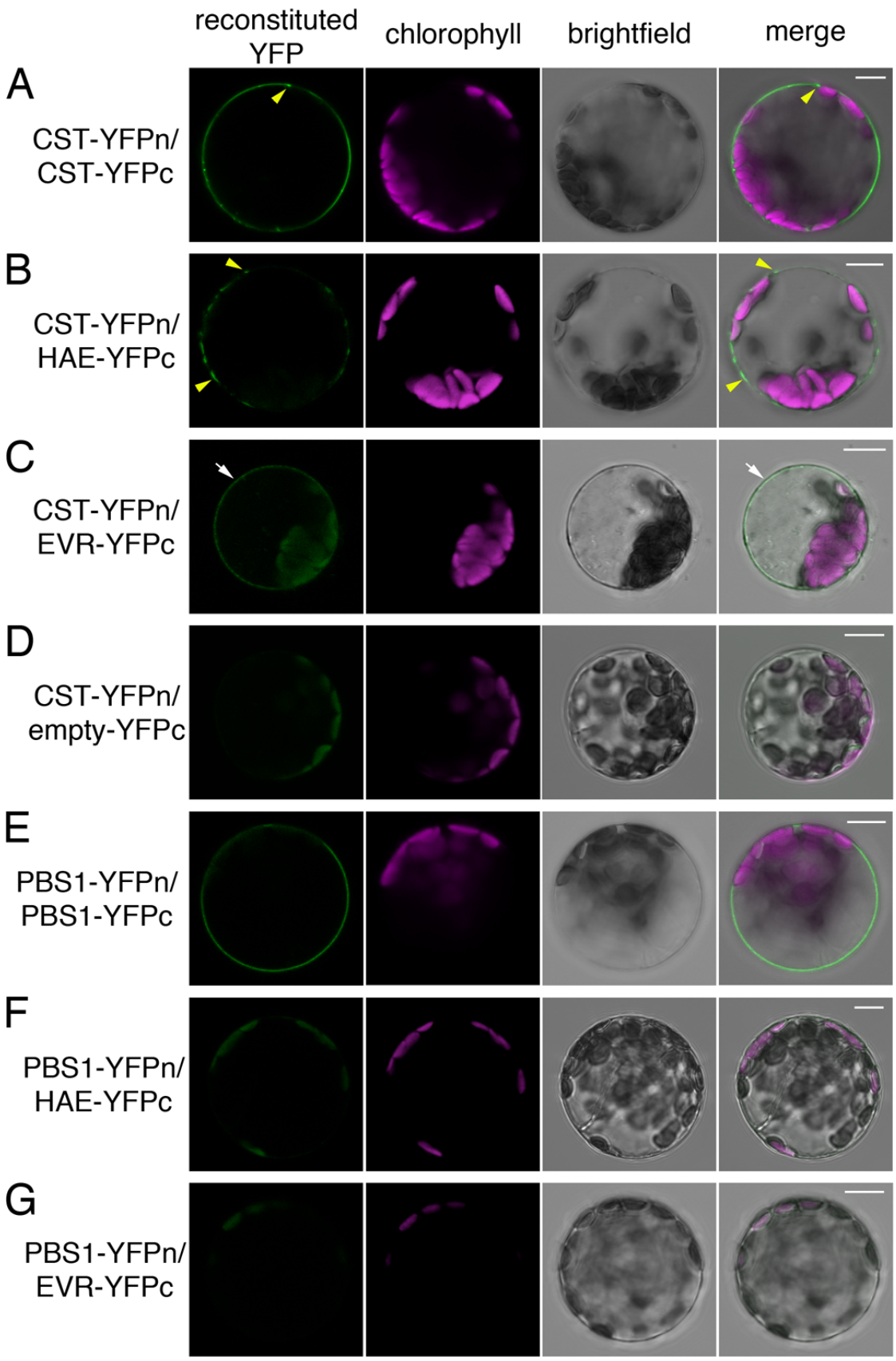
reconstituted YFP
chlorophyll
brightfield
merge
A
CST-YFPn/ CST-YFPc
B
CST-YFPn/ HAE-YFPc
C
CST-YFPn/ EVR-YFPc
D
CST-YFPn/ empty-YFPc
E
PBS1-YFPn/ PBS1-YFPc
F
PBS1-YFPn/ HAE-YFPc
G
PBS1-YFPn/ EVR-YFPc

Figure 2.7. CST interacts with HAE and EVR at the plasma membrane.

Bimolecular fluorescence complementation assays of transfected Arabidopsis protoplasts. Reconstituted YFP fluorescence (green), chlorophyll autofluorescence (magenta), brightfield and merged images are shown for each protoplast. **A,** CST (CST-YFPn/CST-YFPc) homodimerizes at the plasma membrane. An area of increased aggregation is indicated (arrowhead). **B,** CST (CST-YFPn) interacts with HAE (HAE-YFPc) in discrete subdomains (arrowheads) at the plasma membrane. **C,** CST (CST-YFPn) interacts with EVR (EVR-YFPc) in a uniform pattern at the plasma membrane (arrow). **D,** CST (CST-YFPn) does not interact with YFP (YFPc). **E,** The PBS1 class VII RLCK (PBS1-YFPn/PBS1-YFPc) homodimerizes at the plasma membrane. **F-G**, PBS1 (PBS1-YFPn) does not interact with either HAE (HAE-YFPc) or EVR (EVR-YFPc). Scale bars, 10 μm.

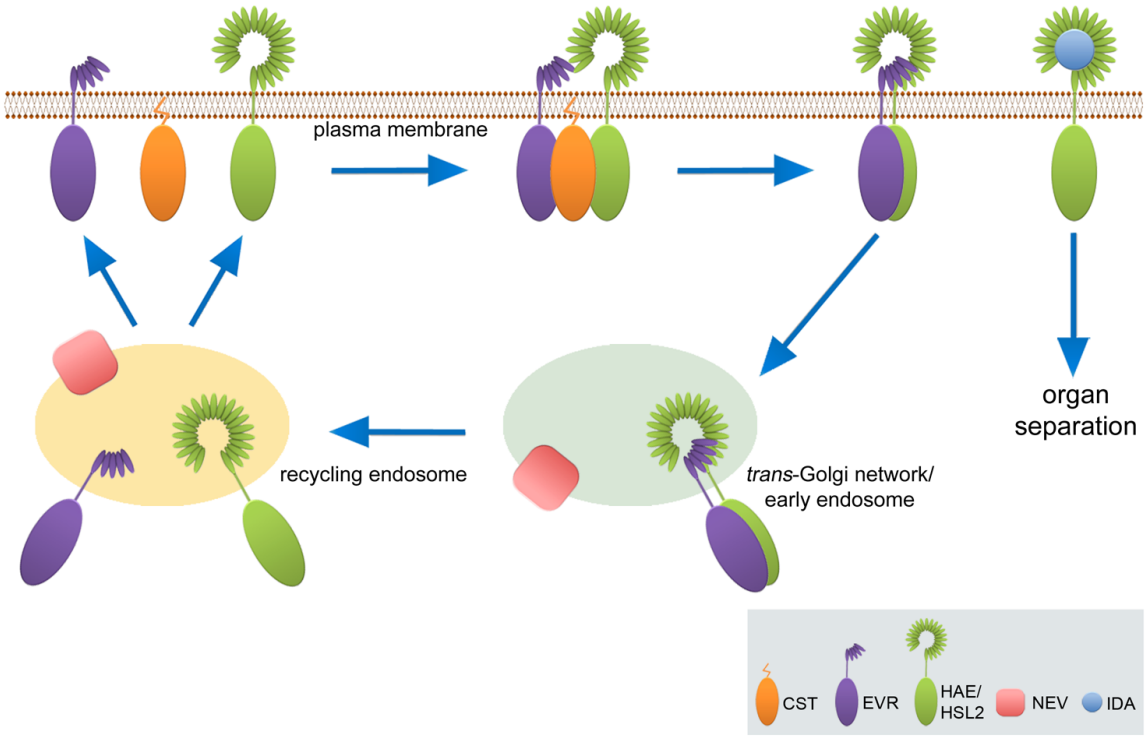

Figure 2.8. An integrated model of RLK function and membrane trafficking during organ abscission.

Interactions between a set of receptor-like kinases may modulate the timing and spatial extent of AZ cell loosening and separation. CST may sequester EVR at the plasma membrane and facilitate formation of HAE/EVR and HSL2/EVR receptor complexes. Interactions between EVR and HAE/HSL2 may trigger internalization of the receptor complexes. NEV may regulate trafficking of the EVR and HAE/HSL2 receptors through the early endosome/*trans*-Golgi network and recycling endosome, eventually restoring EVR and HAE/HSL2 to the plasma membrane. At the appropriate time in a discrete set of AZ cells, the secreted IDA peptide may bind to HAE/HSL2 at the cell surface, stabilizing the receptors and activating the downstream MAPK cascade that leads to cell separation and abscission.

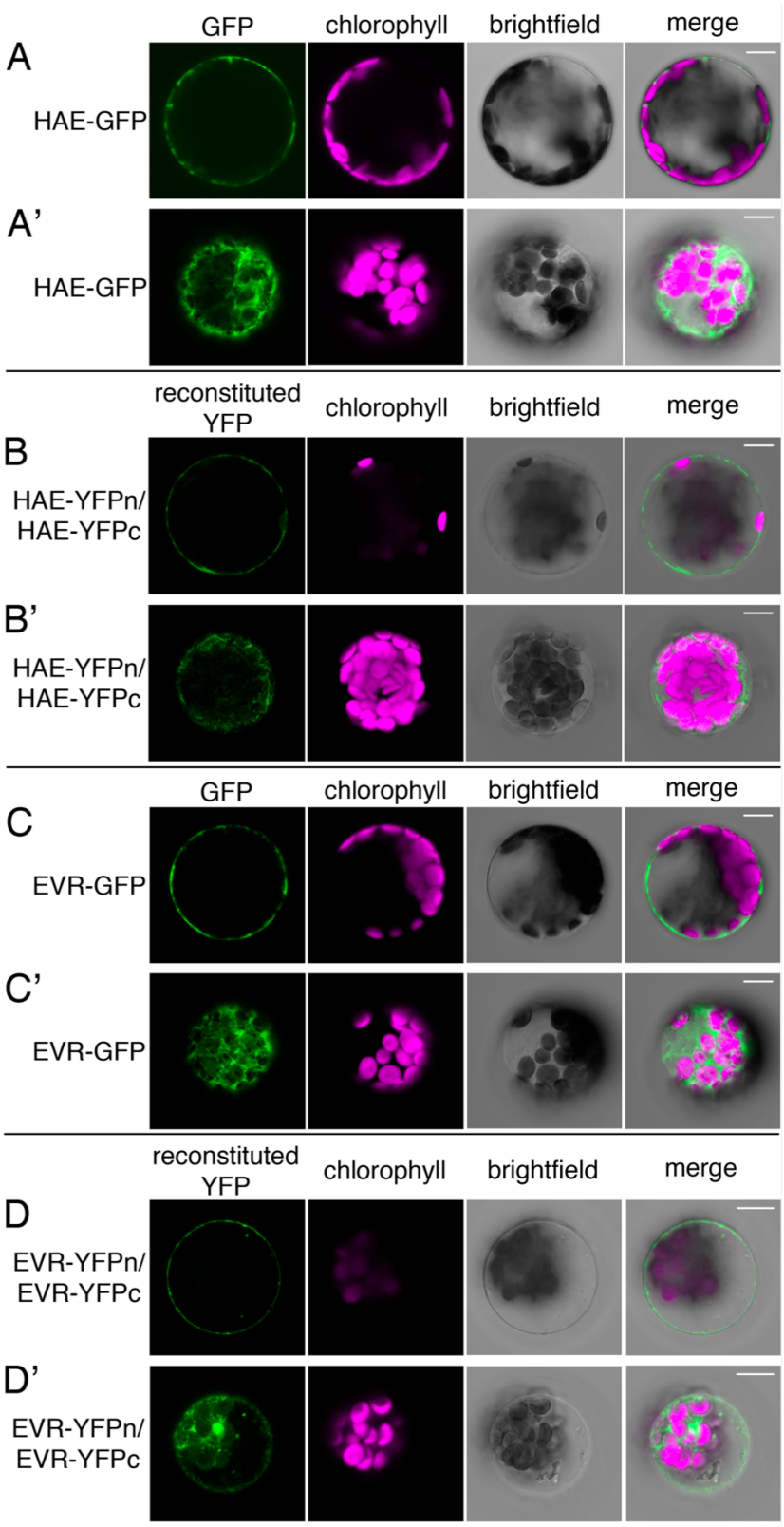
GFP
chlorophyll
brightfield
merge
A
HAE-GFP
A'
HAE-GFP
reconstituted YFP
chlorophyll
brightfield
merge
B
HAE-YFPn/ HAE-YFPc
B'
HAE-YFPn/ HAE-YFPc
GFP
chlorophyll
brightfield
merge
C
EVR-GFP
C'
EVR-GFP
reconstituted YFP
chlorophyll
brightfield
merge
D
EVR-YFPn/ EVR-YFPc
D'
EVR-YFPn/ EVR-YFPc

Figure 2.9. EVR and HAE homodimerize at the plasma membrane.

GFP or reconstituted YFP fluorescence (green), chlorophyll autofluorescence (magenta), brightfield and merged images are shown for each protoplast. Sets show images of the same protoplast at different focal planes. **A-A',** HAE (HAE-GFP) localizes at the plasma membrane (A) and internally (A'). **B-B',** HAE (HAE-YFPn/HAE-YFPc) homodimerizes at the plasma membrane (B) and internally (B'). **C-C',** EVR (EVR-GFP) localizes at the plasma membrane (C) and internally (C'). **D-D',** EVR (EVR-YFPn/EVR-YFPc) homodimerizes at the plasma membrane (D) and internally (D').
Scale bars, 10 μm.

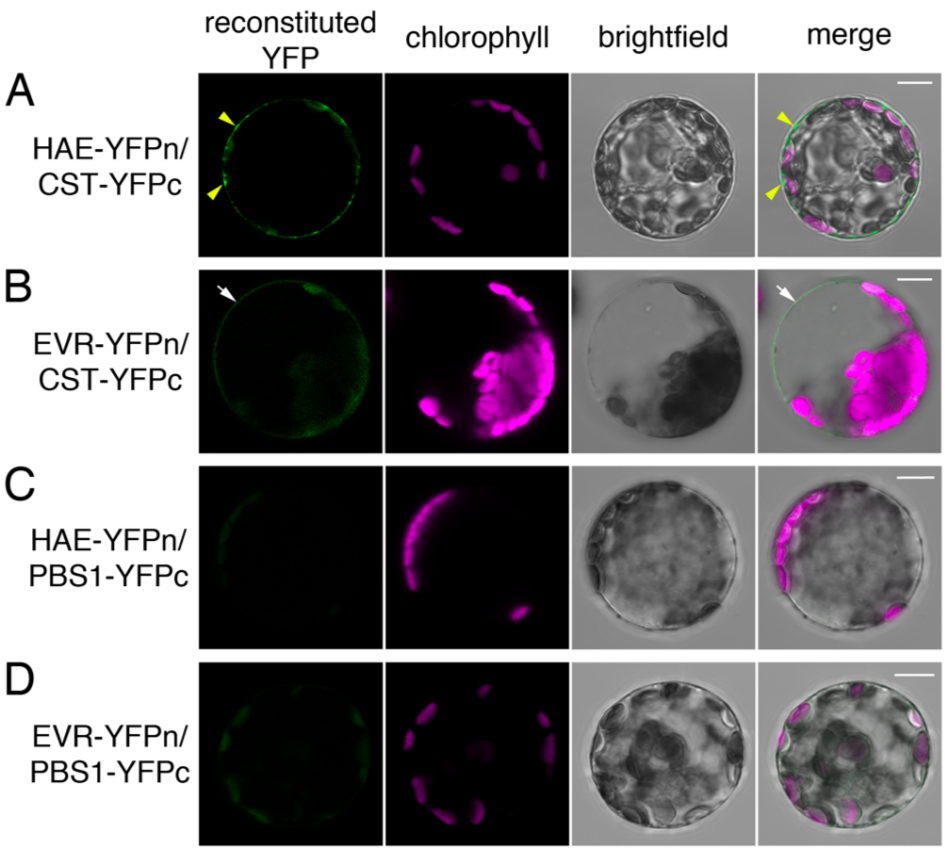

Figure 2.10. CST interacts with HAE and EVR at the plasma membrane.

Receptor-like kinase interactions and non-interactions (Fig. 2.7) were confirmed by performing BiFC assays in protoplasts transfected with the inverse YFP fusions. Reconstituted YFP fluorescence (green), chlorophyll autofluorescence (magenta), brightfield and merged images are shown for each protoplast. **A,** HAE (HAE-YFPn) interacts with CST (CST-YFPc) in discrete subdomains (arrowheads) at the plasma membrane. **B,** EVR (EVR-YFPn) interacts with CST (CST-YFPc) uniformly at the plasma membrane (arrow). **C,** HAE (HAE-YFPn) does not interact with the PBS1 (PBS1-YFPc) class VII RLCK. **D,** EVR (EVR-YFPn) does not interact with PBS1 (PBS1-YFPc).
Scale bars, 10 μm.

REFERENCES

Ade J, DeYoung BJ, Golstein C and Innes RW (2007) Indirect activation of a plant nucleotide binding site-leucine-rich repeat protein by a bacterial protease. Proc Natl Acad Sci USA **104**: 2531-2536

Aker J, Borst JW, Karlova R and de Vries SC (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

Aniento F, Gu F, Parton RG 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

Boisson B, Giglione C and Meinnel T (2003) Unexpected protein families including cell defense components feature in the N-myristoylome of a higher eukaryote. J Biol Chem **278**: 43418-43429

Butenko MA, Patterson SE, Grini PE, Stenvik GE, Amundsen SS, Mandal A and Aalen RB (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, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones JD, Felix G and Boller T (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature **448**: 497-500

Cho SK, Larue CT, Chevalier D, Wang H, Jinn TL, Zhang S and Walker JC (2008) Regulation of floral organ abscission in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **105**: 15629-15634

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

Diévart A, Dalad M, Tax FE, Lacey AD, Huttly A, Li J and Clark SE (2003) *CLAVATA1* dominant-negative alleles reveal functional overlap between multiple receptor kinases that regulate meristem and organ development. Plant Cell **15**: 1198-1211

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

Gao M, Wang X, Wang D, Xu F, Ding X, Zhang Z, Bi D, Chen YT, 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

Goring DR and Walker JC (2004) Self-rejection—a new kinase connection. Science **303**: 1474-1475

Hanks SK (2003) Genomic analysis of the eukaryotic protein kinase superfamily: a perspective. Genome Biol **4**: 111

Hanks SK and Hunter T (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J **9**: 576-96

Heese A, Hann DR, Gimenez-Ibanez S, Jones AM, He K, Li J, Schroeder JI, Peck SC and Rathjen JP (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 MA, Shah K, Russinova E, de Vries SC and Visser AJ (2008) Fluorescence fluctuation analysis of Arabidopsis thaliana somatic embryogenesis receptor-like kinase and brassinosteroid insensitive 1 receptor oligomerization. Biophys J **94**: 1052-1062

Horn MA and Walker JC (1994) Biochemical properties of the autophosphorylation of RLK5, a receptor-like protein kinase from *Arabidopsis thaliana*. Biochim Biophys Acta **1208**: 65-74

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

Jinn TL, Stone JM and Walker JC (2000) *HAESA*, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. Genes Dev **14**: 108-117

Jorgensen R, Snyder C and Jones JG (1987) T-DNA is organized predominantly in inverted repeat structures in plants transformed with Agrobacterium tumefaciens C58 derivatives. Mol Gen Genet **207**: 471-477

Jurca ME, Bottka S and Fehér A (2008) Characterization of a family of Arabidopsis receptor-like cytoplasmic kinases (RLCK class VI). Plant Cell Rep **27**: 739-748

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 transduce self-incompatibility signaling in Brassica rapa. Plant Cell **19**: 3961-3973

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

Leslie ME, Lewis MW, Youn JY, Daniels MJ and Liljegren SJ (2010) The EVERSHED receptor-like kinase modulates floral organ shedding in *Arabidopsis*. Development **137**: 467-476

Lewis MW, Leslie ME, Fulcher EH, Darnielle L, Healy PN, Youn JY and Liljegren SJ (2010) The SERK1 receptor-like kinase regulates organ separation in Arabidopsis flowers. Plant J **62**: 817-828

Liljegren SJ, Leslie ME, Darnielle L, Lewis MW, Taylor SM, Luo R, Geldner N, Chory J, Randazzo PA, Yanofsky MF and Ecker JR (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

Luo R, Ahvazi B, Amariei D, Shroder D, Burrola B, Losert W and Randazzo PA (2007) Kinetic analysis of GTP hydrolysis catalysed by the Arf1-GTP-ASAP1 complex. Biochem J **402**: 439-447

Maranda B, Brown D, Bourgoin S, Casanova JE, Vinay P, Ausiello DA 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

Michels CV (2002) Suppression Analysis. In Genetic techniques for biological research: a case study approach. John Wiley & Sons, Hoboken, NJ, pp 91-97

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H and Shibuya N (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. Proc Natl Acad Sci USA **104**: 19613-19618

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

Muto H, Yabe N, Asami T, Hasunuma K and Yamamoto KT (2004) Overexpression of *Constitutive Differential Growth1* gene, which encodes a RLCKVII-subfamily protein kinase, causes abnormal differential and elongation growth after organ differentiation in Arabidopsis. Plant Physiol **136**: 3124-3133

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

Nelson BK, Cai X and Nebenführ A (2007) A multi-color set of *in vivo* organelle markers for colocalization studies in *Arabidopsis* and other plants. Plant Journal **51**: 1126-36

Punwani JA, Hutchinson CE, Schaller GE and Kieber JJ (2010) The subcellular distribution of the Arabidopsis histidine phosphotransfer proteins is independent of cytokinin signaling. Plant J **62**: 473-482

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

Sessions A, Burke E, Presting G, Aux G, McElver J, Patton D, Dietrich B, Ho P, Bacwaden J, Ko C, Clarke JD, Cotton D, Bullis D, Snell J, Miguel T, Hutchison D, Kimmerly B, Mitzel T, Katagiri F, Glazebrook J, Law M and Goff SA (2002) A high-throughput Arabidopsis reverse genetics system. Plant Cell **14**: 2985-2994

Shao F, Golstein C, Ade J, Stoutemyer M, Dixon JE and Innes RW (2003) Cleavage of Arabidopsis PBS1 by a bacterial type III effector. Science **301**: 1230-1233

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

Shiu SH and Bleecker AB (2001) Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci USA **98**: 10763-10768

Shiu SH, Karlowski WM, Pan R, Tzeng YH, Mayer KF and Li WH (2004) Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. Plant Cell **16**: 1220-1234

Shpak ED, Lakeman MB and Torii KU (2003) Dominant-negative receptor uncovers redundancy in the Arabidopsis ERECTA leucine-rich repeat receptor-like kinase signaling pathway that regulates organ shape. Plant Cell **15:** 1905-1110

Sorek N, Bloch D and Yalovsky S (2009) Protein lipid modifications in signaling and subcellular targeting. Curr Opin Plant Biol **12**: 724-720

Stael S, Bayer RG, Mehimer N and Teige M (2011) Protein N-acylation overrides differing targeting signals. FEBS Letters **585**: 517-522

Stenvik GE, Butenko MA, Urbanowicz BR, Rose JK and Aalen RB (2006) Overexpression of *INFLORESCENCE DEFICIENT IN ABSCISSION* activates cell separation in vestigial abscission zones in *Arabidopsis*. Plant Cell **18**: 1467-1476

Stenvik GE, Tandstad NM, Guo Y, Shi CL, Kristiansen W, Holmgren A, Clark SE, Aalen RB and Butenko MA (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

Swiderski MR and Innes RW (2001) The Arabidopsis PBS1 resistance gene encodes a member of novel protein kinase subfamily. Plant J **26**: 101-112

Veronese P, Nakagami H, Bluhm B, Abuqamar S, Chen X, Salmeron J, Dietrich RA, Hirt H and Mengiste T (2006) The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in Arabidopsis resistance to necrotrophic and biotrophic pathogens. Plant Cell **18**: 257-273

Viotti C, Bubeck J, Stierhof Y-D, Krebs M, Langhans M, van den Berg W, van Dongen W, Richter S, Geldner N, Takano J, Jürgens G, de Vries SC, Robinson DG and Schumacher K (2010) Endocytic and secretory traffic in *Arabidopsis* merge in the trans-Golgi network/early endosome, an independent and highly dynamic organelle. Plant Cell **22**: 1344-1357.

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

Warren RF, Merritt PM, Holub E and Innes RW (1999) Identification of three putative signal transduction genes involved in R gene-specified disease resistance in Arabidopsis. Genetics **152**: 401-412

Wu FH, Shen SC, Lee LY, Lee SH, Chan MT and Lin CS (2009) Tape-Arabidopsis-Sandwich – a simpler Arabidopsis protoplast isolation method. Plant Methods **5**: 16

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

Zappel NF and Panstruga R (2008) Heterogeneity and lateral compartmentalization of plant plasma membranes. Curr Opin Plant Biol **6**: 632-640

Zeuzem S, Feick P, Zimmermann P, Haase W, Kahn RA and Shulz I (1992) Intravesicular acidification correlates with binding of ADP-ribosylation factor to microsomal membranes. Pro Natl Acad Sci USA **89**: 6619-6623

Zhang J, Lei W, Xiang T, Liu Z, Laluk K, Ding X, Zou Y, Gao M, Zhang X, Chen S, Mengiste T, Zhang Y and Zhou JM (2010) Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. Cell Host Microbe **7**: 290-301

Zhu Y, Wang Y, Li R, Song S, Wang Q, Huang S, Jin JB, Liu CM 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

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T and Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell **125**: 749-760

CHAPTER 3

ARF-GAP REDUNDANCY IN *ARABIDOPSIS* DEVELOPMENT

ABSTRACT

ADP-ribosylation factor GTPase activating proteins (ARF-GAPs) play an important role in membrane trafficking and are found in all eukaryotes. In yeast the Age2 ARF-GAP functions redundantly with the Gcs1 ARF-GAP in regulating the traffic leaving the *trans*-Golgi network. This gene family has expanded in *Arabidopsis* where there are two Age2-like ARF-GAPs and two Gcs1-like ARF-GAPs. One of the Age2-like ARF-GAPs, NEVERSHED (NEV), has been characterized and plays a role in the membrane trafficking necessary for floral organ abscission. To determine if NEV has broader roles in plant development which are hidden by functional redundancy, we performed pairwise analysis of *nev* loss-of-function mutations with the other Age2-like and both Gcs1-like ARF-GAPs. We have discovered that the Age2-like NEV and the Gcs1-like ARF-GAP DOMAIN6 (AGD6) ARF GAPs redundantly control plant growth and are essential for reproduction. Plants carrying mutations in both of these ARF-GAPs are sterile, severely dwarfed, and show cell expansion defects in leaves. Additionally, there appear to be significant defects in the root meristem. Our studies provide a unique opportunity to investigate the complex connections between regulation of membrane trafficking and cell growth in a multicellular eukaryotic system.

INTRODUCTION

Membrane trafficking pathways are a highly complex system integral for the growth and development of multicellular organisms. These pathways are critical for the localization of newly synthesized proteins, cycling of receptors and signaling proteins to and from membranes, and targeting proteins for degradation. While trafficking pathways in plants show conservation with those found in mammals, the routes found after the Golgi appear to have become differentiated between plants and mammals (Richter et al., 2009).

ADP-ribosylation factor GTPase-activating proteins (ARF-GAPs) and ADP-ribosylation factor GTPase-exchange factors (ARF-GEFs) are eukaryotic proteins involved in membrane trafficking, specifically at the steps of cargo recruitment and sorting and vesicle budding. Both proteins interact with the ARF G-proteins and function in cycling them between active and inactive forms (Anders and Jurgens, 2008). The ARF-GEFs bind to an inactive ARF and replace GDP with GTP, thereby activating them, while the ARF-GAPs bind to the active form of ARF G-proteins, and facilitate the hydrolysis of GTP, inactivating them

All ARF-GAPs have a common ARF-GAP domain, but the remainder of the protein structure can differ (Nie and Randazzo, 2006). The ARF-GAP domain contains a conserved zinc finger motif and is responsible for facilitating GTP hydrolysis in ARF G-proteins. In animals, there are seven classes of ARF-GAPs based on the presence of additional domains; while in *Arabidopsis* there are three classes (Vernoud et al., 2003; Inoue and Randazzo, 2007). *Arabidopsis* class I ARF-GAPs are the closest to those seen in mammals as they contain the most

similarity in protein structure and domains (Vernoud et al., 2003). In animals, ARF-GAPs have been found to interact with several other kinds of proteins, including lipid-modifying enzymes, protein kinases, SNARE proteins, and coatamer, the vesicle coat protein (Inoue and Randazzo, 2007; Spang et al., 2010). In *Arabidopsis* less in known about the interactions of ARF-GAPs, they have been shown to interact with ARF proteins and to co-localize with proteins that are also active in membrane trafficking, such as SNARE proteins (Liljegren et al., 2009; Stefano et al., 2010).

In yeast Age2 and Gcs1 are a pair of functionally redundant ARF-GAPs that play an essential role in trafficking from the *trans*-Golgi network (TGN) and in trafficking from the TGN to other parts of the cell (Poon et al., 2001). When Age2 and Gcs1 are expressed at insufficient levels, the cells are unable to transport proteins from the *trans*-Golgi network to the vacuole, from the Golgi to the plasma membrane and have difficulties performing endocytosis properly (Poon et al., 2001). In the double mutants there is the formation of membrane structures resembling Berkeley bodies and a loss of the TGN. Berkeley bodies, previously seen in the yeast ARF-GEF mutant, *sec7*, appear to be made up of parts of the Golgi and impede traffic through the Golgi (Poon et al., 2001; Novick et al., 1980). Gcs1 also has overlapping activity with another yeast ARF-GAP, Glo3 (Poon et al., 1999). When inadequate levels of these proteins are available these, cells show a build up of the endoplasmic reticulum (ER) that is lethal. This pair of genes redundantly controls essential retrograde traffic from the Golgi to the ER (Poon et al., 1999).

There are two Age2-like and two Gcs1-like ARF-GAPs in *Arabidopsis.* The two Age2-like ARF-GAPs are NEV and AGD15, and the two Gcs1-like ARF-GAPs

are AGD6 and AGD7. Of these four genes only one, the class II ARF-GAP NEV, has been functionally characterized (Liljegren et al., 2009; Stefano et al., 2010). Mutations in *NEV* result in loss of floral organ shedding, called abscission. NEV localizes to the TGN and the recycling endosome. In *nev* plants there are two detectable trafficking defects, a build up of vesicles between the plasma membrane and the cell wall and circularized multilamellar structures in place or in addition to the Golgi apparatus that are likely chimeric fusions of the Golgi and TGN (Liljegren et al., 2009). The Golgi phenotype is very similar to a defect that has been seen in plants carrying mutations of *V-ATPase-a1* and plants treated with Concamycin-A (Dettmer et al., 2005; Strompen et al., 2005; Viotti et al., 2010). The loss of abscission in *nev* plants are proposed to be due to disruption of trafficking through this chimeric Golgi/TGN structure, as suppressor mutations that restore organ shedding in *nev* flowers selectively restore the independence of the Golgi and TGN (Leslie et al., 2010; Lewis et al., 2010).

Redundancy of *Arabidopsis* ARF-GAPs has been shown among the class I ARF-GAPs. Mutations in VAN3/SCARFACE (VAN3/SCR) results in a highly fragmented vascular network, most likely caused by disruption of intracellular PIN1 transport (Koizumi et al., 2005; Sieburth et al., 2006). This phenotype is enhanced by additional mutations in the *AGD1, AGD2* and *AGD4* ARF-GAP genes. In the higher order mutants there is more fracturing of the venation pattern as well as breaks in the mid-vein and a failure of the mid-vein to attach to the vasculature of the stem, which was not seen in the *van3/scr* single mutant (Sieburth et al., 2006).

To explore the possibility of redundancy similar to that seen between Age2 and Gcs1 in yeast pairwise mutant combinations were made of all the *Arabidopsis* Age2-like and Gcs1-like genes. While most of the double mutants looked very similar to wild type, the *nev agd6* double showed extreme developmental defects. Here we look deeply into the developmental defects displayed in this double mutant to help to provide a deeper understanding between membrane trafficking and plant development.

MATERIALS AND METHODS

Plants and Growth Conditions

agd7-2 (SALK_048407) contains a T-DNA insertion (Alonso et al., 2003). *agd6-2* and *agd15-4* were obtained through screens designed for the Seattle TILLING Project (Erin Friedmann, Lalitree Darnielle, Ji-Young Youn and Sarah Liljegren, unpublished) (Till et al., 2003). *agd6-2* creates an early stop at codon 29, and *agd15-4* affects splicing. The genotyping strategies for these alleles are contained in Table 3.2. The *nev-3* allele was described earlier (Liljegren et al., 2009).

To generate the *pAGD6::*GUS lines a 261 bp section of DNA starting 258 bp upstream of the *AGD6* start site and ending at the second codon of *AGD6* was amplified through PCR from Col DNA using primers 5'-CACCATCTTGGGCATCGTCTAC-3' and 5'-CCCAACTATCCCATCCTCC-3' and was cloned into pENTR/D-TOPO (Life Technologies, Carlsbad, CA) and recombined into pGWB3 (Nakagawa et al., 2007).

The coding region for *AGD6* was amplified from the U87737 (ABRC), using primers 5'-CACCATGGCGGCGAC-3' and 5'-TTAGAGAAAACCTCCACCAGTCCAAG-3'. Using a BamHI site in the first exon of the *AGD6* coding region, the promoter region was combined with the *AGD6* coding region to create *pAGD6::AGD6*. *pAGD6::AGD6* was recombined into pGWB40 (Nakagawa et al., 2007) to generate *pAGD6::AGD6*-YFP (I. Chen and M. Simon).

Root growth was observed by growing plants vertically on 1X Murashige and Skoog (MS) salts supplemented with 0.5% sucrose and 0.8% agar. Plates were scanned daily using a UMAX Powerlook 1000 scanner (Techville Inc., Dallas, TX) and root length was measured using NIH ImageJ software on a Wacom Cintiq tablet (Wacom Company, Ltd., Tokyo, Japan).

Microscopy

For scanning electron microscopy samples were fixed as described (Liljegren et al., 2009), samples were then 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). For transmission electron microscopy the samples were prepared as previously described in Liljegren et al., 2009. Confocal laser scanning microscopy was performed with a Zeiss LSM-710 (Carl Zeiss Thornwood, NY), and the brightness and contrast of the images were adjusted with Adobe Photoshop CS4 (Adobe, Mountain View, CA). Propidium iodide and SYTOX orange staining were performed as previously described (Helariutta et al., 2000; Truernit and Haseloff, 2008). NIH image J software was

used to measure cell areas of leaf epidermal cells within defined areas (M. Simon and S. Liljegren).

Antibody Production

A C-terminal section of the AGD6 protein corresponding to amino acids 243-459 was PCR amplified using 5'-CACCAGAAATAATCAACAGGATG-3' and 5'-TTAGAGAAAACCTCCACCAGTCCAAG-3' using *AGD6* cDNA U87737 (ABRC). This was inserted into pENTR/D-TOPO (Life Technologies, Carlsbad, CA) and then recombined into pDEST17 (Life Technologies, Carlsbad, CA) to produce a 6xHis-tagged recombinant protein in *E. coli*. The recombinant protein was purified using Ni^{2+} affinity chromatography (M. Daniels and S. Liljegren) and used to immunize chickens (Pacific Immunology, Ramona, CA). Antibodies were purified from the egg yolks as previously described (M. Daniels and S. Liljegren) (Araujo et al., 2010).

RESULTS

Identification of *agd15*, *agd6* and *agd7* mutant alleles

A second Age2-like ARF-GAP (AGD15) and two Gcs1-like ARF-GAPs (AGD6 and AGD7) are found in *Arabidopsis* (Fig. 3B). A T-DNA insertion mutant of *AGD7* (*agd7-2;* SALK_048407*)* was found in the Salk T-DNA collection (Alonso et al., 2003) and shown to interrupt transcription (L. Darnielle and S. Liljegren). Loss-of-function point mutations of *AGD6* and *AGD15* were identified through TILLING screens of EMS-mutagenized populations (E. Friedmann, J-Y. Youn, L. Darnielle, and S. Liljegren; Till et al., 2003). The *agd6-2* mutation was found to encode an

mRNA with an early stop codon, and *agd15-4* was confirmed to affect splicing. In the single mutants of each of these ARF-GAPs, no obvious phenotypes were initially detected.

NEV and *AGD6* redundantly control plant growth and reproduction

In order to investigate redundancy among the Age2-like and Gcs1-like ARF-GAPs in *Arabidopsis*, we have generated most of the double and triple mutants. First we made the pair-wise mutant combinations to analyze potential redundancy with *NEV: nev agd6, nev agd7* and *nev agd15.* Interestingly, we found that only the *nev agd6* double mutant has severe developmental defects. These plants did not survive if the seeds were initially planted on soil and required transplantation from nutrient rich plates. The *nev agd6* double mutants were very small and few of them survived long enough to flower. Those that did manage to produce flowers were infertile (Fig. 3.2A). Additionally we produced the other double mutant combinations: *agd6 agd7*, *agd6 agd15* and *agd7 agd15*, and saw no obvious developmental defects. Characterization of *nev agd6* plants shows that *NEV* and *AGD6* together control major aspects of plant growth and development.

Three of the triple mutant combinations have been generated: *agd6 agd7 agd15, nev agd7 agd15,* and *nev agd6 agd7*. Additional growth defects were only seen in the *nev agd6 agd7* triple mutant, these plants were smaller and did not progress as far in their development as the *nev agd6* double mutants. For example, the leaves of *nev agd6 agd7* plants are very small and did not have the appearance of true leaves. Much additional work needs to be done to determine and quantitate

the true extent of the triple mutant developmental defects, but the plants are problematic as we have not as yet been able to keep them alive for longer than three weeks (Fig. 3.2B). The higher order mutants that have yet to be made are *nev agd6 agd15* and *nev agd6 agd7 agd15*, these mutants may prove difficult to produce as adding additional mutations to the *nev agd6* plants has already shown an especially severe degree of developmental problems.

AGD6 is broadly expressed during plant development

NEV is broadly expressed during a plant's life cycle, and the encoded protein localizes to the *trans*-Golgi network/early endosome and the recycling endosome (Liljegren et al., 2009). Analysis of available microarray data indicates that *AGD6* also has a broad expression pattern that overlaps with that of *NEV*, and both genes showed dramatically increased expression in the flowers and floral organs (Fig. 3.3A). To investigate whether *AGD6* is specifically expressed in specific cell-types within tissues, I linked the *AGD6* promoter region to the β-glucuronidase (GUS) reporter gene. The *AGD6* regulatory region was predicted to include the 258 nucleotides upstream of the *AGD6* translational start site, which includes all sequence up to the border of the upstream gene (Fig. 3.3B). The *AGD6::GUS* construct was transformed into wild type plants; 17 of the 51 T1 lines generated were tested for GUS staining in adult plants, and six seedlings from these 17 lines were stained in the next (T2) generation.

GUS staining revealed extensive expression of *AGD6* throughout plant development. In the seedlings high levels of staining were seen in the vasculature

of the roots and the leaves (Fig. 3.3C). The vein expression pattern continued throughout the whole plant, including the mature leaves (Fig. 3.3F) and the sepals (Fig. 3.3D). Expression was also seen in developing flower buds and pollen, the abscission zones, and the mature pollen (Fig. 3.3D,E,G,H). Although this may not be the complete *AGD6* expression pattern, there is much overlap with that of *NEV*. *NEV* expression is also seen in vasculature of the seedlings and leaves, inflorescence stems and developing flower buds (Liljegren et al., 2009).

Epidermal cell size is reduced in *nev agd6* leaves

To investigate the underlying cause of the *nev agd6* growth defects, the size of epidermal cells in *nev agd6* leaves compared to those of wild-type and the single mutants was analyzed (M Simon and S. Liljegren). The area of the five largest cells within a defined area were measured from scanning electron micrographs of the fifth leaf of *nev agd6*, *nev*, *agd6* and wild-type plants. Comparison of the different genotypes showed that *nev agd6* plants have smaller cells, not fewer. This result suggests that the reduced leaf size of *nev agd6* mutants is due to a deficiency in cell expansion, rather than to reduced cell division (Fig. 3.4).

Primary root growth is affected in *nev agd6* mutants

Upon initial observation the growth defects seen in the shoots of the plants seemed to be mirrored in the roots (Fig. 3.5A). To quantify this observation plants were grown vertically on MS plates, and primary root length was measured at daily time points. The data revealed that the difference in growth between *nev agd6* and

wild type or either of the single mutants was significant by Day 6 (Fig. 3.5B). Root growth of the *agd6* single mutant was also significantly affected. As an *erecta* (*er*) mutation was also present in the *agd6* mutant background, additional analysis of the single mutant will be conducted in the presence of wild-type ER. *er* has previously been shown to cause slower growth of roots (Passardi et al., 2007).

The division and/or elongation zones of *nev agd6* roots are compressed

In plants, the root tip is a highly organized area. Cells grow in discrete files and enter distinct phases of growth at highly regulated times. At the lowest part of the root tip is the stem cell niche, above the stem cell niche is the meristematic, or division region. Here the cells stay small and continue to divide, from the meristematic region the cells enter the elongation zone, where the cells may elongate by as much as three times. From the elongation zone, the plants enter the differentiation zone, where the cells differentiate into their final cell fates. This is where root hairs make their first appearance.

When looking closely at the roots of the double mutant, it was clear that the root hairs started appearing much closer to the root tip in the *nev agd6* plants than in the wild type or single mutants (Fig. 3.6A,B). When this distance was measured and graphed, the distance from the root tip to the first root hair was significantly shorter in the double mutant than in the wild type or single mutants (Fig. 3.6C). This indicates that in *nev agd6* roots the differentiation zone is beginning much sooner than in wild type. This points to either or both the division zone and elongation zone being affected in the double mutant. Additional results from propidium iodide staining of

the *nev agd6* roots indicates that the division zone is most likely compressed in the double mutant.

To further examine the organization of the *nev agd6* root tips, the cell walls of mutant and wild-type roots were stained with propidium iodide and examined with confocal microscopy (Fig. 3.7A,B). The width of *nev agd6* roots was notably reduced, and the size of the root meristem or division zone was affected. The area of small cells, indicating the division zone, was abbreviated in the double mutant, giving way to elongated cells much sooner.

Ectopic cell death is seen in *nev agd6* root tips

SYTOX Orange stain was used to look at the amount of cell death seen in *nev agd6* root tips. Preliminary results show increased cell death in *nev agd6* root tips (Fig. 3.8). In order to verify these results, more repetitions need to be done using WT and both *nev* and *agd6* single mutants as controls.

AGD6 is localized in a punctate pattern within root cells

Transgenic *pAGD6::AGD6*-YFP lines were created and are now beginning to be analyzed in the T2 generation (I. Chen and S. Liljegren). Preliminary results show that multiple AGD6-YFP transgenic lines show fluorescent punctate structures throughout the cell (Fig. 3.9). These lines will also be used in rescue experiments to show that the developmental phenotype is the result of mutations in *agd6.*

As a second approach to track AGD6 localization, an antibody has been produced in chicken against the C-terminal region of recombinant AGD6 protein (M.

Daniels, C. Burr, I. Chen and S. Liljegren). The purified antiserum has been found to recognize AGD6 protein produced in *E. coli* (Fig. 3.10) and experiments are currently underway to determine if this antibody specifically recognizes AGD6 from plant seedling extracts.

DISCUSSION

In yeast, Age2 and Gcs1 redundantly control essential trafficking from the *trans*-Golgi network (Poon et al., 2001). Yeast not expressing the necessary amount of these proteins show a variety of trafficking defects such as a loss of the TGN and a build up of membranous structures that show similarity to the circularized Golgi structures called Berkley bodies that are found in other yeast trafficking mutants (Poon et al., 2001). These specific trafficking defects show a striking similarity to the putative chimeric circularized Golgi with fused TGN seen in the *nev* mutant plants (Liljegren et al., 2009). In order to explore the potential of similar redundancy in plants, the Age2-like and Gcs1-like ARF-GAPs in *Arabidopsis* were identified; AGD15, an Age2-like ARF GAP, AGD6 and AGD7, two Gcs1-like ARF-GAPs. The *nev agd6* mutants showed severe developmental defects, indicating that NEV and AGD6 have redundant roles in plant development and, as in yeast, the Age2-like and Gcs1-like ARF-GAPs play a redundant role in plants. We expect that gametophytic development will likely be blocked in the *nev agd6 agd7 agd15* quadruple mutant, since disrupting Age2 and Gcs1 ARF-GAP activity is lethal to single yeast cells.

NEV is highly expressed throughout plant development and is seen in nearly all tissues (Liljegren et al., 2009). However, while *AGD6* has a broad expression pattern and is present throughout the plant throughout development, its expression is limited compared to *NEV*. The areas where these two ARF-GAPs overlap may play a large role in the developmental defects seen in the double mutant. Especially important may be overlap of the expression of *NEV* and *AGD6* throughout the vasculature of the plants. If both of these genes are needed for proper development of the vasculature, this could be a reason for the large effect their loss has on plant development.

Root development in *Arabidopsis* is very carefully coordinated. Root tips have three main developmental zones: meristematic, elongation and differentiation. In the meristematic zone the cells are actively involved in division, from there they move to elongation, where cells may increases in length by three times. After they exit the elongation phase they begin to differentiate, the differentiation phase is clearly marked by the appearance of root hairs (reviewed in Bennett and Scheres, 2010). These distinct developmental zones are maintained by membrane trafficking, such as GN trafficking of the PIN proteins, which controls the protein gradients at specific membranes (Baluska et al., 2010). In the *nev agd6* mutants we see a breakdown in the development of the root tip. Specifically we see a much smaller root tip and suspect the abbreviation of the meristematic zone. Interestingly the reduction of meristem size is similar to the phenotype of some of the weak alleles of *gn* showing that membrane trafficking is important in the maintenance of these root zones (Geldner et al., 2004). Since GN plays such an important role in regulating

the localization of the PIN proteins, it will be essential to determine if AGD6 and NEV also play a role in keeping the PINs polarly localized.

The size of the root meristem is established by the very specific trafficking of many proteins. The PIN proteins need to be trafficked and maintained at specific membranes in order to pump auxin correctly. It is the correct flow of auxin that maintains the root meristem (Wisniewska et al., 2006). The PIN proteins polar localization is maintained through the interplay of the serine/threonine kinase PINOID (PID) and the ARF-GEF GNOM (GN). The loss of either of these proteins causes extreme developmental defects. *pid* mutants show incorrect root and shoot development, while *gn* mutants are unable to create an apical/basal axis and do not develop into seedlings (Mayer et al., 1991; Christensen et al., 2000). Disintegration of trafficking routes caused by the loss of functional NEV and AGD6 could disrupt the trafficking necessary to either establish or maintain the size of the root meristem.

Redundancy in ARF-GAPs has been previously seen in vascular development; *scarface/van3* shows increased vein patterning defects when combined with *agd1*, *agd2,* and *agd4* (Sieburth et al., 2006). The redundancy seen there worsened the phenotype of the single mutants, but new developmental defects were not seen. There is also redundancy among the ARF-GEFs in *Arabidopsis*, where it has been shown that GN can rescue *GNL1* mutants when expressed under the *GNL1* promoter (Richter et al., 2007). The *NEV AGD6* double mutant is the first instance of ARF-GAP redundancy showing profound developmental defects not seen in either single mutant.

The *nev agd6* double mutant offers a unique opportunity to dissect the cell signaling that controls root meristem size that is affected by defects in membrane trafficking. As in plants, in other eukaryotic systems such as worms, mice and flies, ARF-GAP families have likely diverged from yeast and many more genes may need to be knocked out to uncover their functions.

Acknowledgments

We thank M. Daniels I. Chen, M. Simon, E. Friedmann, L. Darnielle and J.Y. Youn for all the work they have put into this project; H. Kizer for help in the lab; We thank M. Duncan, B. Goldstein, S. Ahmed, and J. Reed for helpful discussions; T. Perdue, S. Ray and V. Madden for microscopy assistance; and the ABRC for DNA and seed stocks.

TABLES

Allele	Enzyme	PCR Products (bp)	Digest Products (bp)		Oligos
agd6-2	BsaHI	400	*agd6-2* Col	400 250, 150	5'-GATCTGATTCAGGTTGGATTGATCATCG-3' 5'-GCAGCGTTCGAATTGTACTTGGAG-3'
agd7-2		WT			5'-CCCGATATCGAAGCGACCAAAGC-3' 5'-GCTACGGGTAACACCTAAGAAGAC-3'
		T-DNA			5'-CCCGATATCGAAGCGACCAAAGC-3' 5'-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3'
agd15-4	EcoO109I		*agd15-4* Col		5'-GTTTCTCTCTCATTCTCTGATTCATC-3' 5'-CTCTCGAAATGTTGAGGCAA-3'

Table 3.1 Genotyping the *agd6*, *agd7*, and *agd15* mutant alleles.

Information to identify the loss-of-function *agd6*, *agd7*, and *agd15* mutant alleles is provided.

FIGURES

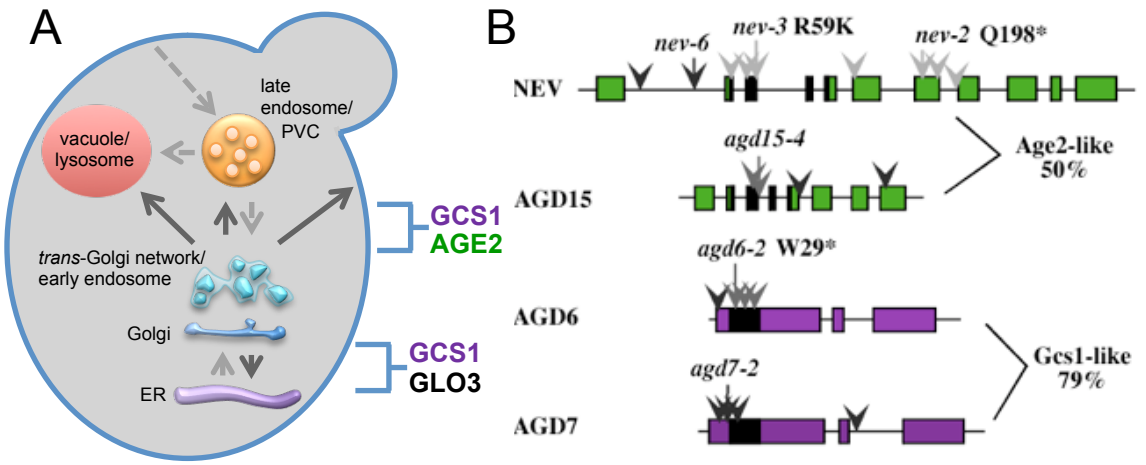

Figure 3.1. Identification of *Age2*-like and *Gcs1*-like ARF-GAP genes in *Arabidopsis*.

(A) In yeast, the Growth cold sensitive1 (Gcs1) and ArfGAP effector2 (Age2) ARF-GAPs act redundantly to regulate traffic leaving the *trans*-Golgi network. Gcs1 also acts together with the Glo3 ARF-GAP to regulate transport between the Golgi and Endoplasmic Reticulum (ER). Figure adapted from Poon et al., 2001.

(B) The *Arabidopsis* NEVERSHED (NEV) ARF-GAP shares 49% amino acid identity within its ARF GAP domain with that of Age2 (Liljegren et al., 2009). Within the *Arabidopsis* genome, another *Age2*-like gene, *ARF-GAP DOMAIN15* (*AGD15*), and two *Gcs1*-like genes, *AGD6* and *AGD7*, were identified. Loss-of-function mutations for *AGD15* and *AGD6* were identified through TILLING screens of EMS-mutagenized populations, and a loss-of-function mutation for *AGD7* was found in the Salk T-DNA collection. T-DNA insertions and EMS mutations within these genes are indicated by dark and light gray arrowheads or arrows, respectively.

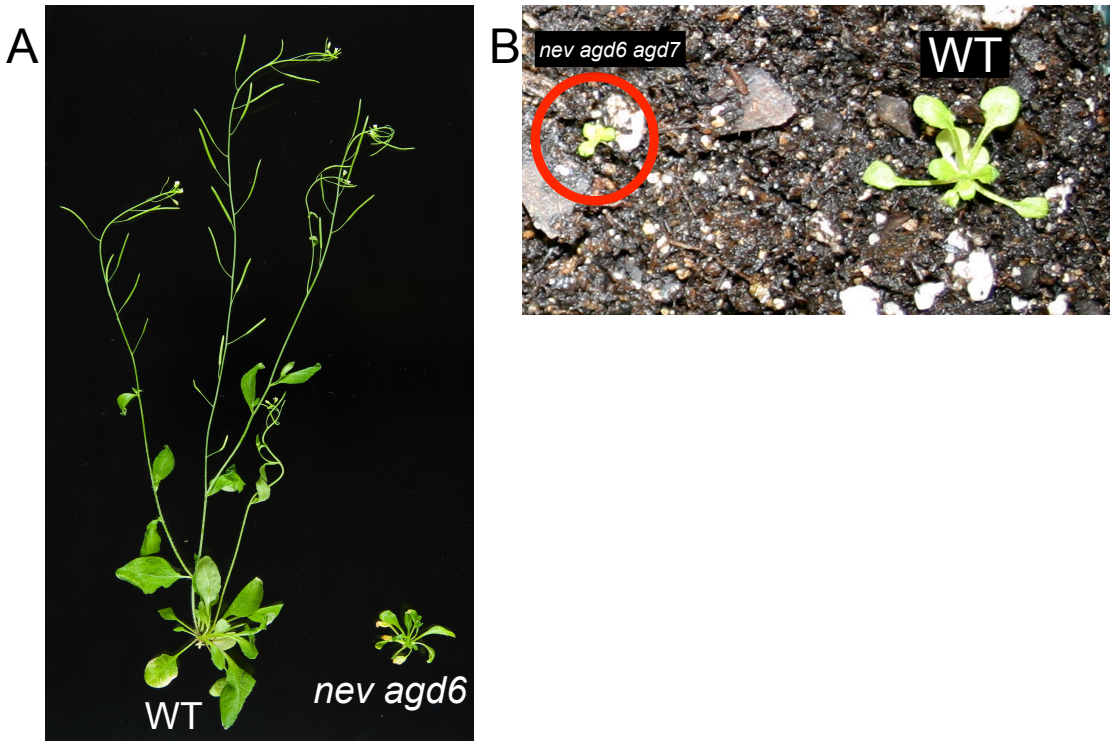

Figure 3.2. The NEV and AGD6 ARF-GAPs redundantly control plant growth and reproduction in *Arabidopsis*.

(A) Growth is severely affected in *nev agd6* double mutants compared to wild-type (WT). While some *nev agd6* plants are able to produce flowers, successful reproduction does not occur. The plants shown are about six weeks old.

(B) Mutations in *AGD7* further enhance the growth defects of *nev agd6* mutants. The wild-type and *nev agd6 agd7* plants shown are 21 days old.

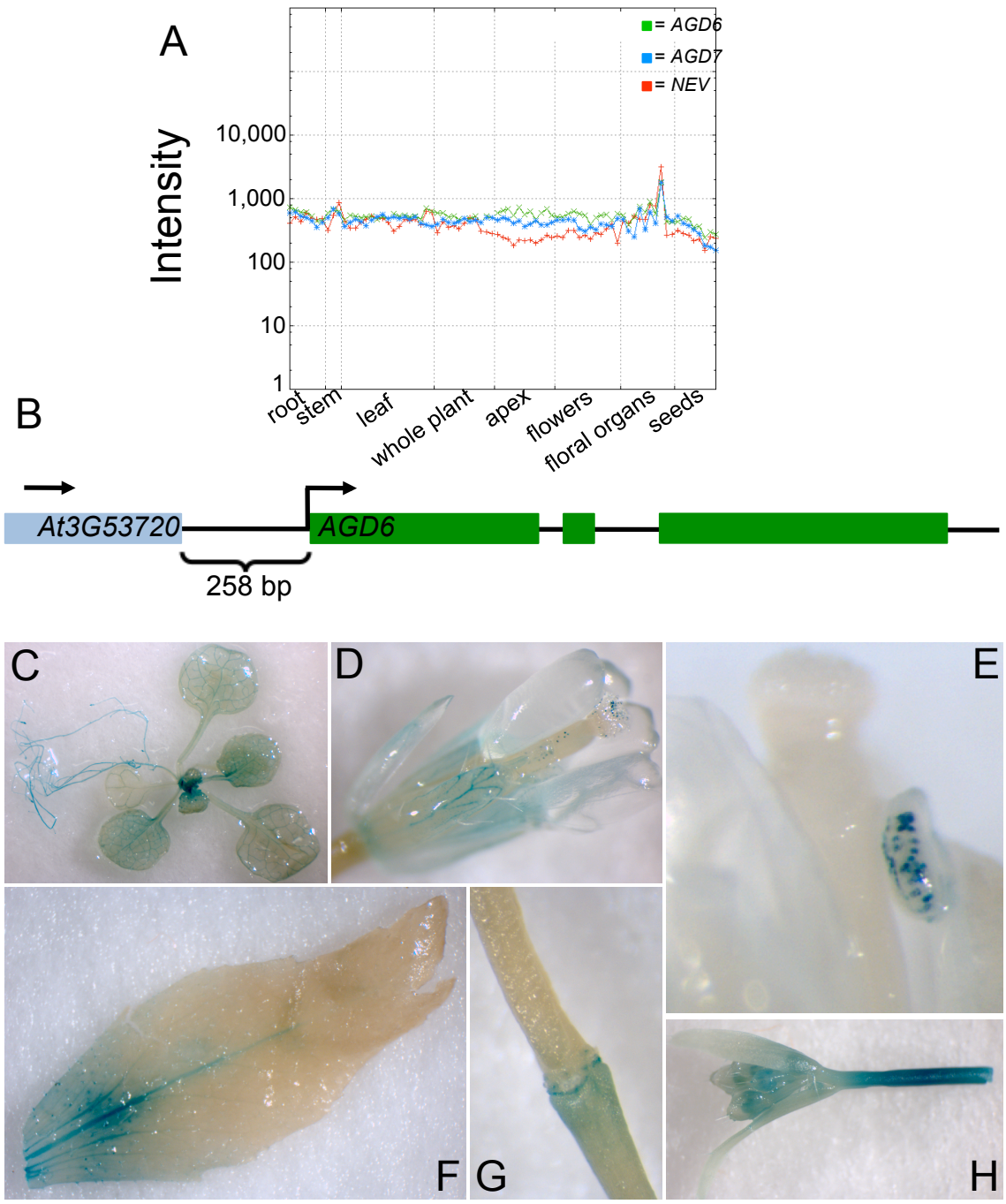

Figure 3.3. *AGD6* is expressed throughout plant development.

(A) Global expression studies in *Arabidopsis* have shown that *NEV, AGD6* and *AGD7* have broad, overlapping transcriptional profiles during plant development (Schmid et al., 2005).

(B) The regulatory region upstream of *AGD6* was used drive expression of the *β-Glucuronidase* reporter gene in transgenic plants.

(C-H) In *AGD6::GUS* plants, *GUS* expression is observed in the roots of developing seedings and the veins of leaves and floral organs (C,D,F), in pollen (E), in the floral abscission zones (G), and in the inflorescence stems and developing flowers (H).

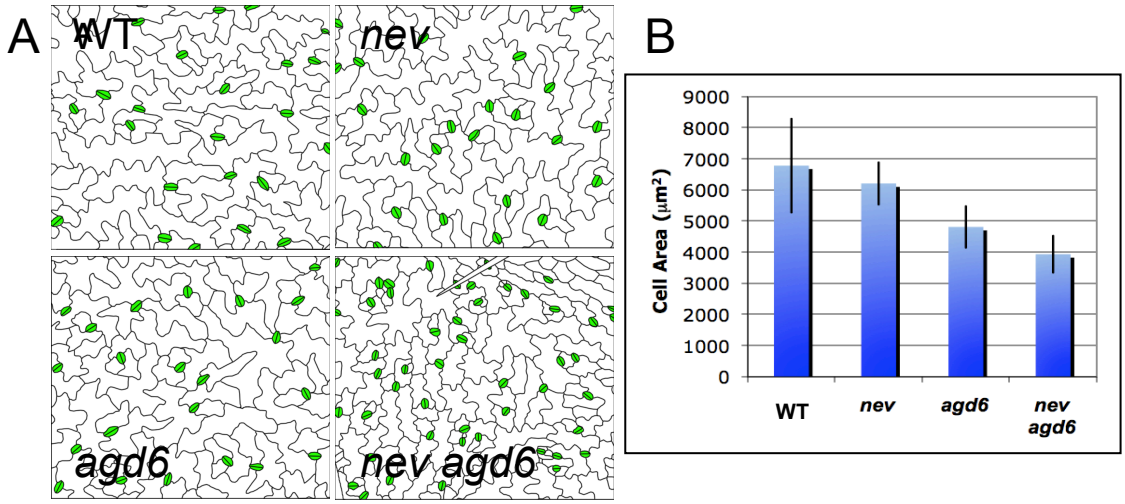

Figure 3.4. Reduced cell expansion is found in *nev agd6* leaves.

(A) Tracings of scanning electron micrographs of wild-type and mutant rosette leaves. Guard cells are shown in green.

(B) The size of the largest epidermal cells is significantly reduced in *nev agd6* leaves compared to those of wild-type. Within defined regions of X μm^2, the area of the largest five cells was measured using NIH Image J software.

Data collected by Matt Simon.

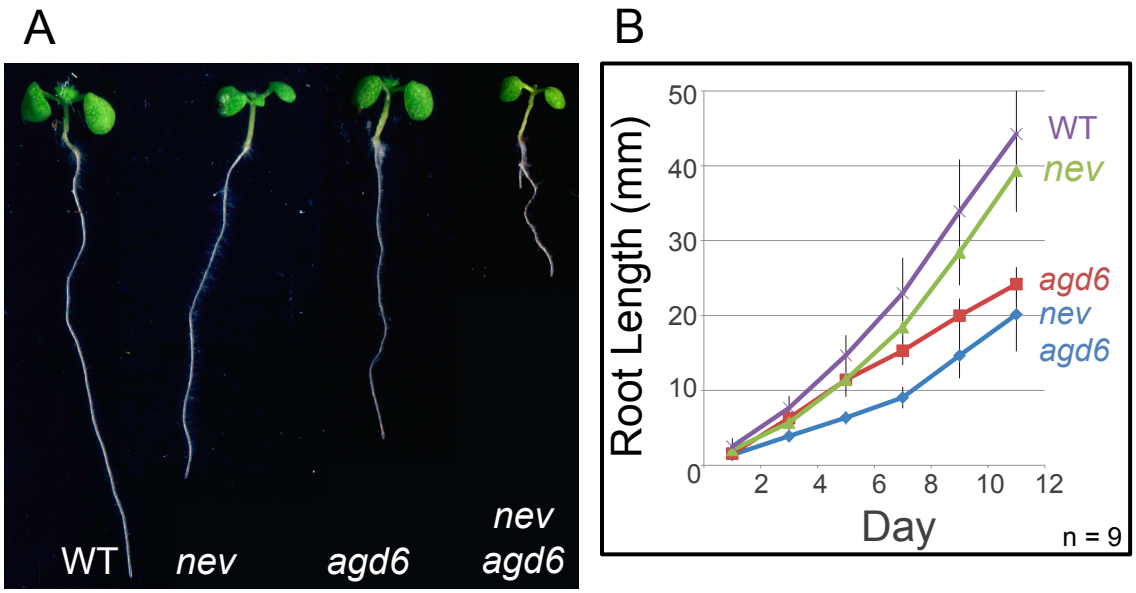

Figure 3.5. Root growth is affected in *nev agd6* plants.

(A) Growth of the primary root is reduced in *nev agd6* mutants compared to wild type (WT). 8-day-old seedlings of each genotype are shown.

(**B**) Significant differences in the root growth of *nev agd6* mutants are detected by Day 6. 9 roots per genotype were analyzed for each timepoint.

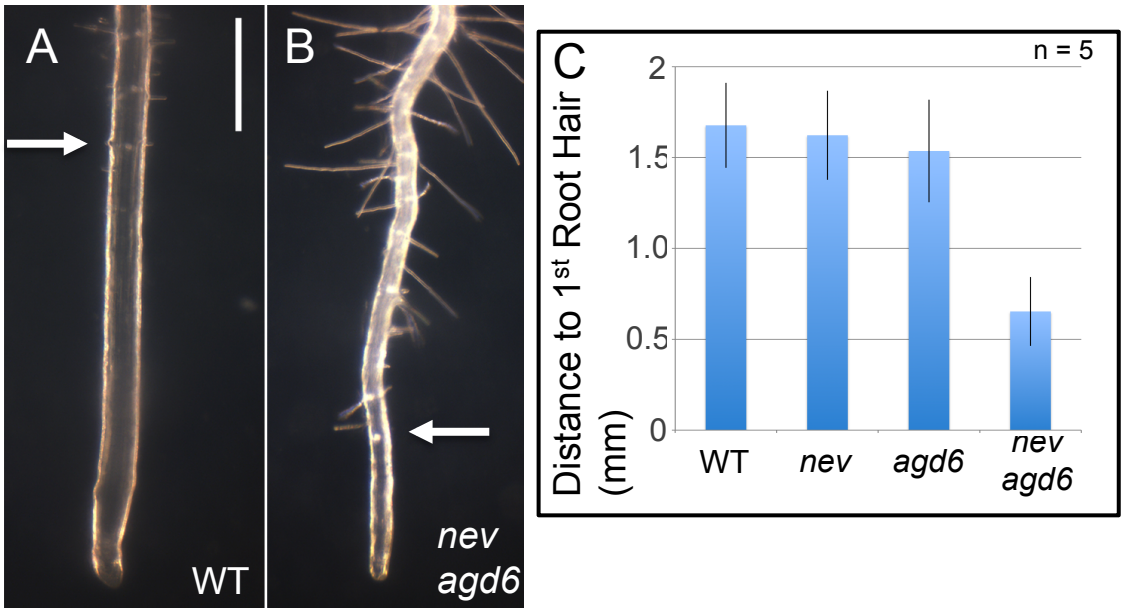

Figure 3.6. The elongation and/or division zones of *nev agd6* root tips are abbreviated.

(A,B) Primary root tips of representative 13 day-old wild-type (A) and *nev agd6* (B) plants. The positions of the first root hairs, which mark the root differentiation zones, are indicated by arrows. Scale bar, 500 μm.

(C) The first detectable root hairs are significantly closer to the tips of *nev agd6* roots than those of wild type. 5 roots per genotype were analyzed.

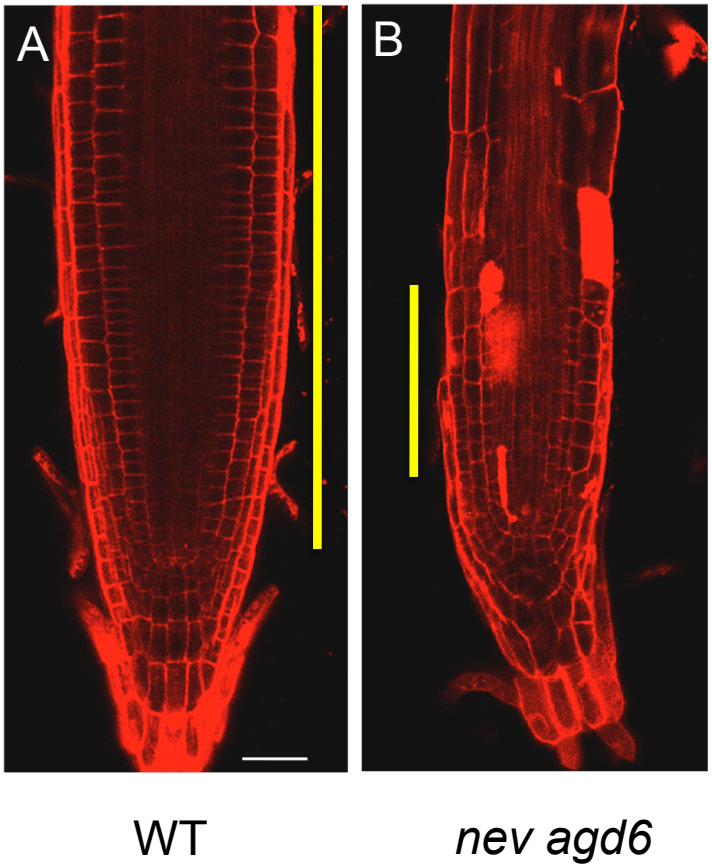

Figure 3.7. The meristems of *nev agd6* roots are smaller than those of wild type.

(A,B) The primary root tips of 13 day-old wild-type (A) and *nev agd6* (B) plants were stained with propidium iodide and viewed with confocal microscopy. The meristems of *nev agd6* roots contain fewer cells than those of wild type. Meristematic regions are indicated by yellow lines. Scale bar, 50 μm.

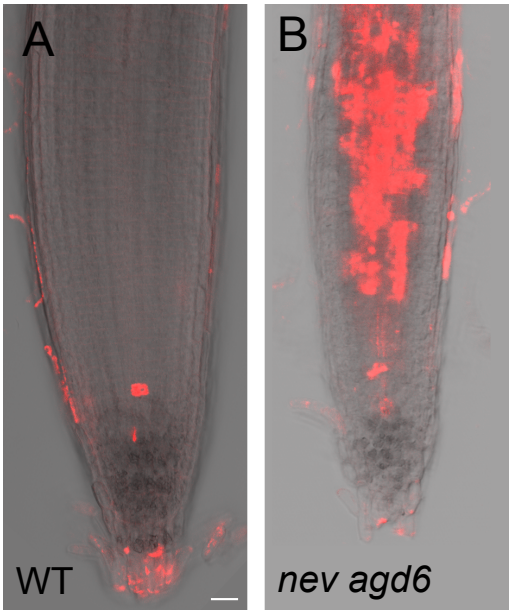

Figure 3.8. *nev agd6* mutant roots show ectopic cell death.

(A,B) The primary root tips of 15 day-old wild-type (A) and *nev agd6* (B) plants were stained with SYTOX Orange and viewed with DIC and confocal microscopy. Increased staining of nucleic acids in the stele region of *nev agd6* mutant suggests that ectopic cell death has occurred. Scale bar, 20 μm.

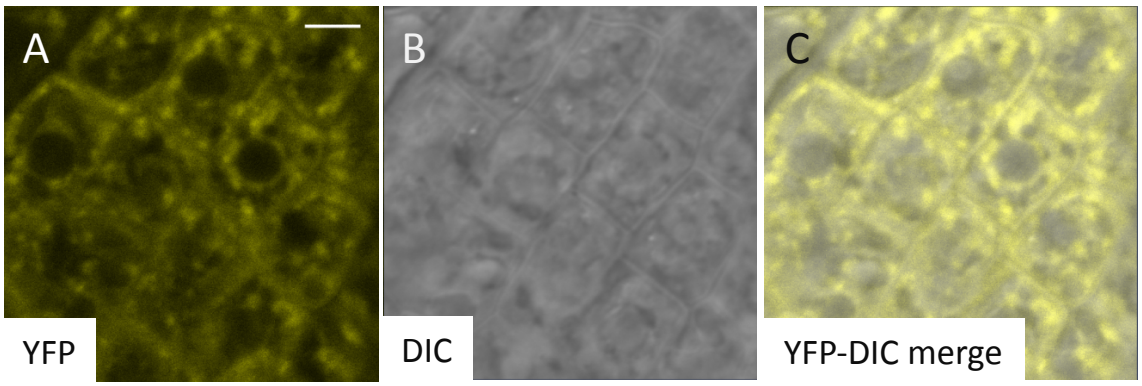

Figure 3.9. An AGD6 marker is expressed in *Arabidopsis* roots.

(A-C) A chimeric fusion of AGD6 to YFP was placed under control of the *AGD6* promoter and transformed into plants. Live roots from *AGD6*::*AGD6-YFP* T2 seedlings were examined using confocal microscopy. YFP expression was observed in the root tips of 16 lines and also in the root vasculature of 12 lines. Fluorescent subcellular speckles were detected in the root epidermal cells of multiple lines. Scale bar, 5 μm.

Data provided by Iris Chen.

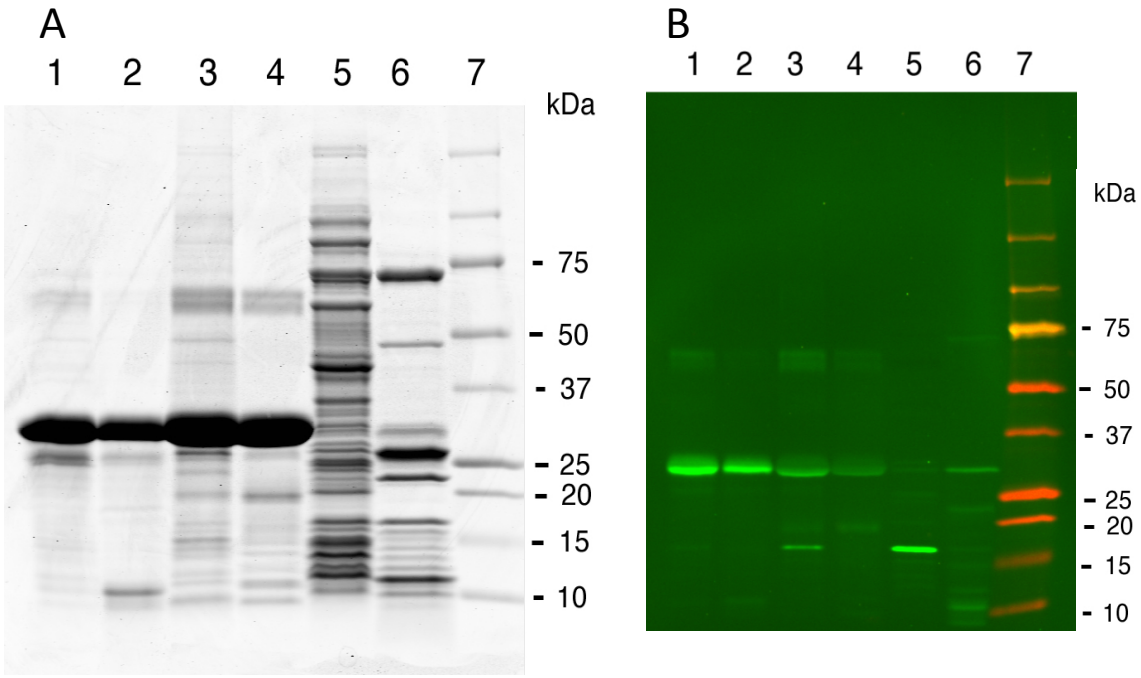

Figure 3.10. The C-terminal region of AGD6 was expressed in *E. coli* for antibody production.

Coomassie-stained gel **(A)** and corresponding immunoblot produced with anti-His tag antisera **(B)** of 6xHis-tagged AGD6 protein purified from *E. coli*. The purified protein was sent for antibody production in chickens.

Lane 1, soluble fraction of Talon resin flow-through from guanidine-HCl solubilized, Triton X-100 washed cell lysate pellet; lane 2, soluble fraction of Talon resin eluate from guanidine-HCl solubilized, Triton X-100 washed cell lysate pellet; lane 3, TBS-insoluble fraction of lane 1 sample; lane 4, TBS-insoluble fraction of lane 2 sample; lane 5, Talon resin flow-through of cell lysate; lane 6, Talon resin eluate of cell lysate; lane 7, prestained protein molecular weight markers.

Data provided by Dr. Mark Daniels (University of Virginia).

References

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R et al: Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science*. 2003, 301: 653-657.

Anders N and Jurgers G: Large ARF guanine nucleotide exchange factors in membrane trafficking. *Cell. Mol. Life Sci.* 2008, 65: 3433-3445.

Araujo A, Lobato Z, Chavez-Olortegui C, and Velarde D: Brazilian IgY-Bothrops antivenom: Studies on the development of a process in chicken egg yolk. *Toxicon*. 2010, 55: 739-744.

Bennett T and Scheres B: Root development-two meristems for the price of one? *Curr Top Dev Biol*. 2010, 91: 67-102.

Christensen SK, Dagenais N, Chory J and Weigel D: Regulation of auxin response by the protein kinase PINOID. *Cell*. 2000, 100: 469-478.

Dettmer J, Schubert D, Calvo-Weimar O, Stierhof YD, Schmidt R and Schumacher K: Essential role of the V-ATPase in male gametophyte development. *Plant J*. 2005, 41: 117-124.

Geldner N, Richter S, Vieten A, Marquardt S, Torres-Ruiz RA, Mayer U and Jurgens G: Partial loss-of-function alleles reveal a role for GNOM in auxin transport-related, post-embryonic development of *Arabidopsis*. *Development*. 2004, 131: 389-400.

Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A and Jurgens G: The Arabidopsis GNOM ARF-GEP mediates endosomal recycling auxin transport, and auxin-dependent plant growth. *Cell.* 2008, 112: 219-230.

Geldner N, Denervaud-Tendon V, Hyman DL, Mayer U, Stierhof YD and Chory J: Rapid, combinatorial analysis of membrane compartments in intact plants with a multicolor marker set. *Plant J*. 2009, 59: 169-178.

Helariutta Y, Fukaki H, Wysocka-Diller J, Nakajima K, Jung J, Sena G, Hauser MT and Benfey PN: The SHORT-ROOT gene controls radial patterning of the Arabidopsis root through radial signaling. *Cell*. 2000, 101: 555-567.

Inoue H and Randazzo PA: Arf GAPs and their interacting proteins. *Traffic*. 2007, 8: 1465-1475.

Jia DJ, Cao X, Wang W, Tan XY, Zhang XQ, Chen LQ and Ye D: GNOM-LIKE 2, encoding an adenosine diphosphate-ribosylation factor-guanine nucleotide exchange factor protein homologous to GNOM and GNL1, is essential for pollen germination in Arabidopsis. *J Integr Plant Biol*. 2009, 51: 762-773.

Koizumi K, Naramoto S, Sawa S, Yahara N, Ueda T, Nakano A, Sugiyama M and Fukuda H: VAN3 ARF-GAP-mediated vesicle transport is involved in leaf vascular network formation. *Development*. 2005, 132: 1699-1711.

Leslie ME, Lewis MW, Youn JY, Daniels MJ and Liljegren SJ: The EVERSHED receptor-like kinase modulate floral organ shedding in Arabidopsis. *Development*. 2010, 137: 467-476.

Lewis MW, Leslie ME, Fulcher EH, Darnielle L, Healy P, Youn JY and Liljegren SJ: The SERK1 receptor-like kinase regulates organ separation in Arabidopsis flowers. *Plant Journal*. 2010, 5: 817-828.

Liljegren SJ, Leslie ME, Darnielle L, Lewis MW, Taylor SM, Luo R, Geldner N, Chory J, Randazzo PA, Yanofsky MF and Ecker JR: Regulation of membrane trafficking and organ separation by the NEVERSHED ARF GAP protein. *Development.* 2009, 136: 1909-1918.

Mayer U, Ruiz R, Berleth T, Miseera S and Jurgens G: Mutations affecting body organization in the *Arabidopsis* embryo. *Nature*. 1991, 353: 402-407.

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T and Kimura T: Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J Biosci Bioeng*. 2007, 104: 34-41.

Nie Z and Randazzo PA: Arf GAPs and membrane traffic. *Journal of Cell Science.* 2006, 119: 1203-1211.

Novick P, Field C and Schekman R: Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell.* 1980, 21: 205-215.

Passardi F, Dobias J, Valerio L, Guimil S, Penel C and Dunand C: Morphological and physiological traits of three major *Arabidopsis thaliana* accession. *J Plant Physiol.* 2007, 164: 980-992.

Poon PP, Cassel D, Spang A, Rotman M, Pick E, Singer RA and Johnston GC: Retrograde transport from the yeast Golgi is mediated by two ARF GAP proteins with overlapping function. *The EMBO Journal*. 1999, 18: 555-564.

Poon PP, Nothwehr SF, Singer RA and Johnston GC: The Gcs1 and Age2ArfGAP proteins provide overlapping essential function for transport from the yeast trans-Golgi network. *J Cell Biol*. 2001, 115: 1239-1250.

Randazzo PA and Hirsch DA: Arf GAPs: multifunctional proteins that regulate membrane traffic and actin remodeling. *Cell Signal*. 2004, 16: 401-413.

Richter S, Geldner N, Schrader J, Wolters H, Stierhof YD, Rios G, Koncz C, Robinson DG and Jurgens G: Functional diversification of closely related ARF-GEFs in protein secretion and recycling. *Nature*. 2007, 448: 488-492.

Richter S, Voss U and Jurgens G: Post-Golgi traffic in plants. *Traffic.* 2009, 10: 819-828.

Richter S, Anders N, Wolters H, Beckmann H, Thomann A, Heinrich R, Schrader J, Singh MK, Geldner N, Mayer U and Jurgens G: Role of the GNOM gene in *Arabidopsis* apical-basal patterning—From mutant phenotype to cellular mechanism of protein action. *Eur J Cell Biol*. 2010, 89: 138-144.

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Scholkopf B, Weigel D and Lohmann JU: A gene expression map of *Arabidopsis thaliana* development. *Nature Genetics*. 2005, 37: 501-506.

Shevell DE, Leu WM, Gillmor CS, Xia G, Feldmann KA and Chua NH: EMB30 is essential for normal cell division, cell expansion and cell adhesion in *Arabidopsis* and encodes a protein that has similarity to Sec7. *Cell*. 1994, 77: 1051-1062.

Sieburth LE, Muday GK, King EJ, Benton G, Kim S, Metcalf KE, Meyers L, Seamen E and Van Norman JM: SCARFACE encodes an ARF-GAP that is required for normal auxin efflux and vein patterning in Arabidopsis. *Plant Cell*. 2006, 18: 1396-1411.

Song XF, Yang CY, Liu J and Yang WC: RPA, a class II ARFGAP protein, activates ARF1 and U5 and plays a role in root hair development in Arabidopsis. *Plant Physiol*. 2006, 141: 966-976.

Spang A, Shiba Y and Randazzo PA: ArfGAPs: gatekeepers of vesicle generation. *FEBS letters.* 2010, 584: 2646-2651.

Stefano G, Renna L, Rossi M, Azzarello E, Pollastri S, Brandizzi F, Baluska F and Mancuso S: AGD5 is a GTPase-activationg protein at the trans-Golgi network. *Plant J*. 2010, 64: 790-799.

Strompen G, Dettmer J, Stierhof YD, Schumacher K, Jurgen G and Mayer U: *Arabidopsis* vacuolar H-ATPase subunit E isoform 1 is required for Golgi organization and vacuole function in embryogenesis. *Plant J*. 2005, 41: 125-132.

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

Till NJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, Johnson JE, Burtner C, Odden AR, Young K, Taylor NE, Henikoff JG, Comai L and Henikoff S: Large-scale discovery of induced point mutations with hig-throughput TILLING. *Genome Research*. 2003, 13: 524-530.

Truernit E and Haseloff J: A simple way to identify non-viable cells within living plant tissue using confocal microscopy. *Plant Methods*. 2008, 4: 15-21.

Turner CE, West KA and Brown MC: Paxillin-ARF GAP signaling and the cytoskeleton. *Curr Opin Cell Biol.* 2001, 13: 593-599.

Vernoud V, Horton AC, Yang Z and Neilsen E: Analysis of the small GTPase gene superfamily of Arabidopsis. *Plant Physiol*. 2003, 131: 1191-1208.

Viotti C, Bubeck J, Stierhof Y, Krebs M, Langhans M, van den Berg W, van Dongen W, Richter S, Geldner N and Takano J: Endocytic and secretory traffic in Arabidopsis merge in the *trans*-Golgi network/early endosome, and independent and highly dynamic organelle. *The Plant Cell*. 2010, 22: 1344-1357.

Wisniewska J, Xu J, Seifertova D, Brewer PB, Ruzicka K, Blilou I, Rouquie D, Benkova E, Scheres B and Friml J: Polar PIN localization directs auxin flow in plants. *Science*. 2006, 312: 883.

Yoo CM, Wen J, Motes CM, Sparks JA, Blancaflor EB: A class I ADP-ribosylation factor GTPase-activating protein is critical for maintaining directional root hair growth in Arabidopsis. *Plant Physiol.* 2008, 147: 1659-1674.

CHAPTER 4

FUTURE DIRECTIONS

In this chapter I discuss the ongoing and future directions of my two thesis projects: characterization of the role of the CAST AWAY receptor-like kinase as an inhibitor of abscission, and exploration of ARF-GAP redundancy during plant development.

The additional work on the *CST* project will possibly contribute to is a further exploration of the abscission pathway, here we will look more deeply into the interactions between all the proteins that play a role in abscission and hope to unravel not only the interactions between the proteins, but what parts of the protein are necessary for these interactions to occur. In doing this we will look carefully at processes like phosphorylation. In addition to the biochemical approach to understanding these proteins action in the abscission pathway we will also use a genetic approach, creating higher order mutants to help understand the way the pathway is set up.

Further work on the *nev agd6* project will be included in a paper characterizing the developmental and trafficking defects of the double mutant as well as work to address what signaling molecules are regulated by NEV and AGD6-mediated membrane trafficking. In this chapter I describe the experiments that have been designed based on our observations of the *nev agd6* root meristem defects. There will likely be an additional paper looking at the quadruple *nev agd15 agd6 agd7* mutant, which will combine loss-of-function mutations of all the Age2-like and Gsc1-like ARF-GAPs present in *Arabidopsis*.

Regulation of organ abscission by CAST AWAY and other receptor-like kinases

Exploration and confirmation of receptor-like kinase interactions.

Some of the future work on CST will involve unraveling the interactions of all the receptor-like kinases that regulate abscission. A first step will be to use the BiFC assay to determine if either EVR or SERK1 interact with HAE, and if SERK1 interacts with CST or EVR. In order to confirm any positive interactions that are found between these receptor-like kinases, we have been creating a set of transgenic plants expressing HA- and Myc-tagged proteins that can be used to confirm these interactions *in vivo* with pull down assays. These tagged lines can also be used to determine if any of the kinases directly interact with NEV. We have not been able to determine using the BiFC assay whether any of the kinases involved in abscission physically interact with NEV, as transfecting protoplasts with YFP-tagged NEV has resulted in protoplast death. We will be able to look for NEV-kinase interactions in the transgenic plants using the NEV antibody (Liljegren et al., 2009). Additionally, by crossing these lines into the *nev-3* mutant background, we can test whether disruption of ARF-GAP activity affects any positive interactions.

What is the role of kinase activity during the inhibition of abscission?

So far the abscission pathway seems to be managed by the abundant RLKs that play a role in abscission. As kinase dead mutations of *CST* and *EVR* have been found through the *nev* screen, it appears that kinase activity will play a role in the abscission pathway. To further investigate how kinase activity may

play a role in abscission we will use kinase dead versions of the RLKs in BiFC assays. In the pathogen resistance pathway involving the RLK FLS2, BIK1 interacts with two LRR-RLKs, FLS2 and BAK1, independent of phosphorylation. However, phosphorlyation of BIK1 is essential to create the complex of all three RLKs necessary for the activation of pathogen resistance(Lu et al., 2010). We are interested in the role that phosphorylation plays in the interaction of the abscission RLKs.

A putative pathway for FLS2 involves a set of three RLKs, two LRR-RLKs, FLS2 and BAK1 and an RLCK BIK1. In this pathway transphosphorylation of these proteins plays a large role in activating downstream signaling (Lu et al., 2010). In order to determine if transphosphorylation plays an important role in the abscission pathway, we would start with finding if transphosphorylation between the kinases in the abscission is possible by incubating different combinations of kinase dead and wild type proteins with radioactive ATP. If these proteins are able to transphosphorylate, the next step would be to determine which residues are being phosphorylated. Rescue experiments using mutant proteins that are unable to be phosphorylated would determine whether this transphosphorylation is necessary for abscission.

How does the membrane association of CST affect its activity?

N-myristoylation is a lipid modification that mediates membrane association, it often works in association with other modifications such as glycosylation and palmitoylation. The CST amino acid sequence indicates a *N*-

myristoylation and palmitoylation domain, and previous work has shown that the *N*-myristoylation domain regulates the ability of CST to associate with the membrane. I've also used a complementation test to show that *N*-myristoylation dead CST (CST^{G2A}) can complement *nev cst* plants, indicating the *N*-myristoylation alone is not necessary for CST function. In order to determine if palmitoylation and *N*-myristoylation are essential for CST function, two additional constructs have been created (I. Chen, C. Burr and S. Liljegren, unpublished). The first creates a palmitoylation mutant (CST^{C4S}) and the second eliminates both *N*-myristoylation and palmitoylation ($CST^{G2A\ C4S}$). In addition to determining if these two modifications are required for membrane association in *Arabidopsis* protoplasts and for CST function via rescue of abscission of *nev cst* plants , we will be able to use these constructs to determine if these modifications are necessary for the interactions of CST with HAE and EVR.

What is the basis for the allele-specific interaction of CST with NEV?

An interesting result of our *CST* genetic studies are the allelic-specific interactions between CST and NEV. While the kinase dead *cst-1* allele suppressed all tested alleles of *nev* recessively*, cst-2* has been found to be unable to suppress some *nev* alleles, even though it suppresses other alleles dominantly. Additional analysis is underway to determine if *cst-2* there is a clear difference in the effect the truncation of CST may have on *nev* alleles whose mutations leave them with an inactive ARF-GAP domain versus those that have truncations that leave them with putatively active ARF-GAP domains. The

preliminary results from this suggest that CST may have direct interactions with NEV, possibly acting as a scaffold mediating interactions with other proteins.

Are there multiple levels of negative regulation during abscission?

EVR, *SERK1*, and *CST* were identified as negative regulators of abscission through a screen of *nev* mutants. As negative regulators they are responsible for the flower maintaining its flower organs to the proper stage, as floral organs abscising early has the serious consequence of reduced fertility. Loss of all negative regulation should lead to early abscission, so we are working towards creating a *cst evr serk1* triple mutant. Once created, we will examine the inflorescences for early abscission and examine the abscission zones for any abnormalities. This work will be able to determine if there are multiple layers of negative regulation working in abscission.

<u>Redundant contributions of ARF-GAPs to plant growth and development</u>

What is the subcellular localization pattern of AGD6?

An interesting question is the extent of the roles of AGD6 in membrane trafficking. NEV is an Age2-like ARF-GAP while AGD6 is a Gcs1-like ARF-GAP. In yeast, Gcs1 has a dual role in regulating traffic leaving the *trans*-Golgi network and retrograde traffic from the Golgi to the ER; Age2 only regulates traffic leaving the *trans*-Golgi network. Will we find that AGD6 plays a similar dual role in plants, or does it have reduced roles that overlap strictly with NEV? In order to

determine the localization pattern of AGD6, we will be conducting co-localization studies using *AGD6::AGD6-YFP* plants (Iris Chen and Sarah Liljegren, unpublished) and a set of fluorescent markers that have been shown to mark different areas of the membrane trafficking system, the WAVE lines (Geldner et al., 2009).

Are the PIN proteins mislocalized in nev agd6 roots?

The PIN proteins are auxin efflux carriers, they are trafficked to distinct membranes and are responsible for maintaining the auxin gradient to aid in plant development and meristem maintenance. If there are defects in the meristems of the *nev agd6* plants, determining if and how PIN proteins are mislocalized may help us to work out what is causing meristem defects. Additionally, PIN2 changes in localization in different developmental zones of the root, switching from basal in the meristemic region to apical in the division zone (Baluska et al., 2010). Preliminary results suggest that the division and/or the elongation zones may be compressed. Determining where PIN2 localizes and changes localization in the double mutant roots may help to determine what areas of the root are not developing normally.

What aspects of root meristem establishment or maintainence are affected in nev agd6 plants?

In addition to looking at the localization of PIN proteins several microscopy methods can be used to examine the *nev agd6* meristems. The roots can be cleared and cortical cell counted on day five, in wild type plants there should be

30 cortical cells between the quiescent center and the transition to the elongation zone (Perilli and Sabatini, 2010). This is a straightforward method to determine if the meristematic region is abbreviated. The plant hormones auxin and cytokinin have distinct effects on meristem size. Auxin increases the rate of cell division and therefore the number of meristematic cells and cytokinin works in the opposite fashion, decreasing the rate of meristematic devision and thereby the number of cells in the meristematic regions (Dello Ioio et al., 2007). By treating the seedlings with auxin or cytokinin before counting the cortical cells we can determine if the double mutants are unable to use these plant hormones correctly in the root meristem.

What trafficking defects are present in nev agd6 plants?

When the *nev* mutant was first characterized two trafficking defects were seen throughout the plant: a circularization of the Golgi and fusion with the TGN, and the build up of vesicles between the cell membrane and the cell wall (Liljegren et al., 2009). Using transmission electron microscopy (TEM) different tissues in the *nev agd6* mutants will be examined for these and other developmental defects. The defects that may be found will most likely relate to the localization pattern of AGD6, if the protein functions in trafficking between the ER and the Golgi we may see defects in trafficking at either of those organelles. For example, in yeast cells deficient in both proteins necessary for retrograde traffic from the Golgi, Gcs1 and Glo3, show a build up of ER (Poon et al., 1999).

Exploring further levels of ARF-GAP redundancy.

In order to fully explore the redundancy between the Age2-like and Gcs1-like ARF-GAPs the rest of the higher order mutants needs to be generated. As seen in the *nev agd6 agd7* triple mutant I expect to see increased defects in plant development in the *nev agd6 agd15* triple mutant. Given the severity of the *nev agd6 agd7* triple mutant I believe there is a strong possibility that the quadruple mutant will be either embryonic or gametophytic lethal. One very important step in the *nev agd6* project will be to create mutants using other alleles of *nev*. This work will show that the phenotype seen is not allele-specific.

References

Baluska F, Mancuso S, Volkmann D and Barlow PW: Root apex transition zone: a signaling-response nexus in the root. *Trends Plant Sci*. 2010, 15: 402-408.

Dello Ioio R, Linhares FS, Scacchi E, Casamitjana-Martinez E, Heidstra R, Costantino P and Sabatini S: Cytokinins determine *Arabidopsis* root meristem size by controlling cell differentiation. *Curr Biol*. 2007, 17: 678-682.

Liljegren SJ, Leslie ME, Darnielle L, Lewis MW, Taylor SM, Luo R, Geldner N, Chory J, Randazzo PA, Yanofsky MF and Ecker JR: Regulation of membrane trafficking and organ separation by the NEVERSHED ARF GAP protein. *Development.* 2009, 136: 1909-1918.

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

Perilli and Sabiatini S: Analysis of root meristem size development. *Methods Mol Biol.* 2010, 655: 177-187.

Poon PP, Cassel D, Spang A, Rotman M, Pick E, Singer RA and Johnston GC: Retrograde transport from the yeast Golgi is mediated by two ARF GAP proteins with overlapping funtion. *The EMBO Journal*. 1999, 18: 555-564.