Regulation of membrane trafficking and organ separation by the NEVERSHED ARF-GAP protein

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

KEY WORDS: Membrane trafficking, ARF-GAP, Abscission, Floral organ shedding, Flower development, *Arabidopsis*, Golgi, Paramural, Exosome

INTRODUCTION

A distinctive feature of plants is their remarkable ability to release entire organs such as leaves, flowers, fruit and seeds by modifying cellular adhesion. Organ separation occurs through the trafficking and secretion of enzymes that locally alter cell walls and dissolve the pectin-rich middle lamella between cells in an abscission zone. The *Arabidopsis* flower has become a model system to investigate several independently regulated separation events, including stamen dehiscence, floral organ separation, fruit opening and seed abscission (Aalen et al., 2006; Lewis et al., 2006; Roberts et al., 2002). Through organ abscission, the three outer whorls of floral organs – the sepals, petals and stamens – are shed after the stamens release their pollen and fertilization occurs (see Fig. 1A,B). The abscission zones consist of a few cell layers at the base of each of these organs adjacent to the floral stem.

Genetic analysis in *Arabidopsis* is revealing an expanding set of factors that are required for, or affect the timing of, organ separation. Integral components include a pair of closely related, redundant leucine-rich repeat receptor-like kinases, HAESA (HAE) and HAESA-LIKE2 (HSL2), which are probably activated by the IDA and IDA-LIKE secreted peptides, and which in turn switch on a conserved mitogen-activated protein kinase signal transduction pathway essential for activating the separation process (Butenko et al., 2003; Cho et al., 2008; Jinn et al., 2000; Stenvik et al., 2008). Two of the polygalacturonase enzymes that contribute to the cell wall remodeling required for floral organ shedding have been identified (González-Carranza et al., 2007; Ogawa et al., 2009). Although factors that directly influence abscission zone differentiation have remained elusive, a pair of redundant transcription factors, BLADE-ON-PETIOLE1 (BOP1) and BOP2, is known to affect the patterning of leaf and floral organ proximal zones, a prerequisite for abscission zone specification (Hepworth et al., 2005; McKim et al., 2008; Norberg et al., 2005). The timing of organ abscission is influenced by signaling via the plant hormones ethylene and auxin (Ellis et al., 2005; Okushima et al., 2005; Patterson and Bleecker, 2004; Patterson et al., 2007) and by transcriptional regulation and chromatin remodeling, which may globally affect both senescence and abscission (Fernandez et al., 2000; Kandasamy et al., 2005a, Kandasamy et al., 2005b).

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

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

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inactive, soluble GDP-bound ARF protein. In addition, interactions of ARF-GAPs with lipids, coat proteins and cargo receptors promote efficient loading of cargo into vesicles and remodeling of the actin cytoskeleton (Inoue and Randazzo, 2007).

Functional characterization of *Arabidopsis* ARF-GAP and ARF-GEF families is revealing roles for these trafficking regulators during specific phases of plant development. The ARF-GAP VASCULAR NETWORK DEFECTIVE3/SCARFACE (VAN3/SCF) and three closely related homologs – ARF-GAP DOMAIN1 (AGD1), AGD2 and AGD4 – together control vein patterning in developing leaves, potentially by affecting auxin signaling and transport (Koizumi et al., 2005; Sieburth et al., 2006). AGD1 also plays a key role in the orientation of root hair growth by influencing cytoskeletal organization (Yoo et al., 2008). ROOT AND POLLEN ARF-GAP (RPA) affects polarized tip growth of root hairs and of pollen tubes (Song et al., 2006). The ARF-GEF GNOM plays a crucial role during embryo development by regulating endosomal cycling of the PIN1 auxin efflux carrier and thereby controlling auxin transport (Geldner et al., 2003; Steinmann et al., 1999). Together with its homolog GNOM-LIKE1 (GNL1), GNOM also functions during gametophytic development and root growth (Richter et al., 2007). With the *Arabidopsis* genome predicted to encode 15 ARF-GAPs and eight ARF-GEFs, potentially regulating 21 ARF-GTPases (Vernoud et al., 2003), much work remains to uncover the unique and overlapping functions of each member of these families in membrane trafficking and their impact on plant development.

Here, we report that the ARF-GAP protein NEVERSHED (NEV) is required for floral organ abscission. Our results suggest that mutations in *NEV* alter the organization of the Golgi apparatus, and block the movement of molecules required for cell separation, providing a link between membrane trafficking and cell separation in plants.

MATERIALS AND METHODS

Plants
Mutant alleles of *NEV* were obtained through ethyl methanesulfonate (EMS) screens as described (Liljegren et al., 2000). The *nev-1*, *nev-3* and *nev-9* alleles contain nucleotide substitutions within codons 51, 59 and 34, which change a cysteine to a tyrosine, an arginine to a lysine, and a cysteine to a tyrosine, respectively. The *nev-2*, *nev-4* and *nev-5* alleles contain nucleotide substitutions within codons 198, 260 and 122, which change a glutamine, a tryptophan and a tryptophan to stop codons, causing production of truncated proteins of 197, 259 and 121 amino acids, respectively. The *nev-8* allele contains a nucleotide substitution at the splice acceptor site of the seventh intron. The *nev-6* (SALK_075680) and *nev-7* (SALK_079928) alleles contain T-DNA insertions in the first intron. With the exception of *nev-6* and *nev-7*, all alleles identified are of the Ler ecotype. The *nev-3* mutant was backcrossed to Ler three times before phenotypic analyses; homozygous mutants can be detected by a BspCNI site introduced by the dCAPs oligo 5′-CTGCATGCAATGTTCTGGGATTCTCA-3′.

Mapping
To map *NEV*, *nev-1* and *nev-2* mutants were crossed to wild type (Col). Using DNA isolated from 515 *nev* F2 plants and PCR-based markers, including several designed from Monsanto Ler polymorphisms (http://www.arabidopsis.org/Cereon/), both mutations were mapped to chromosome 5 between CER436970 and CER465330. The coding regions of 8 of 13 predicted genes in this region were sequenced from *nev* genomic DNA.

Generation of NEV-specific antiserum and immunodetection
NEV cDNA (GenBank Accession number FJ794601) was PCR amplified with 5′-AGATATTGAAGAGCGCTGAAGGCG-3′ and 5′-TCCGTTTA-AGTCTTTTTCAGAGAGAGAG-3′ using Col RNA as template. After

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

Protein extracts were prepared by grinding floral tissue, incubating samples with extraction buffer (50 mM TrisHCl pH 8.5, 2% SDS) at 60°C for 20 minutes, pelleting by centrifugation at 16,000 ***g*** for 5 minutes, and resuspending the supernatant in loading buffer [5 mM EDTA (pH 8.5), 10% glycerol, 50 mM mercaptoethanol, 0.05% Bromophenol Blue]. Proteins were separated by SDS-PAGE and analyzed by western blotting. Anti-NEV antibody was used at a 1:20,000 dilution.

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

For immunofluorescence assays, seedlings were grown vertically on MS media plates for 5-10 days then prepared as described (Lauber et al., 1997) with minor modifications. Primary antibodies were used at the following concentrations: anti-NEV, 1:2500; chicken anti-GFP, 1:500 (Abcam, Cambridge, MA). Fluorochrome-conjugated secondary antibodies were used at a 1:500 concentration: Alexa Fluor 488 anti-rabbit F(ab′)2 and Alexa Fluor 647 anti-rabbit (Molecular Probes, Eugene, OR); anti-chicken Cy2 (Abcam, Cambridge, MA). Confocal laser scanning microscopy was performed with a Zeiss LSM-410 or 510 (Carl Zeiss, Thornwood, NY). Image brightness and contrast were adjusted with Photoshop 7.0 (Adobe, Mountain View, CA). For Brefeldin A (BFA) treatment, 5- to 10-day-old seedlings were incubated in MS liquid media with or without 100 μM BFA for 50-60 minutes, fixed in 4% formaldehyde and prepared for immunofluorescence detection as described above.

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

Generation of transgenic lines

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

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

RESULTS

Mutations in *NEV* prevent floral organ shedding

To identify novel loci required for organ abscission, we carried out mutant screens of *Arabidopsis* plants as flowers matured. Six mutants that retain their floral organs indefinitely represent a unique complementation group that we have named *nevershed* (*nev*) (Fig. 1C). A seventh *nev* mutant allele was kindly provided by Pataradawn Pinyopich (UCSD).

As each of the identified mutant alleles blocks floral organ abscission, we characterized *nev-3* as a representative allele. In examining sections of *nev* flowers at a range of developmental stages compared to wild type (Fig. 1F,G; data not shown), differences in abscission zone morphology were first detected when organ separation normally occurs in wild-type flowers (stage 16). At this point, abscission zone cells expand and become vacuolated in wild type (Fig. 1F), whereas in *nev* flowers the organs stay attached and cells within the sepal and petal abscission zones remain small and densely cytoplasmic (Fig. 1G). As abscission zones are thought to be specified in the proximal regions of the floral organs as they differentiate, enhancer trap lines with early expression profiles at the bases of wild-type floral organs (Campisi et al., 1999) were examined in *nev* flowers (see Fig. S1 in the supplementary material). No temporal or spatial differences were detected for four different markers in *nev* flowers compared to wild type. Taken together, these results suggest that NEV acts during the separation phase of organ abscission, rather than during the initial patterning and differentiation of abscission zone cells.

To determine whether NEV function is specific to abscission, we examined whether other aspects of plant development are affected by mutations in *NEV*. While floral organ separation is tightly linked to the initiation of organ senescence (Fang and Fernandez, 2002), the aerial (cauline) leaves of wild-type *Arabidopsis* plants can be shed well after senescence has occurred (Fig. 1D). Detachment of these leaves is blocked in *nev* mutants (Fig. 1E). Fruit (silique) growth is also significantly affected by mutations in *NEV* (see Fig. S2A,B in the supplementary material). These results indicate that NEV plays a role in multiple developmental processes.

NEV encodes an ADP-ribosylation factor GTPase-activating protein

Using standard mapping procedures, the *nev-1* and *nev-2* mutations were located within a 39 kb interval on the lower arm of chromosome 5, including the BACs MDK4 and GA469. Within one of 13 predicted genes in this interval, At5g54310, a single nucleotide change was identified in the *nev-2* mutant that would introduce a stop codon into the corresponding transcript (Fig. 2A). This gene includes 11 exons and encodes a predicted 483-amino acid protein with a zinc finger ($CX_2CX_{16}CX_2C$) characteristic of ARF-GAP
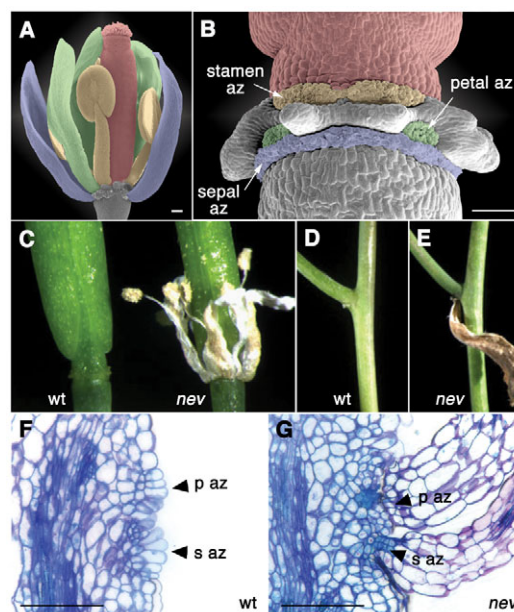

Fig. 1. Mutations in *NEV* prevent organ separation. (**A**) Scanning electron micrograph (SEM) of a wild-type *Arabidopsis* flower before organ separation (stage 13). The sepals, petals, stamens and gynoecium are colored purple, green, yellow and red, respectively. A few organs have been removed for clarity. (**B**) SEM of a wild-type flower after organ separation (stage 17). The remaining abscission zone (az) cells of each organ are colored as in A. (**C**) Floral organs remain attached in *nev* flowers (stage 17) compared with wild type (wt). (**D,E**) Cauline leaves in *nev* plants fail to detach after senescence (E), as compared with wild type (D). (**F,G**) Longitudinal sections of wild-type (F) and *nev* (G) flowers at the time of shedding (stage 16) stained with Toluidine Blue. Adjacent petal (p az) and sepal (s az) abscission zones at the base of each flower are indicated. In wild-type flowers, the remaining abscission zone cells have expanded, which does not occur in *nev* flowers in the absence of organ separation. Scale bars: 100 μm.

proteins located near its N-terminus (Fig. 2A). Three of the nine *nev* alleles introduce missense mutations within the highly conserved 115-amino acid ARF-GAP domain, with two, *nev-1* and *nev-9*, altering crucial cysteine residues in the zinc finger motif to tyrosines, and a third, *nev-3*, changing an invariant arginine in close proximity to the zinc finger to a lysine (Fig. 2B). This particular arginine has been demonstrated to be essential for ARF-GAP activity for all ARF-GAPs examined (Luo et al., 2007), and structural analysis of the PAPβ/ARF1 complex suggests that it may act as an 'arginine finger' directly contacting the active site of the ARF substrate during GTP hydrolysis (Mandiyan et al., 1999). Four additional alleles are predicted to affect the C-terminal region of the protein by introducing premature stop codons (*nev-2*, *nev-4*, *nev-5*) or affecting splicing (*nev-8*) (Fig. 2A). Two alleles of *NEV* identified in the Salk T-DNA mutant collection (Alonso et al., 2003), *nev-6* and *nev-7*, contain insertions in the first intron and are predicted to encode a truncated protein without an ARF-GAP domain (Fig. 2A). As plants carrying either of these null alleles are phenotypically indistinguishable from the other *nev* mutants, all of the identified mutations appear to completely disrupt the function of *NEV* and are considered to be strong loss-of-function alleles.

Comparison of NEV with other eukaryotic ARF-GAPs has revealed that NEV shares 49% amino acid identity within its ARF-GAP domain with yeast ArfGAP effector2 (Age2), 50% identity with
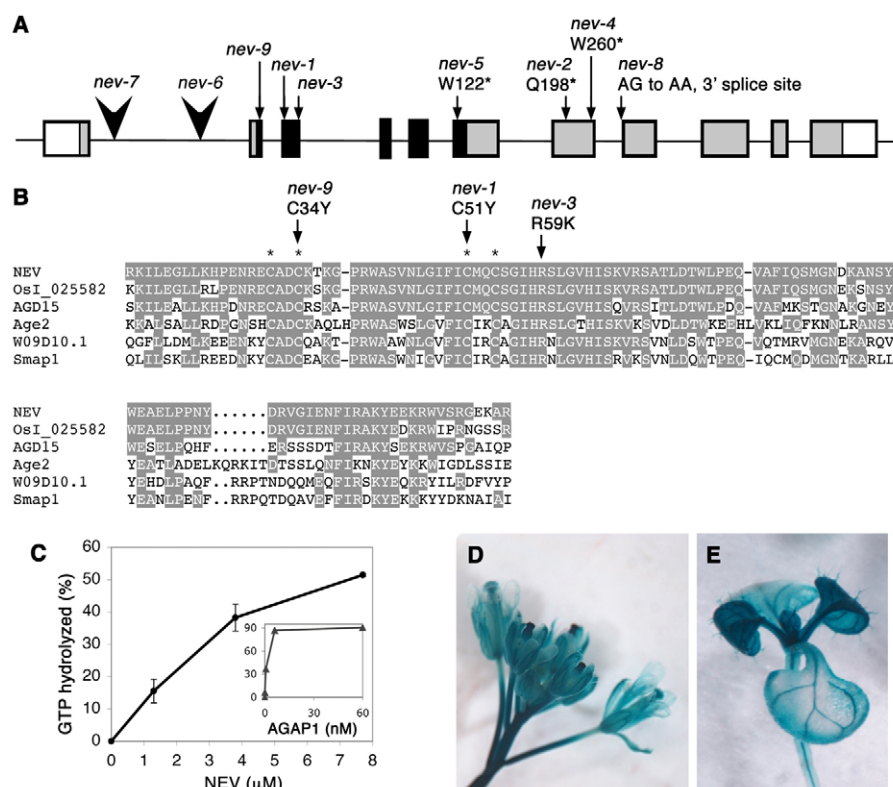
Fig. 2. *NEV* encodes an ARF GAP. (**A**) The At5g54310 locus, showing the sites and sequence alterations of characterized *nev* mutations. Exons are shown as boxes, and the translated regions corresponding to the ARF-GAP domain and the rest of the corresponding protein are indicated in black and gray, respectively. Point mutations are marked by arrows, and T-DNA insertions by arrowheads. (**B**) Sequence alignment of the ARF-GAP domain from NEV and related proteins from plants (OsI_025582, AGD15), yeast (Age2), worm (W09D10.1) and mouse (Smap1). Amino acids conserved between NEV and other proteins are shaded, and the four cysteine residues that constitute the zinc finger are indicated with asterisks. The sites of *nev* missense mutations that affect two of these cysteines and a crucial arginine residue are marked by arrows above the alignment. Characterized ARF-GAP proteins in the alignment include yeast Age2 (Zhang et al., 1998) and mouse Smap1 (Sato et al., 1998). Uncharacterized proteins with closely related ARF-GAP regions include OsI_025582, AGD15 (Vernoud et al., 2003) and W09D10.1, predicted from *Oryza sativa*, *Arabidopsis* and *Caenorhabditis elegans* sequences, respectively. (**C**) NEV promotes GTP hydrolysis of mammalian ARF1. The ARF-GAP activity of recombinant full-length NEV protein was measured using AGAP1, a mammalian ARF-GAP, as a positive control (inset graph). The percentage of GTP bound to Arf1 that was converted to GDP is presented. The data are the summary of two experiments. (**D,E**) *NEV* regulatory regions direct broad expression of β-glucuronidase in flowers and shoot inflorescence stems (D), and in developing leaves and vascular strands (E).

Age2-like ARF-GAPs in worms and mice, and 73% identity with another Age2-like ARF-GAP in *Arabidopsis*, AGD15 (Fig. 2B). In contrast to NEV, Age2 activity has been found to be entirely redundant with that of another yeast ARF-GAP, Growth cold sensitive1 (Gcs1) (Poon et al., 2001). The yeast *age2 gcs1* double mutant disrupts the morphology of the Golgi, and membrane trafficking from the trans-Golgi network (TGN) to the endosome, vacuole and plasma membrane, and is lethal. In mammals, two Age2-related ARF-GAPs, SMAP1 and SMAP2, regulate clathrin-mediated endocytosis from the plasma membrane and retrograde traffic from the early endosome to the TGN, respectively (Natsume et al., 2006; Tanabe et al., 2005). Outside of the ARF-GAP domain, the NEV protein is poorly conserved with Age2 and Age2-like ARF-GAPs, and the conserved clathrin-binding domains of SMAP1 and SMAP2 are not present in the C-terminal regions of either NEV or Age2.

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

To determine whether NEV is able to function as an ARF-GAP, we tested its ability to promote GTP hydrolysis of the mammalian ARF1 G-protein substrate in vitro (Fig. 2C). Recombinant full-length NEV protein was expressed in *E. coli*, purified and used in an ARF-GAP assay, along with a mammalian ARF-GAP, AGAP1, as a positive control. NEV promoted GTP hydrolysis of ARF1 in a concentration-dependent manner with enzymatic activity levels similar to those of other *Arabidopsis* ARF-GAPs acting on heterologous ARF substrates (Koizumi et al., 2005).

NEV is expressed broadly during development

Global analysis of *Arabidopsis* gene expression profiles revealed that *NEV* is expressed ubiquitously during development in floral, leaf, stem and root tissue (see Fig. S4 in the supplementary material) (Schmid et al., 2005). To determine whether expression of *NEV* might be enriched in certain cell types within these tissues, transgenic plants were generated that express a translational fusion of NEV, including 908 nucleotides upstream of the predicted translational start site through part of the second exon, to the complete coding region of β-Glucuronidase (GUS). Of 19 T1 plants analyzed, nine showed broad expression patterns of GUS in the inflorescence stem, abscission zones, stigma, floral and/or leaf

vasculature (Fig. 2D,E), eight showed expression profiles in a subset of these tissues, and two showed no apparent expression. These results together with our phenotypic analysis suggest that *NEV* probably functions broadly during plant development.

NEV localizes to the TGN and endosomes

ARF-GAPs modulate membrane trafficking from multiple sites, including the Golgi, TGN and recycling endosome (Inoue et al., 2008; Min et al., 2008; Poon et al., 1999; Poon et al., 2001). Analysis of NEV using WoLF PSORT (Horton et al., 2007) showed a possible monopartite nuclear localization signal between amino acids 14 and 17 (RHRK) and no other known sorting motifs. To determine the subcellular site of NEV action, we examined its localization using NEV polyclonal antiserum raised against its unique C-terminal region. This antiserum recognized a ~55 kDa protein in wild-type flower extracts, consistent with the predicted size of NEV (53 kDa) (Fig. 3A). Because this protein was not recognized in extracts from *nev-2* flowers containing truncated nev protein, the antiserum appears to be specific for NEV (Fig. 2A; Fig. 3A). Immunolocalization and confocal laser-scanning microscopy revealed that the NEV antiserum labels punctate spots in wild-type root epidermal cells (Fig. 3B). As expected, the signal profile observed in wild-type roots is not consistent with nuclear localization and a substantial reduction of signal is observed in *nev-2* roots (Fig. 3B,C).

We next used a set of markers for specific plant endomembrane compartments (see Table S1 in the supplementary material) to explore the precise localization of NEV (Fig. 3; see Fig. S5 in the supplementary material). Because Age2 localizes to and regulates traffic from the TGN in yeast (Poon et al., 2001), we first examined the co-localization of NEV with markers of the TGN and Golgi (Fig. 3). Whereas NEV localization is distinct from the Golgi marker NAG-GFP (Fig. 3D-F) (Grebe et al., 2003), the punctate spots labeled by NEV antiserum co-localize with spots of a similar size and structure labeled by the TGN/early endosome (EE) marker YFP-VTI12 (Fig. 3G-I, arrows) (Sanderfoot et al., 2001). To further confirm that this marker labels the TGN/EE, co-localization of YFP-VTI12 with the TGN/EE marker VHA-a1-RFP was shown (see Fig. S6 in the supplementary material) (von der Fecht-Bartenbach et al., 2007). In plants, accumulating evidence exists for a unified TGN/EE compartment (Dettmer et al., 2006; Müller et al., 2007; Robinson et al., 2008). These results suggest that NEV is localized at the TGN/EE.

As we observed occasional sites at which NEV fluorescence does not overlap with the YFP-VTI12 marker (Fig. 3G-I, arrowheads), a wider range of endomembrane markers was tested for co-localization with NEV. We discovered that NEV also co-localizes with two novel endosomal markers, YFP-RabA1e and YFP-RabA1g (Fig. 3J-L, arrows; data not shown) proposed to label recycling endosomes (Geldner et al., 2009). The endosomal compartment labeled by these markers is distinct from the TGN/EE and shows a high sensitivity to BFA (Geldner et al., 2009). Rab11, the closest mammalian homolog of the expanded RabA class in *Arabidopsis*, regulates trafficking through the recycling endosome; other characterized RabA family members in *Arabidopsis* and tomato are thought to localize at the TGN/EE (Chow et al., 2008; Nielsen et al., 2008; Rehman et al., 2008). NEV did not co-localize with the YFP-RabF2A marker of the late endosome/pre-vacuolar complex (see Fig. S5A in the supplementary material) (Lee et al., 2004; Ueda et al., 2004) or the YFP-NIP1;1 marker of the ER network (see Fig. S5B in the supplementary material) (Geldner et al., 2009). Vesicles labeled by NEV occasionally overlapped with
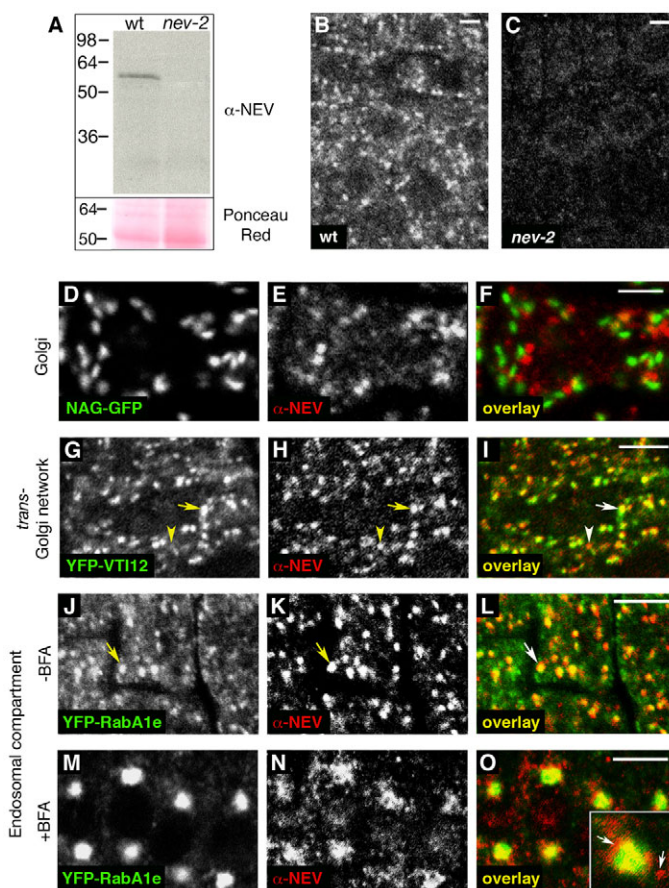

Fig. 3. NEV localizes to the trans-Golgi network (TGN) and endosomes. (**A**) NEV antiserum recognizes a ~55 kDa protein in wild-type flower extracts. As the *nev-2* allele encodes a truncated protein lacking the C-terminal recognition sequence of the NEV antiserum, no protein is detected in *nev-2* flower extracts. Ponceau Red staining was used as a protein-loading control. (**B-O**) Immunofluorescent localization of NEV and endomembrane markers in primary root epidermal cells of wild-type (B), *nev* (C) and transgenic marker (D-O) plants. In M-O, the primary roots of YFP-RabA1e plants were incubated for 1 hour with 100 μM BFA before fixation and immunofluorescent staining. (B,C) NEV antiserum detects NEV protein in punctate structures in wild-type cells (B). Background fluorescence is shown for *nev-2* cells (C). (D-I) NEV localization is distinct from the Golgi (D-F), and shows more precise localization with YFP-VTI12, a marker of the TGN (arrows, G-I). Occasional spots labeled by NEV (arrowheads, G-I) do not co-localize with VTI12. (J-O) NEV also co-localizes with YFP-RabA1e, a novel endosomal marker proposed to localize to the recycling endosome (arrows, J-L). BFA treatment of the primary root causes both NEV and YFP-RabA1e to localize to BFA bodies (M-O). Whereas the localization of YFP-RabA1e is confined to the core of the BFA bodies, NEV is also found at the periphery (arrows, O). Individual channels: YFP markers, green; NEV, red. Scale bars: 5 μm.

YFP-NPSN12, a marker of the plasma membrane (see Fig. S5C in the supplementary material) (Zheng et al., 2002). NEV also partially localized with YFP-RabD2a, a marker associated with the Golgi network and endosome (see Fig. S5D in the supplementary material) (Zheng et al., 2005). Taken together, these results are consistent with roles for NEV in regulating trafficking at the TGN/EE and a distinct endosomal compartment, which may correspond to recycling endosomes.

To further explore NEV localization, we used BFA, a fungal toxin that interferes with the activity of a subset of BFA-sensitive ARF-GEFs, inhibiting ARF-mediated vesicular transport from the target membrane (Peyroche et al., 1999). In *Arabidopsis* roots, BFA inhibits recycling of vesicles back to the plasma membrane without blocking endocytosis or ER-Golgi traffic, resulting in the accumulation of the TGN/EE and rapidly cycling proteins such as PIN1 into BFA bodies (Geldner et al., 2003; Grebe et al., 2003; Richter et al., 2007; Robinson et al., 2008; Teh and Moore, 2007). Golgi, by contrast, tend to cluster around the BFA bodies while remaining functionally intact. When we treated primary roots with 100 μM BFA before immunodetection of NEV, NEV localized to structures resembling BFA bodies rather than the punctate spots characteristic of untreated cells (Fig. 3N; see Fig. S7 in the supplementary material). In the presence of BFA, NEV localization within the BFA compartment overlaps with but is more disperse than that of the endosomal marker YFP-RabA1e found at the core of the BFA compartment (Fig. 3N-O, arrows). These results suggest that NEV is not only localized to the TGN/EE and distinct endosomes, but also to additional BFA-resistant membranes.

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

As *nev* mutations affect a member of a protein family known to regulate membrane trafficking, we examined whether subcellular defects could be detected in *nev* mutant cells. Transmission electron microscopy was performed on *nev* flowers compared to wild type at the time of organ shedding (stage 16). We discovered that cells in the region of *nev* sepal abscission zones predominantly exhibited circularized or cup-shaped multilamellar structures (Fig. 4B; Fig. 5D; see Fig. S8D-F in the supplementary material) rather than the flat stacks of Golgi cisternae typical of wild type (Fig. 4A; Fig. 5D; see Fig. S8A-C in the supplementary material). We also often observed the TGN closely associated with the trans-face of the Golgi in wild-type cells (Fig. 4A; see Fig. S8A-C in the supplementary material), but did not readily observe these tubular-vesicular networks near the circularized structures in *nev* abscission zone cells (Fig. 4B; see Fig. S8D-F in the supplementary material). In older *nev* flowers (stage 17), a mixture of Golgi with a wild-type appearance and circularized structures were observed in sepal abscission zones (data not shown). These unusual compartments were also observed in the two other regions of *nev* flowers (stage 16) examined – fruit walls and floral stems (pedicels) – although vesicular structures resembling the TGN were more apparent in the fruit cells examined (Fig. 5C,D). Taken together, these results suggest that defects in NEV-regulated trafficking can severely affect the structure of the Golgi apparatus and alter the location of the TGN.

Extensive accumulation of vesicles between the plasma membrane and cell wall was also observed in the sepal abscission zone regions of *nev* flowers (Fig. 4D). Vesicle clusters associated with pockets of the plasma membrane have been termed paramural bodies (PMBs) by researchers who have also observed them in the embryos of the *Arabidopsis vps9a* Rab-GEF mutant and in barley leaf cells upon fungal infection (An et al., 2007; Goh et al., 2007). Although PMBs are present in wild-type cells (Fig. 4C,E), far more PMBs containing numerous vesicles were found in *nev* flowers (Fig. 4D,F) [0.002±0.01 PMB/μm plasma membrane in wild-type cells ($n=20$) compared with 0.082±0.066 PMB/μm plasma membrane in nev cells ($n=28$)]. Some PMBs in *nev* flowers have the appearance of a recent fusion of a multivesicular body to the plasma membrane (Fig. 4D). Additional PMBs were also observed in the fruit walls and
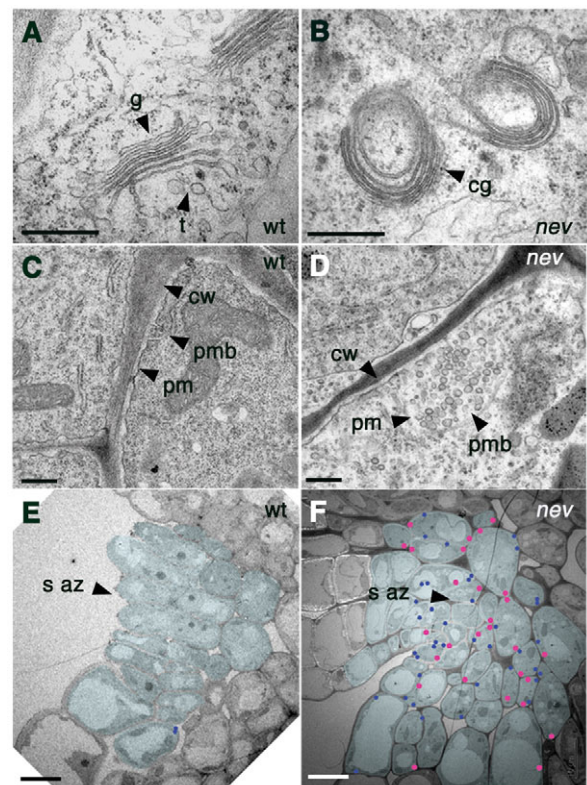

Fig. 4. Mutations in *NEV* alter Golgi structure and location of the TGN and cause an accumulation of paramural vesicles. Transmission electron micrographs of cells at the base of wild-type and *nev* sepals at the time of shedding (stage 16). (**A,B**) Cup-shaped and circularized multilamellar structures are predominantly found in *nev* cells (B) instead of the typically flat Golgi cisternae of wild-type cells (A). Whereas the TGN is frequently observed (84%, $n=19$) near the Golgi (0.041±0.018 μm, $n=16$) in wild-type cells (A), these tubular-vesicular compartments are not clearly identifiable near the circularized structures of *nev* cells (B). (**C,D**) Whereas paramural vesicles are infrequently observed between the plasma membrane and cell wall of wild-type cells (C), numerous vesicles are frequently observed in large paramural bodies in *nev* cells (D). (**E,F**) Distribution of paramural vesicles in cells at the base of wild-type (E) and *nev* (F) sepals. Abscission zone regions are indicated by arrowheads, analyzed cells are colored light blue, and paramural bodies with more than 30 vesicles or 10-30 vesicles are indicated by pink and blue circles, respectively. cg, circularized multilamellar structures; cw, cell wall; g, Golgi cisternae; pm, plasma membrane; pmb, paramural body; t, TGN. Scale bars: 0.5 μm in A-D; 10 μm in E,F.

pedicels of *nev* flowers (Fig. 5F,H; see Fig. S9B,D in the supplementary material) compared with wild type (Fig. 5E,G; see Fig. S9A,C in the supplementary material). These results suggest that NEV may play a role in PMB turnover or biogenesis.

DISCUSSION

We report here the identification and characterization of NEV, an *Arabidopsis* Age2-like ARF-GAP that is required for floral organ abscission. Our studies suggest that NEV plays an essential role in membrane trafficking during the separation stage of organ abscission by facilitating the movement of key cargo molecules through the TGN/EE and other distinct endosomes. Although mutations in *NEV* do not appear to affect the early differentiation of abscission zone cells, they do alter their fate. Instead of
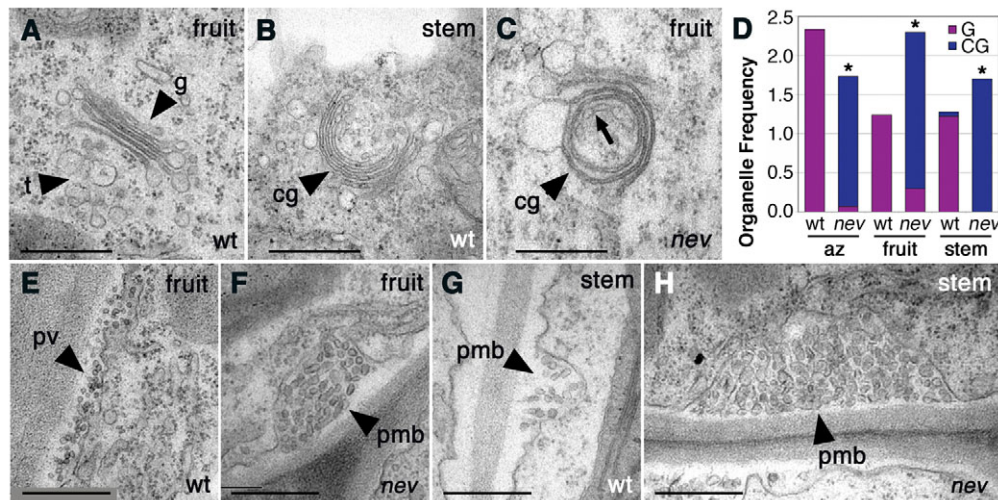
Fig. 5. The fruit and stems of *nev* flowers also show membrane trafficking defects. (A-C,E-H) Transmission electron micrographs of cells in the fruit wall and pedicel (stem) of wild-type and *nev* flowers (stage 16). (**A-C**) Circularized multilamellar structures are enriched in *nev* fruit and stem cells (C,D) by comparison with the linear Golgi cisternae of wild-type cells (A,D). Rarely, Golgi cisternae with a curved appearance were observed in wild-type cells (B,D). Vesicular structures resembling the TGN of wild-type cells (A) are often observed in the vicinity or inside the multilamellar structures of *nev* cells (C, arrow) (73%, *n*=15). (**D**) Frequency of Golgi with a wild-type appearance (G, purple) and circularized multilamellar structures (CG, blue) per cell in sections of *nev* and wild-type sepal AZ regions, fruit walls and pedicels. For each type of tissue analyzed, *n* (cells) ≥15. Statistical differences between *nev* and wild-type tissues are indicated by asterisks (Fisher's exact test, *P*<0.0001). (**E-H**) Large paramural bodies are found in *nev* fruit (F) and stem (H) cells. Paramural vesicles and paramural bodies were also observed in wild-type fruit (E) and stem (G) cells. cg, circularized multilamellar structures; g, Golgi cisternae; pmb, paramural body; pv, paramural vesicles; t, TGN. Scale bars: 0.5 µm.

undergoing cell separation and expansion, cells in the abscission zone regions of *nev* flowers remain small and cytoplasmic (Figs 1 and 4). Disruption of membrane trafficking in *nev* flowers could affect the localization of signaling molecules required to initiate the separation process, the subsequent secretion of cell wall modifying enzymes, or the endocytosis of cell wall materials. Key molecules in the signaling network thought to activate the separation phase of organ abscission include the IDA and IDA-LIKE peptides, and their proposed receptors, the transmembrane HAE and HSL2 leucine-rich repeat receptor-like kinases (Butenko et al., 2003; Cho et al., 2008; Jinn et al., 2000; Stenvik et al., 2008). Cell wall modifying and hydrolytic enzymes – polygalacturonases, cellulases, expansins and others – are secreted by abscission zone cells to loosen their cell walls and and dissolve the pectin-rich middle lamellae. Identifying which cargo molecules are incorrectly trafficked in *nev* flowers will be central to understanding further the role of NEV in abscission.

We discovered two unusual subcellular defects in the cells of *nev* mutant flowers: circularized multilamellar structures and extensive PMBs. Either or both of these defects may be responsible for blocking organ abscission. Because of their resemblance to the Golgi cisternae, and the corresponding lack of Golgi with a wild-type morphology and closely associated TGN (Figs 4 and 5; see Fig. S8 in the supplementary material), we believe the multilamellar structures are Golgi-derived and may represent TGN-Golgi fusions. It is noteworthy that localization of NEV at the TGN and its role in maintaining normal Golgi morphology further support a conserved function with its closest yeast ArfGAP relative, Age2. Although the *age2* single mutant is unaffected, the *age2 gcs1* double mutant accumulates atypical membrane-bound structures due to a fragmentation of the TGN (Poon et al., 2001). The appearance of these structures is clearly distinct from the circularized structures we see in *nev* flowers, which is not surprising as wild-type *Saccharomyces cerevisiae* Golgi are composed of a single disk, in

contrast to the multilamellar organization of the plant Golgi system (Preuss et al., 1992). In the future, we plan to test the potential chimeric nature of the aberrant multilamellar structures we have observed.

Interestingly, the multilamellar structures that we see in *nev* flower cells (Fig. 4; see Fig. S8 in the supplementary material) are similar in appearance to Golgi-derived structures that form when the activity of *Arabidopsis* vacuolar H⁺-ATPase (V-ATPase) complexes are reduced (Brüx et al., 2008; Dettmer et al., 2005; Dettmer et al., 2006; Neubert et al., 2008). The eukaryotic V-ATPase complex is a multi-subunit proton transporter that is crucial for acidification of intracellular organelles and vesicles, thereby affecting secretory and endocytic trafficking (Dettmer et al., 2006; Marshansky and Futai, 2008). The characteristic changes in Golgi organization that are seen in cases of reduced V-ATPase activity (bending of the stacks and swelling at the ends of the cisternae) are thought to be due to defects in vesicle trafficking (Dettmer et al., 2006). Disruption of V-ATPase activity has severe effects on development, as illustrated by the block in pollen development associated with hypomorphic mutations in the TGN/early endosome-localized VHA-a1 subunit (Dettmer et al., 2005; Dettmer et al., 2006). V-ATPases are known to be required for the recruitment of proteins involved in vesicle budding, including ARF G-proteins, ARF-GEFs, and coat components (Aniento et al., 1996; Hurtado-Lorenzo et al., 2006; Maranda et al., 2001; Zeuzem et al., 1992). As ARF-GAPs modulate vesicle formation by promoting GTP hydrolysis of ARF G-proteins and interacting with coat components, the similarity between the multilamellar structures found in *nev* and V-ATPase mutants suggests the possibility that TGN-localized NEV and V-ATPase complexes may jointly regulate the recruitment and activity of the same ARF G-protein(s).

The hyperaccumulation of paramural vesicles in *nev* flowers, fruit and pedicels (Fig. 4F; see Fig. S8B,D in the supplementary material) is a surprising discovery, and would appear to represent a role for NEV

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in membrane trafficking that is not conserved with yeast Age2. NEV and the Rab-GEF VPS9a are the first reported *Arabidopsis* proteins with potential roles in PMB biogenesis or turnover (Goh et al., 2007). The functions and origin of PMBs and paramural vesicles in plants are unknown, although their appearance has been associated with cell-plate formation, secondary cell wall thickening and exposure to pathogens (An et al., 2007). One possibility, previously offered by Goh et al. (Goh et al., 2007), is that PMBs could represent a novel form of endocytosis, by which cell wall materials are taken up into vesicles and transported into the cell for recycling or degradation. Whereas this model is congruent with the need for extensive cell wall modifications during plant cell separation events, the currently favored model is that paramural vesicles in plants may be analogous to the exosomes produced by other higher eukaryotes (An et al., 2007). In animals, exosomes are formed in multivesicular bodies by the reverse budding of membrane into the lumen of late endosomal compartments, and secreted by fusion of the multivesicular body with the plasma membrane rather than with a lytic compartment (van Niel et al., 2006). Exosomes can carry transmembrane signaling molecules and are known to play roles in immunity, development and diseases such as cancer; the multivesicular body pathway of a host cell can also be hijacked by viruses to release viral particles within exosomes (Gould et al., 2003; van Niel et al., 2006; Lakkaraju and Rodriguez-Boulan, 2008). Based on the current understanding of multivesicular body biogenesis, interlumenal vesicles destined for exocytosis versus degradation are indistinguishable in size yet differ in membrane composition (Trajkovic et al., 2008). Analysis of the orientation, content and membrane composition of *nev* and wild-type paramural vesicles will provide further insights into the origin and intended destination of these structures, as well as their potential role in cell separation.

The expression profile of *NEV* and its homology to Age2 suggest that it probably plays a more fundamental role in development than is indicated by the *nev* single mutant phenotype (Fig. 1; see Fig. S2 in the supplementary material). As Age2 activity is redundant with that of the Gcs1 ArfGAP in controlling traffic in yeast cells from the TGN to the endosome, plasma membrane and vacuole (Poon et al., 2001), further functional analysis of the *Arabidopsis* Age2-like and Gcs1-like family members NEV, AGD15, AGD6 and AGD7 may reveal broad, overlapping requirements for these proteins in plant growth and development.

If NEV is indeed regulating membrane trafficking in multiple plant cell types, why do mutations in *NEV* have such a profound effect in abscission zone cells? One possibility is that because these cells rely on a tremendous surge of vesicle trafficking to carry out the extensive cell wall remodeling necessary for cell separation within a short time frame, even a minor delay in traffic could affect this process. More than 30 years ago, ultrastructural studies of leaf abscission zone cells revealed evidence of the importance of vesicle trafficking and protein synthesis in cell separation, with dramatic increases in the area of cytoplasm occupied by the ER and Golgi bodies observed upon induction of abscission (Sexton and Hall, 1974; Sexton et al., 1977). Furthermore, the response of abscission zone cells to *nev*-mediated trafficking defects could be exacerbated by a feedback loop pushing increased vesicle traffic through the system in response to the lack of cell separation. The potential of increased traffic in *nev* mutant flowers suggest that it may represent a sensitized background to screen for mutations in the signaling components NEV may regulate.

To date, analysis of distinct cell separation events during plant development has not uncovered any common regulatory factors (Lewis et al., 2006). Leaf and floral organ abscission may prove to be an exception. Floral organs and leaves have long been considered to be derived from the same basal structure, and many of the same regulatory networks act to establish their early pattern and polarity (Bowman et al., 2002; Smyth, 2005). It is intriguing that neither floral organ shedding nor aerial leaf detachment occurs in *Arabidopsis* plants carrying mutations in *NEV*. Because *Arabidopsis* leaf detachment is not considered a classical form of abscission, future studies to test whether candidate orthologs of *NEV* control the shedding of both types of lateral organs in other angiosperms will be informative.

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

Supplementary material
Supplementary material available online at
http://dev.biologists.org/cgi/content/full/136/11/1909/DC1

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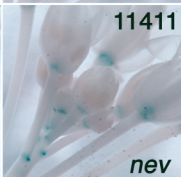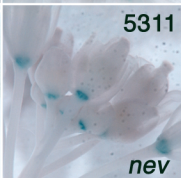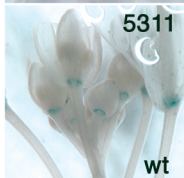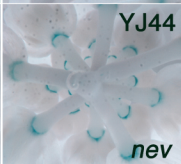
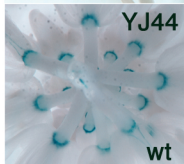| 2870 | 2870 |
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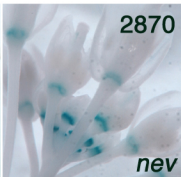
Table S1. Markers used in NEV co-localization experiments

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

*For assessment of localization patterns.

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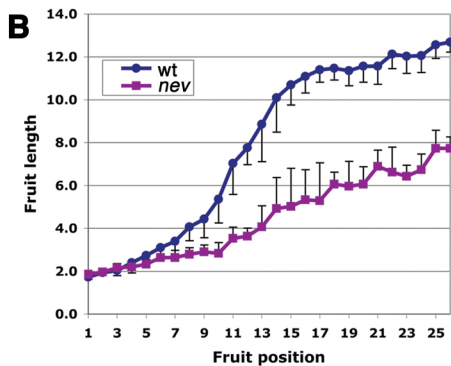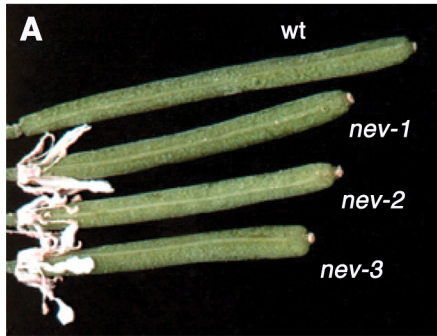
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         _____
NEV        MNEKANVSKELNARHRKILEGLLKHPENRECADCKTKGPRWASVNLGIFICMQCSGIHRS
OsI_025582 MNEKASVSKELNAKHKKILEGLLRLPENRECADCKSKGPRWASVNLGIFICMQCSGIHRS
                                                        _____

NEV        LGVHISKVRSATLDTWLPEQVAFIQSMGNDKANSYWEAELPPNYDRVGIENFIRAKYEEK
OsI_025582 LGVHISKVRSATLDTWLPEQVAFIQSMGNEKSNSYWEAELPPNYDRVGIENFIRAKYEDK
           _____

NEV        RWVSRGEKARSPPRVEQERRKSVERSGPGYEHGHSSSPVNLFEERKTIPASRTRNNVAAT
OsI_025582 RWIPRNGSSRPSSGARDEKSSESQTSVNRGGHNQRSS----FEQHRLSPAAVSKIAPVVS

NEV        RINLPVPPQGPSQVIKEQQK-MESAATEVEREKQAVNVAPASDPPKVDFATDLFNMLSMD
OsI_025582 RTPTQAPHQPKAQPSVEKVSPPQPEKSEPNATPPKVEKPSVAPPPKVDYATDLFNMLSMD

NEV        DSTITNTSEATPGDTPADDNSVAGFQSAGSGQTAEKIVTAKPAESSSPPASSSDFEDLFKD
OsI_025582 GTTEKEAESSS----NDDSAVEGFQSAEPVPSSDKDSAKPVESK--PQSTSGIEDLFKD

NEV        TPNLTTQQAEKD----VKGDIMSLFEKTNIVSPFAMHQQQVAMLAQQQALYMAAAKAAGG
OsI_025582 SPAVTVSSAEAAPQVNVNKDIMSLFEKSSMVSPYAVQQQLAFMTPQQLALLSQQQALLM

NEV        TPNGVN--QQATANALNVASATWSNPGGYQIFGMTNPVGGQADLQKLMQNMNMNANMNTR
OsI_025582 AALKAGNAPQMIPGNASLLNGAGSNPANGGLFSQS----WTNLAYQNPGLAPVAAQNGAT

NEV        PAQPQENTLQYPSSSEYTMCQANQVNGMTPNSTGKPQSSSATQPTSTTPSSQSGKDFDFS
OsI_025582 KVANNNQEFSFGNFNFSIPGAYNTSSSVPANGAASAAANKSTSPTSSSLPSQSGKEYDFS

NEV        SIMDGMFTKH
OsI_025582 SLTQGLFSKR
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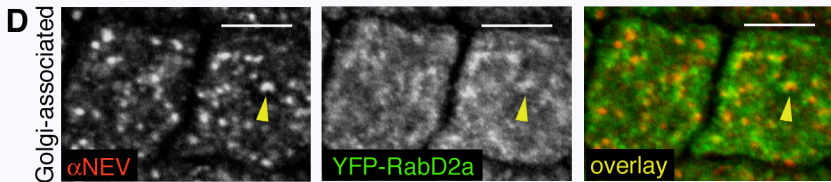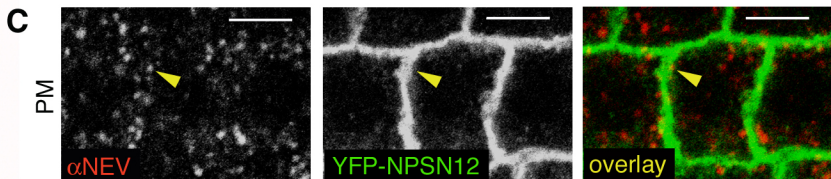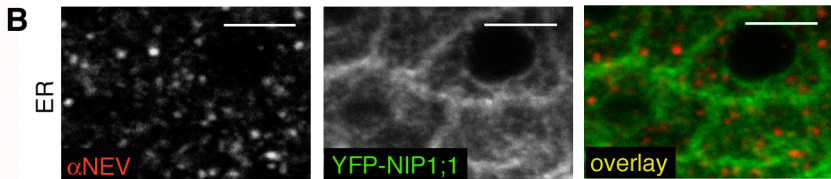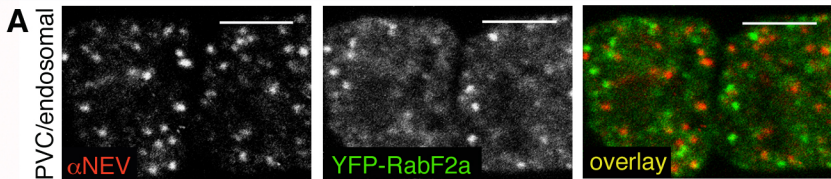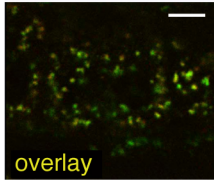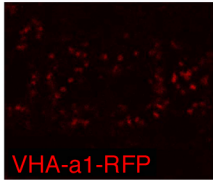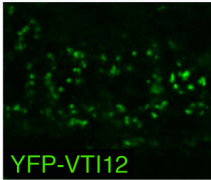
A PVC/endosomal — αNEV | YFP-RabF2a | overlay

B ER — αNEV | YFP-NIP1;1 | overlay

C PM — αNEV | YFP-NPSN12 | overlay

D Golgi-associated — αNEV | YFP-RabD2a | overlay

trans-Golgi network

YFP-VTI12   VHA-a1-RFP   overlay

Col WT
αNEV

A | B | C

g, t labels (wt)

D | E | F

cg labels (nev)

A — fruit — wt
B — fruit — nev
C — stem — wt
D — stem — nev