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The EVERSHED receptor-like kinase modulates floral organ shedding in *Arabidopsis*

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SUMMARY

Plant cell signaling triggers the abscission of entire organs, such as fruit, leaves and flowers. Previously, we characterized an ADP-ribosylation factor GTPase-activating protein, NEVERSHED (NEV), that regulates membrane trafficking and is essential for floral organ shedding in *Arabidopsis*. Through a screen for mutations that restore organ separation in *nev* flowers, we have identified a leucine-rich repeat receptor-like kinase, EVERSHED (EVR), that functions as an inhibitor of abscission. Defects in the Golgi structure and location of the trans-Golgi network in *nev* abscission zone cells are rescued by a mutation in *EVR*, suggesting that EVR might regulate membrane trafficking during abscission. In addition to shedding their floral organs prematurely, *nev evr* flowers show enlarged abscission zones. A similar phenotype was reported for plants ectopically expressing INFLORESCENCE DEFICIENT IN ABSCISSION, a predicted signaling ligand for the HAESA/HAESA-LIKE2 receptor-like kinases, indicating that this signaling pathway may be constitutively active in *nev evr* flowers. We present a model in which EVR modulates the timing and region of abscission by promoting the internalization of other receptor-like kinases from the plasma membrane.

KEY WORDS: SOBIR1, Receptor-like kinase, Membrane trafficking, Abscission, ARF-GAP, NEVERSHED, *Arabidopsis*

INTRODUCTION

Plants have evolved expansive groups of transmembrane receptor-like kinases (RLKs) that allow complex sampling of environmental conditions and developmental status. With over 600 RLKs annotated in the *Arabidopsis* genome, signaling complexity is enhanced by genetic redundancy (Shiu and Bleecker, 2001). Allowing for even further complexity, RLKs containing extracellular leucine-rich repeats (LRRs) can oligomerize and participate in multiple receptor complexes (Dievárt and Clark, 2003). For example, by interacting with the ligand-binding LRR-RLKs BRASSINOSTEROID INSENSITIVE1 (BRI1) and FLAGELLIN-SENSITIVE2 (FLS2), BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) functions in two discrete signaling complexes that regulate cell growth and pathogen defense, respectively (Li et al., 2002; Chinchilla et al., 2007).

Although RLKs are expected to play crucial roles in plant development, deduction of these roles has often required the phenotypical analysis of multiple loss-of-function mutants (Shpak et al., 2004; DeYoung et al., 2006; Morillo and Tax, 2006; Xu et al., 2008). A recent breakthrough is the discovery that a pair of LRR-RLKs, HAESA (HAE) and HAESA-LIKE2 (HSL2), are redundantly required for floral organ abscission (Jinn et al., 2000; Cho et al., 2008; Stenvik et al., 2008). Whereas the outer whorls of the *Arabidopsis* flower, containing the sepals, petals and stamens, are shed following fertilization in wild type, floral organs remain attached indefinitely in *hae hsl2* flowers. As shown for other LRR-RLKs (Li et al., 2002; Kinoshita et al., 2005; Karlova et al., 2006; Chinchilla et al., 2007), HAE and HSL2 probably function as members of larger receptor complexes that are activated through interactions with an extracellular ligand. A small, secreted peptide, INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), is required for abscission and is likely to be the ligand for HAE/HSL2 (Butenko et al., 2003; Cho et al., 2008; Stenvik et al., 2008; Butenko et al., 2009).

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

Membrane trafficking is emerging as an additional means of regulating plant transmembrane RLKs and is likely to modulate signaling during complex developmental events (Geldner and Robatzek, 2008). We recently reported that NEVERSHED (NEV), an ADP-ribosylation factor GTPase-activating protein, is

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

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

MATERIALS AND METHODS

Plants

The *evr-1* and *evr-2* alleles were obtained through ethyl methanesulfonate screens of *nev-3* (Landsberg *erecta*, L*er*) plants as described (Liljegren et al., 2000). The *evr-3* (*sobir1-12*; SALK_050715) and *evr-4* (*srrlk-2*; SALK_009453) alleles (Col) contain T-DNA insertions within codons 516 and 600, respectively (Alonso et al., 2003; Gao et al., 2009; Katiyar-Agarwal et al., 2007). The *At1g17750* (SALK_098161) allele contains an insertion within codon 137. Genotyping primers and restriction enzymes are listed in Table S1 in the supplementary material. Mutant alleles described previously include *nev-2*, *nev-3*, *nev-6*, *ida-2*, *hae-1*, *hsl2-1* and *pepr1-1* (SALK_014538) (Yamaguchi et al., 2006; Cho et al., 2008; Stenvik et al., 2008; Liljegren et al., 2009).

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

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

To generate *35S::IDA-GFP/GUS* plants, a tandem repeat of the 35S promoter was excised as an *Eco*RI/*Hin*dIII fragment from pBIN-JIT (Ferrandiz et al., 2000) and cloned in pBluescriptIISK(+) (Stratagene, La Jolla, CA, USA). The *IDA* cDNA was amplified with 5′-AAGCTTGACCCTTCATTCATTTACTC-3′ and 5′-GTCGACTGAGGAAGAGAGTTAACAAAAGAG-3′ from Col DNA, cloned into pCR2.1, excised with *Hin*dIII/*Sal*I, and cloned into the 35S/pBluescript construct. The *35S::IDA* fragment was excised with *Sac*I/*Sal*I, and replaced the *35S::CLV3* fragment in the pBGF-0 vector (Chytilova et al., 1999; Rojo et al., 2002) to create a translational fusion of IDA to GFP/GUS. Twelve out of 68 T1 lines showed the described phenotype.

Mapping

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

Kinase activity

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

To generate EVR-specific antiserum, a C-terminal peptide, CTLDDPKQRPNSKDVRTMLSQIKH, was synthesized and used to immunize chickens (Aves Labs, Tigard, Oregon, USA). Antisera were used at the following dilutions: anti-EVR (1:10,000), anti-phosphoserine (Sigma-Aldrich; 1:2000), anti-phosphotyrosine (Sigma-Aldrich; 1:2000), anti-phosphothreonine (Zymed/Invitrogen; 1:800). HRP-conjugated goat anti-chicken (Abcam, Cambridge, MA, USA) and chicken anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA, USA) secondary antibodies were used at a 1:10,000 dilution.

Microscopy

For scanning electron microscopy, flowers were fixed in 2% glutaraldehyde in 0.05 M sodium phosphate buffer, treated with 2% osmium tetroxide and dehydrated through an ethanol series. Samples were dried using a Samdri-795 critical point dryer (Tousimis Research Corporation, Rockville, MD, USA) and coated in gold-palladium using a Hummer X sputtering system (Anatech, Alexandria, VA, USA). Images were captured using a Zeiss Supra 25 scanning electron microscope with SmartSEM acquisition and imaging software. For transmission electron microscopy, flowers were analyzed as described (Liljegren et al., 2009). Confocal laser scanning microscopy of leaf petiole (stem), root, and cotyledon epidermal cells was performed with an LSM-510 (Carl Zeiss, Thornwood, NY, USA). Image brightness and contrast were adjusted with Adobe Photoshop CS4.

RESULTS

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

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

Mutations in *NEV* block abscission during the separation phase; the early patterning and differentiation of *nev* abscission zone (AZ) cells are unaffected (Liljegren et al., 2009). The secondary loss of *EVR* restored cell separation within *nev* AZs, such that all of the outer floral organs were shed from *nev evr* flowers (Fig. 1C; see also Fig. S1A in the supplementary material). As the densely cytoplasmic cells of *nev* sepal and petal AZs fail to become vacuolated and expand as they do in wild type (Fig. 1E,F) (Liljegren et al., 2009), we examined longitudinal sections of *nev evr* flowers at the time of shedding to see whether these defects were rescued by mutations in *EVR*. We found that cell vacuolation and expansion are not only rescued, but that *nev evr* AZ cells expand to a greater extent than do those of wild type (Fig. 1E-G).

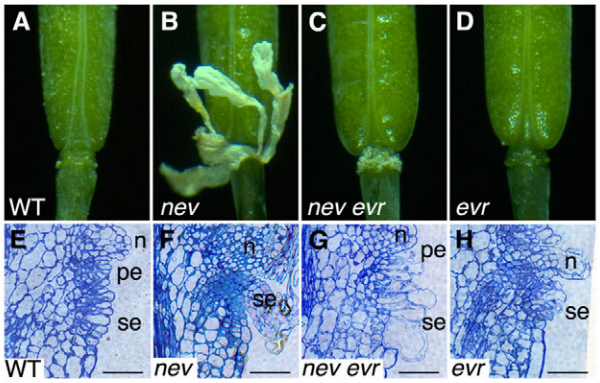

Fig. 1. Mutations in *EVR* rescue organ separation in *nev* flowers. (**A**) Wild-type flower after abscission (stage 17). (**B**) *nev* mutant flowers (stage 17) retain their floral organs indefinitely. (**C**) Organ shedding is rescued in *nev evr* flowers (stage 17). (**D**) *evr* flower after abscission (stage 17). (**E-H**) Longitudinal sections of wild-type (E), *nev* (F), *nev evr* (G) and *evr* (H) flowers (stage 16) stained with Toluidine Blue. In *nev evr* flowers (G), the remaining abscission zone (AZ) cells expand to a greater extent than do those of wild type (E). The sepal (se) and petal (pe) AZ regions are indicated, as are the nectaries (n). Scale bars: 50 μm.

To determine whether mutations in *EVR* affect organ separation in otherwise wild-type flowers, we isolated *evr-1* and *evr-2* single mutants. Floral organ shedding and AZ expansion appeared to be unaffected by loss of *EVR* alone compared with wild type (Fig. 1A,D,E,H; see also Fig. S1B in the supplementary material).

Organ abscission occurs prematurely in *nev evr* flowers

By examining the progression through flower development, we discovered that *nev evr* floral organs are shed prematurely (Fig. 2A,C,E,G). In wild-type flowers, buds open and pollen grains are released by the anthers (stage 13); fertilization of the ovules occurs shortly thereafter (stage 14) (Müller, 1961; Smyth et al., 1990). After the fruit begins to elongate (stage 15), the outer floral organs start to senesce and undergo abscission (stage 16) (Fang and Fernandez, 2002). To compare these developmental stages in wild-type and mutant flowers, the youngest open bud (stage 13) at the inflorescence apex was defined as the first floral position, the next older flower produced as the second position, and so on (Patterson, 2001). The percentage of flowers at each floral stage (13-17) was assessed for positions one to ten. Whereas all floral organs were shed (stage 17) from wild-type inflorescences by position 8, shedding was complete in *nev evr* inflorescences by position 6 (Fig. 2A,C). In *nev evr* flowers, abscission occurred before the petals wither, at a developmental timepoint resembling stage 15 (Fig. 2C, stage 15*) rather than stage 16. Similar results were obtained with petal breakstrength assays (Lease et al., 2006) of wild-type, *nev* and *nev evr* flowers. Whereas a similar force was required to remove petals from *nev* flowers at each position, the force required to remove *nev evr* petals dropped to zero by position 4, one position earlier than for wild type (see Fig. S2 in the supplementary material). These results suggest that *EVR* prevents premature organ abscission during the transition between fertilization and early fruit development.

nev and *nev evr* mutants show other growth defects, including reduced stature (see Fig. S3A in the supplementary material) and decreased fruit growth (Fig. 2F,G) (Liljegren et al., 2009). To confirm that premature shedding of *nev evr* floral organs was not due to a general defect in the timing of floral meristem formation, we also tracked individual wild-type and mutant flowers over the course of 5 days, from bud opening (day 0; stage 13) to the completion of abscission (stage 17). Whereas wild-type floral organs began shedding 3 days post-bud opening, *nev evr* floral organs began shedding one day earlier (see Fig. S3B in the supplementary material). These results suggest that *nev evr* floral organs abscise prematurely with respect to both floral age and stage of development.

Mutations in *EVR* alone did not substantially affect the timing of abscission (Fig. 2D,H; see also Fig. S3B in the supplementary material). However, *evr* fruit were significantly shorter (79%) than wild type (Fig. 2E,H), and *nev evr* fruit were shorter (88%) than *nev* fruit (Fig. 2F,G).

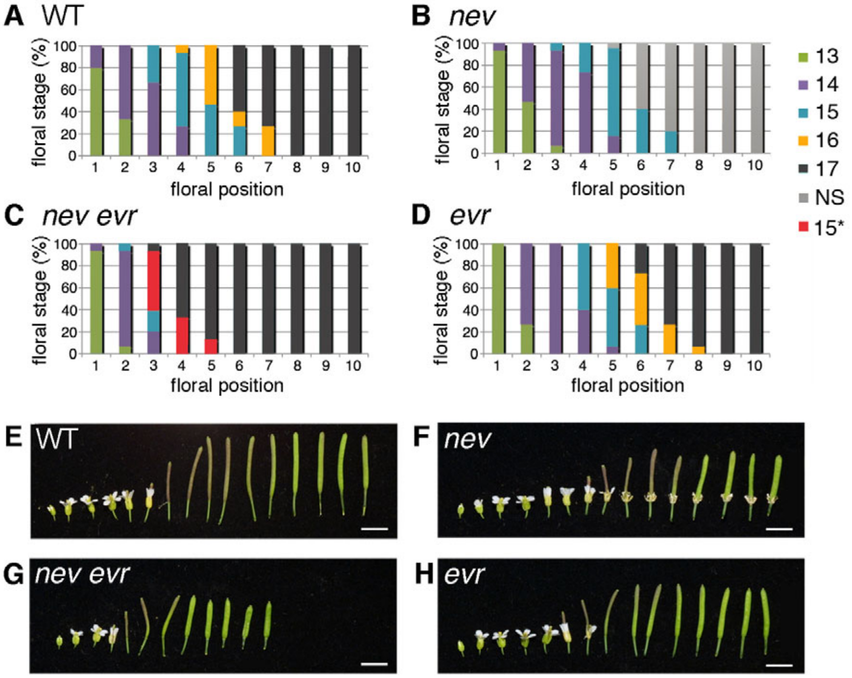

Fig. 2. Abscission occurs prematurely in *nev evr* flowers. Progression of flower development in wild-type, *nev*, *nev evr* and *evr* plants from the first open flower to maturing fruit. (**A-D**) From the first open flower (position 1), stages were assessed for up to 10 flowers per inflorescence (*n*=15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

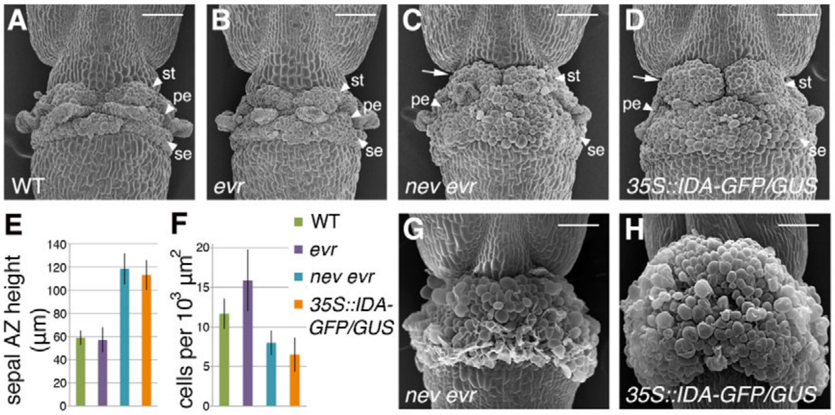

Fig. 3. Ectopic AZ cell expansion occurs in *nev evr* flowers. (**A-D**) Scanning electron micrographs (SEMs) of flowers immediately after organ separation (first stage 17 flower). Owing to increased cell expansion and the presence of additional cells, *nev evr* flowers (C), like flowers constitutively expressing *IDA* (D), develop larger AZs than do wild-type (A) and *evr* (B) flowers, which cover the stem-like gynophore of the fruit (arrows). (**E**,**F**) Quantification of AZ size (E) and cell expansion (F) in wild-type and mutant flowers ($n\geq4$, second stage 17 flower). In *nev evr* and *35S::IDA-GFP/GUS* flowers, the average heights of the sepal AZs are 2-fold greater than those of wild-type or *evr* flowers (E). A decrease in the number of sepal AZ cells in a defined area was observed for *nev evr* and *35S::IDA-GFP/GUS* flowers compared with wild-type and *evr* flowers, suggesting that cell expansion contributes in part to the increase in AZ size (F). (**G**,**H**) The expanding AZs of older *nev evr* (G) and *35S::IDA-GFP/GUS* (Col) (H) flowers (stage 17) envelop the nectaries and form visible collars of tissue at the fruit bases. The sepal (se), petal (pe) and stamen (st) AZs cannot be distinguished. Scale bars: 100 µm.

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

We found that expansion of *nev evr* AZ cells is increased in comparison to wild type at the time of shedding (Fig. 1E,G). To further characterize this phenotype, we took scanning electron micrographs (SEMs) of wild-type, *evr* and *nev evr* flowers after organ separation was complete (youngest stage 17). In wild-type flowers, discrete AZs were found at the base of the developing fruit (Fig. 3A). Loss of *EVR* alone did not appear to affect AZ development in comparison to wild type (Fig. 3B). By contrast, *nev evr* AZs were less defined and appeared to expand into the neighboring fruit and stem tissues (Fig. 3C). In a comparison of sepal AZ height in stage 17 SEMs, we found that *nev evr* sepal AZs were 2-fold taller than either wild-type or *evr* sepal AZs (Fig. 3E). In addition, the stem-like base of the fruit known as the gynophore was hidden owing to expansion of the stamen AZs in mature *nev evr* flowers (Fig. 3C, arrow). Cells within *nev evr* sepal AZs were also significantly larger in comparison to wild-type and *evr* cells (cells/10^3 µm^2 measurement, Fig. 3F), indicating that cell expansion contributes in part to the increase in AZ size. In older flowers, the cell expansion was even more striking – discrete AZs were less recognizable and the floral nectaries became enveloped in the expanding tissue (Fig. 3G). Later on, the expanded *nev evr* AZ cells burst, leaving behind a visible collar of rough tissue around the base of the fruit (Fig. 1C).

A similar phenotype was reported for flowers misexpressing the putative signaling ligand INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) (Stenvik et al., 2006; Cho et al., 2008). We generated plants that constitutively express an IDA-GFP/GUS translational fusion under the control of the viral *35S* promoter. We also observed enlargement of the floral AZs and overexpansion of individual AZ cells (Fig. 3D-F,H). Similar to what we observed in *nev evr* flowers, premature abscission of the sepals, petals and stamens has been reported for *35S::IDA* flowers (Stenvik et al., 2006). These results suggest that the regulation of AZ size and cell expansion by *NEV*, *EVR* and *IDA* is important for the proper timing of organ shedding in *Arabidopsis*.

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

The *nev evr* phenotype suggests an important role for EVR in abscission. To clarify its mechanism of action, we identified single nucleotide changes for the *evr-1* and *evr-2* alleles within At2g31880, one of the 34 predicted genes in the mapping interval. This intronless gene encodes a 641 amino acid protein of the leucine-rich repeat receptor-like kinase (LRR-RLK) family (Fig. 4A). Eleven additional alleles of *EVR* were identified in a screen for mutations that rescue seedling lethality in *bak1-interacting receptor-like kinase1* (*bir1*) mutants (Gao et al., 2009). Loss of *BIR1* is predicted to cause constitutive activation of a SUPPRESSOR OF BIR1 (SOBIR1)/EVR-mediated signaling pathway for disease resistance (Gao et al., 2009). Also encoded within the *EVR* open-reading frame is a heterogeneous cluster of small RNAs that are expressed upon pathogen infection, and for which *EVR* has also been named *small RNA-generating RLK* (Katiyar-Agarwal et al., 2007) (see Fig. S4A in the supplementary material).

Based on the sequence of its kinase domain, EVR has been assigned to the 28-member LRR-RLK subfamily XI (Shiu and Bleecker, 2001). This subfamily includes the HAESA (HAE) and HAESA-LIKE2 (HSL2) RLKs known to redundantly promote organ shedding (Jinn et al., 2000; Cho et al., 2008; Stenvik et al., 2008), the CLATAVA1 and BARELY ANY MERISTEM RLKs that control stem cell differentiation in shoot and flower meristems (Clark et al., 1997; DeYoung et al., 2006; DeYoung and Clark, 2008), the HAIKU RLK that regulates seed size (Luo et al., 2005) and the PEP1 RECEPTOR1 (PEPR1) that is involved in pathogen response (Yamaguchi et al., 2006). Although most members of subfamily XI have extensive sets of 19-32 LRRs in their extracellular domains (Shiu and Bleecker, 2001), EVR has only five predicted LRRs (Fig. 4A). However, in the context of the entire LRR-RLK family, EVR is not unique; the majority of *Arabidopsis* LRR-RLKs have fewer than ten LRRs (Shiu and Bleecker, 2001).

The *evr-1* and *evr-2* mutations are both predicted to be loss-of-function alleles that are unlikely to affect transcriptional regulation of *EVR* or the alternatively encoded small RNAs. The *evr-1* allele

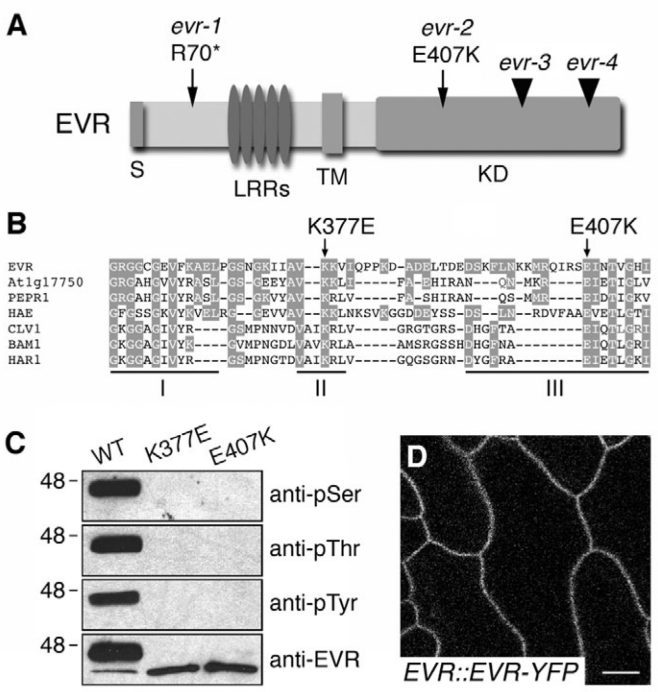
A
evr-1
R70*
evr-2
E407K
evr-3
evr-4
EVR
S
LRRs
TM
KD
B
K377E
E407K
EVR GRGGCGEVFKAELPGSNGKIIAV--KKVIQPPKD-ADELTDEDSKFLNKKMRQIRSEINTVGHI
At1g17750 GRGAHGVVYRASL-GS-GEEYAV--KKLI-----FA-EHIRAN---QN-MKR----EIETIGLV
PEPR1 GRGAHGIVYRASL-GS-GKVYAV--KRLV-----FA-SHIRAN---QS-MMR----EIDTIGKV
HAE GFGSSGKVYKVELRG--GEVVAV--KKLNKSVKGGDDEYSS-DS--LN--RDVFAAEVETLGTI
CLV1 GKGGAGIVYR----GSMPNNVDVAIKRLV-----GRGTGRS-DHGFTA---------EIQTLGRI
BAM1 GKGGAGIVYK----GVMPNGDLVAVKRLA-----AMSRGSSHDHGFNA---------EIQTLGRI
HAR1 GKGGAGIVYR----GSMPNGTDVAIKRLV-----GQGSGRN-DYGFRA---------EIETLGKI
I
II
III
C
WT
K377E
E407K
48
anti-pSer
48
anti-pThr
48
anti-pTyr
48
anti-EVR
D
EVR::EVR-YFP

Fig. 4. *EVR* encodes a membrane-localized LRR-RLK. (**A**) Diagram of the EVR protein, with the sites of the identified *evr* mutations indicated. The regions corresponding to the signal peptide (S), leucine-rich repeats (LRRs), transmembrane domain (TM) and kinase domain (KD) are indicated. Point mutations are marked by arrows, and T-DNA insertions by arrowheads. (**B**) Sequence alignment of kinase subdomains I-III from EVR and related LRR-RLKs from *Arabidopsis* and *Lotus japonicus*. Amino acids conserved between EVR and other proteins are shaded. The sites of the conserved glutamic acid in subdomain III affected by the *evr-2* mutation and an invariant lysine in subdomain II required for kinase activity are marked by arrows above the alignment. (**C**) The recombinant EVR KD autophosphorylates at serine, threonine and tyrosine residues in vitro. Mutations in subdomains II (K377E) and III (E407K) of the kinase domain block the kinase activity of EVR. EVR antiserum recognizes ~46 and ~40 kDa phosphorylated and unphosphorylated proteins, respectively. (**D**) EVR localizes to the plasma membrane. Fluorescent localization of the EVR-YFP marker in epidermal cells of wild-type leaf petioles (stems). Scale bar: 10 μm.

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

Although up to 20% of *Arabidopsis* RLKs are predicted to be kinase dead (Castells and Casacuberta, 2007), isolation of the *evr-2* mutation suggests that EVR kinase activity is required to regulate abscission (Fig. 4A,B). To facilitate the analysis of EVR kinase activity, we generated EVR-specific antiserum using a 24-amino acid peptide corresponding to its unique C terminus. The EVR antibody recognizes the EVR-KDs of wild type (WT), a kinase-dead mutant (K377E) (Horn and Walker, 1994) and the *evr-2* mutant (E407K) expressed as N-terminal 6×His-tagged fusion proteins in *E. coli* (Fig. 4B,C). Whereas the purified $\mathrm{KD}^{\mathrm{K377E}}$ and $\mathrm{KD}^{\mathrm{E407K}}$ proteins migrate as single bands of ~40 kDa near the predicted size of 39 kDa, the purified $\mathrm{KD}^{\mathrm{WT}}$ migrates as two distinct bands of ~40 and 46 kDa, suggesting that the wild-type protein is phosphorylated (Fig. 4C).

To test whether EVR is a functional serine/threonine kinase, we used phosphoserine and phosphothreonine antisera to detect phosphorylated residues on the recombinant KDs. Both antisera recognized the upper $\mathrm{KD}^{\mathrm{WT}}$ band but not the lower, presumably unphosphorylated, $\mathrm{KD}^{\mathrm{WT}}$ band. Neither $\mathrm{KD}^{\mathrm{K377E}}$ nor $\mathrm{KD}^{\mathrm{E407K}}$ appear to have kinase activity, as the antisera did not recognize these mutant proteins (Fig. 4C). As some LRR-RLKs have also been demonstrated to be dual-specificity kinases (Mu et al., 1994; Oh et al., 2009), we also tested the ability of the recombinant EVR-KDs to undergo autophosphorylation at tyrosine residues. We found that phosphotyrosine antiserum exhibited the same recognition pattern as that of the phosphoserine and phosphothreonine antisera (Fig. 4C), and that treatment with calf intestinal alkaline phosphatase was able to completely dephosphorylate the tyrosine residues of EVR-$\mathrm{KD}^{\mathrm{WT}}$ (see Fig. S4B in the supplementary material). These results suggest that EVR is a dual-specificity kinase that autophophorylates serine, threonine and tyrosine residues in vitro.

EVR, like other transmembrane receptor-like kinases, is predicted to localize to the plasma membrane (Alexandersson et al., 2004). To visualize EVR localization in vivo, we generated transgenic plants expressing *EVR-YFP* under control of the *EVR* promoter (see Fig. S4A in the supplementary material). Using confocal laser scanning microscopy, six out of six independent transgenic lines showed localization of EVR-YFP chimeric protein to the periphery of epidermal cells (Fig. 4D), and early colocalization of EVR-YFP with the lipophilic dye FM4-64 was observed (Fig. S4C in the supplementary material). These results suggest that EVR is localized to the plasma membrane and/or internal membrane compartments (Vida and Emr, 1995).

EVR is expressed in floral organ AZs prior to cell separation

RT-PCR and global expression experiments indicate that *EVR* transcripts are present in multiple tissues, with increasing expression in older flowers (see Fig. S5A,B in the supplementary material) (Schmid et al., 2005). To determine whether *EVR* is expressed at the right time and place to modulate organ separation, we generated transgenic plants that express β-glucuronidase (GUS) under the control of the predicted *EVR* promoter (see Fig. S4A in the supplementary material). Of 83 T1 plants examined, 58 showed GUS expression in floral organ AZs, typically just prior to organ shedding (stage 15) with strengthened expression in older flowers (stages 16 and early 17; Fig. 5A,C). This profile is similar to the reported expression patterns of *HAE::GUS* and *HSL2::GUS* (Fig. 5A-D) (Jinn et al., 2000; Cho et al., 2008), and is consistent with a role for EVR in modulating the timing of organ shedding.

Two independent *EVR::GUS* lines with single transgene insertions were characterized in the T2 generation. In addition to expression in floral organ AZs, each line showed GUS expression within the style of the developing fruit (stage 15; Fig. 5E), at the bases of the cauline leaves (Fig. 5G), and in the stems of the first rosette leaves (see Fig. S5C in the supplementary material). Because *evr* mutants showed defects in fruit growth (Fig. 2H), and global

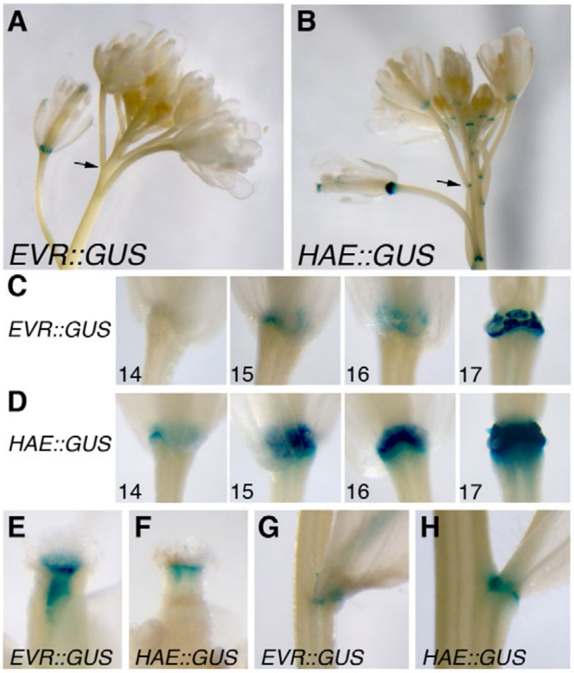

Fig. 5. *EVR* is expressed in organ AZs. (**A-H**) The regulatory regions of *EVR* and *HAE* direct expression of β-glucuronidase (GUS) in AZs (A-D), in internal tissues of the floral style (E,F), and at the junction between the cauline leaves and the inflorescence stem (G,H). The *HAE::GUS* marker is also expressed at the bases of the floral pedicels (B, arrow). Within floral AZs (C), the *EVR* promoter directs GUS expression prior to organ separation (stage 15), during abscission (stage 16), and as the remaining cells form protective scar tissue (stage 17). GUS expression directed by the *HAE* promoter shows a similar, yet stronger profile in AZs, with an earlier stage of initiation (Jinn et al., 2000).

transcriptional analysis suggests that *EVR* has a broader expression profile than our *EVR::GUS* markers (see Fig. S5B in the supplementary material) (Schmid et al., 2005); these might not reflect the complete expression profile of *EVR*. The *HAE* promoter was also observed to direct GUS expression in the style (Fig. 5F), at the cauline leaf bases (Fig. 5H), and at the junction between the floral and inflorescence stems (Fig. 5B, arrow). These observations suggest that EVR and HAE may co-regulate the development of a set of plant tissues.

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

As mutations in *EVR* rescue abscission in *nev* flowers, we examined AZ cells at the time of organ shedding to determine whether the membrane trafficking defects observed in *nev* flowers were also rescued. Loss of *NEV* leads to a distinctive bending of the Golgi cisternae, disruption of the trans-Golgi network (TGN) and the accumulation of clusters of vesicles known as paramural bodies (PMBs) between the plasma membrane and cell wall of AZ cells (Liljegren et al., 2009). Unlike *nev* cells, *nev evr* cells contain flat stacks of Golgi cisternae with a wild-type appearance and closely associated TGN (Fig. 6A-C,E). The Golgi stacks and TGN of *evr* cells resemble those of wild type (Fig. 6D-E). As with *nev* cells, PMBs with more than 30 vesicles were observed in *evr* cells, but not in wild-type or *nev evr* cells (Fig. 6F-J; see also Fig. S6 in the supplementary material). As the defects in Golgi morphology and TGN location correlate with non-shedding floral organs, these might represent the primary cellular changes associated with and potentially responsible for the block of organ shedding in *nev* flowers. Furthermore, the restoration of Golgi/TGN structure in *nev evr* flowers and the defects in PMB formation in *evr* flowers suggest the possibility that, like NEV, EVR might regulate membrane trafficking at the transition to floral organ shedding.

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

To potentially identify additional regulators of abscission, we evaluated whether mutations in two *EVR*-related genes could also rescue *nev*-mediated defects. EVR shares 42 and 45% amino acid identity within the kinase domains of the PEPR1 and At1g17750 LRR-RLKs, respectively (Fig. 4B). PEPR1 was previously found to be a receptor for the PEP1 peptide involved in the innate immune response of plants to pathogen attack (Yamaguchi et al., 2006), and At1g17750 has been shown to be transcriptionally induced by the fungal protein, Nep1 (Necrosis and ethylene-inducing peptide1) (Qutob et al., 2006). *EVR* is also expressed in response to viral and bacterial infection (Whitham et al., 2003; Katiyar-Agarwal et al., 2007; Ascencio-Ibanez et al., 2008). Despite the similarities within the kinase domain and the overlapping expression patterns (see Fig. S5B in the supplementary material), predicted loss-of-function mutations in *PEPR1* and At1g17750 did not rescue organ shedding in *nev* flowers (Fig. 7). These results suggest that these closely related kinases do not regulate organ separation, or that their roles are hidden by additional genetic redundancy.

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

As mutations in *NEV*, *IDA*, and the redundant genes *HAE* and *HSL2* all appear to block floral organ shedding at a similar point during the cell separation stage (Butenko et al., 2003; Cho et al., 2008; Stenvik et al., 2008; Liljegren et al., 2009), we sought to use mutations in *EVR* to test the genetic relationship between these genes. We found that loss of *EVR* does not rescue shedding in either *ida* or *hae hsl2* flowers (Fig. 8A-D). These results suggest that *NEV* and *EVR* could act upstream of *IDA* and *HAE/HSL2*, or in a parallel pathway that converges at the point of *HAE/HSL2* function or further downstream (Fig. 8E).

DISCUSSION

Here, we report the characterization of EVR, an *Arabidopsis* LRR-RLK that modulates floral organ abscission and promotes fruit development. Our studies suggest that EVR functions to inhibit organ separation by regulating signaling that affects the timing of AZ activity, AZ size and cell expansion.

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

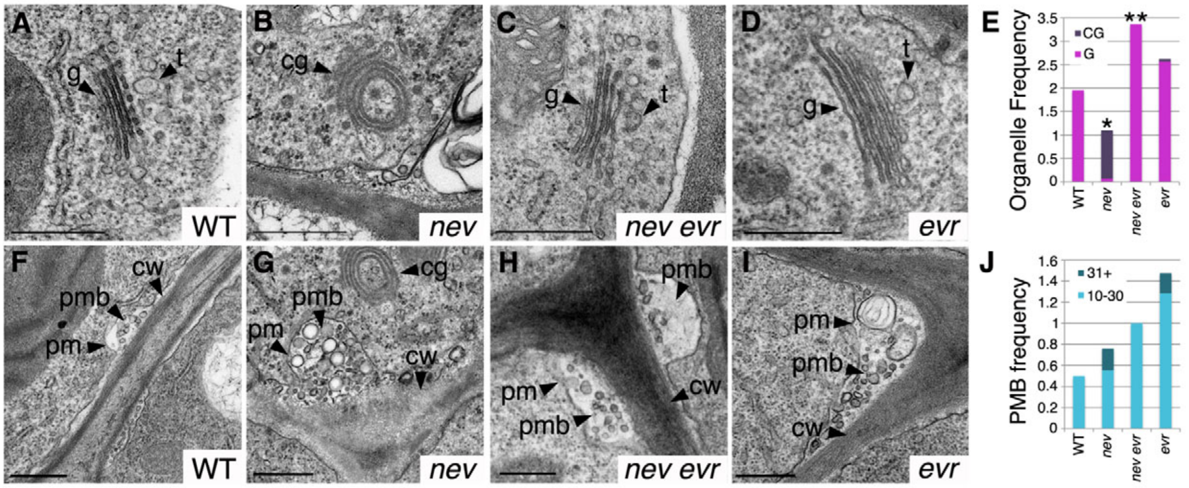

Fig. 6. Mutations in *EVR* restore Golgi structure and location of the TGN in *nev* flowers. Transmission electron micrographs and analysis of cells in sepal AZ regions at the time of organ separation for wild-type (stage 16), *nev* (stage 16 non-shedding), *nev evr* (stage 15*) and *evr* (stage 16) flowers. (**A-D**) Instead of the flat stacks of Golgi cisternae characteristic of wild type (A), circularized multilamellar structures are observed in *nev* cells (B). Golgi with a wild-type appearance are found in *nev evr* (C) and *evr* cells (D). We frequently observed vesicular-tubular structures characteristic of the trans-Golgi network (TGN) (81%, *n*=16) closely associated with the Golgi cisternae (34±21 nm, *n*=13) in wild-type cells (A), whereas the TGN (15%, *n*=13) was less often observed near the circularized multilamellar structures (40±25 nm, *n*=2) of *nev* cells (B). The location of the TGN was restored in *nev evr* cells (83% associated with Golgi, *n*=24; 37±12 nm, *n*=20) and was unaffected by loss of *EVR* alone (76% associated with Golgi, *n*=21; 38±25 nm, *n*=15). (**E**) Frequency of flat Golgi cisternae (G, pink) and circularized multilamellar structures (CG, purple) per cell in sections of wild-type and mutant sepal AZ regions. For each genotype, *n* (cells)≥11. Statistical differences between *nev* and wild type, and between *nev evr* and *nev* tissues are indicated by single and double asterisks, respectively (Fisher's exact test, $P<0.0001$). A statistical difference was not detected between *evr* and wild-type tissues. (**F-I**) Paramural bodies (PMBs) were observed in the cells of wild-type (F), *nev* (G), *nev evr* (H) and *evr* (I) flowers. Whereas PMBs were observed in cells from each genotype, PMBs with greater than 30 vesicles were only observed in *nev* and *evr* cells. (**J**) Frequency of PMBs (10-30 and 31+ vesicles) per cell in sections of wild-type and mutant sepal AZ regions. For each genotype, *n* (cells)≥11. Statistical differences in PMB accumulation were not detected. cg, circularized multilamellar structures; cw, cell wall; g, Golgi cisternae; pm, plasma membrane; pmb, paramural body; t, trans-Golgi network. Scale bars: 0.5 μm.

We have discovered that EVR acts as both a temporal and spatial regulator of abscission. *EVR* regulatory regions first direct *GUS* expression in floral AZs during the transition from fertilization to fruit growth (stage 15; Fig. 5A,C). In wild-type flowers of the L*er* ecotype, organ separation (stage 16) occurs about three days after the buds open and the stamens release their pollen (see Fig. S3B in the supplementary material). Loss of *EVR* causes premature abscission of floral organs in *nev* flowers (stage 15*) two positions earlier than in wild-type inflorescences (Fig. 2C), and about two days after bud opening (see Fig. S3B in the supplementary material). These results indicate that younger flowers are competent to respond to signals to shed their organs, as was previously observed for flowers treated with ethylene or misexpressing IDA (Butenko et al., 2003; Stenvik et al., 2006). More importantly, our results suggest that EVR is one of the factors that acts to inhibit this response. Although mutations in *EVR* alone do not affect the timing of abscission, multiple levels of genetic redundancy probably exist to ensure that organ separation does not occur prematurely.

Mutations in *EVR* appear to alter the spatial restriction of AZ signaling. Whereas smooth, scar-like surfaces form at the sites of organ detachment in wild-type flowers (Fig. 3A), increased expansion of individual AZ cells leads to visible collars of rough, broken tissue at the bases of *nev evr* fruit (Fig. 1C, Fig. 3F,G). AZ regions are also enlarged in *nev evr* flowers compared with wild type (Fig. 3E). These AZ phenotypes strongly resemble those of plants constitutively expressing *IDA* (Fig. 3E-H) (Stenvik et al., 2006; Cho et al., 2008), suggesting that excess levels of the IDA ligand or an activated HAE/HSL2 receptor complex might be present in *nev evr* flowers. An intriguing possibility is that ectopic AZ signaling causes cell wall loosening and expansion of cells both within and neighboring the original AZs of *nev evr* flowers. Although the relationship between AZ cell expansion and organ separation in wild-type *Arabidopsis* flowers is unclear (Patterson, 2001), cell expansion might physically enable the shedding of floral organs during the cell wall loosening process. In this capacity, the

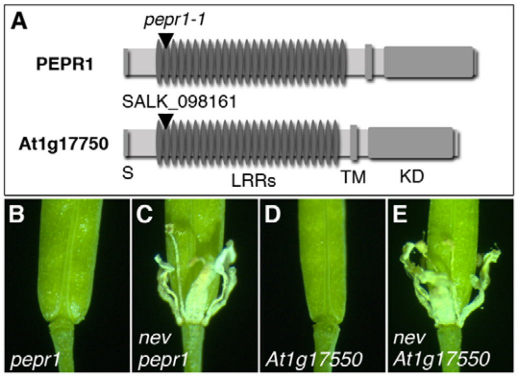

Fig. 7. Mutations in *PEPR1* or At1g17750 do not rescue shedding in *nev* flowers. (**A**) Diagrams of the predicted PEPR1 and At1g17750 proteins, with the sites of the T-DNA mutations indicated by arrowheads. The regions corresponding to the signal peptide (S), leucine-rich repeats (LRRs), transmembrane domain (TM, and kinase domain (KD) are indicated. (**B-E**) Floral organ shedding occurs normally in *pepr1* (B) and *At1g17750* (D) mutant flowers (stage 17), but is blocked in *nev pepr1* (C) and *nev At1g17750* (E) flowers (stage 17).

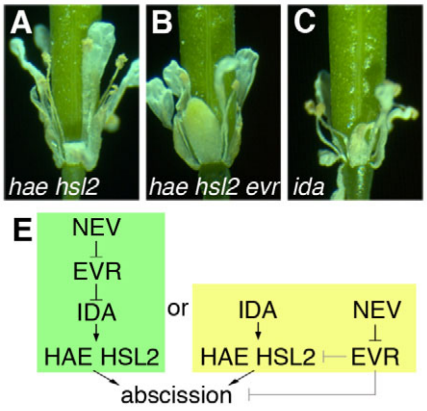

Fig. 8. Mutations in EVR do not restore abscission in *ida* or *hae hsl2* mutant flowers. (**A-D**) Organ separation is blocked in *ida* (C) and *hae hsl2* (A) mutant flowers (stage 17), and is not rescued in *ida evr* (D) or *hae hsl2 evr* (B) flowers (stage 17). (**E**) Alternative pathways for the relationships between NEV, EVR, IDA and HAE/HSL2. In both, EVR is predicted to act as a negative regulator of abscission and to function downstream of NEV. In the simplest model, NEV and EVR act upstream of both IDA and HAE/HSL2. Alternatively, NEV and EVR could function in a converging pathway, in which EVR acts upon HAE/HSL2, or further downstream.

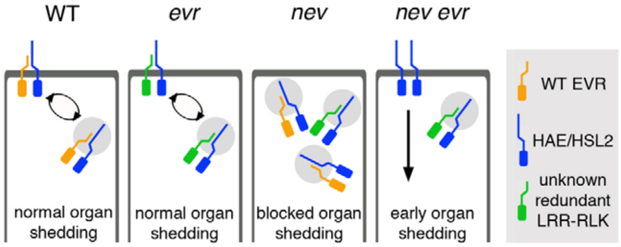

Fig. 9. A model for EVR function during the transition to floral organ separation. In wild-type AZ cells before organ shedding, EVR might inhibit cell separation by interacting with ligand-binding LRR-RLKs, such as HAE/HSL2, that trigger cell wall loosening and separation. This interaction could promote the internalization and recycling of inactive receptor complexes through the endosomal system. Continued signaling from a ligand-activated HAE/HSL2 complex would lead to organ shedding in older flowers. The EVR RLK may act redundantly with another LRR-RLK(s), such that loss of *EVR* alone would not alter the timing of abscission. Disrupting NEV activity might alter the trafficking of receptor complexes containing EVR or EVR-like RLKs, thereby blocking a signal required for cell separation. Mutations in *EVR* could bypass the requirement for *NEV* in floral organ shedding, resulting in constitutive signaling of the HAE/HSL2 LRR-RLKs, premature activation of organ separation, the enlargement of AZ regions, and deregulated AZ cell expansion.

contribution of AZ cell expansion to organ abscission might be analogous to that of lignified fruit cells to *Arabidopsis* pod dehiscence (Liljegren et al., 2004).

With five LRRs in its extracellular domain, EVR may inhibit organ separation by regulating the behavior of ligand-binding LRR-RLKs. One of the best characterized interactions between non-ligand and ligand-binding LRR-RLKs is that of BAK1 (SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE3) and FLAGELLIN-SENSITIVE2 (FLS2) during pathogen-triggered immunity (Geldner and Robatzek, 2008; Boller and Felix, 2009). Recognition of the flagellin-derived flg22 peptide by FLS2 triggers interaction with BAK1 (Chinchilla et al., 2007; Heese et al., 2007). As a co-receptor for FLS2, BAK1 promotes both flg22-induced signaling and internalization of FLS2 from the plasma membrane (Chinchilla et al., 2007). Although EVR may function similarly to BAK1 – by mediating the membrane trafficking of a ligand-binding receptor – we predict that it plays a unique role by acting as an inhibitor of its LRR-RLK partner prior to ligand binding, rather than as a co-receptor after ligand binding. Further studies will be necessary to pinpoint the location(s) of EVR activity; our initial results suggest that EVR functions at the plasma membrane and in closely associated vesicles (Fig. 4D; see also Fig. S4C in the supplementary material).

A model for EVR function during the transition from fertilization to floral organ shedding is shown in Fig. 9. In wild-type AZ cells, plasma membrane-localized EVR may interact with and inhibit the activity of ligand-binding LRR-RLK(s), such as HAE and HSL2. In contrast to BAK1, for which FLS2 binding is enhanced by the presence of ligand, EVR may promote the internalization of an inactive receptor complex, thereby limiting the pool of available receptors and delaying abscission signaling. Increased availability of the HAE/HSL2 receptors, or of the putative ligand IDA, may trigger cell separation in older flowers. Loss of EVR function alone would not alter the timing of floral organ abscission owing to the activity of a redundant LRR-RLK(s). This RLK would also be predicted to promote internalization of inactive receptor complexes in wild-type cells. Loss of NEV function in the TGN and other endosomal compartments could alter the trafficking of receptor complexes containing EVR and other inhibitory RLKs, such that the components required to activate cell separation are not recycled back to the plasma membrane. Secondary loss of EVR function could bypass the requirement for NEV in floral organ shedding, potentially resulting in a constitutive signal for cell separation that is no longer internalized or targeted for degradation. Increased signaling might be responsible for the premature organ shedding, enlarged AZs and deregulated cell expansion that we observe in *nev evr* flowers.

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

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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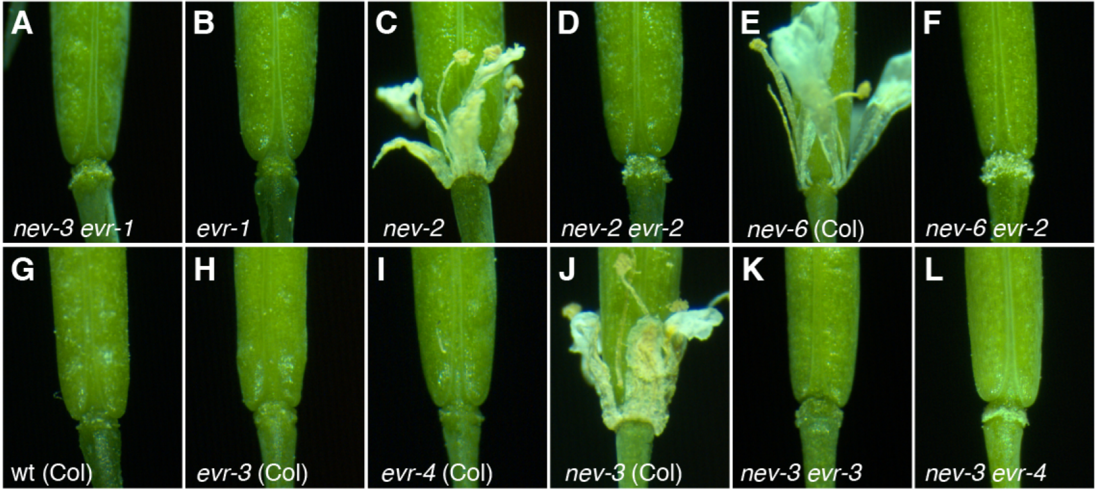
A
nev-3 evr-1
B
evr-1
C
nev-2
D
nev-2 evr-2
E
nev-6 (Col)
F
nev-6 evr-2
G
wt (Col)
H
evr-3 (Col)
I
evr-4 (Col)
J
nev-3 (Col)
K
nev-3 evr-3
L
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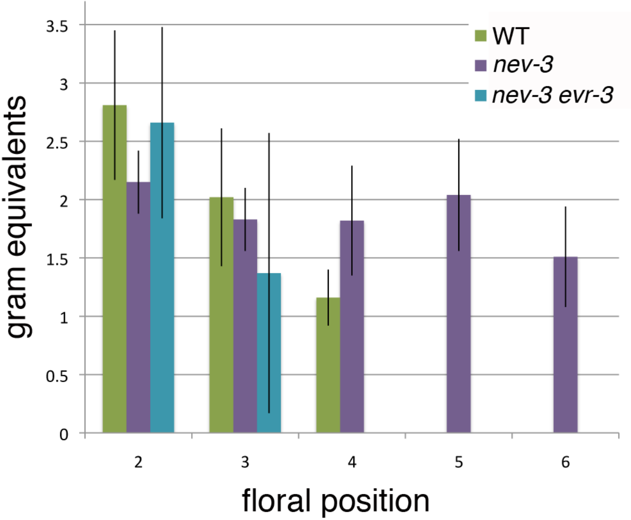
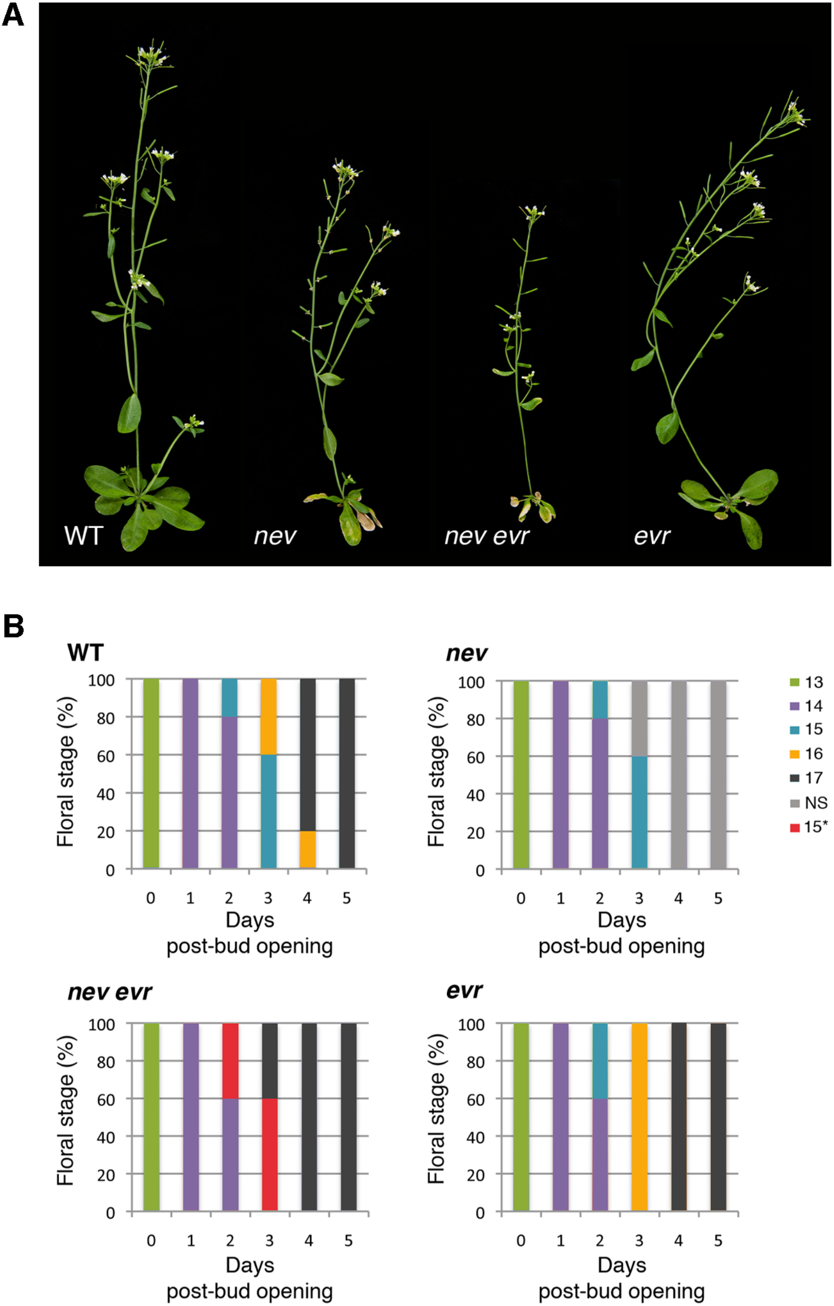
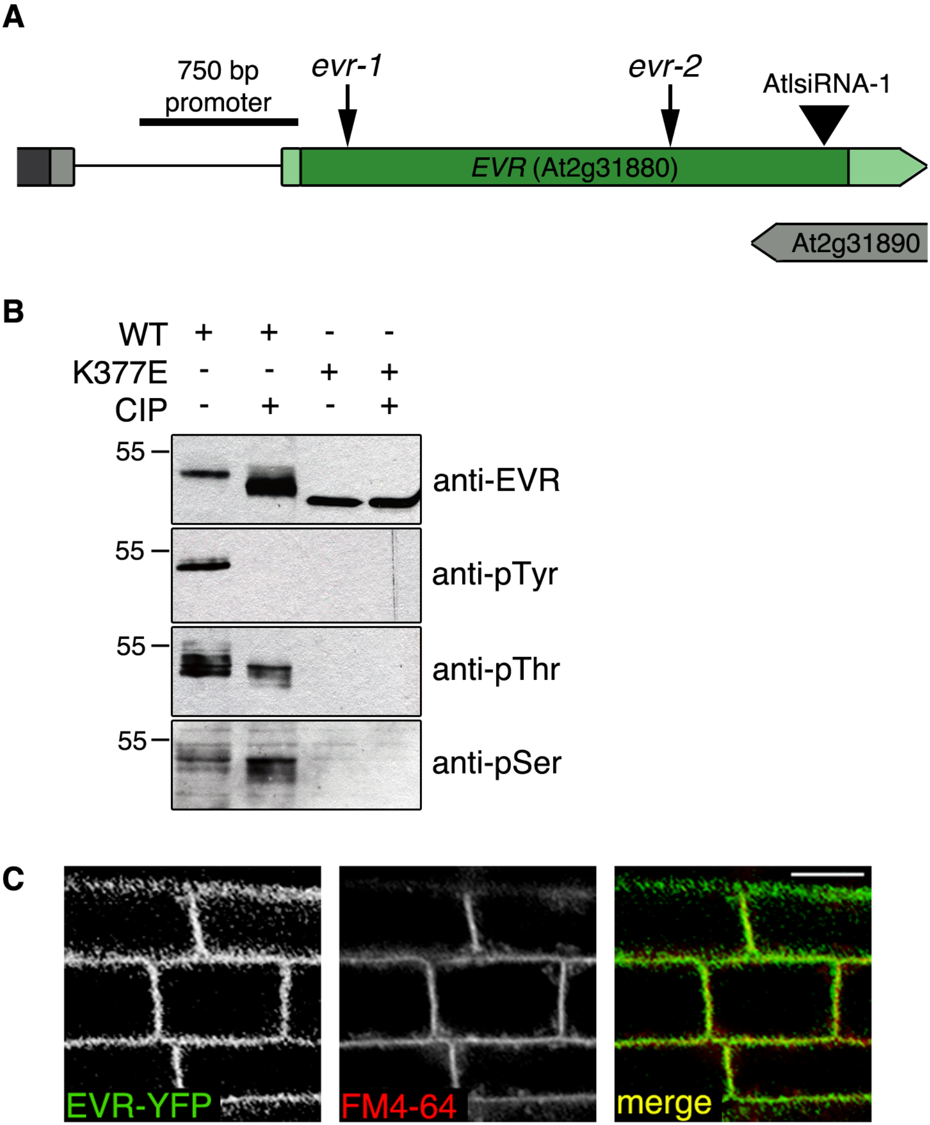
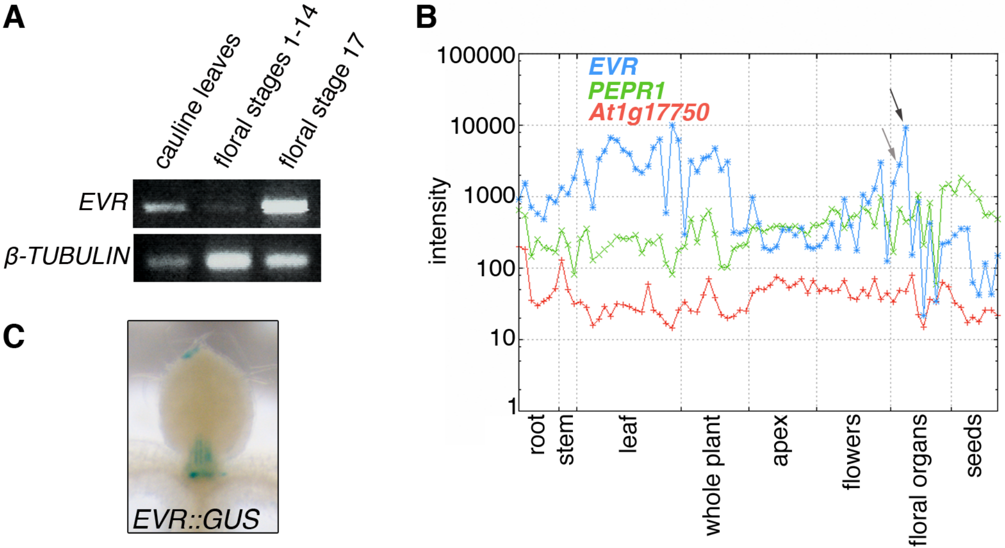

WT
nev-3
nev-3 evr-3
gram equivalents
3.5
3
2.5
2
1.5
1
0.5
0
2
3
4
5
6
floral position


A
WT
nev
nev evr
evr
B
WT
nev
nev evr
evr
Floral stage (%)
0
20
40
60
80
100
Days post-bud opening
0
1
2
3
4
5
13
14
15
16
17
NS
15*


A
750 bp promoter
evr-1
evr-2
AtlsiRNA-1
EVR (At2g31880)
At2g31890
B
WT + + - -
K377E - - + +
CIP - + - +
55
anti-EVR
55
anti-pTyr
55
anti-pThr
55
anti-pSer
C
EVR-YFP
FM4-64
merge


A
cauline leaves
floral stages 1-14
floral stage 17
EVR
β-TUBULIN
B
EVR
PEPR1
At1g17750
intensity
100000
10000
1000
100
10
1
root
stem
leaf
whole plant
apex
flowers
floral organs
seeds
C
EVR::GUS

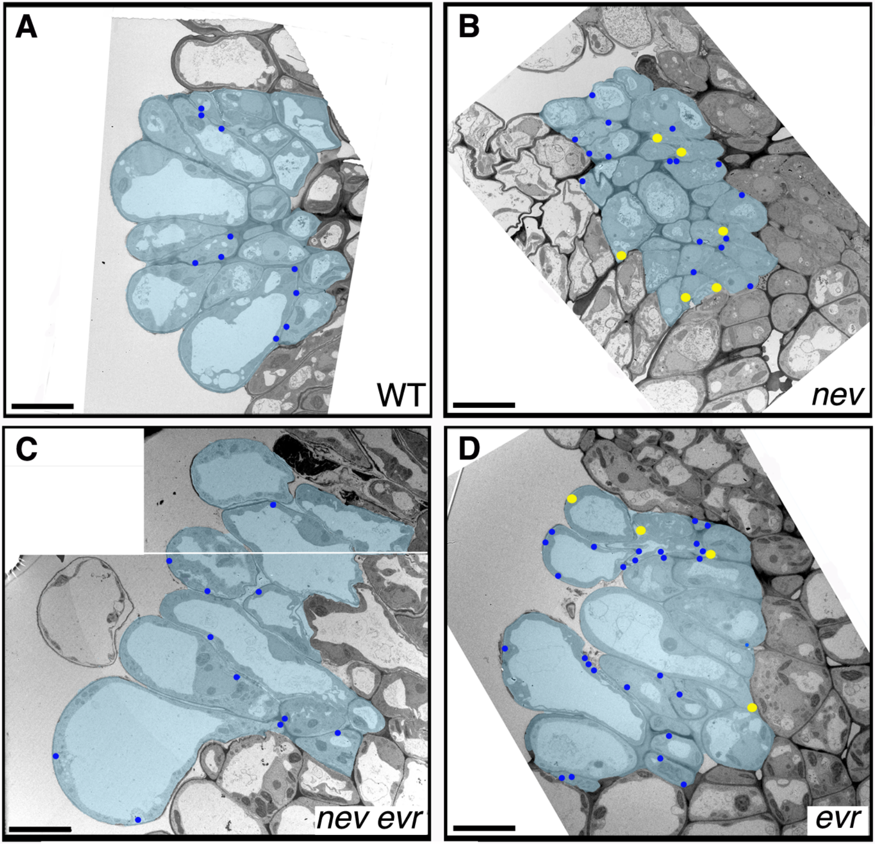

A
WT
B
nev
C
nev evr
D
evr

Table S1. Genotyping mutant alleles

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