NEVERSHEDE and INFLORESCENCE DEFICIENT IN ABSCISSION are differentially required for cell expansion and cell separation during floral organ abscission in Arabidopsis thaliana

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Abstract
Floral organ shedding is a cell separation event preceded by cell-wall loosening and generally accompanied by cell expansion. Mutations in NEVERSHEDE (NEV) or INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) block floral organ abscission in Arabidopsis thaliana. NEV encodes an ADP-ribosylation factor GTPase-activating protein, and cells of nev mutant flowers display membrane-trafficking defects. IDA encodes a secreted peptide that signals through the receptor-like kinases HAESA (HAE) and HAESA-LIKE2 (HSL2). Analyses of single and double mutants revealed unique features of the nev and ida phenotypes. Cell-wall loosening was delayed in ida flowers. In contrast, nev and nev ida mutants displayed ectopic enlargement of abscission zone (AZ) cells, indicating that cell expansion alone is not sufficient to trigger organ loss. These results suggest that NEV initially prevents precocious cell expansion but is later integral for cell separation. IDA is involved primarily in the final cell separation step. A mutation in KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1 (KNAT1), a suppressor of the ida mutant, could not rescue the abscission defects of nev mutant flowers, indicating that NEV-dependent activity downstream of KNAT1 is required. Transcriptional profiling of mutant AZs identified gene clusters regulated by IDA-HAE/HSL2. Several genes were more strongly downregulated in nev-7 compared with ida and hae hsl2 mutants, consistent with the rapid inhibition of organ loosening in nev mutants, and the overlapping roles of NEV and IDA in cell separation. A model of the crosstalk between the IDA signalling pathway and NEV-mediated membrane traffic during floral organ abscission is presented.

Key words: ARF-GAP; cell wall degradation; cell wall loosening; cell-to-cell communication; petal breakstrength; scanning electron microscopy; signalling peptide.
Introduction

Plants have the ability to abscise whole organs once their purpose has been served (Roberts et al., 2002; Lewis et al., 2006). Sepal, petal, and stamen abscission in Arabidopsis thaliana flowers has emerged as a model system to investigate developmentally controlled cell separation processes (Bleecker and Patterson, 1997; Patterson, 2001; Aalen, 2011; Liljegren, 2012). Understanding the underlying genetic and molecular mechanisms has been facilitated by the identification of mutants delayed or deficient in abscission (Lewis et al., 2006; Aalen et al., 2006; Aalen, 2011; Liljegren, 2012). Mutations in certain genes associated with ethylene perception and auxin response result in a delay in abscission (Ecker, 1995; Wilkinson et al., 1997; Ellis et al., 2005), suggesting that hormones contribute to modulating the timing of an underlying hormone-independent developmental process (Patterson and Bleecker, 2004; Aalen et al., 2006; Butenko et al., 2006). Abscission does not occur without an abscission zone (AZ), as illustrated by the double mutant of the BLADE-ON-PETIOLE1 (BOP1) and BOP2 genes (Ha et al., 2003; Hepworth et al., 2005). Scanning electron microscopy (SEM) investigations and measurements of the force needed to remove petals (petal breaskrength, pBS), as well as as transcriptomic data, have revealed that a cell-wall loosening stage is initiated shortly after anthesis, and leads to rounding of the AZ cells (Fernandez et al., 2000; Patterson, 2001; Butenko et al., 2003; Cai and Lashbrook, 2008; Aalen, 2011; Shi et al., 2011). Subsequently, cell separation within the AZ takes place. After the organs are shed, the remaining AZ cells expand further, and the AZ is covered by a protective layer (Patterson and Bleecker, 2004).

Sensitized AZs are responsive to the small peptide INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), which relays a signal through the two closely related leucine-rich-repeat receptor-like kinases (LRR-RLKs) HAESA (HAE) and HAESA-LIKE2 (HSL2) (Cho et al., 2008; McKim et al., 2008; Stenvik et al., 2008; Butenko et al., 2009). Both the ida mutant and the hae hsl2 double mutant retain their floral organs, despite having intact AZs (Butenko et al., 2003; Cho et al., 2008; Stenvik et al., 2008). Cell-wall loosening is delayed, and cell separation is blocked in these mutants. In contrast, overexpression of IDA (IDA4OE) using the cauliflower mosaic virus 35S promoter leads to early abscission of floral organs with enlargement of the AZ region, secretion of arabinogalactan protein (AGP), and ectopic abscission of branches and cauline leaves (Stenvik et al., 2006). These phenotypes are lost for IDA4OE in an hae hsl2 background, consistent with IDA serving as the ligand of HAE/HSL2 (Cho et al., 2008; Stenvik et al., 2008). A MAP kinase cascade has been identified that transduces the signal from IDA-HAE/HSL2 (Cho et al., 2008). A recent screen for mutations that reverse the ida mutant phenotype identified KNOTTED-LIKE FROM ARABIDOPSIS THALLIANA/BREVIPEDICELLUS (KNAT1/BBP) as a downstream component in the IDA-HAE/HSL2 signalling pathway (Shi et al., 2011). The knat1 mutant showed early abscission and enlarged AZs. KNAT1, a class I KNOTTED-LIKE HOMEOBOX (KNOX) transcription factor, is known to restrict the expression of two other KNOX genes, KNAT2 and KNAT6. Delayed abscission is observed in the knat2 knat6 double mutant, even when IDA is overexpressed (Shi et al., 2011). Therefore, as an inhibitor of precocious organ abscission, KNAT1 restricts AZ cell enlargement and represses the expression of KNAT2 and KNAT6, which promote abscission (Shi et al., 2011).

The nevershed (nev) mutant is also deficient in abscission, despite the presence of differentiated AZs (Liljegren et al., 2009). NEV encodes an ADP-ribosylation factor GTPase-activating protein (ARF-GAP) that is localized in the trans-Golgi network/early endosomes and in distinct compartments probably corresponding to recycling endosomes (Liljegren et al., 2009; Stefano et al., 2010). NEV activity is assumed to regulate the movement of cargo molecules required during abscission. Possible cargo includes signaling molecules located at the plasma membrane or within the apoplastic space, internalized and recirculated receptors, and enzymes directly partaking in the breakdown of AZ cell walls (Liljegren et al., 2009; Aalen, 2011; Bryan et al., 2012).

Mutations in the EVERSHED (EVR) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1) LRR-RLK genes, and in the CAST-AWAY (CST) receptor-like cytoplasmic kinase (RLCK) gene, restore organ abscission in nev mutant flowers (Leslie et al., 2010; Lewis et al., 2010; Burr et al., 2011). Interestingly, most of the nev evr, nev serk1, and nev cst double-mutant combinations show an abscission phenotype with enlarged AZs similar to that of IDA4OE (Stenvik et al., 2006; Leslie et al., 2010; Lewis et al., 2010; Burr et al., 2011). It has therefore been suggested that the IDA-HAE/HSL2 signalling pathway is ectopically activated in these double mutants (Leslie et al., 2010; Lewis et al., 2010; Burr et al., 2011).

We have used morphological and genetic analyses to investigate the functional relationship between the IDA and NEV pathways. We conclude that IDA and NEV function during both the cell-wall loosening and the cell separation stages of the abscission process. In addition, NEV acts to inhibit premature cell expansion.

Materials and methods

Plant material

Columbia (Col-0), Landsberg erecta (Ler), and C24 ecotypes of Arabidopsis were used for this study and grown in chambers with 16 h light/8 h dark at 22–24 °C.

The following crosses were carried out: ida-1 (C24) (Butenko et al., 2003) was crossed to nev-3 (Ler) (Liljegren et al., 2009); nev-3 (Ler) introgressed into Col-0 (Liljegren et al., 2009) was crossed to ida-2 (Col-0) (SALK_133209) (Cho et al., 2008); nev-7 (Col-0) (SALK_079928) (Liljegren et al., 2009) was crossed to ida-2 (Col-0), ida-1 (C24), and bp-3 (Col-0) (Rim et al., 2009); nev-3 evr-2 (Ler) (Leslie et al., 2010) was crossed to ida-2 (Col-0); and evr-4 (Col-0) was crossed to bp-3 (Col-0). The genotyping primers are given in Table S1A at JXB online (Liljegren et al., 2009; Leslie et al., 2010). The IDA4OE construct 35S:IDA (Stenvik et al., 2008) was transformed into nev-3 (Col-0) and nev-7 (Col-0) plants, and IDA:GUS (Butenko et al., 2003) was transformed into wild-type and nev-3 (Ler) plants.
using the Agrobacterium tumefaciens-mediated floral dip method (Clough and Bent, 1998). See Table S2 at JXB online for an overview of the lines used.

**RNA extraction and quantitative reverse transcriptase PCR (qRT-PCR)**

RNA was extracted from AZs using a Spectrum Plant Total RNA kit (Sigma). First-strand cDNA was synthesized with QuantiTect reverse transcriptase (Qiagen). Quantitative PCR was performed using LightCycler 480 SYBR Green I Master (Roche) on a LightCycler 480 (Roche), with PP2A as an internal standard (Czechowski et al., 2005). Relative gene expression levels were analysed using REST 2009 software (Qiagen; Pfaffl et al., 2002). For primers, see Table S1B.

**Breakstrength measurements**

pBS analyses were conducted with two measurements per position for 15 inflorescences per genotype, as described previously (Stenvik et al., 2008).

**SEM**

At least 15 AZs of each position analysed were collected from wild-type and mutant plants when the inflorescences had approximately 20 flowers. Individual flowers were fixed in 4% glutaraldehyde (w/v) in a 0.05 M potassium phosphate buffer (pH 7.5) at 4 °C, rinsed four times in buffer, dehydrated in a graded ethanol series, critical point dried in liquid CO2, and stored desiccated. Samples were mounted on steel stubs and sputter coated with gold palladium, and images were collected at 5 kV on an LEO DSM 1530 field emission scanning electron microscope in the Materials Science Center, University of Wisconsin-Madison, WI. Wild-type Ler and nev-3 evr-2 ida-2 flowers were prepared and imaged as described previously (Leslie et al., 2010).

**Staining procedures**

β-Glucosyl Yariv reagent (β-GlcY; Biosupplies) and β-glucuronidase (GUS) staining were performed as described previously (Grini et al., 2002; Stenvik et al., 2006). Samples were inspected with a Nikon SMZ1500 or Zeiss Axioplan2 imaging microscope.

**Microarray analysis**

Gene expression profiles for four biological replicates of ida-2 (Col-0) and hae hsl2 (Col-0) AZ regions of siliques positions 4–8 were compared with their wild-type counterparts using two-colour microarrays and statistical analysis according to Kasnerczyk et al. (2007). Differentially expressed genes were identified using the LIMMA (Linear Models for Microarray Data) software package (Smyth, 2004). All parts of the data analysis were performed using R (R Development Core Team, 2007). For identification of genes co-regulated by IDA and HAE/HSL2, an absolute cut-off value of log2 >0.7 for at least one of the mutants (P <0.2) was employed. Downregulated genes were further analysed for Gene Ontology (GO) terms (AmiGO, http://amigo.geneontology.org/cgi-bin/amigo/term_enrichment) and for co-regulation using the ATTED-II database (Obayashi et al. 2007; http://atted.jp/).

**Results**

**nev and ida mutants display different pBS profiles**

Studies of the force required to remove petals have revealed a gradual loosening of the cell-to-cell adhesion in the petal AZs of wild-type flowers at positions 4–6, followed by organ shedding at positions 8–10, depending on the ecotype (Patterson, 2001; Butenko et al., 2003; Aalen, 2011). Position 1 is defined as the flower at anthesis at the top of the inflorescence. For a detailed comparison of the nev and ida mutant phenotypes, pBS was measured at every other position of 15 mutant and wild-type inflorescences until position 20, where possible (Fig. 1).

The original ida-1 and nev-3 mutant alleles characterized were identified in the C24 and Ler ecotypes, respectively (Butenko et al., 2003; Liljegren et al., 2009). Both mutants show a reduction of pBS followed by an increase back to the initial level (Butenko et al., 2003; Leslie et al., 2010), but the position and contour of these V-shaped profiles were unique for each mutant (Fig. 1A, B). In ida-1, the decrease in pBS was significantly reduced in comparison with C24 wild type at positions 6–8 (Fig. 1A; Student’s t-test, P=0.0005 and P=8.3 × 10−7, respectively), showing that IDA contributes to cell-wall loosening. In contrast, at position 4, the nev-3 mutant showed a significant reduction in pBS compared with Ler wild type (Fig. 1B; P=0.003), indicating that NEV-mediated membrane traffic may repress precocious initiation of the abscission process. These differences suggested that cell-wall loosening within nev and ida mutant AZs slightly precedes and succeeds, respectively, that of wild type.

However, in nev-3, the decrease in pBS values was rapidly reversed (Fig. 1B). Whereas Ler wild-type abscises at position 8, the lowest point in the nev-3 profile was position 6, with a 40% reduction in the pBS level compared with the initial level, which was restored at position 16. In contrast, the ida pBS level was reduced by 90% at position 10, when organs abscise in C24 wild-type plants, and increased thereafter to regain the initial pBS level at position 20 (Fig. 1A). Thus, while NEV is needed for progression of cell-wall loosening as well as cell separation, IDA seems primarily to be needed for the actual cell separation step.

A partial suppressor of the ida and nev mutants is present in the Col-0 ecotype

To evaluate whether the difference in pBS profiles between nev-3 (Ler) and ida-1 (C24) was due to ecotype differences, ida-1 was crossed to Ler. The ida-1 pBS profile was distinctly different from that of nev-3 also in the Ler background (Fig. S1A, B at JXB online). Position 10 had the lowest pBS level for ida-1 (Ler), where it was even weaker than in C24 (P=1.9 × 10−4). Similarly, the overall pBS profile of nev-3 was maintained when this allele was introgressed into Col-0, although the pBS values were significantly weaker than in the Ler background at positions 4–6 (P=0.03 and 4.2 × 10−6) just before abscission takes place in Col-0 wild type (Fig. S1B). Thus, the major characteristics of the nev-3 and ida-1 phenotypes are gene dependent, but ecotype differences in the abscission process seem to modulate the pBS values.

Weaker phenotypes were observed in the nev-7 and ida-2 T-DNA mutants isolated in the Col-0 ecotype (Cho et al., 2008; Liljegren et al., 2009). While the mutant flowers...
Liu et al. retained their organs indefinitely if undisturbed (Fig. S1C), the force needed for organ removal fell below the detection limit from positions 12 and 16 (Fig. S1D). However, ida-2 crossed into Ler behaved like ida-1 (Fig. S1D) and nev-7 in C24 resembled nev-3 in Ler (Fig. S1E). Hence, the weaker pBS levels in ida and nev mutants in the Col-0 background seemed to be due to unknown ecotype factors that partially suppress the ida and nev mutant phenotypes and enhance abscission.

Mutations in NEV can enhance the ida mutant breakstrength profile

Double mutants were used to elucidate further the relationship between NEV and IDA (Table S2). Combining the two strongest alleles, nev-3 ida-1, in the Ler background, transformed the pBS profile into a nearly flat plateau, only significantly different from the initial pBS level at positions 4 and 6 (Fig. 1C; P=0.002 and 0.0001). At positions 8 and 10, the pBS values were 1.3 and 1.2 times the summed values of the single mutants. When the phenotype of a double mutant deviates from both single mutants suggests the two genes act in different pathways (Koornneef et al., 1998). The pBS profile of nev-3 ida-2 in Col-0 largely mirrored that of nev-3 (Fig. 1D). Also, for nev-7 ida-1 in C24, position 6 was the lowest point in the pBS profile, and pBS values were significantly higher than for the ida-1 single mutant (Fig. 1E). Surprisingly, combining the two weakest alleles, nev-7 and ida-2, in Col-0 resulted in lower pBS values than either of the single mutants; from position 8, pBS could not be measured (Fig. S2A at JXB online).
when the plants were left untouched, the floral organs abscised, although four positions later than wild type (Fig. S2B).

AZ cells expand ectopically in nev mutant flowers

To compare the altered morphology of nev and ida mutants, single and double mutant AZs were characterized using SEM (Fig. 2 and Fig. S3 at JXB online). In wild-type plants, removal of petals after anthesis (positions 1–2) revealed broken cells, followed by a flattened fracture plane (position 5) and gradual expansion of AZ cells (positions 6–10) (Patterson, 2001; Patterson and Bleecker, 2004). In ida-1 (C24), the rounding of cells is less pronounced and delayed (Butenko et al., 2003). Consistent with the weaker ida-2 (Col-0) abscission phenotype, ida-2 AZ cells were similar to wild type at position 7/8 when organ shedding takes place, and phenotypically similar to wild type (expanding slightly) through later positions (Fig. 2).

nev-3 (Ler) flowers displayed oblong, irregular AZ cells from position 7/8, and at later stages broken cells were evident, consistent with the high pBS values in this mutant (Fig. S3). In nev-3 (Col-0) and nev-7 (Col-0) AZs, cell enlargement and expansion appeared uncontrolled (Fig. 2 and Fig. S3). A pronounced progression of abnormal development was evident in nev-3 as well as the nev-3 ida-2 (Col-0) double mutant comparing position 7/8 and position 14/15 (Fig. 2). In nev-7, excessively enlarged AZs with giant irregular cells were already present at position 7/8 (Fig. S3). An even more pronounced AZ cell enlargement at positions 10/11 and 14/15 was observed in the nev-7 ida-2 double mutant (Fig. S3). The enhancement of cell expansion in these mutants might underlie the weaker pBS.

nev and nev ida AZs resemble the AZ phenotype of plants overexpressing IDA

The prominent AZ cell enlargement in nev and nev ida mutants is reminiscent of the phenotype found in IDA OE (Stenvik et al., 2006), with the distinct difference that floral organs most often remained attached (Fig. 3A, B and Fig. S2B). The enlarged AZ cells of IDA OE plants have been shown to secrete AGP, which produce a red precipitate when exposed to the β-GlcY reagent (Stenvik et al., 2006). Interestingly, both nev and nev ida enlarged AZ cells showed a strong signal upon β-GlcY treatment (Fig. 3C and Fig. S2C), indicating that nev...
Fig. 3. nev mutants show features reminiscent of IDA overexpression. Floral organs were forcibly removed from wild-type Ler position 5 flowers and from all nev-3 flowers. (A) Close-up of AZs in Col-0 wild type; 35S:IDA, nev-7, and ida-2 in Col-0; and nev-3 (Ler). Note enlarged AZ in the nev mutants. (B) Phenotype of nev-3 ida-2 (Col-0) with close-up of AZs. Inflorescence positions are indicated by numbers. (C) β-GlcY staining detects AGP (red precipitate) in the AZ of nev-3 (Ler). C indicates staining with the negative control, α-GlcY. (D) Overexpression of IDA in nev-3 (Col-0). Three representative 35S:IDA nev-3 plants with a moderate (a), medium (b), and strong (c) 35S:IDA phenotype with representative AZ regions after forcible removal of floral organs shown (inserts). (E) IDA expression levels of the plants in (D) determined by qRT-PCR relative to Col-0 wild type, which was set to 1. The expression levels of the standard 35S:IDA overexpression line (Stenvik et al., 2006; McKim et al., 2008; Stenvik et al., 2008) and 35S:IDA in nev-7 (Fig. S2D) were included for comparison.
mutant AZ cells, regardless of IDA activity, share some of the characteristics of plants with constitutive IDA signalling.

Disruption of NEV activity prevents abscission of flowers overexpressing IDA

To determine whether ectopic expression of IDA could rescue the nev abscission deficiency, an 35S:IDA transgene was transformed into nev-3 (Col-0) and nev-7 (Col-0) mutants (Fig. 3D, E and Fig. S2D). Compared with wild type, nev-7 mutant flowers show about a twofold increase in IDA expression level (Fig. S4 at JXB online), while the 35S promoter-driven IDA expression typically resulted in 100- to 10 000-fold higher transcript levels (Fig. 3E). The size of 35S:IDA nev AZs was larger than those of nev or nev ida plants in a dose-dependent manner (Fig. 3A, D, E and Fig. S2D). pBS was difficult to measure in most 35S:IDA nev flowers, although the floral organs typically remained attached. Therefore, our results indicated that ectopic IDA activity cannot fully override the block in abscission conferred by disruption of NEV function, and furthermore, that NEV activity leading to cell separation is required parallel to and/or downstream of IDA signalling.

A set of genes are co-downregulated in ida and hae hsl2 mutant AZs

Transcriptional profiling of wild-type stamen AZs prior to organ loss has revealed eight gene clusters with different expression profiles (Cai and Lashbrook, 2008). HAE, HSL2, and IDA were identified within clusters 1, 1, and 2, respectively. Both clusters were enriched for cell-wall-modelling genes with increasing expression levels from anthesis to organ separation. To identify genes potentially regulated by the IDA-HAE/HSL2 signalling pathway, we assessed the transcriptional profiles of wild-type, ida-2, and hae hsl2 AZ regions isolated from flowers at positions 4–8 (stages 14–16) (see Materials and methods). Most of the genes with expression levels deviating from the wild type were downregulated (Table S3 at JXB online), and among these, GO analyses identified a significant over-representation of genes encoding cell-wall components and proteins involved in cell-wall organization and modification, such as xyloglucan endotransglucosylase/hydrolase (XTH) enzymes (Table S4 at JXB online). Co-expression analyses of downregulated genes using the ATTED-II database (Obayashi et al., 2007) identified a co-regulated cluster rich in the XTH genes (Table 1). Furthermore, a substantial number of the downregulated genes were demonstrated recently by RNA sequencing to have reduced transcript levels in hae hsl2 AZs of floral stage 15 (position 6) (Niederhuth et al., 2013) (Table S3). Full overlap between our data and the data of Niederhuth et al. (2013) was not to be expected due to differences in sample dissection, floral stages, and the methods used.

Twenty-four genes, including genes belonging to the XTH cluster and clusters identified by Cai and Lashbrook (2008) and Niederhuth et al. (2013), were downregulated both in the ida and the hae hsl2 mutants (log2 <-0.8 for at least one of the mutants, and/or belonging to a cluster). These represent candidate targets of the IDA-HAE/HSL2 signalling pathway.

We carried out qRT-PCR on RNA from wild-type and mutant AZ regions undergoing cell-wall loosening (positions 3–6) and organ separation (positions 7–10) to confirm and further characterize the expression profiles of a subset of these potential IDA-HAE/HSL2 target genes (Table 1), most of which showed increasing transcript levels in stamen AZs from anthesis to abscission (Fig. S5 at JXB online) (Cai and Lashbrook, 2008). This subset of genes encoded proteins involved in cell-wall remodelling (TCH4/XTH22) (Xu et al., 1995), cell expansion [the extracellular EXORDIUM (EXO) and the closely related EXL1] (Schröder et al., 2009), Ca2+ binding (GAD4 and PINOID BINDINGPROTEIN1-LIKE, PPB1-LIKE) (Delk et al., 2005), ethylene response (ERF012), and regulation of auxin-inducible genes (MYB77) (Shin et al., 2007). For both stages the qRT-PCR data showed, with some exceptions, a difference in log2 values (Δlog2) of less than ±0.5 between the ida and hae hsl2 samples (Fig. 4 and Table S5 at JXB online). These concurrent measurements are consistent with genes that are downstream components of the same signalling pathway.

A subset of genes regulated by IDA-HAE/HSL2 signalling are differentially expressed in new mutant AZs

We also investigated whether the expression profiles of the selected genes differed in the nev-7 mutant compared with the hae hsl2 and ida mutants (Fig. 4 and Table S5). In this case, the Δlog2 ratios between either nev-7 and ida, or nev-7 and hae hsl2, ranged from ±1.0 to ±2.8. Compared with wild type, MYB77 and GAD4 were upregulated instead of downregulated in position 3–6 samples, while the other genes tested were more strongly downregulated in nev-7 (Table S5). This suggested differential involvement of NEV and IDA-HAE/HSL2 during the cell-wall loosening stage. For samples from position 7–10, the genes investigated were more strongly downregulated in nev-7 than in ida and hae hsl2 (Fig. 4B). In these positions, the petals of nev mutants are more strongly attached to the plant body than petals of ida flowers (Fig. 1C–E and Fig. S2A). Downregulation of a subset of genes in both the nev and ida mutants may reflect the influence of NEV activity on the IDA-HAE/HSL2 signalling pathway or simply indicate that some genes are regulated by parallel pathways that activate cell separation.

Loss of EVR activity rescues organ abscission in nev ida flowers

Genetic suppressor screens of the nev and ida mutants have uncovered several inhibitors of organ abscission. Mutations in the EVR, SERKI, or CST receptor-like kinases are able to restore organ shedding in nev but not in ida flowers (Leslie et al., 2010; Lewis et al., 2010; Burr et al., 2011). To investigate whether rescue of organ shedding in nev flowers can occur independently of IDA, we generated a nev-3 ida-2 evr-2 triple mutant. While nev-3 ida-2 mutant flowers did not shed their floral organs (Figs 1D and 3B), abscission was restored by the further loss of EVR activity (Fig. 5A). Furthermore, nev-3 ida-2 evr-2 flowers display enlarged abscission zones and
Table 1. Genes downregulated in both ida-2 and hsl2a

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<td>XTH</td>
<td>Interaction with ARF7</td>
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</table>

a Included are downregulated genes belonging to identified clusters and/or with a log2 value < –0.8 in at least one of the mutants (P < 0.2).
b The expression levels of genes indicated in bold were investigated by qRT-PCR.

Loss of KNAT1 activity does not rescue organ abscission in new flowers

Both EVR and KNAT1/BP prevent precocious abscission (Leslie et al., 2010; Shi et al., 2011). A mutation in the latter suppresses the abscission deficiency of the ida mutant (Shi et al., 2011). Interestingly, microarray analysis of the knat1 mutant suggests that expression of EVR in pedicels and flowers is positively regulated by KNAT1 (Wang et al., 2006). Using qRT-PCR, we were able to confirm this result, and found that the EVR expression level was reduced to <10% compared with that of the wild type in both the bp-3 and bp-3 ida-1 mutants (Fig. 5B). Furthermore, the pBS of the bp-3 evr-4 double mutant did not deviate from that of bp-3 (Fig. 5C), which would be consistent with EVR acting downstream of KNAT1.

To investigate whether KNAT1 might represent a link between the IDA-HAE/HSL2 and NEV pathways, we examined whether a mutation in BP could rescue the abscission defects of nev-7 flowers. Although the nev-7 (Col-0) phenotype is relatively weak, and nev-7 petals were easily detached after position 14, the pBS profile of bp-3 nev-7 flowers was similar that of to nev-7, both in the cell-wall loosening and cell separation phases (Fig. 5D). The bp-3 nev-7 double mutant displayed AZ enlargement, as in bp-3 flowers (Fig. 5E).

Therefore, disruption of KNAT1 activity was not sufficient to suppress the abscission deficiency of the nev mutant. These results suggested that NEV activity is required in parallel and/or downstream of KNAT1.

Discussion

NEV inhibits and IDA promotes abscission-related cell expansion, and both are necessary for cell separation

An increasing number of mutants affecting floral organ abscission in Arabidopsis have been identified over the last decade. These mutants have provided insights into the molecular and cellular mechanisms that control organ separation. One such mutant is nev, which was discovered in a screen for genes involved in stamen abscission (Lewis et al., 2010). NEV promotes abscission-related cell expansion and inhibits and/or downstream of KNAT1.

Results suggest that NEV activity is required in parallel and/or downstream of KNAT1.
Decade (reviewed by Aalen, 2011; Liljegren, 2012). Distinct pBS profiles indicate where in the abscission process a given gene exerts its influence. hop1 hop2 mutant flowers with defects in AZ formation display relatively high and constant pBS levels that reflect an absolute block in the abscission process (McKim et al., 2008), while delayed abscission (dab4) flowers show an initial increase in pBS followed by a decline, suggesting decreased sensitivity to triggers of the process, including changes in response to auxin, ethylene, and jasmonic acid (Patterson and Bleecker, 2004; Patterson et al., 2007; Kim et al., 2013). Many ethylene receptor mutants display a delay in the initial decrease in pBS; however, the profile is similar to wild type, suggesting a role in the timing of abscission (Patterson et al., 2007). ida and hae hsl2 represent a fourth, V-shaped profile with a delayed pBS decrease, and a subsequent increase starting one to two positions after organ shedding in the wild type (Butenko et al., 2003; Cho et al., 2008). This profile indicates that the IDA-HAE/HSL2 signalling module plays a positive regulatory role in cell-wall loosening in addition to the later role in promoting cell separation. This is consistent with the increasing expression levels of HAE, HSL2 and IDA (Butenko et al., 2003; Cai and Lashbrook, 2008), as well as genes with IDA-HAE/HSL2-dependent expression profile, throughout the cell-wall loosening stage.

nev mutants display a fifth novel V-shaped pBS profile clearly distinguishable from that of ida. After an initial pronounced reduction in pBS at position 4, cell-wall loosening in nev mutants was reversed one or two positions before organ separation takes place in the wild type, and the pBS levels from position 6–16 were substantially higher in nev mutants compared with ida mutants. This tighter attachment of nev organs may reflect the fact that several potential target genes of the IDA-HAE/HSL2 pathway were more strongly down-regulated in nev-7 than in ida and hae hsl2 flowers, demonstrating the importance of NEV for the progression of the abscission process and final cell separation step. Reduced expression levels of genes in the XTH-enriched cluster in ida, hae hsl2 and nev mutants at the time of organ shedding in the wild type furthermore suggest an involvement of members of this cluster in the actual cell separation process. XTH17, -18, and -19 have recently been demonstrated to have xyloglucan endotransglucosylase (XET) activity, i.e. they cleave a xyloglucan strand that bridges two cellulose

Fig. 4. Differential gene expression in the ida-2, hae hsl2, and nev-7 abscission mutants. Differences in expression levels (\( \Delta \log_2 \)) were analysed by qRT-PCR on mRNA from mutant inflorescences at (A) positions 3–6 and (B) positions 7–10. (This figure is available in colour at JXB online.)

Fig. 5. Genetic analysis of NEV, IDA, EVR, and KNAT1 interactions. (A) SEM of the AZ region of Ler wild-type and nev-3 ida-2 evr-2 triple mutant in Ler immediately after organ shedding. Se, Sepal; st, stamen; pe, petal. (B) qRT-PCR of EVR expression level relative to the C24 wild type in bp-3 and bp-3 ida-1 mutants. (C) pBS of bp-3 evr-4 compared with wild type, bp-3, and evr-4 in Col-0. (D) pBS of nev-7 bp-3 compared with wild type and nev-7 in Col-0. (E) Phenotype of bp-3 nev-7 mutant plant with comparison with bp-3 in close-up images of siliques, and of AZ regions after forcible removal of bp-3 nev-7 floral organs. Standard deviations are indicated by bars in (B)–(D). Mutant values that were significantly lower at a given position than the wild type (blue) or nev-7 (green) values according to Student's t-test are indicated by <.
microfibrils and connects the cleavage product with the end of another xyloglucan strand (Maris et al., 2011). The outcome can be both cell-wall loosening and cell-wall thickening depending on the conditions (Maris et al., 2009). In contrast to ida AZs, nev AZs showed enhanced cell enlargement. This suggests that NEV-regulated membrane traffic is also required to inhibit precocious cell expansion. The nev AZ cells are highly variable in size and shape, which may reflect the intercellular variation of abnormal vesicle accumulation and defects in the integrity of the trans-Golgi network and Golgi apparatus in the AZs of the nev mutant (Liljegren et al., 2009).

Interestingly, MYB77 was downregulated in both the ida and hae hsl2 mutants but oppositely affected in the nev-7 mutant (positions 3–6). MYB77 interacts in vivo with AUXIN RESPONSE FACTOR7 (ARF7), which is known to promote cell expansion, and with ARF1 and ARF2 in vitro (Wilmoth et al., 2005; Shin et al., 2007). These ARFs have been identified as positive regulators of abscission (Ellis et al., 2005), suggesting a possible role for auxin in AZ cell enlargement.

Although the expansion of AZ cells may exert a mechanical pressure on the overlaying layers (Singh et al., 2011) and affect the timing of abscission (Leslie et al., 2010; Lewis et al., 2010; Shi et al., 2011), our analyses of nev and 35S:IDA nev flowers indicate that an increase in AZ cell expansion alone is not sufficient for floral organ separation to occur. While ectopic expression of IDA influences the degree of rounding and results in secretion of AGP, NEV function is required to achieve complete floral organ abscission. This finding suggests that AZ enlargement and cell separation represent genetically distinct phases of the abscission process. Mutations in nev block the latter but promote the former.

Activities of NEV and the EVR, SERK1, and CST receptor-like kinases may influence the IDA-HAE/HSL2 pathway upstream and downstream of KNAT1

Enlarged AZs have been observed for nev single mutants, the double mutants nev-3 serk1, nev-3 cst, and nev-3 evr, and the triple mutants nev-3 serk1 ida-2 and nev-3 evr-2 ida-2 in the present study and in previous publications (Leslie et al., 2010; Lewis et al., 2010; Burr et al., 2011). As the enlargement is independent of a functional IDA, a suggested activation of the IDA-HAE/HSL2 module should occur downstream of IDA. If NEV only influenced early steps in the IDA-HAE/HSL2 pathway, downstream suppressors of ida should also be able to rescue the nev mutation. At least for bp-3, this is not the case, as the bp-3 nev-7 double mutant retains its nev-7 abscission-deficient phenotype. The reduced expression level of EVR in a bp-3 background is not sufficient to rescue organ shedding in nev flowers. Therefore, our data suggest that NEV is also needed downstream of KNAT1 or in a parallel pathway of IDA to promote cell separation.

Fig. 6. Model for networks controlling floral organ abscission. The spatial AZ expression patterns of the genes encoding IDA, HAE, KNAT1, KNAT2, KNAT6, and the polygalacturonases PGAZAT and PGLR are illustrated by the respective promoter:GUS reporter lines (Butenko et al., 2003; Shi et al., 2011; Kumpt et al., 2011). Components that regulate organ abscission are located in the apoplastic space, the plasma membrane, the cytoplasm, and the nucleus, as indicated. IDA has a somewhat broader expression pattern than its receptors and the secreted IDA peptide signals through the HAE/HSL2 receptors expressed specifically in the AZ cells and triggers a MAP kinase cascade in these cells. A subsequent inhibition of KNAT1 activity is obtained, resulting in reduced EVR expression and induced expression of KNAT2 and KNAT6, which promote abscission. The end result of the signal is induction of genes encoding cell-wall remodelling and degradation enzymes facilitating cell-wall loosening and dissolution of the middle lamella between adjacent cell layers in the AZ. In the absence of IDA peptide, KNAT1 blocks premature organ shedding by repressing KNAT2 and KNAT6, and by promoting transcription of EVR, which together with SERK1 and CST modulate the activation of the IDA-HAE/HSL2 signalling pathway, possibly by interaction with HAE/HSL2. NEV is localized to the trans-Golgi network (TGN)/early endosome (EE) and may be involved in transport/recycling of receptors to the plasma membrane. NEV is needed to prevent premature abscission and uncontrolled cell expansion, and is additionally required in the cell separation step, possibly by repressing/removing EVR, SERK1, and CST, to allow IDA signalling. NEV may also be involved in the secretion of cell-wall degradation enzymes facilitating organ separation. After organ loss, the AZ scar displays rounded cells covered by a protective layer. P, petal; s, sepal; f, filament.
On the basis of our findings, we present a working model of how IDA and NEV may mediate floral organ abscission (Fig. 6). Our results suggest that key processes associated with abscission—cell-wall loosening between cell layers in the AZ, cell expansion, and cell separation—are under differential control. In recognition that the activity of signalling pathway components must be coordinated at distinct subcellular locations, we have illustrated the locations of signalling components at the apoplastic space, plasma membrane, cytoplasm, and nucleus. The receptors are confined to the AZ cells, while the expression pattern of IDA is somewhat broader; IDA may therefore be secreted from cells neighbouring the AZ cells and bind the extracellular LRRs of the plasma-membrane-bound HAE/HSL2 receptors, triggering a MAP kinase cascade in the cytoplasm, which presumably suppresses the transcriptional activity of KNAT1 in the nucleus (Cho et al., 2008; Stenvik et al., 2008; Shi et al., 2011). The enlarged AZ phenotypes of nev and knat1 mutants suggest that NEV-dependent membrane trafficking and KNAT1 transcription factor activity are required for suppression of precocious activation of the pathway. EVR, SERK1, and CST have been hypothesized to act as repressors of HAE and HSL2, and NEV may be needed for their removal (Leslie et al., 2010; Lewis et al., 2010; Burr et al., 2011). Consistent with such a hypothesis, mutations in NEV can be compensated by mutations in CST, SERK1, or EVR. When KNAT1 activity is suppressed as a downstream effect of IDA-HAE/HSL2 signalling, expression of EVR is turned off and the transcriptional repression of KNAT2 and KNAT6 is lifted, leading to the expression of cell-wall remodelling and degradation genes (Shi et al., 2011). Downstream of KNAT2 and KNAT6, cell-wall modifying proteins are hypothesized to require NEV for transport to the middle lamella, where they facilitate cell-wall loosening, cell expansion, and finally cell separation. Assuming the NEV and IDA are expressed in the same cells, it is also conceivable that NEV is involved in the secretion of IDA to the apoplastic space at the onset of the abscission process.

Our working model suggests that a more complete understanding of the abscission process will be provided by future studies focused on additional components and target genes of the IDA-HAE/HSL2 signalling pathway. HAE/HSL2 receptor complexes and receptor cycling, and the trafficking of abscission-related components in AZ cells.

Supplementary data

Supplementary data are available online.

Supplementary Fig. S1. Ecotype background influences the pBS profile of nev and ida mutants.

Supplementary Fig. S2. Enlarged AZ cells and delay in abscission in nev-7 irrespective of the expression level of IDA.

Supplementary Fig. S3. SEM analyses reveal extensive expansion of nev AZ cells.

Supplementary Fig. S4. IDA expression levels in nev mutant flowers.

Supplementary Fig. S5. Expression patterns of putative IDA-HAE/HSL2 targets during stamen AZ development.

Supplementary Table S1. Primers used for genotyping and qRT-PCR.

Supplementary Table S2. Single and double mutants investigated in this study.

Supplementary Table S3. Genes up- and downregulated in ida-2 and hae hsl2 mutant AZs.

Supplementary Table S4. Enriched GO annotations for genes downregulated in ida-2 and hae hsl2 mutant AZs.

Supplementary Table S5. Expression levels of potential IDA-HAE-HSL2 target genes.

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