Running Head: KD1 Regulates Abscission

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Research Area:
Genes, Development and Evolution
A KNOTTED1-LIKE HOMEBOX Protein, KD1, Regulates Abscission in Tomato by Modulating the Auxin Pathway

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One Sentence Summary:
A KNOTTED1-LIKE HOMEBOX Protein, KD1, regulates abscission through modulating auxin concentration and response gradient.
Footnotes:
This work was supported by BARD, the U.S.-Israel Binational Agricultural Research and Development Fund (No. IS-4073-08C), and the National Natural Science Foundation of China (Grant 91317312).

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Abstract:

*KD1*, a gene encoding a KNOTTED1-LIKE HOMEOBOX protein, is highly expressed in both leaf and flower abscission zones. Reducing abundance of transcripts of this gene in tomato, both by virus induced gene silencing and by stable transformation with a silencing construct driven by an abscission-specific promoter, resulted in a striking retardation of pedicel and petiole abscission. In contrast, *Pts*, a semi-dominant *KD1* mutant, showed accelerated pedicel and petiole abscission. cDNA microarray and qRT-PCR analysis indicated that regulation of abscission by *KD1* was associated with changed abundance of genes related to auxin transporters and signaling components. Measurement of auxin content and activity of a *DR5::GUS* auxin reporter assay demonstrated that changes in *KD1* expression modulated the auxin concentration and response gradient in the abscission zone.
**Introduction**

Abscission is a highly programmed process and plays a critical role in the physiology and survival of plants, allowing plants to shed nonfunctional, stressed, or infected organs, and to disperse progeny (Bleecker and Patterson, 1997; Roberts et al., 2002). Shedding of plant organs occurs at predetermined positions called abscission zones (AZs). The AZ tissue is composed of small isodiametric cells with dense cytoplasm, and is anatomically distinct long before the initiation of abscission (Roberts and González-Carranza, 2007). In tomato, genetic analysis has revealed that several genes are involved in AZ differentiation. Three members of the MADS-box family, JOINTLESS, MACROCALYX, and SlMBP21, play a key role in differentiation of the pedicel AZ in tomato (Szymkowiak and Irish, 1999; Nakano et al., 2012; Liu et al., 2013). Studies of the tomato mutants *lateral suppressor (ls)* and *blind (bl)* have demonstrated that GRAS and MYB family transcription factors are also involved in formation of the pedicel AZ (Schumacher et al., 1999; Szymkowiak and Irish, 1999; Mao et al., 2000; Ampomah-Dwamena et al., 2002).

Once the AZ is formed, it remains in a quiescent state from days to months until receiving the signals that initiate abscission (Roberts and González-Carranza, 2007; Nakano et al., 2013). Abscission initiation is triggered by developmental and environmental cues (Taylor and Whitelaw, 2001), and is mediated by the interaction of two hormones, auxin and ethylene (Roberts et al., 2002; Estornell et al., 2013). Abscission cannot occur while there is a continuous polar flow of auxin passing through the AZ. Auxin depletion results in the initiation of abscission by making the AZ sensitive to ethylene (Abeles and Rubinstein, 1964; Addicott, 1982; Taylor and Whitelaw, 2001; Roberts et al., 2002; Meir et al., 2006). In tomato, elimination or reduction in auxin flow by removal of the subtending organ (leaf or flower), or application of polar auxin transport inhibitors, initiates abscission (Meir et al., 2010). Reduced auxin flow changes the transcript abundance of many genes involved in auxin biosynthesis, transport and signal transduction. In *Arabidopsis*, it has been reported that IAA signaling in the AZ is essential for organ shedding (Basu et al., 2013). Functional studies of Auxin Response Factors (ARFs) 1, 2, 7, and 19 demonstrate that these transcriptional regulators have functions in floral organ abscission (Ellis et al., 2005; Okushima et al., 2005). However, it is still unclear what mechanism modulates auxin level and auxin signaling in the AZ during the onset of abscission.
A putative peptide ligand-receptor signal transduction pathway plays a role in the control of the onset of abscission in *Arabidopsis* (Cho et al., 2008; Stenvik et al., 2008). Analysis of *inflorescence deficient in abscission* (ida), *haesa* (hae) and *haesa-like2* (hsl2) mutants in *Arabidopsis* indicated that IDA, a putative ligand, interacts with the receptor-like kinases HAE and HSL2 in regulating flower organ abscission (Walker et al., 2000; Cho et al., 2008; Stenvik et al., 2008). In addition, several genes that may affect *Arabidopsis* floral organ abscission through interaction with the IDA-signaling pathway have been identified, including an ADP-ribosylation factor-GTPase-activating gene *NEVERSHEDE* (NEV) (Liljegren et al., 2009; Liu et al., 2013), and three receptor-like kinases *SERK1* (Lewis et al., 2010), *EVERSHEDE* (Leslie et al., 2010) and *CAST AWAY* (Burr et al., 2011). In *Arabidopsis*, evidence suggests that IDA signaling represses the *KNOTTED-LIKE HOMEOBOX* (KNOX) family member *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA* (KNAT) 1, which in turn induces KNAT2 and KNAT6 to activate abscission (Shi et al., 2011).

KNOX proteins comprise a small family of three amino acid loop extension (TALE) homeobox proteins, which fall into three subclasses: Class I, Class II, and Class M (Hay and Tsiantis, 2010). In our previous microarray studies, we found that three KNOX genes, *TKN3*, *TKNA4*, and *KD1* were highly expressed in the pedicel AZ of tomato (Meir et al., 2010). *TKN3* and *TKN4* are Class I KNOX genes whose *Arabidopsis* homologs, *KNAT6* and *KNAT2*, have been shown to be involved in the IDA abscission signaling pathway (Shi et al., 2011). Apart from our demonstration of high *KD1* transcript abundance in the tomato AZ, there has been no suggestion of a role for Class M KNOX proteins in abscission. Rather, these proteins have been associated with formation of compound leaves (Hay and Tsiantis, 2010). For example, *Petroselinum* (*Pts*), a semi-dominant *KD1* mutant in tomato with multiply compound leaves, results from a single nucleotide deletion in the promoter region of the *KD1* gene that results in increased abundance of *KD1* transcripts in the leaves (Kimura et al., 2008). Koenig et al. (2009) showed that auxin gradients play a primary role in controlling morphogenesis in compound leaves. Given the primary importance of an auxin gradient in determining the onset of abscission, we hypothesized that the KD1 protein might also play a role in the initiation of abscission. We report here the results of experiments designed to test that hypothesis. We examined the AZ transcriptome and physiology resulting from reduction in KD1 expression by virus-induced gene silencing (VIGS) or silencing in stably-transformed plants under the control of an
abscission-specific promoter. In addition, we examined the effect, on abscission, of up-regulation of KD1 in the semi-dominant Pts mutant.

Results

KD1 is specifically expressed in abscission zones

In previous microarray studies we found, in tomato, that KD1 is predominately expressed in the pedicel AZ (Meir et al., 2010). To clarify the involvement of KD1 in tomato abscission, we first assayed KD1 transcripts in the pedicel and petiole AZs by qRT-PCR. We divided the pedicel into three parts: AZ, distal region (between the flower and the AZ), and proximal region (between the AZ and the peduncle). Transcripts of KD1 were detected in the AZ (Fig. 1A), but in neither the distal region nor the proximal region. We then analyzed the transcript abundance of KD1 in the petiole AZ, and adjacent regions, the petiole and the main axis. Transcripts of KD1 were much more abundant in the AZ than in the adjacent tissues (Fig. 1B). This AZ-specific expression pattern suggests that KD1 could be involved in the abscission process.

Silencing KD1 delays abscission

To understand the function of KD1 in abscission, we first examined the effect of silencing KD1 on abscission using VIGS. A purple transgenic tomato (cv. New Yorker) overexpressing a maize anthocyanin gene Leaf colour (Lc) (Goldsbrough et al., 1996), was used as silencing reporter. Silencing the Lc gene reversed the color of the transgenic plants from purple to green, providing a visual ‘reporter’ of silenced tissue (Jiang et al., 2008). In this study, we used a silencing construct designed to silence both KD1 and Lc in the purple tomato line. Apart from the color change, there was no visible effect of silencing KD1 on the plants. Only 23% of the pedicels had abscised 16 h after flower removal in pedicels of green plants where both Lc and KD1 genes were silenced (Fig. 2A). In contrast, by that time almost all pedicels had abscised in the controls, whether the purple pedicels of the Lc plants, or the green pedicels of plants where only Lc was silenced (Fig. 2A).

We further generated transgenic tomato plants (cv. New Yorker) in which the KD1 antisense construct was driven by an abscission specific promoter, TAPG4 (Kalaitzis et al., 1997; Hong et al., 2000). Ten independent transgenic lines were generated and three representative transgenic lines (Lines A, E and H) were selected for further analysis. The transformed plants
were visually indistinguishable from the wild type (WT, cv. New Yorker) controls. *KD1*
transcript abundance, measured in pedicel AZs 4 h after flower removal was significantly less in
*TAPG4::antisense KD1* transgenic lines than in WT plants (Fig. 3A). Among them, Line E
showed the lowest *KD1* transcript abundance (Fig. 3A). Delayed abscission was seen in both
pedicel and petiole abscission of the *TAPG4::antisense KD1* transgenic plants (Fig. 2B, C). At
18 h after flower removal, all the pedicels of WT plants had abscised, while only 56% pedicels
had abscised in Lines A and E (Fig. 2B), and 66% in Line H. Most (92%) of the petioles in WT
plants had abscised after 72 h of ethylene treatment, while only 44% of both Lines A and E
petioles were abscised, and 50% in Line H (Fig. 2C).

**Overexpression of KD1 promotes abscission**

To further test the function of KD1 in abscission, we examined the abscission phenotype
of the semi-dominant *KD1* mutant *Pts* (Kimura et al., 2008). The transcript abundance of *KD1*
in the AZ of the *Pts* mutant plants was higher than that in WT (cv. VF36) plants (Fig. 3B). *KD1*
transcripts were undetectable in non-AZ pedicel tissues of both *Pts* and WT plants. In the *Pts*
mutant plants, 81% of the pedicels had abscised 8 h after flower removal, but only 43% of the
pedicels of WT plants (Fig. 4A). In the petiole abscission assay, 22% of the petioles of *Pts* plants
had abscised after 36 h of ethylene treatment, but few of those in the WT plants had (Fig. 4B).

**KD1 expression affects fruit set**

Fruit set percentage was higher in *TAPG4::antisense KD1* transgenic plants than in WT
plants (cv. New Yorker). Under our greenhouse conditions, 59.7 % of flower buds set fruits in
*TAPG4::antisense KD1* transgenic plants, while 44.8% of WT flower buds set fruits (Fig. 5A).
Moreover, fruit set percentage was significantly lower in the *Pts* mutant than in WT plants (cv.
VF36). 70% of flower buds set fruits in the WT plants, but only 9% of *Pts* flower buds set fruits
(Fig. 5B).

**Gene expression analysis identifies genes regulated by KD1 in the abscission zone**

To investigate the transcriptional mechanisms underlying the delay of pedicel abscission
in *TAPG4::antisense KD1* plants, we compared gene expression in the AZ 4 h after flower
removal in WT and *TAPG4::antisense KD1* plants using a custom microarray. In
TAPG4::antisense KD1 AZs, we identified 555 up-regulated and 593 down-regulated genes with known function (Tables S1 and S2). Among these, 19 auxin-related genes were up-regulated, including Aux/IAA2 (SL_NP000208), Aux/IAA4 (SL_TC198378), GH3.3 (SL_TC192282), and two SAUR family genes (SL_TC202903, SL_TC206415) (Table S3), and 11 auxin-related genes were down-regulated, including PIN-like 3 (SL_TCI97872), ARF5 (SL_TZXJ369TF), ARF8 (SL_BF097763), ARF19 (NM_001247676), and two IAA-amino acid hydrolase ILR1 precursors (SL_AW929186, SL_AI781477) (Table S4). In addition, two KNOX family members, KNAT3 and STM, were up-regulated and down-regulated respectively in the AZs of the TAPG4::antisense KD1 line (Table S1 and S2).

As confirmation, we measured transcript abundance of selected genes in abscission zones of TAPG4::antisense KD1 plants using qRT-PCR (Fig. 6A). The results were consistent with the data obtained from the microarray (Fig. 6A) showing decreased abundance of auxin-efflux transporter (PIN) family and ARF genes, and increased abundance of Aux/IAA, GH3.3, and SAUR genes compared to WT (cv. New Yorker) control plants. We also examined transcript abundance of the same genes in AZs of the Pts mutant (Fig. 6B) and found that it was consistently the inverse of the pattern from the antisense plants, with higher abundance of transcripts of efflux transporter and ARF genes, and reduced abundance of Aux/IAA, GH3.3, and SAUR genes compared to the VF36 control plants.

**KD1 regulates the auxin content of the abscission zone**

To test whether the observed differential gene expression related to auxin transport affects the auxin content, we examined IAA concentrations in tomato pedicel AZs by liquid chromatography-tandem mass spectrometry (LC-MS). IAA concentrations in the petiole AZs of TAPG4::antisense KD1 plants were approximately 80% greater than those in the AZs of WT (cv. New Yorker) plants (Fig. 7A). In contrast, IAA concentrations in the AZs of Pts mutant plants were 45% less than those in the AZs of WT (cv.VF36) plants (Fig. 7B).

**KD1 regulates auxin response gradient in the abscission zone**

We introgressed the DR5::GUS auxin reporter transgene into VF36 and into the Pts mutant background, and compared β-glucuronidase (GUS) staining in pedicels of inflorescences from Pts DR5::GUS and VF36 DR5::GUS plants and in pedicels from VF36 DR5::GUS
inflorescences treated with the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) (Fig. 8). The presence of GUS indicates activity of the auxin response pathway (Ulmasov et al., 1997; Koenig et al., 2009). In the distal region (between the flower and the AZ), GUS staining was similar in the VF36 and Pts mutant plants (Fig. 8). However, in the pedicel AZ zone itself, there was a clear gradient in GUS staining between the distal and proximal sides of the pedicel AZ in the VF36 plants, but no detectable staining in the AZs from the Pts mutant (Fig. 8).

We also examined GUS staining in VF36 DR5::GUS plants treated with NPA, which accelerates pedicel abscission in intact inflorescences (Fig. 9). Application of NPA resulted in a GUS staining pattern similar to that seen in the Pts DR5::GUS plants, with staining in the distal region, but none in the AZ or the proximal region (Fig 8).

**Discussion**

Auxin is known to be a key hormone in the initiation of abscission; the conventional model suggests that a change in the transport of auxin through the AZ results in sensitization of the AZ to ethylene, which then induces the hydrolytic enzymes and other components of the separation process (Abeles and Rubinstein, 1964; Addicott, 1982; Taylor and Whitelaw, 2001; Roberts et al., 2002; Meir et al., 2006). Our data are consistent with this model: treatment of tomato inflorescences with the auxin transport inhibitor NPA not only dramatically changed the activity of the auxin response pathway across the abscission zone, but also stimulated pedicel abscission (Fig. 8, Fig. 9).

Our data support the hypothesis that in addition to its well-documented role in leaf development, KD1 plays a role in abscission. KD1 is known to modulate boundary separation and proximal-distal axis development in leaves (Kimura et al., 2008; Magnani and Hake, 2008; Peng et al., 2011), and our previous microarray analysis suggested a role for KD1 in leaf and flower abscission (Meir et al., 2010). Manipulating expression of KD1 by silencing or overexpression affects various aspects of plant development. In Arabidopsis, transgenic lines carrying KNATM (a KD1 ortholog) under control of the 35S promoter showed a series of leaf development defects (Magnani and Hake, 2008). However, Magnani and Hake (2008) reported difficulty in reducing expression of KNATM by RNA interference or artificial microRNA constructs. To overcome this problem and to test the function of KD1 in abscission, we silenced KD1 in tomato using VIGS. VIGS analysis avoids potential lethality, since we inoculate plants
that have already reached the seedling stage. Our results show that silencing KD1 expression significantly delays abscission (Fig. 2A). For further analysis, we created KD1 antisense plants driven by an abscission-specific promoter TAPG4 (Hong et al., 2000; Meir et al., 2010). Abscission was significantly delayed in the TAPG4::antisense KD1 transgenic plants, which otherwise were normal in their growth and development. Although reducing KD1 transcript abundance delayed abscission, it did not prevent it, suggesting that KD1 is part of a complex system controlling the abscission process. In our experiments, reduction in KD1 transcript abundance delayed abscission of both pedicels and petioles. In some tomato mutants, the abscission of pedicels and petioles is uncoupled. For example, in the Jointless1 and mc mutants, pedicels do not abscise, but petiole abscission is normal (Szymkowiak and Irish, 1999; Nakano et al., 2012). These MADS-box mutants seem to affect early events in establishment of the AZ, whereas KD1 appears to be involved in the later physiological events leading to organ separation. It would be interesting to determine the genetic interaction among the Pts, jointless1, and mc mutants.

Plants bearing the semi-dominant KD1 mutation Pts, which has a single nucleotide deletion in the promoter region of the KD1 gene, have high KD1 transcript levels in their leaves (Kimura et al., 2008). We found that KD1 is also up-regulated in the AZ of the mutant plants, but the tissue specificity seen in WT plants remains - transcript abundance is much lower in the non-AZ pedicel tissues (Fig. 3B). The importance of KD1 in the abscission process is also suggested by the fact that pedicel abscission was accelerated in the Pts plants (Fig. 4). The possibility that KD1 is involved in auxin responses is suggested by the phenotype of the mutant plants where growth alterations include not only the reported changes in leaf morphogenesis (which also involves auxin) but also greatly reduced apical dominance (Fig. S1). The Pts mutant was introgressed from the wild species (Solanum galapagense) into VF36 with only two back-crosses, so its genetic background is only 75% VF36. This means that we need to be cautious in interpreting results that might reflect the genome of the source species, rather than effects of the mutation. However, apart from KD1 and its downstream genes, transcripts of genes that we tested in the AZ showed similar abundance in the Pts mutant and in VF36 (data not shown).

Altered auxin contents (Fig. 7) were observed in petiole AZs from plants with enhanced (Pts mutant) or reduced (TAPG4::antisense KD1) KD1 expression, suggesting that KD1 may play a role in modulating auxin levels. We detected no differential expression of auxin
biosynthesis genes in KD1 antisense plants, and three genes encoding for IAA-amino acid conjugate hydrolases ILR were found to be down-regulated (Table S4), so neither increased synthesis nor increased release of auxin from conjugates (Woodward and Bartel, 2005) can explain the increased concentration of auxin in the antisense plants. Our microarray analysis showed that an auxin efflux transporter PIN-like 3 (SL_TC197872, Fig. 6) was down regulated in KD1 antisense plants. Recently, ten members of the auxin efflux transporter gene family were identified in tomato (Pattison and Catala, 2012). Since few of these genes were included in our custom microarray, we examined the transcript levels of other PIN genes in tomato plant AZs by qRT-PCR. In addition to PIN-like 3, one other auxin efflux transporter, PIN9, was down-regulated in KD1 antisense plants (Fig. 6). The observed effect of manipulation of KD1 expression on the transcript abundance of these two auxin efflux transporters in the AZ is consistent with our hypothesis that KD1 plays a role in modulating auxin levels in the AZ, perhaps by controlling auxin efflux transport. In Arabidopsis, reduced auxin concentrations and reduced response to auxin are required for fruit valve margin separation. Reduced auxin concentrations in that system are also attributed to an increase in auxin efflux transporters (Sorefan et al., 2009).

Our microarray results show that transcript abundance of four auxin response genes ARFs was down-regulated in KD1 antisense transgenic plants (Table S2). In Arabidopsis, genetic evidence suggests that ARFs play important roles in modulating the abscission process. ARFs 1, 2, 7 and 19 were identified as being involved in abscission (Ellis et al., 2005). Knock out or silencing of ARF2 in Arabidopsis delays flower senescence and organ shedding, and the delay is enhanced by suppressing the activity of ARF1, ARF7, or ARF19. The tomato homolog of the Arabidopsis ARF19 was also down-regulated in our TAPG4::antisense KD1 transgenic plants (Table S4). Interestingly, transcript abundance of the tomato ARF2 homologue increased in KD1 antisense plants (Table S3), implying a complex interplay between different components of the auxin response pathway during abscission.

Two AUX/IAA gene family members and two SAUR gene family members were also up-regulated in the AZs of TAPG4::antisense KD1 plants (Table S3). This is consistent with our previous microarray analysis showing that seven Aux/IAA genes were down-regulated during tomato pedicel abscission (Meir et al., 2010). It seems highly likely that KD1 modulates the
abscission process through the auxin-signaling pathway perhaps by controlling the auxin response gradient through the AZ.

KNOX-type proteins are known to be involved in other auxin responses; in maize, the KNOX protein KN1 directly controls the auxin pathway at all levels, including auxin synthesis, transport, and signaling (Bolduc et al., 2012). Koenig et al. (2009) suggested that an auxin gradient plays fundamental roles in controlling morphogenesis in the compound leaves of tomato (Koenig et al., 2009). The fact that KD1 has been demonstrated to be a genetic determinant of compound leaves leads us to postulate a role for KD1 in auxin gradient responses.

A transcript profiling study on tomato pedicels demonstrated a gradient in auxin-induced gene expression in the pedicel, which may maintain the AZ in its quiescent state (Nakano et al., 2013). Our results indicate that enhanced expression of KD1 in the Pts mutant reduces auxin level and inhibits the auxin response gradient in the AZ. Our DR5::GUS auxin reporter assay (Fig. 8) revealed that the distal-to-proximal auxin response gradient was greatly reduced in the Pts mutant, especially in the proximal region, suggesting that KD1 modulates abscission by controlling the flow of auxin through the AZ. We hypothesize that enhanced expression of KD1 in the Pts mutant modulates an auxin flow ‘gate’ in the AZ, thereby reducing the auxin gradient in the pedicel. Intriguingly, the elimination of the DR5::GUS response in the Pts mutant occurred even though there was only a 45% reduction in gross IAA content of the AZ (Fig. 7). It is known that the auxin response gradient across AZ is more important in the initiation of abscission than the auxin concentration itself (Nakano et al., 2013). Consistent with this result, NPA treatment, which disrupts the auxin polar transport, also eliminated the DR5::GUS auxin response signals in the AZ (Fig. 8). It appears, therefore, that disruption of the auxin response gradient is a key to the onset of abscission.

Canonical KNOX proteins contain a MEINOX domain at the N terminus and a homeodomain at the C terminus (Hake et al., 2004). The KD1 protein lacks a DNA binding homeodomain, which suggests that the function of KD1 might depend on other proteins that bind to DNA. This possibility is indicated by presence of the MEINOX domain which is known to mediate interaction with transcription factors, for example the BELL transcription factor BIP (Kimura et al., 2008; Magnani and Hake, 2008). Further studies are required to determine whether BIP or similar proteins have a function in abscission. In addition, it will be interesting to determine whether homologs of KNOX proteins known to have a function downstream from
KD1 in *Arabidopsis*, such as KNAT1, 2, and 6 (Shi et al., 2011), play a role in organ abscission in tomato.

**Materials and Methods**

**Plant Materials and Treatments**

Tomato (*Solanum lycopersicum*) germplasm cv New Yorker (LA2009), *Pts* (LA2532), and VF36 (LA0490) plants were provided by the Tomato Genetics Resource Center, University of California Davis. Pedicel and petiole abscission assays were performed as previously described (Jiang et al., 2008; Meir et al., 2010).

For the pedicel abscission assay, tomato inflorescences were harvested at 10 a.m. from plants grown in the greenhouse. Inflorescences with at least two newly opened flowers were placed in a vial containing 10 ml water and held in a high humidity chamber. Flowers were removed with a sharp razor blade, and abscission of the remaining pedicel from the peduncle was monitored at intervals.

To assay petiole abscission, the middle (2\textsuperscript{nd} or 3\textsuperscript{rd}) petioles from young plants with four or five expanded leaves were used to prepare explants. Explants comprised a petiole and its subtending internode. The leaf and lateral shoots at the node were removed using a sharp razor blade. The explants were placed in a vial containing 10 ml water, and then exposed continuously to 3 ppm of ethylene. Petiole abscission was monitored at intervals.

For N-1-napthylphthalamic acid (NPA) treatment, NPA was prepared as a 100 mM stock solution in dimethyl sulfoxide (DMSO), and diluted with water to 25 \( \mu \)M NPA. Tomato inflorescences with at least two newly opened flowers were placed in a vial containing 10 ml of the NPA solution. Control inflorescences were placed in a vial containing a solution of the equivalent concentration of DMSO.

**Determination of transcript abundance**

For examining transcript abundance in the pedicel AZs, 20 segments containing the AZ (less than 2 mm in length) were excised from pedicel AZs for each time point. 20 segments of similar size were also excised from the distal and proximal regions of the pedicel. For examining transcript abundance in the petiole AZs, similar segments were excised from the petiole AZ, the distal region of the petiole, and the main stem axis.
Total RNA was extracted from the tissue samples using TRIZol reagent (Invitrogen), and
was treated with RNase-free DNase I (Promega) to remove any contaminating genomic DNA.
First strand cDNA was synthesized using 2 μg total RNA, oligo d(T) primers, random hexamers,
and Superscript reverse transcriptase (Invitrogen).

Quantitative real-time PCR was performed with the 7300 Real Time PCR System
(Applied Biosystems) using SYBR Green Real-time PCR Master Mix (Applied Biosystems). To
normalize sample variance, 26S ribosomal RNA was used as the internal control. Relative
quantification of the transcript abundance of each gene was performed using the $2^{-\Delta\Delta CT}$ method.
Primers used for determining transcript abundance are listed in Table S5.

**Virus induced gene silencing**

A 203 bp fragment of the Lc gene was PCR-amplified from tomato cDNA generated
from Lc overexpressed transgenic tomato lines using primers
5’-AGCGACGAGAGAAGCTCAAC-3’ and 5’-GGAGGGGCTTGTTATTAGCC-3’. The
resulting product was cloned into pTRV2 to form the pTRV2-Lc construct. A 227 bp fragment of
the KD1 gene was PCR-amplified from tomato cDNA using the primers
5’-TCTCAGCTCAGTGAACTCATGG-3’ and 5’-TTGTGGCAATCTAGCCATACAT-3’, and
then sub-cloned into pTRV2-Lc to generate the pTRV2-KD1+Lc construct. Purple seedlings of
transgenic tomato plants overexpressing Lc were infected with a mixed culture of Agrobacterium
tumefaciens containing the pTRV1 vector and the pTRV2-KD1+Lc or pTRV2-Lc vector. The
infection method has previously been described in detail (Jiang et al., 2008).

**Vector construction and plant transformation**

To generate TAPG4::antisense KD1 transgenic plants, a 2379 bp fragment of the TAPG4
abscission-specific promoter and the same fragment of the KD1 gene used in VIGS was
amplified, and then sub-cloned into the modified binary vector GSA1285, which carried the
NPTII gene conferring kanamycin resistance to positive transformants. The plasmid construct
used for transformation was introduced into Agrobacterium LBA4404 by electroporation.
Tomato (cv. New Yorker) was transformed by the tissue-culture method (Fillatti et al., 1987).
Custom oligonucleotide microarrays were fabricated by NimbleGen, Inc. (Madison, WI, USA) using photolithography directed by the Maskless Array Synthesizer (MAS) (Singh-Gasson et al., 1999). The custom oligonucleotide microarray contained probe sets for a total of 46024 gene models derived from annotation of Solanum lycopersicum (The Dana Farber Cancer Institute (DFCI) Tomato Plant Gene Index http://compbio.dfci.harvard.edu/cgi-bin/tgi/tgi/gimain.pl?gudb_Tomato_plant). Oligonucleotide probes (60-mer) were designed based on the NimbleGen standard procedure that optimizes the uniqueness of the targeted region and GC content, while minimizing self-complementarity and homopolymer runs. The highest-ranking six probes (probe set) were selected to represent each gene model, with optimal probe spacing leading to uniformly distributed, non-overlapping coverage. The 4-plex models used were based on the Nimblegen 385K design format (NimbleGen Systems, Inc., Madison, WI, USA).

10 μg total RNA from mixed samples of three biological replicates was used for microarray hybridization. Concentration and purity of the RNA was evaluated using the 2100 Bioanalyzer (Agilent, USA). Double-stranded cDNA was synthesized using the Invitrogen SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, USA). The cDNA samples were labelled with one colour Cy3 random nonamers using the NimbleGen One-Colour DNA Labelling Kit (Roche, USA), followed by hybridizing to NimbleGen customized array slides according to the manufacturer’s instructions. Array slides were scanned by an MS 200 Microarray Scanner, and data was collected and analyzed using the MS 200 Data collection and NimbleScan Software (Roche, USA). Probe signal summarization, normalization, and background subtraction were performed using Robust Multichip Analysis (Irizarry et al., 2003) using the default parameters. Genes with an expression ratio of more than 2 or less than 0.5 between TAPG4::antisense KD1 and WT tissues were identified as up-regulated or down-regulated respectively.

IAA quantification

IAA content was measured by a liquid chromatography-tandem mass spectrometry system (LC20AD-MS/MS 8030 plus, Shimadzu, Japan). 30 pedicel AZ segments were frozen in liquid nitrogen and ground with a mortar and pestle, then freeze-dried in a low temperature vacuum.
oven. 10 mg of each sample was extracted in 1 ml 80% methanol overnight at 4°C. Supernatants were collected after centrifugation at 15,200 g for 10 min, and then dried using a Jouan RCT-60 concentrator. The dried extract was dissolved in 200 µL of sodium phosphate solution (0.1 mol·L\(^{-1}\), pH 7.8) and passed through a Sep-Pak C\(_{18}\) cartridge (Waters, USA). The cartridge was eluted with 1.5 ml of 50% methanol, and the eluent was collected and vacuum dried again. The samples were re-dissolved in 80 µL of 10% methanol. 10 µL of this solution was loaded onto the LC20AD-MS/MS 8030 plus system. Liquid chromatography was performed using a 2.0 mm×75 mm Shim-pack XR-ODS II column (2.2 µm, Shimadzu) with a column temperature of 40°C. The mobile phase comprising solvent A (0.05% v/v aqueous acetic acid) and solvent B (100% v/v methanol) was employed in a gradient mode [time/concentration of A/concentration of B (min/%/%) for 0/80/20; 6.0/35/65; 7.0/0/100; 7.01/80/20] at an eluant flow rate of 0.3 mL·min\(^{-1}\). MS/MS was performed under previously optimized conditions. The mass system was set to multiple reaction monitoring (MRM) mode using electrospray ionization (ESI) in the positive ion mode. Operating conditions were a nebulizing gas flow of 3L·min\(^{-1}\), drying gas flow of 15L·min\(^{-1}\); desolvation temperature of 180°C and a heating block temperature of 480°. For IAA, a quadrupole 1 pre-bias of -18eV, a quadrupole 3 pre-bias of -24V, a collision energy of -16 eV, and a mass-to-charge ratio (m/z) of 176/130 were employed; while for deuterium labeled IAA, a quadrupole 1 pre-bias of -19eV, a quadrupole 3 pre-bias of -24eV and a collision energy of -18eV, m/z of 181/134 were employed. The IAA contents of the samples were calculated using a calibration curve established using an internal \(^2\)H\(_5\)-IAA standard (Olchemim).

**GUS staining**

Tomato inflorescences were fixed in 90% acetone for 20 min, and then placed into GUS staining buffer [0.5 mM X-gluc, 0.15 M NaH\(_2\)PO\(_4\) (pH 7), 2 mM K\(_3\)Fe(CN)\(_6\), 2mM K\(_4\)Fe(CN)\(_6\), and 0.05% Triton X-100]. The inflorescences were infiltrated in a capped 60 ml syringe by depressing the plunger for 3 min, and then vacuuming for 1 h. After incubation in the dark at 37 °C for 16 h, GUS-stained tissues were cleared and stored in 70% ethanol. Images are representative of >20 observed samples stained in three independent experiments.
Supplementary material

Table S1. List of genes that are up-regulated in TAPG4::antisense KD1 plants
Table S2. List of genes that are down-regulated in TAPG4::antisense KD1 plants
Table S3. List of auxin related genes that are up-regulated in TAPG4::antisense KD1 plants
Table S4. List of auxin related genes that are down-regulated in TAPG4::antisense KD1 plants
Table S5. Primers used for Quantitative PCR analysis.

Figure S1. Phenotypes of the KD1 semi-dominant mutant PETROSELINUM (Pts).

Acknowledgments

We thank the Tomato Genetics Resource Center (TGRC), University of California Davis for providing the tomato seeds, the Ralph M. Parsons Foundation Plant Transformation Facility (University of California Davis) for conducting tomato transformations, Dr. Andrew Macnish for initial characterization of KD1 using VIGS, Dr. Junping Gao for consultation of Auxin measurement, Dr. John Yoder for the transgenic purple tomatoes expressing Lc, and Dr. Neelima Sinha for the DR5::GUS tomato line.

Author contribution

Cai-Zhong Jiang, Chao Ma and Michael S. Reid conceived and designed the experiments. Chao Ma performed the experiments and analyzed the data. Langtao Xiao, Jianhua Tong, and Qing Liu contributed to auxin content measurement. Chao Ma, Cai-Zhong Jiang, and Michael S. Reid wrote the manuscript. Shimon Meir helped to analyze the data and revise the manuscript.

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Figure legends

Figure 1. Abundance of KD1 transcripts in pedicel (A) and petiole (B) abscission zones. (A) Abundance of KD1 transcripts in AZ, distal, and proximal tissues of the pedicel. (B) Abundance of KD1 transcripts in the petiole AZ, petiole, and main stem tissues. The abundance of KD1 transcripts was determined by qRT-PCR. Abundance of tomato 26S ribosomal RNA was used as an internal control. The abundance of KD1 transcripts was normalized against the abundance of 26S ribosomal RNA. Different letters indicate significant differences among tissues (Student’s t–test, P < 0.05). The results are the means of three biological replicates ± SD.

Figure 2. Effects of silencing KD1 on the kinetics of abscission. (A) VIGS silencing of KD1. The effect of flower removal on pedicel abscission after 16 h was determined in purple pedicels of control (Con) Lc overexpressed plants, and in green (silenced) pedicels of Lc overexpressed plants infected with TRV vectors containing fragments of the Lc gene alone (Lc), or combined with a fragment of KD1 (KD1+Lc). Different letters indicate significant differences among Con, Lc, and KD1+Lc (Student’s t–test, P < 0.05); (B) Pedicel and (C) petiole abscission in WT (cv. New Yorker) plants and TAPG4::antisense KD1 transgenic plants (Line A, Line E and Line H). The percentages of pedicel and petiole abscission were determined at intervals following flower removal or during ethylene treatment (3 ppm) respectively. Different letters at each time point indicate significant differences between WT and transgenic lines (Student’s t–test, P < 0.05). The results are the means of three replicates ± SD, with at least 15 samples per replicate.

Figure 3. Abundance of KD1 transcripts in TAPG4::antisense KD1 transgenic plants and in the Pts mutant. The abundance of KD1 transcripts was measured by qRT-PCR (A) in AZ tissues of WT (cv. New Yorker) and TAPG4::antisense KD1 transgenic lines (Line A, Line E and Line H) 4 h after flower removal. Different letters indicate significant differences between WT and transgenic lines (Student’s t–test, P < 0.05); (B) in freshly-harvested AZ tissues of wild type (VF36) and Pts mutant plants. RNA was extracted from pedicel AZ or non-abscission zone (NAZ) pedicel tissues. Abundance of tomato 26S ribosomal RNA was used as an internal control. Results are the means of three biological replicates ± SD. Different letters indicate significant differences among tissues (Student’s t–test, P < 0.05).

Figure 4. Kinetics of pedicel and petiole abscission in the KD1 semi-dominant mutant Pts. Pedicel (A) or petiole (B) abscission assays compared wild type (cv. VF36) plants and plants of the Pts mutant. The percentages of pedicel and petiole abscission were determined at intervals
following flower removal or during ethylene treatment (3 ppm), respectively. Results are the means of three replicates ± SD, with at least 15 samples per replicate. Different letters indicate significant differences (Student’s t–test, P < 0.05).

**Figure 5.** Fruit set in the **TAPG4::antisense KD1 transgenic and Pts mutant.** (A) The percentage of accumulated fruit set was monitored in wild type (cv. New Yorker (NY)) and **TAPG4::antisense KD1** transgenic plants (Line E) in the greenhouse; (B) The percentage of accumulated fruit set was monitored in wild type (cv. VF36) and **Pts** mutant plants in the greenhouse. The results are the means of three replicates ± SD. Letters indicate significant differences (Student’s t–test, P < 0.05).

**Figure 6.** Abundance of transcripts of KD1 downstream genes. Total RNA isolated from pedicel AZs, (A) of wild type (cv. New Yorker (NY)) and **TAPG4::antisense KD1** transgenic plants (Line E) 4 h after flower removal, and (B) of freshly-harvested wild type (cv. VF36) and **Pts** mutant plants, was used to determine abundance of transcripts of each gene by qRT-PCR. Abundance of tomato 26S ribosomal RNA was used as an internal control. Results are the means of three biological replicates ± SD. The expression ratio of each gene from microarray analysis is shown above the corresponding qRT-PCR column. * indicates that the data representing **PIN9** were absent in the microarray. Different letters indicate significant differences between NY and Line E, or VF36 and **Pts** at each time point (Student’s t–test, P < 0.05).

**Figure 7.** Auxin concentrations in pedicel AZs. Tissues isolated from pedicel AZs were used to analyze the free IAA content of petiole AZs. (A) AZs from wild type (cv. New Yorker (NY)) and **TAPG4::antisense KD1** transgenic plants (Line E). (B) AZs from wild type (cv. VF36) and **Pts** mutant plants. Values are the means of five replicates ± SD. Different letters indicate significant differences (Student’s t–test, P < 0.05).

**Figure 8.** Expression of **DR5::GUS in AZ tissues of Pts mutant and NPA treated WT plants.** **DR5::GUS** expression in wild type (VF36), **Pts** mutant, and from flower inflorescences of tomato (cv. VF36) placed in a 25 μM NPA solution for 8 h (VF36+NPA). Transverse sections were taken from the proximal region (between the AZ and the peduncle), the proximal side of the AZ, the distal side of the AZ and the distal region (between the flower and the AZ).

**Figure 9.** Effects of N-1-naphthylphthalamic acid (NPA) treatment on the kinetics of pedicel abscission. The percentage of pedicel abscission was determined at intervals from inflorescences placed in 25μM NPA solution (+) or control (-). The results are means of three
biological replicates ± SD, with at least 15 samples per replicate. Different letters indicate significant differences between treatments at each time point (Student’s t–test, P < 0.05).