Title

Role of the KNOTTED1-LIKE HOMEOBOX protein (KD1) in regulating abscission of tomato flower pedicels at early and late stages of the process

Journal

Physiologia Plantarum, 173(4)

ISSN

0193-0648

Authors

Sundaresan, Srivignesh

Philosoph-Hadas, Sonia

Ma, Chao

et al.

Publication Date

2021-12-01

DOI

10.1111/ppl.13560

Peer reviewed

Received: 1 May 2021 | Revised: 6 July 2021 | Accepted: 13 September 2021

DOI: 10.1111/ppl.13560

Physiologia Plantarum

Role of the KNOTTED1-LIKE HOMEOBOX protein (KD1) in regulating abscission of tomato flower pedicels at early and late stages of the process

Srivignesh Sundaresan[1,2] | Sonia Philosoph-Hadas[1] | Chao Ma[3] | Cai-Zhong Jiang[4,5] | Joseph Riov[6] | Betina Kochanek[1] | Shoshana Salim[1] | Michael S. Reid[5] | Shimon Meir[1]

[1]Department of Postharvest Science, Agricultural Research Organization (ARO), Volcani Institute, Rishon LeZiyon, Israel

[2]Department of Horticulture, Neelakudi Campus, School of Life Sciences, Central University of Tamil Nadu (CUTN), Thiruvarur, India

[3]State Key Laboratory of Agrobiotechnology, Beijing Key Laboratory of Development and Quality Control of Ornamental Crops, Department of Ornamental Horticulture, China Agricultural University, Beijing, China

[4]Crops Pathology and Genetic Research Unit, USDA-ARS, Davis, California, USA

[5]Department of Plant Sciences, University of California at Davis, Davis, California, USA

[6]The Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel

Correspondence
Shimon Meir, Department of Postharvest Science, Agricultural Research Organization (ARO), Volcani Institute, Rishon LeZiyon 7505101, Israel.
Email: shimonm@volcani.agri.gov.il

Funding information
The Chief Scientist of the Israeli Ministry of Agriculture Fund, Grant/Award Number: 203-0898-11; United States-Israel Binational Agricultural Research and Development Fund (BARD), Grant/Award Number: US-4571-12C

Edited by S. Pelaz

Abstract

The *KNOTTED1-LIKE HOMEOBOX PROTEIN1* (*KD1*) gene is highly expressed in flower and leaf abscission zones (AZs), and KD1 was reported to regulate tomato flower pedicel abscission via alteration of the auxin gradient and response in the flower AZ (FAZ). The present work was aimed to further examine how KD1 regulates signaling factors and regulatory genes involved in pedicel abscission, by using silenced *KD1* lines and performing a large-scale transcriptome profiling of the FAZ before and after flower removal, using a customized AZ-specific microarray. The results highlighted a differential expression of regulatory genes in the FAZ of *KD1*-silenced plants compared to the wild-type. In the *TAPG4*::*antisense KD1*-silenced plants, *KD1* gene expression decreased before flower removal, resulting in altered expression of regulatory genes, such as epigenetic modifiers, transcription factors, posttranslational regulators, and antioxidative defense factors occurring at zero time and before affecting auxin levels in the FAZ detected at 4 h after flower removal. The expression of additional regulatory genes was altered in the FAZ of *KD1*-silenced plants at 4–20 h after flower removal, thereby leading to an inhibited abscission phenotype, and downregulation of genes involved in abscission execution and defense processes. Our data suggest that KD1 is a master regulator of the abscission process, which promotes abscission of tomato flower pedicels. This suggestion is based on the inhibitory effect of *KD1* silencing on flower pedicel abscission that operates via alteration of various regulatory pathways, which delay the competence acquisition of the FAZ cells to respond to ethylene signaling.

1 | INTRODUCTION

It is well documented that removal of leaf blades or flowers, the major sites of auxin biosynthesis, or application of polar auxin transport inhibitors, induce leaf petiole and flower pedicel abscission, respectively, as a result of reduction in auxin transport to the organ abscission zone (AZ; Ma et al., 2021). This leads to altered expression of auxin-related genes and upregulation of ethylene-related genes, as well as to

differential expression of other regulatory genes in the AZ as demonstrated in the flower AZ (FAZ) of the model system of tomato (*Solanum lycopersicum*) plants (Meir et al., 2015). These events result in the acquisition of the FAZ cell competence to respond to ethylene signaling (Ma et al., 2021; Meir et al., 2010; Meir et al., 2015; Nakano et al., 2013, 2014; Wang et al., 2013).

By performing a transcriptome microarray analysis of the tomato FAZ following abscission induction by flower removal, several genes were shown to be specifically expressed in the FAZ and not in the pedicel non-AZ (NAZ) region (Meir et al., 2010). Therefore, these genes were suggested to be involved in the regulation of the acquisition of the FAZ cell competence to respond to ethylene signaling following auxin depletion. Of these FAZ-specific expressed genes, two genes were further studied for their functional roles in abscission. These genes were *KD1*, which encodes the KNOTTED1-LIKE HOMEOBOX PROTEIN1 that belongs to the class I KNOX family (Ma et al., 2015), and *THyPRP*, which encodes the Tomato Hybrid Proline-Rich Protein, and its silencing delayed pedicel abscission by regulating the FAZ cell competence to respond to ethylene signaling (Sundaresan et al., 2018). We previously showed that downregulation of *KD1* by antisense significantly delayed pedicel and petiole abscission in tomato plants by affecting auxin levels in the AZ and auxin gradient from the distal to the proximal region of the FAZ, without impairing their growth and development (Ma et al., 2015). The *KD1* gene was silenced, under the control of the AZ-specific promoter, Tomato Abscission Polygalacturonase4 (TAPG4; Hong et al., 2000; Meir et al., 2010; Ma et al., 2015), to study the changes in gene expression profiles in the tomato FAZ shortly after flower removal (4 h) in the *TAPG4::antisense KD1* plants compared to the wild-type (WT). Reducing *KD1* transcript abundance, which is highly expressed in the AZs, delayed organ abscission, but it did not prevent the process, suggesting that KD1 is part of a complex system controlling the abscission process. The data obtained showed that the involvement of KD1 in abscission regulation was associated with auxin transporters and signaling components, and that the changes in *KD1* expression modulated the auxin concentration and response gradient in the FAZ. The above results are in accordance with previous reports demonstrating that manipulation of *KD1* expression affected various aspects of tomato plant development, which were all related to the KD1 role in auxin gradient responses (Koenig et al., 2009), and that the KNOX protein Knotted1 directly controlled auxin homeostasis and signaling in maize meristems (Bolduc et al., 2012).

Three knotted proteins, KNAT1, KNAT2, and KNAT6 in Arabidopsis, as well as the knotted TKN4 in tomato, were previously demonstrated to be involved in flower organ abscission, acting as downstream factors of the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA)-HAESA pathway (Estornell et al., 2013; Liljegren, 2012; Shi et al., 2011). An inhibitory effect of KNOX proteins on fruit abscission was found in litchi (Li et al., 2015; Zhao et al., 2020). Thus, ethephon treatment, that induced fruitlet abscission, resulted in the downregulation of four *KD1* genes in the AZ of litchi fruits (Li et al., 2015). Recently, it was reported that the litchi (Lc) KNOX-like protein LcKNAT1 repressed fruitlet abscission in litchi via a negative regulation of ethylene biosynthesis, which resulted from the inhibition of the expression of the litchi ethylene biosynthetic genes, *1-Aminocyclopropane-1-Carboxylate* (*ACC*) *Synthase* (*ACS*) and *ACC Oxidase* (*ACO*; Zhao et al., 2020). It seems therefore, that the regulation of ethylene biosynthesis by a KNAT1-like protein, LcKNAT1, is involved in litchi fruitlet abscission.

Our previous report provided evidence that KD1 promoted tomato flower pedicel abscission induced by flower removal (Ma et al., 2015). However, the data obtained in that study were quite limited, and demonstrated its effect only on the expression of auxin-related genes at one time point after flower removal (4 h). The aim of the present study was to acquire additional information on the involvement of KD1 in flower pedicel abscission, in order to further elucidate its role in this process and better understand its mode of action. Accordingly, we studied the effect of KD1 on the expression of a wide range of genes before flower removal and at various time points after flower removal. For this purpose, we performed a detailed transcriptome analysis of the FAZ and the NAZ of tomato WT plants compared to the FAZ of the *TAPG4::antisense KD1* plants, using a customized AZ-specific microarray (Sundaresan et al., 2016). The use of this customized AZ-specific microarray in a time course analysis allowed to expand the database of changes in gene expression occurring at the early stage of pedicel abscission, since it contains more gene probes than those that were previously used by Ma et al. (2015). The results obtained in the present study highlighted the expression of regulatory genes in the FAZ, which seem to be controlled by KD1 before and after abscission induction. These genes might have a role in regulating the acquisition of the FAZ cell competence to respond to ethylene signaling, and in controlling abscission in a different mode of action.

2 | MATERIALS AND METHODS

2.1 | Plant material and abscission induction treatments

The experiments were performed with tomato plants (*S. lycopersicum*, cv. "New Yorker"), grown from seeds obtained from the Tomato Genetics Resource Center, University of California, Davis, USA. All plants were grown in a greenhouse located in The Volcani Institute, Israel, under a controlled temperature of 25°C and natural daylight. The flower abscission experiments were carried out with plants grown in 10 l containers. The inflorescences of both WT and the *KD1*-antisense transgenic lines were harvested from 4-month-old plants as previously described (Meir et al., 2010; Sundaresan et al., 2018). The experimental protocols and pedicel abscission assays were performed in a controlled observation room maintained at 20°C and 60–70% relative humidity with continuous light of 14 μmol m^{-2} s^{-1}, as previously detailed (Kim et al., 2015; Meir et al., 2010; Sundaresan et al., 2018). The number of abscised pedicels was monitored daily by touching the distal side of the FAZ, for calculating the accumulated percentage of pedicel abscission.

2.2 | Vector construction and plant transformation

In the present work, we used *KD1* antisense lines A and E (T4 generation) that were previously generated by Ma et al. (2015)

from *TAPG4::antisense KD1* transgenic plants. These two representative transgenic lines were selected out of 10 independent transgenic lines, generated as previously reported (Ma et al., 2015).

2.3 | Gene expression profiling

For expression and microarray studies, samples of the FAZ and the NAZ of the WT and the FAZ of *TAPG4::antisence KD1* line E were collected in duplicates at various time points (0, 4, 8, 12, 16, and 20 h) after flower removal. The RNA was extracted, treated with DNase, processed, and checked for its quality, using the Agilent platform (Agilent, Palo Alto), as previously described (Sundaresan et al., 2016, 2018).

2.4 | Microarray

The experimental procedures for microarray labeling, hybridization, and scanning were performed as previously detailed (Sundaresan et al., 2018), using the AZ-specific microarray chip, AMADID: 043310 (Genotypic Technology, Pvt. Ltd.) for hybridization of the labeled cRNA samples (Sundaresan et al., 2016). The Feature Extraction software of Agilent V-11.5 was used for the data extraction from images. The analysis of the microarray data, using duplicate samples for each time point, to identify significant genes that were up or down-regulated within the group of samples, was basically performed as previously described (Sundaresan et al., 2018).

2.5 | Validation of gene expression by quantitative reverse transcription (qRT-PCR)

All the procedures for validation of gene expression were performed as previously detailed (Sundaresan et al., 2018), according to the primer sequences, amplicon length, and annealing temperature (Tm) presented in Table S1. These primers matched the microarray probes, and the qRT-PCR and microarray analyses were performed with the same RNA samples, as previously described (Sundaresan et al., 2016). *ACTIN* was used as the reference gene for determination of the relative expression levels of the identified genes. More qRT-PCR results for validation of microarray gene expression used in the present study can be found in a similar microarray experiment, performed with the same set of genes at the same time points after flower removal with other transgenic plants of *TAPG4::antisense THyPRP*, as described in our previous article (Sundaresan et al., 2018).

2.6 | Sequence deposition

The data for the *TAPG4::antisense KD1* FAZ samples (12 arrays) were submitted under the Gene Expression Omnibus database NCBI-GEO accession id: GSE64564. The microarray data for WT (cv. "New Yorker") FAZ and NAZ samples (12 arrays each) were submitted as previously detailed (Sundaresan et al., 2018) under the accession id: GSE64221, and the data are publicly available.

3 | RESULTS AND DISCUSSION

3.1 | Downregulation of *KD1* in the AZ inhibits tomato pedicel and petiole abscission

Quantitative reverse transcription (qRT-PCR) demonstrated that the *KD1* transcript was predominately expressed in the FAZ, and its expression was downregulated to very low levels within 8 h after flower removal (Figure 1). This rapid decrease in *KD1* expression in the tomato FAZ after flower removal was already demonstrated in previous reports (Ma et al., 2015; Meir et al., 2010). Pretreatment with the ethylene inhibitor 1-methylcylopropene (1-MCP) did not affect the expression of *KD1*, but the application of IAA after flower removal prevented the decrease in its expression (Meir et al., 2010). These data indicate that *KD1* expression in the FAZ is IAA-dependent, and that the decrease in its expression is a result of IAA depletion after flower removal. Similar results were reported for other Knotted family members, such as *knotted TKN2/LET6* (AF000141) and *knotted TKN4* (AF533597; Meir et al., 2010). The involvement of three knotted proteins, KNAT1, KNAT2, and KNAT6, was also reported for the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) – HAE/HSL1-dependent flower organ abscission in Arabidopsis (Estornell et al., 2013; Liljegren, 2012; Shi et al., 2011). These observations suggest that KD1 and TKN4 could function as regulators of the acquisition of AZ cell competence to respond to ethylene signaling after IAA depletion.

The important role of KD1 in tomato flower and leaf abscission was previously demonstrated by virus-induced gene silencing, and by antisense silencing under the AZ-specific promoter *TAPG4*, which led

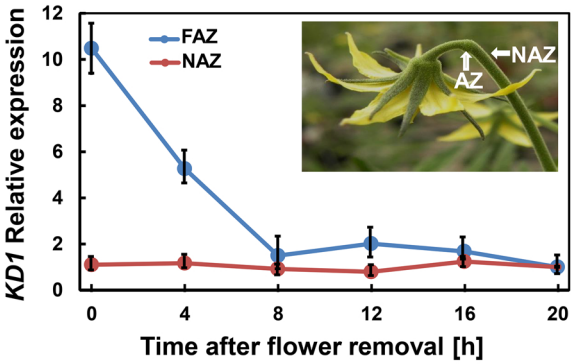

FIGURE 1 Effect of flower removal on the expression of *KD1* in the wild-type FAZ and NAZ. *KD1* expression was determined by qRT-PCR at 0, 4, 8, 12, 16, and 20 h after flower removal. The relative quantification of the gene expression level by the qPCR assay was determined by the comparative CT method $2^{-\Delta\Delta C}$T, using *Actin* as a reference gene. The ΔCT values were compared to the 20-h ΔCT value for *KD1* to generate $2^{-\Delta\Delta C}$T values. Results are means of three biological replicates ± SD. Inset, localization of the FAZ and NAZ along the flower pedicel, from which tissue samples were taken

to delayed pedicel and petiole abscission (Ma et al., 2015). The regulation of abscission by KD1 was found to be associated with modulation of the auxin level and response in the FAZ, leading to changes in the abundance of genes related to auxin transporters and signaling components (Ma et al., 2015). We used the same tomato *KD1* antisense lines A and E for the generation of T4 in the present study.

The efficacy of *KD1* silencing is demonstrated by the qRT-PCR results (Figure 2A). It is evident that the *TAPG4* promoter was very active in the FAZ (Bolduc et al., 2012), and *KD1* expression was downregulated by about 60% at time zero, and by about 70% at 4 h after flower removal, approximately at the same rates that were previously reported (Ma et al., 2015). The microarray antisense probe showed increased expression of *KD1* between 4 and 20 h after flower removal (Figure 2B). This antisense probe spanned the cloned fragment of *KD1* (Figure 2C). We expected a higher expression of the antisense probe also at zero time (Figure 2B), because the expression of *KD1* in the transgenic plants decreased by 60% at this time point (Figure 2A). At present, we do not have a reasonable explanation for these results.

The antisense transgenic lines exhibited a very significant delay in pedicel and petiole abscission following abscission induction (Figure 3). All the pedicels in the WT plants abscised at 20 h after flower removal, compared to about 40% of abscised pedicels in the *TAPG4::antisense KD1* lines (Figure 3).

The molecular events in the process of tomato pedicel abscission were organized in two phases: early events (0–4 h after flower removal) and late events (8–14 h after flower removal; Meir et al., 2010). It is important to note that the previous transcriptomic experiment was performed with *S. lycopersicum*, cv. "Shiran," in which 100% of pedicel abscission was obtained already at 14 h after flower removal, and therefore the time point of 8 h was considered as a late stage. In the present study, we used *S. lycopersicum*, cv. "New Yorker," in which 100% of pedicel abscission was obtained after 20 h (Figure 3), and therefore the time point of 8 h is considered as an early stage in the pedicel abscission execution process. The early events (0–4 h) probably lead to acquisition of the competence of FAZ cells to respond to ethylene signaling, and to increased endogenous ethylene biosynthesis. The late events include the execution of pedicel abscission and the development of the defense layer (Meir et al., 2010).

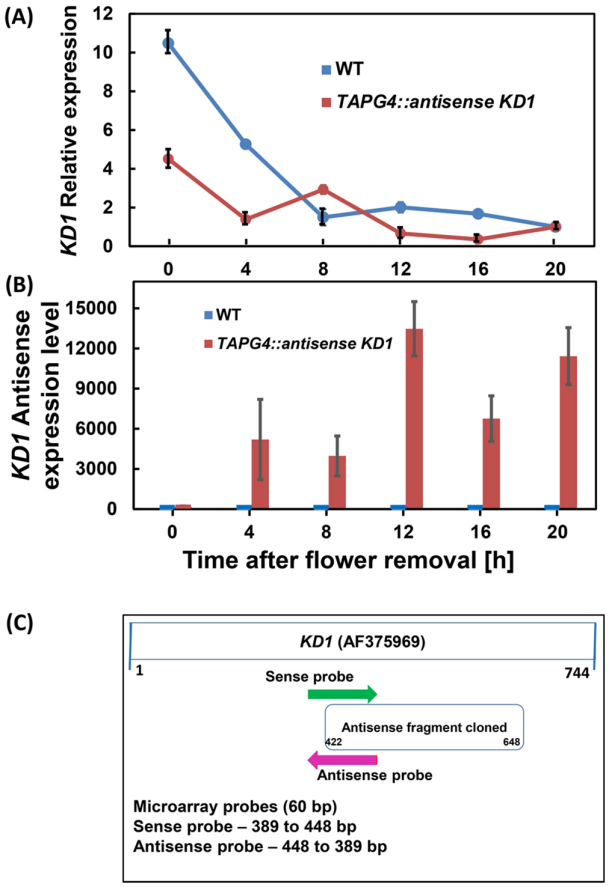

FIGURE 2 Effect of flower removal on the qRT-PCR expression levels of *KD1* (A) and on the array-measured expression levels of the antisense probe of *KD1* (B) in the FAZs of the wild-type and *TAPG4::antisense KD1*—line E. Results in (A) are means of three biological replicates ± SD. Results in (B) are means of two biological replicates ± SD. The schematic illustration of the sense and antisense microarray probes spanning the *KD1* gene is presented in (C). The qRT-PCR assay was performed as detailed in Figure 1. The fragment (422–648th bp) of the *KD1* gene (AF375969) was cloned for antisense into the vector. The sense probe from the microarray spans the region of 389–448th bp, and the antisense probe from the microarray spans the region of 448–389th bp

3.2 | Comparative transcriptome analysis in the FAZ of WT and of *KD1*-silenced plants using a customized AZ-specific array

The microarray experiments were performed with two independently prepared samples, and probes showing at least twofold changes in signal intensity were selected for analysis, except for cell wall genes, for which lower intensities were considered. We observed that numerous transcripts were upregulated and downregulated in the FAZ samples of the *KD1*-silenced versus WT plants, at 0, 4, 8, 12, 16, and 20 h after

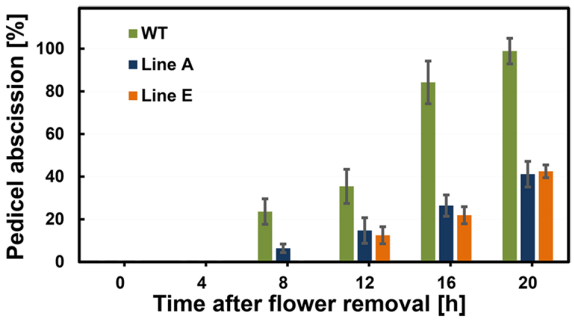

FIGURE 3 Effect of *TAPG4::antisense KD1* silencing on the abscission kinetics of flower pedicels. The accumulated percentage of pedicel abscission was monitored at 0, 4, 8, 12, 16, and 20 h following flower removal. Results are means of three experiments ± SD ($n = 40$ explants for each experiment). *KD1* antisense lines A and E were used in these experiments

flower removal (Table 1). The complete list of differentially expressed genes showing a twofold change was generated and is presented in Tables S2–S7. To validate the microarray assay results, we selected the following five genes, *Cellulase1* (*Cel1*; Solyc08g081620/U13054), *TAPG1* (Solyc02g067630/U23053), *TAPG4* (Solyc12g096750/U70481), *KD1* (Solyc06g072480/AF375969) and *THyPRP* (Solyc07g043000/X57076), and analyzed their expression by qRT-PCR. The qRT-PCR results were similar to the microarray results (Figure S1).

Our microarray results reconfirmed the auxin-related gene expression profiles reported by Ma et al. (2015) in the FAZ at 4 h after flower removal (Table S8). Thus, 40 auxin-related genes were upregulated by a 1.2- to 3.1-fold ratio in *TAPG4::antisense KD1* versus WT plants, while only 13 auxin-related genes were downregulated by a ratio of 0.4- to 0.8-fold. The results revealed the potential role of other regulatory genes, in addition to auxin regulatory genes, which are associated with KD1 at the early stage of the pedicel abscission process, as demonstrated in the following sections. In addition, our results showed significant changes in regulatory genes occurring before flower removal (at time zero). Such regulatory changes may affect the changes in auxin homeostasis demonstrated by Ma et al. (2015) at 4 h after flower removal.

3.3 | Mode of action of the inhibition of abscission in *KD1*-silenced plants

The determination of the role of KD1 in the regulation of abscission in the tomato flower model system based on the microarray results should be related to changes in the expression of regulatory genes occurring specifically in the FAZ of the silenced plants at the early stages of pedicel abscission. Therefore, we focused mainly on genes, the expression of which was altered at time zero and between 0 and 4 h after flower removal, when the competence of the FAZ cells to respond to ethylene is acquired (Meir et al., 2010, 2015; Sundaresan et al., 2018). Later regulatory effects can occur between 4 and 8 h after flower removal, coinciding with the execution of abscission, but it is difficult to distinguish at this stage between cause and result of abscission inhibition. The results presented in Figures 4–8 are organized according to the pattern and timing of gene expression rather than according to the function of the related proteins. Figures 4 and 5 present genes that were downregulated or upregulated following the antisense silencing of *KD1*, respectively, at time zero. Figures 6, 7A–D present genes that were downregulated or upregulated, respectively, at 4 h after flower removal and later on. Figures 7E–L7 and 8 present genes that were transiently upregulated or downregulated, respectively, mainly at 8 h after flower removal. Figure 9 presents genes encoding cell wall degrading enzymes, and Figure 10 presents genes encoding defense and boundary layer-related proteins. All these genes function in the late abscission execution phases, C and D, and not in the early regulatory phases (Estornell et al., 2013; Kim et al., 2015).

TABLE 1 Transcriptome responses of the FAZ tissues at various time points after flower removal in the transgenic line (*TAPG4::antisense KD1*) compared to the wild-type

	Number of differentially expressed transcripts in the FAZs of *TAPG4::antisense KD1* versus WT plants	
Time after flower removal (h)	Upregulated	Downregulated
0	40	31
4	48	49
8	61	97
12	39	26
16	183	270
20	126	51

Note: The data represent the total numbers of differentially expressed transcripts (fold changes: Downregulated ≤ −2; upregulated ≥2) with $P < 0.05$ at different time points after flower removal during the abscission process.

3.4 | Genes whose expressions were altered specifically in the FAZ of *KD1*-silenced plants at time zero

As a result of the decrease in *KD1* expression in the FAZ of the transgenic plants at time zero (Figure 1), FAZ-specific changes in the expression of some regulatory genes occurred at this time point (Figures 4 and 5). Eight genes were downregulated in the transgenic plants, and their expression remained low up to the end of the experiment (Figure 4). The downregulated genes included: two *MADS-box* genes (Figure 4A1–A2); a high mobility group (HMG) TF (*HMG-type nucleosome assembly factor*; Figure 4B); an ortholog gene of the Arabidopsis *Receptor-Like Kinase* (*RLK*; Figure 4C); *Enoyl-CoA Hydratase* (Figure 4D); *NAD-specific Glutamate Dehydrogenase* (*GLDH*; Figure 4E) and two *Unknown Protein* genes (Figure 4F1–F2). All the downregulated genes in the FAZ of the silenced plants were not FAZ-specific, and were also significantly expressed in the WT FAZ and NAZ (Figure 4), except for *RLK* that was expressed at a relatively low level in the WT NAZ (Figure 4C).

Twenty genes were specifically upregulated in the FAZ of *KD1*-silenced lines at time zero and their expression remained high or gradually decreased later on (Figure 5). The upregulated genes included: four *F-box family protein* genes (Figure 5A1–A4); the *Regulatory Particle* (*RP*) *RPN3* (Figure 5A5); genomic DNA *Chromosome 5P1 clone MBG8* (Figure 5B1); *Chromodomain Helicase DNA-binding protein4* (*CHD4*; Figure 5B2); *Histone-lysine N-methyl transferase SETDB1* (Figure 5B3); three genes related to ethylene biosynthesis—two *ACO-like proteins* (Figure 5C1–C2), and one *ACO-homolog* (Figure 5C3); one *AP2-like Ethylene Responsive Factor* (*ERF*; Figure 5C4); six *Unknown Protein* genes (Figure 5D1–D6); a *Serine–Threonine-Protein Phosphatase7* (Figure 5E) and *Glutaredoxin* (Figure 5F). All these genes were significantly upregulated in the FAZ of the *KD1*-silenced lines from time zero up to at least 8 h after flower removal.

Our results revealed that *TAPG4::antisense KD1*-silencing specifically altered the expression of the following major regulatory genes in the FAZ at different levels of regulation: four genes related to plant

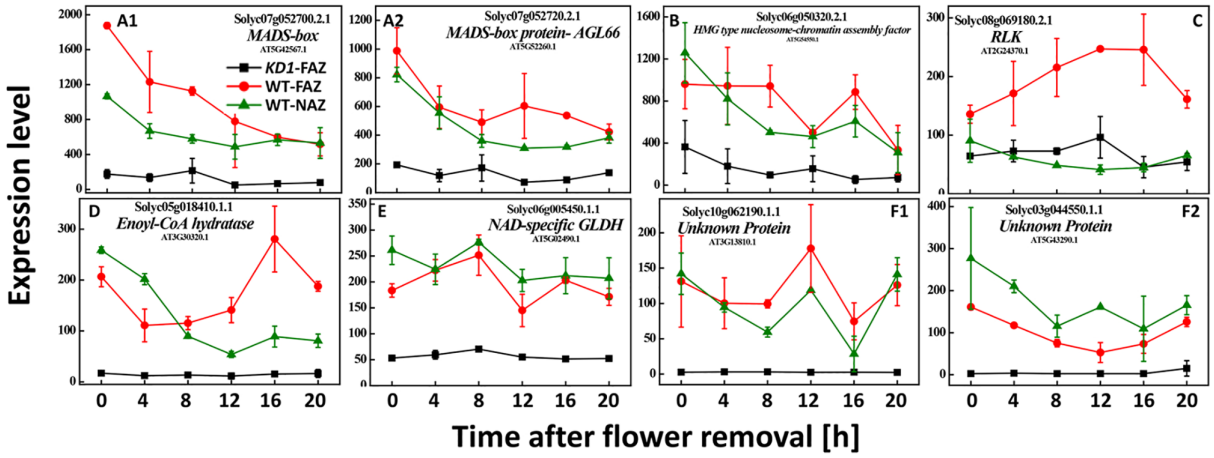

FIGURE 4 Effect of antisense silencing of *KD1* on the kinetics in array-measured expression levels of genes that were specifically downregulated in the FAZ of *TAPG4::antisense KD1*-silenced plants (line E), before (at time zero) and after flower removal compared to the FAZ of wild-type plants. *TAPG4::antisense KD1* silenced line E was used. Expression levels were measured for the following genes: *MADS-box transcription factor1* (A1) and *MADS-box protein-AGL66* (A2); *High Mobility Group* (*HMG*) *type nucleosome/chromatin assembly factor* (B); *Receptor-Like protein Kinase* (*RLK*; C); *Enoyl-CoA Hdratase* (D); *NAD-specific Glutamate Dehydrogenase* (*GLDH*; E); and *Unknown Protein* genes (F1, F2). Transcript identities are indicated in the graphs by their gene ID and their Arabidopsis gene number (At) and/or their nucleotide accession number. Results are means of two biological replicates ± SD. The red and green lines represent the gene expression in the FAZ and NAZ of the WT plants, and the black lines represent the gene expression in the FAZ of *KD1*-silenced plants, respectively

epigenetics, two MADS-box homeobox TFs, eight posttranslational regulation genes related to ubiquitin-based protein degradation or phosphorylation/de-phosphorylation and four ethylene-related genes. Some of these altered genes might trigger a cascade of molecular events, which lead to an alteration of the auxin levels and response in the FAZ at the early stages of the abscission process. These results led in turn to reduced competence of the FAZ cells to respond to ethylene, as demonstrated in our previous study (Ma et al., 2015). Four ethylene-related genes, three *ACO* genes (Figure 5C1–C3) and one *ERF* gene (Figure 5C4) were unexpectedly upregulated in the FAZ of the silenced plants from time zero up to 20 h. On the other hand, the transient upregulation of one *ACO* and two *ERF* genes in the WT FAZ was inhibited in the FAZ of the *KD1*-silenced plants (Figure 6B1–B3). The expression pattern in the WT FAZ and NAZ at different time points of other ethylene-related genes involved in its biosynthesis (*ACS*s and *ACO*s) and perception (*ETR*s and *CTR*s; data not shown), resembled the previously reported expression pattern for these genes (Meir et al., 2010). The expression of these ethylene-related genes, as well as the expression of other *ERF* genes in the FAZ (21 out of 24 *ERF*s) after flower removal, was not affected by *KD1* silencing (data not shown). These data suggest that the inhibition of pedicel abscission induced by *KD1* silencing might not necessarily be ethylene-mediated.

3.5 | Genes whose expressions were altered specifically in the FAZ of *KD1*-silenced plants at 4 h after flower removal

The differentially regulated genes in the FAZ of the silenced plants at 4 h after flower removal included various regulatory genes at different levels of regulation: epigenetics, TFs, post-translational such as kinase/phosphatase and protein degradation/ubiquitination, transporters, signal transduction and oxidase/reductase gene families. The expression of the TF gene encoding the Plant Homeodomain (PHD)-finger family protein, which was specifically upregulated in the WT FAZ at 4 h after flower removal and remained high up to 16 h, was significantly inhibited in the FAZ of the silenced plants (Figure 6C). The PHD finger protein has a metal binding RING domain (Cys3-His-Cys4) motif. The PHD domain has a conserved Zinc finger (Znf) domain in eukaryotic organisms. PHD finger domains in proteins related to epigenetics are involved in the interaction among proteins, especially the modification on histones of nucleosomes, such as methylation, acetylation, and phosphorylation (Li & Li, 2012). A similar expression pattern, i.e. upregulation in the WT FAZ and inhibition in the FAZ of the silenced plants, was also observed for two *bHLH* and *Znf* TF genes, *SlbHLH048* and *SlbHLH046* (Figure 6D1–D2). Some *bHLH* and *Znf* TF genes were previously reported to be involved in the abscission of olive fruit and tomato flower pedicels (Gil-Amado & Gomez-Jimenez, 2013; Meir et al., 2010).

Six genes related to the Ca^{2+} signal transduction, two kinases and four Ca^{2+}/Calmodulin (CaM)-related, which were upregulated at 4 h after flower removal in the WT FAZ, were inhibited in the FAZ of the silenced plants (Figure 6E1–E4, F1–F2). Calcium ions (Ca^{2+}) serve as a universal messenger involved in the modulation of diverse developmental and adaptive processes in response to various physiological stimuli (DeFalco & Bender, 2009; Batistič & Kudla, 2012). Our results support a role for the Ca^{2+}/CaM-mediated signal transduction in the control of the abscission process, and it is probably regulated by KD1. In citrus leaf abscission induced by a cycle of water stress/rehydration, a *CaM* gene was upregulated in the laminar AZ at 1 h after

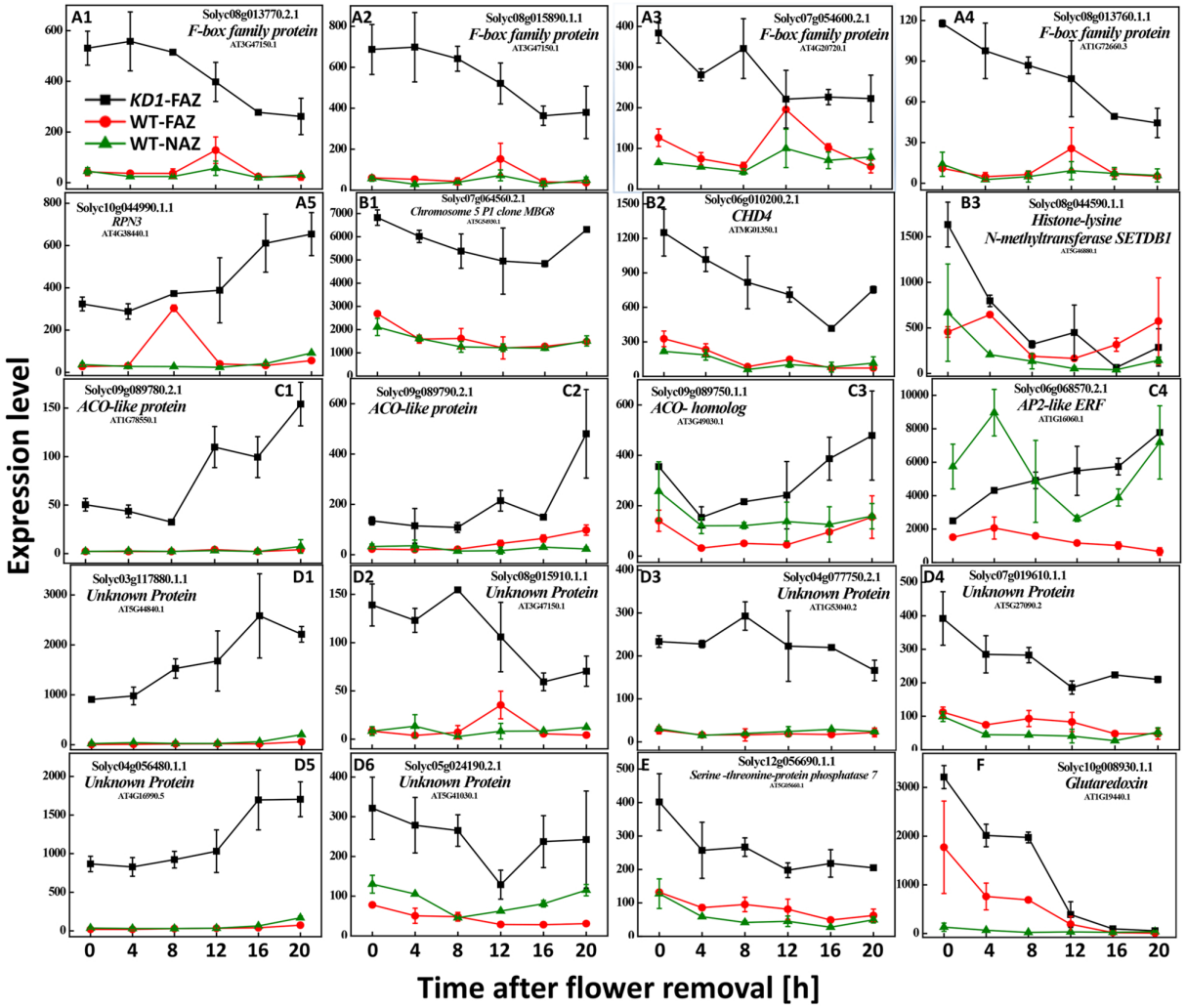

FIGURE 5 Effect of antisense silencing of *KD1* on the kinetics in array-measured expression levels of genes that were specifically upregulated in the FAZ of *TAPG4::antisense KD1*-silenced plants, before (at time zero) and after flower removal, compared to the FAZ of wild-type plants. Expression levels were measured for the following genes: *F-box family proteins* (A1–A4), and *26S proteasome non-ATPase regulatory subunit3* (*RPN3*; A5); *genomic DNA chromosome 5P1 clone MBG8* (B1); *Chromodomain Helicase DNA-Binding protein4* (*CHD4*) (B2), and *Histone-lysine N-methyltransferase SETDB1* (B3); two *1-Aminocyclopropane-1-Carboxylate Oxidase* (*ACO*)*-like protein* genes (C1, C2), *ACO-homolog* (C3), and *AP2-like–Ethylene Response Factor* (*ERF*; C4); six *Unknown Protein* genes (D1–D6); *Serine–threonine–protein phosphatase7* (E); and *Glutaredoxin* (F). All other details are as described in Figure 4

rehydration (Agusti et al., 2012). During ethephon-induced litchi fruitlet abscission, 52 transcripts related to calcium transport and perception displayed altered expression changes. Among them, 19 and 33 genes were up and downregulated, respectively, including *CNGC* genes that were upregulated three days after treatment (Li et al., 2015). In addition, *CaM*, *CML*, and *Calcium-binding protein kinase* genes were upregulated in the AZ during mature olive fruit abscission (Gil-Amado & Gomez-Jimenez, 2013). The above data demonstrate that Ca^{2+}/CaM signaling plays an important role in the regulatory pathways of organ abscission. Our results, demonstrating an inhibition of the upregulation of Ca^{2+}/CaM signaling-related genes in the *KD1*-silenced plants, suggest that KD1 plays a significant role in regulating the Ca^{2+}/CaM signal transduction during tomato pedicel abscission induced by flower removal.

Of particular interest is the exocytosis-related gene, *Syntaxin*, which encodes a membrane integrated protein, Q-Soluble *N*-Ethylmaleimide-sensitive Factor Attachment Protein Receptor (Q-SNARE), required for vesicle trafficking. This gene, which was upregulated in the WT FAZ, was significantly downregulated in the FAZ of the silenced plants (Figure 6F3). The primary role of SNARE proteins is to mediate vesicle fusion with their target membrane bound compartments participating in exocytosis (Lipka et al., 2007). Previous reports showed that mobilization of the secretory pathway leads to the release of cell wall modifying enzymes to implement abscission (Sexton et al., 1977; Sexton & Hall, 1974). Additionally, Agusti et al. (2012) reported the induction of several genes involved in vesicle trafficking, such as *SNARE-like protein* and *Syntaxin*, in citrus laminar AZ during leaf abscission induced by a cycle of water stress

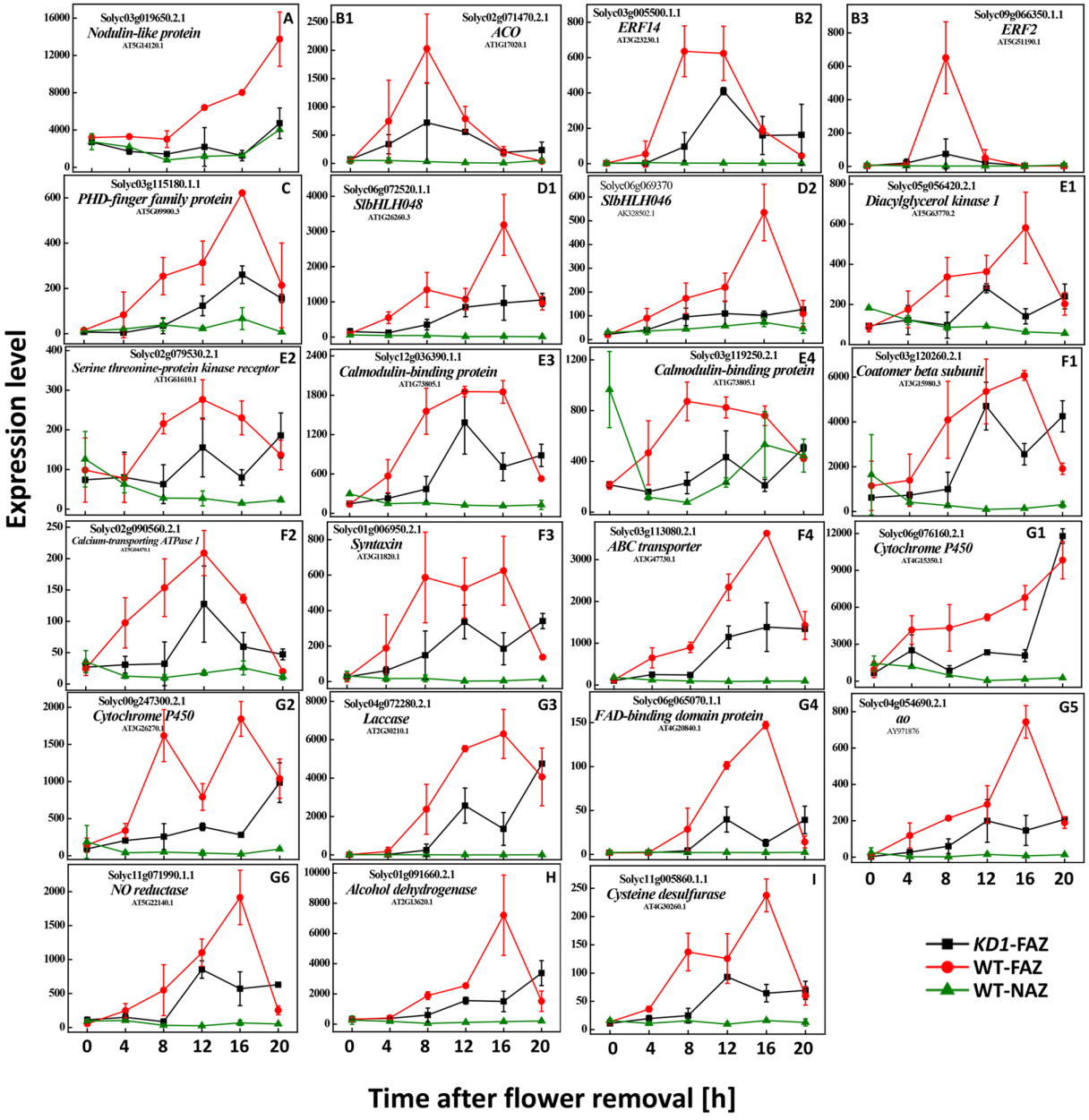

FIGURE 6 Effect of antisense silencing of *KD1* on the kinetics in array-measured expression levels of genes that were specifically modified in the FAZ of wild-type plants at 4 or 8 h after flower removal. Expression levels were measured for the following genes: *Nodulin-like protein* (A); *1-Aminocyclopropane-1-Carboxylate oxidase1* (*ACO1*; B1); *Ethylene-Responsive Factor14* (*ERF14*; B2); *ERF2* (B3); *PHD-finger family protein* (C); *Solanum lycopersicum Basic Helix-Loop-helix048* (*SlbHLH048*; D1), *SlbHLH046* (D2); *Diacylglycerol kinase1* (E1); *Serine/threonine-protein kinase receptor* (E2); two *Calmodulin-Binding protein* genes (E3, E4); *Coatomer beta subunit* (F1), *Calcium-transporting ATPase1* (F2), *Syntaxin* (F3), and *ABC transporter* (F4); two *Cytochrome P450* genes (G1, G2), *Laccase* (G3), *FAD-binding domain-protein* (G4), *Ascorbate Oxidase* (*AO*) (G5), and *Nitric Oxide* (*NO*) *reductase* (G6); *Alcohol Dehydrogenase* (H); and *Cysteine Desulfurase* (I). All other details are as described in Figure 4

and rehydration. Analysis of gene expression in melon fruits AZ revealed that a sequential induction of cell wall-degrading genes was associated with the upregulation of genes involved in endo- and exocytosis during mature fruit abscission (Corbacho et al., 2013). Recently, we reported results that clearly show how the processes of protein secretion by vesicle trafficking are regulated, programmed, and orchestrated at the level of gene expression in the present tomato FAZ model system (Sundaresan et al., 2020). These data further confirm the important role of KD1 in vesicle trafficking, which mediates tomato pedicel abscission.

Our analysis showed that the *ABC transporter* gene, an ATP-binding cassette transporter, which belongs to the ABCA sub-family, was downregulated in the *KD1*-silenced plants compared to the WT (Figure 6F4). This gene was shown to be involved in auxin transport and is specifically expressed in the root system (Andolfo et al., 2015). Similarly, the *Coatomer beta subunit* gene was also downregulated in

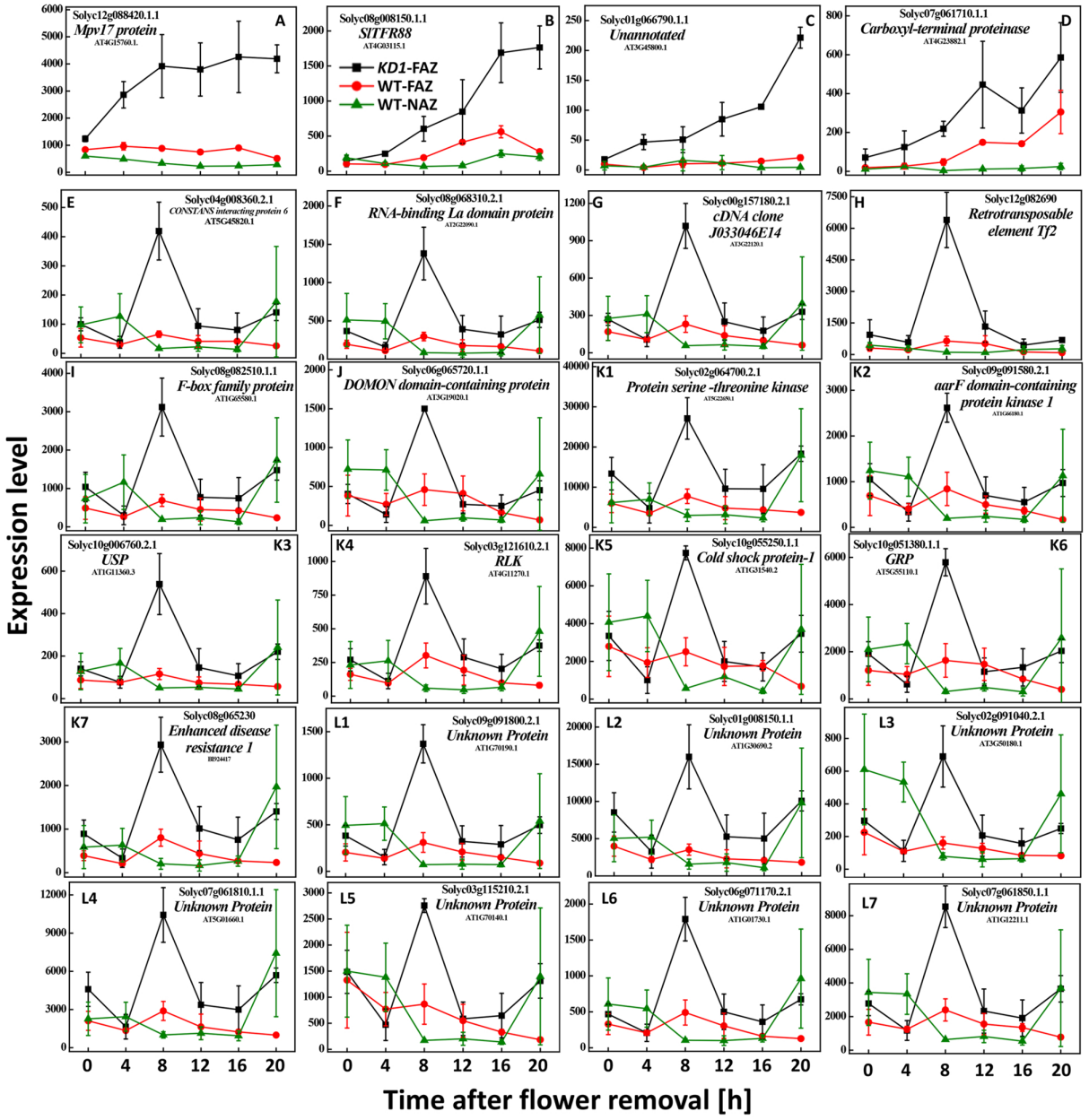

FIGURE 7 Effect of antisense silencing of *KD1* on the kinetics in array-measured expression levels of genes that were specifically continuously (A–D) or transiently (E–L7) upregulated between 0 and 20 h or at 8 h after flower removal, respectively, only in the FAZ of *TAPG4::antisense KD1*-silenced plants. Expression levels were measured for the following genes: *MpV17 protein* (A); *SlTFR88* (B); *Unannotated* (C); *Carboxyl-terminal proteinase* (D); *CONSTANS interacting protein6* (E); *RNA-binding La domain protein* (F); *cDNA clone* (G); *Retrotransposable element Tf2* (H); *F-box family protein* (I); *DOMON domain-containing protein* (J); *Protein serine–threonine kinase* (K1), *aarF domain-containing protein kinase1* (K2), *Universal Stress Protein* (*USP*; K3), *Receptor-Like Kinase* (*RLK*; K4), *Cold Shock protein-1* (K5), *Glycine-rich RNA-binding Protein* (*GRP*; K6), and *Enhanced Disease Resistance1* (K7); and seven *Unknown Proteins* genes (L1–L7). All other details are as described in Figure 4

the silenced plants (Figure 6F1). The coat protein complex genes are responsible for reverse transport of recycled proteins from the Golgi and pre-Golgi compartments back to the ER and vice versa (Barlowe, 2000).

In the present work, two *Cytochrome P450* genes were downregulated in the FAZ of the *KD1*-silenced plants up to 16 h after flower removal, reaching a similar level of expression to that observed in the WT FAZ at 20 h (Figure 6G1-G2). Another *Cytochrome P450* gene was also downregulated in the silenced plants up to 12 h after flower removal and reached a similar level of expression as in the WT FAZ during 12–20 h (see Figure 8D). In Arabidopsis, the cytochrome P450s were shown to be involved in catalyzing the first step of tryptophan-dependent IAA biosynthesis (Hull et al., 2000). The involvement of auxin and auxin-related gene expression in pedicel abscission of the WT and the *KD1*-silenced plants was previously reported (Ma et al., 2015).

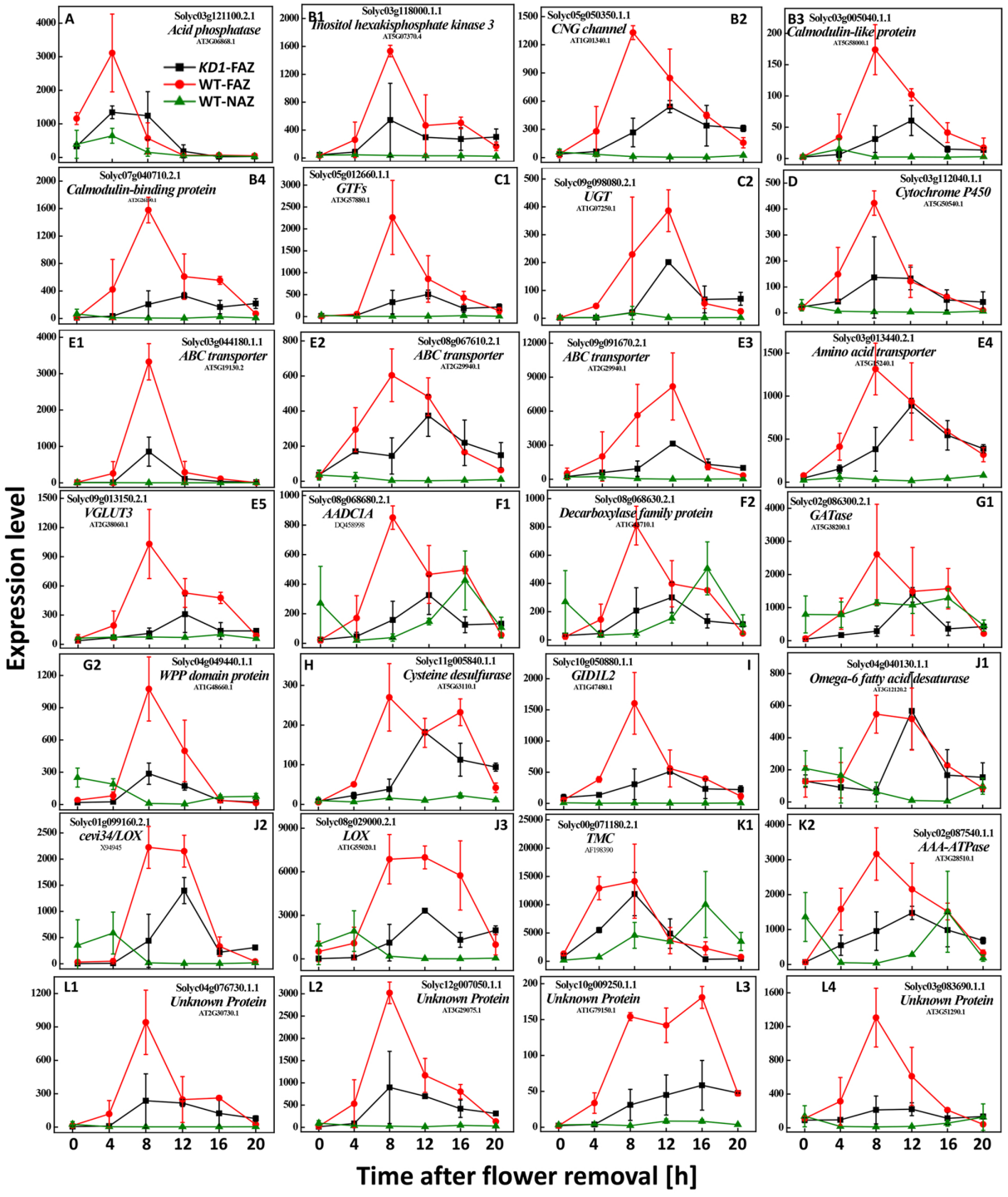

FIGURE 8 Effect of antisense silencing of *KD1* on the kinetics in array-measured expression levels of genes that were specifically and transiently upregulated in the FAZ of wild-type plants, peaking at 4, 8, or 12 h after flower removal. Expression levels were measured for the following genes: *Acid phosphatase* (A); *Inositol hexakisphosphate kinase3* (B1), *Cyclic Nucleotide Gated channel* (*CNG channels*; B2), *Calmodulin-like protein* (B3), and *Calmodulin-Binding protein* (B4); *glycosyltransferases* (*GFTs*; C1), and *UDP-glucosyl transferase* (*UGT*; C2); *Cytochromes P450* (D); three *ABC transporter family protein* genes (E1–E3), *Amino acid transporter protein* (E4), and *Vesicular Glutamate Transporter3* (*VGLUT3*; E5); *Aromatic Amino acid Decarboxylase1a* (*AADC1A*; F1), and *Decarboxylase family protein* (F2); *Glutamine amido transferases* (*GATase*; G1), and *Tryptophan-Proline-Proline (WPP) domain* (G2); *Cysteine Desulfurase* (H); Gibberellin receptor *GID1L2* (I); *Omega-6 fatty acid desaturase* (J1), *cevi34/Lipoxygenase* (*LOX*; J2), *LOX* (J3); *Multicystatin* (*TMC*; K1), and *AAA-ATPase* (K2); and four *Unknown Protein* genes (L1–L4). All other details are as described in Figure 4

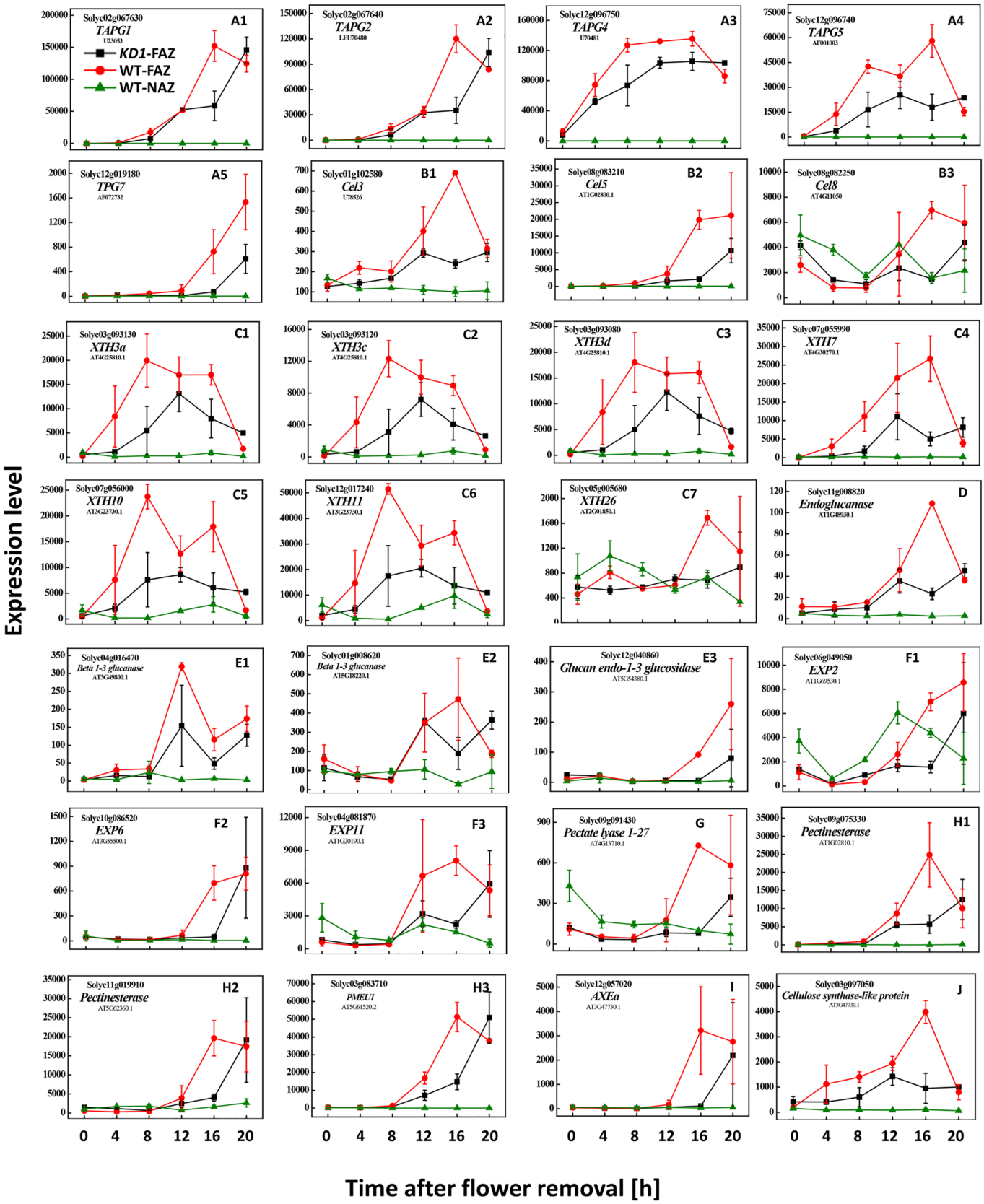

FIGURE 9 Effect of antisense silencing of *KD1* on the kinetics in array-measured expression levels of genes encoding cell wall modifying enzymes, that were specifically upregulated in the FAZ of wild-type plants at specific time points after flower removal: Early—4 or 8 h or late 12–20 h. Expression levels were measured for the following genes: Five *Tomato Abscission Polygalacturonase* (*TAPG*) genes (A1–A5); three *Cellulase* (*Cel*) genes (B1–B3); seven *Xyloglucan endo Transglucosylase/Hydrolase* (*XTHs*) genes (C1–C7); *Endoglucanase* (D); three *β-1,3-Glucanase* genes (E1-E3); three *expansin* (*EXP*) genes (F1-F3); *Pectate lyase 1–27* (G); two *Pectinesterase* genes (H1-H2), and *Pectinesterase/Pectinesterase inhibitor U1* (*PMEU1*; H3); *Acetyl Xylan Esterase a* (*AXEa*; I); and *Cellulose Synthase-Like protein* (J). All other details are as described in Figure 4

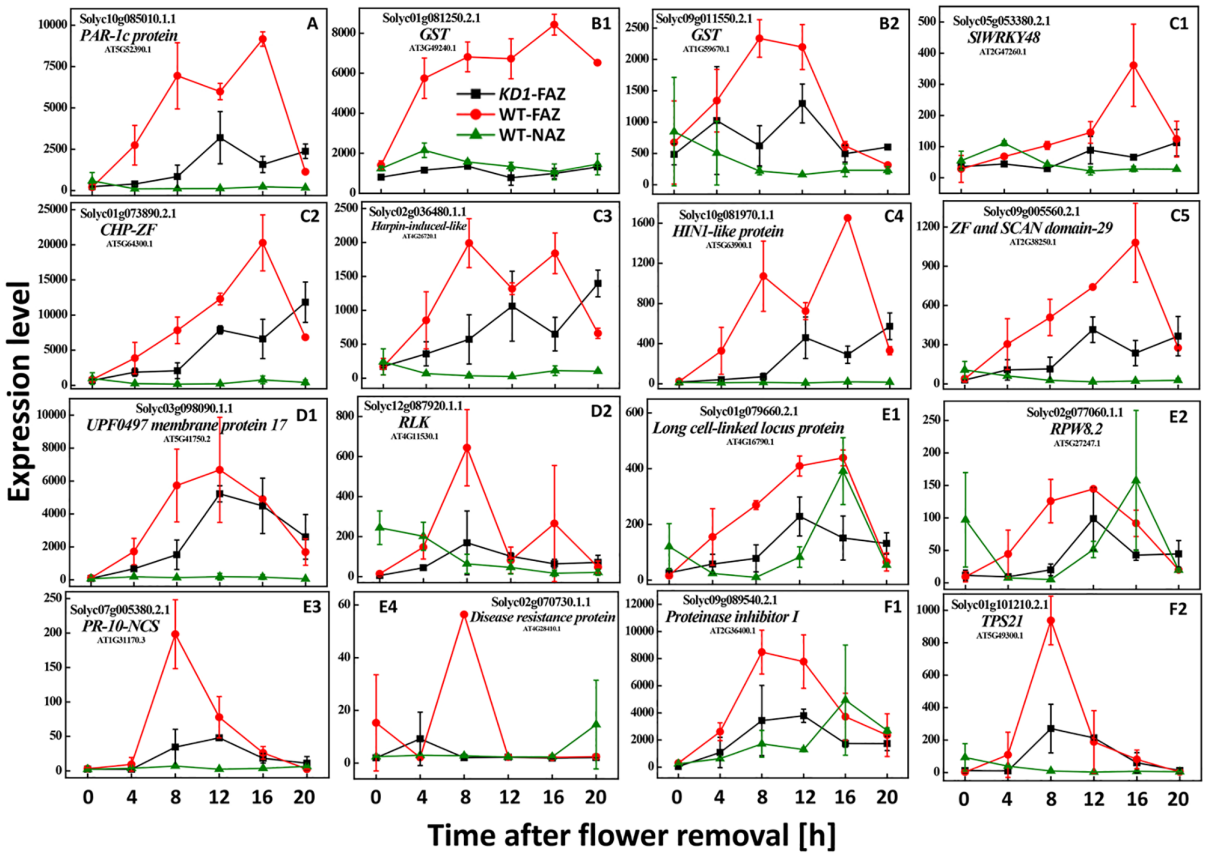

FIGURE 10 Effect of antisense silencing of *KD1* on the kinetics in array-measured expression levels of genes specifically transiently upregulated in the FAZ of wild-type plants at the early stage of abscission, 4 or 8 h after flower removal. Expression levels were measured for the following genes: *Photo Assimilate-Responsive1c protein* (*PAR-1*; A); two *Glutathione S-transferase protein* (*GST*) genes (B1, B2); *Solanum lycopersicum SlWRKY48* (C1), *Cysteine/Histidine-rich Zinc Finger protein-like* (*CHP-ZF*; C2), *Harpin-Induced-Like* (C3), and *HIN1-like protein* (C4); *ZF and SCAN domain-29* (*ZSCAN29*; C5); *UPF0497 membrane protein17* (D1), and *Receptor-Like Kinase* (*RLK*; D2); *Long cell-linked locus protein* (E1), *Resistance to Powdery Mildew8* (*RPW8.2*; E2), *PR-10-related Norcoclaurine Synthase-like protein* (*PR-10-NCS*; E3), and *Disease Resistance protein* (E4); *Proteinase Inhibitorl* (F1), and *α-humulene/(-)-(E)-β-caryophyllene Synthase* (*TPS21*; F2). All other details are as described in Figure 4

Gene expression of several oxidase/reductase-related genes, such as *Laccase* (Figure 6G3), *Ascorbate oxidase* (*AO*; Figure 6G5), *Nitric oxide* (*NO*) *reductase* (Figure 6G6), and *Alcohol dehydrogenase* (Figure 6H) was downregulated in the FAZ of the silenced plants, whereas in the WT FAZ these genes were upregulated during the abscission process. This suggests the involvement of oxidative processes in pedicel abscission.

3.6 | Genes whose expressions were altered specifically and transiently in the FAZ of *KD1*-silenced plants at 8 h after flower removal

Several genes were specifically and transiently altered in the FAZ of *KD1*-silenced plants at 8 h after flower removal, when the execution of cell separation had already started (22% of pedicel abscission was obtained in the WT; Figure 3). One set of genes represents diverse gene families that were transiently upregulated at 8 h in the silenced plants compared to the WT (Figure 7E–L), as detailed below. *CONSTANS interacting protein6* (Figure 7E), which was shown to control flowering in response to photoperiod in Arabidopsis (Ben-Naim et al., 2006; Wenkel et al., 2006), and leaf induction by cytokinins in tomato (Shi et al., 2013); *RNA-binding La domain protein* (Figure 7F), in which La acts as an RNA polymerase III (RNAP III) TF; *cDNA clone* (Figure 7G), that also encodes a RNA-binding protein; and *Dopamine-Monooxygenase N-terminal* (*DOMON*) *domain-containing protein*, also called DoH (Figure 7J), which was originally identified in a group of several secreted or cell surface proteins from plants and animals (Shi et al., 2013). It is usually associated with other redox domains in large proteins, such as cytochrome b561, and has a suggested capability of transmembrane electron transport (Iyer et al., 2007; Ponting, 2001; Verelst & Asard, 2003). Seven *Unknown Protein* genes with a similar expression pattern are seen (Figure 7L1–L7). All these genes were transiently upregulated at 8 h in the FAZ of the silenced plants, in which pedicel abscission was significantly inhibited, whereas in the WT pedicel abscission had already started (Figure 3). Therefore, some of these genes might have an inhibitory role in the abscission process. Interestingly, seven stress defense-associated genes had a similar

expression pattern (Figure 7K1–K7). However, it is not yet clear why these genes were upregulated in the silenced plants, which showed an inhibited abscission phenotype.

A second set of genes, which were specifically and transiently upregulated in the WT FAZ at 8 h after flower removal, were significantly inhibited in the silenced plants (Figure 8). These data suggest that some of these genes encode proteins that regulate the abscission execution, as detailed below: *Acid phosphatase* (Figure 8A), whose activity was detected in AZs of various species, such as sour and sweet cherry fruit, bean leaves, and hibiscus flower pedicels (Gilliland et al., 1976; Poovaiah et al., 1973; Poovaiah & Rasmussen, 1974); *Inositol Hexakisphosphate Kinase3* (Figure 8B1), to which the inositol hexakisphosphate kinase (InsP6) serves as a cofactor that recognizes auxin and the Auxin/IAA polypeptide substrate; transport inhibitor response1 (TIR1; Tan et al., 2007); four Ca^{+2}/CaM-signal transduction regulatory genes, *SlCNGC3* (Figure 8B2), *CaM-like protein* (Figure 8B3), *CaM-binding protein—SlCML35* (Figure 8B4), and an *AAA-ATPase family protein* (Figure 8K2). ATPases associated with diverse cellular activities (AAA^{+}-ATPases) are AAA-type ATPase-family proteins, which are involved in cellular functions, such as vesicle transport, organelle assembly, membrane dynamics, and protein unfolding (White & Lauring, 2007). The Arabidopsis ATPase Family gene1, (AFG1)-like protein 1 (AFG1L1), which belongs to the extended superfamily of AAA^{+}-ATPase proteins, binds to CaM in a calcium-dependent manner through a CaM-binding site in its catalytic AAA-domain (Bussemer et al., 2009). The function of these genes and the involvement of Ca^{+2}/CaM in abscission were discussed above in detail. Our data indicate that different Ca^{+2}/CaM genes regulate early and late events in the abscission process. Additional genes, which were specifically and transiently upregulated in the WT FAZ at 8 h after flower removal and were significantly inhibited in the silenced plant include: different transporter-related genes, such as *Glycosyltransferase* (*GTF*; Figure 8C1); *UDP-glycosyltransferase* (Figure 8C2); three *ABC/ABC-2* type transporters (Figure 8E1–E3); an *Amino Acid Transporter* (Figure 8E4) and *Vesicular Glutamate Transporter3* (*VGULT3*) (Figure 8E5); two decarboxylase genes, *AADC1A* (Figure 8F1) and *Decarboxylase Family Protein* (Figure 8F2); amino acid biosynthesis/metabolism genes—*Glutamine Amido Transferase* (*GATase*; Figure 8G1) and *WPP Domain-Associated Protein* (Figure 8G2), which encodes WPP-domain proteins that are developmentally associated with the nuclear envelope and promote cell division in Arabidopsis (Patel et al., 2004); *Cysteine Desulfurase1*

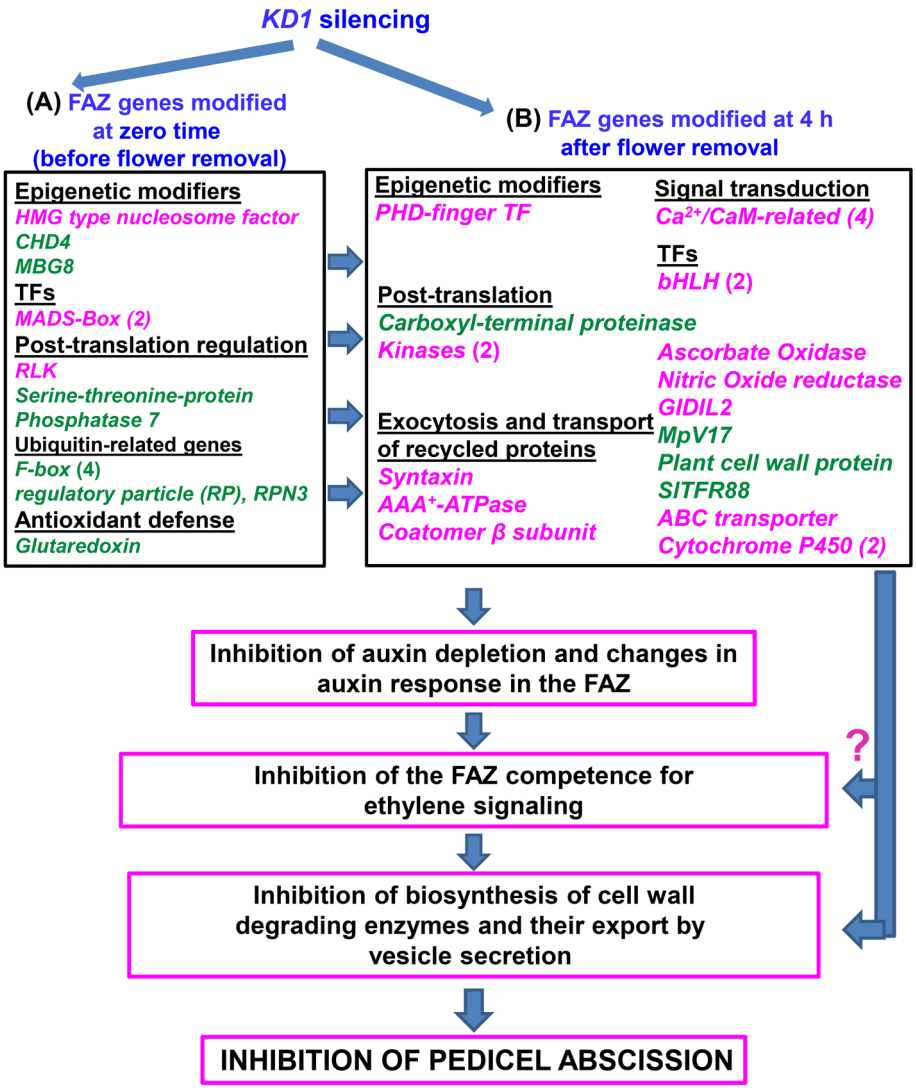

FIGURE 11 Summary of the genes with altered expression in the FAZ of *TAPG4::antisense KD1* plants before (at time zero) and at 4 h after flower removal, in response to *KD1* downregulation during abscission induced by flower removal. The altered gene expressions led to a decrease in the postulated events, resulting in the inhibition of pedicel abscission. Inhibition of auxin depletion and changes in the auxin response in the FAZ at the early abscission stages were demonstrated by Ma et al. (2015). Genes that were specifically upregulated or downregulated in the FAZ of *KD1*-silenced plants compared to the wild type are marked in green or magenta, respectively. The numbers listed besides the gene names indicate the number of genes in the gene family that were affected by *KD1* silencing

(*DES1*; Figure 8H), which encodes the enzyme L-cysteine desulfhydrase that catalyzes the desulfurization of L-cysteine. The GFP fused to the *DES1* promoter was reported to be highly expressed in the AZ of seeds and siliques, and the *des1* mutants exhibited an altered leaf senescence phenotype (Laureano-Marin et al., 2014). Another gene which was upregulated in the WT FAZ at 4 h and peaked at 8 h after flower removal was the gibberellin (GA) receptor gene, *GID1L2* (Figure 8I). This suggests that the inhibition of abscission by *KD1* silencing might be also mediated by the inhibition of *GIDIL2*. Additionally, some other *Unknown Protein* genes, which were specifically upregulated in the WT FAZ, were downregulated in the KD1-AZ (Figure 8L1–L4). Taken together, this figure demonstrates that the expressions of all these genes were significantly inhibited in the *KD1*-silenced plants.

3.7 | *KD1* silencing decreased the expression of cell wall modifying genes

It is well established that cell wall loosening and cell separation occurring in the AZ are controlled by distinct sets of cell wall degrading enzymes. Dissolution of the middle lamella or the shared cell wall in the AZ is a fundamental stage in the abscission process. Enzymes and proteins associated with disassembly and modification of the cell wall include PGs, cellulases, endoglucanases, pectin methylesterases, pectate lyases, xyloglucan endotransglucosylase/hydrolases (XTH), and expansins (EXP; Patterson, 2001; Roberts et al., 2002; Lashbrook & Cai, 2008, Lashbrook, 2009; Estornell et al., 2013). Therefore, the expression pattern of these cell wall modifying enzymes occurring at the late stages of abscission was used as an additional marker for confirming the effects of *KD1* silencing in delaying pedicel abscission.

Our transcriptome analysis showed that 28 genes encoding cell wall modifying enzymes belonging to 11 families, which were strongly upregulated in the WT FAZ, were downregulated in the FAZ of the *KD1*-silenced plants. This was manifested by both lower levels and delayed expression of these genes (Figure 9). These genes were specifically upregulated in the WT FAZ at different time points after flower removal, whereas in the NAZ, the expression of most of them did not change and remained very low during the entire experimental period (Figure 9A1–J). Only *Cel8*, *XTH26*, and *EXP2* genes exhibited some increased expression in the NAZ (Figure 9B3, C7 and F1). Among the genes encoding cell wall hydrolyzing enzymes, *TAPG4*,*5* and *XTH3a*,*3c*,*3d*,*7*,*10* were upregulated in the WT FAZ at 4 h after flower removal, and their expression remained high up to 16 h (Figure 9A3, A4, C1–C5). On the other hand, *Glucanase*, *Pectin Esterase*, and *EXP* genes were upregulated only at 8–12 h after flower removal (Figure 9E1–J). The tomato AZ-specific *TAPG* genes, *TAPG1*,*2*,*4*,*5*, were downregulated in the silenced plants compared to the WT (Figure 9A1–A4). The expression patterns of *TAPG1*,*2*,*4* in the WT FAZ were identical to their patterns reported previously (Meir et al., 2010), thereby confirming the microarray results obtained by the tomato AZ-specific microarray chip. The expression of cell wall genes usually peaked at 8–16 h after flower removal, but at lower rates, and therefore some of them were not included in the Supporting Information Tables, which used a cut-off of twofold change and a $P < 0.05$. Our results further demonstrate that organ abscission execution, manifested by upregulation of cell wall degrading proteins at phase C of the abscission process (Estornell et al., 2013; Kim, 2014; Patterson, 2001; Roberts et al., 2002), is a programmed event, in which these proteins are sequentially increased (Figure 9).

3.8 | *KD1* silencing decreased the expression of boundary layer- and defense-related genes

Phase D of the abscission process, in which the production of a protective defense layer occurs (Estornell et al., 2013; Kim, 2014; Patterson, 2001; Roberts et al., 2002), was shown to significantly overlap with the execution phase C of abscission (Kim et al., 2015). We present here only selected data of the numerous defense genes that were specifically upregulated in the tomato WT FAZ and were significantly inhibited in the *KD1*-silenced plants. Of the set of genes that were altered specifically and transiently in the WT FAZ at 8 h after flower removal, three genes should be mentioned: *Omega-6 Fatty Acid Desaturase* (Figure 8J1) and two *Lipoxygenase* (*LOX*) genes, *Cevi34/LOX* (Figure 8J2) and *LOX* (Figure 8J3). Some of the defense-related genes were upregulated in the WT FAZ very early after flower removal (Figure 10A–E2), while others were upregulated only later on (Figure 10E3–E4, F1–F2). The expression of all these genes was inhibited in the *KD1*-silenced plants (Figure 10). These genes are related to abscission, as they were upregulated only in the WT FAZ. These results confirm the overlapping between phases C and D of the abscission process.

4 | CONCLUSIONS

Based on the results presented in this study, we could further elucidate how KD1 promotes flower pedicel abscission in tomato plants. Figure 11 summarizes the postulated events leading to the inhibition of pedicel abscission in the *TAPG4::antisense KD1* plants, based on the expression of regulatory genes that was altered in the FAZ of the silenced plants at time zero and at 4 h after flower removal, compared to the WT FAZ. These regulatory genes are active at different levels of regulation, and their postulated roles in the abscission process are described and discussed. *KD1* expression decreased in the silenced plants at time zero (before flower removal). As a result, alteration of the expression of regulatory genes, including epigenetic modifiers, TFs, post translation regulators, and antioxidative defense factors occurred before flower removal (Figure 11A). Therefore, it can be concluded that KD1 is a master regulator of the abscission process, which promotes pedicel abscission. It should be noted that the reduction in KD1 activity after flower removal does not contradict its general accelerating effect on abscission, as KD1 operates at the beginning of the abscission process.

The effects of *KD1* silencing, which result in the inhibited abscission phenotype, include also indirect effects leading to the down-regulation of genes involved in abscission execution and defense processes. Thus, the inhibited expression of exocytosis and protein transporters such as AAA-ATPase and syntaxin, which inhibited the export of Golgi-derived vesicles containing cell wall degrading enzymes across the plasma membrane of AZ cells, might have an important role in the inhibited abscission phenotype of the *KD1*-silenced plants. It seems therefore, that the enhancing effect of KD1 on flower pedicel abscission is not limited to manipulation of auxin levels and response as previously reported, but it probably also operates via alteration of other regulatory pathways that promote the acquisition of the competence of the FAZ cells to respond to ethylene signaling, thereby enhancing abscission execution (Figure 11B).

Since KD1 belongs to the class I KNOX family, its role in organ abscission agrees with the various regulatory roles of class I KNOX family proteins, which were found to regulate several genes involved in biosynthesis and signal transduction of various plant hormones and TFs. Hence, this large class I KNOX family contains various proteins and genes, which can either promote or inhibit various processes within the same plant.

Taken together, the present study shades light on the role of KD1 as a master regulator of various signaling factors and regulatory genes involved in organ abscission processes, in addition to its well-documented involvement in leaf development, shoot apical meristem maintenance, and differentiation of flower meristems.

ACKNOWLEDGMENTS

Contribution from the ARO, Volcani Institute, Rishon LeZiyon, Israel. Srivignesh Sundaresan would like to thank the Indian Council of Agricultural Research for providing him with an International Fellowship (ICAR-IF) to support his PhD studies. We acknowledge the Genotypic Technology Private, Ltd. Bangalore, India, for the microarray processing and the data analysis reported in this publication. This research was funded by the United States-Israel Binational Agricultural Research and Development Fund (BARD), grant number US-4571-12C, and by the Chief Scientist of the Israeli Ministry of Agriculture Fund, grant number 203-0898-11.

AUTHOR CONTRIBUTIONS

Srivignesh Sundaresan and Shimon Meir conceived and designed the experiments. Srivignesh Sundaresan, Chao Ma, Cai-Zhong Jiang, Betina Kochanek, and Shoshana Salim performed the experiments and assisted with the data analysis. Srivignesh Sundaresan investigated and validated the data. Chao Ma and Cai-Zhong Jiang provided resources. Srivignesh Sundaresan, Shimon Meir and Sonia Philosoph-Hadas wrote the original draft. Sonia Philosoph-Hadas, Joseph Riov, and Michael S. Reid reviewed and edited the manuscript. Shimon Meir, Sonia Philosoph-Hadas, and Joseph Riov supervised the experimental performance. Shimon Meir and Sonia Philosoph-Hadas administered the project. Shimon Meir, Cai-Zhong Jiang and Sonia Philosoph-Hadas were responsible for funding acquisition. All authors read and approved the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Sonia Philosoph-Hadas https://orcid.org/0000-0001-8417-5024
Shimon Meir https://orcid.org/0000-0002-5792-6862

REFERENCES

Agusti, J., Gimeno, J., Merelo, P., Serrano, R., Cercos, M., Conesa, A. et al. (2012) Early gene expression events in the laminar abscission zone of abscission-promoted citrus leaves after a cycle of water stress/rehydration: involvement of CitbHLH1. *Journal of Experimental Botany*, 63, 6079–6091.

Andolfo, G., Ruocco, M., Donato, A.D., Frusciante, L., Lorito, M., Scala, F. et al. (2015) Genetic variability and evolutionary diversification of membrane ABC transporters in plants. *BMC Plant Biology*, 15, 51.

Barlowe, C. (2000) Traffic COPs of the early secretory pathway. *Traffic*, 1, 371–377.

Batistič, O. & Kudla, J. (2012) Analysis of calcium signaling pathways in plants. *Biochimica et Biophysica Acta*, 1820, 1283–1293.

Ben-Naim, O., Eshed, R., Parnis, A., Teper-Bamnolker, P., Shalit, A., Coupland, G. et al. (2006) The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. *The Plant Journal*, 46, 462–476.

Bolduc, N., Yilmaz, A., Mejia-Guerra, M.K., Morohashi, K., O'Connor, D., Grotewold, E. et al. (2012) Unraveling the KNOTTED1 regulatory network in maize meristems. *Genes & Development*, 26, 1685–1690.

Bussemer, J., Chigri, F. & Vothknecht, U.C. (2009) Arabidopsis ATPase family gene 1-like protein 1 is a calmodulin-binding AAA^{+}-ATPase with a dual localization in chloroplasts and mitochondria. *The FEBS Journal*, 276, 3870–3880.

Corbacho, J., Romojaro, F., Pech, J.C., Latché, A. & Gomez-Jimenez, M.C. (2013) Transcriptomic events involved in melon mature-fruit abscission comprise the sequential induction of cell-wall degrading genes coupled to a stimulation of endo and exocytosis. *PLoS One*, 8, e58363.

DeFalco, T.A. & Bender, K.W. (2009) Breaking the code: Ca^{2+} sensors in plant signaling. *The Biochemical Journal*, 425, 27–40.

Estornell, L.H., Agusti, J., Merelo, P., Talon, M. & Tadeo, F.R. (2013) Elucidating mechanisms underlying organ abscission. *Plant Science*, 199-200, 48–60.

Gil-Amado, J.A. & Gomez-Jimenez, M.C. (2013) Transcriptome analysis of mature fruit abscission control in olive. *Plant & Cell Physiology*, 54, 244–269.

Gilliland, M.G., Bornman, C.H. & Addicott, F.T. (1976) Ultrastructure and acid phosphatase in pedicel abscission of Hibiscus. *American Journal of Botany*, 63, 925–935.

Hong, S.B., Sexton, R. & Tucker, M.L. (2000) Analysis of gene promoters for two tomato polygalacturonases expressed in abscission zones and the stigma. *Plant Physiology*, 123, 869–881.

Hull, A.K., Vij, R. & Celenza, J.L. (2000) Arabidopsis cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 2379–2384.

Iyer, L.M., Anantharaman, V. & Aravind, L. (2007) The DOMON domains are involved in heme and sugar recognition. *Bioinformatics*, 23, 2660–2664.

Kim, J. (2014) Four shades of detachment: regulation of floral organ abscission. *Plant Signaling & Behavior*, 9, e976154.

Kim, J., Sundaresan, S., Philosoph-Hadas, S., Yang, R., Meir, S. & Tucker, M. L. (2015) Examination of the abscission-associated transcriptomes for soybean, tomato, and Arabidopsis highlights the conserved biosynthesis of an extensible extracellular matrix and boundary layer. *Frontiers in Plant Science*, 6, 1109.

Koenig, D., Bayer, E., Kang, J., Kuhlemeier, C. & Sinha, N. (2009) Auxin patterns *Solanum lycopersicum* leaf morphogenesis. *Development*, 136, 2997–3006.

Lashbrook, C.C. (2009) Functional genomic approaches to abscission. *Stewart Postharvest Review*, 5, 1–7.

Lashbrook, C.C. & Cai, S. (2008) Cell wall remodeling in Arabidopsis stamen abscission zones: temporal aspects of control inferred from transcriptional profiling. *Plant Signaling & Behavior*, 3, 733–736.

Laureano-Marin, A.M., Garcia, I., Romero, L.C. & Gotor, C. (2014) Assessing the transcriptional regulation of L-cysteine desulfhydrase1 in *Arabidopsis thaliana*. *Frontiers in Plant Science*, 5, 683.

Li, C., Wang, Y., Ying, P., Ma, W. & Li, J. (2015) Genome-wide digital transcript analysis of putative fruitlet abscission related genes regulated by ethephon in litchi. *Frontiers in Plant Science*, 6, 502.

Li, Y. & Li, H. (2012) Many keys to push: diversifying the 'readership' of plant homeodomain fingers. *Acta Biochimica et Biophysica Sinica (Shanghai)*, 44, 28–39.

Liljegren, S.J. (2012) Organ abscission: exit strategies require signals and moving traffic. *Current Opinion in Plant Biology*, 15, 670–676.

Lipka, V., Kwon, C. & Panstruga, R. (2007) SNARE-ware: the role of SNARE-domain proteins in plant biology. *Annual Review of Cell and Developmental Biology*, 23, 147–174.

Ma, C., Jiang, C.Z. & Gao, J. (2021) Regulatory mechanisms underlying activation of organ abscission. *Annual Plant Reviews*, 4, 27–56.

Ma, C., Meir, S., Xiao, L., Tong, J., Liu, Q., Reid, M.S. et al. (2015) A KNOTTED1-LIKE HOMEOBOX protein regulates abscission in tomato by modulating the auxin pathway. *Plant Physiology*, 167, 844–853.

Meir, S., Philosoph-Hadas, S., Sundaresan, S., Selvaraj, K.S., Burd, S., Ophir, R. et al. (2010) Microarray analysis of the abscission-related transcriptome in the tomato flower abscission zone in response to auxin depletion. *Plant Physiology*, 154, 1929–1956.

Meir, S., Sundaresan, S., Riov, J., Agarwal, I. & Philosoph-Hadas, S. (2015) Role of auxin depletion in abscission control. *Stewart Postharvest Review*, 11, 1–15.

Nakano, T., Fujisawa, M., Shima, Y. & Ito, Y. (2013) Expression profiling of tomato pre-abscission pedicels provides insights into abscission zone properties including competence to respond to abscission signals. *BMC Plant Biology*, 13, 40.

Nakano, T., Fujisawa, M., Shima, Y. & Ito, Y. (2014) The AP2/ERF transcription factor SlERF52 functions in flower pedicel abscission in tomato. *Journal of Experimental Botany*, 65, 3111–3119.

Patel, S., Rose, A., Meulia, T., Dixit, R., Cyr, R.J. & Meier, I. (2004) Arabidopsis WPP-domain proteins are developmentally associated with the nuclear envelope and promote cell division. *Plant Cell*, 16, 3260–3273.

Patterson, S.E. (2001) Cutting loose. Abscission and dehiscence in Arabidopsis. *Plant Physiology*, 126, 494–500.

Ponting, C.P. (2001) Domain homologues of dopamine beta-hydroxylase and ferric reductase: roles for iron metabolism in neurodegenerative disorders? *Human Molecular Genetics*, 10, 1853–1858.

Poovaiah, B.W. & Rasmussen, H. (1974) Localization of dehydrogenase and acid phosphatase in the abscission zone of bean leaves. *American Journal of Botany*, 61, 68–73.

Poovaiah, B.W., Rasmussen, H. & Bukovac, M. (1973) Histochemical localization of enzymes in the abscission zones of maturing sour and sweet cherry fruit. *Journal of the American Society for Horticultural Science*, 98, 16–18.

Roberts, J.A., Elliott, K.A. & Gonzalez-Carranza, Z.H. (2002) Abscission, dehiscence, and other cell separation processes. *Annual Review of Plant Biology*, 53, 131–158.

Sexton, R. & Hall, J.L. (1974) Fine structure and cytochemistry of the abscission zone cells of Phaseolus leaves: I. Ultrastructural changes occurring during abscission. *Annals of Botany*, 38, 849–854.

Sexton, R., Jamieson, G.G.C. & Allan, M.H.I.L. (1977) An ultrastructural study of abscission zone cells with special reference to the mechanism of enzyme secretion. *Protoplasma*, 91, 369–387.

Shi, C.L., Stenvik, G.E., Vie, A.K., Bones, A.M., Pautot, V., Proveniers, M. et al. (2011) Arabidopsis class I KNOTTED-like homeobox proteins act downstream in the IDA-HAE/HSL2 floral abscission signaling pathway. *Plant Cell*, 23, 2553–2567.

Shi, X., Gupta, S., Lindquist, I.E., Cameron, C.T., Mudge, J. & Rashotte, A.M. (2013) Transcriptome analysis of cytokinin response in tomato leaves. *PLoS One*, 8, e55090.

Sundaresan, S., Philosoph-Hadas, S., Ma, C., Jiang, C.Z., Riov, J., Mugasimangalam, R. et al. (2018) The tomato hybrid proline-rich protein regulates the abscission zone competence to respond to ethylene signals. *Horticulture Research*, 5, 28.

Sundaresan, S., Philosoph-Hadas, S., Riov, J., Mugasimangalam, R., Kuravadi, N.A., Kochanek, B. et al. (2016) De novo transcriptome sequencing and development of abscission zone-specific microarray as a new molecular tool for analysis of tomato organ abscission. *Frontiers in Plant Science*, 6, 1258.

Sundaresan, S., Philosoph-Hadas, S., Riov, J., Salim, S. & Meir, S. (2020) Expression kinetics of regulatory genes involved in the vesicle trafficking processes operating in tomato flower abscission zone cells during pedicel abscission. *Life*, 10, 273.

Tan, X., Calderon-Villalobos, L.I., Sharon, M., Zheng, C., Robinson, C.V., Estelle, M. et al. (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature*, 446, 640–645.

Verelst, W. & Asard, H. (2003) A phylogenetic study of cytochrome b561 proteins. *Genome Biology*, 4(6), R38.

Wang, X., Liu, D., Li, A., Sun, X., Zhang, R., Wu, L. et al. (2013) Transcriptome analysis of tomato flower pedicel tissues reveals abscission zone-specific modulation of key meristem activity genes. *PLoS One*, 8, e55238.

Wenkel, S., Turck, F., Singer, K., Gissot, L., Gourrierec, L.J., Samach, A. et al. (2006) CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of Arabidopsis. *Plant Cell*, 18, 2971–2984.

White, S.R. & Lauring, B. (2007) AAA^{+}-ATPases: achieving diversity of function with conserved machinery. *Traffic*, 8, 1657–1667.

Zhao, M., Li, C., Ma, X., Xia, R., Chen, J., Liu, X. et al. (2020) KNOX protein KNAT1 regulates fruitlet abscission in litchi by repressing ethylene biosynthetic genes. *Journal of Experimental Botany*, 71, 4069–4082.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Sundaresan, S., Philosoph-Hadas, S., Ma, C., Jiang, C.-Z., Riov, J., Kochanek, B. et al. (2021) Role of the KNOTTED1-LIKE HOMEOBOX protein (KD1) in regulating abscission of tomato flower pedicels at early and late stages of the process. *Physiologia Plantarum*, 173(4), 2103–2118. Available from: https://doi.org/10.1111/ppl.13560