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Microarray analysis of the abscission-related transcriptome in tomato flower abscission zone in response to auxin depletion

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ABSTRACT

The abscission process is initiated by changes in the auxin gradient across the abscission zone (AZ), and is triggered by ethylene. Although changes in gene expression have been correlated with the ethylene-mediated execution of abscission, there is almost no information on the molecular and biochemical basis of the increased AZ sensitivity to ethylene. We examined transcriptome changes in the tomato (*Solanum lycopersicum* Mill, cv. 'Shiran' 1335) flower AZ during the rapid acquisition of ethylene sensitivity following flower removal, which depletes the AZ from auxin, with or without pre-exposure to 1-methylcyclopropene (1-MCP) or application of indole-3-acetic acid (IAA) after flower removal. Microarray analysis, using the Affymetrix Tomato GeneChip®, revealed changes in expression, occurring prior to and during pedicel abscission, of many genes with possible regulatory functions. They included a range of auxin- and ethylene-related transcription factors (TFs), other TFs and regulatory genes that are transiently induced early, 2 h after flower removal, and a set of novel AZ-specific genes. All gene expressions initiated by flower removal and leading to pedicel abscission were inhibited by IAA application, while 1-MCP pretreatment inhibited only the ethylene-induced expressions, including those induced by wound-associated ethylene signals. The results confirm our hypothesis that acquisition of ethylene sensitivity in the AZ is associated with altered expression of auxin-regulated genes, resulting from auxin depletion. Our results shed light on the regulatory control of abscission at the molecular level, and further expand our knowledge of auxin-ethylene crosstalk during the initial controlling stages of the process.
INTRODUCTION

Abscission, senescence and ripening are plant developmental processes that their timing is determined by tissue sensitivity to ethylene (Trewavas, 1986; Bleecker and Patterson, 1997; Zegzouti et al., 1999). The biological basis for this increased ethylene sensitivity is still not known, but it has been shown to be modulated also by other plant hormones. In abscission, the interplay between indole-3-acetic acid (IAA) and ethylene is well established (Abeles and Rubinstein, 1964; Sexton, 1997; Taylor and Whitelaw, 2001; Roberts et al., 2002). The generally accepted model is that a basipetal IAA flux through the abscission zone (AZ) prevents abscission by rendering the AZ insensitive to ethylene. Unlike various auxin-mediated physiological processes that are a result of transient and local changes in auxin levels (Woodward and Bartel, 2005), prevention of abscission has been found to require a continuous and constant polar supply of auxin to the AZ (Taylor and Whitelaw, 2001). If the source of IAA is removed, the AZ becomes sensitized to the action of ethylene and abscission commences (Rubinstein and Leopold, 1963; Abeles and Rubinstein, 1964; Addicott, 1982; Sexton and Roberts, 1982; Meir et al., 2003, 2006). Accordingly, the activities of cell wall degrading enzymes, including cellulase (Cel), polygalacturonase (PG), expansin (EXP) and xyloglucan endohydrolase endotransglycosylase (XET) have been shown to increase dramatically with the onset of abscission (Lashbrook et al., 1994; Kalaitzis et al., 1997; Agusti et al., 2008, 2009; Cai and Lashbrook, 2008; Roberts and Gonzalez-Carranza, 2009).

The molecular mechanisms leading to increased tissue sensitivity to ethylene in response to IAA deficiency in the AZs are still unknown. Some insights were provided by a study of abscission in Mirabilis jalapa, identifying differentially regulated genes in AZs (Meir et al., 2003, 2006). Auxin depletion led to down-regulation of several auxin-responsive genes, while application of auxin prevented their decrease. Some genes, up-regulated by auxin depletion, were homologous with known ethylene-responsive (ER) genes such as peroxidase (PER) and ER6. Since the experiments were done in the presence of the ethylene action inhibitor, 1-methylocyclopropene (1-MCP), it is likely that these effects are independent of changes in ethylene perception. Based on this study, our hypothesis postulates that acquisition of ethylene sensitivity in the AZ is associated with altered expression of auxin-regulated genes.

It seems that in the last years we are beginning to get insight into regulatory control of abscission at the molecular level. For example, the tomato mutation jointless, which does not form pedicel AZs, is now attributed to a mutation in a MADS-box gene, LeMADS (Mao et al., 2000). Abscission of Arabidopsis floral organs has been shown to be delayed by mutations in a
receptor-like kinase, HAESA (Jinn et al., 2000), or over-expression of a MADS-box gene, AGL15 (Fernandez et al., 2000), and prevented by a mutation in the gene inflorescence deficient in abscission (IDA/IDL) that is thought to encode a receptor ligand (Butenko et al., 2003). HAESA (HAE) and HAESA-Like 2 (HLS2) that serve as receptors for the IDA, were identified as receptor-like kinases (RLKs) (Cho et al., 2008; Stenvik et al., 2008). IDA, HAE and HAESA influenced abscission in Arabidopsis in an ethylene-independently manner (Jinn et al., 2000; Butenko et al., 2006; Binder and Patterson, 2009). However, no studies have yet reported on genes that are involved in sensing the change in auxin gradient and inducing the sensitivity of the AZ cells to ethylene.

Our previous study with *Mirabilis jalapa* relied on differential subtractive hybridization (Meir et al., 2003, 2006), and may have missed important regulatory genes that are expressed in low copy number. The use of microarrays to analyze abscission-related gene expression was significantly promoted during the last years (Lashbrook, 2009). The release of the Affymetrix GeneChip® Tomato Genome Array in 2005 provides a powerful analytical tool to explore the role of auxin in regulation of abscission in a species where abscission has been well characterized physiologically and biochemically (Roberts et al., 1984; Del Campillo and Bennett, 1996). The tomato genome array, which consists of over 10,000 probe sets, allows expression analysis for about one third of the currently identified tomato genes.

We describe here the results of studies in tomato (*Solanum lycopersicum* Mill, cv. 'Shiran' 1335) flower AZ compared to the non-AZ (NAZ) tissue, and examining changes in the transcriptome in response to auxin depletion, with or without of 1-MCP pretreatment. 1-MCP is an effective inhibitor of ethylene action that prevents ethylene effects in a broad range of fruits, vegetables and floriculture crops (Watkins, 2006). Our results describe global changes in gene expression in the tomato flower AZ tissue at an early stage following induction of the abscission process, when the AZ becomes sensitive to ethylene, as well as at later stages in the process during execution of pedicel abscission and the development of a defense layer. Accordingly, the results allow the study of auxin-ethylene relations at a wide scope of the abscission process with the aid of the newly identified abscission-related genes. Furthermore, to distinguish the observed changes in the transcriptome as a result from auxin depletion from changes which may occur due to wounding or other non-related signals, the transcriptome changes in the AZ were analyzed also under the same conditions but with application of exogenous IAA to the cut surface of the remaining tissues after flower removal. The results obtained further support our hypothesis that the observed changes in gene expression induced by flower removal are due to auxin depletion.
RESULTS AND DISCUSSION

Effect of Flower Removal, 1-MCP Pretreatment, and IAA Application after Flower Removal on Kinetics of Pedicel Abscission

Tomato flower bunch explants placed in chlorine solution (Fig. 1A) did not show any pedicel abscission at the flower AZ during 60 h of incubation following cluster detachment (data not shown). Flower removal induced pedicel abscission (Fig. 1, B and C) in control explants, which was already visualized 10 h later, with 15% of the pedicels abscised following a very delicate touch at pedicel (Fig. 2). However, although no abscission was yet visible in control explants up to 8 h after flower removal (Fig. 2), we have observed that cell separation was already initiated. This was evident by the observed separation in about 20% of the pedicel AZs (data not shown), which occurred as a result of the manipulations performed during sampling the tissues for the RNA extraction. This indicates that the abscission process was already initiated at least 8 h after flower removal. Later on, 12 h after flower removal, the abscission rate increased sharply, reaching 75% of pedicel abscission at 18 h (Fig. 2). These results are in good agreement with previous results obtained with tomato flower explants (Roberts et al., 1984). It was also shown in this system that pedicel abscission induced by flower removal could be prevented by application of IAA on the tomato pedicel following flower removal (Roberts et al., 1984; Del Campillo and Bennett, 1996). Application of IAA (10⁻³ M) in lanolin paste to the cut surface of the pedicle after flower removal nullified the abscission during 38 h after flower removal in our system as well (Fig. 2). We assume that pedicel abscission continued to be inhibited even after 38 h of IAA treatment, as it was previously shown that application of 10⁻³ M IAA to tomato explants inhibited pedicel abscission for 21 d even when the explants were exposed to 10 µl l⁻¹ ethylene (Roberts et al., 1984). A similar effect of IAA application to the cut end of the petiole or stump following leaf deblading or stem decapitation was obtained also in Mirbailis jalapa (Meir et al., 2003, 2006). These results indicate that the main effect of organ removal in inducing abscission is due to auxin depletion.

Pretreatment with the ethylene action inhibitor 1-MCP (applied before flower removal) (Fig. 1D) prevented completely pedicel abscission induced by flower removal for at least 20 h (Fig. 2). This result demonstrates again the involvement of ethylene in the tomato flower abscission process, as 1-MCP is well known to bind with higher affinity than ethylene to the ethylene receptor and thereby inhibit its action (Sisler et al., 1997; Watkins, 2006). After about
30 h, the full inhibitory effect of 1-MCP was not maintained any more, possibly due to synthesis of new ethylene receptors in the AZ (Klee, 2002, 2004). It should be noted that samples for the RNA extraction were taken only during the period in which 1-MCP completely inhibited pedicel abscission, up to 14 h following flower removal (Fig. 1D).

Microarray Analysis and Clustering of Differentially Expressed Genes after Flower Removal

A microarray containing about 10,000 tomato probe sets was used to measure the expression of genes in the AZ following induction of abscission. This was compared to gene expression in the NAZ control tissue at various time points following flower removal and in response to pretreatment with 1-MCP or IAA application after flower removal (Fig. 1D). Based on similar kinetic patterns of expression following flower removal, genes were grouped into 22 types of clusters. Each cluster included genes with similar patterns of expression kinetics modified in control AZ (without 1-MCP or IAA) following induction of abscission due to flower removal. Clustered genes were annotated for functional categories. These 22 clusters were divided into five main groups based on the effect of flower removal on their temporal pattern of expression (Fig. 3). The types of expression kinetics used for dividing the deferentially expressed genes into the different groups included: **Group 1** - early (2 to 4 h) and transiently up- or down-regulated genes (Fig. 3.1; Supplemental Table S3.1, A to F; Supplemental Table S4.1, A to F); **Group 2** - late (8 to 14 h) up- or down-regulated genes (Fig. 3.2; Supplemental Table S3.2, A to D; Supplemental Table S4.2, A to D); **Group 3** - genes up- or down-regulated at the interval time of 4 to 14 h (Fig. 3.3; Supplemental Table S3.3, A to D; Supplemental Table S4.3, A to D); **Group 4** - genes up- or down-regulated early (1 to 2 h) and whose expression is maintained constant during the 2 to 14 h period (Fig. 3.4; Supplemental Table S3.4, A to D; Supplemental Table S4.4, A to D); **Group 5** - genes transiently up- or down-regulated during the 4 to 8 h time period (Fig. 3.5; Supplemental Table S3.5, A to D; Supplemental Table S4.5, A to D).

The classification of the clusters into five groups suggests that the abscission process may be separated into two main phases: the early phase occurring at the period of 0 to 4 h after flower removal that probably leads to acquisition of ethylene sensitivity and abscission competence, and the late phase, which occurs between 8 to 14 h after flower removal, when active abscission processes start leading to the execution of pedicel abscission.

The total number of genes in each cluster, the expression of which was affected by flower removal, is summarized in Table I, including the number of genes affected following
flower removal, following 1-MCP-pretreatment and flower removal, and the number of overlapping genes found to be affected in their expression by both treatments. All genes included in the 22 clusters are listed in Supplemental Table S3 and Table S4, with their fold-changes in the AZ at the different time points (h) after flower removal.

Assessing Microarray Data Reliability and the Effect of Flower Removal on Expression of Genes Related to Cell Wall Modification

Experiments were performed to validate the obtained microarray results. The first validation approach was to compare the results obtained in the microarray analysis with those reported before for genes encoding cell wall hydrolyzing enzymes associated with the tomato abscission process, including tomato abscission PG1 (TAPG1), TAPG2, TAPG4 (Kalaitzis et al., 1997) and Cell (Lashbrook et al., 1994). These four abscission-regulated genes displayed expression profiles (Fig. 4) similar to those previously described. TAPG4 mRNA was detected much earlier than TAPG1 and TAPG2 mRNAs (Kalaitzis et al., 1997) or Cell (Lashbrook et al., 1994) during both leaf and flower abscission in tomato. Results from our microarray confirm this sequential expression pattern of these genes. TAPG1, TAPG2, Cell and Cel5 were up-regulated starting from 8 h after flower removal, and were further up-regulated dramatically (by 60 to 110 fold) at 14 h (Fig. 4, A, B, E and F, respectively). On the other hand, TAPG4 was significantly up-regulated much earlier than these four other genes, showing the first increase already at 2 h after flower removal (Fig. 4C). It should be noted that this sharp increase in gene expression in the AZ is even more significant, taking into consideration that AZ cells represent only a small fraction of the total amount of cells in the sampled tissue. Recently, it was shown that silencing TAPGs delayed abscission and increased break strength of the AZ in tomato explants treated with ethylene (Jiang et al., 2008). The expression of the other Cel genes: Cel2, Cel3, Cel7 and Cel8 were not affected in the AZ following flower removal, and their transcript level was very low, except for Cel3 that had a higher level of expression (Supplemental Fig. S1A). The role of two Cel genes, Cell in floral abscission (Lashbrook et al., 1998) and Cel2 in fruit abscission (Brummell et al., 1999), was already demonstrated previously by means of antisense suppression.

The increase in the expression of all the six cell wall hydrolysis-related genes induced by flower removal was highly specific to the AZ and was almost completely prevented by the 1-MCP pretreatment (Fig. 4). These results are in agreement with the published reports on expression of genes encoding for cell wall-hydrolyzing enzymes associated with abscission and their regulation by ethylene (Lashbrook et al., 1994; Kalaitzis et al., 1997; Roberts and...
A novel cell wall-related gene, \textit{XET-BRI}, was found in our analysis to be up-regulated specifically in the AZ after flower removal (Fig. 4D) in a similar pattern as \textit{TAPG4} (Fig. 4C). This gene encodes for xyloglucan endotransglycosylase that was found before to be regulated by brassinosteroid treatment (Koka et al., 2000). The brassinosteroid receptor BRII1 is a receptor kinase that transduces steroid signals across the plasma membrane and has an extracellular domain containing 25 Leu-rich repeats (LRRs) (Wang et al., 2001). The possible role of this domain in regulating abscission will be discussed later on.

To measure their mRNA expression levels, a semi-quantitative real-time PCR (SQ-PCR) analysis was performed for \textit{TAPG1}, \textit{TAPG2}, \textit{TAPG4}, \textit{Cell} and \textit{XET-BRI} (Fig. 4G) in order to compare it with the results obtained in the microarray analysis. RNA from AZ and NAZ tissues, sampled in experiments independent from those used for the microarray assays, was extracted and used for the SQ-PCR assay. The expression pattern revealed by this analysis (Fig. 4G) was in good agreement with the expression patterns obtained by the microarray experiments (Fig. 4, A to E). Thus, these results confirm that the microarray analysis of gene expression at the AZ reflects true molecular events induced by flower removal, as detailed below. In addition, expression analyses using SQ-PCR were performed for few additional genes including: two ethylene responsive factors (ERFs) - \textit{ERF2} and \textit{ERF1}; four novel AZ-specific genes highly expressed in the AZ at zero time (before flower removal) - \textit{TKN4}, \textit{PHANTASTICA} and \textit{OVATE}; and a gene which encodes for a protein phosphatase that is up-regulated 4 h after flower removal in the AZ without being affected by the 1-MCP pretreatment (Supplemental Fig. S3). In this case, as well, results from the SQ-PCR expression analysis (Supplemental Fig. S3G) confirmed the expression patterns of the same genes from the microarray analysis (Supplemental Fig. S3, A to F). While the pattern of gene expression in the AZ measured by SQ-PCR generally matched nicely the expression measured by the microarray analysis, there was some discrepancy in the expression data in the NAZ obtained by the two methods. For example, the expression of \textit{Cell} and \textit{XET-BRI} genes in the NAZ analyzed by SQ-PCR showed a slight increase at the late time points (Fig. 4G), while the microarray analysis did not indicate such an increase (Fig. 4D and E). This difference is not significant for our analysis and may be ascribed either to biological variations or to the higher sensitivity of the SQ-PCR method.

An additional validation of the microarray analysis was performed using quantitative real time PCR (Q-RT-PCR) for seven other genes: \textit{ERT10}, \textit{ERF4}, \textit{TKN4}, \textit{TAGL2}, \textit{HB-13}, \textit{Homeobox-Leu zipper} and \textit{TPRP-F1}. RNA was extracted from either the AZ or the NAZ tissues originating from two biologically independent experiments, and the measured
expression pattern using Q-RT-PCR was compared to the microarray data analysis. Similar results were obtained by the two different methods for these seven genes (Supplemental Figs. S4 to S6), with highly significant regression coefficients obtained between the Q-RT-PCR and the microarray data (Supplemental Figs. S4, E-F; S5, G to I; S6, E-F), thereby validating the microarray analysis. The Q-RT-PCR analysis was also used to validate the microarray data obtained in the experiments examining the effects of 1-MCP pretreatment or IAA application on expression of the eight following genes in the AZ: **ERT10, ERF4, Homeobox-Leu zipper, Protein phosphatase 2c, Proline transporter, Putative PK, PK7 and Ubiquitin-protein ligase (RGLG2).** The results presented in Supplemental Figs. S17 to S20, and the high significant correlations obtained between the microarray and PCR expression data for these eight genes (Supplemental Figs. S17, E-F; S18, E-F; S19, E-F; S20, E-F), further support the validity of the microarray data.

Taken together, the agreement found among the expression results obtained by the different methods, including SQ-PCR, Q-RT-PCR, the published information for several genes and the microarray analyses with the high reproducibility, confirmed the reliability of this microarray-based results of the tomato AZ gene expression.

Recently, a stamen AZ transcriptome profiling study in Arabidopsis was reported, which followed the stamen abscission global gene expression during flower development (Cai and Lashbrook, 2008). This study coupled laser capture microdissection of Arabidopsis thaliana stamen AZs tissue with GeneChip Microarray profiling, to reveal the stamen AZ transcriptome responding to developmental shedding cues. This study resulted in the classification of the identified differentially expressed genes into eight clusters according to their expression. Among the identified genes, were genes with cell wall modification functions including: **EXPs** (three genes), extensin (**EXT4**), glycosyl hydrolase (three genes), pectin methylesterase (**PME**) (five genes), **PER** (11 genes), **PG** (three genes) and xyloglucan endotransglycosylase/hydrolase (**XTH**) (four genes). Differential expression of genes associated with cell wall metabolism during abscission was demonstrated also during ethylene-induced abscission of citrus leaves (Agusti et al., 2008, 2009). These included genes for two different PGs, cellulase and two different XTHs - **XTH1** and **XTH2**, which were over expressed in leaf AZ-enriched tissue (Agusti et al., 2008). The expression of cell wall-metabolism related genes was also studied using real-time PCR and Affymetrix GeneChip hybridization in soybean leaf AZ taken from explants exposed to ethylene (0, 1 or 2 days). This analysis showed strong up-regulation of **Cel1, Cel6, Cel9, pectate lyase (PL1)**, **PL2, PG9, PG11, EXP3**, **www.plantphysiol.org**on September 1, 2017 - Published by www.plantphysiol.org Copyright © 2010 American Society of Plant Biologists. All rights reserved.
EXP8, XET1 and XET2, and strong down-regulation of EXP1, PG7 and PG16 (Tucker et al., 2007).

Our tomato flower AZ microarray analysis revealed that out of 11 XET and XTH genes analyzed, the expression of only XET-BRI gene increased significantly, and remained high in the AZ after flower removal (Supplemental Fig. S1, B and C). The expression of LeXET-B2 (Supplemental Fig. S1, B) and XTH6 (Supplemental Fig. S1, C) increased transiently 2 and 4 h, respectively, after flower removal, while there was no change in the expression of the other XETs and XTHs in the tomato flower AZ (Supplemental Fig. S1, B and C). Still, the transient increase in LeXET-B2 and XTH6 expression was not specific to the AZ and was not affected by 1-MCP pretreatment (data not shown). Therefore, we assume that these genes are not involved in the process of flower abscission in tomato. During Arabidopsis flower development, expression of XTH12 and XTH28 increased continually in the stamen AZ, while expression of XTH14 and XTH7 decreased during early flower development stages and increased during the progress of stamen abscission (Cai and Lashbrook, 2008). In both tomato (Catalá et al., 1997) and Arabidopsis (Xu et al., 1995), the expression of XET genes was found to be restricted to expanding tissues, up-regulated by auxin and brassinosteroids treatments and down-regulated by ethylene (Catalá et al., 1997; Campbell and Braam, 1999).

Expansin has the unique ability of inducing cell-wall extension without hydrolytic breakdown of the major structural components of the cell wall. It has been demonstrated that transcript abundance and activities of expansin increase in the AZ during the abscission process (Cho and Cosgrove, 2000; Belfield et al., 2005; Tucker et al., 2007). On the other hand, the levels of EXPs transcripts in the stamen AZ were reduced during flower development and progress of stamen abscission (Cai and Lashbrook, 2008). Similarly, we have found that the level of expression of four EXPs was reduced in the tomato AZ 2 h after flower removal and remained low during the abscission progress, without being affected by the 1-MCP pretreatment (Supplemental Fig. S2, A to D). Even for EXP9, which was highly expressed in the AZ compared to the NAZ tissue at zero time (before flower removal), the same pattern of expression was observed (Supplemental Fig. S2D). These results suggest that these four genes of the EXP family are not involved in the execution of the tomato flower abscission process.

**Effect of Flower Removal on Expression of Auxin-Related Genes**

Three genes encoding for proteins homologues to IAA-amino acid conjugate hydrolases (ILRs) family were found to be up-regulated within 2 h after flower removal (Fig. 5). The expression of these ILRI homologue genes continually increased in the AZ, and 1-
MCP pretreatment inhibited this expression (Fig. 5A). On the other hand, the two other expressed sequence tags (ESTs), which are homologous to *ILR3*, had the same peak of expression 4 h after flower removal in both the AZ and the NAZ tissues, and 1-MCP pretreatment even further increased their expression (Fig. 5, B and C). IAA can exist in the cells either as the hormonally active free acid or in a bound form in which the carboxyl group is conjugated either to sugars via ester linkages or to amino acids or peptides via amide linkages (Cohen and Bandurski, 1982; Bartel et al., 2001; Woodgard and Bartel, 2005). IAA conjugates have a role in storage, transport and compartmentalization of IAA. The IAA conjugates have auxin activity when applied exogenously, and have a physiological activity in regulating different developmental processes such as seed germination and root elongation. This activity is mediated by the free IAA which is released following hydrolysis of IAA conjugates (Cohen and Bandurski, 1982; Meir et al., 1984; Bartel et al., 2001; Woodgard and Bartel, 2005). Using mutant screens in Arabidopsis, a family of ILRs was identified and characterized (LeClere et al., 2002; Rampey et al., 2004; Woodgard and Bartel, 2005). ILR1 protein specifically hydrolyses IAA-Leu (Bartel and Fink, 1995), while ILR3 specifically hydrolyses IAA-Ala (Davies et al., 1999). The microarray results suggest that the flower AZ tissue can sense reduction in auxin flow and react by increasing ILRs needed for the release of stored conjugated auxins. 1-MCP pretreatment had no effect on *ILR* initial expression, which was low before flower removal (Fig. 5). The effect of 1-MCP pretreatment on expression of *ILRs* after flower removal suggests that a cross-talk mechanism between ethylene and auxin exists in the AZ. The observation showing that IAA application after flower removal prevented the increase in *ILR1* and *ILR3* expression (Supplemental Fig. S7) supports this suggestion. If indeed the increase in expression of *ILRs* after flower removal results in hydrolysis of IAA-conjugates and release of active IAA, it does not seem to be sufficient to keep the AZ insensitive to ethylene and to prevent abscission. This is supported by the findings showing that exogenous IAA application on the cut end after flower removal is still required for inhibition of abscission (Fig. 2 and Roberts et al., 1984). The observed effect of flower removal on expression of other auxin-related genes supports the conclusion that auxin is decreased in the AZ after flower removal, as will be shown and discussed further on.

It should be noted that in abscising organ systems, a continuous auxin flow through the AZ is required for preventing the increase in ethylene sensitivity and abscission (Taylor and Whitelaw, 2001), which presumably also results in continuous expression of *Aux/IAA* genes. Indeed, results from our microarray analysis show that seven *Aux/IAA* genes were down-regulated following flower removal (Fig. 6, A to G). Thus, expression levels of *IAA1* (Fig. 6A),
IAA3 (Fig. 6B), IAA4 (Fig. 6C), IAA7 (Fig. 6D) and IAA10 (Fig. 6G) decreased sharply within 2 h after flower removal, and remained low. The decrease in expression of these Aux/IAA genes was similar in the AZ and NAZ tissues and was not affected by 1-MCP pretreatment (Fig. 6, A to D and G). This indicates that the decrease in the Aux/IAA gene expression as a result of IAA depletion following flower removal is neither AZ-specific nor affected by ethylene. The only two exceptions from this general pattern were observed for IAA8 (Fig. 6E) and IAA9 (Fig. 6F), the expression of which decreased more gradually within 8 h compared to the sharp and immediate decrease in the NAZ tissue (Fig. 6F). The expression of IAA9 decreased more slowly, as it remained high specifically in the AZ within 2 h after flower removal and only subsequently decreased. On the other hand, pretreatment with 1-MCP resulted in a sharp and immediate decrease of IAA9 expression within 4 h (Fig. 6F). Among the tomato Aux/IAA genes, the IAA3 is an interesting gene in functional terms, as it is thought to represent a molecular link between ethylene and auxin signaling. This hypothesis was suggested by the results showing that down-regulation of IAA3 expression in tomato fruit resulted in both auxin and ethylene-related developmental defects (Chaabouni et al., 2009).

One tomato EST registered in the GenBank as an auxin-regulated protein which is expressed in roots (NCBI, AF416289.1; Zurek DM, Franke P, Rayle DL, 2001), was found in our analysis to be transiently up-regulated 2 h after flower removal in both AZ and NAZ samples (Fig. 6H). This gene is probably an auxin-repressed gene, since its expression was increased very dramatically after flower removal. This protein is probably not related to abscission.

It is now well established that the Aux/IAA proteins are actually repressors of auxin-induced transcription, and auxin promotes the degradation of this large family of transcriptional regulators (TFs), leading to diverse downstream effects (Worley et al., 2000; Gray et al., 2001; Overvoorde et al., 2005). This allows Auxin Responsive Factors (ARF) proteins to bind to the Auxin Responsive Elements (ARE) within the promoters and either activate or repress expression of target genes. Rapid induction of the Aux/IAA genes is a response to the reduced levels of the Aux/IAA proteins, which ensures a tightly controlled transient response to changes in auxin concentrations via a negative feedback (Leyser, 2002; Woodward and Bartel, 2005).

Genetic evidence supporting a role for auxin in regulating Arabidopsis floral organ shedding has been elusive. Recently, functional studies of ARF2, ARF1, ARF7 and ARF19 suggested that these transcriptional regulators act with partial redundancy to promote senescence and floral abscission (Ellis et al., 2005; Okushima et al., 2005a,b). Mutations in
ARF2 alone delayed the onset of floral senescence and organ shedding, which are further inhibited by loss of ARF1 activity, or by the loss of both ARF7 and ARF19 activities (Ellis et al., 2005). Changes in auxin gradients across AZs might promote abscission, and one possibility is that the activities of ARF2, ARF1, ARF7 and ARF19 might be modulated by similar gradients in floral organs (Taylor and Whitelaw, 2001; Ellis et al., 2005). Changes in these activities might also play essential roles in auxin-mediated plant development by regulating both unique and overlapping functions of ARF gene family members in Arabidopsis (Okushima et al., 2005b). Since the expression levels of ARF genes were not affected by flower removal (data not shown), it is suggested that the abscission regulation is mediated via an effect on Aux/IAA expression.

Effect of Flower Removal on Expression of Ethylene-Related Genes

Ethylene Biosynthesis-Related Genes

Eight genes related to different steps of the ethylene biosynthetic pathway were modified for their expression following flower removal (Fig. 7). These genes are involved in: Met biosynthesis – homocystein S-methyltransferase (Fig. 7A), S-adenosylmethionine (SAM) biosynthesis - SAM synthase (Fig. 7B); 1-aminocyclopropane-1-carboxylic acid (ACC) synthesis – four ACC synthase (ACS) genes (Fig. 7, C to F), and ACC oxidation to ethylene - two ACC oxidase (ACO) genes (Fig. 7, G and H). All these genes were transiently up-regulated and peaked on the 2 h time point following flower removal. Thus, these genes are probably involved in the ethylene evolution known to peak in tomato 2 h after flower removal (Roberts et al., 1984). The expression induction of ACS (EST – X58885) (Fig. 7E), ACS6 (Fig. 7F) and ACO5 (Fig. 7G) was completely inhibited by 1-MCP pretreatment, suggesting that they are probably regulated by ethylene production through the autocatalytic action of ethylene. Out of the examined different ACS genes, the expression of one ACS (Accession number = M34289) (Fig. 7D), seemed to be more relevant for the abscission process. This suggestion is based on its expression which was induced during 8 to 14 h after flower removal, was inhibited by 1-MCP pretreatment and was highly AZ-specific (Fig. 7D). This conclusion is in accordance with the widely accepted view that Met and SAM production are not limiting steps in the ethylene biosynthesis pathway, and therefore, probably have no controlling role (Kevin et al., 2002). The late induction of this ACS gene also suggests a second increase in ethylene production, coinciding with the abscission development and execution. To the best of our knowledge, the only report on ethylene production during abscission of tomato flower explants was reported by Roberts et al. (1984). Although not showing directly this expected second
increase in ethylene, these authors observed a consistent reduction of ethylene production by aminoethoxyvinylglycine (AVG) treatment that delayed abscission, but was not as effective as the 1-MCP pretreatment, which completely inhibited abscission (Fig. 2). ACO1 expression sharply increased 2 h following flower removal and then leveled off during the subsequent 4 to 14 h, showing higher expression in the AZ during this period (Fig. 7H). The initial increase in expression of ACO1 was not affected by 1-MCP pretreatment, while 1-MCP partially inhibited the later expression during 4 to 14 h, which still remained high (Fig. 7H). This observation suggests that ACO1 does not serve as a controlling factor of ethylene biosynthesis in the AZ during tomato flower abscission.

**Ethylene Signal Transduction-Related Genes**

Our hypothesis postulates that acquisition of ethylene sensitivity in the AZ is associated with alteration in the expression of auxin-regulated genes. Therefore, we have examined the effect of flower removal leading to auxin depletion, on expression of genes related to ethylene-signal transduction pathway. The microarray results show that out of six genes encoding for the tomato ethylene receptors (Klee, 2002, 2004), the expression of five of them was not affected by flower removal (data not shown). Interestingly, *ethylene resistant 4* (ETR4) expression increased transiently following 2 h and again at 8 to 14 h (Fig. 8A) following flower removal, when abscission has already initiated (Fig. 2). Both the early and the late increases in ETR4 expression were inhibited by 1-MCP pretreatment (Fig. 8A). The late increase of ETR4 expression was AZ-specific (Fig. 8A), implying that ETR4 is directly involved in the late stages of the abscission process. Expression of *constitutive triple response 1* (CTR1) (Fig. 8B) was affected by flower removal and by 1-MCP pretreatment in a very similar pattern to that of ETR4 (Fig. 8A). The similar patterns of ETR4 and CTR1 expression observed following flower removal and in response to 1-MCP pretreatment might be due to the functional link which exists between the two encoded proteins. Both yeast two-hybrid system and *in vitro* biochemical experiments in Arabidopsis indicate that the predicted transmitter domain of ETR1 can interact directly with the regulatory domain of CTR1 (Clark et al., 1998; Gao et al., 2003; Huang et al., 2003; Binder, 2008). The late and AZ-specific increase in expression of both ETR4 and CTR1 (Fig. 8, A and B) suggests that this receptor complex is required for function in the late stages of the abscission process. However, the results presented in Fig. 2 demonstrate that 1-MCP, which was bound irreversibly to the available ethylene receptors before flower removal, prevented abscission for a relatively long period of time following flower removal. This suggests that the acquisition of ethylene sensitivity at the AZ in response
to flower removal cannot be gained via modification of the ethylene receptors. It seems, therefore, that \textit{ETR4} and \textit{CTR1} are involved in the late stages of the abscission process, but do not play a regulatory role in acquisition of sensitivity to ethylene in the AZ.

Another set of genes associated with ethylene signaling are the \textit{ethylene responsive factor (ERF)} which are TF genes. Analysis of the promoters of ethylene-responsive genes revealed a common \textit{cis}-acting ethylene responsive element called the GCC box (Fujimoto et al., 2000). This element was shown to be necessary and sufficient for ethylene regulation in a variety of plant species. The first type of the \textit{trans}-acting factors isolated from tobacco which bind to the GCC box was termed ethylene-responsive element binding proteins (EREBPs) (Ohme-Takagi and Shinshi, 1995). EREBPs play a role in plants’ responses to phytohormones, pathogens attack and environmental stresses (references cited in Hu et al., 2008). Five different ERF proteins were described for Arabidopsis: AtERF1, AtERF2 and AtERF5, which function as activators of GCC box-dependent transcription, and AtERF3 and AtERF4 which act as repressors. The \textit{AtERF} genes were differentially regulated by ethylene and abiotic stress conditions, via the ethylene insensitive 2 (EIN2)-dependent or EIN2-independent pathways (Solano et al., 1998; Fujimoto et al., 2000; Nakano et al., 2006). Over-expression of rice \textit{OsERF1} in Arabidopsis resulted with up-regulation of the expression of two known ER genes, \textit{plant defensin (PDF1.2)} (low-molecular-weight Cys-rich 77) and \textit{b-chitinase} (Hu et al., 2008).

Our microarray analysis revealed five different \textit{ERF} genes, the expression of which was altered following flower removal (Fig. 8C-8G). The homologue gene to \textit{AtERF4} repressor was down-regulated early (2 h) following flower removal, and remained at this low expression throughout the subsequent period at 4 to 14 h (Fig. 8G). This decrease in expression was not affected by 1-MCP pretreatment and seemed to be down-regulated even more in the AZ than in the NAZ tissue at 4 h (Fig. 8G). The other ERFs tended to transiently increase in expression after flower removal: \textit{ERF2} increased early (2 h) and transiently and was not affected by 1-MCP pretreatment (Fig. 8E); \textit{ERF3} increased early (2 h) and late (14 h) and its later increase was affected significantly by 1-MCP pretreatment (Fig. 8F); \textit{ERF1b} increased early (2 h) and transiently and was affected by 1-MCP pretreatment (Fig. 8C); \textit{ERF1c} expression increased early (2 h) and transiently and increased again at 8 to 14 h following flower removal; this increase was highly AZ-specific and was not inhibited by 1-MCP pretreatment (Fig. 8D). Based on its expression pattern, which was highly AZ-specific at 14 h (Fig. 8D), and the prevention of its increased expression by IAA application (Supplemental Fig. S11B), \textit{ERF1c} can be considered as a good candidate for encoding an ERF involved in abscission regulation.
The linkage between ERFs and auxin signaling is further supported by published results obtained in peaches, showing that the active ethylene and auxin signaling cross-talk throughout fruit development and ripening is mediated by ERFs and the Aux/IAA genes (Trainotti et al., 2007). Additionally, LeERF1 was reported to mediate the ethylene signals in tomato, as it was positively related with ethylene triple response, plant development and fruit ripening and softening (Li et al., 2007).

**Other Ethylene-Responsive Genes**

The expression of six more ethylene-responsive genes was modified following flower removal (Fig. 9). ER1 and ER49 are tomato ripening-related genes with yet unclear functions. ER49 was suggested to function as a post-transcriptional regulator (Zegzouti et al., 1999). Our microarray data show that ER1 (Fig. 9A), and ER49 (Fig. 9C) were up-regulated and down-regulated, respectively, after flower removal. This effect on expression was maintained throughout the abscission process, was not affected by 1-MCP pretreatment and the expression was not AZ-specific (Fig. 9, A and C). ER5 is a tomato ripening-associated gene, and its expression increases in mature green and breaker fruit development stages as the fruit becomes sensitive to ethylene, and is also activated by ethylene treatment (Zegzouti et al., 1999). ER5 expression increased early and transiently after flower removal, and this increase in expression was not affected by 1-MCP pretreatment, and was three-fold higher in the AZ tissue compared to the NAZ (Fig. 9B). Another ripening-related gene, ERT10, was up-regulated transiently at 2 h and later at 8 and 14 h after flower removal (Fig. 9D). These early and late increases in expression were inhibited by 1-MCP pretreatment and IAA application (Supplemental Fig. S12D), and the second increase was highly AZ-specific (Fig. 9D), similar to the pattern observed for ACS gene (Fig. 7D). This suggests that it can serve as a good marker for ethylene response that is regulated by IAA levels in the AZ. It is interesting to note that the competence of tomato fruit to ripen and to respond to ethylene while undergoing the transition phases from a green fruit (which does not respond to ethylene) to a mature-green fruit (which is ethylene-responsive), is very similar to the abscission process. Therefore, genes associated with tomato ripening and which are modified upon transition between these two ripening stages, such as ER5 and ERT10, may be significant to the general phenomenon of acquisition of ethylene sensitivity manifested in these two systems.

Chitinases are pathogenesis-related (PR) proteins that had been shown to be transcriptionally regulated by ethylene, and very often their induction is considered as a marker for ethylene activity (Broglie et al., 1989; Díaz et al., 2002; Hall and Bleecker, 2003; Taira et
including in abscission systems (Butenko et al., 2006). Two chitinase genes were up-regulated in the AZ 2 h after flower removal and remained highly expressed during 14 h, while in the NAZ their observed increase of expression was only transient and peaked at 2 h after flower removal (Fig. 9, E and F). The increase in expression of the gene coding for basic endochitinase (Fig. 9E) was inhibited by 1-MCP pretreatment at all time points. On the other hand, for the gene encoding chitinase class II, 1-MCP pretreatment inhibited only the late (8 and 14 h) high expression (Fig. 9F). The late high expression of both chitinase genes was AZ-specific. The results suggest that the early (2 h) increase in the *chitinase* expression which is not AZ-specific, is a wounding response which is transient only in the NAZ.

**Effect of Flower Removal on Expression of Transcription Factor Genes**

The regulation of gene expression at the transcription level has a profound role in the control of many biological processes. TFs are acting as major switches of regulatory cascades during development, and alterations in the expression of such genes may affect various developmental processes (Riechmann et al., 2000). Recently, the developments in identifying and assigning roles to various TFs involved in regulation of organ abscission and dehiscence processes were reviewed (Nath et al., 2007). Also reviewed recently is the association of TFs with the development of abscission, including the development of the leaf, floral and pedicel AZs, the protective layers and the dehiscence zone (DZ) (Van Nocker, 2009). The results of our microarray analysis regarding TFs, thought to be directly involved in ethylene and auxin signal transduction such as *ERF*, *Aux/IAA* and *ARF*, were already presented and discussed (Fig. 6 and Fig. 8).

The results from our tomato flower AZ microarray show that the expression of different TF genes was affected in different ways by flower removal (Figs. 10 and 11). Two genes belonging to the *KNOX* family TFs were sharply down-regulated in the AZ 2 h after flower removal (Fig. 10, A and B). The gene showing homology to *class I knotted-like homeodomain* gene was expressed similarly in the AZ and the NAZ, was down-regulated in a similar rate in both tissues and was not affected by 1-MCP pretreatment (Fig. 10A). On the other hand, *TKN4* was expressed initially and before flower removal three-fold higher in the AZ compared to the NAZ tissue, and 1-MCP pretreatment slowed moderately the rate of its expression reduction in the AZ (Fig. 10B). The AZ cells differ considerably from NAZ cells since they are non-differentiated cells, suggesting that cell growth and differentiation is arrested at an early stage in the AZ (Van Nocker, 2009). It was shown that cells in the shoot apical meristem (SAM) are prevented from differentiation through the activity of the *KNOX* family of TFs. For example,
the closest Arabidopsis KNOX TF (At1g62360; Fig.10A) which is *SHOOTMERISTEMLESS* (*STM*), was shown to be required for SAM formation during embryogenesis (Long et al., 1996). It was speculated by Van Nocker (2009) in his review that the apparent lack of development and differentiation of AZ cells may result from the persistent expression of the KNOX gene in this region. Our results support this speculation, as one KNOX gene, *knotted TKN4*, was preferentially expressed in the AZ, and this expression decreased 2 h after auxin depletion in the AZ from flower removal (Fig. 10B). The fact that IAA application after flower removal prevented the decrease in the expression of both knotted genes (Supplemental Fig. S13, A and B) suggests that these increases resulted from auxin depletion. A previous study showed a connection between the expression of *class I knotted-like* gene, (*STM*) and auxin transport in the SAM (Heisler et al., 2005). It was shown that cycles of auxin build-up and depletion, caused by rapid reversal in the polarity of the auxin efflux carrier PIN1, accompanied and may direct different stages of primordium development (Heisler et al., 2005). On the other hand, the possibility that STM may act upstream to PIN1 to influence its behavior, was also suggested (Heisler et al., 2005).

The expression of three *Homeobox-Leu zipper* TF genes was down-regulated within 2 h after flower removal and remained low later on (Fig. 10, C, D and E). 1-MCP pretreatment delayed the reduction of one of these genes (Fig. 10E), and the reduction of one was more AZ-specific (Fig. 10D). Three TGA-type basic Leu zipper TFs were suggested to be involved in abscission and to regulate the expression of abscission-related genes, as indicated by their binding to bean abscission cellulase promoter (Tucker et al., 2002). The promoter of this cellulase gene includes a *cis* DNA element that can function both in negative or positive regulation of the gene (*ERF*). Based on the observed reduction in their expression following flower removal found in our analysis, it is possible that the three *Homeobox-Leu zipper* TF encoding genes (Fig. 10, C, D and E) act as negative regulators in abscission. This is further supported by the results showing that IAA application prevented the decrease induced by flower removal of the expression of the two *Homeobox-Leu zipper* TF encoding genes (Supplemental Fig. S13, D and E), and restored the expression of *Homeobox-Leu zipper HB-13* between 8-14 h after flower removal (Supplemental Fig. S13C).

Another TF gene that was highly expressed in the AZ before flower removal and was sharply down-regulated in the AZ after flower removal is a *basic helix-loop-helix* (*bHLH*) (Fig. 10F). The closest homologous gene in Arabidopsis (At3g26744; Fig. 10F) is *SCREAM/ICE1*, which was reported to be involved in regulation of freezing tolerance and stomata differentiation in the epidermis (Kanaoka et al., 2008). A myc/bHLH TF *ALCATRAZ* (*ALC*),
expressed in the valve-replum margin of Arabidopsis siliques, was found to have an important role in dehiscence, as indicated by the consequence of its inactivation with a disruption of dehiscence and the separation of valve cells from the replum (Rajani and Sundaresan, 2001).

An additional TF gene, bZIP, had a different pattern of expression, showing initially a transient down-regulation until 4 h after flower removal, followed by a continuously increased expression later on. This expression was AZ-specific and was inhibited by the 1-MCP pretreatment (Fig. 11A).

The expression of a gene encoding for a TF containing APETALA2 (AP2) domain was transiently up-regulated specifically in AZ without any effect of the 1-MCP pretreatment (Fig. 11B). AP2 plays an important role in the control of Arabidopsis flower and seed development, and encodes a putative TF that is distinguished by a novel DNA binding motif referred to as the AP2 domain (Okamuro et al., 1997). It has also been reported that expression of ERF genes, including Arabidopsis thaliana ethylene-responsive element binding protein (AtEBP), was regulated by the activity of AP2, a floral homeotic factor (ERF). Over-expression of AtEBP caused up-regulation of AP2 expression in leaves. AP2 expression was affected by EIN2, but was not regulated by ethylene treatment (Ogawa et al., 2007). Actually ERF2 also contains a conserved AP2 domain. The role of this gene in the regulation of flower abscission remains to be examined.

AGAMOUS-like 12 (TAGL12) and AGAMOUS-like 2 (TAGL2), which are members of the MADS domain family and are known to be expressed during tomato seed and fruit development (Busi et al., 2003), were found in our study to be up-regulated in a highly AZ-specific manner only at a late stage of abscission, at 8 to 14 h after flower removal (Fig. 11, C and D), when the abscission process was already initiated (Fig. 2). The induction of TAGL12 was inhibited by 1-MCP pretreatment (Fig. 11C) or IAA application (Supplemental Fig. S14C), while that of TAGL2 was not affected by 1-MCP (Fig. 11D). TAGL12 pattern of expression matches exactly the pattern of expression found for the cell wall modifying genes: TAPG1, TAPG2, Cell and Cel5 (Fig. 4, A, B, E and F, respectively). In Arabidopsis, a MADS-box TF AGL15 and AGL18 were found to be involved in floral abscission (Fernandez et al., 2000, Adamczyk et al., 2007). Over-expression of AGL15, under the 35S promoter, resulted in delay of abscission of petals and sepals, but did not block the development of functional AZ in the flower or the DZ. On the other hand, the MADS-box TF JOINTLESS has a central role in coordinating gene expression underlying the differentiation of the pedicel AZ in tomato (Mao et al., 2000). STK (SEEDSTICK), that encodes a MADS domain TF known to be required for seed abscission (Pinyopich et al., 2003), is closely related to an AGAMOUS SHATTERPROOF
- *SHP1* and *SHP2* which are required for silique dehiscence (Liljegren et al., 2000, Pinyopich et al., 2003).

The WRKY is a super family of TF proteins with up to 100 representatives in Arabidopsis, which are highly divergent and are categorized into distinct groups possibly reflecting their different functions (Eulgem et al., 2000; Eulgem and Somssich, 2007). WRKY factors hold central positions mediating fast, positive and negative regulation of disease resistance. Two *WRKY* genes were found in our analysis to be up-regulated transiently at 2 h after flower removal, followed by an additional increase at 8 and 14 h (Fig. 11, E and F), which coincides with abscission development (Fig. 2). The first increase of both *WRKY1* and *WRKY lld-1* genes was not AZ-specific and was not affected by the 1-MCP pretreatment. However, the second rise of both genes was inhibited by the 1-MCP pretreatment, while only *WRKY lld-1* expression was AZ-specific (Fig. 11, E and F). This suggests that the first transient increase of these *WRKY* genes is a wounding response, while the second increase, mainly that of *WRKY lld-1*, may be involved in regulation of a pathogen defense response in the separation layer. In accordance with our observation, specific expression of *AtWRKY33*, which shows high homology to *WRKY1* and *AtWRKY6* encoding genes, was described to occur in Arabidopsis at the flower base around the AZs of petals, sepals and stamens (Robatzek and Somssich, 2001; Lippok at al., 2007).

Overall, our results show differential expression of genes coding for TFs belonging to different families, including *ARF, Aux/IAA, KNOX, Homeobox Leu zipper, bHLH, AP2, NAC* (AY498713, Supplemental Table S2.4C), *AGL* and *WRKY* in tomato pedicel AZ, which exhibit different patterns of expression after flower removal. Different members of all these TF families were shown to be expressed in plants AZ or DZ, and were suggested to participate in different sub-processes of abscission or dehiscence, such as the development of the AZ, the execution of AZ separation, and regulation of defense-related processes in the abscission layer. In some of these TF encoding genes the changes in transcript levels are observed early, such as 2 h after auxin depletion due to flower removal. This quick response may indicate their involvement in the early regulatory events associated with the development of ethylene sensitivity in the AZ. The exact roles of these regulatory factors in pedicel abscission responding to auxin depletion due to flower removal remain to be established.

**Effect of Flower Removal on Expression of Some Other Regulatory Genes**

The expression of a gene coding for a Leu-rich repeat trans-membrane receptor-like kinase (*LRR-RLK*) was found to be down-regulated specifically in the AZ 8 and 14 h after flower removal.
removal (Fig. 12A). *HAESA* (*HAE*) and *HAESA-Like 2* (*HLS2*) that serve as receptors for the *inflorescence deficient in abscission* (*IDA/IDL*), were identified as receptor-like kinases (RLKs) (Cho et al., 2008; Stenvik et al., 2008). RLKs are components of signal transduction pathways that elicit cellular responses to extracellular information. In plants, the RLKs have been implicated in prevention of self-pollination, pathogen response, hormone perception and signaling and plant development and defense responses (Becraft, 1998; Lease et al., 1998). Very recently, Leslie et al. (2010) have reported that *EVERSHED* (*EVR*), identified as *LRR-RLK*, can function as an inhibitor of abscission. Defects in the Golgi structure and location of the trans-Golgi network in the *NEVERSHED* mutant, *nev*, in which AZ cells were rescued by a mutation in *EVR*, suggested that *EVR* might regulate membrane trafficking during abscission. NEV, an ADP-ribosylation factor-GTPase-activation protein (ARF-GAP), was suggested to be required for the proper trafficking of cargo molecules, such as cell wall modifying enzymes required for cell separation (Liljegren et al. 2009). The tomato LRR-RLK identified in this work seems to belong to a different LRR-RLK family than those into which the previously abscission-related LRR-RLKs are grouped. Based on the grouping of its closest Arabidopsis ortholog in the classification of Shiu et al. (2004), the identified tomato protein is grouped into the LRR III family, while the HAE and EVR are grouped into the LRR XI and LRR XVI families, respectively.

A Ser/Thr protein kinase (PK) encoding gene, *PK7*, was up-regulated specifically in the AZ at 4 and 14 h after flower removal, and 1-MCP inhibited this increase (Fig. 12B). This pattern of induction follows the progress of the abscission process (Fig. 2) and matches the pattern of expression induction found for the cell wall modifying genes: *TAPG1, TAPG2, Cell* and *Cel5* (Fig. 4, A, B, E and F, respectively). These results suggest the possibility that *PK7* is involved in abscission regulation, similar to our earlier observation regarding the MADS-box protein encoding gene, *TAGL12* (Fig. 11C). Similar to *PK7*, some other kinases were also found to be involved in the abscission process. This is based on the data showing that the expression of a gene (AF332960) coding for the auxin-regulated dual specificity cytosolic kinase was found to be up-regulated in the AZ 2 h after flower removal (Supplemental Table S3.4C). The expression of another gene (BM410830) coding for the PK/peptidoglycan-binding LysM domain-containing protein was also up-regulated in the AZ following flower removal (Supplemental Table S3.4C), as well as that of a Putative PK (Supplemental Fig. S20, C and D). 1-MCP pretreatment or IAA application prevented the late increase (4-14 h) in expression of this gene (Supplemental Fig. S20, C and D).

The expression of the *AGO1* gene, encoding for argonaute-like protein, was rapidly
down-regulated within 2 h after flower removal, and remained low later on. This decrease in
AGO1 expression was not AZ-specific, was not affected by IAA application (Supplemental
Fig. S15E), and was only partially relieved by the 1-MCP pretreatment (Fig. 12C). Argonaute
proteins are the catalytic components of the RNA-induced silencing complex (RISC), the
protein complex involved in the gene silencing phenomenon known as RNA interference
(RNAi). Argonaute proteins bind small interfering RNA (siRNA) fragments and have an
endonuclease activity directed against mRNA strands that are complementary to their bound
siRNA fragment. These proteins are also partially responsible for selection of the guide strand
and destruction of the passenger strand of the siRNA substrate (Hutvagner and Simard, 2008).

The gene encoding for a Pro-rich protein, TPRP-F1, was found to be specifically
expressed in the AZ tissue at a high level before abscission induction, but was dramatically
inhibited after flower removal (Fig. 12D). Pretreatment with 1-MCP reduced to some extent
the TPRP-F1 transcript initial level in the AZ, but did not have any effect on its decrease once
the flower was removed (Fig. 12D). The TPRP-F1 was originally identified as a gene encoding
for a Pro-rich protein preferentially expressed in young tomato fruit (Salts et al., 1991). While
the specific functions of TPRP-F1 and related Pro proteins in plants are not yet clear, studies
focusing on various members of this plant gene family indicate functions related to different
developmental aspects or responses to environmental factors (Goodwin et al., 1996; Holk et al.,
2002; Battaglia et al., 2007). In accordance with our observations, a gene encoding for a Pro-
rich protein was previously identified to be up-regulated specifically in the DZ of Brassica
napus pods during dehiscence (Coupe et al., 1994).

Effects of IAA Application After Flower Removal on Expression of Genes Modified by
Flower Removal

Application of IAA to the cut surface of the remaining tissue after flower removal nullified pedicel abscission during 38 h after flower removal (Fig. 2). Indeed, IAA application
clearly inhibited during the late (8-14 h) time points after flower removal the increased
expression of genes encoding for cell wall modifying enzymes (Fig. 13), which are known to
be induced in the AZ following induction of the abscission process, Thus, IAA
supplementation completely inhibited the expression of TAPG1 (Fig. 13A), TAPG2 (Fig. 13B),
Cell (Fig. 13E), and Cel5 (Fig. 13F) at all time points after flower removal, and prevented the
tremendous increase in their expression induced by flower removal, which is AZ-specific (Fig.
4). This further confirms the role of IAA in preventing organ abscission. Similarly, this
increase in expression of these genes, induced by flower removal, was also prevented by 1-
MCP pretreatment (Fig. 4A, B, E, F). On the other hand, IAA treatment had no effect on the early (2-4 h) increase in TAPG4 expression in the AZ, but it reduced TAPG4 expression during the 8-14 h after flower removal (Fig. 13C). This lack of IAA effect on TAPG4 expression during the early phase after flower removal does not contradict the role of IAA in inhibiting abscission, as TAPG4 induction was detected much earlier than TAPG1 and TAPG2 mRNAs (Kalaitzis et al., 1997) or Cell (Lashbrook et al., 1994) during both leaf and flower abscission in tomato, and as demonstrated in Fig. 4. The increase in XET-BRI induced by flower removal in the AZ was prevented by IAA application at all time points after flower removal (Fig. 13D). To the best of our knowledge, this is the first report showing an AZ-specific increase in XET-BRI expression (Fig. 4D), which is also inhibited by IAA treatment (Fig. 13D). The role of XET-BRI in the abscission process remains to be determined.

As demonstrated for the cell wall modifying enzymes, the effect of IAA application after flower removal on gene expression can help us to clarify which genes are likely to be regulated by IAA (Table II). Genes that were down-regulated following flower removal, with IAA application preventing this reduction, are probably genes positively induced by IAA. Genes that were up-regulated by flower removal, with IAA application preventing this induction, are probably genes that are repressed by IAA. Genes whose expression is modified, either induced or inhibited, by flower removal but IAA application does not affect their expression are probably not regulated by IAA. Therefore, any observed modification in the expression of such genes could stem from events not directly related to the abscission process. Such events may include reduced levels of signals originating from the flower, or events resulting from the wounding effect due to flower removal.

Aux/IAA genes are well known auxin-induced genes (Leyser, 2002; Woodward and Bartel, 2005), and therefore, it is anticipated that IAA application will prevent the reduction in their expression induced by IAA depletion. Indeed, the reduction in the expression of five Aux/IAA genes was prevented by IAA application (Fig. 14, A to E). In our study in Mirabilis jalapa we identified, among the different genes whose expression was associated with the leaf AZ, two genes which showed homology to members of the Aux/IAA gene family (MJ-Aux/IAA1 and MJ-Aux/IAA2). The expression of these two genes was similarly repressed by leaf deblading or stem decapitation, and this repression was prevented by application of IAA to the cut end of the petiole or stump, respectively, which also inhibited abscission (Meir et al., 2003, 2006). These results further confirmed that organ removal results in auxin depletion, manifested in down-regulation of Aux/IAA genes. Further support for this conclusion is provided by our observations showing a correlation between the effectiveness of various auxins
in delaying floret abscission and induction of Aux/IAA gene expression in the floret AZ of *Cestrum elegans* cut flowers (Abebie et al., 2005). Application of the synthetic auxin 2,4-diclorophenoxy acetic acid (2,4-D), which delayed floret abscission in this system (Abebie et al., 2005), induced a higher expression of Aux/IAA genes in the floret AZ as compared with naphthalene acetic acid (NAA), which did not delay floret abscission (Abebie et al., 2008). On the other hand, the transient increase in *Auxin-regulated protein* after flower removal was not affected at all by IAA application and only a small decrease in its expression was obtained between 4-14 h after flower removal (Fig. 14F). This suggests that the *Auxin-regulated protein* may not be regulated by IAA.

The effects of IAA application after flower removal on the changes in expression of various genes associated with the abscission process, some of which were identified in this work, are presented in Supplemental Figs. S7 to S20. This analysis includes genes whose products are involved with cell wall modification, ethylene biosynthesis, signaling and action, auxin signaling and metabolism, TFs and other regulatory factors operating during the abscission process. Some genes, whose products are known to be associated with wounding, were also included. The results obtained following IAA application were summarized in Table II, which clustered these genes into six groups, based on the effect of IAA on their modified expression after flower removal.

**Group 1:** Includes genes whose expression was modified in parallel to the abscission progress and mainly in its late stage at 8-14 h after flower removal. IAA application prevented the otherwise observed change in their expression, as well as 1-MCP pretreatment. The modification was AZ-specific (Fig. 4, A, B, E and F; Fig. 11C; not shown for *RBOH1*), except for *ER49* (Fig. 9C). These results suggest that these genes are probably regulated by IAA, but downstream to the ethylene-induced events leading to abscission (Fig. 15).

**Group 2:** Includes genes whose expression was modified early after flower removal and did not recover to initial basal levels observed at 0 h before flower removal. For some of these genes expression was observed to continue and change later on (8-14 h) in the same direction. All those changes in expression of the genes in this group were prevented by IAA application. Therefore, all these genes can be considered as IAA-regulated genes, either induced or repressed. These genes can be classified into three sub-groups: 2I – Genes whose expression was neither modified specifically in the AZ, nor was affected by 1-MCP pretreatment, such as: *ILR3* (Fig. 5B), *IAA* genes (Fig. 6), *Class 1 knotted-like* (Fig. 10A), *Homeobox-Leu zipper* (BG627748) (Fig. 10D), *Cystein protease inhibitor1* and *Remorin2* (data not shown); 2II – Genes whose expression was modified specifically in the AZ and was
affected by 1-MCP pretreatment, such as: ILR1 (Fig. 5A), XET-BRI (Fig. 4D), PK7 (Fig. 12B), 
Ubiquitin-protein ligase – RGLG2 (Supplemental Fig. S19, C and D), and Cystein-type peptidase - RD19 (data not shown); 2II - Genes whose expression was modified specifically in the AZ but was not affected by 1-MCP pretreatment, such as: Knotted TKN4 (Figs. 10B), Protein phosphatase (Supplemental Fig. S3E), Homeobox-Leu zipper HB-13 (Fig. 10C), that 
were expressed specifically in the AZ at 0 h, and Homeobox-Leu zipper (Fig. 10E). Based on 
this classification, we suggest that sub-group 2I includes genes which are generally IAA-
regulated, while sub-groups 2II and 2III include IAA-regulated genes involved in abscission. 
Among these two abscission-related sub-groups, sub-group 2II includes genes which are also 
ethylene-regulated downstream to IAA, and sub-group 2III includes genes which are 
specifically IAA-regulated in the AZ.

**Group 3:** Includes genes whose expression was transiently up-regulated early after 
flower removal, and was followed by a continuous second increase from 8 to 14 h, with IAA 
application preventing these changes. Therefore, genes in this group can be considered as IAA-
repressed genes, whose expression increased due to IAA depletion following flower removal. 
The second increased expression of three of these genes, ACS6, ERT10 and WRKY lld-1, was 
prevented by the 1-MCP pretreatment (Figs. 7F, 9D and 11F), and was AZ-specific for three of 
them, ERF1c, ERT10 and WRKY lld-1 (Figs. 8D, 9D and 11F). This suggests that all these 
genes are involved in abscission regulation.

**Group 4:** Includes two genes, ETR5 and ETR6, encoding for ethylene receptors, whose 
expression was not affected by flower removal, but IAA application induced their expression 
(Supplemental Fig. S10, B and C). It is suggested that these receptors are not involved in 
abscession regulation, but they may contribute to the IAA effect expressed in reducing the 
sensitivity of the AZ to ethylene. It is widely accepted that the level of the ethylene receptor 
proteins is negatively correlated with sensitivity to ethylene (Binder, 2008; Kevany et al. 
2007).

**Group 5:** Includes genes whose expression was modified early during the 2-4 h period 
after flower removal, either transiently or continuously. IAA application did not affect this 
early gene modification, but affected the expression at the later period of 8-14 h after flower 
removal. Therefore, it is suggested that the modification in expression of these genes does not 
result from IAA depletion but rather from the wounding and/or other signals omitted due to 
flower removal. Indeed, this group includes genes whose products are involved in the 
regulation of the systemic signaling during wound response such as lipoxygenase and JA2 
(León et al. 2002, Howe and Schilmiller 2002, Schilmiller and Howe 2005, Wasternack et al.
2006), and genes associated with ethylene biosynthesis and signaling involved in wound ethylene responses (Saltveit and Dilley 1978, Boller and Kende 1980, Dourtoglou et al. 2000, Wasternack et al. 2006). The pattern of ethylene evolution in tomato AZ explants was characterized by a sharp peak at the AZ 2 h following flower removal, which then decreased to the basal level within the subsequent hour (Roberts et al., 1984). This initial burst of ethylene evolution induced by flower removal probably exhibits a typical wounding response. Our microarray results support the occurrence of such a wounding response, since numerous wound-related genes, such as lipoxygenase (LOX), wound-induced proteinase inhibitor I, jasmonic acid 2, protease inhibitor II, osmotin-like protein and wound-inducible carboxypeptidase, were up-regulated in our tomato system within 2 h after flower removal (cluster groups 1 and 4 in Supplemental Table S3 and Table S4; Supplemental Fig. S16).

The effects of IAA on the expression of some genes modified at the late phase of 8-14 h after flower removal, may operate via affecting ethylene sensitivity of the AZ. This possible explanation is supported by the findings showing that 1-MCP pretreatment also inhibited or prevented the modified expression for some of the genes classified in this group, such as: TAPG4 (Fig. 4C), Auxin-regulated protein (Fig. 6H), SAM synthase (Fig. 7B), and ACS (Fig. 7D). The observation showing that IAA application after flower removal did not affect the increase in Chitinase genes expression after 2 h in the AZ (Supplemental Fig. S12, E and F), strongly suggests that alteration in the expression of chitinases may not result from IAA depletion due to flower removal. On the other hand, the late (8 to 14 h) high gene expression of Chitinase which is AZ-specific (Fig. 9, E and F), indicates possible participation of these chitinases in the defense against microorganism occurring in the defense layer formed after tissue separation, which is an ethylene-dependent process and was inhibited by IAA treatment (Supplemental Fig. S12, E and F).

**Group 6:** Includes genes whose expression was modified early during 2-4 h after flower removal, either transiently or continuously, but IAA application did not affect their expression at any time. It is suggested that the modification in expression of these genes after flower removal does not result from IAA depletion, but rather from wounding and/or other signals omitted due to flower removal. Indeed, most of the genes classified in this group are associated with ethylene biosynthesis, signaling and response. Also the AP2 domain-containing TF that belongs to the genes included in Group 6, can be associated with the wounding response (Okamuro et al., 1997).
CONCLUSIONS

The aim of this research was to further explore the molecular changes occurring during acquisition of abscission competence in the AZ following auxin depletion, by using the Affymetrix Tomato GeneChip. Application of IAA after flower removal, that prevented the abscission process, enabled us to differentiate between genes whose expression was affected by IAA due to flower removal, which are the interest of this research, from genes whose expression was modified by flower removal and were not affected by re-supplement of IAA. Based on the kinetics of pedicel abscission, the identity and kinetics of expression of the genes affected by flower removal, the effects of IAA application and of 1-MCP pretreatment, we can separate the sequence of events which occur during tomato flower abscission into two phases: Early events (0 to 4 h after flower removal) that probably lead to acquisition of ethylene sensitivity and abscission competence, and late events (8 to 14 h after flower removal) when processes leading to the execution of pedicel abscission and development of the defense layer occur (Fig. 15). The late events, which are ethylene-induced, are inhibited by 1-MCP pretreatment, while the early events are not necessarily so. On the other hand, IAA application immediately after flower removal inhibited all the cascade of abscission events.

The sequence of molecular events occurring after flower removal is summarized in Fig. 15. Genes showing early modified expression might be involved in mediating auxin regulation of ethylene sensitivity in the AZ. These include three sets of genes (1, 2 and 3 in Fig. 15): Set 1 includes genes that are directly regulated by auxin and are therefore down-regulated early-on after IAA depletion, such as: *Aux/IAA* genes such as *IAA1,3,4,7,8* and some of the TFs whose expression is early down-regulated after flower removal, such as the *knotted*, *Homeobox-Leu zipper* genes and *bHLH*. Set 2 includes genes that are directly IAA-repressed which were up-regulated early-on after IAA depletion, such as *PK7*, *ERF1c*, *WRKY lld-1*, *Protein phosphatase*. Set 3 includes other TF and/or post transcriptional regulators, which are probably regulated by the modified IAA-related genes as their expression is modified at a relatively late stage of the process (Groups 1 and 2 in Table II), such as *LRR-RLK*, *PK7*, *TPRP-F1*, *Phantastica*, and *Ovate*.

As the AZ becomes sensitive to ethylene, the basic level of ethylene production together with its autocatalytic increase is mediated specifically in the AZ by specific expression of ethylene biosynthesis-related genes (e.g. *ACS - M34289*, Fig. 7D). This induction of ethylene levels leads in turn to activation of AZ-specific genes involved in the late stage of abscission and its excution after 4 h (Set 4 in Fig. 15; Groups 1-4 in Table II). The genes included in Set 4 can be classified into three sub-groups, based on their putative functions: I –
TF genes or genes belonging to ethylene signal transduction or abscission regulators such as: *ETR4, CTR1, ERF1c, TAGL12, LRR-RLK,* and *PK7*; **Genes encoding cell wall modifying proteins;** **Genes involved in the PR defense and development of the defense layer such as WRKY TFs, ERT10, Chitinase, and Peroxidases.**

The analysis of the microarray results for the flower AZ allowed us to establish a clear sequence of events occurring during acquisition of tissue sensitivity to ethylene, and to confirm our hypothesis that acquisition of ethylene sensitivity in the AZ is associated with altered expression of auxin-regulated genes. These results shed light on the mechanism of increased sensitivity of the AZ to ethylene, and further expanded our knowledge of auxin-ethylene cross talk during the abscission process.

The present study has established a powerful platform for further analysis of possible regulatory abscission-related genes involved in acquisition of ethylene sensitivity at the AZ. Based on this study, microarray experiments, aimed to examine the effects of IAA and 1-MCP on gene expression in the leaf AZ after leaf deblading, are in progress for comparing the two types the AZs. In parallel, we have initiated functional analysis of selected candidate genes and some function in abscission was strongly suggested, based on the phenotypic consequences of modifying their expression. These genes are currently specifically inhibited using RNAi in stably transformed tomato plants, as regulated by an abscission-specific promoter. This functional analysis will enable us to further reveal the role of key regulators in the early events of the abscission process.

**MATERIALS AND METHODS**

**Plant Material and Treatments**

Flower bunches of cherry tomato (*Solanum lycopersicum* Mill, cv. 'Shiran' 1335, Hazera Genetics Ltd., Israel) were harvested between 10 to 12 AM from a commercial greenhouse in Israel. Bunches containing at least 2 to 4 fresh open flowers, were brought to the laboratory under high humidity conditions. Senesced flowers and young flower buds (unopened) were removed and the stem ends were trimmed. Groups of 3 to 4 bunch explants (Fig. 1, A and B) were placed in a vial containing 10 mL of organic chlorine (50 µL L⁻¹ TOG-6, Milchan Bros, Ltd., Israel) in water to prevent microorganism development. The bunch explants in vials were kept in a covered box containing a moistened paper to maintain high humidity, and were divided into two groups: one was incubated at 20°C (control), and the
second group was exposed to the ethylene action inhibitor, 1-MCP (0.4 µl L⁻¹) in a sealed 200-L chamber at 20°C for 12 h, before flower removal. IAA (10⁻³ M) was applied in lanolin paste immediately after flower removal to the cut surface of the remaining tissues.

Flowers were removed with a sharp razor blade (Fig. 1C, b), and pedicel abscission was monitored in control, 1-MCP-pretreated and IAA-treated explants at various time intervals after flower removal up to 60 h. Groups of 15 vials (containing about 50 explants with ~120 flowers) were used for each treatment.

Tissue samples for RNA extraction were taken from the AZ (100 segments of less than 1 mm thickness for each time point, excised less than 0.5 mm from each side of the visible AZ), and from the NAZ (20 segments of 3 mm for each time point) (Fig. 1C). The AZ tissue was sampled at five time points (0, 2, 4, 8 and 14 h), and the NAZ tissue was sampled at four time points (0, 2, 4 and 14 h) following flower removal (Fig. 1D). Since no major abscission-related changes were expected to occur in the NAZ with or without 1-MCP, less time points were sampled from these tissues. Samples for time zero were taken from entire explants without flower removal (Fig. 1C, a). Tissues were sampled from control, 1-MCP-pretreated and IAA-treated explants. All samples were frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

**RNA Extraction**

RNA was extracted from tissue segments collected as described above. The tissue was grounded in liquid nitrogen and extracted in extraction buffer (100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 2 M NaCl, 2% [w/v] cetyl trimethyl ammonium bromide (CTAB), 2% [w/v] polyvinyl pirolidone (PVP), 2% [v/v] ß-mercaptoethanol). Following 10 min incubation at 65°C, the mixture was extracted twice with chloroform: isoamylalcohol (24:1, v/v). Total RNA was precipitated with 2.5 M LiCl for 12 h at 4°C and following re-suspension in water the RNA was treated with RQ1 RNase-free DNase (PROMEGA, Madison, WI, USA) for removal of any residual DNA. The RNA was further purified using RNeasy Plant mini kit (QIAGEN), resulting with a pure and high quality RNA preparation based on spectroscopic and gel electrophoresis analyses.

For SQ-PCR and Q-RT-PCR experiments total RNA was isolated from AZ and NAZ tissues of flowers sampled at the same time intervals as for the microarray experiments. The RNA was extracted using Spectrum Plant total RNA kit (SIGMA-ALDRICH, St Louis, USA) and cleaned from DNA using RQ1RNase-Free DNase (PROMEGA, Madison, WI, USA).
cDNA was synthesized using the Reverse Transcription System (PROMEGA, Madison, WI, USA) using 2 µg of total RNA from each sample.

**Microarray Analysis**

The microarray analysis was employed to measure global gene expression in the AZ and NAZ tissues of tomato flower pedicel, sampled at various time intervals after flower removal (Fig. 1D). The samples were taken from control explants, from explants pretreated with 1-MCP before flower removal, to block any direct effects of ethylene, and from explants treated with IAA immediately after flower removal. The use of 1-MCP should allow us to distinguish between IAA-related genes that affect ethylene sensitivity of the AZ, and ethylene-related genes that induce the abscission process. IAA application after flower removal should allow us to distinguish between changes in gene expression resulting from auxin depletion or from wounding or other non-related signals.

For microarray analysis we have used the Affymetrix GeneChip® Tomato Genome Array which is designed specifically to monitor gene expression in tomato. The comprehensive array consists of over 10,000 tomato probe sets to interrogate over 9,200 transcripts. We have used RNA extracted from biological duplicates of two independent experiments performed in a 3-week interval for the 1-MCP pretreatment and three for the other treatments. All procedures for probe preparation, hybridization, washing, staining and scanning of the GeneChip® Tomato arrays, as well as data collection were performed at the Microarray Core Facility, Department of Biological Services, The Weizmann Institute of Science, Rehovot, Israel. We have used the Affymetrix GeneChip Exp 3′ One-Cycle kit according to the relevant Affymetrix GeneChip® Expression Analysis Technical Manual (No. 701021 Rev. 5) and Data Analysis Fundamentals booklet (P/N 701190) manual. cDNA was prepared using the two-cycle target labeling procedure and was used for further synthesis of biotin-labeled target cRNA by \textit{in vitro} transcription as described in the Affymetrix GeneChip® Manual. The cRNA was fragmented before hybridization and hybridized to the probe array for 16 h at 45°C. Specific experimental information was defined using Affymetrix® GeneChip Operating Software (GCOS) on a PC-compatible workstation. Immediately following hybridization, the probe array went an automated washing and staining protocol on the fluidics station using the fluorescent molecule streptavidin-phycoerythrin that binds to biotin, and for signal amplification anti-streptavidin and biotinylated goat IgG antibodies. Probe Array scan was also controlled by the GCOS software to define the probe cells and to compute intensity for each cell. The Data image was analyzed for probe intensities as described in the Data Analysis booklet.
Initially, probe signal summarization, normalization, and background subtraction were performed using robust multichip analysis (RMA; Irizarry et al., 2003) in the ‘affy’ package with default parameters. The statistical test for differentially expressed genes was performed using the linear models for microarray (LIMMA) package (Smyth, 2004), which allows a better variance estimation by calculating the moderated t-statistic using empirical Bayesian techniques. These moderated t-statistics were calculated separately for each of the following comparisons. Four time points of IAA-treated samples (2, 4, 8, 14 h) against non-treated sample (AZ before treatment). Four time points of 1-MCP-pretreated samples (2, 4, 8, 14 h) against non-treated samples (1-MCP samples at 0 h). To control the level of false discoveries that result due to multiple comparisons, the approach of Benjamini and Hochberg (1995) was applied, to generate adjusted P-values (q-values).

We defined the following criteria for significantly differentially expressed genes: 1) genes that are statistically significant at the level of p < 0.05 after FDR correction; 2) genes showing a two-fold change in the expression level between treatment and control; 3) Present call (defined by GCOS-software output) and a signal level > 20 at least in one of the experiments. These criteria were applied to each comparison, and groups of gene sets were created by selecting for significantly differentially expressed genes in combinations of comparisons. The combinations are described in details in the Results and Discussion section in the paragraph entitled: “Microarray Analysis and Clustering of Differentially Expressed Genes After Flower Removal”.

In order to enable a gene ontology analysis, we had to assign each tomato gene with its nearest homologous gene from Arabidopsis, as the FatiGo Gene Ontology analysis tools (http://fatigo.bioinfo.cipf.es/) we have used are available only for Arabidopsis. This was done by using the BLAST tool in order to match the tomato transcripts to the best Arabidopsis homologues. The criterion for finding the best Arabidopsis homologue was chosen as genes with less than E value of 1e⁻⁵.

Validation of Microarray Analysis of the Tomato Flower AZ by SQ-PCR

Validation of the microarray expression results was performed for few genes which exhibited abscission-specific type of expression in the tomato flower AZ. Beside validation of the microarray results for newly discovered abscission-specific genes, we have followed the expression of genes encoding for cell wall hydrolases known to be associated with abscission. Expression levels of the following genes were monitored using SQ-PCR: Novel AZ-specific genes - Phantastica, TAGL12 (MADS-box), Knotted protein - TKN4, Ovate protein; Ethylene
RNA was extracted from the flower AZ and NAZ tissue samples at different time intervals (0, 2, 4, 8, 14 h) after flower removal, which induced pedicel abscission. The expression validation experiments were repeated twice in two different experiments that were not used for the microarray analysis. The Expression results obtained in the SQ-PCR analysis were normalized against the expression of the \(\beta\)-Tubulin2 gene at the different time intervals. The gene specific primers were designed using IDT Primerquest tools (http://www.idtdna.com/Scitools/Applications/Primerquest/) and their sequences, annealing temperatures and product sizes are presented in Supplemental Table S1. The number of PCR cycles was optimized. The optimal number of cycles for expression analysis of genes was determined when the amplification level was in the lag phase (Supplemental Fig. S21), to allow easy comparison of the resulting PCR products. The PCR product was run on 0.8% agarose gel in 0.5% TAE buffer for 25 min at 110 V. The total time varied according to the size of the PCR product and recorded with the Image Master VDS 1208 system. The relevant results are shown in Fig. 4G and a similar PCR cycle calibration was done for the data presented in Supplemental Fig. S3G.

We have used for PCR the AmpliQon Taq DNA Polymerase Master Mix (2.0 Master Mix Kit -1.5 mM MgCl\(_2\)). The PCR products were analyzed in 0.8% agarose gel run in X 0.5 Tris, Acetate, EDTA (TAE) buffer for 25 min at 110V. The total running time varied according to the length of the PCR products, which were documented using an Image Master VDS system.

**Validation of Microarray Analysis of the Tomato Flower AZ by Quantitative Real-Time-PCR (Q-RT-PCR)**

Flower samples for AZ and NAZ tissues were collected as described above. The Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). The RNA was treated with RQ1 RNase-Free DNase I (Promega) according to the manufacturer’s instructions. A sample of 1 \(\mu\)g of RNA was reverse transcribed with Moloney Murine Leukemia Virus Reverse Transcriptase kit (Promega). The Q-RT-PCR was performed with Corbett Rotor-Gene 3000 (Corbett Life Research, Australia) using SYBR Green Master mix detection chemistry (AB gene company/ Amplicon company) and gene specific primers. The primers were designed using the Primer Express software 1.0 and Primerquest tools (http://www.idtdna.com/Scitools/Applications/Primerquest/), and are listed in Supplemental Table S2. The thermal profile for SYBR Green Real-Time PCR was 95°C for 15 min, followed by 40 cycles
of 95°C for 15 s, 65°C for 15 s, and 72°C for 20 s. To generate the standard curves, cDNA isolated from tomato flower AZ, NAZ and other treated tissues were mixed, serially diluted by a factor of 10, and aliquots of the dilutions were used in standard Real-Time PCRs. Each value determination was repeated three times to ensure the slope of the standard curves and to determine the SD. The $\beta$-tubulin2 (609267), SL-Actin (U60481/Q96483) and GAPDH (U97257) genes were used as internal controls, and relative expression levels of these genes were computed by the $2^{-\Delta\Delta Ct}$ method of the relative quantification (Livak and Schmittgen, 2001). All experiments were carried with Non template control and Negative control (RNA sample), were repeated at least three times and yielded similar results. Linear regression analyses between the microarray and the Q-RT-PCR expression data were performed, using the statistical program SigmaStat (Jandel Scientific).

SUPPLEMENTAL DATA
The following materials are available in the online version of this article:

Supplemental Figure S1. Kinetics of changes in array-measured expression of genes encoding cell wall-related enzymes following flower removal.

Supplemental Figure S2. Effects of flower removal, 1-MCP pretreatment, and tissue type on the kinetics of changes in array-measured expression levels of cell wall-related genes encoding enzymes belonging to the Expansin family (A-D) and Peroxidases (E, F).

Supplemental Figure S3. Effects of flower removal, 1-MCP pretreatment, and tissue type on the kinetics of changes in array-measured (A-F) and SQ-PCR-validated (G) expression levels of genes encoding some TFs and a protein phosphatase.

Supplemental Figure S4. Effects of flower removal and tissue type on the kinetics of changes in array-measured (A, C) and Q-RT-PCR-validated (B, D) expression levels of ethylene-related genes, ERT10 (A, B) and ERF4 (C, D), and the linear regressions with the appropriate coefficients obtained between the microarray and the Q-RT-PCR expression data for these genes (E, F).

Supplemental Figure S5. Effects of flower removal and tissue type on the kinetics of changes in array-measured (A, C, E) and Q-RT-PCR-validated (B, D, F) expression levels of TF genes, TKN4 (A, B), TAGL12 (C, D), and Homeobox Leu-zipper HB-13 (E, F), and the linear regressions with the appropriate coefficients obtained between the microarray and the Q-RT-PCR expression data for these genes (G, H, I).

Supplemental Figure S6. Effects of flower removal, and tissue type on the kinetics of changes
in array-measured (A, C) and Q-RT-PCR-validated (B, D) expression levels of other TF and regulatory genes, Homeobox-Leu zipper (A, B) and TPRP-F1 (C, D), and the linear regressions with the appropriate coefficients obtained between the microarray and the Q-RT-PCR expression data for these genes (E, F).

**Supplemental Figure S7.** Effects of flower removal and IAA application after flower removal on the kinetics of changes in array-measured expression levels of IAA-amino acid conjugate hydrolases genes, *ILR1* (A) and *ILR3* (B) in the AZ.

**Supplemental Figure S8.** Effects of flower removal and IAA application after flower removal on the kinetics of changes in array-measured expression levels of cell wall-related genes encoding enzymes belonging to the Expansin family (A-D) and Peroxidases (E, F) in the AZ.

**Supplemental Figure S9.** Effects of flower removal and IAA application after flower removal on the kinetics of changes in array-measured expression levels of ethylene biosynthesis-related genes in the AZ.

**Supplemental Figure S10.** Effects of flower removal and IAA application after flower removal on the kinetics of changes in array-measured expression levels of the ethylene receptor homologues - ethylene resistant *ETR4* (A), *ETR5* (B), *ETR6* (C) and constitutive triple response 1 (*CTR1*) (D).

**Supplemental Figure S11.** Effects of flower removal and IAA application after flower removal on the kinetics of changes in array-measured expression levels of ethylene responsive factor (ERF) genes.

**Supplemental Figure S12.** Effects of flower removal and IAA application on the kinetics of changes in array-measured expression levels of ethylene responsive (ER) genes in the AZ.

**Supplemental Figure S13.** Effects of flower removal and IAA application after flower removal on the kinetics of changes in array-measured expression levels of early down-regulated TF genes in the AZ.

**Supplemental Figure S14.** Effects of flower removal and IAA application after flower removal on the kinetics of changes in array-measured expression levels of TF genes in the AZ.

**Supplemental Figure S15.** Effects of flower removal and IAA application after flower removal on the kinetics of changes in array-measured expression levels of *WRKY lld-1* (A), *Phantastica* (B), *Protein phosphatase* (C), *Ovate* (D), *AGO1* (E) and *NADPH oxidase – RBOH1* (F) genes in the AZ.
**Supplemental Figure S16.** Effects of flower removal and IAA application after flower removal on the kinetics of changes in array-measured expression levels of *Lipoxygenase - LOXD* (A), *Jasmonic acid 2 – JA2* (B), *Remorin2* (C), *Cysteine protease inhibitor 1 precursor* (D), *Cysteine protease* (E) and *Cystein-type peptidase – RD19* (F) genes in the AZ.

**Supplemental Figure S17.** Effects of flower removal (control), 1-MCP pretreatment or IAA application after flower removal on the kinetics of changes in array-measured (A, C) and Q-RT-PCR-validated (B, D) expression levels of ethylene-related genes, *ERT10* (A, B) and *ERF4* (C, D) in the AZ, and the linear regressions with the appropriate coefficients obtained between the microarray and the Q-RT-PCR expression data for these genes (E, F).

**Supplemental Figure S18.** Effects of flower removal (control), 1-MCP pretreatment or IAA application after flower removal on kinetics of changes in array-measured (A, C) and Q-RT-PCR-validated (B, D) expression levels of *Homeobox-Leu zipper* (A, B) and *Protein phosphatase 2c* (C, D) genes in the AZ, and the linear regressions with the appropriate coefficients obtained between the microarray and the Q-RT-PCR expression data for these genes (E, F).

**Supplemental Figure S19.** Effects of flower removal (control), 1-MCP pretreatment or IAA application after flower removal on the kinetics of changes in array-measured (A, C) and Q-RT-PCR-validated (B, D) expression levels of *Proline transporter* (A, B) and *Ubiquitin-protein ligase – RGLG2* (C, D) genes in the AZ, and the linear regressions with the appropriate coefficients obtained between the microarray and the Q-RT-PCR expression data for these genes (E, F).

**Supplemental Figure S20.** Effects of flower removal (control), 1-MCP pretreatment or IAA application after flower removal on the kinetics of changes in array-measured (A, B) and Q-RT-PCR-validated (B, D) expression levels of *PK7* (A, B) and *Putative PK* (C, D) genes in the AZ, and the linear regressions with the appropriate coefficients obtained between the microarray and the Q-RT-PCR expression data for these genes (E, F).

**Supplemental Figure S21.** SQ-PCR cycle calibration showing the amplified expression levels of various genes, isolated from the flower AZ 8 h after flower removal.

**Supplemental Table S1.** The primer sequences, annealing temperatures and product sizes for each gene used for the SQ-PCR analyses presented in Fig. 4 and Supplemental Fig. S3.

**Supplemental Table S2.** The primer sequences, annealing temperatures and product sizes for each gene used for the Q-RT-PCR analyses presented in Supplemental Figures S4 to S6.
and S17 to S20.

**Supplemental Table S3.** Fold-changes of the total number of genes which were affected by flower removal in each cluster presented in Fig. 3 and listed in Table I.

**Supplemental Table S4.** Fold-changes of the total number of genes which were affected by 1-MCP pretreatment and flower removal in each cluster presented in Fig. 3 and listed in Table I.

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Table I. *The total number of genes affected in the AZ by flower removal and/or 1-MCP pretreatment in each cluster presented in Fig. 3*

The symbols of the cluster types are detailed in the legend of Fig. 3.

| Cluster Group | Cluster type | Number of genes affected | Flower removal | 1-MCP-pretreated and flower removal | Overlapping between the two treatments |
|---------------|--------------|--------------------------|----------------|-----------------------------------|---------------------------------------|
|               | 2 4 8 14 h   | Total                    |                |                                   |                                       |
| 1             | 1A + 0 0 0   | 145                      | 45             | 82                                | 18                                    |
|               | 1B - 0 0 0   | 202                      | 82             | 93                                | 27                                    |
|               | 1C 0 + 0 0   | 35                       | 22             | 11                                | 2                                     |
|               | 1D 0 - 0 0   | 56                       | 23             | 30                                | 3                                     |
|               | 1E + + 0 0   | 34                       | 10             | 18                                | 6                                     |
|               | 1F - - 0 0   | 63                       | 17             | 42                                | 3                                     |
| 2             | 2A 0 0 -1 -2 | 105                      | 63             | 31                                | 11                                    |
|               | 2B 0 0 - -   | 130                      | 76             | 36                                | 18                                    |
|               | 2C 0 0 1 2   | 57                       | 44             | 10                                | 3                                     |
|               | 2D 0 0 + +   | 68                       | 49             | 13                                | 6                                     |
| 3             | 3A 0 1 2 3   | 37                       | 17             | 12                                | 8                                     |
|               | 3B 0 + + +   | 52                       | 29             | 20                                | 13                                    |
|               | 3C 0 -1 -2 -3| 50                       | 25             | 20                                | 5                                     |
|               | 3D 0 - - -   | 101                      | 40             | 45                                | 16                                    |
| 4             | 4A 1 2 3 4   | 40                       | 22             | 12                                | 6                                     |
|               | 4B -1 -2 -3 -4| 38                     | 12             | 20                                | 6                                     |
|               | 4C + + + +   | 188                      | 87             | 51                                | 50                                    |
|               | 4D - - - -   | 207                      | 86             | 67                                | 54                                    |
| 5             | 5A 0 0 + 0   | 26                       | 13             | 13                                | 0                                     |
|               | 5B 0 0 - 0   | 98                       | 42             | 45                                | 11                                    |
|               | 5C 0 + + 0   | 12                       | 6              | 6                                 | 0                                     |
|               | 5D 0 - - 0   | 45                       | 9              | 22                                | 14                                    |
**Table II.** Summary of the effects of IAA application after flower removal on genes modified at the early (2-4 h) or late (8-14 h) phases after flower removal

The table shows how IAA application affected the modifications in gene expression induced by flower removal. The sign (-) indicates no effect. The genes listed in Group 2 were classified into three sub-groups, I, II and III.

| Gene name                              | Changes in gene expression in response to Flower removal | Changes in gene expression in response to Flower removal + IAA | Fig. No. |
|----------------------------------------|--------------------------------------------------------|-------------------------------------------------------------|----------|
|                                        | Flower removal                                         | Flower removal + IAA                                         |          |
|                                        | Early at 2-4 h                                         | Late at 8-14 h                                             |          |
|                                        | Late at 8-14 h                                         | Early at 2-4 h                                             |          |
|                                        | Late at 8-14 h                                         |                                                             |          |
| **Group 1**                            |                                                        |                                                             |          |
| TAPG1                                  | - increase                                             | - prevention                                               | 13A      |
| TAPG2                                  | - increase                                             | - prevention                                               | 13B      |
| Cel1                                   | - increase                                             | - prevention                                               | 13E      |
| Cel5                                   | - increase                                             | - prevention                                               | 13F      |
| TAGL12                                 | - increase                                             | - prevention                                               | S14C     |
| NADPH oxidase – RBOH1                  | - increase                                             | - prevention                                               | S15F     |
| ER49                                   | - decrease                                             | - increase                                                 | S12C     |
| **Group 2**                            |                                                        |                                                             |          |
| I ILR3                                 | transient increase                                     | decrease but higher than 0 h                               | lower transient increase | S7B      |
| I IAA1                                 | decrease                                               | decrease                                                   | prevention | 14A      |
| I IAA4                                 | decrease                                               | decrease                                                   | inhibition | increase to original | 14C      |
| I IAA7                                 | decrease                                               | -                                                         | inhibition | increase to original | 14D      |
| I IAA8                                 | decrease                                               | decrease                                                   | increase   | remain high | 14B      |
| I IAA10                                | decrease                                               | -                                                         | prevention | increase | 14E      |
| I Class I knotted-like homeodomain     | decrease                                               | -                                                         | prevention | - | S13A      |
| I Homeobox-Leu zipper                  | decrease                                               | -                                                         | prevention | - | S13D      |
| (BG627748) | Decrease | Decrease | Prevention | Increase | S16C |
|------------|----------|----------|------------|----------|------|
| I Remorin2 |           |          |            |          |      |
| I Cystein protease inhibitor1 precursor | Increase | Increase | Prevention | Prevention | S16D |
| II XET-BRI | Increase | -        | Prevention | -        | 13D  |
| II ILR1    | Increase | Increase | Prevention | Prevention | S7A  |
| II PK7     | Increase | Increase | Prevention | Prevention | S20A |
| II RGLG2   | Increase | Increase | Prevention | Prevention | S19C |
| II Cystein-type peptidase - RD19 | Increase | Increase | Prevention | Prevention | S16F |
| III Protein phosphatase | Increase | -        | Prevention | -        | S15C |
| III Knotted TKN4 | Decrease | -        | Prevention | -        | S13B |
| III Homebox-Leu zipper (CK715706) | Decrease | -        | Prevention | Increase | S13E S18A |

**Group 3**

| ACS6 | Transient Increase | Increase | Prevention | Prevention | S9F |
|------|-------------------|----------|------------|------------|-----|
| ERF1c | Transient Increase | Increase | Prevention | Prevention | S11B |
| ERT10 | Transient Increase | Increase | Prevention | Prevention | S12D S17A |
| WRKY lld-1 | Transient Increase | Increase | Prevention | Lower Increase | S15A |

**Group 4**

| ETR5 | - | - | - | Increase | S10B |
|------|---|---|---|----------|------|
| ETR6 | - | - | Increase | Increase | S10C |

**Group 5**

| TAPG4 | Increase | Increase | - | Prevention / Decrease | 13C |
|-------|----------|----------|---|-----------------------|-----|
| Auxin-regulated protein | Transient Increase | - | - | Lower Transient Increase | 14F |
| Gene/Protein                  | Regulation | Time Course | Transient | Comparison | Quantity |
|------------------------------|------------|-------------|-----------|------------|----------|
| SAM synthase                | transient  | -           | higher    | transient  | S9B      |
| ACS (M34289)                | increase   | -           | prevention| S9D        |
| ACO5                        | increase   | -           | prevention| S9G        |
| ERF1b                       | decrease   | similar in 2 h | remain high | S11A      |
| ERF4                        | decrease   | transient decrease | higher | S11E S17C |
| ERI1                        | increase   | increase    | -         | decrease  | S12A     |
| Proline transporter         | increase   | increase    | -         | prevention| S19A     |
| Protein phosphatase 2c      | increase   | increase    | -         | prevention| S18C     |
| Putative PK                 | increase   | increase    | -         | prevention| S20C     |
| Chitinase class II          | increase   | -           | -         | decrease  | S12F     |
| Basic endochitinase         | increase   | -           | -         | similar but lower | S12E     |
| Homeobox-Leu zipper HB-13   | decrease   | -           | -         | increase  | S13C     |
| bZIP                        | decrease   | increase    | -         | inhibition| S14A     |
| Phantastica                 | decrease   | -           | -         | increase  | S15B     |
| Ovate                       | decrease   | -           | -         | increase  | S15D     |
| Peroxidase21                | increase   | -           | -         | decrease  | S8F      |
| Expansin3                   | decrease   | -           | -         | increase  | S8A      |
| Expansin4                   | decrease   | -           | -         | increase  | S8B      |
| Expansin5                   | decrease   | -           | inhibition| increase  | S8C      |
| Expansin9                   | decrease   | -           | similar   | increase  | S8D      |
| Lipoxygenase - LOXD         | increase   | -           | similar in 2 h | decrease | S16A     |
| Jasmonic acid 2 – JA2       | increase   | increase    | -         | decrease  | S16B     |

**Group 6**

| ACS1A                        | transient | -           | -         | -         | S9C      |
| Genes/Molecules                | Transient Activity | Change 1 | Change 2 | Change 3 | Change 4 |
|-------------------------------|--------------------|----------|----------|----------|----------|
| ACS2                          | transient increase | increase | -        | -        | S9E      |
| ETR4                          | transient increase | -        | -        | -        | S10A     |
| CTR1                          | transient increase | -        | -        | -        | S10D     |
| ER5                           | transient increase | decrease | -        | -        | S12B     |
| AP2 domain-containing TF      | transient increase | -        | -        | -        | S14B     |
| ACO1                          | increase           | -        | -        | -        | S9H      |
| ERF2                          | transient increase | decrease | -        | -        | S11C     |
| ERF3                          | transient increase | -        | -        | -        | S11D     |
| bHLH                          | decrease           | -        | -        | -        | S13F     |
| AGO1                          | decrease           | -        | -        | -        | S15E     |
| Peroxidase precursor          | increase           | -        | similar but higher | decrease to a similar level | S8E     |
| Cystein protease              | increase           | decrease | -        | -        | S16E     |
FIGURE LEGENDS

Figure 1: Experimental outline: Appearance of flower explants of cherry tomatoes (*Solanum lycopersicum* Mill, cv. 'Shiran' 1335) held in water, before (A) and after (B) flower removal; C, schematic presentation of the AZ and non-AZ (NAZ) tissue sampling for RNA extraction before (a) and after (b) flower removal (abscised pedicel is indicated by the gray bar); D, table of pretreatments and timing of tissue sampling for RNA extraction. Samples for time zero were excised from plants without flower removal (C, scheme a). 1-MCP pretreatment was performed by exposing the flower explants to 0.4 nL L⁻¹ 1-MCP for 12 h in the dark at 20°C, prior to flower removal. IAA (10⁻³ M) was applied immediately after flower removal in lanolin paste to the cut surface of the remaining tissues. ns, not sampled.

Figure 2: Effect of flower removal, 1-MCP pretreatment, and IAA application after flower removal on the kinetics of pedicel abscission. Tomato flower explants held in water were exposed to 0.4 nL L⁻¹ 1-MCP for 12 h in the dark at 20°C. Control flower explants were kept without 1-MCP under similar conditions for the same period. Then, flowers were removed, and IAA (10⁻³ M) in lanolin paste was immediately applied to the cut surface of the remaining tissues in control explants. The percentage of accumulated pedicel abscission was monitored at various time intervals following flower removal, 1-MCP pretreatment or IAA application. The results are means of four replicates (30 flowers each) ± SE.

Figure 3: Gene expression profiles obtained by kinetics-based clustering of Groups 1-5: Group 1 - clusters of differentially expressed genes with early and transient changes of expression in the AZ following flower removal; Group 2 - clusters of genes with expression kinetics exhibiting late changes in the AZ following flower removal; Group 3 - clusters of genes modified in their expression in the AZ during 4-14 h following flower removal; Group 4 - clusters of genes modified in their expression in the AZ during 2-14 h following flower removal; and Group 5 - Clusters of genes with transient changes in their expression in the AZ following 4-8 h after flower removal. Numbers in red above each graph indicate the sampling time points (in hour) after flower removal. The (+) and (-) signs below the time points represent up- or down-regulation of genes, respectively, while the (0) sign represents no
change. The (1), (2), (3) or (4) and the (-1), (-2), (-3) or (-4) signs below the time points represent continuously up- or down-regulated genes, respectively. All of these changes were based on a two-fold change criterion (1 log ratio).

**Figure 4:** Effects of flower removal, 1-MCP pretreatment, and tissue type on the kinetics of changes in array-measured (A-F) and semi-quantitative PCR (SQ-PCR)-validated (G) expression levels of genes encoding cell wall hydrolyzing enzymes in control samples. Expression levels were measured for tomato abscission polygalacturonases (TAPGs) (A, B, C), xyloglucan endohydrolase endotransglycosylase (XET-BRI) (D) and cellulases (Cel) (E, F). RNA samples were extracted from flower AZ or NAZ tissues taken from untreated (control) or 1-MCP-pretreated tomato flower explants, at the indicated time points after flower removal. The results are means of two or three biological replicates ± SD. Transcript identities are indicated in the graphs by their tentative consensus sequence (TC) number in The Institute for Genomic Research (TIGR) and/or accession numbers. The microarray and the SQ-PCR analyses were performed with different control samples taken from independent biological replicates of two separate experiments.

**Figure 5:** Effects of flower removal, 1-MCP pretreatment, and tissue type on the kinetics of changes in array-measured expression levels of genes belonging to the IAA-amino acid hydrolyses (ILR) family (A-C). RNA samples were extracted from the flower AZ or NAZ tissues taken from untreated (control) or 1-MCP-pretreated tomato flower explants, at the indicated time points after flower removal. The results are means of two or three biological replicates ± SD. Transcript identities are indicated in the graphs by their Arabidopsis thaliana (At) gene number, TC number in TIGR, and/or accession number.

**Figure 6:** Effects of flower removal, 1-MCP pretreatment, and tissue type on the kinetics of changes in array-measured expression levels of auxin-related genes including: IAA1 (A), IAA3 (B), IAA4 (C), IAA7 (D), IAA8 (E), IAA9 (F), IAA10 (G) and Auxin-regulated protein (H). The experiment was performed as detailed in Figure 5. The results are means of two or three biological replicates ± SD. Transcript identities are indicated in the graphs by their Arabidopsis thaliana (At) gene number, TC number in TIGR, and/or accession number.
**Figure 7:** Effects of flower removal, 1-MCP pretreatment, and tissue type on the kinetics of changes in array-measured expression levels of ethylene biosynthesis-related genes. The gene names are listed as follows: Homocystein S-methyltransferase (A), S-adenosylmethionine (SAM) synthase (B), 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS1A) (C), ACS (D, E), ACS6 (F), ACC oxidase (ACO5) (G) and ACO1 (H). The experiment was performed as detailed in Figure 5. The results are means of two or three biological replicates ± SD. Transcript identities are indicated in the graphs by their Arabidopsis thaliana (At) gene number, TC number in TIGR, and/or accession number.

**Figure 8:** Effects of flower removal, 1-MCP pretreatment, and tissue type on the kinetics of changes in array-measured expression levels of genes encoding the ethylene receptor homolog – ethylene resistant 4 (ETR4) (A), constitutive triple response 1 (CTR1) (B) and ethylene responsive factor (ERF): ERF1b (C), ERF1c (D), ERF2 (E), ERF3 (F) and ERF4 (AP2 TF) (G). The experiment was performed as detailed in Figure 5. The results are means of two or three biological replicates ± SD. Transcript identities are indicated in the graphs by their Arabidopsis thaliana (At) gene number, TC number in TIGR and/or accession number.

**Figure 9:** Effects of flower removal, 1-MCP pretreatment, and tissue type on the kinetics of changes in array-measured expression levels of ethylene responsive (ER) genes. The gene names are listed as follows: fruit ripening-related ERI - Ser protease inhibitor 1 (ER1) (A), ER5 (B), ER elongation factor (ER49) (C), ripening-related burst oxidase protein D (RbohD) (D), basic endochitinase (E) and chitinase class II (F). The experiment was performed as detailed in Figure 5. The results are means of two or three biological replicates ± SD. Transcript identities are indicated in the graphs by their Arabidopsis thaliana (At) gene number, TC number in TIGR and/or accession number.

**Figure 10:** Effects of flower removal, 1-MCP pretreatment, and tissue type on the kinetics of changes in array-measured expression levels of early down-regulated TF genes. The gene names are listed as follows: Class I knotted-like homeodomain (A), Knotted TKN4 (B), Homeobox-Leu zipper HB-13 (C), Homeobox-Leu zipper (D, E), and Basix helix-loop-helix TF (bHLH) (F). The experiment was performed as
detailed in Figure 5. The results are means of two or three biological replicates ± SD. Transcript identities are indicated in the graphs by their *Arabidopsis thaliana* (At) gene number, TC number in TIGR, and/or accession number.

**Figure 11:** Effects of flower removal, 1-MCP pretreatment, and tissue type on the kinetics of changes in array-measured expression levels of TF genes. The gene names are listed as follows: *bZIP TF* (A), *AP2 domain-containing TF* (B), *TAGL12 MADS-box protein* (C) and *TAGL2 MADS-box protein* (D), *WRKY1 TF* (E) and *WRKY lld-1* (F). The experiment was performed as detailed in Figure 5. The results are means of two or three biological replicates ± SD. Transcript identities are indicated in the graphs by their *Arabidopsis thaliana* (At) gene number, TC number in TIGR, and/or accession number.

**Figure 12:** Effects of flower removal, 1-MCP pretreatment, and tissue type on the kinetics of changes in array-measured expression levels of different regulatory genes including: *Leu-rich repeat trans-membrane receptor-like kinase (LRR-RLK)* (A), *Ser/Thr-protein kinase 7 (PK7)* (B), *argonaute-like protein (AGO1)* (C) and *Pro-rich protein (TPRP-F1)* (D). The experiment was performed as detailed in Figure 5. The results are means of 2 or 3 biological replicates ± SD. Transcript identities are indicated in the graphs by their *Arabidopsis thaliana* (At) gene number, TC number in TIGR, and/or accession number.

**Figure 13:** Effects of flower removal, and IAA application on the kinetics of changes in array-measured expression levels for tomato abscission polygalacturonases (*TAPGs*) (A, B, C), xyloglucan endohydrolase endotransglycosylase (*XET-BRI*) (D) and cellulases (*Cel*) (E, F) in flower AZ. IAA was applied immediately after flower removal to the cut surface of the remaining tissues. RNA samples were extracted from flower AZ tissues taken from untreated (control) or IAA-treated tomato flower explants, at the indicated time points after flower removal. The results are means of three biological replicates ± SD. Transcript identities are indicated in the graphs by their TC number in TIGR, and/or accession number.

**Figure 14:** Effects of flower removal, and IAA application on the kinetics of changes in array-measured expression levels of auxin-related genes including: *IAA1* (A), *IAA8* (B), *IAA4* (C), *IAA7* (D), *IAA10* (E), and *Auxin-regulated protein* (F). The experiment
was performed as detailed in Figure 15. The results are means of three biological replicates ± SD. Transcript identities are indicated in the graphs by their *Arabidopsis thaliana* (At) gene number, TC number in TIGR, and/or accession number.

**Figure 15:** Summary of the postulated events leading to tomato pedicel abscission in response to auxin depletion following flower removal, and the possible effects of exogenous application of IAA or 1-MCP.
Fig. 1

D

| Treatments                           | Time after flower removal (h) | Flower AZ |
|--------------------------------------|------------------------------|-----------|
| Flower removal                       | 0 2 4 8 14                   |           |
| 1-MCP pretreatment + flower removal  | 0 2 4 8 14                   |           |
| Flower removal + IAA application      | 2 4 8 14                     | Non-AZ (NAZ) |
| Flower removal                        | 0 2 4 ns 14                  |           |
| 1-MCP pretreatment + flower removal  | 0 ns 4 ns 14                 |           |
Fig. 2

Time after flower removal (h)

Pedicel abscission (%)

Control
IAA
1-MCP

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Fig. 3
**Fig. 5**

(A) Expression levels of *ILR1* (At3g02875, BG734768) over time after flower removal (h) with AZ control and AZ 1-MCP treatments.

(B) Expression levels of *ILR3* (TC162468, At1g27980, BG129507) over time after flower removal (h) with AZ control, AZ 1-MCP, NAZ control, and NAZ 1-MCP treatments.

(C) Expression levels of *ILR3* (TC162468, At1g51760, AW649713) over time after flower removal (h) with AZ control, AZ 1-MCP, NAZ control, and NAZ 1-MCP treatments.
Fig. 6

A. IAA1
   TC154653
   At5g43700
   BI209735

B. IAA3
   TC156698
   At5g04240
   AF522014

C. IAA4
   TC162280
   At5g59790
   AF416289

D. IAA7
   TC155214
   At3g23050
   AF022018

E. IAA8
   BT014412

F. IAA9
   TC156891
   At5g65750
   AF022020

G. IAA10
   TC158767
   At5g65670
   BG628584

H. Auxin-regulated protein
   TC162697
   At5g59790
   AF416289

Time after flower removal (h)

Expression level

0 2 4 6 8 10 12 14 16
Fig. 9

A: ER1
TC164904, At4g09000
J04099

Time after flower removal (h)
0 2 4 6 8 10 12 14 16

Expression level
0 2000 4000 6000 8000

B: ER5
U77719

Time after flower removal (h)
0 2 4 6 8 10 12 14 16

Expression level
0 500 1000 1500 2000 2500 3000

C: ER49
TC154901, At4g11120
AF096247

Time after flower removal (h)
0 2 4 6 8 10 12 14 16

Expression level
0 1000 2000 3000 4000 5000 6000 7000

D: ERT10
TC162187, At3g46170
X72730

Time after flower removal (h)
0 2 4 6 8 10 12 14 16

Expression level
0 200 400 600 800 1000 1200 1400 1600

E: Basic endochitinase
TC154384, At3g12500
Z15141

Time after flower removal (h)
0 2 4 6 8 10 12 14 16

Expression level
0 2000 4000 6000 8000 10000

F: Chitinase class II
TC155645, At3g12500
U30465

Time after flower removal (h)
0 2 4 6 8 10 12 14 16

Expression level
0 500 1000 1500 2000 2500 3000
Fig. 12

(A) LRR-RLK
TC290650 (TC109650)
At5g58300
Time after flower removal (h)
0 2 4 6 8 10 12 14 16
Expression level
0 1000 2000 3000 4000
AZ control
AZ 1-MCP
NAZ control
NAZ 1-MCP

(B) PK7
At13p127500
BT013735
Time after flower removal (h)
0 2 4 6 8 10 12 14 16
Expression level
0 500 1000 1500 2000 2500

(C) AGO1
TC162675
CD002775
Time after flower removal (h)
0 2 4 6 8 10 12 14 16
Expression level
0 200 400 600 800 1000 1200 1400 1600 1800

(D) TPRP-F1
BG627766
Time after flower removal (h)
0 2 4 6 8 10 12 14 16
Expression level
0 200 400 600 800 1000 1200 1400
Fig. 13
Fig. 14

A  
IAA1  
TC154635::At5g43700::BI209735  
Expression level  
0  200  400  600  800  1000  1200  1400  1600  
0  2  4  6  8  10  12  14  16  
Time after flower removal (h)  
A2 control  
A2:IAA  

B  
IAA8  
BT014412  
Expression level  
0  500  1000  1500  2000  2500  3000  
0  2  4  6  8  10  12  14  16  
Time after flower removal (h)  

C  
IAA4  
TC162280::At5g65670::BG628584  
Expression level  
0  1000  2000  3000  4000  5000  6000  7000  8000  
0  2  4  6  8  10  12  14  16  
Time after flower removal (h)  

D  
IAA7  
TC 155214::At3g23050::AF022018  
Expression level  
0  500  1000  1500  2000  2500  3000  
0  2  4  6  8  10  12  14  16  
Time after flower removal (h)  

E  
IAA10  
TC158767::At3g04730::AJ785373  
Expression level  
0  200  400  600  800  1000  1200  
0  2  4  6  8  10  12  14  16  
Time after flower removal (h)  

F  
Auxin-regulated protein  
TC162977::At5g57970::AF416289  
Expression level  
0  500  1000  1500  2000  2500  3000  
0  2  4  6  8  10  12  14  16  
Time after flower removal (h)
Fig. 15

Flower removal

IAA
No IAA supply to AZ

Set 1
IAA-requiring genes are down-regulated

Set 2
IAA-repressed genes are up-regulated

Set 3
Modification of transcription factors, post translation regulators and AZ-specific genes

AZ becomes ethylene-sensitive: Acquisition of abscission competence

Ethylene

Set 4
Genes involved in the abscission process are specifically up-regulated in the AZ

Pedicel abscission development of defense layer

Early events
Regulatory responses

0 h
4 h
8 h
14 h

Late events
Abscission execution

IAA-requiring genes are down-regulated
IAA-repressed genes are up-regulated

Pedicel abscission development of defense layer

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