Microarray Analysis of the Abscission-Related Transcriptome in the Tomato Flower Abscission Zone in Response to Auxin Depletion

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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 ‘Shiran 1335’) flower AZ during the rapid acquisition of ethylene sensitivity following flower removal, which depletes the AZ from auxin, with or without preexposure to 1-methylcyclopropene or application of indole-3-acetic acid 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, other transcription factors 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 indole-3-acetic acid application, while 1-methylcyclopropene pretreatment inhibited only the ethylene-induced expressions, including those induced by wounding-associated ethylene signals. These 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 cross talk during the initial controlling stages of the process.

Abscission, senescence, and ripening are plant developmental processes whose 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).

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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-methylcyclopropene (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 recent years, we are beginning to get insight into the regulatory control of abscission at the molecular level. For example, the tomato (Solanum lycopersicum) 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 (Arabidopsis thaliana) floral organs has been shown to be delayed by mutations in a receptor-like kinase, HAESA (Jinn et al., 2000), or by overexpression of a MADS box gene, AGL15 (Fernandez et al., 2000), and prevented by a mutation in the gene INFLORESCENCE DEFICIENT IN ABSCSSION (IDA/IDL) that is thought to encode a receptor ligand (Butenko et al., 2003). HAESA (HAE) and HAESA-Like2 (HLS2), which serve as receptors for the IDA, were identified as receptor-like kinases (RLKs; Cho et al., 2008; Stenvik et al., 2008). IDA, HAE, and HLS2 influenced abscission in Arabidopsis in an ethylene-independent 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 the auxin gradient and inducing the sensitivity of the AZ cells to ethylene.

Our previous study with M. 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 in recent 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 the 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 (cv Shiran 1335) flower AZ compared with the non-AZ (NAZ) tissue and examination of changes in the transcriptome in response to auxin depletion, with or without 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 of auxin depletion from changes that may occur due to wounding or other nonrelated signals, the transcriptome changes in the AZ were analyzed also under the same conditions but with the 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 the 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 the pedicel (Fig. 2). However, although no abscission was yet visible in control explants up to 8 h after flower removal (Fig. 2), we 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 of the tissues for 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 the 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 pedicel 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 $10^{-2}$ M 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 in M. 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) completely prevented 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 and Serek, 1997; Watkins, 2006). After about 30 h, the full inhibitory effect of 1-MCP was not maintained any longer, possibly due to the 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 with 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 differentially expressed genes into the different groups are as follows: group 1, early (2–4 h) and transiently up- or down-regulated genes (Fig. 3, group 1; Supplemental Tables S3.1, A–F, and S4.1, A–F); group 2, late (8–14 h) up- or down-regulated genes (Fig. 3, group 2; Supplemental Tables S3.2, A–D, and S4.2, A–D); group 3, genes up- or down-regulated at the time interval 4 to 14 h (Fig. 3, group 3; Supplemental Tables S3.3, A–D, and S4.3, A–D); group 4,
Figure 3. Gene expression profiles obtained by kinetics-based clustering of groups 1 to 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 to 14 h following flower removal; group 4, clusters of genes modified in their expression in the AZ during 2 to 14 h following flower removal; group 5, clusters of genes with transient changes in their expression in the AZ following 4 to 8 h after flower removal. Numbers in red above each graph indicate the sampling time points (h) after flower removal. The + and − signs below the time points represent up- and down-regulation of genes, respectively, while 0 represents no change. The numbers 1, 2, 3, or 4 and −1, −2, −3, or −4 below the time points represent continuously up- and down-regulated genes, respectively. All of these changes were based on a 2-fold change criterion (1 log ratio). [See online article for color version of this figure.]
genes up- or down-regulated early (1–2 h) whose expression is maintained constant during the 2- to 14-h period (Fig. 3, group 4; Supplemental Tables S3.4, A–D, and S4.4, A–D); and group 5, genes transiently up- or down-regulated during the 4- to 8-h time period (Fig. 3, group 5; Supplemental Tables S3.5, A–D, and S4.5, A–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 and 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 Tables S3 and 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 the 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 Cel1 (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 Cel1 mRNA (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, Cel1, 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 the 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, was not affected in the AZ following flower removal, and their transcript levels were very low, except for Cel3, which had a higher level of expression (Supplemental Fig. S1A). The roles of two Cel genes, Cel1 in floral abscission (Lashbrook et al., 1998) and Cel2 in fruit abscission (Brummell et al., 1999), were already demonstrated previously by means of antisense suppression.

The increase in the expression of all six cell wall hydrolysis-related genes induced by flower removal was highly specific to the AZ and was almost completely prevented by 1-MCP pretreatment (Fig. 4). These results are in agreement with published reports on the 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 Gonzalez-Carranza, 2009). A novel cell wall-related gene, XET-BRI1, was found in our analysis to be up-regulated specifically in the AZ after flower removal (Fig. 4D) in a similar pattern as TAPG4 (Fig. 4C). This gene encodes for xyloglucan endotransglycosylase, which was found before to be regulated by brassinosteroid treatment (Koka et al., 2000). The brassinosteroid receptor BRI1 is a receptor kinase that transduces steroid signals across the plasma membrane and has an extracellular domain containing 25 leucine-rich repeats (LRRs; Wang et al., 2001). The possible role of this domain in regulating abscission will be discussed below.

To measure their mRNA expression levels, a semi-quantitative real-time PCR (SQ-PCR) analysis was performed for TAPG1, TAPG2, TAPG4, Cel1, and XET-BRI1 (Fig. 4G) in order to compare 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–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 a few additional genes, including two ETHYLENE-RESPONSIVE FACTORS (ERFs), ERF2 and ERF1; four novel AZ-specific genes highly expressed in the AZ at time 0 (before flower removal), TKN4, PHANTASTICA, OVATE, and a gene that 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. S5G) confirmed the expression patterns of the same genes from the microarray analysis (Supplemental Fig. S3, A–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...
There was some discrepancy in the expression data in the NAZ obtained by the two methods. For example, the expression of Cell1 and 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. 4, D 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: ERT10, ERF4, Homeobox-Leu zipper (HAT), HAT HB-13, Pro 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 and F, S18, E and F, S19, E and F, S20, E and 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 the microarray-based results of the tomato AZ gene expression.

Recently, a stamen AZ transcriptome profiling study in Arabidopsis was reported that followed the stamen abscission global gene expression during flower development (Cai and Lashbrook, 2008). This study coupled laser-capture microdissection of Arabidopsis stamen AZ 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 following genes in the AZ: ERT10, ERF4, HAT, Protein phosphatase2c (PP2C), Pro 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 and F, S18, E and F, S19, E and F, S20, E and F), further support the validity of the microarray data.

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

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

<table>
<thead>
<tr>
<th>Cluster Group</th>
<th>Cluster Type</th>
<th>No. of Genes Affected</th>
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<td>Flower Removal</td>
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<td>4 h</td>
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<td>1B</td>
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<td>202 82 93 27</td>
</tr>
<tr>
<td>1C</td>
<td>0 + 0 0</td>
<td>35 22 11 2</td>
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<tr>
<td>1D</td>
<td>0 − 0 0</td>
<td>56 23 30 3</td>
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<tr>
<td>1E</td>
<td>+ + 0 0</td>
<td>34 10 18 6</td>
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<td>1F</td>
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<td>63 17 42 3</td>
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<td>2</td>
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<td>105 63 31 11</td>
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<td>2B</td>
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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 (Agustí et al., 2008, 2009). These included genes for two different PGs, cellulase, and two different XTHs (XTH1 and XTH2) that were overexpressed in the leaf AZ-enriched tissue (Agustí et al., 2008). The expression of cell wall metabolism-related genes was also studied using real-time PCR and Affymetrix GeneChip hybridization in soybean (Glycine max) leaf AZ taken from explants exposed to ethylene (0, 1, or 2 d). This analysis showed strong up-regulation of Cel1, Cel6, Cel9, PECTATE LYASE (PL1), PL2, PG9, PG11, EXP3, 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, expres-
sion of only the 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. S1B) and XTH6 (Supplemental Fig. S1C) 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 brassinosteroid 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 the 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 EXP transcripts in the stamen AZ were reduced during flower development and the 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 process without being affected by the 1-MCP pretreatment (Supplemental Fig. S2, A–D). Even for EXP9, which was highly expressed in the AZ compared with the NAZ tissue at time 0 (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 the Expression of Auxin-Related Genes

Three genes encoding for protein homologs of the IAA-amino acid conjugate hydrolase (ILR) family were found to be up-regulated within 2 h after flower removal (Fig. 5). The expression of these ILR1 homolog genes continually increased in the AZ, and 1-MCP pretreatment inhibited this expression (Fig. 5A). On the other hand, the two other 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; Woodward 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 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 the hydrolysis of IAA conjugates (Cohen and Bandurski, 1982; Meir et al., 1936)
ment had no effect on release of stored conjugated auxins. 1-MCP pretreatment suggested that the flower AZ tissue can sense a reduction in auxin flow and react by increasing ILRs needed for the release of stored conjugated auxins. The microarray results suggest that the flower AZ tissue can sense a 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 the 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 the hydrolysis of IAA conjugates and the 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 the inhibition of abscission (Fig. 2; Roberts et al., 1984). The observed effect of flower removal on the expression of other auxin-related genes suggests the conclusion that auxin is decreased in the AZ after flower removal, as will be shown and discussed below.

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 downregulated following flower removal (Fig. 6, A–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–D and G). This indicates that the decrease in 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 with 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, 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 GenBank as an auxin-regulated protein (accession no. AF416289.1), which is expressed in tomato ovary (Vriezen et al., 2008) and fruit (Martelli et al., 2009), 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 Factor (ARF) proteins to bind to the Auxin-Responsive Elements (AREs) within the promoters and either activate or repress the expression of target genes. Rapid induction of the Aux/IAA genes is a response to the reduced levels of the Aux/IAA proteins that ensures a tightly controlled transient response to changes in auxin concentration 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 TFs act with partial redundancy to promote senescence and floral abscission (Ellis et al., 2005; Okushima et al., 2005a, 2005b). 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 levels of ARF genes are mediated via an effect on Aux/IAA expression.

**Effect of Flower Removal on the 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 (homocysteine S-methyltransferase [HMT; 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–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 at 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 no. 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 abscission development and execution. To the best of our knowledge, the only report on ethylene production during abscission of tomato flower explants was 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 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 the expression of genes related to the 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 resistant4 (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 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 response1 (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 that exists between the two encoded proteins. Both the 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 increases in expression of both ETR4 and CTR1 (Fig. 8, A and B) suggest that this receptor complex is required for function in the late stages of the abscission process. However, the results presented in Figure 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. Therefore, it seems that ETR4 and CTR1 are involved in the late stages of the abscission process, but they do not play a regulatory role in the acquisition of sensitivity to ethylene in the AZ.

Another set of genes associated with ethylene signaling are the ERFs, which are TF genes. Analysis of the promoters of ethylene-responsive genes revealed a common 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 trans-acting factors isolated from tobacco (Nicotiana tabacum) that bind to the GCC box were termed ethylene-responsive element-binding proteins (EREBPs;
Ohme-Takagi and Shinshi, 1995). EREBP s play a role in plant responses to phytohormones, pathogen attack, and environmental stresses (Hu et al., 2008, and refs. therein). 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 AtERF genes were differentially regulated by ethylene and abiotic stress conditions via ethylene-insensitive2 (EIN2)-dependent or EIN2-independent pathways (Solano et al., 1998; Fujimoto et al., 2000; Nakano et al., 2006). Overexpression of rice OsERF1 in Arabidopsis resulted in up-regulation of the expression of two known ER genes, plant defensin (low-molecular-weight Cys-rich 77) and B-chitinase (Hu et al., 2008).

Our microarray analysis revealed five different ERF genes the expression of which was altered following flower removal (Fig. 8, C–G). The homolog gene to 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: ERF2 increased early (2 h) and transiently and was not affected by 1-MCP pretreatment (Fig. 8E); ERF3 increased early (2 h) and late (14 h), and its later increase was affected significantly by 1-MCP pretreatment (Fig. 8F); ERF1b increased early (2 h) and transiently and was affected by 1-MCP pretreatment (Fig. 8C); ERF1c expression increased early (2 h) and transiently and increased again at 8 to 14 h following flower removal, and 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), 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 peach (Prunus persica) 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 to the 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-TF (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; it 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 3-fold higher in the AZ tissue compared with 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 the 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 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 that are modified upon the transition between these two ripening stages, such as ER5 and ERT10, may be significant to the general phenomenon of the acquisition of ethylene sensitivity manifested in these two systems.

Chitinases are pathogenesis-related (PR) proteins that have 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; Diaz et al., 2002; Hall and Bleecker, 2003; Taira et al., 2005), including in abscission systems (Butenko et al., 2006). Two Chitinase genes were up-regulated in the AZ at 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 encoding 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. These results suggest that the early (2 h) increase in chitinase expression, which is not AZ specific, is a wounding response that is transient only in the NAZ.

**Effect of Flower Removal on the Expression of TF Genes**

The regulation of gene expression at the transcription level has a profound role in the control of many...
biological processes. TFs act 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, developments in identifying and assigning roles to various TFs involved in the regulation of organ abscission and dehiscence processes were reviewed (Nath et al., 2007). Also reviewed recently was 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 ERFs, Aux/IAA, and ARFs, were already presented and discussed (Figs. 6 and 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 at 2 h after flower removal (Fig. 10, A and B). The gene showing homology to the class I knotted-like homeodomain gene was expressed similarly in the AZ and the NAZ, which 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 3-fold higher in the AZ compared with 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 nondifferentiated cells, suggesting that cell growth and differentiation are arrested at an early stage in the AZ (Van Nocker, 2009). It was shown that cells in the shoot apical meristem are prevented from differentiation through the activity of the KNOX family of TFs. For example, the closest Arabidopsis KNOX TF (At1g62360; Fig. 10A), SHOOT-MERISTEMLESS (STM), was shown to be required for shoot apical meristem 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 due to 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 shoot apical meristem (Heisler et al., 2005). It was shown that cycles of auxin buildup and depletion, caused by rapid reversal in the polarity of the auxin efflux carrier PIN-FORMED1 (PIN1), accompany and may direct different stages of primordium development (Heisler et al., 2005). On the other hand, the possibility that STM may act upstream of PIN1 to influence its behavior was also suggested (Heisler et al., 2005).

The expression of three HAT TF genes was downregulated within 2 h after flower removal and remained low later on (Fig. 10, C–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 (bZIP) TFs were suggested to be involved in abscission and to regulate the expression of abscission-related genes, as indicated by their binding to bean (Phaseolus vulgaris) abscission cellulase promoter (Tucker et al., 2002). The promoter of this cellulase gene includes a cis-DNA element that can function both in negative and positive regulation of the gene. Based on the observed reduction in their expression following flower removal found in our analysis, it is possible that the three HAT TF-encoding genes (Fig. 10, C–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 HAT TF-encoding genes (Supplemental Fig. S13, D and E) and restored the expression of HAT HB-13 between 8 and 14 h after flower removal (Supplemental Fig. S13C).

Another TF gene that was highly expressed in the AZ before flower removal and was sharply downregulated in the AZ after flower removal is 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 the regulation of freezing tolerance and stomata differentiation in the epidermis (Kanaoka et al., 2008). A myc/bHLH TF, ALCATRAZ, 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 downregulation 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 an 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 the expression of ERF genes, including Arabidopsis ethylene-responsive element-binding protein (AtEBP), was regulated by the activity of AP2, a floral homeotic factor. Overexpression 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 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). The TAGL12 pattern of expression matches exactly the pattern of expression found for the cell wall-modifying genes TAPG1, TAPG2, Cel1, and Cel5 (Fig. 4, A, B, E, and F, respectively). In Arabidopsis, two MADS box TFs, AGL15 and AGL18, were found to be involved in floral abscission (Fernandez et al., 2000; Adamczyk et al., 2007). Overexpression of AGL15, under the control of the 35S promoter, resulted in a 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). SEEDSTICK, which encodes a MADS domain TF known to be required for seed abscission (Pinyopich et al., 2003), is closely related to AGAMOUS SHATTERPROOF1 (SHP1) and SHP2, which are required for silique dehiscence (Liljegren et al., 2000; Pinyopich et al., 2003).

The WRKY family is a superfamily of TF proteins with up to 100 representatives in Arabidopsis that are highly divergent and are categorized into distinct groups, possibly reflecting their different functions.

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 as follows: bZIP TF (A), AP2 domain-containing TF (B), TAGL12 MADS box protein (C), TAGL2 MADS box protein (D), WRKY1 TF (E), and WRKY Ild-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 (At) gene number, TC number in TIGR, and/or accession number. [See online article for color version of this figure.]
including genes coding for TFs belonging to different families, 2001; Lippok et al., 2007).

petals, sepals, and stamens (Robatzek and Somssich, in Arabidopsis at the flower base around the AZs of AtWRKY6 established. auxin depletion due to flower removal remain to be regulatory factors in pedicel abscission responding to ethylene sensitivity in the AZ. The exact roles of these regulatory events associated with the development of auxin depletion due to flower removal. This quick transcript levels are observed early, such as 2 h after some of these TF-encoding genes, the changes in defense-related processes in the abscission layer. In

in the prevention of self-pollination, pathogen response, hormone perception and signaling, and plant development and defense responses (Bécraft, 1998; Lease et al., 1998). Very recently, Leslie et al. (2010) reported that EVERSHEDE (EVR), identified as a LRR-RLK, can function as an inhibitor of abscission. Defects in the Golgi structure and the location of the trans-Golgi network in the NEVERSHEDE 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, 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 discovered abscission-related LRR-RLKs are grouped. Based on the grouping of its closest Arabidopsis ortholog in the classification of Shi et al. (2004), the identified tomato protein is grouped into the LRR III family, while HAE and EVR are grouped into the LRR XI and LRR XVI families, respectively.

Overall, our results show differential expression of genes coding for TFs belonging to different families, including ARF, Aux/IAA, KNOX, HAT, bHLH, AP2, NAC (AY498713; Supplemental Table S3.4C), 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 plant AZ or DZ and were suggested to participate in different subprocesses of abscission or dehiscence, such as the development of the AZ, the execution of AZ separation, and the 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 the Expression of Some Other Regulatory Genes

The expression of a gene coding for a LRR-RLK was found to be down-regulated specifically in the AZ at 8 and 14 h after flower removal (Fig. 12A). HAE and HLS2, which serve as receptors for IDA/IDL, were identified as 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 the prevention of self-pollination, pathogen response, hormone perception and signaling, and plant development and defense responses (Bécraft, 1998; Lease et al., 1998). Very recently, Leslie et al. (2010)
of the guide strand and the 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 initial TPRP-F1 transcript level in the AZ but did not have any effect on its decrease once the flower was removed (Fig. 12D). The TPRP-F1 gene was originally identified as a gene encoding 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 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 the 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), Cel1 (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 the expression of these genes, induced by flower removal, was also prevented by 1-MCP pretreatment (Fig. 4, A, B, E, and 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 8 to 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 Cel1 (Lashbrook et al., 1994) during both leaf and flower abscission in tomato, as demonstrated in Figure 4. The increase in XET-BR1 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-BR1 expression (Fig. 4D), which is also inhibited by IAA treatment (Fig. 13D). The role of XET-BR1 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 re-
moval 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); 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–E). In our study in M. jalapa, we identified, among the different genes whose expression was associated with the leaf AZ, two genes that 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 the 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-dichlorophenoxyacetic acid, 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, 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 and 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 Figures 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...
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. –, No effect. The genes listed in group 2 were classified into three subgroups: I, II, and III.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Changes in Gene Expression in Response to:</th>
<th>Figure No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flower Removal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Early at 2 to 4 h</td>
<td>Late at 8 to 14 h</td>
</tr>
<tr>
<td>TAPG1</td>
<td>Increase</td>
<td>Prevention 13A</td>
</tr>
<tr>
<td>TAPG2</td>
<td>Increase</td>
<td>Prevention 13B</td>
</tr>
<tr>
<td>Cel1</td>
<td>Increase</td>
<td>Prevention 13E</td>
</tr>
<tr>
<td>Cel5</td>
<td>Increase</td>
<td>Prevention 13F</td>
</tr>
<tr>
<td>TAGL12</td>
<td>Increase</td>
<td>Prevention 14C</td>
</tr>
<tr>
<td>NADPH oxidase-RBOH1</td>
<td>Increase</td>
<td>Prevention 15F</td>
</tr>
<tr>
<td>ER49</td>
<td>Decrease</td>
<td>Increase 12C</td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 ILR3</td>
<td>Transient increase</td>
<td>Decrease but higher than 0 h</td>
</tr>
<tr>
<td></td>
<td>Prevention</td>
<td>Lower transient increase S7B</td>
</tr>
<tr>
<td>1 IAA1</td>
<td>Decrease</td>
<td>Prevention 14A</td>
</tr>
<tr>
<td>1 IAA4</td>
<td>Decrease</td>
<td>Inhibition Increase to original 14C</td>
</tr>
<tr>
<td>1 IAA7</td>
<td>–</td>
<td>Inhibition Increase to original 14D</td>
</tr>
<tr>
<td>1 IAA8</td>
<td>Decrease</td>
<td>Increase Remain high 14B</td>
</tr>
<tr>
<td>1 IAA10</td>
<td>Decrease</td>
<td>Prevention Increase 14E</td>
</tr>
<tr>
<td><strong>Class 1 knotted-like homeodomain</strong></td>
<td>Decrease</td>
<td>Prevention – S13A</td>
</tr>
<tr>
<td>1 Homeobox-Leu zipper (HAT; BG627748)</td>
<td>Decrease</td>
<td>Prevention – S13D</td>
</tr>
<tr>
<td>1 Remorin2</td>
<td>Decrease</td>
<td>Prevention Increase S16C</td>
</tr>
<tr>
<td>1 Cys protease inhibitor1 precursor</td>
<td>Increase</td>
<td>Prevention Prevention 16D</td>
</tr>
<tr>
<td>11 XET-BR1</td>
<td>Increase</td>
<td>Prevention – 13D</td>
</tr>
<tr>
<td>11 ILR1</td>
<td>Increase</td>
<td>Prevention Prevention 17A</td>
</tr>
<tr>
<td>11 PK7</td>
<td>Increase</td>
<td>Prevention Prevention S20A</td>
</tr>
<tr>
<td>11 RGLG2</td>
<td>Increase</td>
<td>Prevention Prevention 19C</td>
</tr>
<tr>
<td>11 Cys-type peptidase-RD19</td>
<td>Increase</td>
<td>Prevention Prevention 16F</td>
</tr>
<tr>
<td>11 Protein phosphatase</td>
<td>Increase</td>
<td>Prevention Prevention 15C</td>
</tr>
<tr>
<td>11 Knotted TKN4</td>
<td>Decrease</td>
<td>Prevention – S13B</td>
</tr>
<tr>
<td>11 Homeobox-Leu zipper (HAT; CK715706)</td>
<td>Decrease</td>
<td>Prevention Increase S13E, S18A</td>
</tr>
<tr>
<td><strong>Group 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACS6</td>
<td>Transient increase</td>
<td>Increase Prevention Prevention S9F</td>
</tr>
<tr>
<td>ERF1c</td>
<td>Transient increase</td>
<td>Increase Prevention S11B</td>
</tr>
<tr>
<td>ERT10</td>
<td>Transient increase</td>
<td>Increase Prevention S12D, S17A</td>
</tr>
<tr>
<td>WRKY IId-1</td>
<td>Transient increase</td>
<td>Increase Prevention Lower increase S15A</td>
</tr>
<tr>
<td><strong>Group 4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETR5</td>
<td>–</td>
<td>Increase S10B</td>
</tr>
<tr>
<td>ETR6</td>
<td>–</td>
<td>Increase S10C</td>
</tr>
<tr>
<td><strong>Group 5</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAPG4</td>
<td>Increase</td>
<td>– Prevention/decrease 13C</td>
</tr>
<tr>
<td>Auxin-regulated protein</td>
<td>Transient increase</td>
<td>– Lower transient 14F</td>
</tr>
<tr>
<td>SAM synthase</td>
<td>Transient increase</td>
<td>– Higher transient S9B</td>
</tr>
<tr>
<td>ACS (M34289)</td>
<td>Transient increase</td>
<td>Increase Prevention 9D</td>
</tr>
<tr>
<td>ACOS</td>
<td>Transient increase</td>
<td>Increase Prevention 9G</td>
</tr>
<tr>
<td>ERF1b</td>
<td>Transient increase</td>
<td>Decrease Similar in 2 h S11A</td>
</tr>
<tr>
<td>ERF4</td>
<td>Decrease</td>
<td>Transient Higher S11E, S17C</td>
</tr>
<tr>
<td><strong>ER1</strong></td>
<td>Increase</td>
<td>Increase Decrease S12A</td>
</tr>
<tr>
<td>Pro transporter</td>
<td>Increase</td>
<td>Increase Decrease S19A</td>
</tr>
<tr>
<td>Protein phosphatase2c (PP2C)</td>
<td>Increase</td>
<td>Increase Decrease S18C</td>
</tr>
<tr>
<td>Putative PK</td>
<td>Increase</td>
<td>Increase Decrease S20C</td>
</tr>
<tr>
<td>Chitinase class II</td>
<td>Increase</td>
<td>Increase Decrease S12F</td>
</tr>
<tr>
<td>Basic endochitinase</td>
<td>Increase</td>
<td>Increase Similar but lower S12E</td>
</tr>
<tr>
<td>Homeobox-Leu zipper (HAT) HB-13</td>
<td>Decrease</td>
<td>Increase S13C</td>
</tr>
<tr>
<td>bZIP</td>
<td>Decrease</td>
<td>Increase Inhibition S14A</td>
</tr>
</tbody>
</table>

(Table continues on following page.)
Abscission-Related Transcriptome following Auxin Depletion

following IAA application are 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 with the abscission progress and mainly in its late stage at 8 to 14 h after flower removal. IAA application prevented the otherwise observed change in their expression, as did 1-MCP pretreatment. The modification was AZ specific (Figs. 4, A, B, E, and F; data not shown); and 2II, genes whose expression was modified specifically in the AZ but was not affected by 1-MCP pretreatment, such as ER5 (Fig. 10A), Protein phosphatase (Supplemental Fig. S3E), HAT HB-13 (Fig. 10C), which was expressed specifically in the AZ at 0 h, and HAT (Fig. 10E). Based on this classification, we suggest that subgroup 2I includes genes that are generally IAA regulated, while subgroups 2II and 2III include IAA-regulated genes involved in abscission. Among these two abscission-related subgroups, subgroup 2II includes genes that are also ethylene regulated downstream of IAA, and subgroup 2III includes genes that 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 IId-1, was prevented by the 1-MCP

### Table II. (Continued from previous page.)

<table>
<thead>
<tr>
<th>Group 6</th>
<th>Gene Name</th>
<th>Changes in Gene Expression in Response to:</th>
<th>Figure No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Early at 2 to 4 h</td>
<td>Late at 8 to 14 h</td>
</tr>
<tr>
<td></td>
<td>Phantastica</td>
<td>Decrease –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Ovate</td>
<td>Decrease –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Peroxidase21</td>
<td>Increase –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Expansin3</td>
<td>Decrease –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Expansin4</td>
<td>Decrease –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Expansin5</td>
<td>Decrease –</td>
<td>Inhibition Increase</td>
</tr>
<tr>
<td></td>
<td>Expansin9</td>
<td>Decrease –</td>
<td>Similar Increase</td>
</tr>
<tr>
<td></td>
<td>Lipoygenase, LOXD</td>
<td>Increase –</td>
<td>Similar in 2 h</td>
</tr>
<tr>
<td></td>
<td>Jasmonic acid2 (JA2)</td>
<td>Increase Increase</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Group 6</td>
<td>ACS1A</td>
<td>Transient increase –</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACS2</td>
<td>Transient increase In</td>
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<tr>
<td></td>
<td></td>
<td>ETR4</td>
<td>Transient increase –</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTR1</td>
<td>Transient increase –</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ER5</td>
<td>Transient increase Decrease</td>
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<tr>
<td></td>
<td></td>
<td>AP2 domain-containing TF</td>
<td>Transient increase –</td>
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<td></td>
<td></td>
<td>ACO1</td>
<td>Increase –</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ERF2</td>
<td>Transient increase Decrease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ERF3</td>
<td>Transient increase –</td>
</tr>
<tr>
<td></td>
<td></td>
<td>bHLH</td>
<td>Decrease –</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGO1</td>
<td>Decrease –</td>
</tr>
<tr>
<td></td>
<td>Peroxidase precursor</td>
<td>Increase</td>
<td>Similar but higher Decrease to a similar level</td>
</tr>
<tr>
<td></td>
<td>Cys protease</td>
<td>Increase Decrease</td>
<td>–</td>
</tr>
</tbody>
</table>
pretreatment (Figs. 7F, 9D, and 11F), and it was AZ specific for three of them, ERF1c, ERT10, and WRKY IId-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 abscission 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 (Kevany et al., 2007; Binder, 2008).

Group 5 includes genes whose expression was modified early during the 2- to 4-h period after flower removal, either transiently or continuously. IAA application did not affect this early gene modification, but it affected the expression at the later period of 8 to 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 systemic signaling during the wound response, such as lipoxygenase (LOX) and jasmonic acid2 (JA2; Howe and Schilmiller, 2002; Len et al., 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 at 2 h following flower removal, which then decreased to the basal level within the subsequent 1 h (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 LOX, wound-induced proteinase inhibitor I, JA2, 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 Tables S3 and S4; Supplemental Fig. S16).

The effects of IAA on the expression of some genes modified at the late phase of 8 to 14 h after flower removal may operate via affecting the ethylene sensitivity of the AZ. This possible explanation is supported by 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
CONCLUSION

The aim of this research was to further explore the molecular changes occurring during the acquisition of abscission competence in the AZ following auxin depletion by using the Affymetrix Tomato GeneChip. Application of IAA after flower removal, which prevented the abscission process, enabled us to differentiate between genes whose expression was affected by IAA due to flower removal, which are the subject of this research, and genes whose expression was modified by flower removal and were not affected by resupplementation of IAA. Based on the kinetics of pedicel abscission, the identity and kinetics of expression of the genes affected by flower removal, and the effects of IAA application and 1-MCP pretreatment, we can separate the sequence of events that occur during tomato flower abscission into two phases: early events (0–4 h after flower removal) that probably lead to the acquisition of ethylene sensitivity and abscission competence; and late events (8–14 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 Figure 15. Genes showing early modified expression might be involved in mediating the auxin regulation of ethylene sensitivity in the AZ. These include three sets of genes (sets 1–3 in Fig. 15). Set 1 includes genes that are directly regulated by auxin and are therefore down-regulated early after IAA depletion, such as the Aux/IAA genes IAA1, -3, -4, -7, and -8, and some of the TFs whose expression is down-regulated early after flower removal, such as knotted, HAT, and bHLH. Set 2 includes genes that are directly IAA repressed that were up-regulated early after IAA depletion, such as PK7, ERF1c, WRKY 11d-1, and Protein phosphatase. Set 3 includes other TFs and/or post-TFs, 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-FI, Phantastica, and Ovate.

As the AZ becomes sensitive to ethylene, the basic level of ethylene production together with its autocatalytic increase are mediated specifically in the AZ by the specific expression of ethylene biosynthesis-related genes (e.g. ACS [M34289]; Fig. 7D). This induction of ethylene levels leads in turn to the activation of AZ-specific genes involved in the late stage of abscission and its execution 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 subgroups, based on their putative functions: I, TF genes or genes belonging to ethylene signal transduction or abscission regulators, such as synthase (Fig. 7B), and ACS (Fig. 7D).

The observation showing that IAA application after flower removal did not affect the increase in Chitinase gene 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 (8–14 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 microorganisms 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).
ETR4, CTRL, ERFlc, TAGLI2, LRR-RLK, and PK7; II, genes encoding cell wall-modifying proteins; and III, genes involved in PR defense and the 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 the 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.

This study has established a powerful platform for the further analysis of possible regulatory abscission-related genes involved in the 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 a 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 RNA interference in stably transformed tomato plants, as regulated by an abscission-specific promoter. This functional analysis will enable us to further reveal the roles 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* ‘Shiran 1335’; Hazera Genetics) were harvested between 10 and 12 AM from a commercial greenhouse in Israel. Bunches containing at least two to four 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 three- to four-bunch explants (Fig. 1, A and B) were placed in a vial containing 10 mL of organic chlorine (50 µL 2 M NaCl, 2% [w/v] cetyl trimethyl ammonium bromide, 2% [w/v] polyvinyl pyrrolidone, and 2% [v/v] β-mercaptoethanol). Following 10 min of incubation at 65°C, the mixture was extracted twice with chloroform:isoamyl alcohol (24:1, v/v). Total RNA was precipitated with 2.5 M LiCl for 12 h at 4°C, and following resuspension in water, the RNA was treated with RQ1 RNase-free DNase (Promega) for removal of any residual DNA. The RNA was further purified using the RNeasy Plant Mini Kit (Qiagen), resulting in a pure and high-quality RNA preparation based on spectrophotometric 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 the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and cleaned from DNA using RQI RNase-free DNase (Promega). cDNA was synthesized using the Reverse Transcription System (Promega) 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 the 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 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 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, Weizmann Institute of Science in Rehovot, Israel. We used the Affymetrix GeneChip Expert Suite One-Cycle kit according to the relevant Affymetrix GeneChip Expression Analysis Technical Manual (No. 701021, Rev. 5) and the Data Analysis Fundamentals manual (P/N 701190). cDNA was prepared using the two-cycle target labeling procedure and was used for further synthesis of biotin-labeled target copy RNA by in vitro transcription as described in the Affymetrix GeneChip Manual. The copy RNA 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 personal computer-compatible workstation. Immediately following hybridization, the probe array went on automated washing and staining protocol on the fluidics station using the fluorescent molecule streptavidin-phycocerythrin 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 the 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 (Irizarry et al., 2003) in the affy package with default parameters. The statistical test for differentially expressed genes was performed using the LIMMA (linear models for microarray) 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, 12, and 16 h) and for 1-MCP-treated samples (2, 4, 8, and 12 h) and control explants.
and 14 h) against nontreated samples (AZ before treatment), and four time points of 1-MCP-pretreated samples (2, 4, 8, and 14 h) against nontreated 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 false discovery rate correction; (2) genes showing a 2-fold change in the expression level between treatment and control; and (3) present call defined by GCOS software output) and a signal level of 20 or greater in at least 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 detail in “Results and Discussion.”

In order to enable a Gene Ontology analysis, we had to assign each tomato gene to its nearest homologous gene from Arabidopsis, as the FatiGo Gene Ontology analysis tools (http://fatiigo.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 homologs. The criterion for finding the best Arabidopsis homolog was chosen as genes with an E value of less than 1e \(^{-5} \).

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

Validation of the microarray expression results was performed for a few genes that exhibited an abscission-specific type of expression in the tomato flower AZ. Beside validation of the microarray results for newly discovered abscission-specific genes, we 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, and Ocate; ethylene signal transduction-related genes ERF2 and ERF10; regulatory gene *Protein phosphatase*; and abscission-related cell wall degradation genes TAPG1, TAPG2, TAPG4, Cell1, and XET-BR1. RNA was extracted from the flower AZ and NAZ tissue samples at different time intervals (0, 2, 4, 8, and 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 β-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 1. 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. S2) to allow easy comparison of the resulting PCR products. The PCR product was run on a 0.8% agarose gel in 0.5× Tris-acetate-EDTA buffer for 25 min at 110 V. The total time varied according to the size of the PCR product and was recorded with the Image Master VDS 1208 system. The relevant results are shown in Figure 4G, and a similar PCR cycle calibration was done for the data presented in Supplemental Figure S3G.

We used for PCR the Amplicon Taq DNA Polymerase Master Mix (2.0 Master Mix Kit; 1.5 mM MgCl\(_2\)). The PCR products were analyzed on a 0.8% agarose gel run in 0.5× Tris-acetate-EDTA buffer for 25 min at 110 V. 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 Q-RT-PCR

Flower samples for AZ and NAZ tissues were collected as described above. 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 μg of RNA was reverse transcribed with the Moloney Murine Leukemia Virus Reverse Transcriptase Kit (Promega). Q-RT-PCR was performed with a Corbett Rotor-Gene 3000 (Corbett Life Research) using SYBR Green Master Mix detection chemistry (Agene Ltd.) and gene-specific primers. The primers were designed using 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, cDNAs isolated from tomato flower AZ, NAZ, and other treated tissues were mixed and serially diluted by a factor of 10, and aliquots of the dilutions were used in standard real-time PCR. Each value determination was repeated three times to ensure the slope of the standard curves and to determine the sd. The β-Tubulin2 (609267), SL-Actin (U00481/Q96843), and GAPDH (U97257) genes were used as internal controls, and relative (2) gene levels of these genes were computed by the 2\(^{-ΔΔCt}\) method of relative quantification (Livak and Schmittgen, 2001). All experiments were carried with nontemplate control and negative control (RNA sample) and 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 and F).

Supplemental Figure S3. 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 and F).

Supplemental Figure S4. Effects of flower removal and tissue type on the kinetics of changes in array-measured (A and C) and Q-RT-PCR-validated (B and D) expression levels of ethylene-related genes, ETR10 (A and B) and ERF4 (C and D), and the linear regressions with the appropriate coefficients obtained between the microarray and the Q-RT-PCR expression data for these genes (E and F).

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

Supplemental Figure S6. Effects of flower removal and tissue type on the kinetics of changes in array-measured (A and C) and Q-RT-PCR-validated (B and D) expression levels of other TF and regulatory genes, HAT’ (A and B) and TPRP-F1 (C and D), and the linear regressions with the appropriate coefficients obtained between the microarray and the Q-RT-PCR expression data for these genes (E and 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 and 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 homologs *ETR4* (A), *ETR5* (B), and *ETR6* (C) and *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 ERF genes.
Supplemental Figure S12. Effects of flower removal and IAA application on the kinetics of changes in array-measured expression levels of 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 Hld-I (A), Phantastica (B), Protein phosphatase (C), Oste4 (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 LOXD (A), I2A (B), Remorin2 (C), Cys protease inhibitor1 precursor (D), Cys protease (E), and Cys-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 and C) and Q-RT-PCR-validated (B and D) expression levels of ethylene-related genes, ERT10 (A and B) and ERF4 (C and 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 and F).

Supplemental Figure S18. Effects of flower removal (control), 1-MCP pretreatment, or IAA application after flower removal on the kinetics of changes in array-measured (A and C) and Q-RT-PCR-validated (B and D) expression levels of HAT (A and B) and PP2C (C and 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 and 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 and C) and Q-RT-PCR-validated (B and D) expression levels of Pro transporter (A and B) and Ubiquitin-protein ligase-RGLG2 (C and 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 and 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 and C) and Q-RT-PCR-validated (B and D) expression levels of PK7 (A and B) and Putative PK (C and 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 and F).

Supplemental Figure S21. SQ-PCR cycle calibration showing the amplified expression levels of various genes isolated from the flower AZ at 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 Figure 4 and Supplemental Figure 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 that were affected by flower removal in each cluster presented in Figure 3 and listed in Table I.

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

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