Molecular Changes Occurring during Acquisition of Abscission Competence following Auxin Depletion in Mirabilis jalapa

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To understand how auxin regulates sensitivity of abscission zone (AZ) tissues to ethylene, we used a polymerase chain reaction-based subtractive approach to identify gene transcripts in Mirabilis jalapa AZs that changed in abundance during the time the zones became competent to abscise in response to exogenous ethylene. Transcript expression was then examined in leaf and stem AZs over the period they became ethylene competent following indole-3-acetic acid (IAA) depletion either by leaf deblading, treatment with the IAA transport inhibitor naphthylphthalamic acid, or cutting the stem above a node (decapitation). Transcripts down-regulated by deblading/decapitation included Mj-Aux1/IAA1 and Mj-Aux2/IAA2, encoding Aux/IAA proteins, and three other transcripts showing highest identity to a polygalacturonase inhibitor protein, a β-expansin, and a β-tubulin. Application of IAA to the cut end of petioles or stumps inhibited abscission, and prevented the decline in the levels of transcripts in both AZs. Transcripts up-regulated in the AZ following deblading/decapitation or treatment with naphthylphthalamic acid were isolated from plants pretreated with 1-methylcyclopropene before deblading to help select against ethylene-induced genes. Some of the up-regulated transcripts showed identity to proteins associated with ethylene or stress responses, while others did not show homology to known sequences. Sucrose infiltration of stem stumps enhanced abscission following ethylene treatment and also up-regulated transcripts showing identity to proteins associated with ethylene or stress responses, while others did not show homology to known sequences. Sucrose infiltration of stem stumps enhanced abscission following ethylene treatment and also enhanced the induction of some of the up-regulated genes. Our results demonstrate a correlation between acquisition of competence to respond to ethylene in both leaf and stem AZs, and decline in abundance of auxin regulatory gene transcripts.

The timing of developmental processes in plants, such as abscission, senescence, and ripening, is affected by increased tissue sensitivity to ethylene (Trewavas, 1986; Bleecker and Patterson, 1997; Zegzouti et al., 1999). The cause of this increased ethylene sensitivity is still not known, but it has been shown to be affected by other plant hormones. In abscission, the interplay between indole-3-acetic acid (IAA) and ethylene is important (Abeles and Rubinstein, 1964; Sexton, 1997; Taylor and Whitelaw, 2001). The generally accepted model is that a basipetal polar IAA flux through the abscission zone (AZ) prevents abscission by rendering the AZ insensitive to ethylene. If the source of IAA is removed, the AZ becomes sensitized to the presence of ethylene and abscission commences (Rubinstein and Leopold, 1963; Abeles and Rubinstein, 1964; Addicott, 1982; Sexton and Roberts, 1982). A classic experiment in support of the above model has been to deblade leaves (removing the IAA source), which leads to premature formation of an AZ and rapid petiole abscission in response to exogenous ethylene (Abeles and Rubinstein, 1964). The direction of the auxin flux is also important for determining whether abscission occurs (Morris, 1993; Roberts et al., 2002). Thus, unlike various auxin-mediated physiological processes that are a result of transient and local changes in auxin levels (Woodward and Bartel, 2005), prevention of abscission has been found to require a continuous and constant polar supply of auxin at the AZ (Taylor and Whitelaw, 2001). Auxin affects abscission not only by regulating sensitivity to ethylene, but also by affecting the intracellular transport of the hydrolytic enzyme polygalacturonase (PG) associated with cell wall degradation. A specific mechanism by which auxin delays or even prevents cell separation in silhouette dehiscence of oilseed rape (Brassica napus) has been reported. In this system the auxin analog 2-methyl-4-chloroephenoxyacetic acid retarded the secretion of PG to the cell wall, thereby inhibiting pod dehiscence (Dal Degan et al., 2001).

Our knowledge of the biology of abscission has been increased greatly through anatomical, physiological, and biochemical studies (Sexton and Roberts, 1982;...
Osborne, 1989; Sexton, 1997; Taylor and Whitelaw, 2001), and progress has been made in identifying genes that encode hydrolytic enzymes involved in cell separation processes in the AZ, e.g. PG and β-1,4 endoglucanases (Taylor and Whitelaw, 2001). Nevertheless, the molecular mechanisms leading to increased tissue sensitivity to ethylene in response to IAA deficiency in abscising systems have not been studied much, and their interactions are still unknown. Several previous studies may provide useful hints as to the possible molecular mode of action. A tomato (Lycopersicon esculentum) ethylene receptor gene ETR1 homolog (tETR) was found to be specifically up-regulated in the flower AZ, as well as in other ethylene-mediated processes in other tissues (Payton et al., 1996). Delayed abscission has been demonstrated in tomato plants with suppressed expression of the ethylene receptor (LeETR; Barry et al., 2005) and in Arabidopsis (Arabidopsis thaliana) plants that express the mutant ethylene receptor allele etr1-1 (Bleecker and Patterson, 1997; Patterson and Bleecker, 2004). Likewise, anther dehiscence was shown to be delayed in tobacco (Nicotiana tabacum) plants rendered ethylene insensitive either by treatment with the ethylene-perception inhibitor 1-methylcyclopropene (1-MCP) or by Arabidopsis etr1-1 allele expression (Rieu et al., 2003). These examples suggest an association between ethylene, its receptor, and timing of organ abscission, and support a role for the ethylene receptor controlling the competence of the AZ to respond to ethylene.

However, it has also been shown in Arabidopsis (Bleecker and Patterson, 1997; Patterson and Bleecker, 2004) and tomato (Barry et al., 2005) that although ethylene accelerates abscission of flower organs, ethylene is not essential for the abscission process to occur. Similarly, a lack of an ethylene requirement in abscission has also been shown for tulip (Tulipa kaufmanniana; Sexton et al., 2000), cocoa (Theobroma cacao; Aneja et al., 1999), and orchid (Cymbidium spp.; Van Doorn, 2002) flowers, although ethylene in these systems has been demonstrated to accelerate abscission. In addition, another tomato ETR1 homolog (eETR1) was constitutively expressed in the AZ, independent of IAA or ethylene, suggesting that other ethylene receptors (like ERS1) may act during abscission (Zhou et al., 1996). It is generally accepted that ethylene receptors work as negative regulators of ethylene response and therefore there would be an inverse correlation between receptor levels and ethylene sensitivity of a tissue (Klee, 2002, 2004). Although most published results are in agreement with this, some inconsistencies have been reported. For instance, the increased ethylene receptor expression as tomato fruit approach maturity, a phase which is associated with their highest ethylene levels and sensitivity (Klee, 2004); suppression of LeETR1 delaying tomato leaf abscission rather than increasing it, attributed to its effect on auxin movement that led to increased auxin levels in the petiole AZ (Whitelaw et al., 2002); enhanced transcription of ERS1 in response to ethylene in delphinium (Delphinium spp.) florets associated with their increased sensitivity to ethylene during their abscission (Kuroda et al., 2003, 2004); and the positive correlation obtained between the sensitivity of miniature rose (Rosa hybridra) varieties to ethylene and the expression of ETR gene family (RhETR1–4) resulting in flower abscission (Müller et al., 2000a, 2000b).

Considering these inconsistencies between expression of ethylene receptor genes in the AZ and tissue sensitivity to ethylene, it is still possible that IAA may control AZ sensitivity to ethylene by affecting components downstream to ETR1 or ERS1 receptors in the ethylene signal transduction pathway.

It seems that in the last years we are only beginning to get insight into regulatory control of abscission at the molecular level. For example, the tomato mutation jointless, which does not form pedicel AZs (Butler, 1936), is now attributed to a mutation in a MADS-box gene, LeMADS (Mao et al., 2000). Abscission of Arabidopsis floral organs has been shown to be delayed by mutations in a receptor-like kinase, HAESA (formerly named receptor-like protein kinase 5; Jinn et al., 2000), or overexpression of a MADS-box gene, AGAMOUS-like 15 (Fernandez et al., 2000), and prevented by a mutation in a gene (INFLORESCENCE DEFICIENT IN ABSCIDION) that is thought to encode a receptor ligand (Buitenko et al., 2003). However, to our knowledge, no studies have yet reported on genes that are involved in sensing the change in auxin gradient and inducing the sensitivity of the AZ cells to ethylene.

We hypothesize that IAA regulates genes that control the sensitivity of the AZ tissue to ethylene. To examine this hypothesis we identified in this study transcripts that changed in abundance in the Mirabilis AZ as it became competent to respond to ethylene. We used the AZ tissue of Mirabilis jalapa for this purpose, as this plant has long been used as a model for abscission studies due to its easily identifiable and large AZs (Lloyd, 1916). One of the Mirabilis AZs is located at the base of the petiole, and the other well-defined and large adventitious AZ is located in each node of the stem, whose abscission is regulated by IAA (Beal and Whiting, 1945). Recently, there has also been some progress in vitro regeneration, propagation, and transformation of this plant (M. Zaccar, G. Jia, X. Chen, O. Genis, D. Feibin, and R. Gesua, unpublished data), which may also enhance its use as a model system for molecular studies. Using a PCR-based subtractive approach we were able to isolate gene transcripts that were either up- or down-regulated in the leaf and stem AZs in response to auxin deficiency gained by leaf debalding or cutting the stem above a node (decapitation). The association of these clones with the development of AZ sensitivity to ethylene was studied by examining their expression following leaf debalding or stem decapitation, and application of abscission-modifying treatments such as auxins, naphthalhthalamic acid (NPA), an auxin transport inhibitor, and 1-MCP, an inhibitor of ethylene action (Sisler and Serek, 1997). Our results show that transcripts...
isolated from the leaf AZ had similar expression patterns in both leaf and stem AZs following deblading/decapitation or application of abscission-modifying treatments. In this way we could demonstrate a correlation between acquisition of competence to respond to ethylene in both leaf and stem AZs, and decline in abundance of auxin regulatory gene transcripts. The pool of transcripts we identified may serve in turn as a promising source out of which we will be able to isolate those that affect the tissue competence to abscise.

RESULTS

Physiological Characterization of the *M. jalapa* Abscission Systems

The petiole AZ of *M. jalapa* became sensitive to the abscission-inducing effects of ethylene when the leaf blades were removed (Fig. 1). The rate of petiole abscission was dependent upon the time between leaf deblading and ethylene exposure, with higher abscission rates obtained as this period increased. The petioles of debladed leaves in the absence of exogenous ethylene eventually abscised 6 d after deblading, but their abscission rate was much slower than that of debladed petioles exposed to ethylene. Non-debladed petioles did not abscise over the time frame examined, even when exposed to ethylene (Fig. 1).

Naphthalene acetic acid (NAA) or IAA applied to the cut surface of the debladed plants reduced the number of petioles that abscised following ethylene exposure (Fig. 2). Both native (IAA) and synthetic (NAA) auxins were tested to guarantee a clear auxin effect. IAA was substantially more effective than NAA, resulting in close to complete inhibition of abscission. The ethylene action inhibitor 1-MCP applied prior to leaf deblading, completely prevented petiole abscission caused by exogenously applied ethylene (Fig. 2).

Cutting the stem above a node (decapitation) induces the formation of an AZ at the base of the cut internode that leads to abscission of the internodal stem section (stump) above the newly formed AZ (Beal and Whiting, 1945). In our experiments, the stumps abscised in response to 2 d of ethylene exposure (8–5 \( \mu L^{-1} \)) administered 4 (Fig. 3), 6, or 9 (data not shown) d after they had been decapitated. The percentage of stump abscission increased with time reaching approximately 90% 5 d after the ethylene treatment. Ethylene did not cause stump abscission in intact plants. 1-MCP applied before decapitation or IAA applied after (in lanolin paste), effectively prevented stump abscission. By contrast, NAA applied to the cut surface either in a lanolin paste (Fig. 3) or by water infiltration (data not shown) was comparatively ineffective.

Suc was applied to the decapitated stumps to examine two possibilities: (1) whether elimination of Suc transport to *Mirabilis* roots and tuber via the node AZ might in part be responsible for the enhanced abscission, and (2) whether Suc has a possible signaling effect on abscission as a modulator of processes controlled by hormones (León and Sheen, 2003). Surprisingly, application of 2% Suc to the cut end of the decapitated stump increased the sensitivity of the AZ to ethylene, causing 100% of the stumps to abscise 1 d after ethylene treatment (data not shown).
was performed 4 d before exposure to ethylene. NAA (2 or decapitated at 20°C) was monitored in each experiment. Samples of 10 to 12 stems per treatment were presented, and data represent means ± s of two experiments performed with young plants. Samples of 10 to 12 stems per treatment were monitored in each experiment.

Isolation and Characterization of Gene Transcripts That Decreased in the Petiole AZ of *M. jalapa* following Deblading/Decapitation

We used a PCR-based subtractive procedure to isolate gene transcripts that declined in the petiole AZ of *Mirabilis* during the time the AZ became sensitive to respond to ethylene. This was notably 3 d before the debladed petioles without ethylene treatment would abscise (Fig. 1). To accomplish this we used RNA isolated from the petiole AZ of non-debladed leaves as tester RNA, and RNA isolated from the AZ of petioles that had been debladed for 3 d as driver RNA. The PCR-select procedure selected for those transcripts present in the tester RNA pool and not present in the driver RNA pool.

Sequencing and homology searches of 140 insert-containing clones revealed that the deduced amino acid sequence of seven transcripts had significant similarities to a diverse array of auxin-related genes in the GenBank database (Table I). SM-01 (AY137966) showed 54% identity to *Arabidopsis* auxin response factor 6 (ARF6 [At1g30330]), SM-32 (AY137965) displayed 62% identity to a *Robinia pseudoacacia* auxin-repressed protein (ARP) gene (AY009694), and clones *Mj-Aux/AIA1* to 5 (AY137961–4 and DQ070872) showed 47% to 93% identity to various members of the *Aux/AIA* gene family.

RNA gel-blot analyses indicated that the IAA-related genes were expressed differently in the AZ in response to deblading. The expression of some of these genes, such as *Mj-Aux/IAA4* (AY137964) and clone SM-32 (AY137965) was not affected by deblading or stem decapitation (data not shown). On the other hand, the expression of certain genes, such as *Mj-Aux/AIA1* (AY137961) and *Mj-Aux/AIA2* (AY137962) was substantially down-regulated within 1 d of deblading (Fig. 4) and within 6 to 9 d of stem decapitation (Fig. 5).

The deblading-induced decline in *Mj-Aux/AIA1* transcripts could be prevented by applying IAA, but not NAA, to the cut end of the debladed petiole (Fig. 4), whereas the reduction in *Mj-Aux/IAA2* transcripts was unaffected by application of either auxin after deblading (Figs. 4 and 5). These transcripts were not specific to the AZ and were also detected in petioles of intact (control) leaves and adjacent stem tissues (Fig. 4). The expression of two of the *Aux/AIA* genes, *Mj-Aux/AIA1* and *Mj-Aux/AIA4*, was also examined in a number of plant tissues and the data are shown in Supplemental Figure 1. Their expression patterns were very different. *Mj-Aux/AIA1* was predominantly expressed in the petal-like calyx tissue during opening and senescence of the flowers, and was also expressed, but to a lesser extent, in young stems. *Mj-Aux/AIA4*, by contrast, was expressed in young leaf, stem, ovary, fruit, root, and tuber tissues, and in flowers only at the small bud stage.

The expression patterns of 12 additional clones from the subtracted cDNA library were examined by RNA gel-blot analysis (data not shown). Only three clones (MSR-27, MSR-127, and MSR-212) were found to be down-regulated in the petiole AZ following deblading (Fig. 4). MSR-27 (AY589714) showed 53% identity to the *Actinidia delicosa* PG inhibitor protein (PGIP) gene (Z49063), MSR-127 was found to encode expansin gene ExpB2.1 (AY147412.1), and MSR-212 (AY589713) displayed 84% identity to the *Arabidopsis* β-tubulin gene (At1g75780; Table II). The rate of reduction of all three gene transcripts was slower than that observed for *Mj-Aux/AIA1*, but like *Mj-Aux/AIA1*, the decline was prevented by applying IAA, and to a much lesser extent NAA, to the cut end of the petiole (Fig. 4B). The transcripts of all three genes were also down-regulated in the stem AZ within 6 to 9 d after decapitation (Fig. 5). The CT6 and CT9, and within 6 d after decapitation in combination with Suc infiltration. Their expression, however, was not specific to the AZ (Supplemental Fig. 1).

Isolation and Characterization of Gene Transcripts That Increased in the Petiole AZ of *M. jalapa* following Deblading/Decapitation

An additional PCR-based subtractive library was constructed to isolate gene transcripts that were induced in the petiole AZ following deblading. This library was enriched with RNA species that were preferentially transcribed in the petiole AZ of 1-MCP-treated plants 3 d after deblading (tester RNA) and not present in the petiole AZ of nondebladed leaves.
FS-39 (AY589698) displayed 83% identity to a ethylene-responsive transcript ER6 (AF096262.1). FS-25 (AY589695) showed 40% identity to tomato known sequence in the GenBank database, whereas (AY589707) did not show significant homology to any FS-18 (AY589694), FS-44 (AY589704), and FS-55 (Fig. 6, M/D3) or not (Fig. 6, D3). Of the six transcripts, whether the petioles were pretreated with 1-MCP in the petiole AZ tissue within 3 d after deblading, confirmed by RNA gel-blot analysis to be induced FS-87) were chosen for further study, and all were subtraction kit user manual). These transcripts and the deblading induced (according to the PCR-select cDNA gene transcripts, 21 had a 95% probability of being array is shown in Supplemental Figure 2. Of the 94 clones selected from the subtractive library. The resulting dot-blot of deblading on the expression of 94 clones selected (driver RNA). The tester RNA plants were pretreated with 1-MCP prior to deblading since this pretreatment eliminated abscission of the debladed petioles (Fig. 2). Therefore, 1-MCP pretreatment should help select for gene transcripts involved in competency of the AZ to respond to ethylene rather than for those involved in ethylene-mediated cell separation at the AZ.

Using differential screening, we examined the effect of deblading on the expression of 94 clones selected from the subtractive library. The resulting dot-blot array is shown in Supplemental Figure 2. Of the 94 gene transcripts, 21 had a 95% probability of being deblading induced (according to the PCR-select cDNA subtraction kit user manual). These transcripts and the putative enzymes they encode are listed in Table III. Six transcripts (FS-18, FS-25, FS-39, FS-44, FS-55, and FS-87) were chosen for further study, and all were confirmed by RNA gel blot analysis to be induced in the petiole AZ tissue within 3 d after deblading, whether the petioles were pretreated with 1-MCP (Fig. 6, M/D3) or not (Fig. 6, D3). Of the six transcripts, FS-18 (AY589694), FS-44 (AY589704), and FS-55 (AY589707) did not show significant homology to any known sequence in the GenBank database, whereas FS-25 (AY589695) showed 40% identity to tomato ethylene-responsive transcript ER6 (AF096262.1). FS-39 (AY589698) displayed 83% identity to a Cicer arietinum cationic peroxidase (AJ216602.2), and FS-87 (AY589699) showed 66% identity to a Fragaria ananassa osmotin-like protein (AF532965.1). These transcripts differed in their levels of expression both before and after deblading (Fig. 6) or decapitation (Fig. 7). Some transcripts, such as FS-18, were not detected in the AZs of the nondebladed petioles (Fig. 6, lane C) or non-decapped stems (Fig. 7, lane C), whereas others, such as FS-25, FS-39, and FS-87, were detected. FS-44, FS-55, and FS-87 were also expressed in the non-AZ region of the leaf petiole and in the stem. On the other hand, no detectable expression of FS-18, FS-39, and FS-25 transcripts was observed in these tissues (Fig. 6). The abundance of some of the transcripts (FS-25, FS-44, FS-55, and FS-87) increased considerably within 1 d after deblading, whereas for others (e.g. FS-39) it took 2 d before substantially higher expression levels were observed (Fig. 6). All the transcripts showed increased expression in the stem AZ at 6 d after decapitation, which was the shortest time period examined (Fig. 7). The expression of two of the transcripts (FS-18 and FS-87) was also elevated by exposure to 1-MCP in the AZ of petioles that were not debladed (Fig. 6, M/C). Application of Suc to the cut end of the stump hastened the formation of the stem AZ and its sensitivity to ethylene, resulting in accelerated abscission of the stem stumps. In addition, the Suc treatment enhanced the induction of some of the decapitation-induced transcripts (Fig. 7, FS-44 and FS-55). As was found for the deblading-repressed gene transcripts (Fig. 4), applying NAA to the cut petiole end (Fig. 6, N/D3) or to the cut stump end (Fig. 7, N/CT9) did not affect expression of the transcripts. However, application of IAA in lanolin paste to the cut end of the petiole was effective at inhibiting the deblading-induced increase in expression of FS-18 and FS-44 transcripts even 72 h after the treatment (Fig. 8).

The expression of the up-regulated gene transcripts, like that found for the down-regulated ones, was not

### Table 1. Auxin-related gene transcripts isolated from petiole AZ tissue of *M. jalapa* and summary of RNA gel-blot analyses

<table>
<thead>
<tr>
<th>Putative Identity</th>
<th>Clone No. and Accession No.</th>
<th>Homology (Locus Tag) or Accession No.</th>
<th>High Score (E Value)</th>
<th>% Amino Acid Identity</th>
<th>% Amino Acid Positives</th>
<th>RNA Gel-Blot Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auxin-responsive factor protein</td>
<td>SM-01, AY137966</td>
<td>Arabidopsis ARF6, At1g30330</td>
<td>107 (9e-38)</td>
<td>56/102 (54%)</td>
<td>70/102 (67%)</td>
<td>No hybridization</td>
</tr>
<tr>
<td>Auxin-associated protein SM-32, AY137965</td>
<td></td>
<td>R. pseudobaccaia auxin-repressed protein, AY009094</td>
<td>108 (1e-22)</td>
<td>61/120 (62%)</td>
<td>76/120 (62%)</td>
<td>No effect of deblading or decapitation</td>
</tr>
<tr>
<td>*Mj-Aux/IAA1 SM-36, AY137961</td>
<td></td>
<td>Arabidopsis auxin-responsive protein IAA16, At3g04730</td>
<td>181 (2e-45)</td>
<td>83/96 (86%)</td>
<td>91/96 (94%)</td>
<td>Down-regulated by deblading or decapitation; IAA-restored</td>
</tr>
<tr>
<td>*Mj-Aux/IAA2 RS-31, AY137962</td>
<td></td>
<td>Gossypium barbadense ribosomal protein, AY337616.1</td>
<td>166 (2e-40)</td>
<td>76/92 (82%)</td>
<td>82/92 (89%)</td>
<td>Down-regulated by deblading or decapitation</td>
</tr>
<tr>
<td>*Mj-Aux/IAA3 RS-74, AY137963</td>
<td></td>
<td>Tobacco Nt-iaa4.1 deduced protein, AF123509</td>
<td>247 (2e-21)</td>
<td>45/48 (93%)</td>
<td>48/48 (100%)</td>
<td>(–)</td>
</tr>
<tr>
<td>*Mj-Aux/IAA4 MSR-193, AY137964</td>
<td></td>
<td>Cucumis sativus Cs-IAA2, AB026822</td>
<td>98 (1e-20)</td>
<td>44/51 (86%)</td>
<td>49/51 (95%)</td>
<td>No effect of deblading</td>
</tr>
<tr>
<td>*Mj-Aux/IAA5 MSR-282, DQ070872</td>
<td></td>
<td>Glycine max auxin-induced protein AUX22, P13088</td>
<td>54 (3e-07)</td>
<td>34/71 (47%)</td>
<td>39/71 (54%)</td>
<td>(–)</td>
</tr>
</tbody>
</table>
specific to the AZ tissue. Expression of transcripts FS-44 and FS-25 in various tissues is shown in Supplemental Figure 3. The FS-44 transcript showed high expression in flowers at different stages of development, as well as in ovary, root, and tuber tissues. FS-25 showed an increased expression pattern during flower opening and senescence, in mature and senescent leaves, and in young and mature cotyledons, mature stems, and ovaries.

Effect of IAA Depletion by NPA Treatment in M. jalapa Plants

Leaf deblading or stem decapitation are very effective treatments for removing the auxin source, but they can also result in undesirable local and systemic wounding effects caused by the localized tissue damage (León et al., 2001; Schilmiller and Howe, 2005). We used the auxin transport inhibitor NPA to exclude these possible wounding effects. This enabled us to distinguish the abscission-related responses from the ethylene-induced or ethylene-independent wound responses. NPA treatment resulted in gradual leaf abscission following the ethylene exposure, starting on day 1 and reaching 100% abscission on day 3 (Fig. 9A). The abscission rate of NPA-treated leaves during the initial 3 d following exposure to ethylene was lower than the petiole abscission in debladed plants, but significantly higher than in control untreated leaves (Fig. 9A). Application of dimethyl sulfoxide (DMSO; in which the NPA was dissolved) with lanolin paste did not have any effect on petiole abscission (data not shown).

The effect of NPA treatment on expression of some clones that were found to be up-regulated in response to deblading was analyzed by real-time PCR. The expression results of clone FS-44 are presented in Figure 9B, and of an additional five clones in Supplemental Figure 4. In general, NPA treatment clearly caused up-regulation of expression in the leaf AZ of these clones in a similar manner to the deblading treatment. In this experiment we could also see a difference in the kinetics of the increased expression of the various clones, manifested as a 1 d delay in the increased expression of clones FS-39 (Supplemental Fig. 4C) and FS-87 (Supplemental Fig. 4E).

Figure 4. Effect of abscission-modifying treatments on expression of deblading-repressed mRNA transcripts in the petiole AZ, leaf petiole (LP), and stem (ST) tissues of M. jalapa (Mj). Transcripts were isolated from the petiole AZ of M. jalapa by a PCR-based subtractive procedure using RNA isolated from AZ of nondebladed petioles as tester, and RNA isolated from AZ of petioles debladed for 3 d as driver. 1-MCP pretreatment, deblading, and auxin application were performed as detailed in Figure 2. Expression of transcripts in AZ tissues of: C, control nondebladed petioles; D1, D2, and D3, petioles 1, 2, or 3 d, respectively, after deblading; N/D3, debladed + NAA-treated petioles 3 d after deblading; M/D3, 1-MCP-treated petioles 3 d after deblading; M/C, 1-MCP-treated petioles that were never debladed; I/D3, debladed + IAA-treated petioles 3 d after deblading. LP that does not include AZ region and ST tissues were isolated from control (nondebladed) plants. Clone/accession numbers (right side) and putative enzymes encoded (left side) are indicated. Samples of 20 μg of total RNA were loaded onto the RNA gel blot. Equivalence of loading was checked by ethidium bromide staining of 28S rRNA. A representative blot stained with ethidium bromide is shown.

Figure 5. Effect of abscission-modifying treatments on expression of deblading-repressed mRNA transcripts in the stem AZ of M. jalapa (Mj). Transcripts were isolated from the petiole AZ of M. jalapa by a PCR-based subtractive procedure using RNA isolated from AZ of nondebladed petioles as tester, and RNA isolated from AZ of petioles debladed for 3 d as driver. Stem decapitation and auxin application were performed as detailed in Figure 3. Expression of transcripts in AZ tissues of: C, control nondecapitated stumps; CT6 and CT9, stumps 6 or 9 d, respectively, after decapitation; N/CCT9, stumps 9 d after decapitation and NAA treatment; CTSuc6, stumps 6 d after decapitation and infiltration with 2% Suc; I/CCT9, stumps 9 d after decapitation and IAA treatment. Clone/accession numbers (right side) and putative enzymes encoded (left side) are indicated. Samples of 20 μg of total RNA were loaded onto the RNA gel blot. Equivalence of loading was checked by ethidium bromide staining of 28S rRNA. A representative blot stained with ethidium bromide is shown.
DISCUSSION

It is well known that organ abscission is controlled by the interplay between auxin and ethylene. While our knowledge of the biology of organ abscission and genes of hydrolytic enzymes involved in cell separation processes in the AZ has been increased greatly (Sexton and Roberts, 1982; Osborne, 1989; Sexton, 1997; Taylor and Whitelaw, 2001), there is still very little known about the control of abscission competence at the molecular level. This study aimed to identify mRNA transcripts in AZ tissues whose activated or suppressed expression enabled the AZ to gain competence to respond to ethylene. We hoped to accomplish this by isolating transcripts in the AZ whose expression changed in abundance as the AZ became sensitive to ethylene, but was not abscising. Such gene expression might be associated with increased competence to abscise. For this purpose we used the ethylene action inhibitor 1-MCP as a means for focusing our gene libraries on the acquisition of the abscission competence rather than on the ethylene-dependent abscission events.

IAA Depletion Elicits Competence to Abscise by Increasing the Ability of the AZ to Respond to Ethylene

Results from our initial physiological characterization of the abscission process in M. jalapa indicated that petiole abscission of debladed leaves and internodal stump abscission of decapitated shoots were mediated through ethylene, as pretreatment of the plants with 1-MCP prevented shedding of these organs following deblading/decapitation (Figs. 2 and 3). We showed that this abscission was not solely the result of increased ethylene produced by the detachment, because exogenously applied ethylene did not cause petiole (Fig. 1) or stump (Fig. 3) abscission in intact plants. This suggests that deblading or decapitation caused abscission by increasing the competence of the petiole or stump AZs, respectively, to respond to ethylene. Previous research indicates that the increased competence of AZ tissues to respond to ethylene is due to the reduced auxin flux across the AZ, that occurs either during aging or by removing the auxin source (i.e. the leaf blade or the stem above a node; Beal and Whiting, 1945; Rubinstein and Leopold, 1963; Warren-Wilson et al., 1986; Sexton, 1997; Taylor and Whitelaw, 2001). Our results showing that immediate application of IAA to the cut end of the petiole (Fig. 2) or stump (Fig. 3) decreased the effectiveness of ethylene at causing abscission in M. jalapa were consistent with these findings. We tested both native (IAA) and synthetic (NAA) auxins to guarantee a clear auxin effect, because IAA is sometimes ineffective due to its rapid metabolism in tissue (Woodward and Bartel, 2005), and NAA at times is ineffective due to its impaired transport in tissue (Abebie et al., 2005a). Our results further illustrated how the different auxins can vary in their ability to affect abscission processes, with IAA being better than NAA at inhibiting both petiole abscission in debladed leaves (Fig. 2) and stump abscission in decapitated stems (Fig. 3).

Further confirmation for auxin regulation of the process was obtained by application of the IAA transport inhibitor NPA, which caused leaf abscission in response to ethylene similar to the deblading-induced petiole abscission (Fig. 9A). This clearly shows that reducing IAA flux across the AZ without wounding leads to increased competence of the AZ to respond to ethylene. The gradual leaf abscission following the NPA treatment compared to the fast petiole abscission following deblading (Fig. 9A) may be attributed to the relative effectiveness of these two treatments in IAA elimination, as in the presence of NPA the IAA source is still present in the tissue. The NPA results ruled out any local and systemic wounding effects (León et al., 2001; Schilmiller and Howe, 2005) that could be imposed by deblading or decapitation, thereby distinguishing the abscission-related response from possible ethylene-induced or ethylene-independent wound responses. In addition, any wound ethylene action (León et al., 2001) that could exhibit the main wounding effect associated with abscission, would be inhibited by the 1-MCP pretreatment (Sisler and Serek, 1997). Thus, it may be concluded that the wounding by deblading or decapitation did not cause any artifact in our system, and the responses obtained following these treatments could be attributed to auxin depletion.

We applied Suc to the cut end of the decapitated stem to test whether Suc flow to the Mirabilis roots and tuber via the node AZ might also have a role in inhibiting

<table>
<thead>
<tr>
<th>Putative Identity Clone No. and Accession No.</th>
<th>Homology (Locus Tag) or Accession No.</th>
<th>High Score (E Value)</th>
<th>% Amino Acid Identity</th>
<th>% Amino Acid Positives</th>
<th>RNA Gel-Blot Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGIP inhibitor protein MSR-27, AY589714</td>
<td>A. deliciosa PGIP, Z49063</td>
<td>90 (4e-18)</td>
<td>41/77 (53%)</td>
<td>54/77 (69%)</td>
<td>IAA restored</td>
</tr>
<tr>
<td>β-Expansin MSR-127, AY147412.1</td>
<td>Arabidopsis pollen allergen (β-expansin), At3g45970</td>
<td>102 (2e-21)</td>
<td>46/94 (48%)</td>
<td>62/94 (65%)</td>
<td>IAA restored</td>
</tr>
<tr>
<td>β-Tubulin MSR-212, AY589713</td>
<td>Putative tubulin β-1 chain, At1g75780</td>
<td>92 (0.027)</td>
<td>18/18 (100%)</td>
<td></td>
<td>IAA restored</td>
</tr>
</tbody>
</table>
abscission. Surprisingly, we found that rather than inhibiting abscission, the Suc treatment increased the rate at which abscission occurred. Why this is so is unclear, but it may be related to the ability of Suc to affect hormone signaling (León and Sheen, 2003).

**Table III. Gene transcripts isolated from petiole AZ tissue and shown by differential screening to be up-regulated in the AZ by deblading**

Transcripts additionally confirmed by RNA gel blot to be up-regulated by deblading are shown in bold. Genes were isolated by a PCR-based subtractive procedure using RNA extracted from the petiole AZ of 1-MCP-treated plants 3 d after deblading (tester) and RNA extracted from AZ of control leaf (driver). Dot-blot array is shown in Supplemental Figure 2. EDGP, Extracellular dermal glycoprotein.

<table>
<thead>
<tr>
<th>Putative Identity Clone No. and Accession No.</th>
<th>Homology (Locus Tag) or Accession No.</th>
<th>High Score (E Value)</th>
<th>% Amino Acid Identities</th>
<th>% Amino Acid Positives</th>
<th>RNA Gel-Blot Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown FS-01, AY589693</td>
<td>Daucus carota EDGP precursor, D14550</td>
<td>38.1 (0.1)</td>
<td>16/30 (53%)</td>
<td>22/30 (73%)</td>
<td>Up-regulated by deblading/decapitation and Suc; IAA repressed</td>
</tr>
<tr>
<td>Unknown FS-10, AY589700</td>
<td>Arabidopsis hypothetical protein, At4g37030</td>
<td>96 (1e-04)</td>
<td>21/27 (77%)</td>
<td>23/27 (84%)</td>
<td>Up-regulated by deblading</td>
</tr>
<tr>
<td>Unknown FS-11, AY589701</td>
<td>No similarity</td>
<td></td>
<td>16/30 (53%)</td>
<td>22/30 (73%)</td>
<td></td>
</tr>
<tr>
<td>Unknown FS-18, AY589694</td>
<td>No similarity</td>
<td></td>
<td>21/27 (77%)</td>
<td>23/27 (84%)</td>
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</tr>
<tr>
<td><strong>Ethylene-responsive protein FS-25, AY589695</strong></td>
<td>Tomato ethylene-responsive ER6 protein, AF096262.1</td>
<td>108 (3e-24)</td>
<td>66/161 (40%)</td>
<td>93/161 (56%)</td>
<td>Up-regulated by deblading/decapitation and Suc; IAA repressed</td>
</tr>
<tr>
<td>Peroxidase FS-29, AY589696</td>
<td>Arabidopsis peroxidase, X98317.1</td>
<td>327 (7e-89)</td>
<td>159/221 (71%)</td>
<td>182/221 (81%)</td>
<td>Up-regulated by deblading</td>
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<tr>
<td>Terpene-synthase-like FS-35, AY589702</td>
<td>Arabidopsis linalool synthase protein, At1g61120</td>
<td>332 (4e-30)</td>
<td>69/137 (50%)</td>
<td>94/137 (68%)</td>
<td>Up-regulated by deblading/decapitation and Suc; IAA repressed</td>
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<tr>
<td>Kinesin-like protein FS-38, AY589697</td>
<td>Arabidopsis kinesin light chain, At1g27500</td>
<td>301 (3e-81)</td>
<td>147/213 (68%)</td>
<td>181/213 (84%)</td>
<td>Up-regulated by deblading</td>
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<tr>
<td>Peroxidase FS-39, AY589698</td>
<td>C. arietinum cationic peroxidase, AI21660.2</td>
<td>137 (3e-32)</td>
<td>62/74 (83%)</td>
<td>64/74 (91%)</td>
<td>Up-regulated by deblading</td>
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<tr>
<td>FKBPI2-like protein FS-43, AY589703</td>
<td>Arabidopsis FKBPI2 interacting protein, At3g54170</td>
<td>673 (4e-69)</td>
<td>171/249 (68%)</td>
<td>210/249 (84%)</td>
<td>Up-regulated by deblading/decapitation and Suc; IAA repressed</td>
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<tr>
<td><strong>Unknown FS-44, AY589704</strong></td>
<td>No similarity</td>
<td></td>
<td>16/30 (53%)</td>
<td>22/30 (73%)</td>
<td></td>
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<tr>
<td><strong>CAP-binding protein FS-46, AY589705</strong></td>
<td>Arabidopsis nuclear CAP-binding protein, At5g44200</td>
<td>614 (2e-62)</td>
<td>129/146 (88%)</td>
<td>138/146 (94%)</td>
<td>Up-regulated by deblading/decapitation and Suc; IAA repressed</td>
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<tr>
<td><strong>Phosphorylase-domain protein FS-48, AY589706</strong></td>
<td>Populus balsamiterra vegetative storage protein, L20233.1</td>
<td>128 (4e-29)</td>
<td>75/231 (32%)</td>
<td>132/231 (56%)</td>
<td>Up-regulated by deblading/decapitation and Suc; IAA repressed</td>
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<tr>
<td><strong>Unknown FS-55, AY589707</strong></td>
<td>No similarity</td>
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<td>16/30 (53%)</td>
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<tr>
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<td>22/30 (73%)</td>
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<tr>
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<td>No similarity</td>
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<tr>
<td>Unknown FS-80, AY589710</td>
<td>No similarity</td>
<td></td>
<td>16/30 (53%)</td>
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<tr>
<td>Thaumatin-like protein FS-82, AY589711</td>
<td>Tobacco PR protein, AY1745249.1</td>
<td>161 (1e-39)</td>
<td>72/110 (65%)</td>
<td>86/110 (77%)</td>
<td>Up-regulated by deblading and decapitation</td>
</tr>
<tr>
<td><strong>Osmotin-like protein FS-87, AY589699</strong></td>
<td>Fragaria ananassa osmotin-like protein, AF532965.1</td>
<td>176 (5e-74)</td>
<td>120/180 (66%)</td>
<td>136/180 (74%)</td>
<td>Up-regulated by deblading and decapitation</td>
</tr>
<tr>
<td>Lysophospholipase-like protein FS-93, AY589712</td>
<td>Arabidopsis lysophospholipase isolog, At1g11090</td>
<td>205 (1e-52)</td>
<td>98/153 (64%)</td>
<td>119/153 (77%)</td>
<td>Up-regulated by deblading and decapitation</td>
</tr>
</tbody>
</table>

Molecular Analysis of AZ Genes Modified by IAA Depletion

The time interval obtained between the deblading and actual petiole abscission in response to ethylene (Fig. 1) indicates that there was a clear separation in time between the tissue’s ability to respond to ethylene and the occurrence of the hydrolytic processes necessary for separation of cells in the AZ. This suggested that we could isolate RNA from tissue that was undergoing a change in sensitivity to ethylene, but likely not yet undergoing changes associated with cell separation at the AZ. We hypothesized that the increased ethylene sensitivity of the AZ resulting from deblading would be due to changes in gene expression resulting from the altered auxin flux across the AZ.

Decreased gene expression would likely occur for genes whose expression was normally maintained by the auxin flux across the AZ, whereas increased gene expression would likely occur for genes that were normally repressed by this auxin flux.

**Analysis of Aux/IAA AZ Genes Suppressed following IAA Depletion**

We made two subtractive libraries to identify these gene transcripts. In the first we selected for mRNA transcripts in the AZ tissue that were down-regulated within 3 d of deblading, during which the AZ had already gained competence to abscise in response to ethylene. We speculated that these would be the genes whose expression would normally be maintained by the auxin flux. Interestingly, we found that five of the mRNA transcripts we identified in this screen showed homology to members of the *Aux/IAA* gene family (Table I). This gene family contains members that are rapidly induced within 5 to 60 min of IAA application (Abel and Theologis, 1996). It is now known that the *Aux/IAA* proteins are actually repressors of auxin-induced transcription, and auxin promotes the degradation of this large family of transcriptional regulators, leading to diverse downstream effects (Worley et al., 2000; Gray et al., 2001). This allows ARF proteins to bind to auxin response elements within promoters and either activate or repress target gene expression. Rapid induction of the *Aux/IAA* genes is a response to falling levels of the *Aux/IAA* proteins that ensures a tightly controlled transient response to changes in auxin concentrations via a negative feedback (Leyser, 2002; Woodward and Bartel, 2005). However, it should be noted that in abscising systems, a continuous auxin flow through the AZ, resulting in continuous expression of *Aux/IAA* genes, is required for preventing ethylene sensitivity and abscission (Taylor and Whitelaw, 2001).

RNA gel-blot analysis confirmed that the expression of two of the transcripts that showed homology to members of the *Aux/IAA* gene family (*Mj-Aux/IAA1* and *2*) was repressed by deblading or decapitation. This repression was inhibited by applying IAA to the cut end of the petiole (Fig. 4) or stump (Fig. 5), which also prevented abscission (Figs. 2 and 3). A similar correlation between the effectiveness of auxin in delaying abscission and induction of *Aux/IAA* gene expression was reported recently in *Cestrum elegans* (Abebie et al., 2005b). Application of the synthetic auxin 2,4-dichlorophenoxy acetic acid, which delayed floret abscission in this system (Abebie et al., 2005a), induced a higher expression of *Aux/IAA* genes in the floret AZ. In addition, it was recently reported that Arabidopsis mutation in *ARF2* exhibited pleiotropic

![Figure 6](image_url) Effect of abscission-modifying treatments on expression of deblading-induced mRNA transcripts in the petiole AZ, leaf petiole (LP), and stem (ST) tissues of *M. jalapa*. Transcripts were isolated from the petiole AZ of *M. jalapa* by a PCR-based subtractive procedure using RNA isolated from the petiole AZ of 1-MCP-treated plants 3 d after deblading as tester, and RNA isolated from the AZ of nondebladed petioles as driver. 1-MCP pretreatment, deblading, and NAA application were performed as detailed in Figure 2. Expression of transcripts in AZ tissue of: C, control nondebladed petioles; D1, D2, and D3, petioles 1, 2, or 3 d, respectively, after deblading; M/D3, 1-MCP-treated petioles 3 d after deblading; N/D3, NAA-treated petioles 3 d after deblading; M/C, 1-MCP-treated petioles that were never debladed. LP that does not include AZ region and ST tissues were isolated from control (nondebladed) plants. Clone/accession numbers (right side) and putative enzymes encoded (left side) are indicated. Samples of 20 µg of total RNA were loaded onto the RNA gel blot. Equivalence of loading was checked by ethidium bromide staining of 28S rRNA. A representative blot stained with ethidium bromide is shown.

![Figure 7](image_url) Effect of abscission-modifying treatments on expression of deblading-induced mRNA transcripts in the stem AZ of *M. jalapa*. Transcripts were isolated from the petiole AZ of *M. jalapa* by a PCR-based subtractive procedure using RNA isolated from the petiole AZ of 1-MCP-treated plants 3 d after deblading as tester, and RNA isolated from the AZ of nondebladed petioles as driver. 1-MCP pretreatment, stem decapitation, and NAA application were performed as detailed in Figure 3. Expression of transcripts in AZ tissue of: C, control non-decapitated stumps; CT6 and CT9, stumps 6 or 9 d, respectively, after decapitation; N/CT9, stumps 9 d after decapitation and NAA treatment; CTSuc6, stumps 6 d after decapitation and infiltration with 2% Suc. Clone/accession numbers (right side) and putative enzymes encoded (left side) are indicated. Samples of 20 µg of total RNA was loaded onto the RNA gel blot. Equivalence of loading was checked by ethidium bromide staining of 28S rRNA. A representative blot stained with ethidium bromide is shown.
developmental phenotypes, including delayed senescence and abscission (Okushima et al., 2005). ARF2 is thought to function as a transcriptional repressor, and its pleiotropic effects may be mediated by negatively modulating the transcription of downstream genes in the ethylene signaling pathways that are involved in cell growth, senescence, and abscission (Okushima et al., 2005). Based on these findings, it is possible to assume that Mj-Aux/IAA1 and 2 genes are expressed when there is a continuous IAA flow through the AZ and the Aux/IAA proteins form dimers with a possible ARF2 or other ARF homologs in Mirabilis. This situation is associated with low ethylene sensitivity of the AZ.

The regulation by IAA is important in our study, as the cells in the debladed petiole tissue are potentially influenced by numerous signals (e.g. wound and dehydration) resulting from the excision that may not be related to the AZ formation. The restoration of their expression by IAA application provides evidence that it is the removal of the IAA source rather than the stress of excision that causes their altered expression. The expression of Mj-Aux/IAA1 was also examined in other plant tissues, and it was found not to be specific to the AZ. Rather, it was shown to accumulate in the non-AZ portion of the petiole, the stem, and in high amounts in the petal-like calyx (Supplemental Fig. 1), in agreement with the findings of Gookin et al. (2003). This suggests that although the capability to acquire ethylene sensitivity leading to abscission is specific to the AZ tissue (Osborne, 1989), the capability to respond to auxin via expression of Aux/IAA genes exists also in various nonabscising tissues. Taken together, we cannot at this time yet say what role the Aux/IAA members we identified have in the abscission process, but we have shown for the first time a correlation between acquisition of competence to respond to ethylene and decline in abundance of auxin regulatory gene transcripts.

Possible Roles for Some Identified Genes Suppressed following IAA Depletion

Other mRNA transcripts that were down-regulated by deblading showed homology to a PGIP, β-expansin, and β-tubulin, but their association with abscission is not clear. It was surprising to find that the PGIP transcript was down-regulated in the AZ of debladed leaves (Figs. 4 and 5) when increased accumulation of pathogenesis-related proteins is a common feature in AZs (Roberts et al., 2002). PGIPs are Leu-rich repeat cell wall glycoproteins that protect tissues from pathogen attack by inhibiting the action of fungal PGs, but shown...
These proteins, together with microtubule function (kinesin-like protein), and others binding protein, and phosphorylase-domain protein), cellular signaling (FKBP12-like protein, capping protein and osmotin-like protein), abscission (peroxidase-like protein and thaumatin-like protein), stress (ER6-like protein, and osmotin-like protein), abscission (peroxidase), cellular signaling (FKBP12-like protein, capping binding protein, and phosphorylase-domain protein), microtubule function (kinase-like protein), and others whose functions are still unknown (Table III). We used RNA gel-blot or real-time PCR analyses to confirm the deblading or NPA induction, respectively, of a number of the transcripts (Figs. 6 and 9A; Supplemental Fig. 4), including those encoding a putative thaumatin-like protein, peroxidase, and three unknown transcripts, FS-18, FS-44, and FS-55. The roles of these identified proteins in abscission are currently not clear. Among the unknown transcripts, FS-18 was strongly up-regulated in the petiole AZ, apparently independently of ethylene, although the finding that this transcript was induced in nondebladed tissue by 1-MCP (Fig. 6) suggests that it might be actively repressed by endogenous ethylene. The induction of FS-18 was also found to be repressed by IAA in the leaf AZ (Fig. 8), strongly indicating its regulation by IAA.

The addition of Suc to the cut end of the decapitated internode further increased the expression levels of some of the decapitation up-regulated transcripts (Fig. 7). This suggests that the regulation of these decapitation-induced transcripts via carbohydrate signaling is probably additional to their up-regulation by auxin depletion. Suc application also accelerated the abscission of the cut internode (data not shown), confirming previous findings showing that Suc induced abscission of internodal explants of Impatiens sultani (Warren-Wilson et al., 1986).

**Toward Elucidating a General Abscission Mechanism**

In our study we were able to isolate gene transcripts that increase or decrease in the petiole AZ during the time it developed competency to respond to ethylene by petiole abscission. We tested some of these transcripts for their expression in leaf and stem AZs, and for their regulation by abscission-modulating factors such as 1-MCP, IAA, NPA, and Suc. The similarity in the expression patterns of transcripts, isolated only from the leaf AZ, in both leaf and stem AZs following deblading/decapitation and abscission-modifying treatments, points to a general abscission mechanism. This suggests that auxin induces or represses in both AZ tissues the same type of regulatory gene transcripts, which retain the AZ insensitive to ethylene. It is possible that the change in abundance of part of these transcripts leads to changes that affect the ability of the tissue to respond to ethylene. It should be noted that the expression of the majority of the transcripts we studied was found to be unaffected by the 1-MCP pretreatment, suggesting that if they are involved in abscission they are not regulated by ethylene but by IAA. It is possible that this IAA regulation of AZ sensitivity to ethylene may operate by affecting components downstream to

### Table IV. List of primers used for the real-time PCR reactions

<table>
<thead>
<tr>
<th>Mirabilis Clone No.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS-18</td>
<td>5′-AAGACAGTGGAAAGGGGACAA</td>
<td>5′-GAATGACAGCCTAGTGTTTAA</td>
</tr>
<tr>
<td>FS-25</td>
<td>5′-CGATTTGGAGCCGAAGAACCT</td>
<td>5′-GCTGGTTCGGAGAGAGTTTT</td>
</tr>
<tr>
<td>FS-39</td>
<td>5′-ACTGGGTCCACACATGGGTATAC</td>
<td>5′-TGCTTTGCTTGAGACCTGCA</td>
</tr>
<tr>
<td>FS-44</td>
<td>5′-GACCAAGTACATGGGAAAAA</td>
<td>5′-TGGCGATACCTTATGCCTTAA</td>
</tr>
<tr>
<td>FS-55</td>
<td>5′-GCGAACAACAGATGCTCTAGT</td>
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<td>FS-87</td>
<td>5′-TCAATGTGCCCATGACCTTTC</td>
<td>5′-TGCGAGGAACATTGACGT</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5′-GGCACGGCATCACAAATTTC</td>
<td>5′-TCCGAATCGAAGCCCTAATT</td>
</tr>
</tbody>
</table>

to be ineffective against other pectic enzymes of either microbial or plant origin (D’Ovidio et al., 2004). This appears to rule out a role for these proteins in regulating plant PGs involved in forming the AZs of *M. jalapa*.

Our study showed that the β-expansin mRNA of *M. jalapa* was down-regulated in the AZ tissue during the time the AZ became responsive to ethylene (Fig. 4). It appears unlikely that down-regulation of an expansin gene would function to enable the AZ to respond to ethylene. Expansins are proteins that cause cell wall loosening during expansion and growth, and evidence is now accumulating that they also have a role in abscission (Roberts et al., 2002, and refs. therein). Interestingly, the β-expansin transcript is also down-regulated dramatically as the young floral buds of *M. jalapa* open, develop, and senesce (Gookin et al., 2003; Supplemental Fig. 1). The role that β-tubulins would have in the developing AZ is unclear. As it is known that AZ cells undergo distinct morphological changes (Osborne, 1989; Roberts et al., 2002), it is possible that tubulins may be involved in such changes. These proteins, together with α-tubulins, make up the microtubule arrays of the cytoskeleton that determine cellular shape in response to a variety of internal and external cues (Breviario and Nick, 2000).

### Analysis of AZ Genes Induced following IAA Depletion

Our second subtractive library enabled us to isolate gene transcripts that were induced in the AZ by either deblading or NPA treatment during the time the zone became competent to respond to ethylene, but before abscission of the plant organs. We pretreated the plants with 1-MCP to select for genes that increased in expression independently of ethylene. We identified gene transcripts that were induced in the AZ by either deblading/decapitation and abscission-modifying factors such as 1-MCP, IAA, NPA, and Suc. The similarity in the expression patterns of transcripts, isolated only from the leaf AZ, in both leaf and stem AZs following deblading/decapitation and abscission-modifying treatments, points to a general abscission mechanism. This suggests that auxin induces or represses in both AZ tissues the same type of regulatory gene transcripts, which retain the AZ insensitive to ethylene. It is possible that the change in abundance of part of these transcripts leads to changes that affect the ability of the tissue to respond to ethylene. It should be noted that the expression of the majority of the transcripts we studied was found to be unaffected by the 1-MCP pretreatment, suggesting that if they are involved in abscission they are not regulated by ethylene but by IAA. It is possible that this IAA regulation of AZ sensitivity to ethylene may operate by affecting components downstream to

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<td>18S rRNA</td>
<td>5′-GGCACGGCATCACAAATTTC</td>
<td>5′-TCCGAATCGAAGCCCTAATT</td>
</tr>
</tbody>
</table>


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ETR1 or ERS1 receptors in the ethylene signal transduction pathway (Zhou et al., 1996).

Many of the transcripts we isolated encoded proteins of unknown functions and merit further examination. It will be particularly interesting to examine the effect on abscission of the modulated expression of Aux/IAA and other genes that we identified in our study in M. jalapa. However, despite the obvious anatomical advantages that the Mirabilis system provides in studying genes in the AZ, we are aware that other common model systems, such as tomato or Brassica, will likely be more useful for further research and functional analysis. Accordingly, experiments with genes isolated from tomato AŽs and their analysis by tomato microarrays are currently under way.

MATERIALS AND METHODS

Plant Systems and Treatments

Experiments were performed with Mirabilis jalapa plants. The plants were grown in pots filled with a mix of one-third fir (Abies spp.) bark, one-third sand, and one-third peat moss under standard greenhouse conditions. Seedlings were trimmed at the fifth node to initiate side shoots, and flower buds were constantly removed. Both leaf and stem AZ systems of M. jalapa were studied. IAA flow from the leaf blade or stem internode to the corresponding AZs was eliminated by either leaf deblading or stem decapitation, respectively. Leaf deblading was performed by cutting the petiole at 1.5 cm from the AZ with a sharp razor blade. Lanolin paste was applied on the cut surface to prevent water stress and as a control for the auxin treatments (see below). Stem decapitation was performed by cutting the stem 5 cm above the stem node AZ. The cut surface was treated similarly to that of the petioles. To avoid wounding, IAA flow from the leaf blade was also eliminated by application of the auxin transport inhibitor NPA. An aliquot of 0.025 g of NPA (Chem Service) was dissolved in 0.5 mL DMSO and mixed with 5 g of warmed (50°C) lanolin paste to a final concentration of 0.5% (w/w). The lanolin paste containing the NPA was applied by spreading it as a ring around the leaf petiole, in the section close to the leaf blade. The same amount of lanolin paste with DMSO was similarly applied as control. To renew the auxin supply to the leaf blade, in the section close to the leaf blade, the NPA-containing lanolin paste was applied again. To renew the auxin supply following deblading or decapitation, IAA (10⁻³ M) or NAA (2 x 10⁻³ M) was immediately applied in lanolin paste to the cut surface of the remaining tissues. Control plants had lanolin alone applied to the cut surface. Both native (IAA) and synthetic (NAA) auxins were used to guarantee a clear auxin effect. The ethylene action inhibitor 1-MCP (0.2 μL L⁻¹ for 24 h at 20°C) was applied 1 d before leaf deblading or stem decapitation, to block any direct effects of ethylene on gene expression (Sisler and Serek, 1997). Suc was introduced to the AZ with a sharp razor blade. Lanolin paste was applied on the cut surface to prevent water stress and as a control for the auxin treatments (see below).

Subtracted Library Construction and RNA Gel-Blot Analysis

Total RNA was isolated from the stored tissue as outlined by Hunter et al. (2002). Poly A⁺ RNA was isolated from total RNA using the PolyATtract mRNA isolation system IV kit (Promega) according to manufacturer’s instructions. Differentially expressed genes were isolated using the PCR-select cDNA subtraction kit (CLONTECH) according to the manufacturer’s instructions. Putative auxin-maintained (deblading-repressed) clones were isolated using RNA (1 μg Poly A⁺) from the petiole AZ of control nondebladed leaves (as tester RNA), and RNA (1 μg Poly A⁺) from the petiole AZ of leaves debladed for 3 d (as driver RNA). Putative auxin-repressed (deblading-induced) clones were isolated using RNA from the petiole AZ of leaves pretreated with 1-MCP and debladed for 3 d (as tester RNA), and RNA from the petiole AZ of control nondebladed leaves (as driver RNA). Subtracted cDNA sequences were TA cloned into the pGem-T Easy vector system II (Promega) and transformed into JM109 high-efficiency competent cells supplied with the kit. Clones that contained inserts were identified by colony PCR as described by Hunter et al. (2002). The inserts were sequenced with an automated DNA sequencer (model 373, PRISM, Applied Biosystems) at the Plant Genetics Facility (University of California, Davis). The putative identity of the subtracted sequences was obtained by comparing them to sequences in the GenBank database using the BLASTX algorithm (Altschul et al., 1990). The expression of the subtracted sequences was examined by northern analyses as outlined in Hunter et al. (2002). In brief, 20 μg total RNA was isolated from leaf or stem AZ tissues following the various treatments detailed above or from other organs of M. jalapa, including various developmental stages of flowers, leaves, cotyledon, stem, ovary, young fruit, roots, and tuber. RNA samples were separated on a 1% agarose gel containing 0.22 M formaldehyde and blotted onto Hybond N⁺ (Amersham Pharmacia Biotech) using the alkaline downward procedure of Ingelbrecht et al. (1998). The cDNA sequences were radiolabeled with ³²P-dATP using the Strip-EZ DNA labeling kit (Ambion) after purification through G50 PrepQuant columns (Amersham). The labeled probes were denatured and added to UltraHyb Ultrasensitive Hybridization buffer (Ambion), bathing the membranes at 42°C. After overnight incubation, the membranes were washed sequentially for 15 min with 2 x sodium chloride sodium citrate (SSC) buffer (0.3 M NaCl/0.03 M sodium citrate pH 7.2), 1 x SSC, and 0.1 x SSC at 65°C. All SSC solutions contained 0.1% (w/v) sodium lauryl sulfate. Membranes were analyzed for radioactivity with the Molecular Dynamics STORM PhosphorImager system. If the membranes were reused they were first stripped following the instructions of the Strip-EZ DNA labeling kit (Ambion).

Real-Time PCR

Transcription levels were quantified by means of the real-time PCR method, using Sybr-green amplification kits (Absolute QPCR SYBR Green mix) and the Rotor-Gene 3000 sequence detection system (Corbett Research) according to the manufacturer’s instructions. cDNA was prepared by using Moloney murine leukemia virus reverse transcriptase and random primers (Promega). Ribosomal 18S RNA served to normalize expression. The primers used (detailed in Table IV) were designed with the Primer Express software (Applied Biosystems). The amplification efficiency for each primer pair was determined by serial dilutions of the cDNA (1:5, 1:25, 1:125, 1:625, and 1:3,125 for the target genes and 1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000 for the ribosomal 18S). A 1:100 reaction mixture contained 2.5 μL of 1:10 diluted cDNA (or 1:100 diluted cDNA for Ribosomal 18S amplification), 5 μL of Absolute QPCR SYBR Green mix, primers at a final concentration of 2.5 μM, and water to a final volume of 10 μL. PCR conditions were 95°C for 15 min (activation of Thermocycler DNA polymerase), 40 cycles at 94°C for 1 s, at 62°C for 15 s, and at 72°C for 20 s. After the amplification steps, the melting curve was determined for each primer pair to verify the presence of only one specific product. The reactions were performed in triplicate using the same cDNA and the results were averaged. Two different experiments were carried out. The comparative quantitation values were calculated with the analysis software supplied with the detection system.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY137961.4, AY137962.4, and AY1380969-714.

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