Analysis of Gene Promoters for Two Tomato Polygalacturonases Expressed in Abscission Zones and the Stigma

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The tomato (*Lycopersicon esculentum* cv Ailsa Craig) polygalacturonase genes *TAPG1* (*LYCes;Pga1;2*) and *TAPG4* (*LYCes;Pga1;5*) are abundantly expressed in both abscission zones and the pistils of mature flowers. To further investigate the spatial and temporal expression patterns for these genes, the *TAPG* gene promoters were ligated to β-glucuronidase (GUS) reporter genes and transformed into tomato. GUS expression with both constructs was similar and entirely consistent with the expression patterns of the native gene transcripts. GUS activity was observed in the weakening abscission zones of the leaf petiole, flower and fruit pedicel, flower corolla, and fruit calyx. In leaf petiole and flower pedicel zones this activity was enhanced by ethylene and inhibited by indole-3-acetic acid. On induction of abscission with ethylene, GUS accumulation was much earlier in TAPG4:GUS than in TAPG1:GUS transformants. Moreover, TAPG4:GUS staining appeared to predominate in the vascular bundles relative to surrounding cortex cells whereas TAPG1:GUS was more evenly distributed across the separation layer. Like the native genes, GUS was also expressed in the stigma. Activity was not apparent in pistils until the flowers had opened and was confined to the stigma and style immediately proximal to it. A minimal promoter construct consisting of a 247-bp 5'-upstream element from *TAPG1* was found to be sufficient to direct GUS expression in both abscission zones and the stigma.

Polygalacturonase (PG) (EC 3.2.1.15) hydrolyzes pectin in the cell wall and middle lamella of plant cells. Pectin polysaccharides make up approximately 30% of the dry mass of the primary cell wall in flowering plants and a much greater proportion of the middle lamella (Carpita and Gibeaut, 1993). PG expression increases during a number of developmental processes thought to involve cell wall breakdown. These include fruit ripening (Fischer and Bennett, 1991), lateral root emergence (Peretto et al., 1992), root cap cell detachment (Hawes and Lin, 1990), organ separation (abscission) (Tucker et al., 1984), pod dehiscence (Jenkins et al., 1996), and pith autolysis (Huberman et al., 1993). In addition, wounding (Bergey et al., 1999) and attack by plant pathogens (Ryan and Farmer, 1991; Cote and Hahn, 1994) also correlate with an increase in PG expression. Despite a great deal of work to elucidate the role of PG in these processes, its function is still a matter of speculation (Hadfield and Bennett, 1998).

In tomato (*Lycopersicon esculentum* cv Ailsa Craig) there are currently nine different PGs recorded in the public nucleotide databases. One of the nine PGs is specific to fruit ripening (Della Penna et al., 1986). Several are expressed in weakening abscission zones and pistils (Kalaitzis et al., 1997; Hong and Tucker,

1998, 2000), and another is induced by wounding and systemin (Bergey et al., 1999). The expression patterns for two of the nine PG genes have not been determined (Hong and Tucker, 1998).

Our primary interest in the PG genes is their use as tools to study molecular mechanisms that control the abscission process. Abscission and the synthesis of PG in abscission zones in dicotyledonous plants are stimulated by ethylene and suppressed by auxin (Sexton and Roberts, 1982; Sexton, 1995). The balance between these two hormones may determine the timing of abscission (Sexton, 1995). Despite the significance of abscission to agricultural productivity, the molecular mechanisms underlying tissue differentiation and hormonal control are not currently understood.

The weakening of tomato abscission (TA) zones and subsequent organ separation is typical of most abscission systems that have been studied (Sexton, 1995). Abscission of the flower pedicel of tomato involves the breakdown of the connective middle lamella and partial degradation of the primary wall in a four- to 10-cell-wide separation layer (Roberts et al., 1984; Tucker et al., 1984). In common with gene expression examined in abscission zones of other plants, the weakening of tomato zones is correlated with the localized synthesis of both endoglucanases (Lashbrook et al., 1994; del Campillo and Bennett, 1996) and PGs (Roberts et al., 1984; Kalaitzis et al., 1997).

Several of the tomato PG genes expressed during abscission are also expressed in mature pistils (Kalaitzis et al., 1997). The breakdown of the middle lamella between the longitudinal files of cells composing the transmitting tract in pistils resembles the cell separation that occurs during abscission (Cresti et al., 1976; Dumas et al., 1978).

In addition to a role for PGs in the digestion of cell walls, they are also implicated in the elicitation of defense responses and signaling events in growth and development (Ryan and Farmer, 1991; Cote and Hahn, 1994). Oligogalacturonides released from the plant cell wall during infection have been shown to induce the accumulation of several pathogenesis-related (PR) genes (Cote and Hahn, 1994). Moreover, Bergey et al. (1999) recently described the cloning of a PG cDNA induced by wounding. They suggested that PG gene expression might potentiate a defense response elicited by wounding. Defense genes are expressed in ripening fruit (Dopico et al., 1993), mature pistils (del Campillo and Lewis, 1992; Harikrishna et al., 1996), and abscission zones (del Campillo and Lewis, 1992). Expression of defense genes in these tissues may be important in the prevention of opportunistic infection of cells made vulnerable by the loss of a protective cell wall structure (del Campillo and Lewis, 1992).

We previously identified and characterized cDNA and genomic clones encoding four tomato PGs expressed in weakening abscission zones, i.e. *TAPGs 1*, *2*, *4*, and *5* (Kalaitzis et al., 1995, 1997; Hong and Tucker, 1998). *TAPGs 1*, 2, and *4* were also expressed in the upper portion of pistils that included the stigma and style (Kalaitzis et al., 1997). No transcripts for these genes were detected in stems, petioles, or fruit. Expression of multiple PGs in the same tissue suggests that each may have a different enzymatic function and/or different temporal or spatial expression pattern. For simplicity abscission zones are often visualized as identical cells acting in synchrony. However, in reality they contain different classes of cells with different wall chemistries and different temporal patterns of wall degradation (Sexton and Roberts, 1982; Sexton, 1995). It is possible that each PG gene product has a discrete role in this complex process.

The coding regions of the four *TAPGs* expressed during abscission share 72% nucleotide sequence identity. The 5′-upstream promoters of the *TAPGs* are composed of two domains, a 300-bp proximal domain, which is partially conserved in all four *TAPGs*, and an upstream distal domain, which is widely divergent with the exception of *TAPG1* and 2 that share 66% identity in the first 1,140 bp of upstream sequence (Hong and Tucker, 1998). The *TAPG4* transcript accumulates much earlier in abscission zones than that of *TAPG1* and 2. Although all three genes are expressed in abscission zones, the

details of their spatial expression patterns have not been determined.

To better understand processes that regulate hydrolase expression in abscission and pistil development, we prepared chimeric gene fusions between the 5′- and 3′-flanking regions of *TAPG1* and *4* with a β-glucuronidase (GUS) reporter gene. In addition to these full-length promoter constructs, we prepared reduced-length and deletion constructs of the TAPG1 promoter to better define potentially important cis-acting elements in this gene. Transgenic tomato plants containing these chimeric gene fusions were surveyed for GUS expression, and the temporal and spatial distribution of GUS activity was determined in abscission zones and pistils. In addition, the response of the full-length and deletion transgenes to inhibitory concentrations of auxin and silver thiosulfate (STS), an ethylene action inhibitor, was investigated.

RESULTS

Tissue-Specific and Temporal Expression Patterns of GUS Activity in Transgenic Plants

Kalaitzis et al. (1997) demonstrated that *TAPG1*, *TAPG2*, and *TAPG4* were expressed in weakening leaf abscission zones (LAZ), floral abscission zones (FAZ), and pistils. The temporal expression patterns for *TAPG1* and 2 were very similar. *TAPG4* transcript, however, appeared much earlier than *TAPG1* and 2 in both abscission zones and pistils. The coordinated expression of *TAPG1* and 2 is reflected in 66% sequence identity in their 5′-upstream regions between −1 and −1,140 bp (Hong and Tucker, 1998). The same region of the TAPG4 gene is more divergent (Hong and Tucker, 1998). Therefore we have focused our studies on *TAPG1* and *TAPG4*.

The 5′-upstream sequences from *TAPG1* and *4* (2.1 and 2.4 kb, respectively) and 3′-downstream sequences (0.4 and 0.8 kb, respectively) were fused to GUS reporter genes to generate constructs p1-1 and p4-4, respectively (Fig. 1). Putative kanamycin-resistant transformants were screened by PCR analysis followed by histochemical staining to confirm GUS activity in ethylene-induced abscission zones. Three transgenic lines for 1-1 and five for 4-4 were selected for further study.

Histochemical localization and quantitative fluorometric assays of GUS were carried out on the pistils as well as the corolla abscission zone, FAZ, and LAZ from single-copy homozygous second and third generation plants (Fig. 2). There was considerable variation in the magnitude of GUS activity between independent transformants of both constructs, which is reflected in the large SES for the means (Fig. 2). Although the amount of the GUS activity varied greatly, the temporal and spatial patterns of GUS expression were very consistent from one transformant to another.
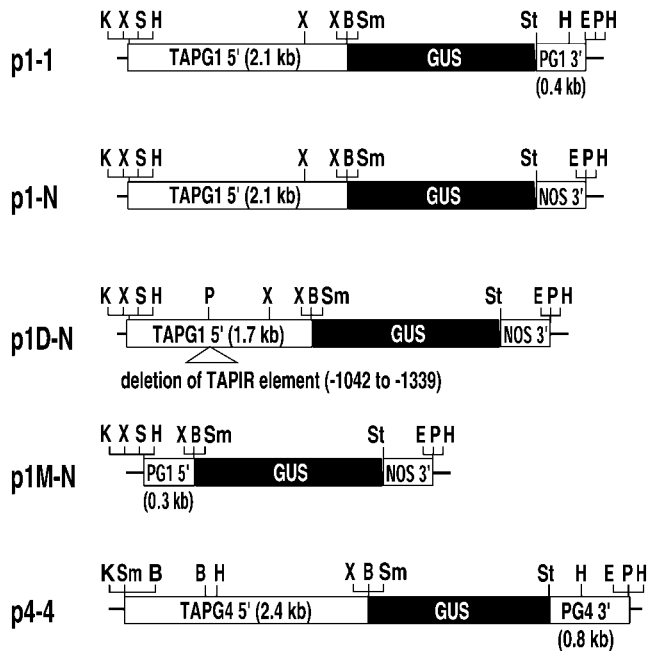
Figure 1. Gene constructs used for *Agrobacterium* transformation of tomato. E, *Eco*RI; K, *Kpn*I; B, *Bam*HI; X, *Xba*I; H, *Hind*III; Sm, *Sma*I; S, *Sal*I; St, *Sst*I; P, *Pst*I; PG1 5', *TAPG1* 5'-flanking region; PG1 3', *TAPG1* 3'-flanking region; PG4 3', *TAPG4* 3'-flanking region.

Expression in the Pistil

Ribonuclease protection assay showed that transcripts of *TAPG1* and *4* are expressed in mature pistils (Kalaitzis et al., 1997). Histochemical and fluorometric assays of the transformants indicated that the GUS expression patterns faithfully followed those of the native genes (Fig. 2; Kalaitzis et al., 1997; Hong and Tucker, 2000). In very young closed buds and buds where only sepals had opened, there was no GUS detected in the stigma or style of either transformant. Once the flower was fully open, staining was apparent in the upper pistil and this increased further in flowers where the petals were senescing (Fig. 2). In TAPG4:GUS transformants stain was present both in the stigma and in the upper 500 μm of the style where stain was most intense in a central core of transmission tract tissue. In some stigmas there were isolated islands of cells that did not stain. Activity in the TAPG1:GUS transformants was considerably lower but had a similar pattern of expression to that for TAPG4:GUS (Fig. 2). Histochemical stain, however, was only detected at the stigma surface of senescing flowers from TAPG1:GUS transformants (Fig. 2).

Expression in the Corolla Abscission Zone

In mature and senescing flowers, staining was present on the receptacle at the base of the flowers. This stain is located in the abscission zone where the corolla detaches. The stained spots represent the tissue around the severed vascular connections in the abscission zone scars (Fig. 2). Neither GUS activity nor staining was apparent in flower buds before weakening of the abscission zones commences; however, activity was detected in some fully open flowers (Fig. 2). GUS activity was more intense in senescent flowers where abscission was in progress and the corollas could be easily detached (Fig. 2). Incubating freshly opened flowers in ethylene for approximately 24 h accelerated petal abscission and enhanced staining of the corolla abscission zones (Fig. 2). The cells expressing GUS are largely located on the proximal side of the abscission zone. GUS expression in these corolla abscission zones was considerably lower in the TAPG1:GUS transformants. Only in one TAPG4:GUS transformant with very high levels of GUS activity was any staining found on the base of the corolla (distal side) after fracture.

Expression in the FAZ

Ribonuclease protection assay demonstrated that transcripts for *TAPG1* and *4* are abundantly expressed in flower pedicel abscission zones and that transcript accumulation occurs earlier for *TAPG4* transcript than that for *TAPG1* (Kalaitzis et al., 1997). GUS activity measurements of ethylene treated transformants demonstrated that the temporal expression characteristics of the native genes had been retained in the respective transgenes (Fig. 2).

The FAZ in the pedicel is easily recognized as a swollen node approximately 5 mm below the calyx. In opening buds and fully open flowers there was no staining in this region. However, as the flowers senesced there was a small population of pedicels that had faintly localized staining (Fig. 3). Since damaged and unfertilized flowers abscise naturally at this stage, this result was predictable.

If the pedicel abscission zones of fully opened nonsenescent flowers were exposed to ethylene for longer than 10 h, staining became obvious in the swollen node of the pedicels where abscission will ultimately occur (Figs. 2 and 3). In median longitudinal sections GUS appears first in the swollen node as a discrete peripheral spot of stained cells that includes a vascular bundle and surrounding parenchyma cells (Fig. 3). These spots frequently appear first on the lower side of the pedicel (Fig. 3) but have also been observed at other positions around the pedicel. After 16 to 20 h of ethylene exposure, when fracture is beginning to occur, GUS staining becomes more intense and spreads to the entire vascular system and associated parenchyma. Cross-sections through different planes in the pedicel revealed that the stained tissue is largely confined to a doughnut-shaped mass of small parenchyma cells that encompass the ring of vascular bundles (Figs. 2 and 3).
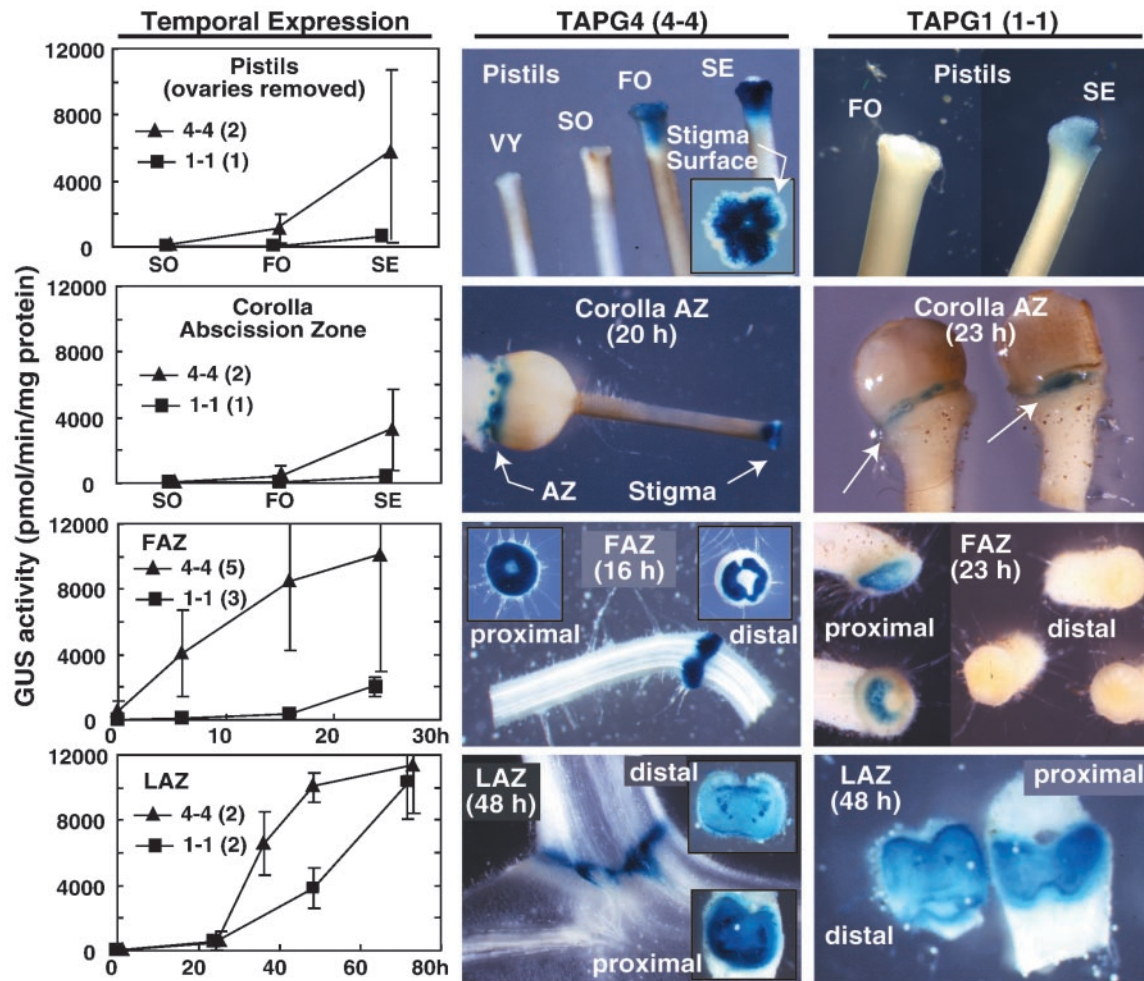
Figure 2. Time course and histochemical staining for TAPG1:GUS and TAPG4:GUS expression in pistils and selected abscission zones from 4-4 and 1-1 transformants. Expression of GUS activity (left side) was measured in protein extracts from pistils (ovaries removed) and corolla abscission zones taken from flower buds with opened sepals but unopened petals (SO), fully open but non-senescent flowers (FO), and senescent flowers (SE). Flower parts used for GUS activity measurements were not exposed to ethylene. GUS activity was measured in LAZ and FAZ, respectively, in explants treated with 25 μL/L ethylene at 25°C for the indicated times at the bottom of each graph. Error bars indicate the SD of the means. The numbers in parentheses to the immediate right of the symbol definitions in each graph indicate the number of independent transformants used to derive the means for each construct. Histochemical staining of pistils from 4-4 and 1-1 plants was performed at the indicated stages described above. In addition, a pistil from a very young (VY) unopened flower bud was also stained for GUS activity. Explants that included the flower corolla abscission zone (AZ), pedicel FAZ, and LAZ were exposed to ethylene for the indicated time intervals (in parentheses under the labels) and histochemically stained for GUS activity.

Examination of the exposed fracture surface showed that the doughnut-shaped ring of stained cells is surrounded by five to seven rows of outer cortical cells and epidermis that are unstained (Figs. 2 and 3). At the center of the ring is a region of larger parenchymatous pith cells that only develop weak staining just before fracture (Fig. 2). If FAZs are broken prematurely after 12 to 14 h of incubation, the doughnut-shaped ring is occasionally incomplete and staining is delimited to a crescent or horseshoe band. This pattern is consistent with the initiation of GUS expression in a localized region of the abscission zone that subsequently spreads until a complete ring of stain is formed.

GUS stain is most intense on the proximal surface of abscising and abscised pedicels (Fig. 2). Higher expression on the proximal surface relative to the distal surface appears to be true for both TAPG4:GUS and TAPG1:GUS transformants. Expression of TAPG1:GUS in the distal half of the FAZ was so low that it was difficult to detect GUS stain on the distal surface of abscission zones exposed to ethylene for 23 h (Fig. 2).

As the fruit develops the pedicel abscission zone thickens. When the fruit is fully ripe and the fruit can be easily separated from the plant, there is a wide band of GUS staining cells on both the proximal and distal sides of the fruit pedicel abscission zone. The
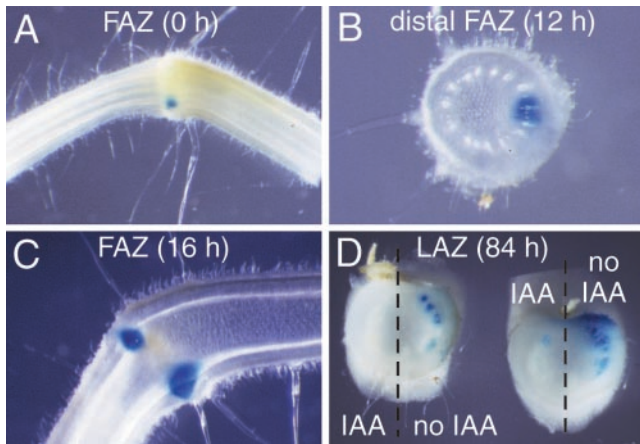
Figure 3. TAPG4-driven GUS activity (construct 4-4) appears first in the vicinity of the vascular bundle and its expression is independent of expression in other vascular bundles in the abscission zone. A, Pedicel FAZ from a senescent flower not exposed to ethylene (0 h). B, Distal FAZ showing initiation of localized GUS expression after 12 h of exposure to 25 μL/L ethylene at 25°C. C, Longitudinal half-section of FAZ showing vascular localization of GUS expression after 16 h of exposure to ethylene. D, 0.1 mM IAA was applied to the distil end of the petiole in a lanolin paste and the petiole sliced half-way through 5 mm distal to the LAZ. The LAZ explant was then exposed to ethylene for 84 h and the LAZ was stained for GUS activity.

doughnut-shaped pattern of the GUS-staining cells in pedicel FAZ (Fig. 2) also occurs in the fruit pedicel abscission zones.

Expression in the Fruit Calyx Abscission Zone

In addition to abscission in the pedicel, ripe tomato fruit can also be detached at a calyx abscission zone at the proximal end of the fruit. GUS stain was also observed in the calyx abscission zone of ripe fruit from TAPG4:GUS transformants. As in the FAZ and corolla abscission zones, staining was most intense in cells within and surrounding the vascular traces.

Expression in the LAZ

In the LAZ exposed to ethylene there is an approximately 20 h lag before GUS activity can be detected in both TAPG1:GUS and TAPG4:GUS transformants. The subsequent increases in GUS activity in LAZ have very similar kinetics to those in the FAZ; the accumulation of GUS in TAPG4:GUS transformants is quicker and approaches maximal levels much earlier than in TAPG1:GUS transformants (Fig. 2). GUS stain first appears in the abscission plane in islands around the vascular tissue much as it does in FAZ (Fig. 3). The stained region spreads with ethylene exposure time. When fracture begins at approximately 48 h, all of the vascular bundles in the U-shaped stele are stained and the central pith parenchyma and the outer cortex are stained too (Fig. 2). Staining in the cortex and pith cells was less apparent when tissues were incubated in a 3.0-mM ferricyanide staining solution compared to a 0.5-mM solution (see "Materials and Methods"). To confirm that the GUS staining was not a diffusion artifact, areas of central and peripheral cortex from abscission zone scar faces were isolated and fluorometrically assayed. GUS activity in the non-vascular tissue taken from the TAPG4:GUS abscission zone was approximately one-third of that in the stele but significantly higher than surrounding nonabscission zone tissue.

Separation of the petiole from the stem is complete after a 72-h exposure of explants to ethylene. If the petiole is sliced into 1-mm transverse sections and then stained, GUS activity is clearly apparent in the proximal and distal fracture surfaces of both transformants and also in the vascular bundles 1 mm distal to the fracture plane (Fig. 4).

The main difference between GUS expression in TAPG4:GUS and TAPG1:GUS transformants is that TAPG4:GUS is expressed before TAPG1:GUS. Moreover, after examination of numerous GUS stained sections we conclude that GUS expression in LAZ from TAPG1:GUS transformants is more uniform
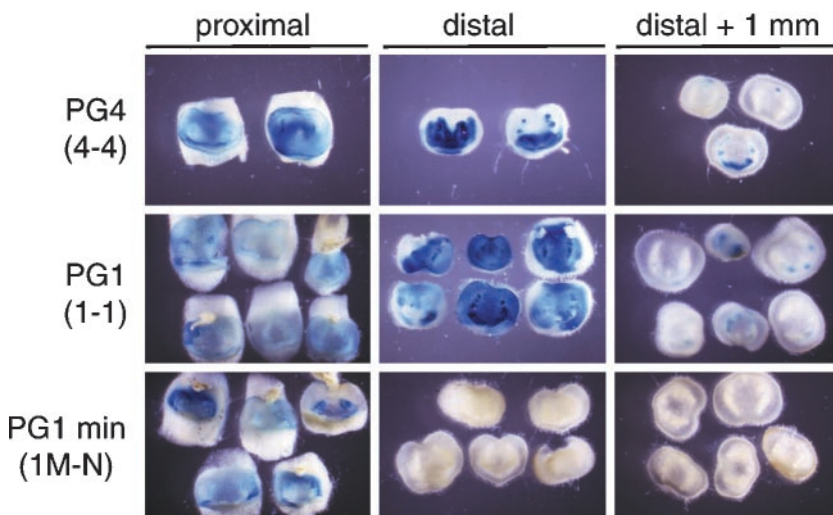

Figure 4. TAPG:GUS expression for the full-length constructs 1-1 and 4-4 but not the minimal construct 1M-N is detected in vascular bundles 1 mm distal to the separation layer of the LAZ. Explants were exposed to 25 μL/L ethylene at 25°C for 72 h and the distal petiole sliced into 1-mm sections. In addition to the fracture surfaces (proximal and distal) the faces of the sections taken 1 mm distal to the fracture are shown (distal + 1 mm).

across the separation layer compared with LAZ from TAPG4:GUS plants, which appear to stain more intensely in the vascular bundles (Fig. 2). Although GUS staining in the LAZ of TAPG1:GUS plants is clearly apparent in vascular bundles, it appears in cortical cells at approximately the same time or very soon after its first appearance in vascular tissue.

Other Organs and Tissues

A number of tissues do not express detectable levels of GUS staining cells. These included ripe fruit pericarp, root caps, anthers, ovaries, and germinating pollen. A very small proportion (0.5%) of ungerminated pollen grains stained blue, but we were unable to find an explanation for this observation. In addition, thin sections through adventitious root initials were examined for GUS staining. In a very few sections from TAPG4:GUS (4-4) transgenic plants a narrow band of cells stained faintly for GUS activity immediately in front of a newly forming root initial.

Hormonal Regulation and Role of the 3′ End and Conserved TAPIR Element in the *TAPG1* and *4* Gene Promoters

To ascertain if the 3′ end of the *TAPG1* gene contained information that was essential for tissue specificity or hormonal control of *TAPG1* gene expression, a construct was prepared in which the nopaline synthase termination sequence (NOS 3′) replaced the *TAPG1* 3′ termination sequence, construct p1-N (Fig. 1). Moreover, sequence comparison of the 5′-upstream sequences for the *TAPG* genes identified a conserved 300-bp inverted repeat (TAPIR) that contained several potential hormone response elements (Hong and Tucker, 1998). The 300 bp containing this inverted repeat was deleted from the p1-N promoter construct to generate construct p1D-N (Fig. 1). Each of these constructs was transformed into tomato. Histochemical staining for GUS activity in transformants containing these constructs, p1-N and p1D-N, were indistinguishable from transformants containing the more complete p1-1 construct. In addition to histochemical staining, GUS activity was quantified in the FAZ of several primary transformants for each of these constructs (Fig. 5). The temporal expression patterns in FAZ for these deletion constructs in transgenic tomato were not significantly different from those for the complete construct, p1-1, in transgenic tomato (Fig. 5).

Treatment of leaf explants with 0.1 mM indole-3-acetic acid (IAA) or 0.5 mM STS (an ethylene action inhibitor) prior to exposure of explants to 25 μL/L ethylene for 72 h inhibits petiole abscission and *TAPG* gene expression (Kalaitzis et al., 1995; P. Kalaitzis and M.L. Tucker, unpublished data). Similarly, treatment of flower explants with 0.1 mM IAA inhibits pedicel abscission (del Campillo and Bennett,
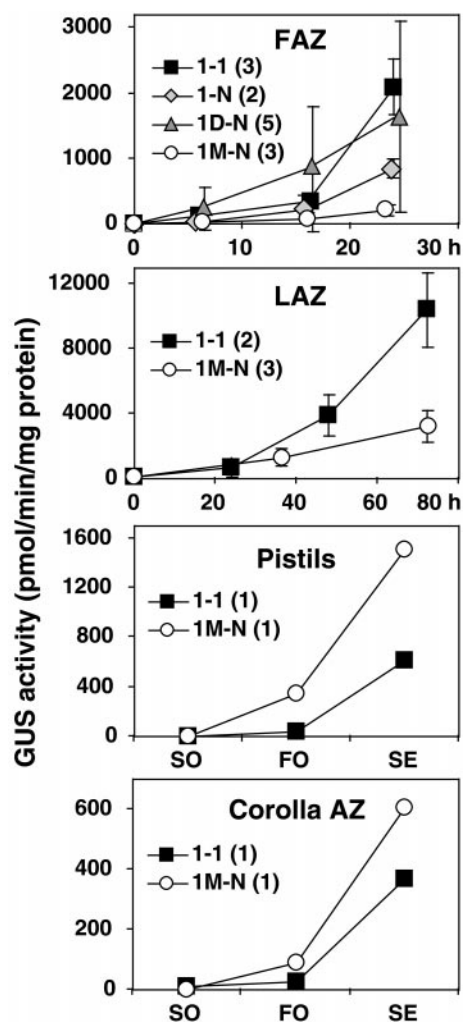

Figure 5. GUS activity in abscission zones and pistils from transgenic tomato containing the *TAPG1* promoter constructs described in Figure 1. Ethylene exposure and stages of flower development are as described in Figure 2. The numbers in parentheses to the immediate right of the symbol definitions in each graph indicate the number of independent transformants used to derive the means displayed.

1996). GUS activity in IAA-treated FAZ and IAA- or STS-treated LAZ from 1-1, 1-N, 1D-N, and 4-4 transgenic explants was reduced by greater than 90% relative to the GUS activity in abscission zones of explants not pretreated with IAA or STS (Fig. 6). There was no significant difference in the inhibitory response of the different promoters in these constructs (Fig. 6).

Although explants treated with IAA show no sign of weakening in the abscission zones after 72 h of exposure to ethylene, abscission zone sections did contain sporadic islands of staining around the vascular tissue. This would happen if the IAA applied onto the petiolar stump does not reach all parts of the abscission zone equally. If IAA is added to the petiole and one side of the petiole cut half-way through to prevent movement of IAA down that side, GUS
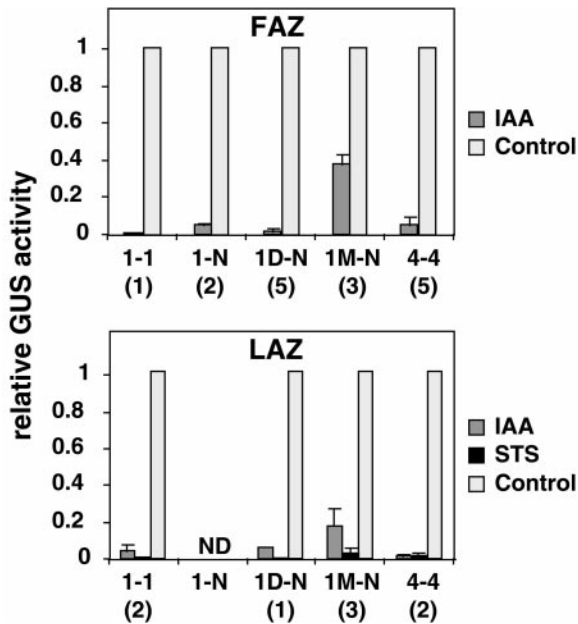
Figure 6. Relative GUS activity in FAZ and LAZ of explants from transgenic tomato not treated (control) or pretreated with 0.1 mM IAA or 2.0 mM then 0.5 mM STS. All FAZ and LAZ explants were exposed to 25 μL/L ethylene at 25°C for 24 or 72 h, respectively. *TAPG1* and *TAPG4* promoter constructs are as described in Figure 1. ND, Not determined. The numbers in parentheses immediately below the construct name indicate the number of independent transformants used to derive the means displayed.

staining develops in the abscission zone predominantly on the same side as the cut where IAA concentration should be lowest (Fig. 3D).

Role of a 320-bp Proximal TAPG1 Sequence in the Regulation of Abscission-Specific Expression

Optimal sequence alignment of the 5′-upstream sequences for the four PG genes known to be expressed during abscission indicated that the first 300 bp is partially conserved in all four PG genes (Hong and Tucker, 1998). Accordingly, to test if this proximal region is sufficient for regulated expression in TA zones, 247 bp of 5′-upstream sequence and 73 bp immediately downstream from the start of transcription of the *TAPG1* gene were fused to a GUS reporter gene to generate construct p1M-N (Fig. 1).

GUS activity was quantified in primary transformants of 1M-N plants and single-copy first or second generation 1-1 plants (Fig. 5). The temporal expression patterns for GUS activity in 1M-N plants containing the minimal promoter construct were similar to the patterns found for 1-1 plants containing the full-length constructs (Fig. 5). However, the level of GUS expression in FAZ and LAZ from the minimal promoter plants was generally less than that measured in plants containing the full-length construct (Fig. 5). Histochemical staining for GUS activity in four independent transformants (1M-N) containing

the minimal construct was generally very faint. However, tissues that stained for GUS activity in the minimal TAPG1:GUS transformants (1M-N) were essentially the same as those that stained in the full-length TAPG1:GUS transformants (1-1), i.e. LAZ, FAZ, fruit abscission zones, and pistils. The most striking difference in the histochemical staining pattern for the full-length TAPG1:GUS transformant (1-1) compared with the minimal construct transformant (1M-N) was that in the minimal transformant very little or no stain was observed in the distal surface of the separation layer after 72 h of exposure to ethylene (Fig. 4). However, the lack of stain in the distal portion may not indicate a total loss in a cell-specific response in the minimal construct but rather a lower rate of accumulation of GUS in the distal abscission zone, which was below the threshold necessary for GUS staining.

The temporal expression patterns for GUS activity in pistils and corolla abscission zones were very similar between the minimal and full-length TAPG1:GUS transformants (Fig. 5). However, the GUS activity for the minimal promoter (1M-N) in pistils and corollas abscission zones was greater than that for the full-length promoter (Fig. 5). Because of the large amount of plant material required and the difficulty in collecting it, GUS activity in the staged flower parts was measured in only one 1M-N transformant. The 1M-N transformant selected had the highest GUS activity in LAZ and FAZ of three 1M-N transformants. The selection process might explain why 1M-N flower parts had higher activity than did those from the full-length 1-1 transformant.

Another difference between the full-length construct (p1-1) and the minimal construct (p1M-N) was that the relative GUS activity in 1M-N transgenic plants treated with IAA was inhibited by only 60% in FAZ and 80% in LAZ (Fig. 6). This is in contrast to approximately 95% inhibition in plants containing the full-length construct (Fig. 6). However, treatment with the ethylene action inhibitor STS inhibited the accumulation of GUS activity by greater than 95% in LAZ from both 1M-N and 1-1 transgenic plants (Fig. 6).

DISCUSSION

Correlation of TAPG-Promoted GUS Expression in Transgenic Plants with TAPG Transcript Accumulation in Wild-Type Plants

Analysis of the spatial and temporal expression of the GUS reporter gene in TAPG1:GUS and TAPG4:GUS transgenic cv Ailsa Craig tomato plants revealed virtually identical patterns to those of the native genes in the tomato cv Rutgers. Kalaitzis et al. (1997) demonstrated using an RNase protection assay that the accumulation of both *TAPG1* and *TAPG4* transcript in cv Rutgers tomato correlated with the weakening of FAZ and LAZ and was spatially de-

limited to the abscission zones. In addition, abscission and accumulation of *TAPG1* transcript in LAZ were induced by ethylene and inhibited by auxin. The hormonal regulation of GUS expression from the *TAPG1* and *4* promoter constructs was retained in the transgenic plants (Fig. 6). In addition to expression in abscission zones, the promoter constructs retained the ability to promote GUS expression in the mature stigma and upper style as was demonstrated for the native genes (Kalaitzis et al., 1997; Hong and Tucker, 2000). Accumulation of GUS in transgenic cv Ailsa Craig plants, however, is slightly slower than that for the PG transcripts in the native cv Rutgers. This may simply be a difference between transcript and protein accumulation or it may reflect different rates of abscission between the two cultivars of tomato.

In addition to LAZ, FAZ, and pistils, which had been previously demonstrated to accumulate *TAPG1* and *4* transcript, we examined fruit pedicel, fruit calyx, and corolla abscission zones. GUS accumulated in all of these abscission zones in explants from both the TAPG1:GUS (1-1) and TAPG4:GUS (4-4) transgenic plants. Peretto et al. (1992) measured PG activity associated with root initials in *Allium porrum*. In a few thin sections from 4-4 transgenic plants a narrow band of cells immediately above an adventitious root initial stained faintly for GUS activity. This staining pattern was difficult to reproduce but may suggest transient expression of the *TAPG4* promoter during adventitious root initiation. Histochemically detectable GUS activity was not observed in fruit, anthers, roots, or root caps. Other genes from the tomato PG family may be expressed in these organs and tissues.

Deletion analysis of the *TAPG1* promoter indicates that the conserved TAPIR element and 3′ end of the *TAPG1* gene, which included multiple auxin and ethylene response elements (Hong and Tucker, 1998), were not essential to the hormonal or tissue-specific regulation of *TAPG1*-promoted GUS expression in abscission (histochemical observations and Figs. 5 and 6). Tissue-specific and hormone-responsive elements in the *TAPG1* gene must reside within the first 247 bp of the promoter or 73 bp of the 5′-upstream untranslated region of the transcript, because the minimal promoter construct (p1M-N) retained these major control characteristics.

Nevertheless, organization of gene promoters can be quite complex. A pertinent example in the context of our studies are those for the tomato fruit PG (TFPG) (Montgomery et al., 1993). GUS expression from a minimal −231 TFPG promoter construct demonstrated expression of GUS in the outer pericarp of ripening fruit very similar to that of the longer 1.4-kb promoter construct, which faithfully reflected that of the native PG transcript. However, when TFPG deletion constructs of −443 and −806 were examined for GUS expression, GUS stain was observed in both the inner and outer fruit pericarp indicating the pres-

ence of a positive regulatory element between −231 and −1,150 that controlled expression in the inner pericarp. Montgomery et al. (1993) further concluded that a negative regulatory element between −1,150 and −1,411 inhibited GUS expression in the inner pericarp re-establishing an expression pattern more like that of the native PG transcript.

Clearly, we cannot be certain that there are no additional regulatory elements between −247 bp and −2.1 kb in the *TAPG1* gene promoter. Nevertheless, the minimal promoter in p1M-N must include elements that can control abscission specificity and hormonal regulation. Examination of this region of the *TAPG1* gene promoter for regulatory motifs did not identify any motifs of particular interest (Hong and Tucker, 1998). Regions upstream of −247 must at the very least play an ancillary role since the overall strength of the *TAPG1* minimal promoter in abscission zones was significantly reduced compared with the longer promoter construct (Fig. 5) and the IAA inhibition of expression less absolute (Fig. 6). This is not unexpected since enhancer elements commonly reside considerable distances from the start of transcription and regulatory elements may be repeated several times (Nicholass et al., 1995; Singh, 1998).

Significance of the Spatial Expression of GUS as Abscission Progresses

In the p1-1 and p4-4 gene constructs we have attempted to include as much of the native gene regulation as possible to create a reporter gene that can be used to study the biology of abscission in tomato. The p1-1 and p4-4 constructs include 2.1 and 2.4 kb, respectively, of native PG sequence 5′ to the start of translation of PG and 0.4 and 0.8 kb, respectively, of 3′ sequence below the stop codon for the end of PG translation (Fig. 1). Sequence analysis of the three introns in each of the PG genes did not highlight any notable motifs or conserved sequences (Hong and Tucker, 1998). Therefore, we did not include any intron sequences in the gene constructs. Observations described above indicate that expression of the GUS reporter gene from the respective constructs faithfully reflects that of the native PG genes.

The localization of GUS presented above allows a detailed insight into the progressive patterns of expression of an abscission-specific gene. GUS expression in the FAZ is initiated in isolated vascular bundles and surrounding parenchyma cells. Examples were found where only one or two bundles were stained and others where a crescent or horseshoe shaped group of bundles stained blue. As weakening progressed in FAZ the stain spread and intensified in a doughnut-shaped ring of parenchyma surrounding the circle of vascular bundles. This ring of smaller parenchyma cells was described by Biain de Elizalde (1980) who suggested it was the result of intense meristematic activity in this region. It has been pro-

posed that these extra cell divisions might be necessary for the programming of cells to respond to the abscission-inducing signal whereas neighboring cells do not (Sexton and Roberts, 1982). The ring of intensely stained cells surround the larger cells of the central pith on the inside that only become stained late in the development of the separation layer. In addition, the FAZ includes five to seven rows of outer cortex cells that do not stain for GUS (Fig. 2).

The LAZ showed a similar progression of GUS expression. Staining begins around isolated vascular bundles and then spreads to the entire U-shaped stele. Eventually GUS activity spreads laterally to the outer cortex where it is restricted to a fracture plane several cell layers deep. A similar spread of cellulase activity from the stele to the cortex has been described in the abscission zones of bean (Sexton et al., 1981; del Campillo et al., 1990).

By surgically removing the stele from the LAZ of bean, Thompson and Osborne (1994) demonstrated that the vascular tissue was essential to initiate β-1,4-glucanase (EGase) and abscission in the surrounding cortical cells. If they delayed removal of the stele for several hours or removed the stele and then immediately replaced it, EGase was expressed in the cortical cells, and cell separation progressed across the outer cortical parenchyma. They concluded that a signal emanates from the stele that then elicits a program of abscission-related gene expression in the cortical cells. They further speculated that this signal could be a diffusible cell wall oligosaccharide arising from hydrolysis of cell walls in the stele. The observation that GUS is first detected in the vascular traces of FAZ and LAZ supports the first tenet of the model, which predicts that the target cells for the initial inductive stimulus are in the stele. The subsequent spread of GUS staining to the cortex is also consistent with the lateral diffusion of a secondary signal.

GUS expression was detected in the fracture plane and in vascular tissues proximal and distal to the separation layer (Fig. 4). A similar distribution was observed for an EGase expressed in bean LAZ (del Campillo et al., 1990; Tucker et al., 1991). The enhanced expression of cell wall hydrolases in vascular bundles has not only been implicated in the separation of the small, tightly packed cells but has also been implicated in cell wall breakdown necessary for the formation of tyloses (Sexton et al., 1981). Tyloses are cells that penetrate and block the xylem vessels preventing both water loss and microbial access after fracture. Close examination of thin sections of vascular tissue from TAPG4:GUS transgenic plants revealed GUS stain in all the cells of the vascular bundle including those next to the vessels that are producing tylose outgrowths (data not shown).

As abscission progresses GUS activity is detected progressively farther away from the separation layer primarily distal to the fracture and is restricted to the stele (Fig. 4). Progressive GUS expression along the vascular traces is not dependent on a secondary signal produced initially in the abscission zone. If the petiole is sliced into sections prior to exposure to ethylene in a manner similar to that shown in Figure 4, GUS stain develops after 72 h of exposure to ethylene in vascular traces of slices closest to where the abscission zone was attached (data not shown). In a similar experiment with bean, cellulase activity increased in petiole sections distal to the LAZ even though there was no contact with the abscission zone (Sexton et al., 1981).

TAPG:GUS Expression in Pistils

Recently, Hong and Tucker (2000) showed that *TAPG4* transcript accumulated only in mature pistils and was limited to the upper one-third of the pistil. This pattern of GUS accumulation was faithfully reproduced in pistils of 4-4 transformants (Fig. 2). TAPG4:GUS and TAPG1:GUS are not expressed in pistils of young or unopened buds (Fig. 2). GUS expression begins in pistils of 4-4 transformants as the flower opens and continues to increase as the flower senesces (Fig. 2). In pistils from 1-1 transformants, GUS activity was very low until flowers began to senesce, and faint GUS staining was observed only at the stigma surface of pistils from senescent flowers (Fig. 2).

Tomato has what is described as a solid style with a wet stigma (Kadej et al., 1985). GUS staining in the stigma and upper style is present in regions corresponding to the outer papillae, the underlying stigmatic zone, and the upper transmitting tract (Dumas et al., 1978). Cell walls in these tissues are degraded to produce longitudinal canals in which large amounts of a stigmatic exudate accumulate and through which the pollen tubes grow (Dumas et al., 1978; Janson et al., 1994). The loss of cohesion between stigma cells anatomically resembles that in abscission zones and could involve PG in a similar role. Although GUS staining was very intense in the stigmatic region and in the upper 500 μm of the style below it, GUS staining was not present in the lower style or ovary. This indicates that *TAPG1* and *4* are not involved in the formation of the lower transmitting track, which passes through the style to the ovary (Cresti et al., 1976).

It is interesting that transcript for another tomato PG gene, *TPG7*, was found to be abundantly expressed in both mature and immature pistils (Hong and Tucker, 2000). In mature pistils, transcript accumulation for *TPG7* was also limited to the upper one-third of the pistil. It appears that PG is important to several stages of pistil development.

Proposed Roles for PG

The patterns of PG expression are consistent with a proposed role in cell separation, which occurs in both

of the abscission zones, in the stigma, and in the style. However, these same cells also share the characteristic that they are unprotected by mechanical barriers and therefore are susceptible to microbial attack. The antimicrobial enzymes, chitinase, β-1,3-glucanase, and several other PR proteins accumulate abundantly in abscission zones at the time of shedding (Gomez et al., 1987; Weiss and Bevan, 1991; del Campillo and Lewis, 1992). Moreover, PR gene expression is evoked in mature pistils in a similar manner to that observed in abscission zones (del Campillo and Lewis, 1992; Atkinson et al., 1993; Harikrishna et al., 1996). Oligosaccharides and specifically oligogalacturonides have been shown to activate plant defensive genes (Baydoun and Fry, 1985; Cote and Hahn, 1994; Doares et al., 1995). Oligosaccharides move only very small distances and therefore are not part of a long-distance signal to mount a systemic defense response in tomato (Baydoun and Fry, 1985). As a result PG may play a secondary role in abscission zones and pistils by releasing oligogalacturonides from cell walls to mount a local defense response.

CONCLUDING REMARKS

Unfortunately, we have not been able to produce reliable in situ hybridization data to confirm the transgenic results. In addition to typical difficulties with in situ hybridization, expression of a family of at least four related PG genes in abscission (Hong and Tucker, 1998) complicates probe selection for gene-specific hybridization. However, the ease of identifying and following the expression of the GUS reporter gene has generated a level of resolution concerning its topographical and temporal expression that we have not achieved for the native gene transcripts.

TAPG1:GUS and TAPG4:GUS expression were observed in all the abscission zones examined, i.e. leaf petiole, flower and fruit pedicel, fruit calyx, and corolla abscission zones. Our model for PG expression, and abscission in general, is that an abscission signal is perceived first in a target cell localized somewhere in the vascular bundle. The target cell then evokes an abscission program that includes *TAPG4* gene expression. As proposed by Thompson and Osborne (1994), the abscission signal is then further propagated to surrounding cortical and pith cells in the separation layer through another secondary signal that regulates the expression of both *TAPG1* and *TAPG4*. Each vascular bundle works independently of each other to propagate a localized abscission signal across the separation layer. In addition to propagation of the signal transversely across the separation layer, an abscission signal is also perceived in the vascular bundle a short distance proximal and distal to the separation layer. Perception of the signal distal to the separation layer is not dependent on a secondary signal, because transverse sections cut

across the petiole 1 to 2 mm distal to the separation layer can be induced by ethylene to express TAPG4:GUS in the vascular bundles without contact with the separation layer cells. The target cells in the distal vascular bundles must be different from those in the separation layer or, as suggested by Thompson and Osborne (1994), the cortical cells in the separation layer must already be predetermined to respond to a secondary signal emanating from the target cells in the vascular bundles. Although the above model fits the current data, the precise location of the target cells in the vascular bundles and the role of a secondary signal in abscission zones need to be rigorously examined with further experimentation.

MATERIALS AND METHODS

Construction of Gene Fusions

The 5′-flanking sequences of *TAPG1* and *4* (accession nos. AF001000 and AF001002, respectively) were contained in a 3-kb *Eco*RI fragment of the genomic clone λPG1-2 and a 7-kb *Eco*RI fragment of the genomic clone λPG3-3, respectively (Hong and Tucker, 1998). The 2.1- and 2.4-kb sequences upstream from ATG start codons of *TAPG1* and *4* were amplified by long PCR, respectively, using *Taq* extender PCR additive (Stratagene, La Jolla, CA) and oligonucleotide primers with restriction sites at their 5′ ends. The resulting fragments were purified, cleaved, and cloned into *Hind*III/*Xba*I sites of the vector pBI221 to make transcriptional fusions with the coding region of the GUS gene with the 3′-terminator sequence from the NOS 3′. The *Hind*III/*Eco*RI fragments from the pBI221 containing the TAPG:GUS:NOS fusions were then subcloned into the pBluescript SK⁺ vector (Stratagene). Finally, the *Kpn*I/*Pst*I fragments of the pBluescript SK⁺ vector containing TAPG:GUS:NOS fusions were inserted into the binary vector pCGN1547 (McBride and Summerfelt, 1990). The resulting binary constructs containing TAPG1:GUS:NOS and TAPG4:GUS:NOS fusions were called p1-N and p4-N, respectively (Fig. 1).

PCR was used to generate a deletion construct in which the TAPIR element (Hong and Tucker, 1998) was deleted from an otherwise normal 2.0-kb 5′-upstream sequence from *TAPG1* and named p1D-N (Fig. 1). In addition, a minimal construct was prepared for the *TAPG1* gene, p1M-N, that contained 247 bp of sequence 5′ upstream and 73 bp downstream of the start of transcription for the *TAPG1* transcript (Fig. 1). This 320-bp *TAPG1* fragment was sequenced and determined not to contain any errors introduced by the PCR amplification. Next, the NOS 3′-terminator sequence of p1-N and p4-N was replaced with 350 and 800 bp of the native 3′-downstream sequences of *TAPG1* and *4*, respectively. The *TAPG1* and *TAPG4* 3′ sequence include 38 and 12 bp, respectively, of 3′-translated sequence upstream from the respective stop codons. However, these *TAPG* translated sequences are downstream of the native stop codon for the GUS open reading frame. These two new constructs, which now contain both 5′- and 3′-flanking sequences of *TAPG1* and *4*, were named p1-1 and p4-4, respectively (Fig. 1).

Tomato Transformation and Propagation

The chimeric TAPG:GUS fusions in the binary vectors p1-1, p1-N, p1D-N, p1M-N, and p4-4 were transformed into *Agrobacterium tumefaciens* (strain EH105) by the freeze-thaw method of Holsters et al. (1978). Transgenic tomato (*Lycopersicon esculentum* cv Ailsa Craig) plants were generated by the method of McCormick et al. (1986). Transformed shoots were selected on kanamycin (100 μg/mL). After rooting of shoots, the plantlets were transferred to sterile potting soils and gradually acclimated before transfer to the greenhouse. The integrity and copy number of the introduced genes was checked by PCR and Southern-blot analysis. GUS activity was quantified fluorometrically in the FAZ of all the selected primary transformants, some of which had several copies of the transgenes. Because of the large amount of plant material required for GUS quantification, single-copy transformants were selected for 1-1 and 4-4 transformants and propagated by seed.

Plant Tissue Preparation

Ethylene, STS, and IAA treatments of tomato explants were performed as described previously (Koehler et al., 1996). For STS treatment, tomato explants with two to three attached leaves were pretreated for 3 h in 2 mM STS. Leaf blades were then removed, leaving the subtending petiole attached, and the explants placed upright in beakers containing 0.5 mM STS and then treated with 25 μL/L of ethylene at 25°C for an additional 72 h.

Petal wilt is an indication that abscission may have already been induced. Floral explants harvested for the time course experiments had fully open and bright yellow petals with no sign of flower senescence or petal wilt. IAA treatment of FAZ and LAZ was done by applying 0.1 mM IAA in lanolin to the cut ends of the pedicels of fully open flowers or petioles of expanded leaves, respectively. After application of lanolin the explants were allowed to stand in air for 4 h before exposure to 25 μL/L ethylene at 25°C. At least 20 FAZ and 10 LAZ were collected for each time point by cutting approximately 2-mm sections that included the abscission zone.

Assay for GUS Activity

Quantitative GUS enzyme assays were performed as described by Jefferson et al. (1987). Pooled tissues were ground in GUS extraction buffer (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 0.1% [w/v] SDS, and 10 mM β-mercaptoethanol) followed by centrifugation. Protein concentrations were estimated using the colorimetric method of Bradford (1976). To reduce endogenous GUS-like activity in tomato, sample extracts were heated to 55°C for 30 min. The volume equivalent to 20 μg of protein was incubated with 4-methylumbelliferyl glucuronide solution for 60 min at 37°C, and the reaction stopped by adding 40 μL of the reaction mixture to 1.0 mL of 0.2 M sodium carbonate. Fluorescence was measured with a Bio-Rad VersaFluor fluorometer (Bio-Rad Laboratories, Hercules, CA). The instrument was calibrated with a solution of 1.0 μM 4-methylumbelliferone in 0.2 M sodium carbonate. Histochemical staining was conducted by incubating hand-cut sections for 4 h at 37°C in a buffer (100 mM NaH$_2$PO$_4$, pH 8.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, and 0.5 mM or 3.0 mM potassium ferro- and ferricyanide) containing 500 μg/mL of X-gluc (5-bromo-4-chloro-3-indolyl-β-glucuronic acid) (Stomp, 1992). Sections were cleared of chlorophyll with several changes of a mixture of ethanol:acetic acid at a 3:1 ratio. Samples were stored in the same 3:1 mixture of ethanol:acetic acid.

GUS histochemical staining can be susceptible to artifacts caused by the diffusion of the reaction product before precipitation. This problem can be overcome by increasing the ferricyanide concentration in the reaction mixture (Guivarc'h et al., 1996). In preliminary experiments we directly compared the two halves of bisected abscising pedicel and leaf base zones incubated in media with either 0.5 or 3.0 mM ferricyanide. The higher concentration markedly inhibited staining, although staining was more discrete at higher concentration. It appeared that the risk of obtaining "false-negative" results when using the higher ferricyanide concentration was greater than the risk of false distributions. As a result, we adopted the strategy that all initial investigative incubations were carried out in 0.5 mM ferricyanide and any observations of significance were checked at a higher concentration.

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