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Stigma- and Vascular-Specific Expression of the *PR-10a* Gene of Potato: A Novel Pattern of Expression of a Pathogenesis-Related Gene

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The tissue- and organ-specific expression of *PR-10a* (formerly *STH-2*), a pathogenesis-related gene of potato, was investigated using an anti-PR-10a antibody and transgenic potato plants transformed with *PR-10a* promoter-β-glucuronidase (GUS) chimeric gene. Strong PR-10a expression was observed in tubers, stolons, stems, and petioles following infection with the potato pathogen *Phytophthora infestans* or elicitation with *P. infestans* homogenate. These tissues also responded to different degrees to wounding. Induction in leaves was weak and detectable only with the elicitor treatment. Histochemical GUS staining indicated that PR-10a induction was associated with vascular bundles. Tuber storage parenchyma responded locally to wounding and infection but to a much smaller extent than vascular bundles. In infected petioles and stems, GUS activity was seen in most cell types beneath the infection site, with strongest expression in vascular cells. PR-10a expression was very vascular-specific in wounded stems and petioles, as well as in elicitor-treated leaves. In healthy, unstressed plants, PR-10a was expressed exclusively in the stigma. Neither GUS activity nor PR-10a protein was detected in vegetative shoot tissues, or in style, ovary, sepal, petal, or anther extracts. The stigma-specific expression was shown to be developmentally regulated, with more PR-10a protein accumulating in the stigmas of fully open than unopened flowers. Histochemical GUS staining indicated strongest PR-10a expression in the papillae and uppermost cell layers of the stigma, with lower levels of expression in cell layers below. Although the general pattern of expression of *PR-10a* resembles the pattern of expression of genes encoding cell wall protein genes, its organ- and tissue-specific expression differentiates it from most other defense-related proteins and genes. This suggests that *PR-10a* plays a unique role in plant defense.

Additional keywords: elicitor, flower, gene expression, plant defense, ribonuclease, wounding.

Plants respond to pathogen attack by transcriptionally activating a broad range of genes thought to help the plant ward off the invader (Lamb *et al.* 1989). These include genes important for cell wall reinforcement, such as those encoding hyroxyproline-rich glycoproteins (Showalter *et al.* 1985), proline-rich proteins, and enzymes of phenylpropanoid metabolism involved in lignification (Hahlbrock and Scheel 1989). Other genes code for products which have antimicrobial activity themselves, such as thionins (Apel *et al.* 1990), chitinases and β-glucanases (Kombrink *et al.* 1988), or which are required for the synthesis of the antimicrobial compounds, the phytoalexins (Dixon 1986). In some cases, genes encoding proteins with defined biochemical activities but unknown role to plant defense have been shown to be expressed after pathogen attack (Dudler *et al.* 1991). Furthermore, a number of genes with unknown function but activated by pathogen stress have been cloned, such as the *ELI* genes of parsley (Somssich *et al.* 1989), and the *win*, *wun,* and *PR1* genes of potato (Stanford *et al.* 1989; Logeman *et al.* 1989; Taylor *et al.* 1990).

As part of our long-term aim of better understanding the defense against pathogens in potato and its control, we have identified genes that are expressed in tubers following elicitor treatment of potato tuber disks (Marineau *et al.* 1987). One strongly induced gene, *PR-10a*, was cloned and found to encode a 17-kDa polypeptide (Matton and Brisson 1989). Sequence comparisons gave no indication of a biochemical role of the gene product, but homologous genes were also described from *Fusarium solani*-infected pea endocarp (Chiang *et al.* 1990) and fungal elicitor-treated parsley and bean cell suspensions (Somssich *et al.* 1988; Walter *et al.* 1990). Recently a related gene from a monocot plant family was identified in mechanically isolated and cultured asparagus cells (Warner *et al.* 1992). The absence of a signal peptide in any of the encoded proteins suggests that, unlike the classic virally induced "pathogenesis-related" proteins, this family of proteins is intracellular. The biological function of these genes remains unknown, but their evolutionary conservation as wound- and pathogen-activated genes in both monocot and dicot families suggests an important role in the plant defense response. In addition, genes expressed during late pea embryogenesis and in auxin-starved soybean cell cultures (Barrat and Clark 1991; Crowell *et al.* 1992), were shown to be related to PR-10a, as was a major birch pollen allergen (Breiteneder *et al.* 1989).

In potato tuber disks, PR-10a mRNA is detectable as early as 2 hr after treatment with arachidonic acid, an elicitor of sesquiterpenoid phytoalexins in potato tuber tissue (Matton and Brisson 1989). Wounding also induced PR-10a transcript accumulation but to much lower levels. The accumulation of the PR-10a gene product was demonstrated following elicita-

tion as well as infection with the potato pathogen *Phytophthora infestans* using a PR-10a-specific antibody (Constabel and Brisson 1992). Whether *PR-10a* is required for resistance to pathogens is not yet clear, since PR-10a protein accumulates after challenge with both compatible and incompatible races of *P. infestans*. However, at low inoculum concentrations, the incompatible race induced more rapid accumulation of PR-10a protein than the compatible race (Constabel and Brisson 1992).

Analysis of the promoter region of the *PR-10a* gene in transgenic potato plants indicates that a 1,015-bp 5′ upstream region of the gene is sufficient to confer elicitor inducibility to the β-glucuronidase (GUS) reporter gene in potato tuber disks (Matton *et al.* 1993). Here we demonstrate that in these transgenic plants the promoter also confers wound and pathogen inducibility to the GUS reporter gene in other plant organs. By using these transgenic plants in conjunction with an anti-PR-10a antibody, we investigated the organ- and tissue-specific expression of *PR-10a* in infected and healthy potato plants. We found that following infection with *P. infestans* or treatment with *P. infestans* homogenate, the *PR-10a* gene was highly expressed in tubers, stolons, stems, and petioles, especially in the vascular tissue of these organs. Furthermore, our findings indicate that in unstressed plants, PR-10a was expressed exclusively in the stigma.

RESULTS

Induction of PR-10a expression in vegetative plant tissues.

Transgenic potato plants transformed with the β-glucuronidase (GUS) reporter gene under the control of 1,015 bp of the PR-10a 5′ upstream sequence have been described previously (Matton *et al.* 1993). In tuber disks prepared from these transgenic plants, GUS activity increased following arachidonic acid elicitor treatment, consistent with the pattern of PR-10a mRNA accumulation (Matton and Brisson 1989). To determine if PR-10a could be induced in other tissues and organs, we looked for the accumulation of PR-10a protein as well as GUS activity in stolons, stems, leaves, and petioles. Since preliminary experiments had indicated that *P. infestans* homogenate was a much more effective elicitor of PR-10a expression in shoot tissues than arachidonic acid, this elicitor was used for routine inductions of PR-10a. Stems, petioles, and stolons were excised and treated with homogenate on the wounded surface to ensure penetration of elicitor into the tissue. Application directly onto unwounded surfaces failed to elicit the defense response. For leaves, the elicitor treatment was performed by floating leaf segments on diluted fungal homogenate. To facilitate a comparison with the tuber response, homogenate-treated tuber disks were also included. After a 4-day induction period, extracts were prepared from the treated as well as untreated and unwounded control tissues. The same extracts were analyzed by immunoblot for PR-10a protein accumulation as well as for GUS activity using the fluorometric assay. This parallel analysis was carried out to ensure that the reporter gene expression accurately reflected PR-10a expression in all plant organs. Figure 1A shows that on immunoblots the antibody strongly recognized two proteins, migrating at 17 and 18 kDa, respectively. We had previously shown that the 17-kDa protein is the PR-10a gene product, while the 18-kDa protein may represent another member of the *PR-10* gene family (Constabel and Brisson 1992).

Extracts of untreated control tissues did not contain detectable PR-10a protein (Fig. 1A, "–" lanes). Although a faint protein band was visible in control tuber extracts in Figure 1A, this band did not comigrate exactly with the 17-kDa band and was never observed in other experiments and therefore may represent a gel artifact. Following treatment with the elicitor, however, tuber disks, stolons, stems, and petioles all accumulated substantial levels of PR-10a protein (Fig. 1A, "+" lanes). The highest levels of PR-10a protein were seen in elicited tubers, petioles, and stems, with somewhat lower amounts detected in stolons. The leaf segments, even though they had clearly responded to the elictor with hypersensitive browning, accumulated very little PR-10a protein. Figure 1B demonstrates that those organs showing a clear PR-10a protein band following elicitation also showed high GUS activity. In control extracts, no GUS activity above background was detected, whereas after elicitor treatment GUS activity was high in tubers, stolons, stems, and petioles, but low in leaves. This confirmed the faithful regulation of the GUS gene by the PR-10a promoter. Wounding and infection also induced PR-10a and GUS expression (not shown) and the correspondence of GUS activity with the PR-10a protein was likewise observed. However, leaves responded to infection

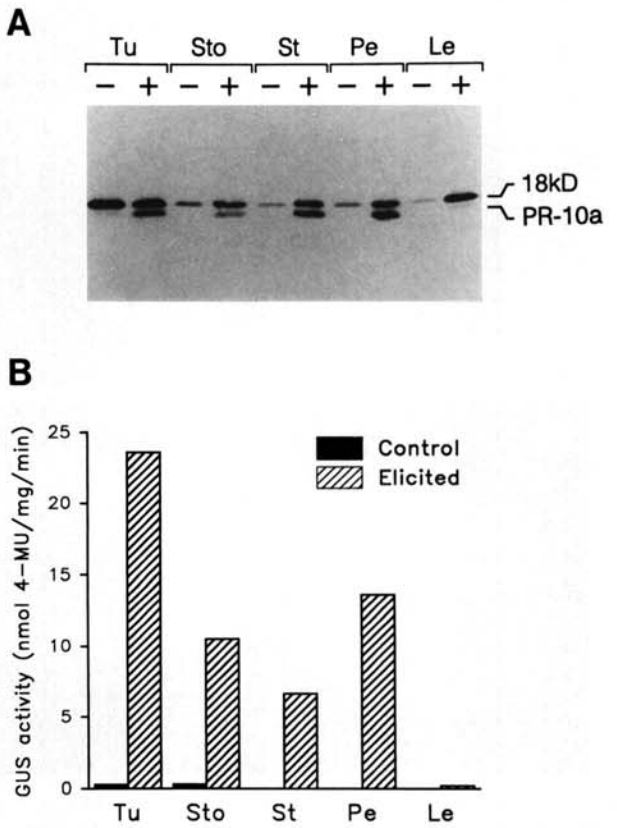

Fig. 1. PR-10a protein and GUS activity in *Phytophthora infestans* homogenate-treated organs of transgenic potato. Plant tissues were detached from the plant, treated with elicitor on the cut surface, and incubated for 4 days. Soluble proteins were extracted and analyzed for **A,** PR-10a protein by immunoblotting and, **B,** GUS activity by fluorometry. Tu, tuber; Sto, stolon; St, stem; Pe, petiole, Le, leaf. +/– indicates fungal elicitor-treated and control (untreated) tissues, respectively.

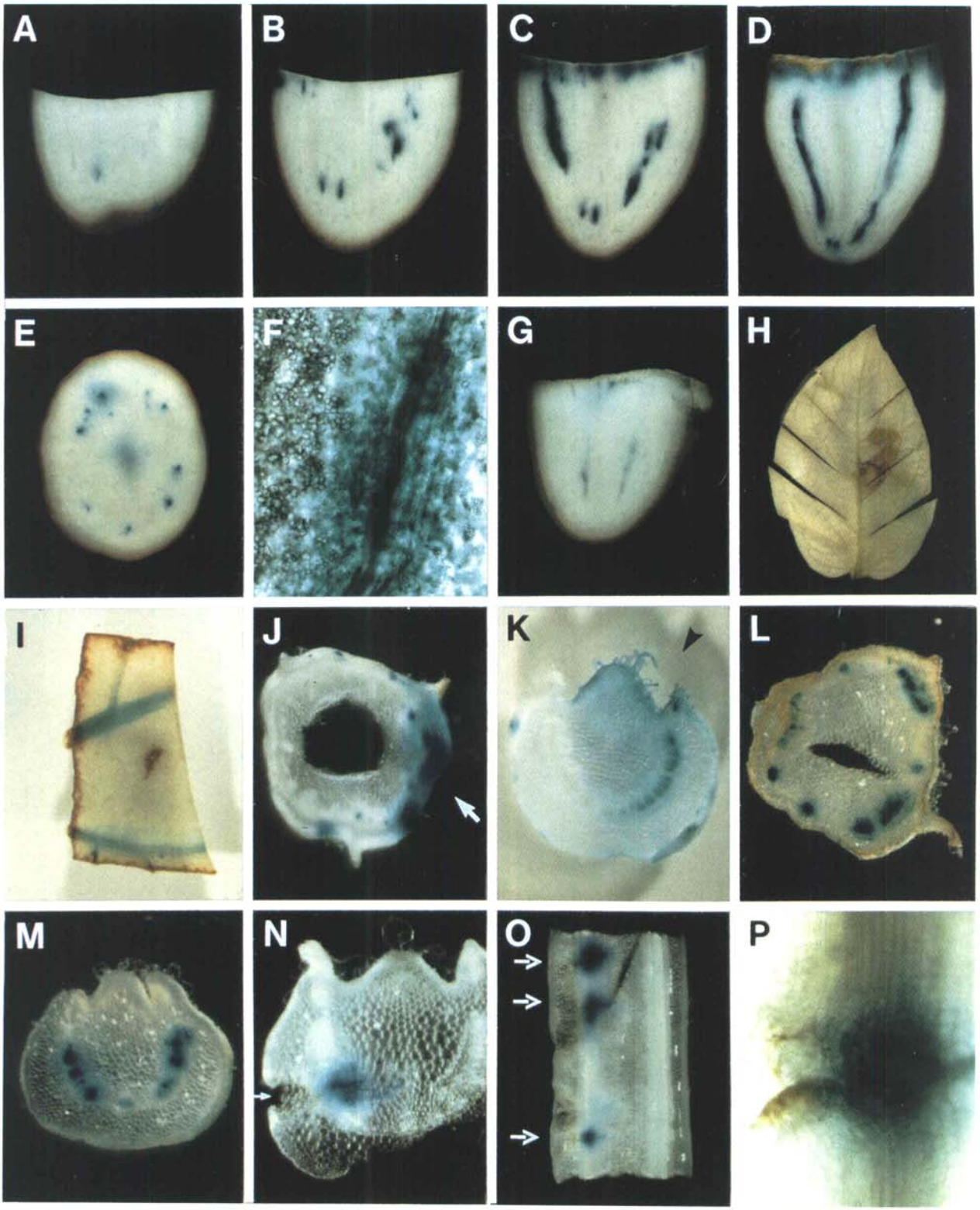

Fig. 2. Histochemical GUS staining to localize PR-10a promoter activity in transgenic potato plants. Tissues were infected with *Phytophthora infestans*, treated with elicitor, or wounded to induce GUS activity. **A–F,** Tubers were halved, the wounded surface inoculated with zoospores of a compatible race of *P. infestans*, and sectioned through the infected surface at different intervals. **A–D,** 1–4 days postinoculation (p.i.). **E,** Same as D but sectioned in parallel to and 1 cm below the inoculated surface. **F,** Enlarged section through tuber vascular tissue 3 days p.i. **G,** Tuber piece mock-inoculated with water (4 days). **H,** Leaflet 4 days p.i. with *P. infestans*. Before staining, the leaflet was sliced to permit even penetration of X-Gluc. **I,** Leaf segment, 4 days floatation on fungal homogenate. **J,** Stem cross section, 4 days p.i. with *P. infestans* (arrow indicates inoculation site). **K,** Petiole cross section, 4 days p.i. (arrow indicates inoculation site). **L,** Wounded surface of stem, 3 days after excision wounding. **M,** Wounded surface of petiole, 3 days after excision wounding. **N,** Cross section through petiole, 3 days after incision wounding (at arrow). **O,** Longitudinal section through petiole, 3 days after incision wounding (at arrows). **P,** same as O, higher magnification.

with *P. infestans* or wounding with neither detectable PR-10a induction nor GUS activity (not shown). Other independently transformed plants gave the same pattern of relative GUS expression in the tissues tested, although variation in absolute levels of GUS activity was observed.

The cross-reacting 18-kDa protein was also induced by elicitation in all organs except tubers, where high constitutive levels of the protein were already present. This protein was also present at low to moderate levels in unstressed stolons, stems, petioles, and leaves (Fig. 1A).

Histochemical GUS localization in tubers, stems, and petioles.

The faithful regulation of the GUS reporter gene by the PR-10a promoter allowed us to pursue a more detailed spatial analysis of PR-10a expression following wounding and pathogen stress using the GUS-specific histochemical stain X-Gluc. We used infection with the live pathogen rather than the *P. infestans* homogenate to avoid the problem of transport of the elicitor within the plant and to approximate the natural situation more closely. GUS reporter gene expression was induced by infecting different organs of transgenic potato plants with a compatible race of *P. infestans*. Figure 2A-D shows sections from a halved tuber, inoculated on the cut (upper) surface with *P. infestans* zoospores and incubated for 1-4 days before being sectioned vertically across the infected surface and incubated with X-Gluc. Figure 2A shows that 1 day postinoculation (p.i.), no GUS activity was visible at the infected surface, but some patchy blue coloration had appeared within the tuber. These patches of GUS expression became enlarged and connected in the following days, until a band covering the vascular cylinder was deeply stained at 4 days p.i. (Fig. 2C and 2D). At 3 days p.i. a zone of blue also became visible at the infected surface (Fig. 2C). Mycelium appeared on the infected surface at this time and expanded rapidly so that at 5 days p.i. the tissue became discolored and the experiment was stopped (not shown). The predominant distribution of the GUS activity in the vascular ring was clearly evident when a tuber piece, infected for 4 days, was sectioned horizontally approximately 1 cm below the infected surface (Fig. 2E). Thin hand-sections observed by light microscopy confirmed the association of GUS activity with vascular cells (Fig. 2F). Control tuber pieces mock-inoculated with water for 4 days showed faint staining below the cut surface and in the vascular area (Fig. 2G), demonstrating the slight wound-induction of the PR-10a promoter.

Leaves infected with *P. infestans* gave no detectable staining at any time during the infection process, even when lesions were clearly visible 4 days p.i. (Fig. 2H). By contrast, leaf pieces floated on fungal homogenate for 4 days showed blue coloration specifically in the veins (Fig. 2I); however, no color was detected in the leaf parenchyma along the cut margin even though the cells were in direct contact with the elicitor. The absence of GUS activity in leaf parenchyma was consistently observed in numerous independent experiments. In contrast to the leaf response, stems and petioles responded strongly to infection (Fig. 2J and K). All cell types near the infected surface (arrows) showed GUS activity, but the degree of coloration was variable between experiments. Observation of many sections indicated that, overall, vascular bundles showed stronger GUS activity than other cell types. In some infection experiments only the vascular tissues showed visible coloration. In all sections examined, GUS activity was limited to the region of the stem or petiole directly below the site of inoculation, suggesting that the *PR-10a* gene is not induced systemically in shoots.

Since preliminary experiments suggested that in petioles and stems, wounding could also induce significant PR-10a expression, we analyzed PR-10a promoter activity in wounded tissues. While wounding the leaf blades did not result in detectable GUS staining, the excision of shoots and leaves resulted in strong GUS expression at the cut end of the stems and petioles, but only at the vascular bundles (Fig. 2L). The coloration was very localized, and GUS activity usually did not extend beyond 1–2 mm of the wounded surface (not shown). Essentially an identical pattern was obtained with excised petioles (Fig. 2M). Replicate experiments performed asceptically on *in vitro*-grown plants gave the same results, confirming that the coloration was not due to localized microbial contamination (not shown). GUS expression could not be localized to a particular cell type; the coloration was seen in both xylem and phloem bundles. We further determined the vascular-specific PR-10a expression by wounding petioles with radial incisions using a sterile needle. Only where the incision (arrows) had damaged a vascular bundle was PR-10a induced (Fig. 2N–P). Epidermal and parenchyma cells at the incision site, though clearly damaged (see browning of cells in Fig. 2P), did not show detectable GUS activity.

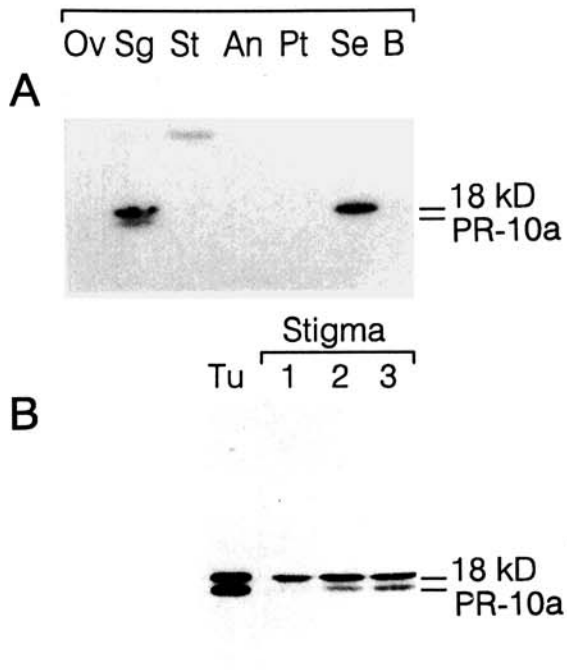

Fig. 3. PR-10a protein accumulation in potato flowers. **A,** Soluble proteins of mature flower tissues were extracted and analyzed for the presence of PR-10a protein by immunoblotting. Ov, ovary; Sg, stigma; St, style; An, anther; Pt, petal; Se, sepal; B, flower bud. **B,** Extracts of stigmas were prepared from flowers at different stages of development and analyzed by immunoblotting. 1, flower closed; 2, flower closed with style protruding; 3, flower fully open; Tu, arachidonic acid-elicited tuber.

Developmental expression of PR-10a in stigmas of healthy potato plants.

As several other PR proteins have been shown to accumulate in flowers (Lotan *et al.* 1989; Neale *et al.* 1990; Memelink *et al.* 1990), we also used the anti-PR-10a antibody to screen floral organs for the constitutive presence of PR-10a protein. Mature flowers were dissected and protein extracts of floral organs analyzed by immunoblot. Figure 3 shows that of all the organs tested, only in extracts of stigmas did the antibody recognize a 17-kDa protein, presumably the *PR-10a* gene product. The antibody did not recognize a corresponding band in extracts of the style or the ovary, nor in extracts of anthers, petals, sepals, or young flower buds (Fig. 3A). Immunoblots of extracts prepared from the stigmas of flowers harvested at different stages of development demonstrated that this 17-kDa protein was already present in small amounts in the stigma before flower opening (Fig. 3B, lane 1), and that levels increased later when the flowers were partially or fully open (Fig. 3B, lanes 2 and 3, respectively). At none of the stages of flower development could the 17-kDa protein be detected in any other floral organ (not shown). The 17-kDa protein in stigma extracts comigrated with the PR-10a protein seen in elicited tuber disks (Fig. 3B, Tu), suggesting strongly that it is the product of the *PR-10a* gene.

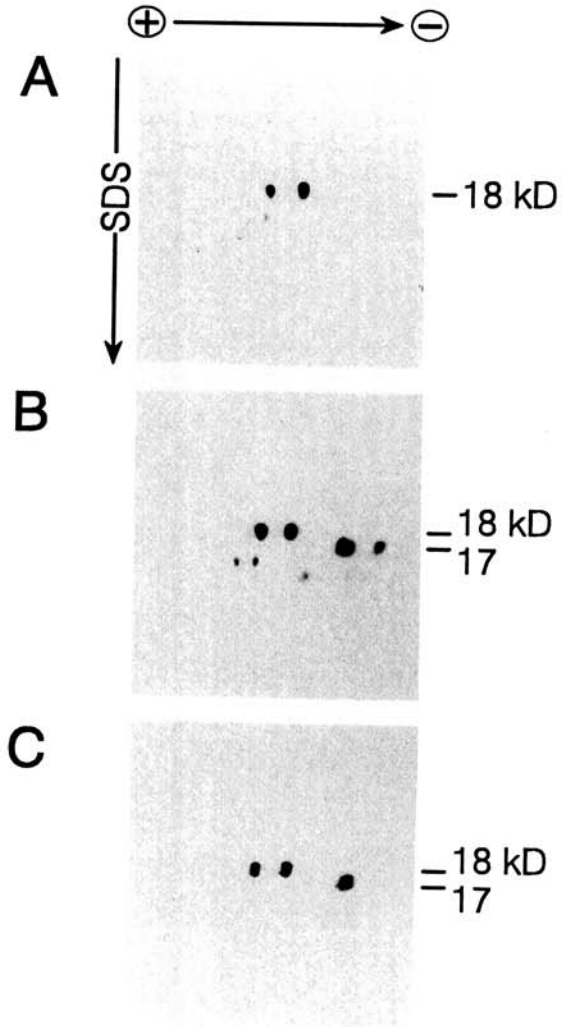

Fig. 4. Two-dimensional immunoblot analysis of tuber and stigma proteins. **A,** Extracts of untreated tubers were separated by isoelectric focussing in the first dimension and by 14% SDS-PAGE in the second dimension before being blotted to nitrocellulose and probed with the anti-PR-10a antibody. **B,** Two-dimensional immunoblot of extracts of tuber disks 4 days after arachidonic acid elicitor treatment. **C,** Two-dimensional immunoblot of extracts of stigmas of mature flowers from healthy potato plants.

In both stigma and sepal extracts the antibody again reacted with a protein of 18 kDa, presumably the same protein we had observed in both control and stressed vegetative tissues (Fig. 1A). The antibody also gave a faint reaction with a 40-kDa protein present in the style. This reaction appeared to be nonspecific, however, since replica Coomassie-stained gels indicated that this band represents the predominant protein of the style. Unlike the 17-kDa protein, levels of the 18 kDa protein in the stigma did not change during flower development (Fig. 3B).

To confirm that the 17-kDa band recognized by the PR-10a antibody in the stigma is indeed the *PR-10a* gene product, we analyzed stigma and tuber extracts by two-dimensional gel electrophoresis followed by immunoblotting. Figure 4A shows that on gels of control tuber extracts, no cross-reacting protein was detected at 17 kDa, while the 18 kDa protein was resolved into at least two major polypeptides. In tuber disks treated with elicitor, however, at least three protein spots corresponding to the single 17-kDa band on one-dimensional gels appeared (Fig. 4B). All three spots are products of *PR-10a* or very closely related genes, since the pattern obtained was identical with the results seen when PR-10a-hybridizing messages were selected and translated *in vitro* in a reticulocyte lysate (Marineau *et al.* 1987). We do not know the identity of the other minor polypeptides recognized by the antibody in Figure 4A and B; they may be breakdown products of PR-10a or the 18-kDa protein or gel artifacts.

Figure 4C shows that the two-dimensional immunoblot of the stigma proteins was similar to that of the elicited tuber proteins. Two polypeptides migrated at 18 kDa, but only a single PR-10a protein spot was visible. The most acidic PR-10a polypeptide was clearly absent, and careful alignment of Figure 4B and C suggested that the more basic of the two spots of the 17-kDa doublet seen in Figure 4B was also absent. Thus, the two-dimensional gels confirmed that PR-10a protein was present in the stigma. Furthermore, additional isoforms of the protein were present in elicited tuber disks.

We also analyzed extracts of floral organs of the transgenic plants for GUS activity. Table 1 shows several transformants in which elevated levels of GUS activity were observed in the stigma, while only low levels were measured in other floral organs. Other transformants, however, in which GUS was stress inducible, failed to show GUS activity in the stigma. This may be due to insertion of the T-DNA into regions of DNA transcriptionally active during the defense response but inactive during flower development. The stigma-specific expression of GUS matches the distribution of the PR-10a protein as determined by immunoblot (Fig. 3A). The levels of GUS activity observed in stigmatic extracts were variable between transformants and significantly lower than those seen in vegetative tissues. However, GUS activity was never observed in other floral organs, indicating that while GUS expression was variable between transformants, the PR-10a promoter is regulating the reporter gene correctly in the flowers. Therefore, we used the histochemical GUS stain to in-

vestigate the spatial pattern of GUS expression in potato stigmas. Mature flowers were harvested, dissected, and incubated in the X-Gluc solution. Figure 5A confirms that the blue reaction product indicative of GUS activity was produced only in the stigma. Closer examination of the stigma indicated that the colored precipitate of the reaction product accumulated near the stigma surface (Fig. 5B). This distribution was not the result of limited diffusion of the X-Gluc substrate, since hand-sectioning the stigma longitudinally prior to incubation with the substrate gave the identical result (not shown). Varying the concentration of ferric ions, recently shown to have a dramatic effect on the localization of GUS activity (de Block and Debrouwer 1992), altered the intensity but not the overall pattern of the stain. In some other experiments, coloration was also seen in anther tissue, but was equally pronounced in untransformed plants. Such endogenous GUS activity had previously been described in tobacco anthers and pollen (Plegt and Bino 1989). In paraffin-embedded thin sections the coloration was clearly most intense near the papillae at the stigma surface (Fig. 5C). GUS activity, however, was not limited to this cell layer and the blue precipitate was also visible in the zone of secretory cells below, as well as in the next zone of cells contiguous with the style. However, no coloration was detected in the vascular tissue, the epidermis of the style adjacent to the stigma surface, nor in the cells of the style itself.

DISCUSSION

Induction of PR-10a in response to wounding and pathogen stress.

With the exception of the stigma (see below), *PR-10a* was not constitutively expressed in any floral or vegetative organ tested in healthy plants. However, PR-10a expression could be induced in all of the vegetative organs assayed (leaves, stems, petioles, stolons, or tubers) by elicitation or infection with *P. infestans*. Wounding also stimulated PR-10a expression in most organs tested, but to lower levels than elicitor treatment. These results contrasted with those reported for a *PR-10a* homologous gene of soybean, which has been shown to be developmentally regulated in roots and cotyledons of seedlings (Crowell *et al.* 1992). Similarly, two abscisic acid-inducible proteins homologous to PR-10a were shown to be expressed in pea during embryogenesis (Barratt and Clark 1991). We have been unable to observe developmental expression of PR-10a in roots of potato, nor does ABA induce PR-10a expression in potato leaves (C. P. Constabel and N. Brisson, unpublished results).

In leaves, GUS activity could not be induced by *P. infestans* or wounding. However, low levels of GUS activity were detected when leaf segments were floated on *P. infestans* homogenate. The lack of PR-10a induction by *P. infestans* infection of leaves is surprising and conflicts with our previous report of detectable PR-10a protein accumulation in potato leaves after inoculation with compatible or incompatible zoospores of *P. infestans* (Constabel and Brisson 1992). In the previous experiments we had used plants of cv. Kennebec, whereas here we used cv. Desiree. The discrepancy may thus result from distinct responses of these cultivars to *P. infestans*. Differences between potato cultivars in defense reactions and in the induction of specific proteins has been documented (Fritzemeier *et al.* 1987; Vaughn and Lulai 1992). However, in cv. Kennebec leaves accumulation of PR-10a proteins was also weak and approximately tenfold lower than in tubers. Since leaves are the first points of contact for many pathogens, strong leaf expression would seem appropriate for plant defense genes. Thus, PR-10a expression differs from the pattern demonstrated by other pathogen-induced genes and proteins of potato which are strongly expressed in leaves as, for example, the phenylpropanoid enzymes, chitinase, β-1,3-glucanase, and other pathogenesis-related proteins (Fritzemeier *et al.* 1987; Schröder *et al.* 1992; Logeman and Schell 1988; Stanford *et al.* 1989; Taylor *et al.* 1990). One explanation for the low level of PR-10a expression in leaves could be the activation of a PR-10a homolog more specific to leaves. A candidate is the 18-kDa protein which strongly cross-reacts with the anti-PR-10a antibody and which accumulates to high levels in *P. infestans*-inoculated leaves (Constabel and Brisson 1992).

An unexpected feature of wound- and pathogen-induced PR-10a expression in potato was its association with vascular bundles. Histochemical GUS staining of infected tubers, stems, and petioles indicated that PR-10a was preferentially expressed in vascular tissue. Experiments with *P. infestans*-infected stems and petioles indicated that in these tissues, most if not all cell types were competent to respond to infection with PR-10a expression, but vascular expression was the strongest. Our data also indicate that in these organs only vascular tissue responded to wounding; this may be an indication of several distinct control mechanisms for PR-10a expression in these tissues. In tubers, infection and wounding both resulted in vascular and localized induction in storage parenchyma, and in leaves the only tissues in which GUS activity could be detected were the leaf veins. Since vascular tissue has been shown in other systems to transport systemic wound and pathogen signals to distal portions of the plant (Davis *et al.* 1991; Yalpani *et al.* 1991), we considered whether the vascular transport of stress signals could explain the pattern of PR-10a expression in vascular tissue. However our data are not consistent with such an explanation for the following reasons: 1) Vascular tissue in infected tubers expressed GUS well before the infected surface itself. Although in this case there may be a signal which induces cells along the vascular bundles, the response of the vascular cells is nonetheless stronger and faster than that of storage parenchyma cells at the infection site itself. 2) Excision and incision wounding of petioles damaged epidermal, parenchyma, and vascular tissue alike, yet only the latter showed significant PR-10a expression. 3) Leaf segments floated on *P. in-*

Table 1. GUS activity in pistil of transgenic potato flowers expressing the GUS gene under the control of the *PR-10a* promoter

	GUS activity (pmole 4-MU mg^{-1} min^{-1})				
Transformant	**Sepal**	**Petal**	**Ovary**	**Style**	**Stigma**
D1	51	63	48	66	90
D9	NT[a]	NT	30	45	357
D17	NT	NT	30	93	171
D22	51	63	42	99	150
D26	33	27	36	24	2,468
Control[b]	15	54	6	36	14

[a] Not tested.
[b] Control is an untransformed potato plant.

festans homogenate showed GUS expression only in veins, but not in the leaf mesophyll cells which were most directly in contact with the elicitor. Despite the differences in response between tissues and stress type, overall our data show that PR-10a expression is specifically induced in vascular cells.

Other PR protein genes such as *win*, *PR-1*, and *PAL* also show expression in vascular bundles (Stanford *et al.* 1990; Carr *et al.* 1987; Liang *et al.* 1989, Zhu *et al.* 1993). However, these differ from *PR-10a* in being strongly inducible in leaves. The essential pattern of organ- and tissue-specific PR-10a expression, strong induction in vascular bundles but very little induction in leaves, differentiates *PR-10a* from most other defense-related proteins and genes. However, the hydroxyproline-rich glycoproteins (HRGPs) and glycine-rich proteins (GRPs), which are wound- and pathogen-inducible in stems, are also induced to a much lesser extent in leaves (Showalter *et al.* 1992; Cassab and Varner 1988). Furthermore, transcriptional activity of the wound-induced *GRP 1.8* gene was localized to the pith adjacent to the xylem cells of tomato stems (Keller *et al.* 1988), and HRPGs were found associated with xylem cells in non-necrotic tobacco mosaic virus-infected tobacco leaves (Benhamou *et al.* 1990). Interestingly, cDNAs encoding HRGP-like proteins have been cloned from tobacco pistils (Chen *et al.* 1992; de S. Goldman *et al.* 1992), and a GRP is expressed in stamens (de Oliveira *et al.* 1993), further linking pathogen and wound-induced genes with flower development (see below). However, none of these respective genes were stress inducible. Nevertheless, the similarities in the expression of PR-10a with genes encoding cell wall proteins is intriguing and could lead to more direct tests of PR-10a function.

Developmental regulation of PR-10a in potato stigmas.

Our results clearly demonstrate that the *PR-10a* gene is expressed and that PR-10a protein accumulates in the stigma of healthy, uninfected potato plants. In no other floral organs or vegetative shoot tissue could we detect significant PR-10a protein. The presence of the PR-10a protein prior to flower opening (Fig. 1B) argues against the possibility of PR-10a induction by a symptomless infection. The increase in PR-10a protein levels after flower opening indicates that in the stigma, control of PR-10a expression is under precise developmental control. The expression of the *PR-10a* gene in the stigma was confirmed in transgenic potato plants by the expected regulation of the GUS reporter gene under control of the PR-10a promoter. Sections of histochemically stained flowers showed that PR-10a expression was strongest at the stigma surface.

The stigma-specific expression of PR-10a is especially intriguing in light of the high similarity existing between the sequence of the major pollen allergen of birch (Breiteneder *et al.* 1989) and PR-10a. However, with our antibody we have never been able to detect any cross-reacting proteins in extracts of pollen or anthers at any time during flower development, in either transgenic or wild-type potato plants. Warner *et al.* (1993) recently reported that the promoter of *AoPr1*, a *PR-10a* homolog from asparagus, drives GUS expression in pollen grains of transgenic tobacco, but it is not clear if AoPR1 mRNA or protein accumulates in asparagus pollen. We do not know if any of the *PR-10a* homologs in other plants species are expressed in either male or female reproductive organs. However, recently the products of several pathogen- or wound-induced genes have been shown to be expressed in the stigma, including tobacco proteinase inhibitors (Atkinson *et al.* 1993) and chitinase (Leung 1992). Other defense-related proteins such as β-glucanase, osmotin, tobacco PR-1, GRPs, and HRGPs have been found in other floral organs (Memelink *et al.* 1990; Neale *et al.* 1990; Ori *et al.* 1990; Côté *et al.* 1991). The role of these proteins in flowers is unknown, but it may not be directly related to plant defense. As Lotan *et al.* (1989) have pointed out, callose, a 1,3-β-glucan polymer and potential substrate of the β-glucanases, is deposited during pollen tube growth through the transmitting tissue. Recently, an essential role of chitinase in carrot embryo development was demonstrated by De Jong *et al.* (1992).

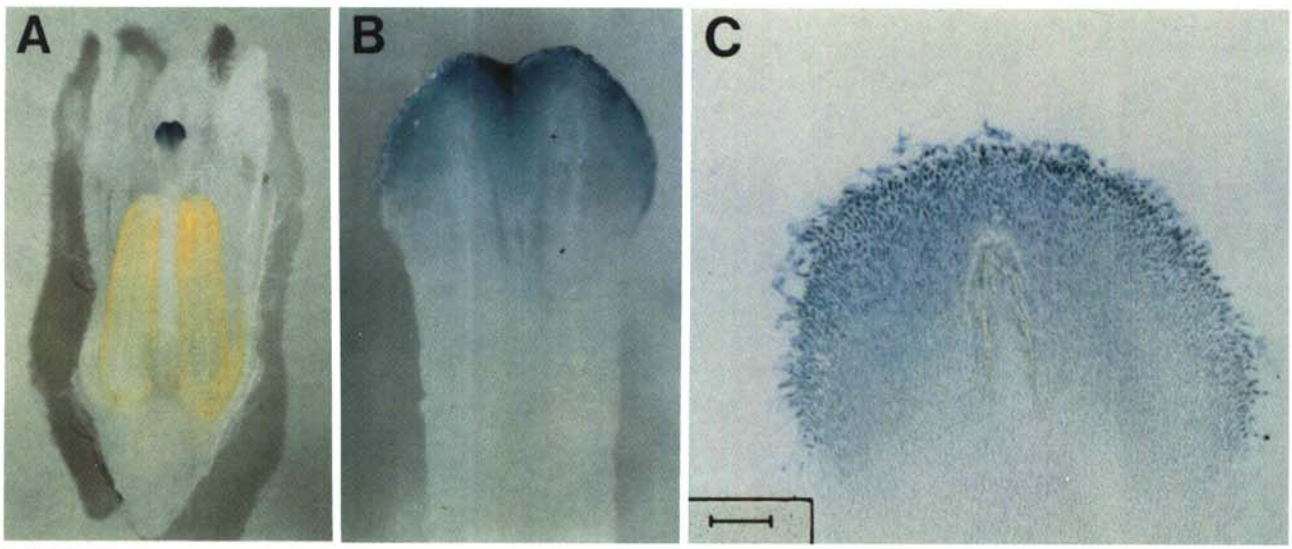

Fig. 5. The PR-10a promoter-GUS chimeric gene expression in transgenic potato stigmas. **A,** Whole transgenic potato flower sectioned longitudinally and incubated for 16 hr in X-Gluc. **B,** As in A, but higher magnification showing stigma-specific coloration. **C,** Thin section (15 μm) of a potato stigma stained by incubation with X-Gluc and embedded in paraffin. Bar represents 100 μm.

Therefore, it is not clear if the presence of a pathogen-induced protein such as PR-10a in the stigma is related to defense against pathogens. While the involvement of specific recognition events during both plant-pathogen interactions and pollination events is well-documented (Keen 1990; Haring *et al.* 1990), it remains to be seen if the superficial similarity between these processes has a biochemical and physiological basis. Interestingly, it has been suggested recently that the PR-10 gene family may encode ribonucleases (Moiseyev *et al.* 1994). This was based on the high sequence similarity between a ribonuclease from ginseng and the parsley PR2-encoded protein. Such a function would establish an interesting parallel between the defense response and events related to pollination since the S-glycoproteins, which are linked to self-incompatibility in solanaceous plants (Cornish *et al.* 1991), also demonstrate ribonuclease activity (McClure *et al.* 1989). Preliminary experiments, using a non-specific RNase assay (Brown and Ho 1986) have failed to demonstrate RNase activity for the PR-10a gene product (R. Xiao and N. Brisson, unpublished results). If PR-10a protein does indeed function as an RNase, it is thus most likely to be specific for certain RNA substrates, or require a specific activation of its enzymatic activity. This would also be most consistent with the lack of visible phenotypical abnormalities of transgenic potato plants overexpressing PR-10a (Constabel *et al.* 1993), something which would not be expected if PR-10a encoded an unregulated or nonspecific RNase activity. Future work will aim to further test these hypotheses.

MATERIALS AND METHODS

Plant material and stress treatments.

Solanum tuberosum L. 'Desiree' and 'Kennebec' plants were maintained in growth chambers under long-day conditions. Transgenic potato plants (cv. Desiree) transformed with the β-glucuronidase (GUS) gene under the control of the PR-10a promoter have been described previously (Matton *et al.* 1993). Of 26 independent kanamycin-resistant primary transformants, 10 were used for further analysis on the basis of elicitor-inducible GUS activity in *in vitro*-grown tubers. Southern blot analysis using a GUS probe confirmed that these lines were due to independent transformation events and indicated that six lines contained a single insertion of the transgene, while four lines contained two insertions. There was no correlation between the level of expression of the transgene and the number of copies of the transgene in those lines (G. Prescott and N. Brisson, unpublished observations). Transgenic plants were acclimated and grown in peat pots in a growth chamber. Tubers from the primary transformants were used as seed for producing the second and third generation plants used in all experiments. Cultures of *Phytophthora infestans* (Mont.) de Bary (race 1,2,3,4) were obtained from the American Type Culture Collection, Rockville, MD, and maintained at 15° C on rye agar (Stolle and Schöber 1980). Zoospores were induced as described by Rohwer *et al.* (1987). *P. infestans* homogenate was prepared from liquid cultures grown on V8 medium (200 ml of V8 vegetable juice, 3 g of $CaCO_3$, 12 g of agar, 800 ml of H_2O) as described by Lisker and Kuc (1977).

Tuber disks were prepared as described previously (Marineau *et al.* 1987) and treated with 50 µl of fungal homogenate spread evenly over the surface, or with sterile water in the case of controls. For GUS histochemistry, medium-sized tubers were halved, and the wounded surface inoculated with *P. infestans* zoospores (20,000 spores/ml). Petiole and stem elicitor treatments were performed by excising whole leaves or shoots and placing small droplets of fungal homogenate on the cut surface. Stolons were treated in the same manner but were cut into 4–5 cm segments. For infection with *P. infestans*, stems, leaves, and petioles were inoculated with 5-µl droplets containing 80–250 zoospores. For elicitor treatment, leaves were cut into segments (1 cm^2) and floated on fungal homogenate (1:20 dilution in sterile H_2O). Wound treatments of petioles and stems were performed using sterile instruments on tissues surface-sterilized with 70% ethanol. Treated tissues were incubated at 19° C on moistened filter paper.

Immunoblotting and GUS fluorometry.

Soluble protein was extracted into 50 mM NaH_2PO_4, pH 7.0, containing 10 mM EDTA, 0.1% v/v Triton X-100, 0.1% Sarkosyl, and 15 mM β-mercaptoethanol. The extracts were cleared by centrifugation, and the protein concentration determined by the method of Bradford using bovine serum albumin as a standard. For immunoblotting, proteins were precipitated with five volumes of cold acetone and protein resuspended in SDS-sample buffer. Proteins were separated by SDS-PAGE (14%) and immunoblots performed as described previously (Constabel and Brisson 1992). The blots were developed using the ECL chemiluminescent detection system (Amersham) according to the manufacturer's instructions, using Tris-buffered saline containing 0.05% Tween-80 for all washes. GUS assays were carried out in 50 µl of extraction buffer with 1 mM 4-methyl umbelliferyl glucuronide (MUG) as described by Jefferson *et al.* (1987). Fluorometric measurements were performed on a Hoefer Scientific DNA Fluorometer.

Two-dimensional gel electrophoresis.

Potato tissues were ground in 50 mM NaH_2PO_4, pH 7, containing 15 mM β-mercaptoethanol, and the extracts clarified by centrifugation and then lyophilized. Twenty five micrograms of total protein was resuspended in first dimension sample buffer, and the first dimension separation was performed with a Bio-Rad Mini-Protean II 2-D cell using the conditions outlined by the manufacturer. The first dimension gel contained 1.6% pH 5–7 and 0.4% pH 3-10 ampholytes. The second dimension separation was carried out on 14% SDS-polyacrylamide gels. The proteins were blotted to nitrocellulose and processed for immunoblotting as described (Constabel and Brisson 1992).

GUS histochemical staining and thin sectioning.

Induced and control tissues were dissected, hand-sectioned, and incubated in 50 mM NaH2PO4 buffer, pH 7.0, containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), and 25 mM each of $K_4Fe(CN)_6 \bullet 3H_2O$ and $K_3Fe(CN)_6$. Cycloheximide (2 mM) was added to prevent the induction of GUS during the staining incubation. For some experiments 0.02% Triton X-100 was added to facilitate penetration of the solution into the tissue, and leaf tissues were vacuum-infiltrated for 30 min. After incubation at 37° C for 16 hr, the tissue was fixed in 70% ethanol/5% glacial acetic acid/5% neutralized

formalin overnight. The tissue was cleared in 95% ethanol, and observed and photographed on a Wild M400 Photomakroscope. For thin sectioning the stained samples were dehydrated in an increasing series of tertiary butanol and embedded in Paraplast. Sections (15 μm) were prepared using a microtome, mounted, and observed and photographed with a Zeiss Axiophot microscope. All histochemistry experiments were repeated several times and performed with at least three independent transformants.

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