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Spatial and temporal patterns of transcription of a wound-induced gene in potato

A.C.Stanford[1], D.H.Northcote[2] and M.W.Bevan

Molecular Genetics Department, IPSR Cambridge Laboratory, Maris Lane, Trumpington, Cambridge CB2 2JB and [2]Biochemistry Department, Cambridge University, Cambridge CB2 1QW, UK

[1]Present address: Laboratoire de Biologie Moleculaire (INRA-CNRS), BP27, 31326 Castanet-Toloson, France

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Transcriptional fusions between the gene encoding *win2* from potato and the reporter gene encoding β-glucuronidase (GUS) have been used to study the spatial and temporal patterns of wound induced gene activity in transgenic potato and tobacco plants. Gene fusions containing a full length *win2* promoter were found to be correctly regulated in response to mechanical wounding in transgenic potato, but not in the heterologous host, tobacco. Sequences >560 bp upstream of the transcription start site of *win2* were shown to be important for wound inducibility. The dramatic induction of GUS activity detected using fluorometric assays of extracts of wounded and aged leaves of several independent *win2*-GUS transformants was consistent with the kinetics of *win2* mRNA accumulation. Histochemical analysis of wounded leaves showed that transcription first occurred in cells immediately adjacent to the wound, and was then progressively induced in cells associated with the vascular system at a distance from the wound site. In tubers, a localized response to wounding was observed, and this only spread to other parts of the tuber if it had started to sprout. It was concluded that active vascular transport was necessary for the spread of wound response. *Win2*-GUS fusions were also expressed as part of normal plant development, as GUS activity was detected in the developing buds and in a layer of cells associated with the lenticels of unwounded tubers.

Key words: β-glucuronidase/systemic response/transgenic potato/vascular system/wound-induced genes

Introduction

Plants respond to mechanical stress and pathogen attack by synthesizing a variety of proteins that are thought to provide protection against further trauma, and to inhibit both the growth and spread of invasive pathogens. The rapid induction of these specific proteins is primarily the result of increased gene transcription. For example, accumulation of mRNA for hydroxyproline-rich glycoproteins (HRGPs; Lawton and Lamb, 1987), for several of the enzymes catalysing steps in phenylpropanoid metabolism (Chappell and Hahlbrock, 1984; Cramer *et al.*, 1985; Bevan *et al.*, 1989), for several Pr proteins (Somssich *et al.*, 1986; Hedrick *et al.*, 1988), for a protein inhibitor (Sanchez-Serrano *et al.*, 1987; An *et al.*, 1989) and for an 18 kd protein of unknown function, *wun*1 (Logemann *et al.*, 1989), have all been shown to be due to a rapid increase in transcription of the respective genes.

Many of the genes involved in plant defence responses exhibit differences in the magnitude, spatial pattern and timing of induction in response to different conditions of stress. In certain cases this reflects the selective activation of individual members of gene families, e.g. those encoding chalcone synthase (Ryder *et al.*, 1987), HRGPs (Corbin *et al.*, 1987), and a family of wound-induced genes (*win*) (Stanford *et al.*, 1989). Differences in the spatial and temporal patterns of expression also occur for different defence genes in response to a single stress stimulus. For example, the highly localized induction of genes encoding enzymes of phenylpropanoid metabolism during a hypersensitive response to pathogen attack (Bell *et al.*, 1986), can be contrasted to the systemic induction, at a distance from the wound site, of genes encoding proteinase inhibitors (Ryan, 1978; Pena-Cortes *et al.*, 1988).

In order to describe and understand the different activation mechanisms which regulate the expression of genes involved in defence responses, it would be useful to examine the timing of induction and cell specificity of the transcription of these genes in response to mechanical and biological stress. Keil *et al.* (1989) have shown that a proteinase inhibitor II gene from potato, when fused to the β-glucuronidase (GUS) reporter gene (Jefferson *et al.*, 1987), is transcribed in tubers, as well as in mechanically wounded leaves. Leaves from plants which had undergone a systemic induction showed transcription occurring in cells associated with the vascular system.

This study has focused on a member of the wound induced *win* gene family which has been characterized at the sequence and RNA level in potato (Stanford *et al.*, 1989), although the functions of the gene products are not known. Transcriptional fusions have been constructed between the 5′ flanking sequences of *win*2 and the GUS reporter gene (Jefferson *et al.*, 1987) and introduced into potato and tobacco by *Agrobacterium* mediated transformation. This has allowed both the spatial and temporal patterns of *win*2-GUS gene transcription to be examined in mechanically wounded potato and tobacco tissues, using fluorometric and histochemical analyses for GUS activity. It was shown that dramatic inductions of GUS occurred upon wounding potato plants, with striking patterns of local and distant induction being observed in different organ types and at different developmental stages. In contrast, chimaeric gene activity was not regulated in a wound specific manner in the leaves of transgenic tobacco. The data indicated that promoter elements upstream of −560 bp from the cap site of *win*2 were necessary for wound induction, that the time course of induction of GUS activity was similar to that of *win*2 mRNA accumulation, that *win*2-GUS fusions were also

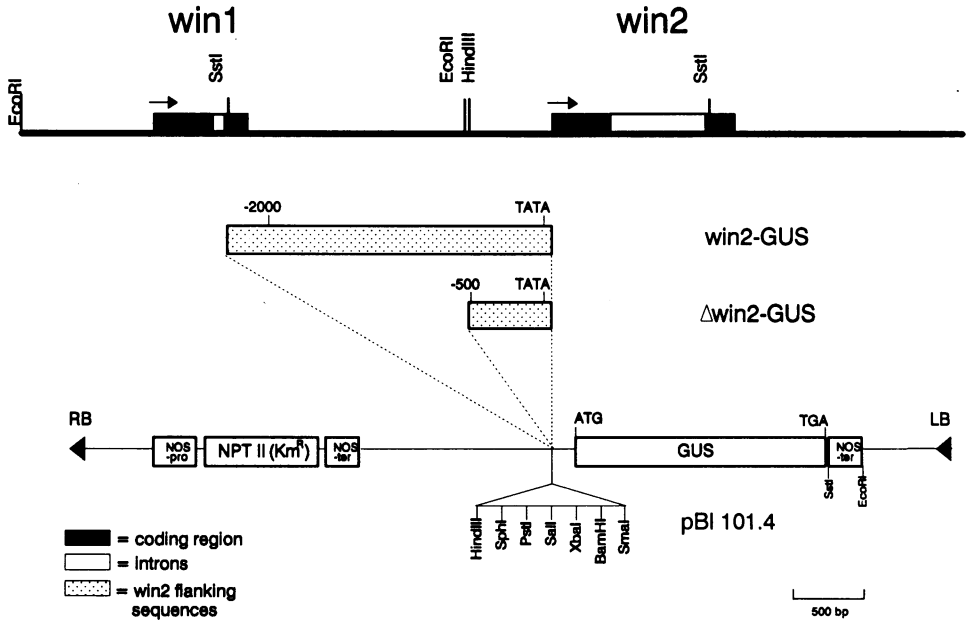

Fig. 1. Organization of *win*1 and *win*2 within the genomic clone λSt511, with the structure of the transcriptional fusions shown beneath. *win*2−GUS and δ*win*2−GUS contained 2177 and 558 bp of the 5′ flanking sequences of *win*2 respectively, cloned in front of the coding region of GUS within the transforming vector pBI101.4.

expressed as part of normal development, and that active vascular transport is important in determining the movement of a putative wound stimulus away from the original wound site.

Results

Win2−GUS fusions are transcriptionally activated in wounded transgenic potato plants

Chimaeric *win*2−GUS gene fusions containing either the full length or truncated *win*2 promoter fused to GUS (Figure 1, *win*2−GUS and δ*win*2−GUS respectively) were introduced into potato plants as described in Materials and methods. In initial screening of putative transformants, extracts of leaves which had been wounded and aged for 0 or 20 h were assayed fluorometrically for GUS activity. Twelve of the 16 putative transformants containing the *win*2−GUS gene fusion showed enhanced GUS activity after wounding. In contrast, no fluorescence was observed in extracts of wounded leaves from any of the eight putative δ*win*2−GUS transformants tested in an identical way. These latter transformants were not analysed further. Five of the *win*2−GUS transformants were replicated and used in subsequent studies. Replicate samples were taken from a single leaf or stem segment of any one transformant and assayed for GUS activity after wounding and ageing for up to 64 h. GUS activity was also assayed in replicate samples taken from the root system of each plant and from *in vitro* grown mini-tubers derived from each individual transformant. The leaves, stems, roots and mini-tubers of a transformant expressing a gene fusion containing GUS and the 35S promoter of cauliflower mosaic virus (CaMV−GUS) (Jefferson *et al.*, 1987) were assayed in a similar way. The results of these analyses are shown in Figure 2, in which the mean specific activity of GUS in three replicate samples has been plotted against time after wounding. There was a dramatic increase in GUS activity after wounding in both the leaves and stems of *win*2−GUS transformants, with the levels of activity consistently higher in stems than in leaves (Figure 2A). Although there was some variation in the magnitude and timing of the response between different transformants, similar profiles of induction were observed both for different organ types and independent transformants, with a characteristic delay of 16 h, before reaching maximal levels 48−64 h after wounding. This later phase of ageing represents a turning-point in the pattern of expression of the *win*2−GUS gene and may account for the relatively large standard error observed for the 64 h time points. Although the amount of variation within replicate samples taken at other time points was not as large, it appeared to be greater than the experimental error introduced during extraction, and during GUS and protein assays, which is shown for the samples taken from the CaMV−GUS transformant (Figure 2A). In contrast to the activity of the *win*2−GUS gene, expression of GUS activity under control of the CaMV promoter remained relatively constant during the wound time course in the organ types studied. This indicated that there were neither general alterations to gene transcription nor differences in the stability of GUS during the time course of wounding. While the magnitude of the induction was found to be reproducible both between experiments and among independent transformants (averaged over 15 plants derived from five independent transformants, GUS activity in leaves and stems increased 430-fold and 450-fold respectively over the first 48 h of ageing), a single but striking aberration was shown for the wounded leaf of transformant PA12. For this transformant, GUS activity was barely detectable in leaves over the entire 64 h of ageing despite a characteristic induction of GUS activity being observed in the wounded stem of the same plant.

The results for root and tuber tissue were considerably less clear-cut (Figure 2B). Although the level of GUS activity in the wounded roots of all five *win*2−GUS transformants was of the same order of magnitude as shown in wounded leaves and stems, it was also relatively high in intact tissue. The magnitude of the induction was consequently reduced

to between 2- and 6-fold after 64 h of ageing. One exception was PA17 which showed a sudden increase in GUS activity after 48 h of ageing, which resulted in an 80-fold induction over basal levels. By comparison with all other organs examined, the data from mini-tubers were extremely variable and little or no induction from high basal levels was observed after wounding, with the exception of transformant PA12, which showed an 8-fold induction in GUS activity between 48 and 64 h of ageing.

In order to confirm that the increases in GUS activity after wounding were mirrored by accumulation of mRNA encoding GUS and *win2*, Northern blots of total RNA taken from unwounded and wounded leaves and stems aged for 24 h were hybridized with probes specific for GUS and *win2*. The results are shown in Figure 2(C). There were low levels of both types of transcript in unwounded tissue (Figure 2C, lanes 1 and 3), and 24 h after wounding there were large increases in the levels of both types of transcript (Figure 2C, lanes 2 and 4). The total levels of *win* RNA, and differences between levels in wounded and unwounded tissues, were much greater than that seen for the GUS RNA.

win2−GUS and δ*win2*−GUS fusion expression in tobacco plants

Extracts from tobacco leaf tissue wounded for 0 or 20 h were screened for GUS activity as described above. Thirteen of the 21 kanamycin resistant *win2*−GUS transformants showed fluorescence in extracts prepared from unwounded tissue. Of these plants, half showed a small increase in the level of fluorescence in wounded tissue and half showed a decreased level. In contrast, no fluorescence was observed in extracts prepared from the unwounded leaves of 16 independent δ*win2*−GUS transformants. However, a very low level of fluorescence was detected in extracts prepared from the wounded leaves of five such plants. Leaves from eight GUS positive *win2*−GUS tobacco transformants, which on initial screening had shown an increase in GUS activity after wounding, were re-assayed over a time course of ageing (0, 12, 24 and 36 h). Four of the eight *win2*−GUS transformants exhibited a similar pattern of expression after wounding, in which GUS activity dropped 2-fold over the first 12 h of ageing before regaining the high basal levels present in intact tissue over the subsequent 24 h. By comparison, GUS activity was barely detectable in the intact leaves of δ*win2*−GUS transformants and only increased very slightly over the first 36 h of ageing (data not shown). No further analyses were performed on the tobacco transformants, as it was considered that the gene fusions were not being appropriately regulated in the heterologous tobacco host plant.

Localization of *win2*−GUS transcription in wounded potato plants

Histochemical analyses were performed on several independently transformed *win2*−GUS potato plants in order to localize the expression of the chimaeric GUS gene in wounded tissues.

In initial experiments, a leaf from each of two replicate plants derived from a single independent transformant was wounded aseptically using the pointed ends of a pair of forceps. One of these leaves was immediately excised and incubated with a histochemical substrate for GUS, bromo-chloro-indolyl-β-D-glucuronide (X-gluc), as described in Materials and methods. The second wounded leaf was left to age on the plant for ~48 h and then excised and stained in the same way. The two leaves were not taken from the same plant since the excision of a leaf at 0 h could potentially lead to secondary wounding effects in other leaves of the plant. There was no staining in the unaged leaf, even after a 16 h incubation with the substrate (Figure 3A). In contrast, an intense indigo staining was visible around many of the wound sites of the second leaf which had been aged for 72 h (Figure 3B). This staining also extended beyond the wound site, predominantly within the veins of the leaf. To verify that the observed pattern of staining was not an artefact caused by the differential penetration of the substrate into the wound, two leaves taken from replicate plants of a CaMV−GUS transformant were treated in the same way. An even staining was observed over the whole surface of leaves which had been wounded and aged for either 0 or 72 h prior to staining, consistent with constitutive expression of the CaMV−GUS gene (Figure 3C). These results confirmed previous observations which showed that wounding had no effect on gross levels of transcription, and also showed that the methods used allow for a fairly even penetration of the substrate into the leaf. The results were reproducible for replicate leaves of a single plant and for the leaves of six independent transformants expressing *win2*−GUS fusions. However, in a few cases, a high level of GUS activity was observed in freshly wounded leaves. This was localized to part of the leaf, e.g. the tip and edges, which appeared to be senescent.

In further experiments, the expression of the chimaeric *win2*−GUS gene was monitored within a single transformed leaf over a time course of ageing. After wounding with a sharp point, the midrib of the leaf was removed and each half of the leaf was cut into two pieces. These quadrants were then aged *in vitro* for 0, 48, 96 and 150 h before incubating with X-gluc. The results of a histochemical examination are shown in Figure 3(D−F). As before, no GUS activity was detected in freshly wounded leaf tissue (not shown) but, after 48 h of ageing, GUS activity was detected in cells adjacent to the cut edge and in minor veins interrupted by the wound (Figure 3D). After 96 h, intense staining was predominantly localized in cells bordering the wound but could now be seen to extend into the veins of the leaf (Figure 3E). After 150 h of ageing, GUS activity was detectable throughout the entire network of vascular tissue of the leaf quadrant and extended over nearly all of the leaf surface (Figure 3F). Other samples taken from longer time courses of ageing showed a decrease in GUS activity in the cells immediately bordering the wound, which had presumably died (data not shown). These experiments were repeated with the leaves of other independent transformants. While the timing and intensity of the wound response varied slightly between different leaves of different transformants, the spatial pattern of expression was essentially the same.

Sections were prepared from potato stems which had been wounded by a radial cut along the length of the stem and aged for either 0 or 48 h on the plant. Freshly cut stems usually did not show any indigo staining (Figure 4A) although in several cases some staining was observed at the base of the petiole. Forty-eight hours after wounding, intense staining was visible within the vascular tissue of the stems (Figure 4B), predominantly within the cells of the internal and external phloem, but also within parenchymatous cells

associated with the xylem (Cutter, 1982; Peterson *et al.*, 1985). In a few places, blue staining also appeared to be localized within the xylem vessels (Figure 4C). This pattern of staining was observed in all three major vascular bundles and also within the interfascicular phloem and xylem. In comparison, sections prepared from the stems of CaMV–GUS transformants showed staining within the phloem alone, and to a lesser extent in the cortical and pith cells, both 0 and 48 h after wounding (data not shown). This pattern of staining has been reported previously in tobacco (Jefferson *et al.*, 1987).

Despite the inconsistent data obtained from fluorometrical assays, a histochemical analysis of GUS activity in wounded mini-tubers was also performed. Mini-tubers were excised from stem cuttings and wounded by cutting longitudinally into either two or four segments. These were then aged with

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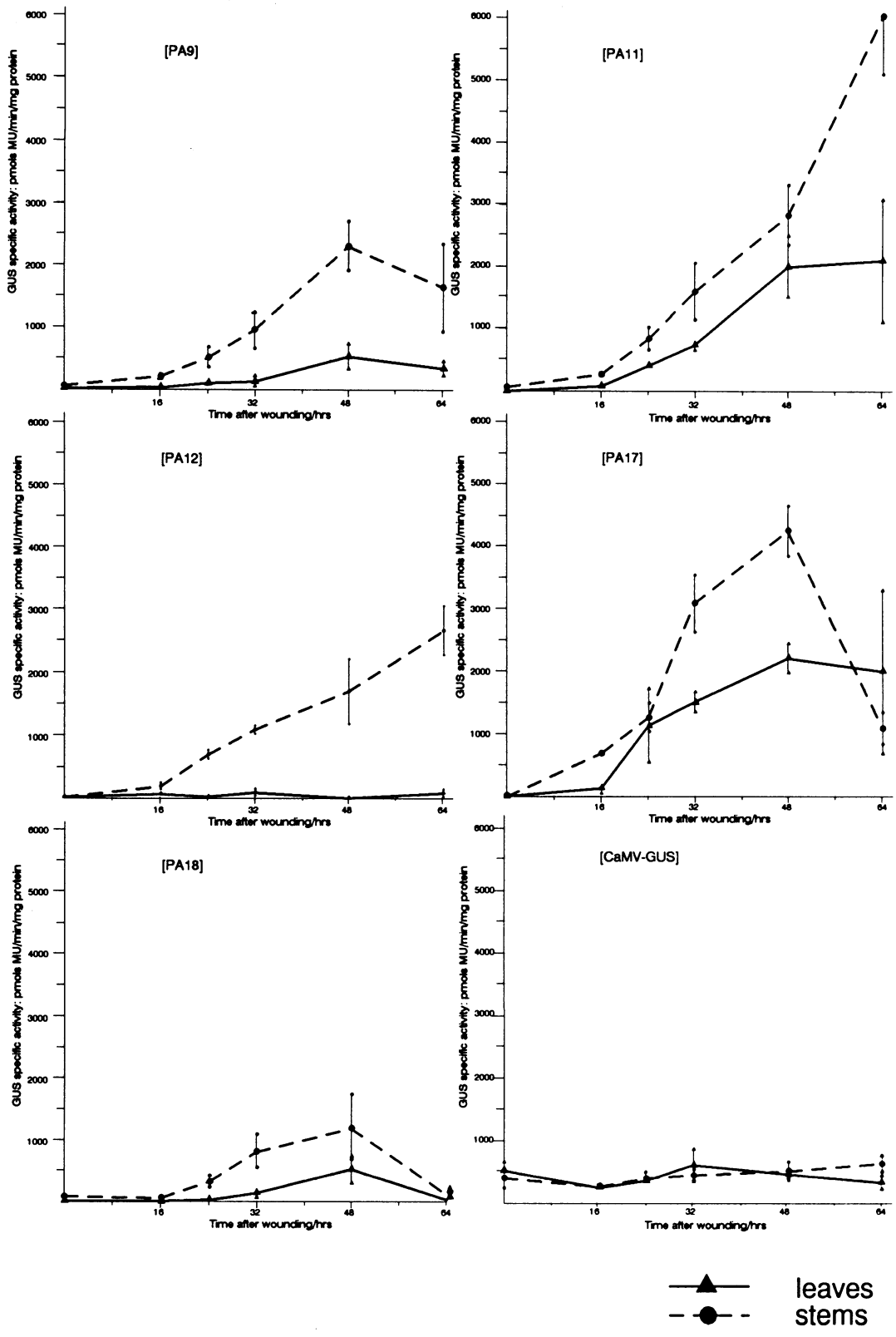

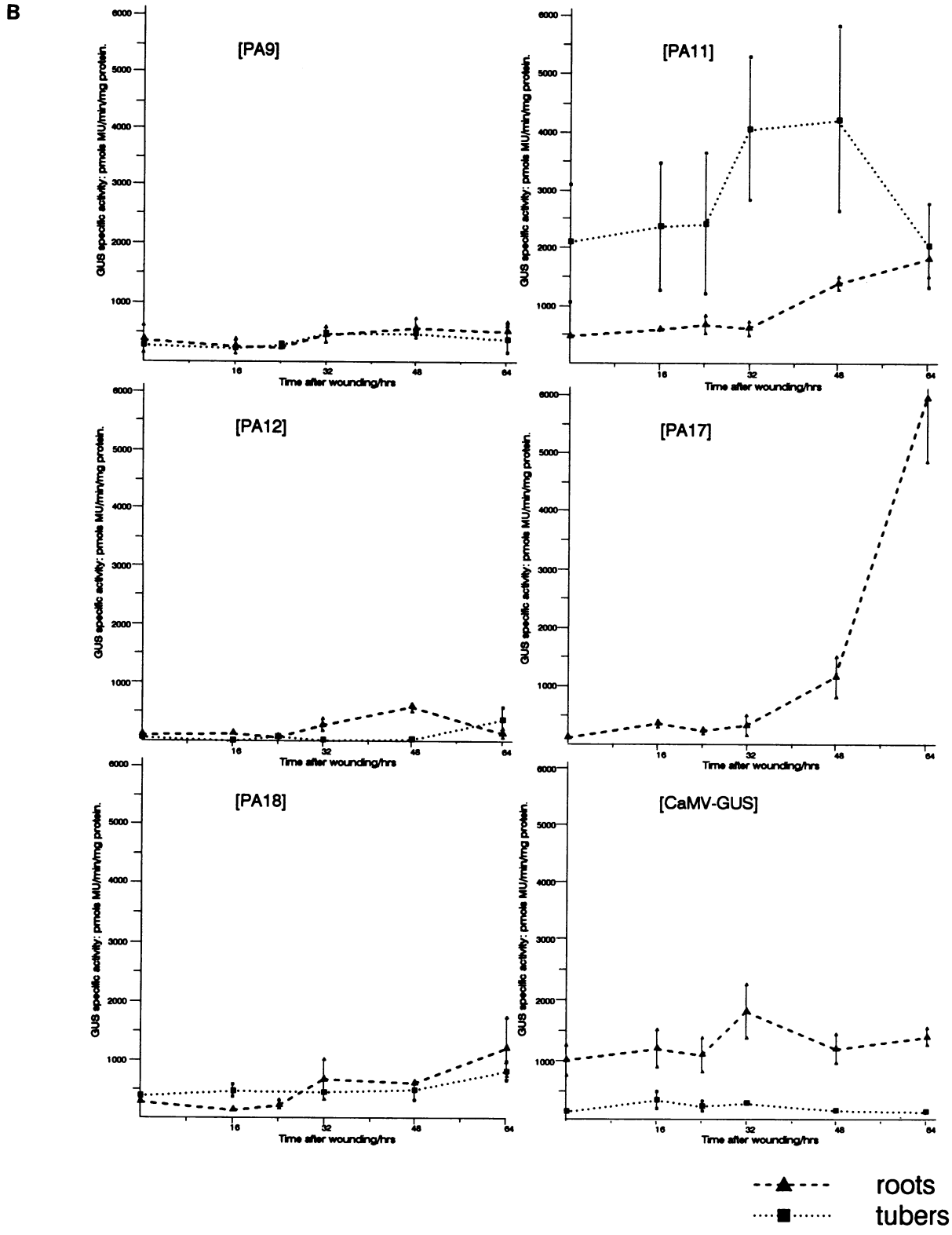

Fig. 2. Time course of *win2*–GUS expression in wounded potato organs. The mean GUS specific activity of three replicate samples taken from wounded leaves and stems (**A**) and roots and tubers (**B**) of five independent transformants, PA9, 11, 12, 17 and 18, and from a transformant expressing a CaMV–GUS fusion, was measured over a 64 h time course of ageing. (**C**) An autoradiograph of a Northern blot of total RNA from intact (**lanes 1** and **3**) tissue and from wounded tissue aged for 24 h (**lanes 2** and **4**). **Lanes 1** and **2** were probed with *win2* specific DNA, and **lanes 3** and **4** were probed with GUS specific sequences. The mol. wt standards used were an RNA ladder.

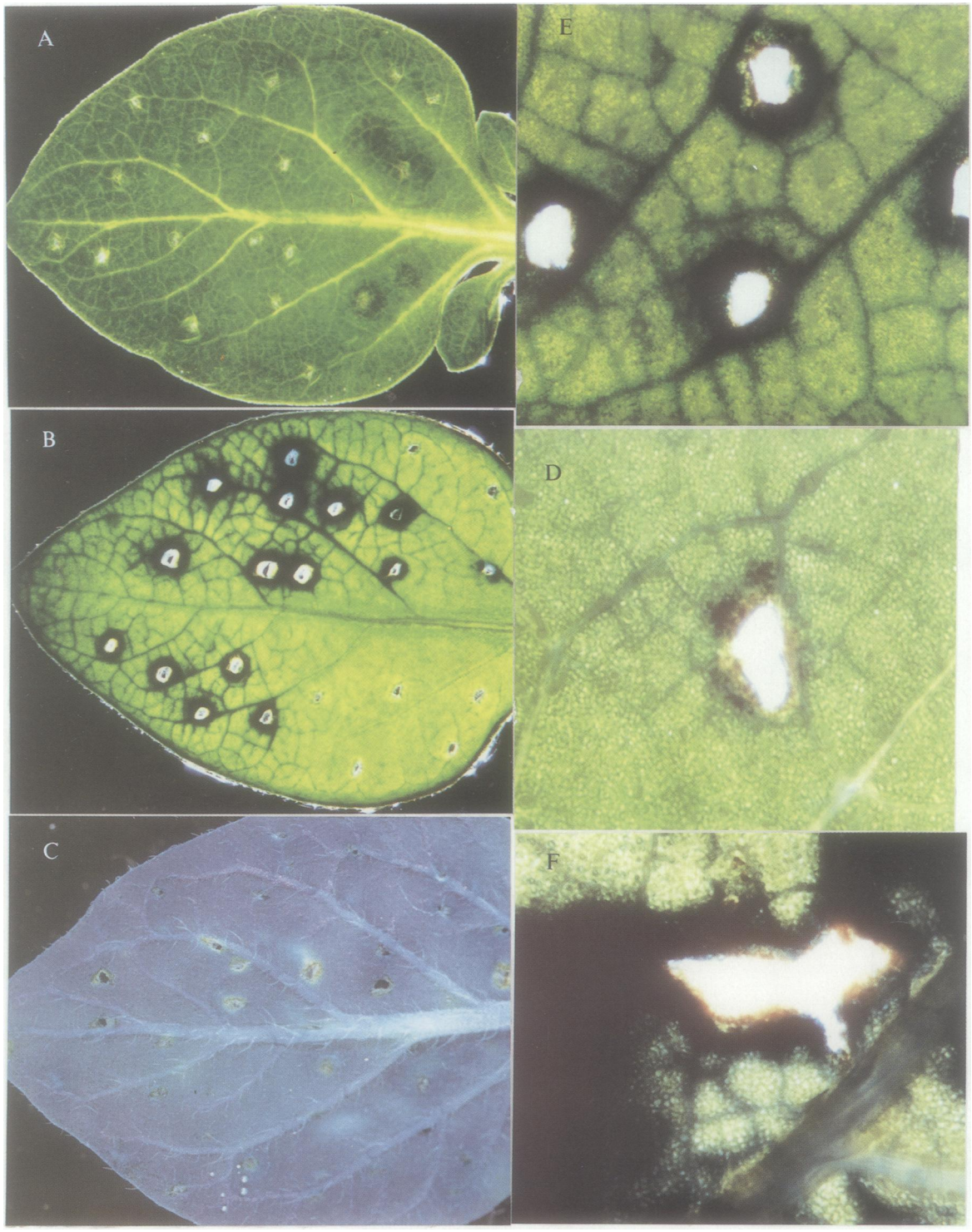

Fig. 3. Histochemical analysis of *win2*−GUS expression in wounded potato leaves. A leaf from each of two replicate plants of transformant PA17 was wounded with a sharp point and one of these leaves was immediately excised and stained (**A**). (**B**) The leaf left to age on the plant for ~72 h before excision and staining. (**C**) A wounded leaf from a CaMV−GUS transformant which was left to age on the plant for 72 h before staining. (**D**), (**E**), (**F**) show quadrants from a single wounded leaf aged for 48, 96 and 150 h respectively after wounding and excision from the mother plant. Staining was for ~16 h in all cases. The samples in (A)−(C) were photographed under dark field illumination at a magnification of ~×75, while those in (D)−(F) were photographed under transmitted light at a magnification of ~×30.

the cut surfaces exposed axenically for 0, 48, 96 and 150 h, prior to sectioning and incubating in X-gluc. In contrast to leaves and stems, all tubers showed a low level of GUS activity in freshly cut tissue that was located just beneath the lenticels in the periderm (Peterson *et al.*, 1985) (Figure 5A). For those tubers which had reached full size but remained dormant, no other blue stain could be seen in freshly cut tissue. However, GUS activity was clearly induced in these tubers after wounding. Figure 5(B) shows half a tuber in cross-section, stained 48 h after wounding. Cells immediately bordering the longitudinally cut wounded surface were intensely stained, as well as those cells

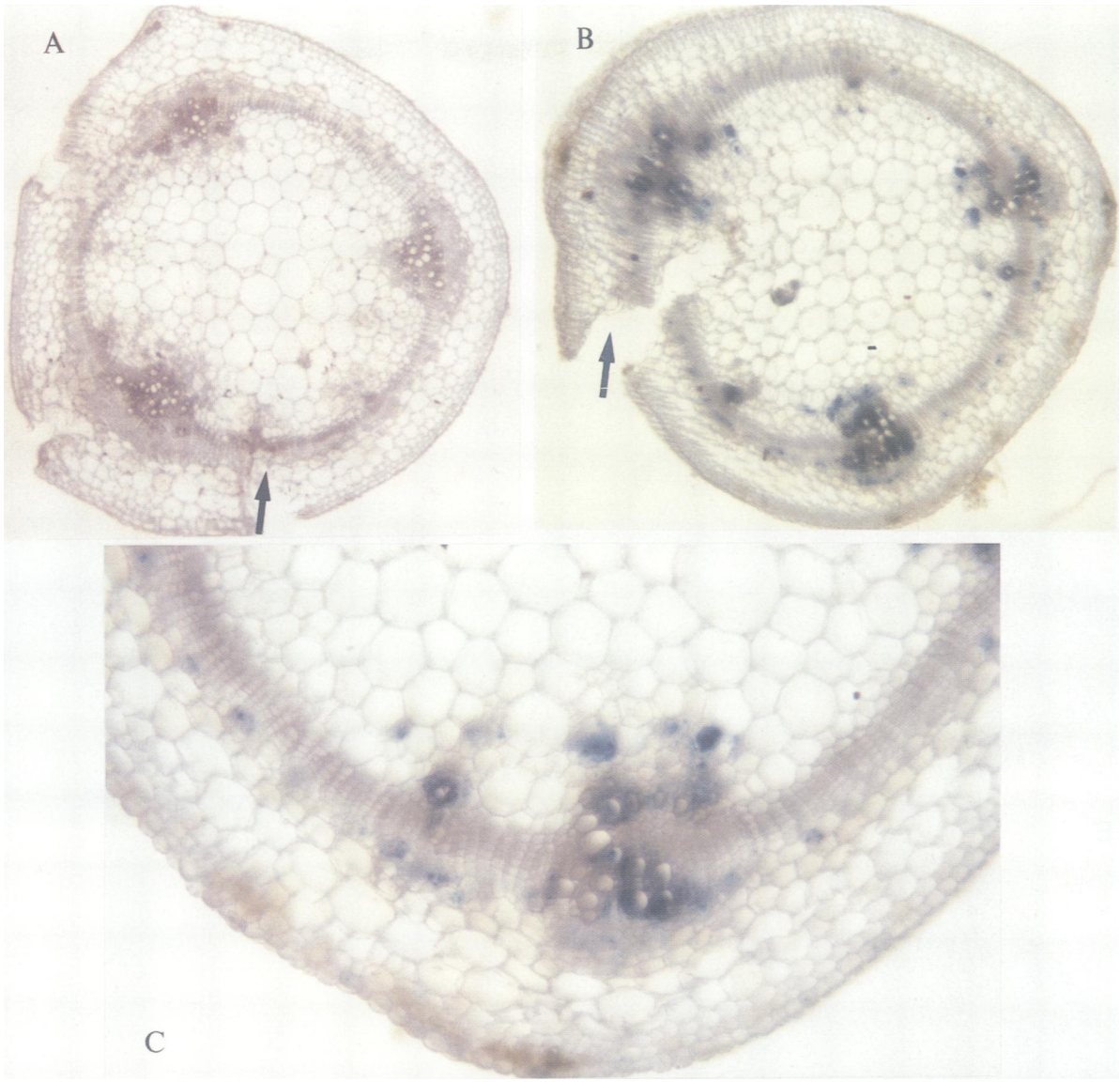

Fig. 4. *Win2*–GUS expression in wounded potato stems. Cross-sections were prepared from the stems of duplicate plants of transformant PA17 which had been wounded by a radial cut (arrowed), and either excised immediately (**A**) or left to age on the plant for 48 h (**B** and **C**), prior to sectioning and incubation with X-gluc. The magnification in (A) and (B) is ~×25, and in (C) is ~×100.

surrounding an incision made with forceps (arrowed). In sections prepared 150 h after wounding, GUS activity was still localized in a single cell layer immediately bordering the cut surface (Figure 5C). Beneath this layer, the first cell divisions in the formation of a wound periderm occurred (Figure 5C, arrowed) (Kahl, 1978; Cutter, 1982). After 240 h, the wound periderm was fully developed and GUS activity was generally no longer detectable in cells at the wound surface (data not shown). Similar observations were made for several mini-tubers derived from each independent transformant, but the pattern of staining varied with the developmental stage of the tuber. For mini-tubers which had started to sprout, blue staining was present within the developing bud as well as beneath the lenticels of freshly wounded tissue (Figure 5D). After 48 h of ageing, staining extended throughout the cortex of the tuber and was predominantly localized within the disperse vascular tissue, as well as at the wounded surface. Figure 5(E) shows an example of this pattern of staining in a tuber which had been aged for 100 h after wounding.

Discussion

In this paper, transcriptional fusions between the regulatory sequences of *win2* and the GUS reporter gene have been used to study the spatial and temporal regulation of wound induced gene expression in transgenic potato and tobacco plants. A *win2*–GUS gene fusion containing the entire 5′ flanking sequences of *win2* was correctly regulated in response to wounding in its homologous host, potato, with a dramatic induction of GUS activity being observed in both the wounded leaves and stems of several independent transformants. In contrast, no induction of *win2*–GUS gene expression was observed in transgenic tobacco. This contrasts with the observations of An *et al.* (1989) and Sanchez-Serrano *et al.* (1987), who showed that a potato proteinase inhibitor was wound inducible in transgenic tobacco. This difference is surprising since tobacco is not thought to encode any proteinase inhibitors similar to those found in potato and tomato, whereas *win2* gene homologues have been detected in tobacco (Stanford *et al.*, 1989). One

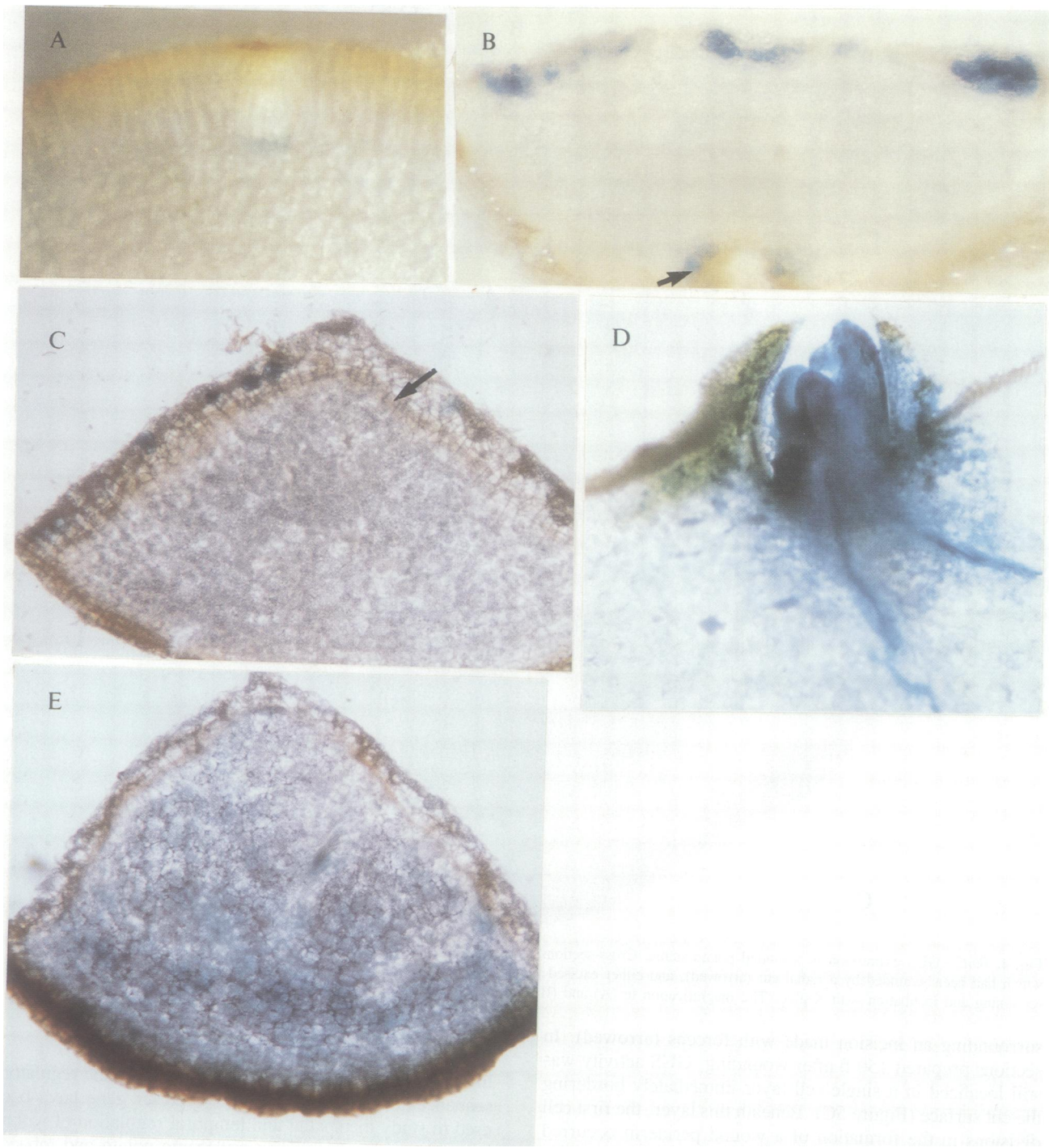

Fig. 5. *Win2*−GUS expression in wounded mini-tubers. *In vitro* grown tubers derived from transformant PA11 were wounded and aged for various times before sectioning and staining. **(A)** Section through the periderm of an unwounded tuber. Staining is visible in a layer of cells beneath a lenticel (×50). **(B)** Cross-section of a tuber aged for 48 h after wounding. The tuber has been sliced in one plane to make a wounded surface, then later sectioned perpendicular to the wound surface (uppermost). A wound made by forceps is arrowed (×30). **(C)** Tuber aged for 150 h after wounding. The developing wound periderm is arrowed (×25). **(D)** Staining in a developing bud of a sprouting tuber. GUS activity localized in a vascular strand leading from the bud is arrowed (×75). **(E)** Pattern of transcription in a sprouting tuber 100 h after wounding (×25).

explanation for this could be that the tobacco plants were stressed prior to the wounding experiment by, for example, the build up of ethylene in the closed containers in which they were grown. This could result in high basal levels of GUS activity which would mask the initial stages of underlying wound induction.

There was considerable variation in the magnitude of the response to wounding between independent *win2*−GUS potato transformants. However, this was probably not significant when compared with the variation observed within replicate samples of a single transformant. It was thought that the primary source of this variation were differences in the degree of mechanical wounding, although developmental differences between leaves may also contribute to the variation observed. Despite this variation, a characteristic profile of induction was observed for several

plants. After an initial lag phase of 16–24 h, GUS activity rose from barely detectable levels in intact tissue to high levels within 32–64 h after wounding, and these values were probably not the maximum induction which might be achieved over an extended time course of ageing. A comparable time course for the accumulation of RNA complementary to *win*2 was performed in wounded leaves or stems of transformants, and similar increases in the steady state levels of RNA complementary to *win*2 and GUS were observed. There were substantial differences in the degree of induction and final levels of transcript between GUS and *win*2. This may be due in part to a larger number of genes expressing *win* related transcripts, to the differential stability of transcripts, and to important quantitative regulatory loci not being present in the GUS fusions. It has also been a common observation that transgenes are not regulated in a quantitatively similar way to endogenous genes.

Although the wound induced expression of *win*2 mRNA in potato tubers has been demonstrated using gene specific probes in S1 nuclease protection experiments (Stanford *et al.*, 1989), no consistent increase in GUS activity after wounding was detected fluorometrically in wounded mini-tubers derived from *win*2–GUS transformants. GUS activity was also present at relatively high basal levels in intact tubers, and this contrasts with the results of S1 nuclease protection experiments (Stanford *et al.*, 1989) which showed *win*2 specific RNA to be undetectable prior to wounding. The reason for this apparent discrepancy is discussed below with reference to histochemical observations.

GUS activity was also present at relatively high basal levels in the intact roots of all transformants tested and in most cases showed only a small 2- to 6-fold increase in response to wounding. Since one of the five transformants displayed a mean increase in GUS activity of 80-fold after 48 h of ageing, a delayed induction might be expected for the other root samples. Although the background GUS activity in intact roots is inconsistent with S1 nuclease protection data (Stanford *et al.*, 1989), the relatively small increase in chimaeric gene expression compared with that observed for leaves and stems of the same plants is compatible with the RNA data which showed only a very slight accumulation of *win*2 specific RNA in roots after 20 h of ageing.

It was concluded that the induction of GUS activity in response to wounding was the result of regulated gene expression under the control of the *win*2 promoter, and was not the result of either a general increase in the rate of transcription or translation within wounded tissue, or a change in the turnover of GUS, since a constitutive expression of a CaMV 35S–GUS fusion was observed and a similar time course of *win*2 and GUS RNA was also observed. As both the *win*2–GUS and CaMV–GUS gene fusions shared the same signal for transcriptional termination, it appeared that sequences present within 2 kb of the 5′ flanking region of *win*2 were sufficient and necessary for wound induced gene expression. This idea is consistent with that of An *et al.* (1989), who demonstrated that the 3′ end of a wound induced proteinase inhibitor from potato contributed to increased mRNA stability, not to an effect on transcriptional activation (Thornburg *et al.*, 1987).

No GUS activity was detected in the leaves of potato plants transformed with *δwin*2–GUS containing a truncated *win*2 promoter, either before or after wounding. Although leaf tissue was assayed after the same 20 h period of ageing as were leaves of *win*2–GUS transformants, it is possible that this was insufficient time for either transcriptional activation of *δwin*2–GUS to have occurred or for a detectable amount of GUS to have accumulated. However, it is likely that important *cis*-acting elements have been deleted in the *δwin*2–GUS construct. Substantial homology exists between the 5′ flanking sequences of *win*2, *win*1 and the wound induced potato gene *wun*1 (Logemann *et al.*, 1989), at −877 to −852 bp upstream of the transcriptional start site of *win*2. This upstream conserved element has been deleted in the *δwin*2–GUS fusions which contain 5′ sequences extending to −558 bp of the transcription start site. The presence of putative *cis*-acting elements distant from the TATA box region of *win*2 is similar to the arrangement of important *cis*-acting elements in the *Phaseolus* chitinase gene, which is also regulated by wounding (Broglie *et al.*, 1989). Current experiments aim to delineate more closely the *cis*-acting elements of the *win*2 promoter.

The marked induction of GUS activity in *win*2–GUS potato transformants after wounding made this an ideal system in which to study the spatial pattern of wound induced transcription using histochemical analysis. This was facilitated by the time scale of the induction as the initial delay of ~16 h allowed freshly sectioned tissue to be considered as a 0 h control, even though staining was carried out for 12–16 h. In leaves, GUS activity could not be detected histochemically in intact tissue but was specifically induced around the wound sites within 48 h after wounding. The activity was initially localized in cells immediately bordering the wound but was progressively induced in neighbouring cells. This flush of chimaeric gene activity then spread throughout the entire vascular system of the leaf until almost all parts of the leaf expressed high levels of GUS activity, while cells at the cut edge ceased to express GUS as they became necrotic. The marked expression of the *win*2–GUS gene within the leaf vasculature was also observed in wounded stems where it was localized within cells of both the phloem and xylem, often at some distance from the incision. However, unlike the pattern of expression in leaves, no local reaction was observed in cells adjacent to the wound in stems, even after 48 h of ageing. One possible explanation for this was that the wound was found to close up tightly after incision, and thus the wound surface of stems may not have aged under the same aerobic conditions as other excised tissues of the potato plant. It is known that atmospheric conditions at the wound surface have an important effect on other defence related genes (Smith and Rubery, 1981; Logemann *et al.*, 1988).

In contrast to the systemic induction of GUS activity in wounded leaves, chimaeric gene expression in wounded mini-tubers remained localized in the cells immediately adjacent to the wound, even after prolonged ageing. However, the pattern of expression was strikingly different in tubers which had started to sprout: a high level of GUS activity was observed in the developing bud of the intact tuber and was rapidly induced throughout the cortex and vascular system after wounding. This marked difference in the response of different tubers to wounding accounts for some of the variability detected fluorometrically, and it may indicate that the developmental stage of the tuber influences the regulation of the *win*2–GUS gene. The greater degree of physical disruption of tuber tissue described previously (Stanford *et al.*, 1989) compared with that carried in the

fluorometric and histochemical assays for GUS activity probably accounts for many of the observed differences. The localized GUS activity in the buds of sprouting tubers and beneath the lenticels of intact mini-tubers accounts for the relatively high basal levels of GUS measured fluorometrically. However, no RNA complementary to *win*2 was detected in unwounded tubers by either Northern analysis or S1 mapping experiments (Stanford *et al.*, 1989). This inconsistency probably arose from removal of the periderm and underlying cortical tissue from the core of potato tissue used in wounding experiments and subsequent RNA extractions.

It is possible that the observed spread of X-gluc staining away from the wound site in leaves was due to the movement of the GUS protein away from the site of chimaeric gene expression in the vicinity of the wound. GUS may have passed out of these cells into neighbouring cells, including the xylem and phloem, which would allow the enzyme to be transported rapidly throughout the plant. However, there was no gradient of staining away from the wound site as would have been expected if this were the sole source of the gene product, but rather an induction to similar levels at more distal sites from the wound. Furthermore, GUS activity continued to spread throughout the leaf tissue even after the cells immediately bordering the wound had died and ceased to express GUS. It is therefore concluded that the observed pattern of staining is a direct indication of the spatial pattern of expression of the *win*2−GUS gene during the wound response, and that chimaeric gene expression under the control of the *win*2 promoter is triggered by a putative wound stimulus. This stimulus is initially generated at the site of the wound but subsequently spread to adjacent cells. The observation that gene expression is predominantly associated with the veins of the leaf strongly suggests that the putative wound stimulus is transported via the vascular system. This would be expected to provide a more rapid signalling of the wound response to parts of the plant distant from the original wound site. Our results indicate that the putative signal molecule(s) is probably amplified or perpetuated by some mechanism during signalling of the systemic response, since there is no apparent gradient of chimaeric gene expression away from the wound site, as would be expected if a signalling molecule released from traumatized cells at the wound site formed a gradient of effective concentration away from the wound site. Since the wound stimulus does not travel from the original wound site in dormant tubers, but does so in tubers that have broken dormancy and are sprouting, it is likely that active vascular transport is required for the movement of the stimulus. In its dormant state, little or no flux is expected from the vascular system of the tuber to other parts of the plant and consequently the wound stimulus would not be rapidly transmitted away from the cells at the wound surface. Upon sprouting there is an increasing flux through the vascular system of the tuber to supply the demands of the rapidly growing bud (Wright and Oparka, 1989). The wound stimulus would therefore be transported throughout the disperse vascular tissue and transcription of the *win*2−GUS gene would be activated in all parts of the cortex.

The expression of other defence related genes or proteins is also associated with the vascular system. For example, PR1 proteins have been shown to accumulate within the xylem of infected tobacco leaves (Carr *et al.*, 1987), and the wound induced expression of a reporter gene under the control of a proteinase inhibitor II promoter is predominantly localized in the phloem of transgenic potato plants (Keil *et al.*, 1989). Systemic induction has been reported for several defence responses in plants. The induction of proteinase inhibitors and PR proteins (Ryan, 1978; Bol, 1988) is thought to be signalled by an oligosaccharide called PIIF (proteinase inhibitor inducing factor) generated from the cell walls of either the plant itself or of an invading pathogen. The spatial pattern of wound induced gene expression described here suggests that the vascular system is a major route for signalling wound induced gene activity.

Future research will attempt to determine the putative function of the *win*2 gene product in potato defence responses, and will use histochemical localization of wound and pathogen induced *win*2−GUS expression to examine the mechanisms of local and systemic responses in more detail. Finally, a detailed examination of the promoter regions of *win*1 and *win*2 will be undertaken to determine *cis* elements important for tissue specificity and wound responsiveness.

Materials and methods

Construction of gene fusions

DNA manipulations were carried out according to Maniatis *et al.* (1982). The 5′ flanking sequence of *win*2 was contained in a 3.5 kb *Sst*I fragment of the genomic clone λSt5111 (Stanford *et al.*, 1989) (Figure 1). This fragment also contained the first 1135 bp of the coding region of *win*2 and 147 bp of the C-terminal coding region of the tandemly repeated and related gene, *win*1. The *win*2 coding sequences were deleted unilaterally. The resulting fragment of 2361 bp, which contained the entire 5′ untranslated region of *win*2 and 2177 bp of 5′ flanking sequences, was cloned into the vector pBI101.4 to make a transcriptional fusion with the coding region of the GUS gene (Jefferson *et al.*, 1987). A shorter promoter fragment truncated at a *Hin*dIII site 591 bp from the deletion end point and containing 560 bp of 5′ flanking sequence, was also ligated into pBI101.4. The structures of these chimaeric genes, called *win*2−GUS and δ*win*2−GUS respectively, are shown in Figure 1.

Plant transformation

The chimaeric *win*2−GUS fusions in the binary vector were mobilized into *Agrobacterium tumefaciens* helper strain LBA4404 as described by Bevan (1984). Potato (variety Desiree) tuber discs were transformed according to Sheerman and Bevan (1988). Transformed shoots were maintained in axenic culture on kanamycin selection. Mini-tubers were obtained by growing cuttings *in vitro* (Bourque *et al.*, 1987). Tobacco leaf explants were transformed according to Horsch *et al.* (1985), and transformants were grown axenically on kanamycin selection.

Wounding methods

Axenic potato or tobacco tissue was used for all experiments, and all experiments were carried out on plants ~2 weeks after subculture. Tissue was excised from the leaves, mini-tubers, stem or root segments, cut into 1 mm slices, and aged aerobically and axenically on filter paper moistened with MS salts (Murashige and Skoog, 1962) in the dark at 26°C. Replicate samples were taken at various times after wounding, and were immediately frozen in liquid nitrogen and stored at −70°C prior to fluorimetric analysis. For histochemical analysis of GUS activity, leaves and stems were wounded *in situ* and excised from the plant at various times after wounding for sectioning, staining and photography. Mini-tubers were wounded by cutting in half, and placed on sterile moist filter paper for different times before sectioning and staining.

GUS was extracted from tissues as described by Jefferson *et al.* (1987) except that the extraction buffer contained insoluble polyvinylpyrollidone and 1 mM phenylmethylsulphonylfluoride. Fluorometric GUS assays were conducted in microtitre plates, using 1 mM 4-methylumbelliferone as substrate, and the fluorescence was measured in a Fluoroskan plate reader. Data were analysed using the program 'Plates' (written by D.W.Computing, Cambridge), and was normalized to the protein content of the extracts.

Histochemical analysis was conducted by incubating hand cut sections for ~12−16 h at 37°C in a solution of 1 mM X-gluc (Biosynth AG, Staad,

Switzerland) in 50 mM sodium phosphate buffer, pH 7.0, 0.1 mM potassium ferro- and ferricyanide. Sections and whole mounts were photographed with a Wild stereomicroscope using Kodak Ektachrome reversal film.

Total RNA was isolated from leaf and stem tissue by phenol extraction and LiCl precipitation. Samples (10 μg) were electrophoresed in MOPS–formaldehyde agarose gels, blotted onto HybondN membranes and hybridized to radiolabelled probes made from the coding region of the GUS gene, and from the coding region of the *win*2 gene, from which the intron had been removed in a PCR reaction.

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