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Abscisic Acid Mediates Wound Induction but Not Developmental-Specific Expression of the Proteinase Inhibitor II Gene Family

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The expression of the potato and tomato proteinase inhibitor II (pin2) gene family is subject to both developmental and environmental control, being constitutively expressed in potato tubers while only being present in the foliage of the potato or tomato plants after mechanical damage. There is evidence that the phytohormone abscisic acid (ABA) is involved in this wound induction of pin2 gene expression. This paper describes experiments that demonstrate that ABA is able to induce the expression of the pin2 gene family, both locally and systemically, at physiological concentrations. The significance of the ABA involvement in the pin2 induction upon wounding has been further strengthened by analyzing the expression of a pin2 promoter-β-glucuronidase gene fusion in transgenic ABA-deficient mutant potato plants. We have analyzed the developmental regulation of pin2 gene expression in wild-type and ABA-deficient potato and tomato plants. The pin2 mRNA level is identical in mutant and wild-type parental *Solanum phureja* tubers. In addition, evidence is presented for pin2 also being constitutively expressed at certain stages in the development of both tomato and potato flowers. Again, the ABA deficiency appears to have little influence in this tissue-specific expression in the mutants. These results suggest the action of separate pathways for the developmental and environmental regulation of pin2 gene expression.

INTRODUCTION

Plants react to changes in the environment by altering their gene expression to meet the newly imposed requirements. Wounding is an especially severe stress, which leads to dramatic changes in the gene expression pattern, some of the genes involved in photosynthesis, for instance, being turned off (Peña-Cortés et al., 1988; Kombrink and Hahlbrock, 1990), whereas transcriptional activation of other genes is triggered. The activity of the latter is mainly concerned with wound healing and the prevention of the invasion of pathogens, which take advantage of naturally occurring wounds to evade the protective layers normally hindering their penetration. It is, therefore, not surprising that most genes involved in the plant defense mechanism against pathogen invasion are activated also by mechanical wounding. Their activation is often confined to the immediate vicinity of the wound site, but some of them show a systemic response being also transcribed in distal tissues, which themselves are not damaged (Bowles, 1990). The mechanisms underlying this different spatial activation are likely to be connected to the distribution of a "wound signal."

The potato and tomato proteinase inhibitor II (pin2) gene families are perhaps the best-studied examples of systemically induced genes. Wounding of a tomato or potato leaf results in the rapid activation of the pin2 gene, whose mRNA can be detected shortly thereafter in the damaged tissue. pin2 homologous RNA also accumulates in the nondamaged leaves, both acropetally and basipetally to the wounded one (Peña-Cortés et al., 1988). The pin2 gene, however, displays two different modes of expression because it is also constitutively present in potato tubers. In this case, wounding has no effect on its steady-state mRNA level, which follows the development of the tuber, starting to accumulate in stolons induced to tuberize and disappearing upon tuber sprouting.

A single pin2 promoter was shown to be active both in leaves upon wounding and constitutively in tubers (Keil et al., 1989). This gene might thus be responding to a signal common to the wounding and tuberization processes or, alternatively, this complex pattern of expression could be modulated by at least two different *cis*-regulatory elements, one reacting to the signals associated with tuberization, the other being wound responsive.

We have recently demonstrated that the phytohormone abscisic acid (ABA) is involved in the activation of the pin2

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gene family upon wounding. First, ABA treatment resulted in the appearance of the pin2 homologous RNA in the foliage of potato plants, in the absence of any wounding. In the case of tomato, incubation of detached leaves in an ABA solution also led to pin2 mRNA accumulation. Second, no activation of the pin2 gene family was observed upon wounding of mutant tomato or potato plants deficient in the synthesis of ABA. This lack of induction could be lifted by ABA treatment, which allowed the accumulation of pin2 mRNA to the levels normally found in wild-type plants (Peña-Cortés et al., 1989).

In the present paper, we have addressed two main questions: (1) Is ABA only involved in the wound-induced expression of the pin2 gene family or is it also necessary in the developmentally programmed expression pattern? (2) How does ABA mediate the wound activation of the pin2 gene?

To ascertain the role of ABA in the constitutive expression of the pin2 gene family, we have analyzed the distribution of pin2 mRNA in the different organs of nonwounded ABA-deficient tomato and potato plants (*sitiens* and droopy, respectively). We have further established transgenic droopy lines to analyze the expression of a pin2 promoter-β-glucuronidase (GUS) reporter gene fusion, which has been shown to be wound inducible and tuber constitutive in wild-type transgenic potato plants. To gain further insight into the physiological role of ABA in the wound-induced transcriptional activation of the pin2 gene family, we have correlated the observed effects on the pin2 gene induction to the concentration of ABA attained in the respective tissues.

RESULTS

Developmentally Controlled Expression of the Potato pin2 Gene Family Is Not Affected by a Deficiency in ABA Synthesis

The pin2 gene exhibits two different modes of expression. On the one hand, it is constitutively transcribed in potato tubers. On the other hand, its mRNA accumulates upon wounding in the aerial part of the potato or tomato plant. We have previously shown that the plant hormone ABA is involved in the wound-induced transcriptional activation of the pin2 gene (Peña-Cortés et al., 1989). To investigate whether ABA also plays a role in its constitutive expression, we have determined the steady-state levels of the pin2 mRNA in the different organs of wild-type potato plants and compared them with the levels observed in mutant plants impaired in ABA synthesis. Figure 1 shows the pin2 gene expression pattern in *Solanum phureja* (a wild-type diploid potato) and its ABA-deficient mutant (droopy). In accordance with previously published data, a wound-induced expression of the pin2 gene in leaves was

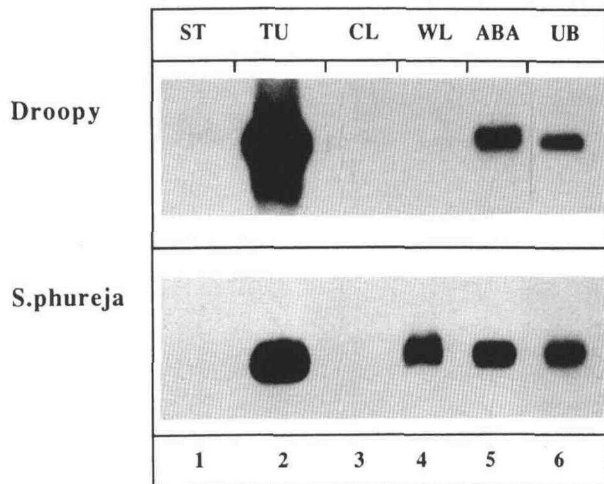

Figure 1. pin2 Expression Pattern in Wild-Type and ABA-Deficient *S. phureja* Plants.

RNA was isolated from stolons of nontuberized plants (ST), tubers (TU), nondamaged leaves (CL), wounded leaves (WL), leaves sprayed with ABA (ABA), and unripe floral buds (UB) from wild-type (*S. phureja*), or ABA-deficient mutant plants (droopy). The autoradiogram shows the result of an RNA gel blot hybridization of total RNA (20 μg per slot) against a radioactive pin2 cDNA probe.

only seen in the wild-type plant but not in the droopy mutant (Figure 1, lane 4). However, this difference in the response was not seen when ABA was sprayed on the leaves, which triggered the accumulation of pin2 mRNA to similar levels in both wild-type and ABA-deficient plants (Figure 1, lane 5).

In contrast to the induction of the pin2 gene upon wounding, no difference between wild-type and ABA-deficient plants was observed with regard to its developmentally controlled expression. Thus, pin2 homologous RNA accumulated to similar levels in both wild-type and droopy tubers (Figure 1, lane 2), despite a 10-fold reduction in the ABA content of the latter. In both cases, the ABA concentration in tubers was lower than in the respective nonwounded leaves, the concentration in droopy leaves being about 10% of the concentration in wild-type leaves, as shown in Figure 2. Whereas the endogenous ABA level of the wild-type leaves rose threefold upon wounding, the concentration of ABA in the droopy leaves showed no such increase after wounding.

Before wounding, pin2 mRNA was present not only in the tubers, as previously described, but also during certain developmental stages of the flower, i.e., in unripe buds (Figure 1, lane 6). The pin2 expression pattern in flowers is described in more detail below. Analogous to the situation in potato tubers, no difference in the pin2 expression

in floral buds was observed between the ABA-deficient and the wild-type plants.

pin2 Is Expressed in Potato and Tomato Flowers

An unexpected result from the above-mentioned experiments was the fact that potato floral buds, as well as tubers, contained high levels of pin2 mRNA in the absence of any wounding. To get more detailed insight into the expression of the pin2 gene during flower development, the distribution of the pin2 homologous RNA in the different parts of fully developed potato flowers was analyzed by RNA gel blot experiments. The expression patterns displayed by wild-type and ABA-deficient flowers were compared to determine whether differences in ABA content have any effect on the organ distribution of the pin2 mRNA.

Figure 3 shows that in potato, pin2 mRNA was constitutively present in the young floral buds but was absent from all organs of the developed flower. Within the limits of detection, the droopy mutant displayed an identical distribution, showing, as in the case of tubers, that the ABA deficiency had no detectable influence in the pin2 constitutive expression. Figure 4 shows the staining of floral buds from wild-type plants transgenic for a pin2 promoter-GUS fusion (Keil et al., 1989). The highest pin2 promoter activity was observed in the developing ovules (Figures 4A and 4B), and in most cases GUS staining could also be detected at the edges of the developing petals (Figure 4C).

To analyze whether the homologous pin2 gene family was also constitutively transcribed in tomato flowers and, if so, whether its developmental regulation was identical to that of its potato counterpart, a series of RNA gel blot experiments was performed. As shown in Figure 3 (lower panel), tomato flowers exhibited a substantially different pin2 mRNA distribution from that in potato flowers. In tomato, the homologous mRNA could be found in all different organs of the adult flower, as well as in the young floral buds (Figure 3). Similar to potato, the deficiency in ABA had little effect in the constitutive transcription of the pin2 gene in most of the floral organs. However, a significant difference is seen with respect to the expression in sepals and ovaries, being much weaker in the mutant flowers. To which extent this is due to the ABA deficiency remains unknown.

The pin2 expression pattern in potato and tomato flowers was not altered by mechanical wounding of the plant foliage (not shown).

Absence of pin2 Expression in Wounded Leaves of Mutant Plants Is Related to Their ABA Deficiency

Data described above have shown that pin2 homologous RNA can be detected in tubers and unripe buds of both

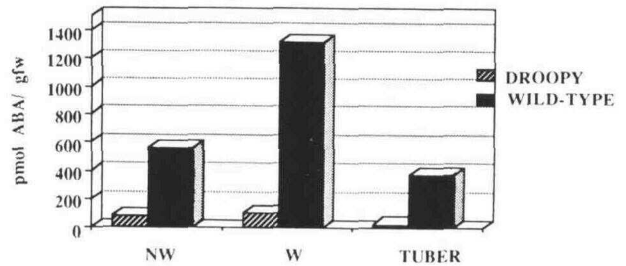

Figure 2. ABA Quantitation in Wild-Type and ABA-Deficient *S. phureja* Plants.

The ABA concentration in tubers and leaves (both before and after wounding) were determined for wild-type *S. phureja* and its ABA-deficient droopy mutant plants. Small, developing tubers and young, fully developed leaves were used as sources. At least three leaves per plant were wounded 24 and 4 hr before harvesting. The ABA quantitations shown are the mean value determined from three independent experiments, except for the wild-type tuber, the value for which was determined only once. NW, nonwounded leaves; W, wounded leaves.

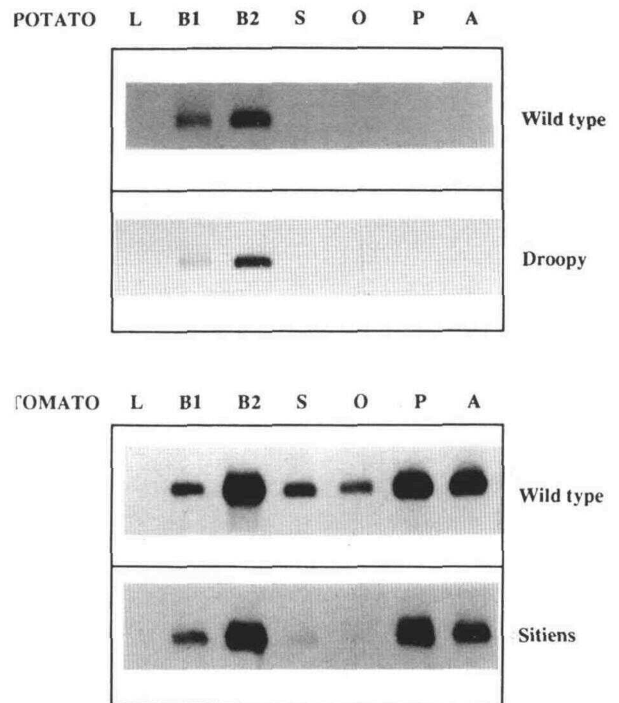

Figure 3. pin2 mRNA Distribution in Potato and Tomato Flowers.

RNA isolated from the different organs of wild-type and ABA-deficient potato and tomato flowers (droopy and *sitiens*, respectively) was blotted and hybridized against a radioactive pin2 cDNA probe. L, nonwounded leaves; B1, floral buds still closed; B2, floral buds recently opened; S, sepals; O, ovaries; P, petals; A, anthers.

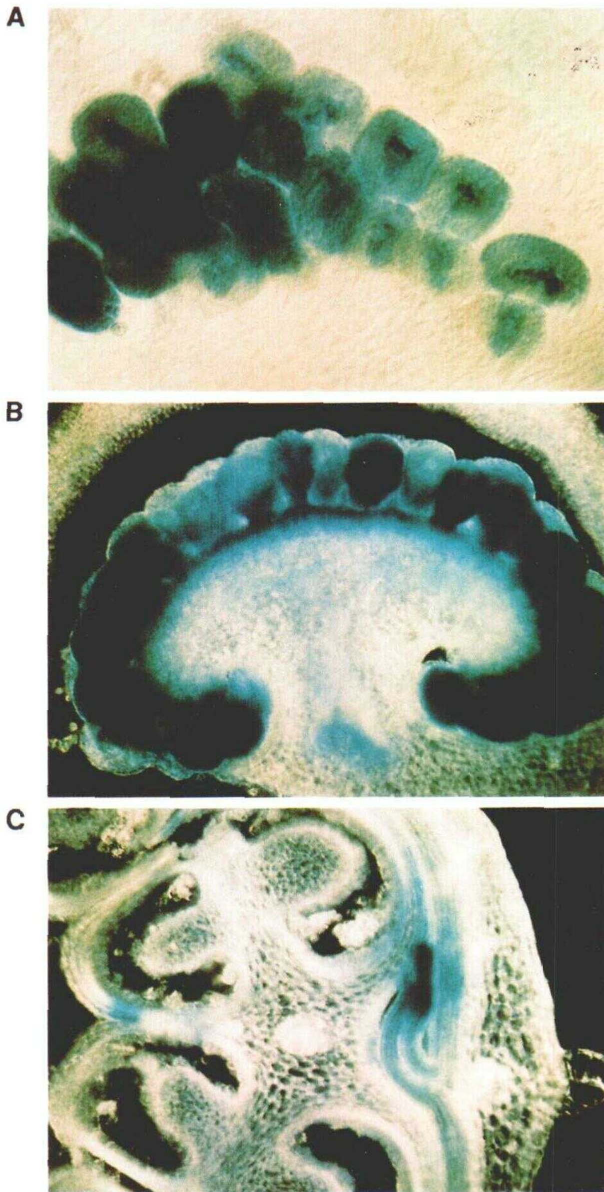

Figure 4. GUS Staining of Transgenic Potato Floral Buds.

Young floral buds from potato plants, transgenic for a pin2 promoter-GUS fusion (Keil et al., 1989), were sliced at different levels and stained to monitor the activity of the reporter GUS enzyme. Blue staining is indicative of GUS activity.

(A) Cross-section of a floral bud close to the pedicel. The blue-stained cells represent the developing ovules.

(B) Blue-stained ovules at later stages of development, when the placenta is already formed.

(C) Upper part of the floral bud showing a cross-section of an anther. Blue staining is seen at the petals' edges.

ABA-deficient and wild-type *S. phureja* plants but not in the leaves of the former upon wounding. This would imply that ABA is only involved in the induction of expression of the pin2 gene after wounding but is not needed for pin2 expression in tubers and floral buds, strongly suggesting the existence of at least two different signal pathways for the expression of this gene. To substantiate this conclusion and to exclude other possible explanations, such as loss of the wound-responsive members of the pin2 gene family in the droopy mutant due to outcrossing, we decided to transfer a chimeric gene consisting of a pin2 promoter fused to the GUS reporter gene to the ABA-deficient plants using Agrobacterium-mediated techniques. This chimeric gene had been shown previously to confer wound-inducible and tuber-constitutive GUS activity in transgenic wild-type potato plants (Keil et al., 1989).

As shown in Figure 5, the chimeric gene is not induced upon wounding in the ABA-deficient plants, whereas it is highly active when present in a wild-type background. This difference vanished upon ABA treatment, when a similar accumulation of GUS mRNA was observed in both wild-type and mutant plants, thus following the expression pattern of the pin2 gene family. The GUS activities in ABA-incubated leaves of the different transgenic droopy lines are indicated in Table 1. These data correlate with the GUS mRNA accumulation (not shown).

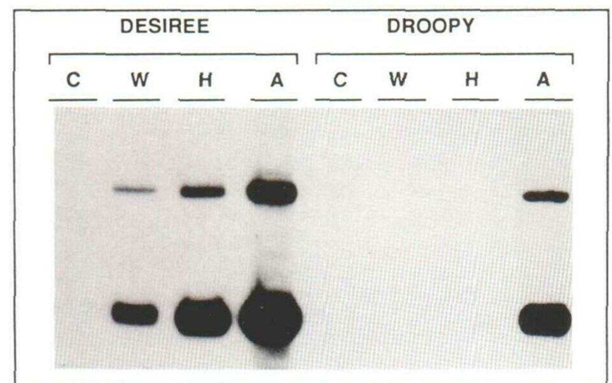

Figure 5. Comparison of the pin2 Promoter Activity in the Wild-Type and ABA-Deficient Backgrounds.

An RNA gel blot analysis was performed using wild-type and droopy plants transgenic for a pin2 promoter-GUS gene fusion (lines S9-114 and 14, respectively). RNA was isolated from leaves before (lanes C), or 24 hr after (lanes W) wounding. RNA was also isolated from detached leaves incubated in water (lanes H) or 50 μM of ABA (lanes A), for 24 hr. As a radioactive probe, a DNA fragment encompassing the 3′ end of the pin2 gene was used because it is shared by both the endogenous pin2 gene family (lower band, 800 nucleotides) and the GUS mRNA transcribed from the transgenic pin2 promoter (upper band, 2000 nucleotides).

Table 1. GUS Activity of the S9 Transgenic Droopy Lines upon ABA Treatment

Transformed Lines	GUS Activity in Leaves (pmol product/mg protein/min)	
	Water	ABA
droopy 12	873	2761
droopy 13	236	958
droopy 14	321	3778
Wild-type (Desiree) S9-114	4370	3168

Detached leaves from droopy plants transgenic for a pin2 promoter-GUS gene fusion (S9, Keil et al., 1989) were incubated for 24 hr in 100 μM of ABA or water alone. The GUS activity present in the respective protein extracts was determined as picomoles of the 4-methyl umbelliferone product per milligram of protein extract per minute of incubation at 37°C. As control, a transgenic S9 line in the wild-type background (Keil et al., 1989) was used.

Whereas the expression pattern of this chimeric gene as a result of wounding differs dramatically between ABA-deficient and wild-type plants, no such difference is seen for the other modes of expression. As in the wild-type transgenic potato, this pin2 promoter conferred constitutive GUS activity to tubers and floral buds in the ABA-deficient background (data not shown). The expression in the floral buds was also confined to the ovary in the early stages of floral development, its pattern of expression thus resembling that in the wild-type plants (cf. Figure 4). Identical results were obtained from RNA gel blot analysis of the GUS mRNA distribution (not shown).

Exogenously Supplied ABA Induces the Expression of the pin2 Gene Family at the Same Internal Concentrations as Normally Reached in Wounded Leaves

The complementation of the lack of induction of the pin2 gene in the ABA-deficient mutants by ABA spraying is a key experiment with regard to the involvement of ABA in the wound-induced expression of the pin2 gene family. The interpretation of data obtained from experiments involving the exogenous application of plant hormones, however, is often hampered by the pleiotropic effects exerted by these substances. Moreover, the hormonal concentration that is actually reached in the tissue under examination is in most cases uncertain, depending on factors such as the rate and ease of absorption, its half-life in the tissue, and its availability to the cells that are actually responding to it.

To exclude the possibility that the induction of the pin2 gene by ABA resulted from the presence of nonphysiological levels of this phytohormone in the leaves, we have determined the minimal time the leaf petiole has to be incubated in an ABA solution, followed by further incubation in water for up to 24 hr, to trigger the induction of the pin2 gene family. We have taken advantage of the ABA-deficient droopy plants where the pin2 mRNA accumulation by incubation of detached leaves in water alone is very low.

We have also related the detected mRNA accumulation to the concentration of ABA actually attained in the leaves because of the exogenously supplied phytohormone. As shown in Figure 6, a 2-min incubation in ABA (the shortest time investigated) was enough to obtain a distinct accumulation of pin2 mRNA. This induction was obtained when the ABA concentration in the mutant leaves was within the range normally observed in wild-type leaves 24 hr after wounding (Peña-Cortés et al., 1989). pin2 mRNA accumulation reached a maximum 2 hr after application, and further incubation in ABA had no effect on the final pin2 mRNA levels detected, despite the fact that the concentration of ABA rose higher upon longer incubation.

The accumulation of pin2 mRNA had a time course that closely followed its induction kinetics upon wounding. Figure 7 shows a typical result of a hormone feeding experiment in which the pin2 mRNA could be clearly detected 30 min after the application of ABA and accumulated until after 8 hr of incubation, thus resembling the described accumulation curve upon wounding (Peña-Cortés et al., 1988).

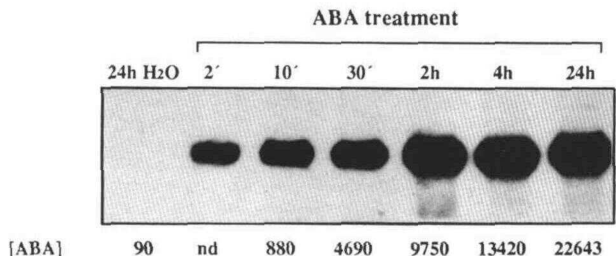

Figure 6. Incubation Time in ABA Required for the Induction of the pin2 Gene Family.

Detached droopy leaves were fed in a 100 μM ABA solution for the indicated time and subsequently incubated in water up to 24 hr. Leaves were then frozen and RNA was isolated. The RNA gel blot was hybridized against a pin2 cDNA probe. To determine the ABA level attained in the leaves upon treatment, part of each leaf was directly frozen after ABA feeding, before any further incubation in water, and the determined concentration is indicated below the respective lanes (in picomoles of ABA per gram, fresh weight). Single ABA quantitations are shown for 10 and 30 min, whereas concentrations for 2, 4, and 24 hr are the mean value of two independent determinations. nd, not determined. As control, droopy leaves were incubated in water alone (24h H_2O, single determination).

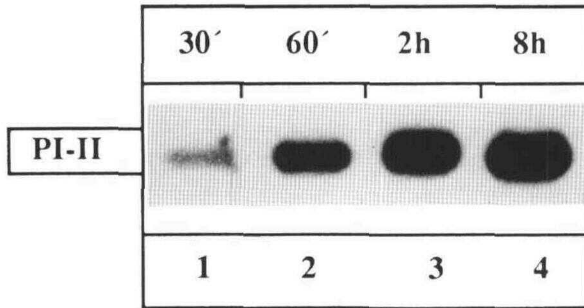

Figure 7. Time Course of the pin2 Induction upon ABA Feeding.

Detached droopy leaves were fed for 15 min in a 100 μM ABA solution and subsequently transferred to water. Leaves were harvested at the indicated time after the ABA treatment and RNA was isolated. The RNA gel blot was probed with the pin2 cDNA.

Increase in ABA Concentration in Damaged Tissue Is Required for Both Local and Systemic pin2 Induction in Leaves

The above-mentioned experiments indicated a specific role for ABA in the induction of the pin2 gene family upon wounding. However, the local expression of genes at the site of the injury could result from the action of signals different from those required for the systemic activation of a subset of wound-induced genes.

We have tested this possibility by comparing the pin2 mRNA level attained in the immediate vicinity of the injury inflicted to both wild-type and ABA mutant plants. As illustrated in Figure 8, after a single wounding, the mRNA accumulation in the systemically induced leaf tissue (lanes S) was much lower than in the tissue surrounding the site of the injury (lanes L). Wounding the leaf a second time, as described previously (Peña-Cortés et al., 1988), strongly enhanced the systemic induction (not shown). Droopy plants showed a distinct local accumulation of pin2 mRNA upon wounding (potato plants 1 and 2, lanes L), which is below the limits of detection in the tomato *sitiens* counterpart. However, both droopy and *sitiens* plants showed a drastically reduced pin2 mRNA level at the site of the injury compared with their respective wild-type lines. These results are consistent with the lack of local ABA increase upon wounding and suggest a role for ABA in the local response of the pin2 gene to wounding.

Exogenously Applied ABA Migrates to Distal Tissues and Is Correlated with the Expression of the pin2 Gene Family

The ability of ABA to migrate through both xylem and phloem vessels has been unequivocally demonstrated (Wolf et al., 1990). Local ABA concentrations increase as a result of injury, and this newly generated ABA could be then distributed throughout the plant, resulting in the activation of the pin2 gene in the nonwounded, systemically induced tissues. We have addressed this point by assessing the ability of exogenously applied ABA to induce the expression of the pin2 gene family in a systemic manner.

To this end, we sprayed an aqueous ABA solution on leaves of droopy plants and analyzed the presence of pin2 mRNA in the directly treated leaves and in the nonsprayed, distal leaves. As shown in Figure 9, the pin2 gene family was activated as a result of the ABA treatment in both the directly sprayed leaves and in the more distal, nonsprayed ones, following a time course that mirrored the induction caused by mechanical wounding. We have correlated this induction to the levels of ABA present in the induced tissue. The directly sprayed leaves had a high ABA concentration (not shown); however, this was most likely because of the phytohormone adsorbed to the leaf cuticle. More importantly, a threefold increase in the ABA concentration in the distal, nontreated leaves was attained 6 hr after spraying, a time point when the pin2 gene family was expressed in this systemically induced tissue.

The results from these experiments demonstrate the ability of ABA to induce the expression of the pin2 gene family in a systemic manner.

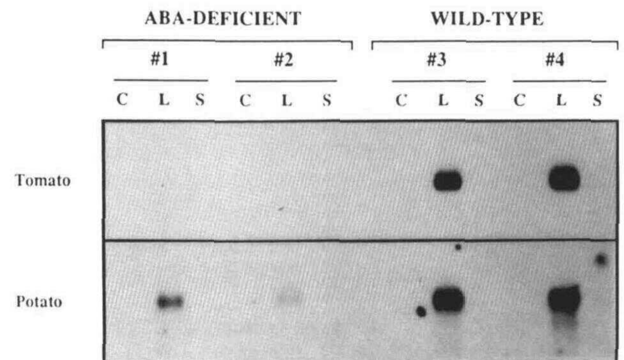

Figure 8. Differences in the Induction of the pin2 Gene Family upon Wounding of Wild-Type and ABA-Deficient Plants.

Two separate leaves of tomato and potato ABA-deficient and wild-type plants were wounded for each plant, leaving a nonwounded leaf in between. After 24 hr, the tissue surrounding the injury (lanes L) and the nonwounded, systemically induced leaf (lanes S) were harvested. A control leaf was collected before wounding (lanes C). The blotted RNA was hybridized against the pin2 cDNA. The figure shows a typical result obtained from two different plants of each category which had been consistently observed in independent experiments. The systemically induced pin2 mRNA could be observed upon longer exposures (not shown). In that case, however, local pin2 mRNA accumulation in *sitiens* was still below detection.

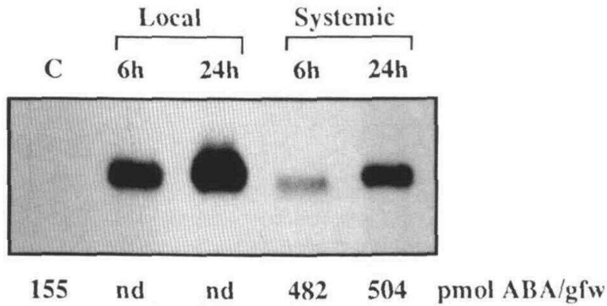

Figure 9. Systemic Induction of the pin2 Gene Family upon ABA Spraying.

Droopy plants were sprayed with a 100 μM ABA solution. Both directly sprayed and nonsprayed systemically induced leaves were harvested at the indicated time after treatment. The RNA gel blot was hybridized against a pin2 cDNA probe. The ABA concentration attained upon treatment in the systemically induced leaves at the time of harvesting was also determined. Concentrations given are the mean value obtained from two (6h) or three (24h) independent experiments. As a control, RNA was isolated from a droopy plant 24 hr after spraying with water (lane C). The ABA concentration was also determined in this control plant in leaves of equivalent age to the systemically ABA-induced ones (single determination). nd, not determined.

DISCUSSION

The pin2 gene family has been implicated in the ecological survival of potato and tomato plants in its action against feeding herbivores (Broadway and Duffey, 1986; Thornburg et al., 1990). This view is supported by the fact that its transcription is activated in the aerial part of the plant upon mechanical wounding and that this activation is not confined to the wound site but is rather a systemic accumulation of its mRNA throughout the plant (Peña-Cortés et al., 1988). We have previously shown that the plant hormone ABA is involved in this wound-induced activation of the pin2 gene (Peña-Cortés et al., 1989). However, the pin2 gene family is constitutively expressed in potato tubers and developing flowers where it might play a developmentally regulated role in preventing premature protein degradation.

One important observation is that, in contrast to its activation upon wounding, the developmentally controlled expression of the pin2 gene family is not influenced by the concentration of ABA in tubers or in flowers. Droopy tubers accumulate pin2 mRNA to levels similar to the *S. phureja* wild-type parental line, and in both cases the ABA concentration determined in tubers was lower than in the respective nonwounded leaves, which do not express the pin2 gene. Constitutive pin2 expression in flowers could be observed in both tomato and potato plants. Again, both wild-type and mutant flowers contained pin2 mRNA, and its organ distribution was nearly identical in the wild-type and the ABA-deficient plants of the relevant species. However, the expression level in the tomato mutant *sitiens* was lower in sepals and ovules compared with the wild type.

The abundance of pin2 mRNA in the plant's reproductive organs, tomato and potato flowers, and potato tubers tends to support its protective role against pathogen attack. It is worth mentioning in this regard that flowers constitutively express other proteins involved in the plant defense mechanism, some of them belonging to the pathogenesis-related (PR) protein group (Lotan et al., 1989; Neale et al., 1990). Interestingly, a protein antigenically related to the major tuber protein patatin, which shares the same cell specificity and induction pattern as pin2 upon tuberization, also has been shown to be present in flowers of potato and other members of the Solanaceae (Vancanneyt et al., 1989). In this case, however, a different tissue distribution is displayed, with patatin being present only in the outermost layer of the anthers, the exothecium.

As described above, an injury inflicted to the plant leads to the induction of the pin2 gene both in the directly damaged tissue and in the distantly located, nonwounded tissue. Two hypotheses can be put forward to explain how the local and systemic inductions are accomplished. One is that at least two signals are produced at the wound site, one activating the pin2 gene locally while the second signal migrates to the distal tissues and triggers the systemic activation of pin2. Alternatively, a single signal produced or released upon wounding activates the pin2 gene both locally and systemically.

Evidence presented here implies that ABA is involved in both the local and the systemic accumulation of the pin2 mRNA upon wounding. The results obtained are consistent with its role as a wound signal for the following reasons: first, its concentration increases locally upon wounding, and this increase correlates with the wound induction of the pin2 gene. This is highlighted by the fact that potato and tomato ABA-deficient mutants, which do not exhibit this rise in their ABA concentration, show neither local nor systemic activation of the pin2 gene upon wounding. The impaired step that results in the ABA deficiency is the conversion of ABA aldehyde to its active acid form as the final step of its synthesis de novo (Taylor et al., 1988; Duckham et al., 1989). This implies that the rise in ABA concentration upon wounding is a result of de novo synthesis and not due to conversion of stored molecules (i.e., ABA glycosides, Zeevaart and Creelman, 1988) to the active form. Second, by spraying ABA on leaves of the droopy mutant, we could show that the exogenously applied ABA migrated to the distal, nonsprayed leaves. The pin2 gene family was transcribed in these leaves at a time when the ABA concentration in this tissue was 3 times the nonwounded level. A similar increase in ABA concentration is also found in wild-type leaves systemically induced by mechanical wounding.

The ABA concentration increases locally after wounding, but if the systemic induction of the pin2 gene family is only mediated by the ABA produced at the wound site, which then migrates throughout the plant, one might expect a decrease in the accumulation of pin2 mRNA the more distal the leaves were from the site of the injury as a consequence of the dilution of the locally formed ABA. This would be in contrast to our previous observations on the pin2 systemic induction in potato (Peña-Cortés et al., 1988), and therefore suggests the existence of a mechanism to amplify the signal generated upon wounding. Such a mechanism might be local synthesis of ABA in the systemically induced organs, which would amplify the wound signal (and maintain a high concentration) while spreading throughout the plant.

We have also shown that ABA synthesis is not only required for the systemic activation but also for the local induction of the pin2 gene. If a different signal were responsible for the local induction of the pin2 gene, a normal accumulation of its mRNA in the tissue surrounding the injury would be expected in the ABA-deficient tomato and potato plants. The local induction of the pin2 gene in these plants, however, is reduced to a similar extent as the systemic induction.

A general picture emerges in which a local increase in ABA concentration upon wounding acts as a trigger of the systemic defense reaction in plants, which in turn is itself dependent upon increased ABA concentration in the distal tissues. Changes in ABA concentrations have been related to differences in the susceptibility to pathogens. Thus, the resistance of bean seedlings toward the fungal pathogen *Colletotrichum lindemuthianum* could be related to elevated ABA levels (Dunn et al., 1990). Changes in the ABA concentration have also been reported in tobacco upon tobacco mosaic virus infection (Fraser, 1979), and in the interaction of soybean and *Phytophtora megasperma* f sp *glycinea*, where differences in concentration were observed between inoculations with compatible and incompatible races both at the site of the lesion and in the apparently healthy distal tissues (Cahill and Ward, 1989).

Other substances shown to induce pin2 activity include sucrose and the hormone-like compound methyl jasmonate (Farmer and Ryan, 1990; Johnson and Ryan, 1990; our unpublished data). Sucrose induction of pin2 has been obtained in detached potato leaves and, in contrast to the ABA induction shown in this paper, requires its continuous presence in the incubation medium. This precludes any effect as a trigger of pin2 transcriptional activation and might be related to the physiological situation in sink tubers, where high carbohydrate levels might induce the transcription of tuber-specific genes. However, differences between pin2 and patatin, the major tuber protein, with regard to sucrose induction in detached leaves require the presence of another regulatory step in addition to high carbohydrate content (H. Peña-Cortés, X. J. Liu, J. J. Sánchez-Serrano, R. Schmid, and L. Willmitzer, manuscript submitted for publication). The effect exerted by methyl jasmonate is of particular interest because ABA and jasmonic acid (JA) treatments induce similar sets of proteins in a number of plant species (Parthier, 1990). Also, other wound-induced genes are responsive to JA treatment (Staswick, 1990). However, it is not known whether JA and ABA metabolic pathways interact, although recent work suggests that this is the case (Abián et al., 1991). The absence of JA-deficient mutants precludes, at this stage, more precise conclusions on the physiological role of JA in pin2 induction to be drawn.

Other signals are also likely to be involved in the systemic defense reaction in plants. It has been shown recently that salicylic acid induces the acquired systemic resistance of tobacco plants upon viral challenge (Malamy et al., 1990). This resistance is likely to involve the activation of several salicylic acid-responsive genes, the PR-1 gene being one of them. The time course of the PR-1 induction, which is much slower than that of the pin2 and other ABA-induced genes, together with the fact that pin2 activation is actually inhibited by salicylic acid (Doherty et al., 1988) suggest a mechanism by which plant defense genes are coordinately expressed upon pathogen attack. The rapid activation of a set of early genes related to wounding and responding to ABA would thus be replaced by the late, salicylic acid-responsive ones.

How this coordinated regulation is achieved and how pathogens still manage to escape the defense mechanisms will be two of the fundamental issues for engineering disease resistance in plants.

METHODS

Plant Material

Potato plants used were *Solanum tuberosum* var Desiree (tetraploid), *S. tuberosum* group *phureja* (diploid, derived from the CPC 4461 × CPC 4463 crossing), and its ABA-deficient droopy mutant (also diploid) (Quarrie, 1982). Seeds of both *S. phureja* and droopy plants were obtained from Dr. S. Quarrie (The Cambridge Laboratory, John Innes Institute, Norwich, United Kingdom), and plants were subsequently vegetatively propagated from cuttings.

Tomato plants used were *Lycopersicon esculentum* var Moneymaker and the ABA-deficient *sitiens* mutant (Taylor et al., 1988). They were propagated from seeds (kindly provided by Dr. M. Koornneef, Agricultural Department, University of Wageningen, The Netherlands).

Growth conditions in the greenhouse were 16-hr light photoperiod (18°C day/10°C night) at 60% to 90% relative humidity for potato, and 14-hr light (26°C day/20°C night) at 70% to 80% relative humidity for tomato.

Plants were wounded by applying dialysis clamps to the third and fifth fully opened leaves (starting from the apical part of the plant). Locally wounded tissue (within 10 mm from the clamp) was

collected 24 hr after wounding. The fourth, nonwounded leaf was taken as systemically induced.

Other sources used for RNA were small, developing tubers and young floral buds (defined as those closed or with petals not yet colored). Plant material was frozen in liquid nitrogen and stored at −80°C or used immediately.

RNA Gel Blot Analyses

Plant total RNA was isolated and subjected to electrophoresis (20 μg of RNA per slot) in agarose-formaldehyde gels as described by Logemann et al. (1987). Blotting and hybridization conditions were as described by Amasino (1986). Probes used for radioactive labeling were a pin2 cDNA fragment (cDNA 1, Sánchez-Serrano et al., 1986) and a 270-bp fragment encompassing the pin2 3′ end (Keil et al., 1989).

Plant Transformation

Agrobacterium tumefaciens S9, harboring a BIN 19-derived vector including a pin2-GUS gene fusion was used to transform droopy mutant plants (*S. phureja*) by the leaf disc infection method as described by Keil et al. (1989). Transformed shoots were selected on kanamycin (50 mg/L). After rooting, transformed plants were transferred to the greenhouse, and the integrity of the transformed gene fusion was checked by DNA gel blot analysis (not shown). Plants containing one or few intact copies of the pin2-GUS gene were subsequently propagated from excised shoots.

ABA Treatment and Quantitation

A 100 mM racemic *cis-trans* ABA in ethanol was used as stock solution and subsequently diluted in water to the concentration indicated for each experiment. Plants were sprayed with a 100 μM of ABA solution every 3 hr (approximately 100 mL per plant per time point) for the duration of the treatment. Leaves to be taken as systemically induced were covered with plastic foil while spraying. ABA concentrations were determined as described by Peña-Cortés et al. (1989).

GUS Assays and Stainings

Whole leaves, or cuttings taken by hand of the different plant organs, were fixed in a 4% paraformaldehyde solution in 50 mM phosphate buffer, pH 7.0, for 30 min on ice. Histochemical analyses and fluorimetric GUS assays in plant protein extracts were as described by Keil et al. (1989).

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