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Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato

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ABSTRACT **Plants respond to wounding or pathogen attack by a variety of biochemical reactions, involving in some instances gene activation in tissues far apart from the actual site of wounding or pathogen invasion. One of the best analyzed examples for such a systemic reaction is the wound-induced expression of proteinase inhibitor genes in tomato and potato leaves. Local wounding of potato or tomato plants results in the accumulation of proteinase inhibitors I and II throughout the aerial part of the plant. In contrast to wild-type plants, abscisic acid-deficient mutants of potato (droopy) and tomato (*sit*) show a drastically reduced induction of these genes in response to plant wounding. High levels of proteinase inhibitor II gene expression are obtained in mutant and wild-type plants upon exogenous application of abscisic acid. Measurements of the endogenous abscisic acid levels in wild-type plants show that wounding results in increased levels of this phytohormone in wounded and nonwounded systemically induced leaves. Thus these results show that the plant hormone abscisic acid is involved in the wound-induced activation of the proteinase inhibitor II gene. Furthermore, they are compatible with a model assuming this hormone to be the actual mediator of the systemic wound response.**

Potato and tomato plants accumulate proteinase inhibitors I and II in leaves as a direct consequence of insect damage or mechanical wounding. Both inhibitors are specifically directed against insect proteases and are considered to be part of the natural defense mechanism of plants against attacking insects (1–3). The accumulation of these proteins is not restricted to the wound site, but it is also observed in nonwounded aerial tissues. This indicates that upon wounding an inducing factor or wound hormone is released that, probably by way of the vascular system, is rapidly transported to other tissues of the plant, thereby inducing the expression of these proteinase inhibitor genes. Pectic polysaccharides derived from the cell wall have been shown to be powerful inducers of the proteinase inhibitor genes when supplied to excised leaves through the cut petiole and have thus been postulated as a possible proteinase-inhibitor-inducing factor (4–6). In a similar way oligosaccharides such as chitosan have been shown to be strong inducers of the proteinase inhibitor II (PI-II) gene (1). By using radiolabeled oligosaccharides, however, Baydoun and Fry (7) have shown that molecules with a degree of polymerization greater than 6 do not travel long distances through the plant vascular system, which argues against them actually mediating the systemic activation of the PI-II gene.

Wounding represents, on the other hand, a special case of stress for the plant cell. Stress conditions elicit a number of physiological responses, often resulting in changes in the internal levels of plant hormones. Thus elevated levels of abscisic acid (ABA) have been reported as a result of water or osmotic stress conditions (8, 9), whereas ethylene biosynthesis has been associated with the initial response of the plant tissue to mechanical wounding (10). Indirect evidence for the involvement of ABA in wound responses has also been obtained from two maize proteins, whose synthesis is induced by water stress and by ABA and in addition shows low wound inducibility (11, 12).

We, therefore, decided to test whether or not ABA is involved in the systemic induction of the PI-II gene. More specifically, we asked the following questions: (*i*) Does treatment of nonwounded plants with ABA lead to an induction of PI-II gene expression? (*ii*) Do ABA-deficient mutant plants show a reduced wound-induced expression of the PI-II gene and, if so, can this defect be complemented by applying exogenous ABA? (*iii*) Do known antagonists of ABA reduce or suppress the wound-related induction of PI-II gene expression? (*iv*) Does wounding lead to an increased level of ABA in wounded and nonwounded parts of the plant?

In this report we present data based on the analysis of ABA-deficient potato and tomato mutants that strongly suggest that the phytohormone ABA is directly involved in the induction of PI-II genes.

MATERIALS AND METHODS

Plant Material and Chemicals. Potato line cv. Berolina, tomato cv. Money maker, ABA-deficient mutant potato (droopy, provided by S. Quarrie, Agriculture and Research Council Institute of Plant Science Research, Cambridge, U.K.), and tomato (*sit*, provided by M. Koornneef, Landbouwuniversiteit Wageningen, The Netherlands) were grown under greenhouse conditions (16°C day/10°C night, 50–80% relative humidity, and normal photoperiod, 14 hr of light). Chitosan (from crab shells), gibberellic acid (GA_3), (±)-ABA, cycloheximide, and chloramphenicol were obtained from Sigma.

Chitosan and GA_3 Application. Chitosan was treated before use with nitrous acid according to Hadwiger and Beckman (13). Leaves were harvested and incubated in a solution containing chitosan, GA_3, or ABA as described by Peña-Cortés *et al.* (1). GA_3 was dissolved in 5 mM KOH.

ABA Application. ABA solutions [10 and 100 μM (±)-ABA in sterile water/0.01% ethanol] were applied to plants by aerial spraying every 6 hr for the entire test period or, alternatively, by direct incubation of detached leaves in the solution.

Wounding of Potato and Tomato Leaves. Potato and tomato leaves were wounded according to Sánchez-Serrano *et al.* (3).

Isolation and Analysis of RNA. Isolation of RNA was performed as described by Logemann *et al.* (14). For gel

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Abbreviations: PI-II, proteinase inhibitor II; ABA, abscisic acid; GA_3, gibberellic acid.

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electrophoretic analysis, RNA was denatured with formaldehyde in the presence of formamide and electrophoresed on 1.5% agarose gels. RNA was transferred to nylon membranes and hybridized using PI-II cDNA-1 (3) as a probe. Hybridization and washing conditions were performed as described (1). The amount of RNA on the membranes was checked by hybridizing them to a rRNA probe (data not shown).

Protein Analysis. Proteins from seeds of tobacco, tomato, and potato plants were extracted according to Racusen and Foote (15), separated by gel electrophoresis (16), and analyzed by the Western blot technique as described (17). The antibody used for this experiment was raised against the potato tuber PI-II protein and purified by affinity chromatography using an oligopeptide representing the 16 carboxyl-terminal amino acids of the PI-II protein, as deduced from the clone cDNA-1 (3).

ABA Quantitation. ABA was extracted from potato leaves. Harvested leaves were immediately frozen in liquid nitrogen after weight determination. Tissue was then homogenized in an extraction solution containing 80% (vol/vol) methanol and butylated hydroxytoluene (2.5 mg/liter) and subsequently mixed for 1 hr at 4°C in darkness. The extract was centrifuged at 3000 × *g* for 7 min at 4°C, and the resulting pellet was reextracted twice with the extraction buffer, as described above. The supernatants were pooled, equilibrated in 70% methanol, and applied to a 2-ml bed volume Sep-Pak C_{18} column (Millipore). Methanol was removed from the fractions by evaporation in a Rotavapor (Büchi Instruments, Geneva). Samples were dried and resuspended in PBS (10 mM sodium phosphate, pH 7.4/0.15 M NaCl). An aliquot was used to determine the amount of ABA in the sample by a monoclonal antibody-based RIA assay, as described (18). A minimum of five plants was used for establishing each ABA concentration value.

RESULTS

ABA Spraying and Wounding Lead to Very Similar PI-II mRNA Accumulation Patterns. A 100 μM aqueous solution of ABA was directly sprayed on the leaves of greenhouse potato plants. As a control, similar plants were sprayed with water. After 24 hr RNA was isolated from different organs of the sprayed and the control plants and analyzed for the expression of the PI-II gene by RNA blot analysis. As shown in Fig. 1, PI-II mRNA accumulates in leaves and stems of sprayed plants. Tissues that were sprayed directly as well as systemically induced tissues, irrespective of ABA being applied to upper or to lower leaves, showed increased PI-II mRNA

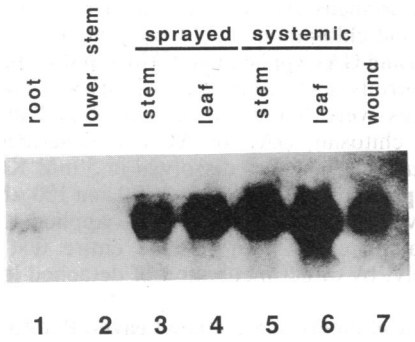

FIG. 1. Effect of ABA on the expression of the PI-II genes in potato plants (*Solanum tuberosum* L.) var. Berolina. Total RNA (50 μg) was applied that had been isolated from roots (lane 1), lower stem segment (lane 2), sprayed stem segment (lane 3), sprayed leaf (lane 4), systemic stem segment (lane 5), and systemic leaves (lane 6). RNA isolated from wounded leaves (lane 7) was included as control. Systemic, regions of the plant not directly sprayed; lower stem, stem segment located directly above the roots.

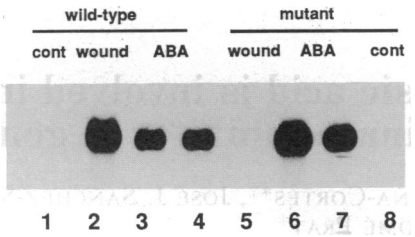

FIG. 2. Accumulation of PI-II mRNA in potato leaves of ABA-deficient mutant and wild-type plants upon wounding and ABA-treatment. Total RNA was isolated from leaves of nonwounded (lane 1, cont), wounded (lane 2), and ABA-sprayed (lanes 3 and 4) wild-type potato plants (*Solanum tuberosum* L.) and from nonwounded (lane 8, cont), wounded (lane 5), and ABA-sprayed (lanes 6 and 7) droopy mutant potato plants (*Solanum tuberosum* L., group phureja), respectively (19).

levels. More significantly, the systemic induction was limited to the aerial parts of the plant with no induction detected in the root and lower part of the stem. Identical responses were obtained with a lower concentration of ABA (10 μM). Control plants sprayed with water did not show any accumulation of PI-II mRNA (data not shown). ABA sprayed to the leaves of a plant is thus able to trigger the systemic induction of the PI-II gene, with a pattern identical to the one described for wounded plants (1).

Potato and Tomato ABA-Deficient Mutant Plants Fail to Accumulate PI-II mRNA upon Wounding but Show High Levels of PI-II Expression as a Result of Exogenous ABA Application. The induction of PI-II genes in nonwounded plants as a direct consequence of ABA application provides preliminary evidence that this hormone is involved in the wound-induced expression of these genes. To obtain more conclusive evidence for this assumption, the wound response of these genes was studied in ABA-deficient mutants of potato (droopy) (19) and tomato (*sit*) plants (20). The altered phenotype of these mutants results from their lower internal level of ABA (9% in case of droopy and 12–15% in *sit*) and can be reversed by exogenous application of the hormone.

As shown in Fig. 2, the induction of PI-II mRNA as a result of wounding is much higher in wild-type potato as compared to the droopy mutant. This result was consistently obtained in several independent experiments. Most important, however, this difference in the expression level is not observed when induction is performed by spraying ABA, thus providing a direct proof for the involvement of the hormone ABA in the wound-induced expression of the PI-II genes.

Similar data were obtained by wounding the tomato wild-type and the *sit* mutant plants (Fig. 3). Again, mutant plants

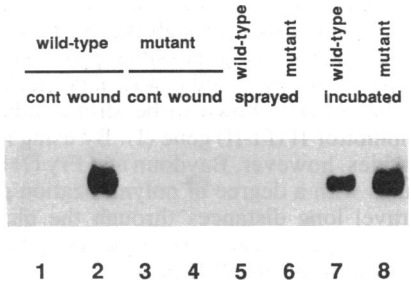

FIG. 3. PI-II mRNA expression in tomato leaves of ABA-deficient mutant and wild-type plants upon wounding, ABA spraying, and ABA feeding through the petiole. Total RNA was isolated from nonwounded (lane 1, cont), wounded (lane 2), ABA-sprayed (lane 5), and ABA-incubated (lane 7) wild-type tomato plants and nonwounded (lane 3, cont), wounded (lane 4), ABA-sprayed (lane 6), and ABA-incubated (lane 8) *sit* mutant tomato plants (*Lycopersicon esculentum*) (20).

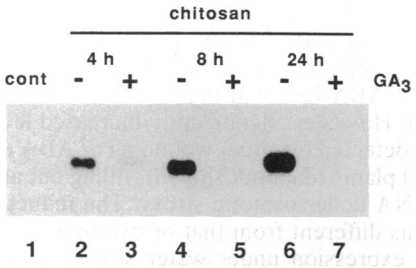

FIG. 4. Time course of GA_3 effects on the chitosan-induced accumulation of PI-II. Detached leaves were incubated through the cut petiole for 4, 8, or 24 hr (h) in chitosan in the presence (+) of GA_3 (lanes 3, 5, and 7) or in the absence (−) of GA_3 (lanes 2, 4, and 6). RNA was isolated from leaves prior to chitosan incubation as a noninduced control (lane 1, cont).

showed a very reduced wound induction of the PI-II genes and no difference in the expression levels of wild-type and mutant plants was seen upon ABA treatment. In this case response to ABA was only observed upon hormone feeding through the cut petiole but not upon spraying. Control leaves incubated for the same time in phosphate buffer did not show any accumulation of PI-II mRNA (data not shown). A similar observation was also made for leaves of transgenic tobacco plants containing a potato-derived PI-II gene (21). Again, this gene could be induced by ABA only when supplied by the cut petiole.

GA_3 Blocks the Chitosan Induction of the PI-II Genes on Detached Leaves. As described above wounding can be mimicked by several oligosaccharides, such as chitosan, by uptake through detached petioles (1). To test whether the chitosan-induced accumulation of PI-II genes is also mediated by ABA, detached leaves were incubated with chitosan in the presence or absence of 10 μM GA_3. This hormone has been found to counteract all ABA effects investigated in barley aleurone cell layers (22). As shown in Fig. 4, detectable levels of PI-II mRNA are observed in leaves incubated with chitosan after 4 hr and increase further for 24 hr. After a 4-hr incubation, the amount of PI-II mRNA is, however, much lower in leaves incubated with chitosan in the presence of GA_3. After an 8-hr incubation, the hormone GA_3 is able to repress completely the induction of the gene by chitosan (Fig. 4, lane 5).

ABA-Induced Accumulation of PI-II mRNA Requires Protein Synthesis. Our initial studies on the molecular mechanism of ABA action investigated whether protein synthesis was required for the PI-II gene expression. Detached leaves were incubated for 1.5 hr with inhibitors of protein synthesis, acting on chloroplast (100 μM chloramphenicol) and cytoplasmatic (10 μM cycloheximide) ribosomes. Incubation with protein synthesis inhibitors clearly blocks the accumulation of PI-II mRNA after chitosan or ABA treatment (Fig. 5). These data suggest that ABA-induced PI-II gene expression requires protein synthesis and that the factors mediating the induction of these genes are newly synthesized upon wounding and/or hormone treatment.

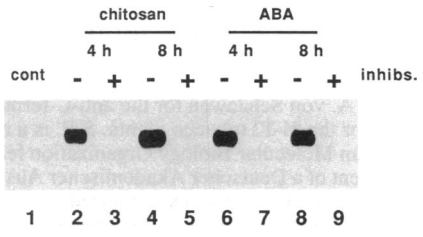

FIG. 5. Effect of inhibitors of protein synthesis on the accumulation of PI-II mRNA in detached leaves of potato incubated with chitosan or ABA. Leaves were incubated with chitosan (lanes 2–5) and ABA (lanes 6–9) for 4 and 8 hr. When inhibitors (inhibs.) of protein synthesis were included (lanes 3, 5, 7, and 9), the leaves were preincubated for 1.5 hr in the presence of cycloheximide (10 μM) and chloramphenicol (100 μM). Inhibitors of protein synthesis were continuously present during the subsequent incubation. RNA isolated from leaves before the incubation started was included as control (lane 1, cont). +, Inhibitors present; −, inhibitors absent.

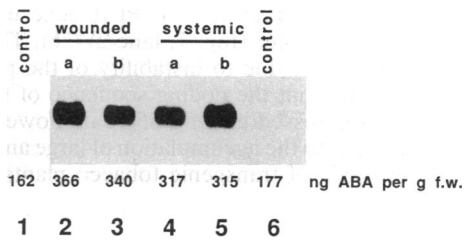

FIG. 6. Increase in the endogenous levels of ABA as response to wound stress. Endogenous concentration of ABA was measured in systemic (lanes 4 and 5) and directly wounded (lanes 2 and 3) leaves of plants that had been injured 24 hr before and in leaves from nonstressed control plants (lanes 1 and 6). ABA measured in these leaves is reported as ng per g of leaves (fresh weight). Total RNA was isolated from the same tissues to test the expression of the PI-II gene.

Plant Wounding Increases Endogenous ABA Levels in Wounded and Systemically Induced Leaves. The results above indicate that the endogenous ABA plays a crucial role in the wound response. To test whether stress results in an increase of the internal levels of ABA in wounded leaves as well as in systemically induced nonwounded leaves, endogenous levels of this hormone were measured after wounding. RNA blot analysis of the same samples were performed as a control to show the induction state of the plants used for the ABA determination. As shown in Fig. 6, wounding leads to an induction of PI-II genes as well as to increased internal levels of ABA not only in wounded leaves but also in systemic nonwounded leaves.

PI-II Protein Is Not Present in Seeds of Potato, Tomato, and Transgenic Tobacco Plants. ABA induces the accumulation of specific mRNAs and proteins late in embryogenesis in seeds of diverse species, playing a critical role in the desiccation process of the embryo (12, 13, 23–25). To test whether or not the PI-II protein also accumulates in seeds, we analyzed the protein content of tomato and potato seeds by Western blot. As shown in Fig. 7, the PI-II protein is not detectable in seeds of either species. The same holds true for a transgenic

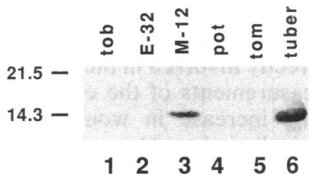

FIG. 7. Western blot analysis of seed proteins of potato, tomato, and transgenic tobacco plants. Proteins isolated from seeds of nontransformed tobacco (lane 1, tob), transgenic tobacco E-32 (lane 2), transgenic tobacco M-12 (lane 3), potato (lane 4, pot), and tomato (lane 5, tom) plants were separated by gel electrophoresis (16) and hybridized by the Western blot technique (17) against an anti-PI-II antibody specifically recognizing this protein. Potato tuber protein was included as control (lane 6, tuber). Tobacco (*Nicotiana tabacum*) var. W38, potato (*Solanum tuberosum*) var. Berolina, and tomato (*Lycopersicon esculentum*) var. MM plants refer to nontransformed plants. Tobacco M-12 is a transgenic tobacco plant that contains the coding region of the genomic proteinase inhibitor clone E-32 under the control of the 35S promoter of cauliflower mosaic virus (M. Keil, personal communication). Tobacco E-32 is a transgenic tobacco plant containing the entire potato-derived PI-II clone E-32 under the control of its own promoter (26). Locations of the 21.5- and 14.3-kDa bands are shown.

tobacco expressing a potato-derived PI-II gene under the control of its own promoter (Fig. 7, lane 2) (26). The possibility that this would be due to instability of the protein is ruled out by the fact that the coding sequence of the same PI-II gene, when expressed under a 35S cauliflower mosaic virus promoter, leads to the accumulation of large amounts of PI-II protein in seeds of transgenic tobacco plants (Fig. 7, lane 3).

DISCUSSION

This study was designed to investigate whether the phytohormone ABA, consistently associated with responses to stress situations, is involved in the induction of the PI-II genes. ABA directly sprayed on to the leaves proved to be sufficient to induce the accumulation of PI-II mRNA. More remarkable, the sprayed plants showed systemic induction of the PI-II gene with a pattern identical to wound-induced plants. Direct evidence for the involvement of the hormone ABA in the wound-induced response of the PI-II genes was obtained by the use of potato and tomato ABA-deficient mutants. These plants present much lower constitutive levels of ABA than wild-type plants and under water-stress conditions do not accumulate ABA, as do the wild-type plants (20). ABA-deficient mutants show a drastically reduced induction of these genes as a result of wounding when compared to wild-type plants. Of greater importance, differences in the PI-II induction between wild-type and mutant plants are completely abolished by ABA application. Upon ABA spraying the mutant potato plants show levels of PI-II expression as high as wild-type plants, demonstrating that lack of wound response of the PI-II genes in the mutant plants is directly related to their low levels of endogenous ABA.

In a similar way, tomato mutants show a very weak wound response, which is restored to normal levels by ABA treatment. In this case, exogenous ABA was applied by feeding through the petiole, since no response was achieved by ABA spraying. Interestingly, tobacco plants show the same behavior as tomato plants. Although the response of mutant tomato plants to sprayed ABA has been reported, such studies were always performed as long-term experiments where the plants were sprayed for 1–2 weeks (27). In short-term experiments, the hormone was always supplied for 12–24 hr by feeding through the petiole (28). The different responses of tomato and tobacco as compared to potato might reflect their different abilities to take up exogenous ABA.

The induction of PI-II genes upon ABA spraying in wild-type and mutant plants clearly demonstrates that the hormone ABA is directly involved in the wound induction of the PI-II genes. Measurements of the endogenous ABA levels show a hormone increase in wounded leaves and non-wounded systemically induced leaves. The systemic increase of ABA, as direct consequence of the wounding, is compatible with a model where this hormone is not only responsible for the local induction but also for the systemic induction of the PI-II genes. This model would require that ABA is released at the wound site and transported through the plant mediating the systemic wound response. One important requirement for this hypothesis is the timing of the ABA transport. Studies on the rate of ABA transport in soybean showed that labeled ABA is found in roots within 15 min after application to the leaf and is recycled by the xylem back to the shoot apex during the next 30 min (29). Assuming a similar rate of ABA transport in potato and tomato plants, these data agree well with the timing of the wound systemic response (1).

Several observations described above suggest at least two mechanisms of gene activation by ABA. (*i*) PI-II protein is not expressed in seeds of tomato and potato, which is different from the expression pattern of most other ABA-induced genes (12, 18, 24, 30). (*ii*) Water stress leads to a low-level accumulation of PI-II mRNA, although it also leads to increased ABA levels in higher plants, including potato and tomato. However, significantly increased levels of PI-II mRNA are detected by either wounding or ABA treatment of the stressed plants (data not shown), ruling out an instability of this mRNA under osmotic stress. The induction of PI-II genes is thus different from that of two maize genes, which show high expression under water stress but showed low expression when wounded (11, 12).

Finally, experiments to determine the molecular mechanism of the ABA action showed that the ABA-mediated induction of PI-II gene can be blocked with protein synthesis inhibitors. This is contrasted by the result of a similar experiment, performed with a water-responsive rice gene, where protein synthesis was not required (30). The different requirements with respect of protein synthesis displayed by both ABA-induced genes strongly support the model that there are at least two mechanisms to mediate ABA responses.

In conclusion, we have presented data based on the analysis of the induction of the PI-II gene by ABA as well as on the analysis of ABA-deficient potato and tomato mutants that prove that ABA is of crucial importance for the induction of the PI-II gene after wounding of the plant. The data are not yet conclusive as to whether or not ABA is actually the signal released from the wound site that directly mediates the systemic induction of the PI-II genes in nonwounded distal tissues. Although this is a very attractive hypothesis compatible with all data described, more complicated models cannot be excluded. Such alternative models would, e.g., involve (an) additional factor(s) released at the wound site and transported throughout the plant, thereby, inducing ABA synthesis at various locations, which in turn would lead to the systemic activation of PI-II genes. Irrespective of the final model, it has to explain the observation that the PI-II gene is only weakly induced under conditions that are known to activate other ABA-responsive gene systems. This suggests the existence of at least two mechanisms for ABA induction of gene activity. One possible mechanism could be a differential accumulation of ABA in the various cell compartments, depending in the stress conditions. The fact that both water and wound stress responses can be induced by application of exogenous ABA alone suggests that probably additional factors (i.e., *de novo*-synthesized polypeptides, polysaccharides, etc.) are not needed for this discrimination. Upon dehydration the ABA levels increase greatly in the apoplastic fluid as a consequence of large changes in apoplastic pH (31). Future work should lead to an understanding of the various signal transduction mechanisms involved in the wound response.

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