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Extensin and Phenylalanine Ammonia-Lyase Gene Expression Altered in Potato Tubers in Response to Wounding, Hypoxia, and *Erwinia carotovora* Infection[1]

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ABSTRACT

Potato (*Solanum tuberosum* L.) tubers are susceptible to infection by *Erwinia carotovora*, causal agent of bacterial soft rot, when wounded and subjected to wet, hypoxic environments. The expression of two putative plant defense genes, extensin and phenylalanine ammonia-lyase (PAL), was examined by monitoring their respective mRNA levels and cell wall hydroxyproline levels in tuber tissues under various conditions leading to susceptibility or resistance and after inoculation with *E. carotovora* in order to assess the possible roles of these genes and their products in this plant-pathogen interaction. Extensin and PAL mRNA levels as well as cell wall hydroxyproline levels accumulated markedly in response to wounding and subsequent aerobic incubation. Extensin and PAL mRNA levels as well as cell wall hydroxyproline levels decreased in response to wounding and subsequent anaerobic incubation; these changes were correlated with high susceptibility of tuber tissue to *E. carotovora* infection. Inoculation of wound sites with *E. carotovora* caused some additional accumulation of the wound-regulated extensin and PAL mRNAs under certain aerobic conditions, but never under anaerobic conditions.

Potato tubers are susceptible to infection by the bacterium, *Erwinia carotovora*, causal agent of bacterial soft rot. *E. carotovora* secretes pectate lyases as well as other degradative enzymes which are responsible for the maceration of tuber tissue (5, 20). Two critical environmental factors govern disease susceptibility in this host-pathogen system: the presence of wound sites on the tuber surface and the oxygen status of the tuber (7, 17). Wounding can be viewed as a prerequisite for infection in this system. Wound sites on the tuber surface are passageways for *E. carotovora* to invade underlying tuber tissue and initiate the infection process (20). Moreover, the type of wound affects susceptibility; Maher and Kelman (17, 18) have shown that tubers are more susceptible to soft rot when infection is initiated at bruise sites rather than shallow cut sites. Furthermore, tubers are susceptible to soft rot when infection proceeds under anaerobic or hypoxic conditions, but not when infection proceeds under aerobic conditions (7, 16). It is unlikely that alteration of bacterial growth and/or metabolism is responsible for such differential susceptibility, since bacterial growth continues under both anaerobic and aerobic conditions (18) and since injection of purified pectate lyase is sufficient to elicit rapid decay of tubers under low oxygen conditions but not under ambient oxygen conditions (16). Consequently, it is likely that susceptibility and resistance are determined by specific host responses.

Although the specific host factors responsible for resistance to bacterial soft rot are unknown, a number of plant responses to wounding and infection are documented (1, 13, 23). These responses, many of which have been demonstrated in potato, include the accumulation of phytoalexins, lignin or lignin-like material, callose, extensins, glycine-rich proteins, suberin, and proteinase inhibitors, as well as increases in the enzymatic activities of chitinase, glucanase, and several enzymes of the phenylpropanoid and terpenoid pathways. Likewise, a number of plant responses to anaerobic stress have been documented, largely based on work with maize (11, 21). These responses include an inhibition of preexisting protein synthesis followed by the accumulation of several enzymes responsible for or associated with glycolysis, including alcohol dehydrogenase, aldolase, glucose phosphate isomerase, pyruvate decarboxylase, and sucrose synthetase, as well as a number of other, as yet, unidentified gene products.

Recently, Vayda and Schaeffer (27) demonstrated that potato tubers accumulate a set of proteins upon bruising under aerobic conditions but not under hypoxic conditions. One member of the set has been identified by immunoprecipitation as PAL,[4] a key enzyme of the phenylpropanoid pathway. Accumulation of this set of proteins may account for disease resistance, whereas the lack of accumulation of these proteins under conditions of low oxygen levels may account for disease susceptibility. In this report, we examine the expression of two putative plant defense genes, extensin and PAL, by monitoring their respective mRNA levels and the cell wall hydroxyproline levels of tuber tissues under various conditions lead-

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[4] Abbreviations: PAL, phenylalanine ammonia-lyase; CFU, colony forming units; kb, kilobase.

ing to susceptibility or resistance and after inoculation with *E. carotovora* to assess the possible roles of these genes and their products in this plant-pathogen interaction.

MATERIALS AND METHODS

Potato Stocks

Potato tubers (*Solanum tuberosum* L. cv Russett Burbank) were stored at 4 to 6°C after sampling from commercial potato storages in Central Wisconsin. Tubers, selected for uniform size and absence of injury or disease, were surface-sterilized by immersion in 10% Clorox for 40 min, rinsed with deionized water, sprayed with 95% ethanol, and allowed to dry before further manipulation.

Tuber Wounding

Three types of wounds were inflicted on tubers in our study: bruises, slices, and pipet tip wounds. Wounds were inflicted at single sites in a midtuber position between stolon and bud ends. Bruising was accomplished by the action of a pendulum bruising device (2); two such bruises per site were inflicted. Slicing was accomplished with a razor blade to make a shallow, lateral cut in the tuber, resulting in exposure of an approximately 1.5 × 2.0 cm wound surface. Pipet tip wounds were inflicted by insertion of sterile yellow, plastic micropipet tips to a depth of 15 mm in the tuber.

Inoculation with *E. carotovora*

Suspension cultures of *E. carotovora* pv. *carotovora* (strain SR 392) were prepared as described previously (18) and used to inoculate wound sites immediately after wounding as follows: 25 μL of inoculum containing 1.9×10^5 CFU for bruised and sliced sites and 10 μL of inoculum containing 74 CFU in sites simultaneously wounded with pipet tips. Sterile distilled water, applied in the same manner and volumes noted above, was used for mock-inoculation controls.

Tuber Incubations

Three different incubation conditions were used: a humidity chamber, a mist chamber, and anaerobe jars. A humidity chamber adjusted to 20°C, 92% RH was used for aerobic incubations. A mist chamber adjusted to 20°C, 100% RH was used to provide a hypoxic environment. Anaerobe jars with palladium catalyst (Baltimore Biological Laboratories) and an 80% nitrogen (v/v), 10% hydrogen (v/v), and 10% carbon dioxide (v/v) atmosphere were used to provide an anaerobic environment. Incubations were carried out immediately following wounding/infection for 0, 8, and 24 h. Unmanipulated tubers were used as controls.

Sample Preparation

Approximately 18 g (fresh weight) of tuber tissue was extracted and pooled from several tubers for each different treatment. Bruised tissue and unmanipulated tissue were collected with a No. 10 core borer (17 mm diameter), removing the periderm, and utilizing the proximal 15 mm of core tissue. Tissue injected with pipet tips was collected in a similar manner, but with a No. 5 core borer (10 mm diameter). Sliced tissue was collected to a depth of 2 to 4 mm below the cut surface using a razor blade. All tissue was immediately frozen in liquid nitrogen and stored at −80°C prior to isolation of cell wall and RNA.

Cell Wall Isolation, Protein Determination, and Hydroxyproline Determination

Tuber tissue was homogenized with a Brinkman PT 10/35 tissue homogenizer in 15 mM sodium phosphate (pH 7.0) and centrifuged at 3000*g* for 5 min to pellet cell walls. Crude wall pellets were extracted four times with 1 M NaCl in order to eliminate ionically bound material and then rinsed three times with distilled water. The wall pellets were lyophilized, and 50 mg of this material was digested with α-amlyase for 18 h at room temperature as described by Harris (12), washed five times with water, and lyophilized. Approximately 5 mg of this material was hydrolyzed in 0.4 mL of 6.0 N HCl at 115°C for 90 min. The hydrolysate was reduced to dryness, resuspended in 0.5 mL of distilled water, and centrifuged to remove insoluble material (humin). Hydroxyproline was determined in duplicate on the clear hydrolysate according to the spectrophotometric assay of Drozdz *et al.* (8).

RNA Isolation and RNA Gel Blot Hybridization Analysis

Total cellular RNA was isolated from 8 g samples of frozen tuber tissue by homogenization in a phenol/0.1 M Tris-HCl (pH 9.0) emulsion as described by Haffner *et al.* (10). Total RNA (10 μg) was denatured with formamide/formaldehyde and separated by electrophoresis on 1% agarose gels containing formaldehyde (19). The amount of RNA and the integrity of ribosomal RNA were confirmed by ethidium bromide staining of the gels. Gels were blotted onto nitrocellulose filters, and the filters were prehybridized overnight at 42°C in 50% formaldehyde, 0.1% SDS, 1 × Denhardt's solution, 2 × SSC, and 50 μg/mL denatured salmon sperm (carrier) DNA. Hybridizations were carried out under these same conditions but with the addition of the appropriate nick-translated DNA probe (see below). Approximately 15 ng of DNA probe per mL of hybridization solution was used in each hybridization. Filters were then washed three times with 2 × SSC, 0.1% SDS at 65°C and a final time with 2 × SSC at 65°C before autoradiography. Each RNA gel blot hybridization experiment was carried out twice with identical results.

DNA Probes

DNA clones encoding carrot extensin [pDC5A1] (4) and bean PAL [pPAL5] (9) were isolated from their respective plasmid vectors and labeled by nick-translation with [α-^{32}P] dATP. The extensin and PAL clones were the kind gifts of Drs. Joseph Varner and Christopher Lamb, respectively.

RESULTS

Wounding of potato tubers resulted in a marked accumulation of cell wall hydroxyproline levels in tissue incubated

under aerobic conditions but not under anaerobic conditions (Table I). The greatest accumulation was observed in response to slicing after 24 h of aerobic incubation (155% over time 0 control), whereas accumulations observed in response to bruising and pipet tip wounding were somewhat less marked (120 and 125% over time 0 controls, respectively). In contrast, tubers wounded with pipet tips and incubated anaerobically did not show any marked accumulation of cell wall hydroxyproline content (108% over time 0 control).

RNA isolated from the same tubers used for cell wall hydroxyproline analysis was subjected to gel blot hybridization analysis with extensin and PAL DNA probes (Figs. 1 and 2, respectively). As seen in Figure 1, extensin mRNA levels were barely detectable in unwounded tubers and in tubers immediately after wounding. However, after wounding of any kind and continued aerobic incubation, marked accumulation of at least five extensin mRNA species, 4.5, 3.6, 2.0, 1.8, and 1.3 kb in size, was observed; these RNA species were more clearly resolved in a subsequent experiment (see Fig. 3). This wound-induced accumulation of extensin mRNAs was observed after 8 h but was more pronounced after 24 h of aerobic incubation. In contrast, tuber tissue which was wounded by pipet tip insertion and incubated anaerobically did not accumulate the two larger extensin mRNA species but did accumulate the smaller-size extensin transcripts, albeit to a lesser extent than in the tip-inserted, aerobically incubated tissue.

In an identical RNA gel blot probed with PAL (Fig. 2), wound-induced accumulation of a 2.7 kb transcript was observed in response to slicing and tip insertion but not in response to bruising. PAL mRNA was barely detectable in unwounded tubers and in tubers immediately following wounding but accumulated markedly after aerobic incubation following slicing and tip injection. Accumulation of PAL message also occurred in response to tip insertion followed by anaerobic incubation but to a lesser extent than in the tip inserted, aerobically incubated tissue. In all cases, this accumulation was more pronounced after 24 h of incubation than after only 8 h.

RNA gel blots were subsequently performed on mock-inoculated and *E. carotovora*-infected tuber tissue following wounding and probed with extensin and PAL DNA clones (Figs. 3 and 4, respectively). Unwounded tubers placed in a humidity chamber and mist chamber for 24 h served as controls in these experiments as did the mock-inoculated tubers. In RNA gel blots probed with the extensin clone (Fig. 3), no mRNA hybridization was detected in the two unwounded control tissues. Wound responses in mock-inoculated tuber tissue in this experiment were qualitatively identical to those observed previously (Fig. 1), although the mRNAs were better resolved during electrophoresis in this experiment. Specifically, wounding and mock-inoculation resulted in the accumulation of five extensin mRNA species (4.5, 3.6, 2.0, 1.8, and 1.3 kb) following aerobic incubation, but only the three smaller mRNAs accumulated under anaerobic conditions. Tubers which were bruised and subsequently incubated in a hypoxic environment, the mist chamber, accumulated extensin mRNA in an essentially identical manner to bruised, aerobically incubated tubers. When *E. carotovora* was inoculated at wound sites, there was little difference in the pattern of extensin mRNA expression from the corresponding mock-inoculated tuber tissues with the exception of the bruised and tip-injected, aerobically incubated tubers, which showed some accumulation of all extensin mRNAs after 24 h of infection. In these bacterial infections, as in earlier studies (16, 18), disease resistance (*i.e.* no tissue maceration) was observed in all sliced, bruised, and tip-injected tubers incubated aerobically in the humidity chamber, while susceptibility (*i.e.* extensive tissue maceration in the infected area) was observed after 2 to 4 d in tubers injected with tips and incubated anaerobically, and limited susceptibility (*i.e.* limited tissue maceration in the infected area) was observed after 2 to 4 d in bruised tubers incubated under hypoxic conditions in the mist chamber.

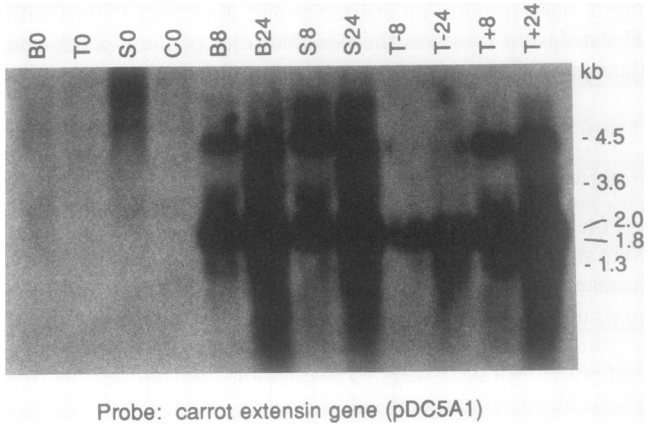

Figure 1. RNA gel blot hybridization of potato RNA extracted from wounded and hypoxically stressed tubers probed with a ^{32}P-labeled extensin gene clone. Total RNA was extracted from tubers 0, 8, and 24 h after bruising (lanes B0, B8, and B24, respectively), slicing (lanes S0, S8, and S24), and injection with yellow pipet tips followed by aerobic incubation (lanes T0, T+8, and T+24) and anaerobic incubation (lanes T-8 and T-24). RNA was also isolated from unmanipulated potato tubers to serve as a control (lane C0). Following gel electrophoresis and transfer to nitrocellulose, these RNAs were hybridized to a ^{32}P-labeled carrot extensin gene clone (pDC5A1). Sizes indicated for the hybridizing RNA species were calculated relative to the mobility of known RNA size standards purchased from Bethesda Research Laboratories.

Table I. *Hydroxyproline Content of the Cell Walls of Wounded Potato Tubers*[a]

Hours[b]	Bruised	Sliced	Tips (Anaerobic)	Tips (Aerobic)
0	0.56	0.62	0.64	0.64
8	0.60 (107)[c]	0.71 (114)	0.69 (108)	0.74 (116)
24	0.67 (120)	0.96 (155)	0.69 (108)	0.80 (125)

[a] Data are presented as μg hydroxyproline/mg dry weight of the cell wall. [b] Hours after wounding. Following wounding, bruised, sliced, and tip-inserted tubers were incubated aerobically in a humidity chamber, while other tip-inserted tubers were incubated anaerobically in anaerobe jars. [c] Numbers in parentheses indicate the % increase over the corresponding 0 h value.

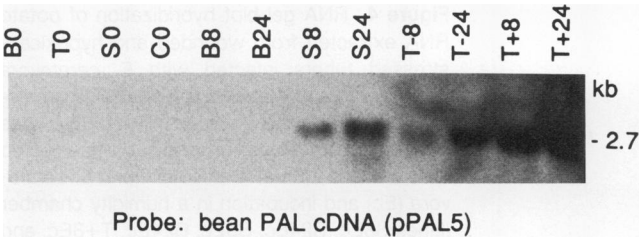

Figure 2. RNA gel blot hybridization of potato RNA extracted from wounded and hypoxically stressed tubers probed with a ^{32}P-labeled phenylalanine ammonia-lyase (PAL) cDNA clone. Total RNA was extracted from tubers 0, 8, and 24 h after bruising (lanes B0, B8, and B24 respectively), slicing (lanes S0, S8, and S24), and injection with yellow pipet tips followed by aerobic incubation (lanes T0, T+8, and T+24) and anaerobic incubation (lanes T-8 and T-24). RNA was also isolated from unmanipulated potato tubers to serve as a control (lane C0). Following gel electrophoresis and transfer to nitrocellulose, these RNAs were hybridized to a ^{32}P-labeled bean PAL cDNA clone (pPAL5). Sizes indicated for the hybridizing RNA species were calculated relative to the mobility of known RNA size standards purchased from Bethesda Research Laboratories.

In identical RNA gel blots probed with the PAL cDNA (Fig. 4), no hybridization was detected in unwounded control tissue nor in any anaerobically incubated tuber tissues. Accumulation of a 2.7 kb PAL transcript, however, was detected in all mock-inoculated, aerobically incubated tubers and in mock-inoculated, hypoxically incubated, bruised tubers. This accumulation was usually maximal at the 24 h time point in these mock-inoculated tissues; however, sliced tubers demonstrated maximal accumulation at the 8 h time point. These results were generally consistent with those seen previously (Fig. 2) except that PAL mRNA was now observed to accumulate in bruised, aerobically incubated tubers. When *E. carotovora* was inoculated at wound sites, further accumulation of PAL mRNA was detected in both the sliced and bruised tissue incubated aerobically and in the bruised tissue incubated hypoxically. PAL mRNA decreased in the tip-inoculated, aerobically incubated tubers with respect to the corresponding mock-inoculated control and remained undetected in the tip-inoculated, anaerobically incubated tissue.

DISCUSSION

Wounding dramatically alters the pattern of gene expression in potato tubers incubated under aerobic or hypoxic environments as demonstrated in our study by changes in extensin and PAL mRNA levels as well as in cell wall hydroxyproline levels, complementing the work of Vayda and Schaeffer (27). Moreover, we find that slicing, bruising, and pipet tip insertion wounds are capable of inducing these alterations. These wound-induced changes include a marked accumulation of five extensin mRNA species, calculated to be 1.3, 1.8, 2.0, 3.6, and 4.5 kb in size, as well as an accumulation of cell wall hydroxyproline, a diagnostic amino acid for extensin. Similarly, a 2.7 kb PAL mRNA also accumulates in response to wounding, consistent with the observation by Vayda and Schaeffer (27) that a 78 kD PAL polypeptide accumulates in potato tubers following wounding (pipet tip insertion) and aerobic incubation. Given that extensin and PAL transcripts have been found to accumulate in carrot roots (4), tomato stems (24), and bean hypocotyls (6, 14) in response to slicing, our findings were expected but were dependent upon the successful cross-hybridization of the heterologous probes to specific potato RNA species. Clearly, these heterologous probes are capable of recognizing specific

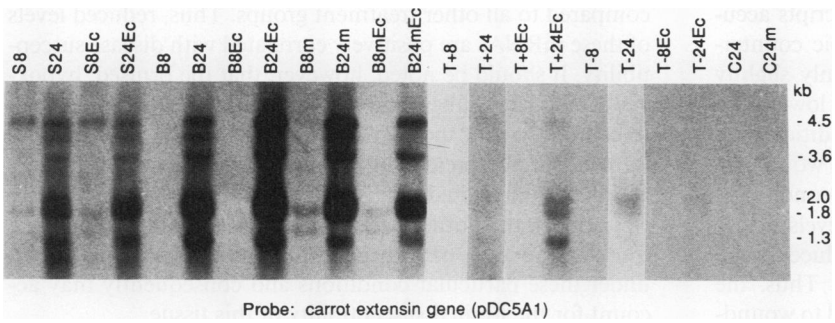

Figure 3. RNA get blot hybridization of potato RNA extracted from wounded and hypoxically stressed tubers infected with *Erwinia carotovora* probed with a ^{32}P-labeled extensin gene clone. Total RNA was extracted from sliced, bruised, and tip-injected tubers 8 and 24 h after infection with *E. caratovora* (Ec) and incubation in a humidity chamber (lanes S8Ec, S24Ec, B8Ec, B24Ec, T+8Ec, and T+24Ec respectively), a mist chamber (lanes B8mEc and B24mEc), and anaerobe jars (lanes T-8Ec and T-24Ec). RNA was also extracted from the corresponding mock-inoculated (*i.e.* sterile distilled water was substituted for *E. caratovora* inoculum) tubers (lanes S8, S24, B8, B24, T+8, T+24, B8m, B24m, T-8, and T-24). RNA extracted from unmanipulated potato tubers incubated in the humidity and mist chambers for 24 h served as controls (lanes C24 and C24m, respectively). Following gel electrophoresis and transfer to nitrocellulose, these RNAs were hybridized to a ^{32}P-labeled carrot extensin gene clone (pCD5A1). Sizes indicated for the hybridizing RNA species were calculated relative to the mobility of known RNA size standards purchased from Bethesda Research Laboratories.

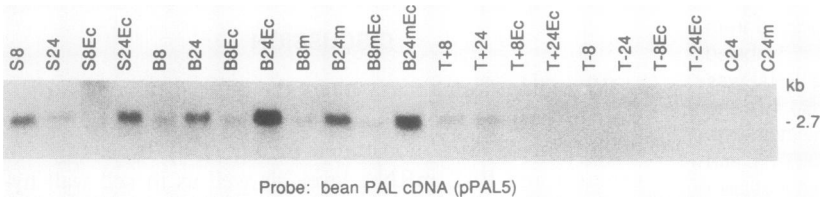

Figure 4. RNA gel blot hybridization of potato RNA extracted from wounded and hypoxically stressed tubers infected with *E. carotovora* probed with a ^{32}P-labeled phenylalanine ammonia-lyase (PAL) cDNA clone. Total RNA was extracted from sliced, bruised, and tip-injected tubers 8 and 24 h after infection with *E. caratovora* (Ec) and incubation in a humidity chamber (lanes S8Ec, S24Ec, B8Ec, B24Ec, T+8Ec, and T+24Ec respectively), a mist chamber (lanes B8mEc and B24mEc), and anaerobe jars (lanes T-8Ec and T-24Ec). RNA was also extracted from the corresponding mock-inoculated (*i.e.* sterile distilled water was substituted for *E. caratovora* inoculum) tubers (lanes S8, S24, B8, B24, T+8, T+24, B8m, B24m, T-8, and T-24). RNA extracted from unmanipulated potato tubers incubated in the humidity and mist chambers for 24 h served as controls (lanes C24 and C24m, respectively). Following gel electrophoresis and transfer to nitrocellulose, these RNAs were hybridized to a ^{32}P-labeled bean PAL cDNA clone (pPAL5). Sizes indicated for the hybridizing RNA species were calculated relative to the mobility of known RNA size standards purchased from Bethesda Research Laboratories.

potato RNAs having properties consistent with those of known extensin and PAL mRNA species. Specifically, the multiple extensin transcripts observed in this study are analogous to the multiple extensin transcripts previously found to accumulate in carrot (4), tomato (24), and bean (6) and are apparently generated in the same manner. In tomato and bean, these multiple transcripts result from the expression of multiple extensin genes; whereas, in carrot, such mRNA polymorphism is due, at least in part, to the utilization of multiple transcription initiation sites. Likewise, the single 2.7 kb PAL mRNA observed in this study is apparently analogous to the single 2.5 kb PAL transcript previously detected in bean (9).

When wounded tuber tissue is incubated under anaerobic conditions instead of aerobic or our hypoxic conditions, the pattern of gene expression is extremely different. Specifically, only the three smallest of the five extensin transcripts accumulate, albeit to a lesser extent than their aerobic counterparts, and cell wall hydroxyproline levels rise only slightly above control levels. PAL mRNA levels are much lower than their aerobic counterparts. Thus, anaerobic conditions are sufficient to eliminate or, at least, diminish the wound-induced responses which we observed under aerobic conditions. This reduction in extensin and PAL mRNA levels under anaerobic conditions is likely the result of reduced gene transcription and/or increased mRNA turnover. Thus, the picture which emerges is that potato tubers respond to wounding under aerobic conditions by the accumulation of a set of proteins, probably caused in large part through increased mRNA levels encoding these proteins. However, anaerobiosis eliminates this wound response, through a reprogramming of tuber metabolism resulting in the accumulation of only a set of anaerobically induced proteins. Recently, Butler *et al.* (3) have found that mRNAs encoding alcohol dehydrogenase and aldolase, two glycolytic enzymes, accumulate in potato tubers in response to anaerobiosis, regardless of whether or not wounds (pipet tip injection) are inflicted. Apparently, there is a hierarchy of stresses, and anaerobiosis supersedes wounding as the tuber attempts to meet minimum energy needs in lieu of inducing the synthesis of proteins involved with defense and wound repair.

Of all the treatments used in this study, the tip-injected, anaerobically incubated tubers are the most susceptible to bacterial soft rot (16); this was also found to be the case in these experiments with maceration occurring 2 to 4 d after inoculation with *E. carotovora.* As mentioned already, tip-injected, anaerobically incubated tubers had the lowest levels of extensin (particularly the two largest transcripts) and PAL mRNAs as well as the lowest levels of cell wall hydroxyproline compared to all other treatment groups. Thus, reduced levels of these mRNAs are positively correlated with disease susceptibility. It should be noted, however, that the bruised, hypoxically incubated tubers showed some limited susceptibility to infection, despite the fact that extensin and PAL mRNAs were essentially present in amounts identical to the bruised, aerobically incubated tubers which showed resistance. Hence, we suspect that other wound-response proteins which are more sensitive to oxygen conditions may not be expressed under these particular conditions and consequently may account for the limited susceptibility of this tissue.

In a number of plant-pathogen systems, where wounding is not a prerequisite for infection, the infection process itself can induce a plant defense response. For example, when the fungus *Colletotrichum lindemuthianum*, causal agent of anthracnose, infects bean plants, it does not require a wound site and yet causes the accumulation of extensin, PAL, chal-

cone synthase, and a number of other mRNAs (13). To determine whether *E. carotovora* causes any changes in potato gene expression in addition to those observed in response to wounding and incubation conditions, we examined extensin and PAL mRNA levels in mock-inoculated and infected tubers. While none of the wounded, anaerobically incubated tissues demonstrated changes upon infection in their characteristically low or undetectable levels of these mRNAs, five of the eight wounded, aerobically or hypoxically incubated, infected tuber tissues did show some further accumulation of extensin and PAL mRNAs, but no consistent pattern emerged and no novel sized transcripts appeared.

In conclusion, extensin and PAL may contribute to disease resistance in potato tubers through their respective actions as structural cell wall proteins (25) and the key enzyme responsible for the production of the polyphenolic constituents of phytoalexins and lignin as suggested for other plant-pathogen interactions (13). In this context, the polymorphism exhibited by the extensin mRNAs detected in the various treatments used in this study may reflect regulatory and structural differences associated with specific functions of the corresponding proteins in defense and wound healing. Clearly, extensin and PAL constitute only a subset of wound-induced proteins in potato tubers (15, 26, 27), and it is likely that they confer disease resistance only by acting in concert with other wound-induced gene products and possibly with preexisting tuber defense proteins such as proteinase inhibitor II (22). In this sense, it will be important to determine how wounding and infection activate these and other putative defense genes, since an understanding of the activation mechanism(s) may allow for the manipulation of these genes so as to enhance the expression of disease resistance.

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