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Activation of plant defense responses and sugar efflux by expression of pyruvate decarboxylase in potato leaves

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Summary

When plants are infected with avirulent pathogens, a selected group of plant cells rapidly die in a process commonly called the hypersensitive response (HR). Some mutations and overexpression of some unrelated genes mimic the HR lesion and associated defense responses. In all of these situations, a genetically programmed cell death pathway is activated wherein the cell actively participates in killing itself. Here we report a developmentally and environmentally regulated HR-like cell death in potato leaves constitutively expressing bacterial pyruvate decarboxylase (PDC). Lesions first appeared on the tip of fully expanded source leaves. Lesion formation was accompanied by activation of multiple defense responses and resulted in a significant resistance to *Phytophthora infestans*. The transgenic plants showed a five- to 12-fold increase in leaf tissue acetaldehyde and exported two- to 10-fold higher amounts of sucrose compared to the wild-type. When plants were grown at a higher temperature, both the lesion phenotype and sucrose export were restored to wild-type situations. The reduced levels of acetaldehyde at the elevated temperature suggested that the interplay of acetaldehyde with environmental and physiological factors is the inducer of lesion development. We propose that sugar metabolism plays a crucial role in the execution of cell death programs in plants.

Introduction

The plant's defense response to incompatible pathogen interactions is often manifested as rapid localized host cell death, termed the hypersensitive response (HR). HR is thought to contribute to the containment of the pathogen and is associated with most but not all incompatible host–pathogen interactions and disease resistance (Dangl *et al.*, 1996; Hammond-Kosack and Jones, 1996). During the HR, a battery of defense reactions are initiated by the plant, and include a burst of reactive oxygen species (Lamb and Dixon, 1997; Levine *et al.*, 1994; Pennell and Lamb, 1997); ion fluxes and activation of H^+/K^+ exchange (Atkinson *et al.*, 1990); callose and lignin deposition and cell wall cross-linking (Bradley *et al.*, 1992; Brisson *et al.*, 1994); lipid peroxidation (Keppler and Baker, 1989); production of antimicrobial phytoalexins (Glazebrook and Ausubel, 1994; Osbourn, 1996); induction of a repertoire of pathogenesis-related (PR) proteins (Bowles, 1990); and disease resistance (see Bent, 1996; Hammond-Kosack and Jones, 1996; Ryals *et al.*, 1996 for reviews). The mechanism of HR is not clear, but it is an active process and involves new transcription and translation by the host (reviewed in Dangl *et al.*, 1996; Greenberg, 1996). Purified bacterial elicitors can induce HR cell death and disease resistance response when applied locally to a plant (He *et al.*, 1993), indicating that HR is a pre-set genetic program that can be activated by external factors. The isolation of maize, *Arabidopsis,* tomato and barley mutants that exhibit HR-like lesions in the absence of a pathogen or an elicitor is strong evidence that cell death during the HR is genetically programmed (Büschges *et al.*, 1997; Dietrich *et al.*, 1994; Greenberg *et al.*, 1994; Walbot *et al.*, 1983).

In a number of the lesion mimic mutants, the onset of lesion formation is subject to developmental and environmental changes (Dietrich *et al.*, 1994; Walbot *et al.*, 1983). It has been argued that alteration of cellular homeostasis in such mutants may be misinterpreted by host cells as pathogen infection (Dietrich *et al.*, 1994; Mittler *et al.*, 1995). The connection of altered cellular homeostasis with the activation of programmed cell death is also supported by experiments in which the ectopic expression of unrelated proteins caused a lesion phenotype. For example, manipulation of the ubiquitin-dependent protein degradation system (Becker *et al.*, 1993), expression of the bacterio-opsin (bO) proton pump in tobacco (Mittler *et al.*, 1995) and potato (Abad *et al.*, 1997), and targeting yeast invertase to the apoplast and vacuole of tobacco (Herbers *et al.*, 1996a) were shown to cause the lesion mimic phenotype. In these examples, not only lesion formation but also the biochemical markers and disease resistance responses were typical of incompatible host–pathogen interactions and HR cell death.

In early experiments, pathogen infection was shown to increase tissue sugar levels (Hall and Loomis, 1972; Watson and Watson, 1951), and this increase was correlated with resistance to pathogen attack (Horsfall and Dimond, 1957).

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Sugar modulated gene expression in plants is thought to be an adaptive response to developmental and environmental changes (see Koch, 1996 for review). The activation or repression of gene expression by sugars is highly selective and involves the use of a sugar-hexokinase complex as a signal (Jang and Sheen, 1994; Jang *et al.*, 1997). Among the sugar inducible genes are a number of the pathogenesis-related proteins (Herbers *et al.*, 1995; 1996b; Johnson and Ryan, 1990; Tsukaya *et al.*, 1991). Obviously, pathogen infection is likely to result in perturbed metabolism and it is plausible, therefore, that plant cells may perceive a metabolic imbalance as intruder-induced disturbance and initiate a cascade of defense reactions in the absence of a pathogen.

Here we report the occurrence of transgene-induced, developmentally regulated and temperature sensitive HR-like lesion formation and disease resistance in potato leaves. We introduced a pyruvate decarboxylase (PDC) protein from the obligate anaerobe *Zymomonas mobilis* into potato. PDC catalyzes the decarboxylation of pyruvate to acetaldehyde and CO_2, and is the first of two enzymes specifically required for ethanolic fermentation. Acetaldehyde is then converted to ethanol by the action of alcohol dehydrogenase (ADH) with the concomitant oxidation of NADH to NAD^+. Transgenic potatoes show a necrotic lesion phenotype on their source leaves that closely correlates with the level of transgene expression. We show that cell death is developmentally and environmentally regulated, and that it is an active process fully comparable to the previously described lesion mimic mutants. Furthermore, the drastic effect on sugar export suggests a link between carbohydrate metabolism and disease susceptibility.

Results

Expression of ZymPDC leads to a lesion mimic phenotype in potato leaves

The *Pdc* gene from the obligate anaerobe *Zymomonas mobilis* was inserted between the alfalfa mosaic virus (AMV) translational enhancer and the nos terminator under the control of the 35S promoter (Bucher *et al.*, 1994). Potato plants (*Solanum tuberosum* var. Désirée) were transformed using the *Agrobacterium* system. Transgene expression was variable and four lines accumulating the PDC protein to different levels (Figure 1a) were maintained in tissue culture by clonal propagation. The *in vitro* PDC enzymatic activity correlated with the level of protein accumulation, and showed more than a sixfold increase in the case of highest expression in line 8 (L-8) as compared to the wild-type (Figure 1b).

The transgenic plants displayed a lesion mimic phenotype, the severity of which correlated with the expression level of the *Zym*PDC protein, the highest expressors showing the most extensive lesions (Figure 2a). Lesions began to develop about 4 weeks after planting in soil. In most cases, the lesion started at the tip of source leaves near the midrib as small brownish spots, and spread in all directions, primarily along the midrib and the veins (Figure 2b). Within 3–5 days of the start of lesion formation, the lesion encompassed most of the leaf area leading to a dry, grey to brownish, shrunken appearance. Finally, the whole leaf collapsed and abscised. The lesion continued to the next fully expanded source leaf and proceeded to the one above even before the complete collapse of the one below it. The timing and progression of the lesion showed dependence on the level of the transgene expression. The lines that expressed the PDC protein at highest levels were the first to show the lesion appearance. The difference in symptom appearance between the highest expressor (L-8) and the lowest expressors (L-21 and L-17) was at least 6–10 days. The progression of the lesion was also dependent on the PDC protein levels. In L-8 and L-25, the lesion spread was rapid and uncontrolled, leading to the death of the entire plant within 2–3 weeks of the onset of lesion formation. In L-17 and L-21, the lesions were localized, spread slowly to consume the entire leaf but remained restricted to only a few source leaves, and never reached the top of the plant (Figure 2a). The transgenic plants were not reduced in height before lesion formation, and L-17 and L-21 grew to a similar height as the wild-type even after lesion development.

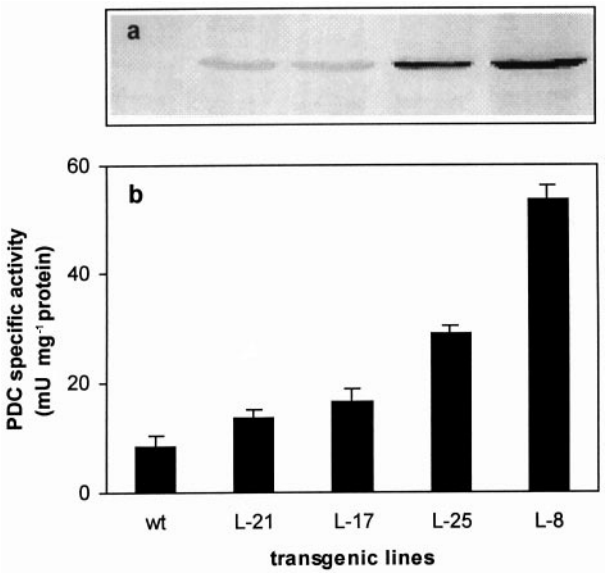

Figure 1. *Zymomonas* PDC expression and *in vitro* enzymatic activity.
(a) *Zymomonas* PDC protein expression in potato leaves. Twenty micrograms of total soluble protein were loaded per lane and probed with anti-*Zymomonas* PDC antibody.
(b) PDC enzymatic activity. PDC activity was measured in the leaf total soluble protein extract in an ADH coupled spectrophotometric assay. One unit is defined as the conversion of 1 μmol of pyruvate into acetaldehyde per minute. Values are the mean ± SE of six measurements. L, transgenic line; wt, wild-type.

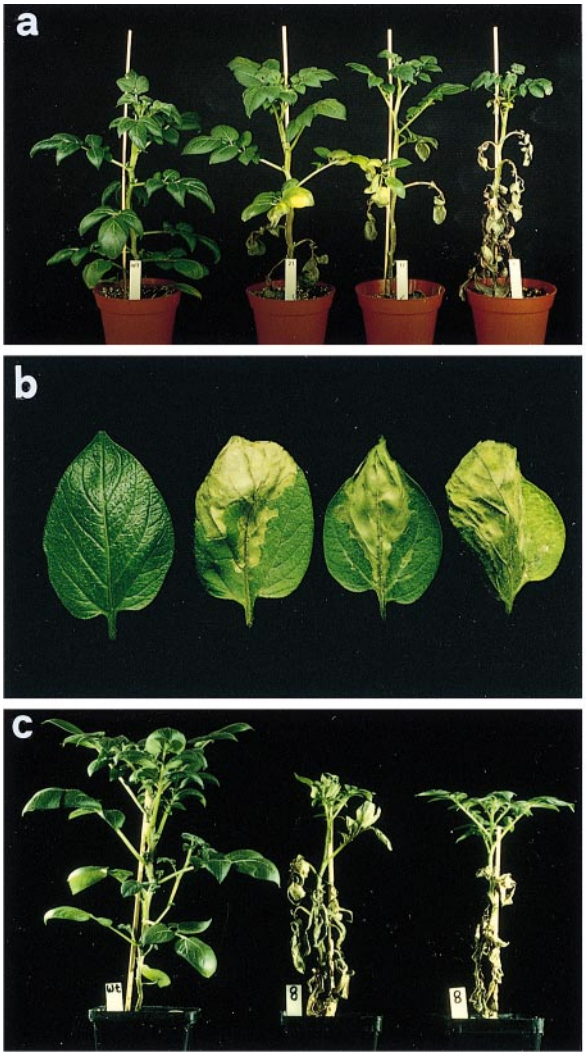

Figure 2. Phenotype of *Zymomonas* PDC expressing potatoes.
(a) Six-week-old transgenic plants grown at 18 ± 3°C. In both (a) and (b) plants from left to right are wt, L-21, L-17, and L-8.
(b) Extensive lesions on leaves from plants grown at 18 ± 3°C.
(c) Seven-week-old plants, wt (left) and L-8 (middle), grown continuously at 18 ± 3°C for 7 weeks; L-8 (right), grown at 18 ± 3°C for 6 weeks and transferred to 25 ± 2°C for 10 days. Note the emergence of new healthy leaves.

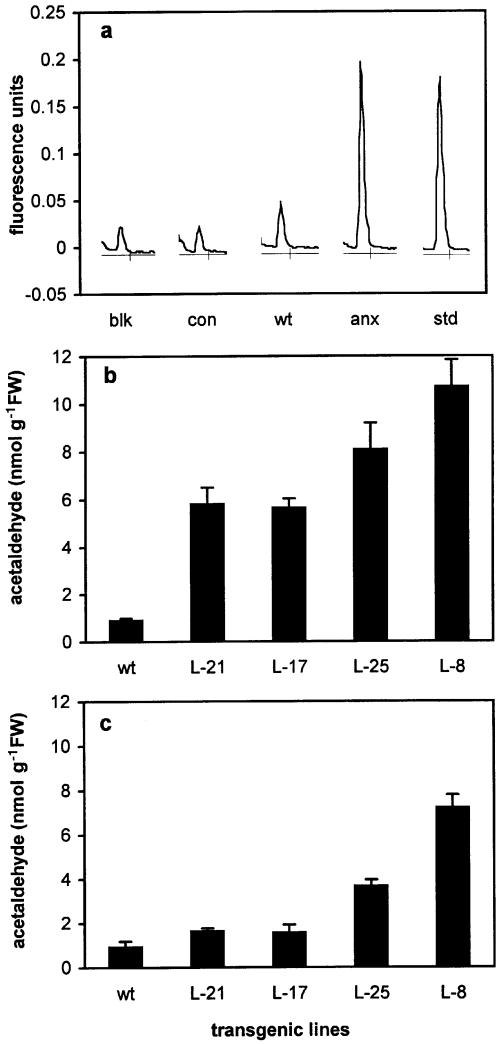

Figure 3. Acetaldehyde concentration in potato leaf tissue.
(a) Chromatograms of control experiments. blk, water blank; con, control in which the wild-type leaf extract was incubated at 37°C for 1 h; wt, wild-type leaf untreated; anx, wild-type leaf incubated under anoxic conditions for 2 h followed by 10 min in air; std, acetaldehyde standard (1 μM).
(b) Acetaldehyde from plant leaves grown at 18 ± 3°C.
(c) Acetaldehyde from plant leaves grown at 25 ± 2°C. Acetaldehyde in a perchloric acid extract of 4-week-old leaves was measured as a fluorescent adduct with 1,3-cyclohexanedione by HPLC. Values represent the mean ± SE of six independent measurements.

These phenotypes were reproducible in all seasons when potatoes were grown at a temperature of 18 ± 3°C in greenhouses or growth rooms at different locations in Europe (Berlin, Berne and Zürich). However, when plants were grown at a temperature of 25 ± 2°C, the lesion phenotype was masked and all the transgenics grew to full maturity (data not shown). Occasionally, localized lesions appeared on one or two leaves of L-8, but these were weaker in magnitude than the lesions of L-17 and L-21 at 18°C, and in most cases did not spread to engulf the entire leaf. When plants were transferred from 18 to 25°C after lesion development, further lesion progression stopped and the transgenics resumed normal growth. Even the highest expressor, severely affected L-8, fully recovered when transferred to 25°C. In this case, the recovery required 1–2 days, and newly developing leaves and shoots could be observed within 1 week (Figure 2c). When plants that had been maintained at 25°C for 4 weeks were transferred to the 18°C growth condition, the lesion started within 2–3 days in the case of L-8 and L-25, and after 1 week in the case of L-17 and L-21 (data not shown). High humidity and too frequent watering shortened the time required for lesion initiation at 18°C, and led to localized lesion formation at 25°C in L-8 and L-25. These lesions could spread

Figure 4. Delay of lesion formation by spraying with PDC inhibitor.
One set of line 8 plants were sprayed with glyoxylate (a PDC inhibitor) and a second set of plants were sprayed once every day with water for controls. After 7 days at 18°C, the 6th leaf from each of these plants is shown. Upper panel, controls sprayed with water; lower panel, sprayed with 100 mM glyoxylate.

within the leaf and resembled those of L-17 and L-21 when grown at 18°C.

Transgenic potatoes accumulate higher levels of acetaldehyde in their leaf tissue

PDC catalyzes the first step in ethanolic fermentation, a decarboxylation of pyruvate yielding acetaldehyde and CO_2. In wild-type leaves, ethanolic fermentation occurs only during oxygen limitation and some other stresses. Constitutive high level expression of bacterial PDC in tobacco leaves did not lead to measurable acetaldehyde production in the presence of oxygen as determined by gas chromatography (Bucher *et al.*, 1994). The techniques for the detection of acetaldehyde in plants have been restricted to gas chromatography and enzymatic methods, both of which are unable to detect low level tissue concentrations. Since the transgenic potato plants analysed here developed lesions under normoxic conditions, and the severity of the lesion correlated with the level of transgene expression, we wished to determine whether acetaldehyde might be produced at levels too low to be detected by conventional methods. We adapted a sensitive high-performance liquid chromatography (HPLC) method (Helander *et al.*, 1993), in which acetaldehyde is measured as a fluorescent adduct with 1,3-cyclohexanedione and ammonium ion (see Experimental procedures). This method enabled us to measure low levels of acetaldehyde in actively respiring normoxic potato leaves.

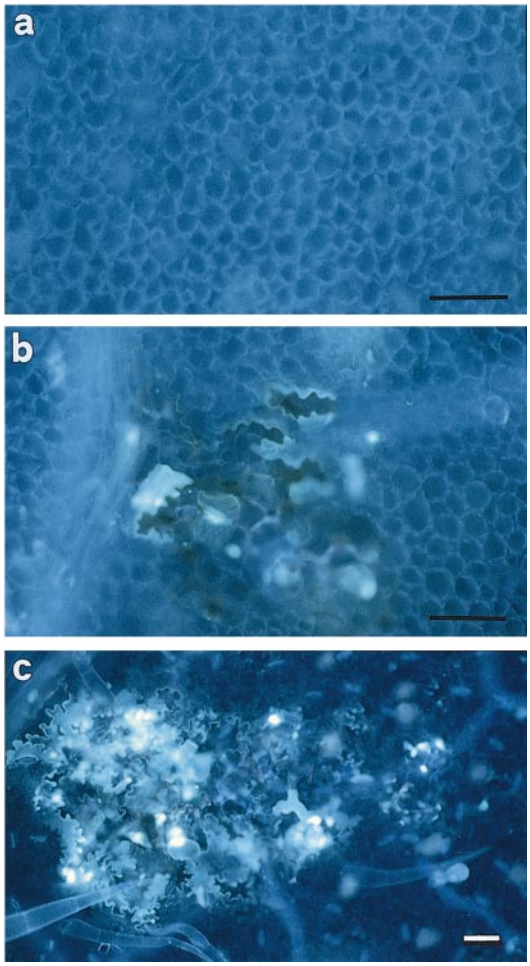

Figure 5. Callose deposition in transgenic potato leaves.
Four-week-old leaves from lines 25 and 8 were stained with aniline blue and viewed under UV light (bar = 100 μm).
(a) Wild-type.
(b) Line 25.
(c) Line 8.

Figure 3(a) shows chromatograms of control experiments used to authenticate the assay conditions. In the water blank and wild-type leaf extract incubated at 37°C for 1 h, a small background peak appeared which has the same retention time as the acetaldehyde adduct. A similar background peak was observed by Helander *et al.* (1993). As positive controls, wild-type potato leaves were incubated under anoxia, in which more than a 10-fold increase in acetaldehyde peak area was observed compared to the untreated wild-type (Figure 3a). In wild-type potato leaves a low but significant amount of acetaldehyde was measured at both 18 and 25°C growing conditions (Figure 3b,c). When the transgenics were grown at 18°C, a five- to 12-fold increase in acetaldehyde levels was measured compared to the wild-type (Figure 3b). This increase in tissue acetaldehyde content was related to the level of PDC protein, the highest being detected in L-8

(Figure 3b). At 25°C the level of acetaldehyde was drastically reduced in all the transgenics, only in L-25 and L-8 were the values significantly higher than in the wild-type (Figure 3c). A comparison between acetaldehyde levels at 18 and 25°C showed that there was no absolute correlation between acetaldehyde levels and lesion formation. For instance, L-21 at 18°C and L-8 at 25°C have comparable acetaldehyde levels, yet L-21 developed severe symptoms at 18°C, whereas L-8 at 25°C was virtually unaffected. This clearly indicates that, in addition to acetaldehyde concentration, other physiological and environmental factors must contribute to lesion formation.

Two experiments were carried out to ascertain that the observed phenotype was caused by the enzymatic activity of PDC and not by some structural determinant of the foreign protein. First, when line 8 transgenics were sprayed with the PDC inhibitor glyoxylate, lesion formation and spread were significantly delayed compared with the untreated controls (Figure 4). Thus, inhibition of PDC activity inhibits symptom development. Second, wild-type potato plants were incubated under intermittent anoxia, a treatment known to lead to a surge in acetaldehyde production (Zuckermann *et al.*, 1997). When plants were returned to aerobic condition at 18°C, the characteristic lesions developed on leaves after 2 days (data not shown). No lesion formation was observed at 25°C. This shows that the lesion phenotype can be reproduced by acetaldehyde production in wild-type plants.

Lesion formation is accompanied by induction of the plant defense response

Since the lesions resembled those occurring during pathogen infection, we were interested to determine whether other classical defense reactions were initiated in transgenic potatoes. Callose deposition and accumulation of autofluorescent phenolic compounds are markers associated with lesion formation during the HR (Koga *et al.*, 1980), in lesion mimic mutants (Dietrich *et al.*, 1994), and in lesion mimic transgenics (Herbers *et al.*, 1996a). Although callose deposition was not an exclusive marker of HR, it represents one of the earliest plant defense responses (Bradley *et al.*, 1992). When 4-week-old leaves were stained with aniline blue and examined under UV-light, a fluorescent material became evident in the transgenics. The intensity of the fluorescence was stronger in the higher expressors of PDC protein and the highest deposition of callose was observed within and around the lesion area (Figure 5). In the area surrounding some lesions, a strong signal was found in the cells bordering the veins, while in the wild-type no signal could be detected.

Callose deposition and cell wall cross-linking are probably general responses meant to limit pathogen spread. In response to pathogens, plants also induce specific antifungal component enzymes such as β-1,3-glucanases and chitinases (Mauch *et al.*, 1988; Zhu *et al.*, 1994), also known as pathogenesis-related (PR) proteins. The mRNA levels of three typical potato PR genes (gst1, glucanase and chitinase) were increased by eight- to 45-fold in the transgenic line 25 compared to the wild-type (Figure 6). Lesion formation enhanced the accumulation of the mRNAs, but healthy leaves also showed significantly higher (2.5- to 20-fold) levels of PR gene induction as compared to the wild-type (Figure 6). These results show that the observed cell death is not a passive process, but is associated with activation of multiple defense responses.

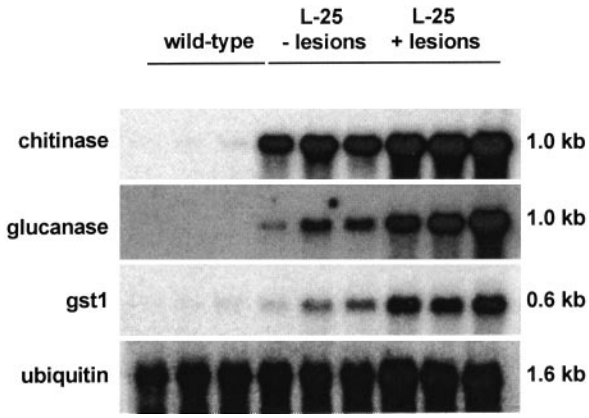

Figure 6. Accumulation of PR gene transcripts in transgenic potato leaves. Total RNA (10 μg) from healthy (– lesion) or lesioned (+ lesion) leaves of line 25 was loaded per lane and probed with specific cDNA probes indicated on the left side. Ubiquitin is a control for equal loading. Sizes are indicated on the right.

Lesion formation is associated with major changes in sugar metabolism

The fact that the lesion phenotype starts in the fully expanded source leaves and progresses towards the sink suggests that sugar metabolism might be involved in the process. Moreover, since the increased acetaldehyde production may divert a considerable amount of pyruvate from the respiratory pathway, imbalance in sugar metabolism might be expected. We therefore measured the sugar content in the fully expanded source leaves at 18°C. Figure 7 shows the amount of soluble sugars and starch at the initiation of lesion formation. A dramatic decrease of starch content in the highest PDC expressors, L-25 and L-8, was found compared to the untransformed wild-type (Figure 7a). The soluble sugars, on the other hand, were found to be somewhat elevated in the transgenics compared to the wild-type (Figure 7a). However, this increase was not consistent with the level of transgene expression and was insufficient to account for the drastic drop in starch content.

Since the decrease in starch content was not fully

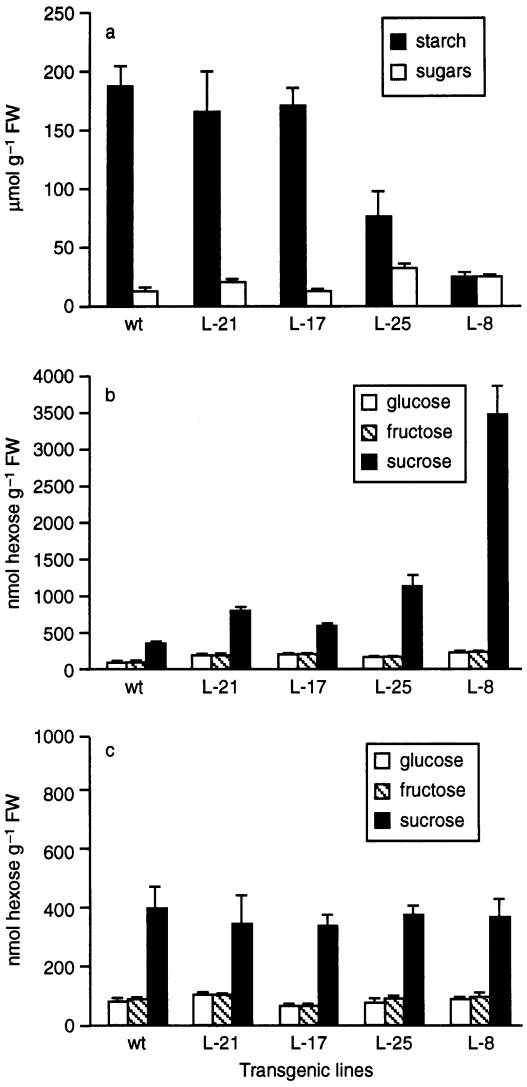

Figure 7. Soluble sugars and starch in potato leaves.
(a) Concentration of starch and soluble sugars in the leaf tissue from plants grown at 18 ± 3°C.
(b) Concentration of soluble sugars in the petiole exudates of plants grown at 18 ± 3°C.
(c) Concentration of soluble sugars in the petiole exudates of plants grown at 25 ± 2°C. Exudates were collected from cut petioles for 8 h under the same conditions as plant growth. Values represent the mean ± SE of five measurements of individual plants.

compensated by a corresponding increase in soluble sugar levels, we suspected that sugars might be exported from the leaf. Fully expanded source leaves were cut from the base of their petiole, placed in an EDTA solution for 8 h, and soluble sugars were measured in the exudate. At this time the leaves looked completely healthy. The exported sucrose increased by approximately two- to 10-fold in the transgenics compared to the wild-type (Figure 7b), suggesting that most of the mobilized starch was transported out of the tissue in the form of sucrose. Although glucose and fructose were slightly elevated in the transgenics compared to the wild-type, the values remained low and comparable in all transgenics (Figure 7b). Thus, transport is specific for sucrose and unlikely to be a passive response of collapsing and dying cells. Petiole exudates from plants grown at 25°C showed no difference between the transgenics and the wild-type in sucrose export (Figure 7c). It is not known why sucrose should be exported in the transgenics, but the conversion of starch into sucrose and its correlation with the PDC expression suggests that sucrose translocation is a component of the developmentally regulated cell death initiation and execution process.

Transgenic potatoes are resistant to Phytophthora infestans

Some of the lesion mimic mutants such as the *lsd1* and *acd2* of *Arabidopsis* (Dietrich *et al.*, 1994; Greenberg *et al.*, 1994), and transgenic tobacco expressing yeast invertase and bacterio-opsin proton pump (Herbers *et al.*, 1996a; Mittler *et al.*, 1995) have been shown to exhibit resistance to virulent pathogens. The results described here suggest that the transgenic potatoes behave in the same way as the lesion mimics, and probably operate by activating the same or a similar genetically programmed cell death pathway. We assessed the response of the PDC transgenics to a virulent fungal inoculation to determine whether the transgenics exhibit disease resistance as well.

Transgenic and wild-type potato leaves were inoculated with the fungal pathogen *Phytophthora infestans,* causative agent of late blight disease, to which the wild-type Désirée variety is susceptible. Both the transgenic and wild-type leaves were completely healthy at the time of inoculation. Lesions spread faster in the transgenics than in the wild-type, the rate of lesion propagation correlating with the level of PDC expression. Spread of the pathogen was assessed 6 days after inoculation. More than 90% of the L-8 leaf area was covered by lesions, whereas in L-25 the proportion of the leaf area covered by the lesions was about 60%. In L-17 leaves, the four inoculation spots could be distinguished but were more irregular and diffuse than in the wild-type (Figure 8b). In the wild-type leaves, the lesion spread slowly covering less than 20% of the total area and the lesions appeared as four distinct areas well separated from one another. On the other hand, the mock inoculated L-25 and wild-type controls showed no lesion at all, although L-8 did show some lesion formation (data not shown), as is expected for this high expressor line. However, microscopic counting of fungal sporangia on the leaves after 6 days of infection showed that the transgenics considerably impaired the fungal propagation. In the transgenics, a 10- to 300-fold decrease in the number of sporangia was found compared to the wild-type (Figure 8a). This indicates that the wild-type is more susceptible to infection than any of the transgenics.

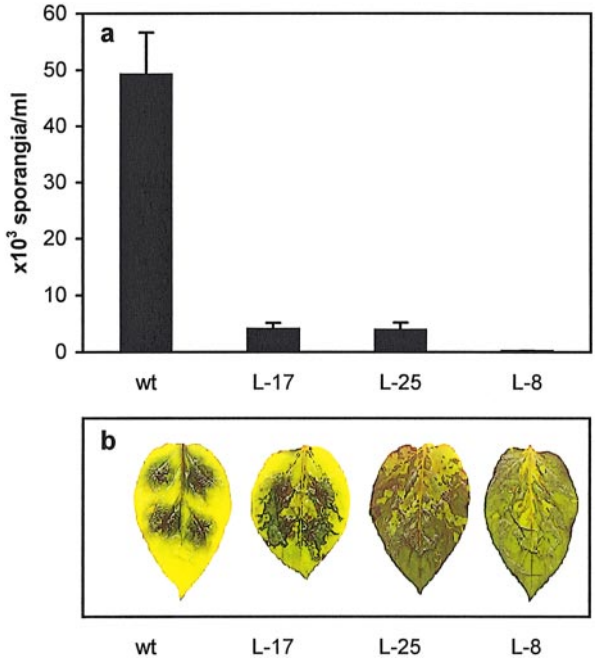

Figure 8. Resistance test to the fungus *Phytophthora infestans*.
(a) Sporulation efficiency 6 days post-infection. Values represent the mean ± SE of three leaves of eight individual plants for each line.
(b) Morphology of the infected leaves.

Discussion

We described bacterial PDC expressing potato plants which exhibit a lesion mimic phenotype. The lesions started to develop at the tip of fully expanded source leaves and spread rapidly in all directions, primarily along the midrib and veins. Lesion formation was developmentally and environmentally controlled. At 18°C, the lesions developed 4 weeks after planting in soil, but this phenotype was completely masked by growth at 25°C (Figure 2). The lesions described here resemble the propagation class lesion mimic mutants, such as the *lls1* of maize (Gray *et al.*, 1997), and the *acd1* and *acd2* of *Arabidopsis* (Greenberg *et al.*, 1994) in the sense that lesion spread was uncontrolled once initiated in responsive cells. The transgenics produced five- to 12-fold higher levels of acetaldehyde (Figure 3), and exported two- to 10-fold more sucrose from the leaves compared to the wild-type (Figure 7b). Starch content, on the other hand, was drastically reduced in the high level PDC expressing potatoes (Figure 7a). The existence of a developmental component in the process and the export of sugars out of the source leaves resembles a senescence response, but it is not clear if the development of this phenotype has any connection with senescence. Analysis of physiological and molecular markers of hypersensitive cell death revealed that defensive reactions similar to the HR were initiated in the potato transgenics. These included deposition of callose in the leaf tissue (Figure 5), high level induction of PR protein encoding transcripts (Figure 6), and increased resistance to *P. infestans* (Figure 8). Taken together, our results suggest that the PDC expressing potatoes activate the same or a similar programmed cell death pathway as do the lesion mimic mutants and/or lesion mimic transgenics.

How could overexpression of PDC initiate a hypersensitive response? We envisage three possibilities. First, lesion is caused by a foreign bacterial protein. Second, that the response is triggered by an interaction of acetaldehyde with a component of the cell death machinery. Third, that the action of PDC leads to an imbalance in carbohydrate metabolism. In the first scenario, *Zymomonas* PDC as a foreign protein could cause lesion formation independent of its enzymatic function. Although we don't have a precedent for developmentally regulated temperature-sensitive lesions caused by the foreignness of a protein, we cannot rule this possibility out. However, lesion formation could be activated by prolonged production of acetaldehyde in wild-type leaves at 18°C, and lesion formation on transgenic leaves is delayed by inhibiting PDC activity (Figure 4). Thus, the foreignness of ZymPDC as the trigger of lesion appears to be unlikely.

In the second scenario, the physical and chemical properties of acetaldehyde will determine its interaction with endogenous molecules. Fortunately, acetaldehyde has been intensely studied because it is produced after ethanol consumption in humans. It binds to proteins and nucleic acids, and causes sister chromatid exchange and mutation in mammalian cells (Dellarco, 1988; He and Lambert, 1990; Stevens *et al.*, 1981). Treatment of human cells with ethanol leads to an increase of acetaldehyde which is detoxified through conjugation with glutathione. Depletion of cellular glutathione (GSH), the major endogenous antioxidant, appears to be one of the early metabolic alterations that form part of the animal apoptotic process (Macho *et al.*, 1997). Human cells treated with low levels of acetaldehyde showed a 95% reduction in GSH content (Olivares *et al.*, 1997). Because acetaldehyde binds very well to GSH, this binding could directly cause depletion of GSH in the PDC-transgenic plants. The observation that acetaldehyde accumulated to higher levels in the transgenics grown at 18°C compared to the wild-type (Figure 3b) and the fact that lesion spread closely correlates with acetaldehyde levels at 18°C support this view. However, the absence of severe lesion at 25°C in L-8 while the acetaldehyde levels are still high points to the importance of temperature in the execution of the whole program. This means that if acetaldehyde is the cause, it is the interaction with environmental and physiological factors that is important and not its mere toxicity like excessive environmental insults.

In the third scenario, the unbalanced biochemical state of the cell imposed by transgene expression may be the trigger common to all lesion phenotypes. This model is

supported by the transgenic approach of Mittler *et al*. (1995) and Becker *et al*. (1993), where they showed that expression of a bacterio-opsin proton pump and perturbation in protein metabolism caused by alteration of the ubiquitin protein degradation system, respectively, lead to the formation of distinct HR-like lesions in transgenic tobacco. Recently, Herbers *et al*. (1996a) showed that a change in sugar metabolism mediated by the expression of a yeast invertase in tobacco resulted in a lesion mimic phenotype on source leaves. Transgenic lines expressing invertase either in the vacuole or apoplast, accumulated high levels of soluble sugars in their tissue and formed necrotic lesions first in fully expanded source leaves accompanied by a disease resistance response. The data presented in Figure 7 show that transgene expression of PDC resulted in up to a twofold increase in soluble sugar and that translocated sucrose increased two- to 10-fold in the transgenics compared to the wild-type. The fact that lesions start first in fully expanded source leaves also suggests the involvement of sugar levels in the process. Interestingly, increased levels of soluble sugars in the tissue during pathogen infections has long been noted (Hall and Loomis, 1972; Watson and Watson, 1951). Horsfall and Dimond (1957) pointed out that plant diseases can be categorized into low and high sugar diseases, and that high levels of tissue sugar confer resistance to low sugar diseases. In addition, pathogenesis related proteins such as PAR-1, PR-Q, chalcone synthase and proteinase inhibitor II are induced by sugars (Herbers *et al*., 1996b; Johnson and Ryan, 1990; Takeuchi *et al*., 1994; Tsukaya *et al*., 1991). Thus, it is conceivable that plants have evolved a mechanism to modulate their tissue sugar levels in response to a pathogen attack, and use altered sugar levels as a signal to initiate subsequent defense reactions.

These last two possibilities are not mutually exclusive and there could be a direct relationship between carbohydrate metabolism, oxidative stress and cell death. In fact, Pugin *et al*. (1997) showed that application of the fungal elicitor cryptogein to tobacco cells causes both an oxidative burst and activation of the oxidative pentose phosphate pathway. According to their model, a plasma membrane NADPH oxidase is activated by cryptogein and produces reactive oxygen species. The depletion of NADPH in turn activates the pentose phosphate pathway which serves to regenerate NADPH. This results in the flow of more sucrose into the system via glucose-6-phosphate, and in the accumulation of glycolytic intermediates such as triose phosphates and phosphoenolpyruvate. Similarly, in our transgenic potatoes the presence of an additional sink for pyruvate and/or the accumulation of acetaldehyde may activate NADPH oxidase and the oxidative pentose phosphate pathway. This may result in starch degradation and sucrose export on one side and oxidative stress on the other. Thus, acetaldehyde could be the first signal for temperature-dependent changes in sugar levels, and the altered sugar level may mediate the execution of subsequent cell death processes. Our results contribute significantly to the emerging realization that metabolic processes not only respond to environmental cues, but can also actively participate in signaling plant responses themselves.

Experimental procedures

Plant growth and transformation

Potato plants (*Solanum tuberosum* var. Désirée) were grown in a greenhouse or growth room at 16/8 h light/dark cycle and a temperature of either 18 ± 3°C or 25 ± 2°C. The cloning of the *Zymomonas mobilis Pdc* gene in the pMON505 expression vector under the control of the CaMV 35S promoter has been described previously (Bucher *et al*., 1994). Potatoes were transformed using the *Agrobacterium* system according to Rocha-Sosa *et al*. (1989) and propagated from tissue cultures.

Anaerobic incubation and inhibitor treatment

Wild-type plants were grown at 25°C for 4 weeks and incubated in an anaerobic work bench for 15 h at room temperature. At the end of the incubation period, one set of plants was placed back at 25°C and the second set of plants was placed at 18°C for 9 h. This was repeated once more the next day and plants were evaluated for the presence or absence of distinct lesions on leaves.

For the PDC inhibitor treatment, the highest PDC expressor transgenics (line 8) were grown at 25°C for 4 weeks and then transfered to 18°C. For the treated plants, 100 mM glyoxylate was sprayed on each leaf 2 h before the transfer and daily during 1 week at 18°C. The control plants were sprayed with water. Lesion appearance and progression was followed in the treated and untreated controls.

RNA and protein analysis

Leaf samples were collected from plants grown at 18 ± 3°C and Northern analysis, Western blotting and PDC enzymatic assays were performed essentially as described previously (Bucher *et al*., 1994).

Detection of callose deposition

Leaf discs for callose examination were bleached in a series of 50, 75 and 96% ethanol overnight. The cleared leaves were rinsed in water and stained with a 0.05% (w/v) solution of aniline blue in 0.15 M K_2HPO_4 for 1 h at room temperature in a humid chamber. Stained leaves were examined under UV-light using excitation filter, 365 nm; dichromic mirror, 396 nm; and barrier filter, 420 nm.

Determination of acetaldehyde in potato leaf tissue

Healthy leaf discs (2 per plant) from leaves 3 and 4 (as counted from the top of a 4-week-old potato) were snap frozen in liquid nitrogen. The leaf discs were extracted with 6% perchloric acid at 4°C and incubated on ice for 2 h. After incubation, the samples were spun at maximum microfuge speed for 10 min at 4°C. The

supernatant was neutralized to pH 6.0–6.5 with 5 M K_2CO_3 on ice. The neutralized extract was spun again for 10 min as above, and the supernatant was transferred to a pre-cooled Eppendorf and kept on ice until derivatization. Acetaldehyde in this extract was measured as a fluorescent adduct formed by a reaction with 1,3-cyclohexanedione (CHD) essentially according to Helander *et al*. (1993). The reaction mixture contained 150 μl ammonium acetate (20%,w/v, in water), 150 μl thiourea (6%, w/v, in water), 50 μl CHD (1.25%, w/v, in water), and 150 μl leaf extract added to a 2 ml glass bottle in this order. Each bottle was immediately sealed after adding the extract, and all samples were incubated at 60°C in a gently shaking water bath for 1 h. The samples were cooled on ice and 20 μl aliquots were analyzed by high-performance liquid chromatography (HPLC). The HPLC system was as described in Helander *et al*. (1993) with the following modifications: System Gold HPLC System (Beckmann, Nyon, Switzerland), a Rheodyne RH 7010 injector with a 100 μl sample loop (Beckmann, Nyon, Switzerland), and Nucleosil 100–5 C18 reverse – phase analytical column (40 × 250 mm i.d., 5 μm particle size; Macherey-Nagel, Oensingen, Switzerland) were used. The column was eluted isocratically at a flow rate of 1.0 ml min^{-1} at ambient temperature with a mobile phase consisting of methanol-water (40: 60, v/v). A Kontron Model SFM-25 fluorescent detector (Kontron, Zürich, Switzerland) was used with excitation and emission wavelengths of 366 and 440 nm, respectively.

We made use of the volatility of acetaldehyde to authenticate our assay conditions. As negative controls, extractions from wild-type leaves were performed at room temperature and the extracts were incubated at 37°C for 1 h before mixing with cyclohexane. In this control, as well as in the water blank a small peak with a comparable height appeared with the same retention time as the acetaldehyde adduct. This was considered to represent the background peak. As positive controls, wild-type potato leaves were incubated for 2 h under anoxia and 10 min in air before extraction, where more than a 10-fold increase in the acetaldehyde peak was observed compared to the untreated wild-type.

Determination of soluble sugars and starch in the leaf tissue

Leaf discs (two per plant) from fully expanded source leaves after 8 h of the light period were collected in liquid nitrogen. Most of the L-8 and L-25 plants growing at 18°C showed lesions at the time of sampling (4–5 weeks after planting to soil), but samples were collected from healthy looking leaves. Leaves were homogenised in an Eppendorf tube with 80% ethanol (v/v) and extracted for 90 min at 70°C. Samples were spun at maximum microfuge speed for 10 min, and the supernatant was stored at –20°C for soluble sugars (sucrose, glucose and fructose) determination. The pellet was washed with 1 ml of 80% ethanol twice and spun in the same way. The washed pellet was resuspended in 400 μl of 0.2 M KOH and incubated at 95°C for 1 h. The starch was solubilised further at 50°C overnight. The volume was adjusted to 0.5 ml with KOH, neutralized with 1 M acetic acid, and centrifuged at maximum speed for 10 min. This supernatant was used to determine starch content. Sucrose, glucose, fructose and starch were measured enzymatically using a Boehringer kit (Mannheim, Germany).

Petiole exudates

Fully expanded source leaves (one leaf per plant) from 4- to 5-week-old potatoes were cut from the base of their petiole and immersed immediately into 3 ml of 5 mM EDTA (pH 6.0) solution according to Riesmeier *et al*. (1994). The petiole exudates were collected for 8 h under the same conditions as plant growth, and soluble sugars were determined enzymatically as above.

Resistance test to Phytophthora infestans

An inoculum of *Phytophthora infestans* strain 94–18 was prepared by adding 15 ml of ice-cold 0.5% glucose to a 3-week-old culture on rye A medium (Caten and Jinks, 1968). After 3 h incubation at 4°C to allow for the release of the zoospores, spores were counted and the concentration was adjusted to 25 000 per ml. Eight plants per line were grown in the greenhouse for 3 weeks at 24°C and transferred to 17°C for the infection test. On each plant, three leaves were infected with four droplets of 5 μl of spore suspension per leaf. Mock inoculations were done in the same way, except that 5 μl droplets of 0.5% glucose were spotted on the leaves. Inoculated plants were kept covered to maintain high humidity for 24 h. Plants were covered again 40 h before scoring. Six days after infection, the three infected leaves per plant were placed in a 50 ml tube containing 30 ml of 10% ethanol and shaken at 300 r.p.m. for 20 min. Leaves were then removed from the tube and photographed. Sporulation was estimated by counting the sporangia in the remaining solution.

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