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Systemic Acquired Resistance Mediated by the Ectopic Expression of Invertase: Possible Hexose Sensing in the Secretory Pathway

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Systemic acquired resistance (SAR) has been reported to be associated with lesion-mimic mutants. Tobacco plants expressing vacuolar and apoplastic yeast-derived invertase (vacInv and cwInv, respectively) develop spontaneous necrotic lesions similar to hypersensitive responses caused by avirulent pathogens. Therefore, SAR and metabolic alterations leading to the activation of defense-related responses were studied in these plants. Defense-related gene transcripts, callose content, peroxidase activities, and levels of salicylic acid were found to be elevated. The defense reactions were accompanied by increased resistance toward potato virus Y and were measured as decreased viral spreading and reduced multiplication in systemic leaves of the transgenic plants. Interestingly, the accumulation of pathogenesis-related (PR) protein transcripts (PR-Q) and repression of photosynthetic gene transcripts (chlorophyll *a/b* binding protein) were inversely correlated and required the same threshold level of hexoses for induction and repression. Expression of a cytosolic yeast-derived invertase in transgenic tobacco plants with equally increased levels of sugars neither displayed SAR responses nor showed decreased levels of photosynthetic genes. It is suggested that hexose sensing in the secretory pathway is essential for mediating the activation of defense-related genes as well as repression of photosynthetic genes in vacInv and cwInv plants.

INTRODUCTION

The hypersensitive response (HR) is a defense reaction of plants to pathogens and occurs in incompatible host–pathogen and non-host–pathogen interactions. It has been defined as rapid and localized tissue collapse resulting in necrotization and immobilization of the intruding pathogen at the site of attack (Klement, 1982). The biochemical basis for HR is not known, but changes in membrane potential, ion fluxes, and lipid peroxidation have been observed (Keppler and Baker, 1989; Atkinson et al., 1990). With the onset of the HR, other defense responses to pathogens are induced. These include strengthening of cell walls through callose deposition, lignin, and related wall-bound phenolics, production of antimicrobial phytoalexins, and biosynthesis of pathogensis-related (PR) and other defense-related proteins (Hahlbrock and Scheel, 1987; Lamb et al., 1989; Bowles, 1990). Activation of defense responses also may extend to uninfected tissue, leading to heightened resistance toward an entire range of unrelated pathogens. This has been termed systemic acquired resistance (SAR; Ross, 1961). SAR is accompanied by elevated levels of endogenous salicylic acid (SA) (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991). Recently, it has been shown that SA is essential for the development of SAR in tobacco plants (Gaffney et al., 1993). Yet, it is still a matter of debate whether SA is the phloem-translocated signal that mediates SAR (Vernooij et al., 1994; Shulaev et al., 1995).

Mutants (disease lesion mimics, lesion-simulating mutants, and accelerated-death mutants) displaying phenotypes similar to lesions in response to pathogen infection have been reported in maize, barley, and Arabidopsis (Emerson, 1923; Neuffer and Calvert, 1975; Hoisington et al., 1982; Walbot et al., 1983; Wolter et al., 1993; Dietrich et al., 1994; Greenberg et al., 1994). The occurrence of these mutants and the universality of the processes associated with the HR and SAR indicate that the HR as well as the ensuing plant defense responses are genetically programmed. Thus far, functions have not been assigned to the respective mutant genes; however, it has been speculated that they might result in an unbalanced biochemical state misinterpreted by the cell as pathogen infection (Dietrich et al., 1994). Biochemical perturbations in plant cells may indeed lead to the activation of programmed cell death and plant defense responses (Takahashi et al., 1989; Becker et al., 1993; Mittler et al., 1995).

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It has been known for a long time that susceptibility of plants to disease is dependent on the sugar content in the leaf tissue (Horsfall and Dimond, 1957). Based on these findings, diseases were classified as high- and low-sugar diseases (Horsfall and Dimond, 1957; Vanderplank, 1984). Low-sugar diseases are characterized by heightened resistance to pathogens when leaves contain increased levels of soluble sugars (Vanderplank, 1984). In this respect, it is interesting that a number of defense-related genes were found to be inducible by soluble sugars, for example, proteinase inhibitor II (Johnson and Ryan, 1990), chalcone synthase (Tsukaya et al., 1991), photoassimilate-responding PAR-1 and PR-3 from tobacco (Herbers et al., 1995), and the cathepsin D inhibitor and leucine aminopeptidase from potato (K. Herbers, unpublished data). These data suggest that sugars play a role not only in the repression of photosynthetic genes (Sheen, 1990; Krapp et al., 1993) but also in the induction of defense responses. Jang and Sheen (1994) suggested a common mechanism of sugar sensing in the repression of photosynthetic genes and activation of stress-related genes.

Previously, transgenic tobacco plants have been engineered for constitutive expression of yeast invertase in the apoplast and in the vacuole and the cytosol. They have been designated cwInv, vacInv, and cytInv, respectively (von Schaewen et al., 1990; Sonnewald et al., 1991). Apoplastic and vacuolar invertases give rise to stunted growth and to development of bleached and/or necrotic regions in older leaves (von Schaewen et al., 1990; Sonnewald et al., 1991). These symptoms are reminiscent of disease lesion-mimic mutants. Biochemically, the plants are characterized by limited sucrose export, resulting in accumulation of photoassimilates and inhibition of photosynthesis in their leaves (von Schaewen et al., 1990; Sonnewald et al., 1991). Stimulated by the observation that vacInv and cwInv plants develop necroses like those of lesion-mimic mutants, we studied defense-related responses and SAR in the transgenic plants. Possible relationships between carbohydrate metabolism and induction of defense-related functions were analyzed. In this study, we show that a preconditioned resistance state comparable to SAR is acquired in vacInv and cwInv plants. Threshold levels of hexoses are necessary to activate PR protein genes as well as to repress photosynthetic genes. Interestingly, these findings are not valid for cytInv plants, suggesting a transduction mechanism for defense-related functions that originates in the secretory pathway.

RESULTS

Spontaneous HR-like Lesions on Source Leaves of cwInv and vacInv Plants

Transgenic tobacco plants expressing yeast invertase in the apoplast and vacuole may develop necrotic lesions spontaneously on their source leaves, in contrast to transgenic tobacco plants in which the yeast invertase is located in the cytosol (von Schaewen et al., 1990; Sonnewald et al., 1991). Formation of necroses starts at the tip and develops to the leaf base as individual necrotic lesions, which in the end may coalesce. A histochemical marker of necrotic lesions associated with plant resistance responses is the presence of autofluorescent material. Autofluorescence is due to the biosynthesis of soluble phenolic compounds, such as phytoalexins, and to cell wall–bound phenolics responsible for reinforcement of the cell wall by suberization and lignification. To identify HR-like lesions, autofluorescence was investigated in leaves of wild-type, cwInv, and vacInv plants (Figure 1). Bright yellow fluorescence, indicating phenolic material of lesions, was detectable in the cwInv and vacInv plants (Figures 1B and 1C) but not in the leaves of wild-type plants (Figure 1A). Healthy tissue is characterized by the red emission of chlorophyll. To demonstrate cell wall–bound phenolics, leaves were cleared of soluble material by chloral hydrate. Strong fluorescence of cell walls was observed in the cwInv and vacInv plants (Figures 1E and 1F) in contrast to wild-type plants (Figure 1D), demonstrating that at least part of the overall cellular autofluorescence is due to cell wall–bound phenolics in the invertase-expressing plants.

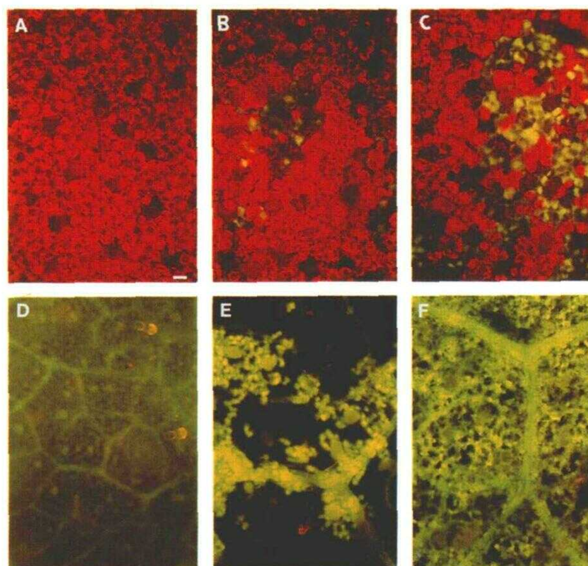

Figure 1. Detection of Autofluorescent Material Associated with Necrotic Lesions.

(A) to **(C)** UV-stimulated autofluorescence of untreated tobacco leaves. **(D)** to **(F)** UV-stimulated autofluorescence of chloral hydrate–treated tobacco leaves.

Shown in **(A)** and **(D)** are wild-type leaves. vacInv and cwInv leaves are shown in **(B)** and **(E)** and **(C)** and **(F)**, respectively. The exposure time for the leaves shown in **(E)** and **(F)** was ~10 times less than for the leaf in **(D)**. Small and enlarged lesions were chosen randomly. Bar in **(A)** = 50 μm for **(A)** to **(F)**.

Table 1. Callose Content in Wild-Type, vacInv, and cwInv Plants

	Relative Fluorescence[a]	
Plant	Nonnecrotic Leaves	Partially Necrotic Leaves
Wild type	6.0 ± 1.7	
Wild type/PVY[b]	16.3 ± 1.5	
vacInv	11.9 ± 1.3	24.5 ± 3.1
cwInv	20.3 ± 2.3	29.8 ± 4.1

[a] Values represent the means ± SE of six samples taken from mature source leaves of different plants before and after necrotic lesion formation.
[b] Systemic leaves of PVY-infected wild-type plants 12 dpi.

Callose content, another marker for structural changes associated with plant defense responses, was found to be elevated by factors of approximately two and three in nonnecrotic leaves of vacInv and cwInv plants, respectively, compared with the wild type (Table 1). This increase in callose was in the same range as that found in leaves of the wild-type plants treated with potato virus Y (PVY) (Table 1). Callose content in healthy parts of leaves that were partially necrotic was fairly variable and increased fourfold to fivefold (Table 1).

Accumulation of Defense-Related Transcripts in Leaves of cwInv and vacInv Plants

A molecular marker for plants prevailing in a state of alarm is the accumulation of genes encoding defense-related proteins. RNA isolated from mature source leaves of 9-week-old invertase-expressing plants was analyzed for the expression of PR protein genes (Figure 2) and genes involved in the biosynthesis of phytoalexins (Figure 3). RNA from tobacco leaves infected with PVY was used as a control in both cases. A strong accumulation of the PR protein transcripts PAR-1 (Herbers et al., 1995), PR-1b, and PR-Q was found in PVY-infected leaves as well as in cwInv and vacInv tobacco plants but not in cytInv plants (Figure 2). Tobacco suspension cells respond to elicitor treatment with the synthesis of sesquiterpenoids, primarily the phytoalexin capsidiol (Chappell et al., 1987). Central to capsidiol biosynthesis is the induction of 5-*epi*-aristolochene synthase (EAS) (Facchini and Chappell, 1992). EAS transcripts were induced in cwInv and vacInv genotypes by factors ranging between four and 10, and in PVY-infected wild-type plants by a factor of 15 (Figure 3). EAS transcripts were not induced in cytInv plants (data not shown). RNA levels of phenylalanine ammonia-lyase (PAL), one of the three core enzymes in phenylpropanoid metabolism, were slightly induced (Figure 3). Whereas in PVY-infected leaves the level of PAL increased by a factor of eight, it increased only by a factor of 1.2 to 1.5 in vacInv and cwInv plants. PAL transcripts accumulate particularly in tissue surrounding hypersensitive cell death (Hahlbrock and Scheel, 1989). Thus, the different expression levels may be explained by the fact that PVY-infected leaves used in this experiment were already heavily affected.

Peroxidases are involved in several different defense-related processes, that is, clearance of H_2O_2, suberin synthesis, and lignin synthesis (Bowles, 1990). The induction of at least two different peroxidase activities in vacInv and cwInv plants could be visualized after electrophoretic separation of protein extracts from leaves, using guaiacol as the substrate (Figure 4; Coll et al., 1993).

cwInv and vacInv Plants Accumulate SA

The development of lesions indicates the presence of SA. Recently, it has been shown that the application of SA to tobacco leaves leads to necroses (van der Straeten et al., 1995). Furthermore, SA is essential for SAR development (Gaffney et al., 1993). To study whether the general state of defense in vacInv and cwInv plants might be mediated by SA, free and bound SA levels were determined. Leaves of wild-type plants systemically infected with PVY (12 days postinfection [dpi]) accumulated ~4.3 and 40.8 ng of free and bound SA, respectively, per square centimeter of leaf area. This accumulation corresponds to increases of 8.5- and 24.5-fold, respectively, as

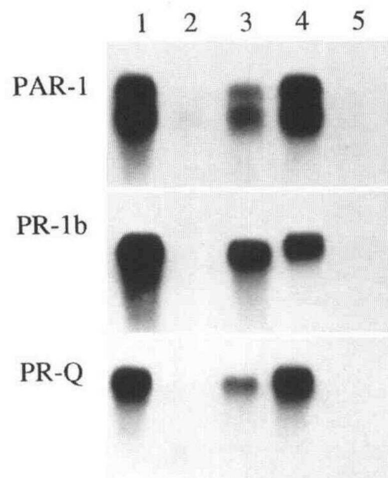

Figure 2. PR Protein Transcripts Accumulate in cwInv and vacInv but Not in cytInv Plants.

Gel blots contain RNA isolated from mature source leaves of wild-type (lane 2), cwInv (lane 3), vacInv (lane 4), and cytInv plants (lane 5). RNA was extracted 9 weeks after germination, and the plants had 12 to 14 leaves. For comparison, total RNA was isolated from wild-type leaves 12 days after infection with PVY (lane 1). RNA gel blots were hybridized with the indicated cDNA fragments. Each lane contains 15 μg of RNA.

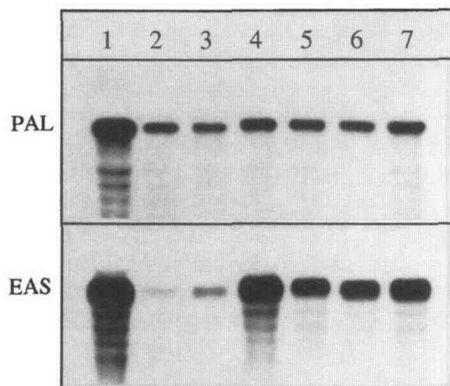

Figure 3. Induction of EAS and PAL in cwInv and vacInv Plants.

Gel blots with RNA isolated from source leaves of noninfected wild-type (lanes 2 and 3), vacInv (lanes 4 and 5), and cwInv plants (lanes 6 and 7). RNA was extracted from two plants of each type. For comparison, total RNA was isolated from leaves of tobacco plants 12 days after infection with PVY (lane 1). RNA gel blots were hybridized with cDNA fragments encoding PAL-1 from potato and EAS-3 from tobacco. Each lane contains 15 μg of RNA.

compared with uninfected controls (Figures 5A and 5B). Although SA levels in cytInv plants did not differ from wild-type plants, SA in leaves of vacInv and cwInv plants increased by 14-fold and fivefold (free SA) and by 33- and 13-fold (bound SA), respectively (Figures 5A and 5B). By comparison, tobacco mosaic virus (TMV)–infected leaves of Xanthi NN accumulate SA at least 20-fold (Malamy et al., 1990) or even 48- to 70-fold (Vernooij et al., 1994). The relatively low levels of SA observed in the PVY-infected plants reflect the susceptibility to the virus. For comparison, no increase was observed in the compatible reaction of TMV with Xanthi nn (Malamy et al., 1990).

A Preconditioned Resistance State Induced in cwInv and vacInv Plants

PVY spreads systemically throughout cultivar Samsun NN tobacco plants. Infected leaves are characterized by browning of midribs followed by crinkling and necrotizing of the laminae. Final evidence for SAR in cwInv and vacInv plants mediated by the defense-related responses was obtained when their resistance to PVY was analyzed. Viral extract was applied to mature source leaves of 32 wild-type, 69 cwInv, and 24 vacInv plants. After 10 days, visual analysis showed that all wild-type plants were systemically infected, whereas many vacInv and cwInv plants did not display disease symptoms. The relative amount of virus in systemic leaves was determined 12 dpi by ELISA, using monoclonal antibodies raised against the PVY coat protein. The relative optical densities reflecting the amount of PVY coat protein were 0.010 $\pm$ 0.013 (n = 6) and 0.243 $\pm$ 0.113 (n = 32) for noninfected and infected wild-type plants, respectively. (The numbers given are mean values $\pm$ SD and refer to dilutions of 1:16 of the initial extract.)

From a comparison with the ELISA data for noninfected plants, it was concluded that 50% of the cwInv and 46% of the vacInv plants were not systemically infected. The amounts of virus coat protein in those plants infected were approximately five (cwInv) and two times lower (vacInv) than those in wild-type plants (cwInv: 0.049 $\pm$ 0.042, n = 35; vacInv: 0.127 $\pm$ 0.119, n = 13). VacInv and cwInv plants are thus more resistant to PVY infection. In an independent experiment, wild-type and cytInv plants at the 12-leaf stage were treated with PVY, and viral spreading and multiplication followed in systemic leaves. No significant difference was found between wild-type (0.247 $\pm$ 0.151; n = 20) and cytInv plants (0.230 $\pm$ 0.152; n = 20), showing that cytInv plants do not possess increased resistance to PVY.

Another remarkable feature of the transgenic cwInv and vacInv plants was that the directly infected leaves developed stronger necrotic lesions as compared with the wild type (Figure 6). Infected leaves of wild-type plants 8 dpi were devoid of disease symptoms, whereas cwInv and vacInv plants had already started to develop necroses (Figure 6A compared with Figures 6B and 6C). By 12 dpi, the necroses had already coalesced, whereas infected leaves of wild-type plants had just begun to develop lesions (Figure 6D as compared with Figures 6E and 6F). Necrotic lesions also evolved more readily on leaves of the transgenic plants after mechanical wounding (data not shown).

Inverse Correlation and Requirement of Hexose Threshold Levels for the Regulation of PR Protein and Chlorophyll *a/b* Binding Protein Transcripts

Based on the hypothesis promoted by Jang and Sheen (1994) that activation of defense-related genes and repression of photosynthetic genes might be subject to the same signal

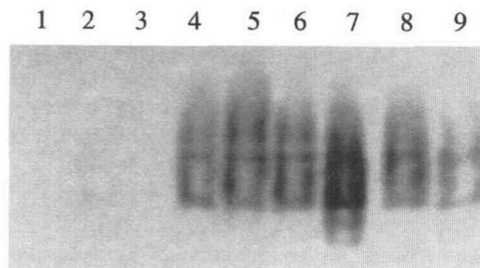

Figure 4. Peroxidase Activities Are Induced in cwInv and vacInv Plants.

Leaf tissue corresponding to a 0.8-cm^2 area was homogenized in 90 μL of 50 mM Tris-HCl, 10% glycerol, 5% β-mercaptoethanol, 250 mM NaCl, pH 6.8. The extracts (30 μL) were analyzed on SDS–polyacrylamide gels. Samples were taken from the following leaves: wild type, mature source (lane 1); senescent (lane 2); sink (lane 3); PVY-infected necrotic (lane 8); PVY-infected nonnecrotic (lane 9); cwInv nonnecrotic (lanes 4 and 5); cwInv necrotic (lane 6); and vacInv nonnecrotic (lane 7).

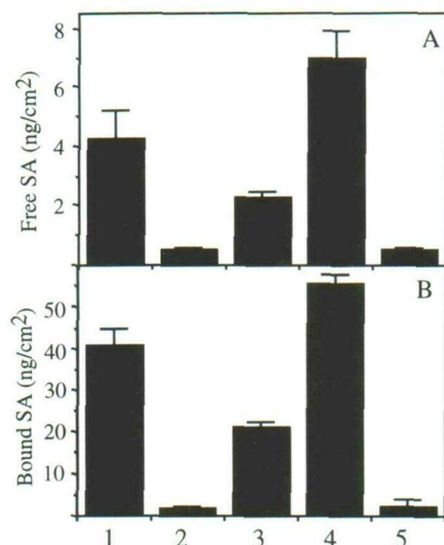

Figure 5. SA Levels in cwInv, vacInv, and cytInv Plants.

(A) Free SA.

(B) Bound SA.

Bar 1 represents the PVY-infected wild type; bar 2, the noninfected wild type; bar 3, cwInv plants; bar 4, vacInv plants; and bar 5, cytInv plants. Values are means ± SD of three independent extracts and are given in nanograms per square centimeter of leaf area. Each extract represents a pool of samples taken from source leaves of seven different plants. Plants were 9 weeks old and had 12 to 14 leaves.

transduction pathway with sugars as signal molecules, the expression of transcripts encoding the chlorophyll *a/b* binding protein (CAB) was included in our studies. RNA from different leaves of cwInv, vacInv, and wild-type plants was hybridized with PAR-1, PR-1b, PR-Q, SAR8.2, and *cab* cDNAs (Figure 7). Transcript levels encoding PR proteins gradually increased from sink to source leaves, whereas the levels of transcripts encoding CAB decreased to the same extent as PR protein transcripts accumulated (Figure 7).

To determine whether sugars might be involved in PR protein gene activation and *cab* gene repression, steady state levels of sugar and starch as well as PR-Q and *cab* transcripts were studied in cwInv plants. Thirty leaf samples of transplants with invertase activities between 0.2 and 4 mmol of hexose per min per m^2 were analyzed (in wild-type plants, neutral invertase activity was undetectable). Samples for invertase measurements and sugar/starch determinations were derived from the same samples used for RNA analysis. Due to the wide range of invertase activities, contents of soluble sugars and starch were variable in the cwInv plants. Levels ranged between 0.5 and 18.5 mmol/m^2 for glucose, 0.8 and 5.5 mmol/m^2 for fructose, 0.5 and 1.7 mmol/m^2 for sucrose, and 1 to 31 mmol of hexose per m^2 for starch. For comparison, levels of glucose, fructose, sucrose, and starch in wild-type plants (n = 6) grown under the same conditions were 0.96 ± 0.36 mmol/m^2, 0.74 ± 0.23 mmol/m^2, 0.82 ± 0.17 mmol/m^2, and 2.35 ± 0.88 mmol of glucose/m^2, respectively. Quantified *cab* and PR-Q transcripts did not correlate with steady state levels of starch and sucrose of the corresponding extracts (data not shown). However, comparisons between hexose levels and levels of PR-Q and *cab* transcripts revealed that above ~2.5 mmol/m^2 of glucose and 2 mmol/m^2 of fructose, respectively, PR-Q transcripts accumulated, whereas *cab* transcripts decreased (Figure 8). The data suggest a threshold level of either fructose or glucose (or both) for the activation of PR protein transcripts and repression of *cab* transcripts. This indicates that a common sensor is involved in both activation and repression of transcription. Interestingly, despite elevated levels of soluble sugars (Sonnewald et al., 1991), neither accumulation of PR protein transcripts nor repression of *cab* transcripts was observed in cytInv plants (Figure 2 and data not shown), illustrating the significance of the compartment in which the hexoses are primarily produced and possibly accumulate to some degree.

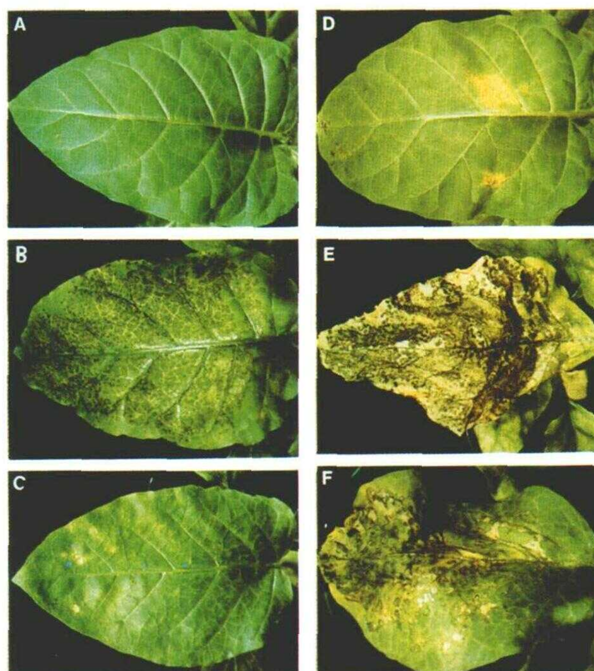

Figure 6. Accelerated Development of HR-like Lesions in cwInv and vacInv Plants.

Source leaves of wild-type, vacInv, and cwInv plants at the 12-leaf stage were treated with PVY.

(A) to **(C)** Infected leaves photographed 8 days after infection.

(D) to **(F)** Infected leaves photographed 12 days after infection.

(A) and **(D)** show wild-type leaves. **(B)** and **(E)** show vacInv leaves. cwInv leaves are shown in **(C)** and **(F)**.

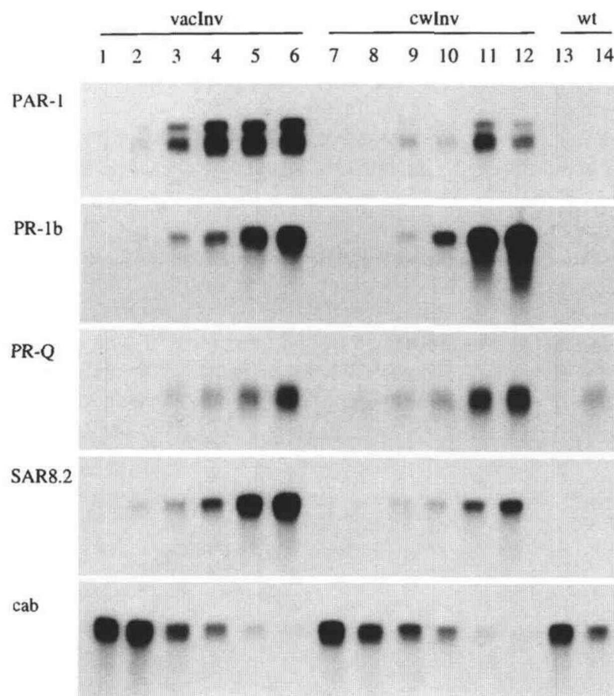

Figure 7. Expression of PR Protein and *cab* Transcripts in Different Leaves of vacInv and cwInv Plants.

RNA gel blots from different leaves of vacInv, cwInv, and wild-type (wt) plants were hybridized with PAR-1, PR-1b, PR-Q, SAR8.2, and *cab* cDNAs as indicated. RNA was isolated from different leaves of 11-week-old plants at the 17-leaf stage. Fifteen micrograms of total RNA from sink leaves (lanes 1, 7, and 13); from leaves undergoing the sink-to-source transition (lanes 2 and 8); from source leaves without any phenotypic alteration (lanes 3 and 9), with necrotic lesions starting at the tip (lanes 4 and 10), and with a fully developed phenotype (lanes 5 and 11); and from senescing leaves (lanes 6 and 12) was loaded per lane. Lane 14 contains RNA from mature source leaves of wild-type plants.

Transgenic Tobacco Plants with Reduced Expression of the H^+-Sucrose Transporter Accumulate PR Protein Transcripts

Invertase glycopeptides have been shown to act as elicitors in tomato cell suspension cultures in vitro (Basse and Boller, 1992; Basse et al., 1992). The possibility that these invertase fragments would be responsible for eliciting the defense-related reactions observed in the cwInv and vacInv plants had to be ruled out. Transgenic tobacco plants with reduced levels of the H^+-sucrose transporter (αNtSUT1) (L. Bürkle, J. Hibbert, P.W. Quick, C. Kühn, B. Hirner, and W.B. Frommer, submitted manuscript) are characterized by a metabolic disturbance similar to that seen in cwInv plants. In both cases, phloem loading of sucrose is inhibited, and as a consequence, leaves accumulate carbohydrates (von Schaewen et al., 1990; L. Bürkle, J. Hibbert, P.W. Quick, C. Kühn, B. Hirner, and W.B. Frommer, submitted manuscript). Furthermore, αNTSUT1 plants also develop chlorotic and necrotic regions on their source leaves (data not shown). To verify the similarity at the molecular level, RNA from different αNtSUT1 genotypes was hybridized to PR-Q cDNA. Figure 9 illustrates that transcripts encoding PR-Q clearly were induced, showing that the perturbation in carbohydrate metabolism and not any specific feature of the transgene leads to the acquired resistance state of the vacInv and cwInv plants.

DISCUSSION

Transgenic tobacco plants constitutively expressing yeast invertase in the cytosol, the vacuole, or the apoplast were analyzed with respect to defense-related reactions and their possible relationships with the accumulation of soluble sugars and starch. Our investigations were inspired by the previous observations that necrotic lesions developed spontaneously on mature leaves of vacInv and cwInv plants reminiscent of lesion-mimic mutants associated with increased resistance toward fungal and bacterial pathogens (von Schaewen et al., 1990; Sonnewald et al., 1991; Dietrich et al., 1994; Greenberg et al., 1994). We were interested in determining whether perturbed carbohydrate metabolism might lead to a cellular situation that would resemble viral or pathogen attack resulting in SAR. The observation that resistance and susceptibility of plants to a number of diseases often are dependent on the

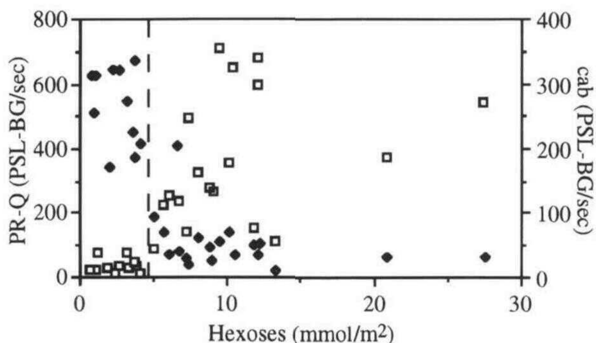

Figure 8. Threshold Levels of Hexoses for PR-Q Induction and *cab* mRNA Repression.

Source leaves of different cwInv plants of the 12- to 14-leaf stage were used to isolate total RNA and to measure invertase activity, soluble sugars, and starch (see text). RNA blots were hybridized with PR-Q and *cab* cDNA fragments. The respective signals were quantified using an imaging analyzer and plotted against steady state levels of sugars. Hexoses are the sum of the measured glucose and fructose content and represent the means of four independent samples. Signal strengths are given in PSL-BG/sec, which stands for the radiation dose of the signal minus background per second. The dashed line indicates the hexose threshold level. Filled diamond, *cab*; open square, PR-Q.

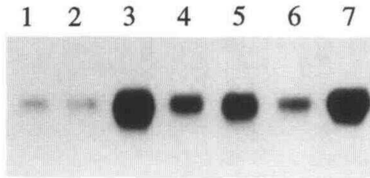

Figure 9. Accumulation of PR-Q–Specific Transcripts in Sucrose Transporter Antisense Plants.

RNA from source leaves of wild-type, αNtSUT1, and cwInv plants was hybridized with PR-Q cDNA. Plants had been grown for 4 weeks in the greenhouse after transfer from tissue culture and had 10 to 12 leaves. Fifteen micrograms of total RNA was loaded per lane. Lanes 1 and 2 contain RNA from wild-type leaves. RNA samples from different lines of αNtSUT1 are as follows: lane 3, $35S_{46}$; lane 4, $35S_{55}$; lane 5, $35S_{12}$; lane 6, $35S_{50}$; and lane 7, cwInv. Antisense constructs, tobacco transformation, and analysis of the different lines are described by L. Bürkle, J. Hibbert, P.W. Quick, C. Kühn, B. Hirner, and W.B. Frommer (submitted manuscript).

sugar levels of leaf tissue ("sink-induced loss of resistance") (Horsfall and Dimond, 1957; Vanderplank, 1984) hints at a possible role of sugars in the inducible defense systems of plants.

Perturbed Carbohydrate Metabolism Leads to SAR

Our investigations demonstrated that a number of important defense-related reactions are constitutively present in vacInv and cwInv plants and result in SAR. Both transgenic plant lines displayed necroses similar to HR-like lesions with respect to accumulation of phenolic compounds and cell death, as determined by chlorophyll loss using fluorescence microscopy. Callose, a structural marker for induced resistance responses, was elevated two- to threefold in leaves before lesion formation and four- to fivefold in tissue surrounding necrotic lesions. As a molecular marker, constitutive gene expression of a novel class of PR proteins termed PAR-1 (Herbers et al., 1995) and of PR-1b, PR-Q, and SAR8.2, which have been shown to be induced coordinately after TMV infection and SA treatment (Ward et al., 1991), was observed. Moreover, all acidic PR proteins expressed after PVY infection were detectable in intercellular fluids of cwInv and vacInv plants (Sonnewald et al., 1995). Other defense-related protein gene transcripts, such as PAL and EAS, as well as peroxidase activities also were induced in the transgenic plants. Free SA, which is an essential molecule in transducing SAR (Gaffney et al., 1993; Delaney et al., 1994), was elevated by factors of ~14 and five in source leaves of vacInv and cwInv plants, respectively. Concomitantly, bound SA increased by 33- (vacInv) and 13-fold (cwInv). By using chimeric plants consisting of wild-type scions grafted on salicylate hydroxylase–expressing rootstocks, it has been shown that an increase of only 1.2-fold in the scion leaves of TMV-treated rootstocks still was sufficient to mediate SAR (Vernooij et al., 1994). Thus, SA levels in vacInv and cwInv plants are more than sufficient to mediate SAR responses.

The defense-related responses of vacInv and cwInv plants were accompanied by increased resistance toward the viral pathogen PVY. Necrotic lesions developed more rapidly on leaves of vacInv and cwInv plants after inoculation with PVY or after wounding. This pattern is similar to the development of HR lesions in TMV-infected tobacco carrying the *N* gene (Whitman et al., 1994). However, the reversal from a susceptible to a resistant interaction is not complete in vacInv and cwInv plants because the virus can still move through the plant. This could be due to the timing of the appearance of the necroses in relation to virus multiplication, because some virus particles are able to escape the necrotizing cells. Nevertheless, our results show a significant decrease in systemic virus spread, leading to a limitation of pathogen damage, which is a typical feature of the SAR preconditioned state. Thus, the genetic program underlying HR and SAR processes is triggered in vacInv and cwInv plants. Transgenic plants with repressed levels of H^+-sucrose transporter (αNtSUT1) are biochemically similar to cwInv plants because both transgenic lines are characterized by reduced phloem loading of sucrose and accumulation of photoassimilates in their source leaves (von Schaewen et al., 1990; L. Bürkle, J. Hibbert, Paul W. Quick, C. Kühn, B. Hirner, and W.B. Frommer, submitted manuscript). The αNtSUT1 plants developed spontaneous necrotic lesions and accumulated PR protein transcripts (Figure 9), showing that the perturbation in carbohydrate metablism and not any specific feature of the transgene leads to the SAR state of cwInv and vacInv plants.

Hexose Sensing for Defense Gene Activation and Photosynthetic Gene Repression May Occur at Secretory Membranes

To determine whether carbohydrates might be the causal agents for the induction of SAR responses, the relationship between carbohydrate accumulation and expression of PR protein genes was investigated. No linear relationship could be detected between steady state levels of sucrose and starch, respectively, and PR-Q transcripts. Yet, above a certain level of glucose and fructose (~4.5 mmol/m^2), PR-Q transcripts accumulated, indicating that a defined threshold level of hexoses is required for defense gene expression. Sugars often have been shown to repress photosynthetic genes (Sheen, 1990; Goldschmidt and Huber, 1992; Krapp et al., 1993). Interestingly, *cab*-specific transcripts used as a representative example of photosynthetic genes were down-regulated above the very same threshold level found to give rise to increased defense-related transcripts. This finding supports the hypothesis forwarded by Jang and Sheen (1994) that sugars suppress photosynthetic genes by the same mechanism used to activate defense-related genes. In addition, they also suggested that hexoses require transport across the plasma membrane, subsequent phosphorylation, and no further metabolism. Thus,

they proposed that sugar sensing is intracellular and mediated by hexokinase.

The data presented here do not support a role of cytosolic hexokinase in signal initiation. cytInv plants hydrolyze newly formed sucrose directly by cytosolic invertase into fructose and glucose. Thus, the hexoses are available immediately for phosphorylation in the cytosol. However, despite elevated sugar levels in these transgenic plants (Sonnewald et al., 1991), *cab* transcripts were not repressed (data not shown). Similarly, cytInv plants do not acquire SAR: plants accumulated neither SA nor PR proteins and were as susceptible to PVY as were wild-type plants. Therefore, we propose that sugar signaling for both activation of defense-related genes and repression of photosynthetic genes is associated with sensing mechanisms located at the secretory membrane system, possibly at the endoplasmic reticulum or Golgi apparatus. This is the only explanation for why plants expressing a heterologous invertase in either the vacuole or the apoplast behave similarly but differently from plants expressing the invertase in the cytosol.

Studies on the subcellular distribution of hexoses and sucrose by means of nonaqueous fractionation revealed that 97 to 98% of the steady state level of sugars were located in the vacuole of all three invertase-expressing lines (Heineke et al., 1994). This result is not surprising because the vacuole is considered to be the compartment in which sugars are taken up by transporters and are finally stored. The data support our hypothesis that hexose sensing must occur along the secretory pathway because the vacuole itself can be excluded. The postulate implies that (1) yeast invertase is active in the secretory pathway, and (2) sucrose is present there to be metabolized by the invertase. There is no need to assume that yeast invertase activity might be affected by differences in glycosylation. The enzyme is highly active without any glycosyl residues, as in the cytInv plants and in yeast in which the enzyme contains glycosylation branches of the high-mannose type. The endoplasmic reticulum/Golgi forms are intermediary, and thus full activity of the enzyme is to be expected. The second argument currently is being addressed by creating transgenic plants that express yeast invertase in the endoplasmic reticulum.

The physiological relevance of photosynthetic gene repression as a way of regulating photosynthesis according to sink demands has been discussed often (e.g., Krapp et al., 1993). With respect to defense reactions, sugars may play a dual role. (1) Plants produce a wide range of phenolic compounds (including SA) in response to invading pathogens. The availability of metabolizable sugars is required for the synthesis of these compounds. (2) Sugars are known to alter gene expression, leading to the activation of defense-related genes. These two aspects have been dissected in transgenic tobacco plants expressing *Escherichia coli* pyrophosphatase behind the constitutive cauliflower mosaic virus 35S promoter (*ppa-1*; Sonnewald, 1992). The *ppa-1* plants are characterized by even higher contents of soluble sugars than those of the invertase-expressing plants (Sonnewald, 1992). Recently, we isolated 12 different cDNAs from the *ppa-1* plants, of which four cDNAs could be identified as coding for PR proteins (Herbers et al., 1995). However, *ppa-1* plants do not exhibit SAR, as judged by unchanged levels of SA as compared with wild types (K. Herbers, P. Meuwly, J.P. Métraux, and U. Sonnewald, unpublished data). In *ppa-1* plants, sucrose biosynthesis is favored strongly over sucrose breakdown (Sonnewald, 1992). This suggests that one prerequisite for the formation of SA and possibly other phenolic defense products might be the presence or even local excess of metabolizable sucrose.

Further support for this hypothesis comes from data obtained with transgenic plants expressing the *E. coli* pyrophosphatase behind the phloem-specific *rol-C* promoter (*ppa-4*; Lerchl et al., 1995). In the mesophyll cells of sugar-accumulating *ppa-4* plants, sucrose metabolism is not influenced by the transgene, and levels of free and bound SA in the *ppa-4* plants were elevated by ~3.5- and 43-fold, respectively, as compared with wild types (K. Herbers, P. Meuwly, J.P. Métraux, and U. Sonnewald, unpublished data). Thus, degradation of sucrose and the cross-talk between primary and secondary metabolism appear to be important for the production of SA.

Infection of leaf tissues by viruses and pathogens may result in elevated carbohydrate levels (Watson and Watson, 1951; Steudel and Heiling, 1954; Hall and Loomis, 1972). Thus, local accumulation of sugars caused by the pathogen may in turn be exploited by the plant cell in the way described above. Further support for this interactive relationship between pathogen and plant comes from the observation that cell wall invertases have been observed to be inducible in viral, bacterial, and fungal infections (Joosten et al., 1990; Sturm and Chrispeels, 1990; K. Herbers, unpublished data). The phenomenon of high-sugar resistance (Horsfall and Dimond, 1957; Vanderplank, 1984) may thus be explained by the constitutive presence of defense mechanisms due to elevated sugar levels.

METHODS

Plant Maintenance

Plants (*Nicotiana tabacum* cv Samsun NN) were obtained from Vereinigte Saatzuchten AG (Ebstorf, Germany). Greenhouse-grown plants were maintained in soil with a 16-hr-light and 8-hr-dark regime. Sunlight was supplemented with artificial lamps ranging between 200 and 250 μmol m^{-2} sec^{-1}. Relative humidity was between 60 and 70%, and temperatures were 20/15°C in the light/dark cycle. The transgenic plants used were A-41 (cwInv), U-In6 (vacInv), U-In5 (cytInv) (von Schaewen et al., 1990; Sonnewald et al., 1991), and αNtSUT1 (L. Bürkle, J. Hibbert, P.W. Quick, C. Kühn, B. Hirner, and W.B. Frommer, submitted manuscript).

Microscoscopy

For determination of autofluorescence of cell wall–bound phenolics, leaves were cleared with chloral hydrate–water (8:3[v/v]) for 2 hr at 70°C and then rinsed in water. Autofluorescence of cleared and noncleared leaves was observed from the top side of leaves, using ultraviolet epifluorescence (excitation filter of 365 nm, dichroic mirror of 395 nm, and barrier filter of 420 nm).

Determination of Callose

Callose was essentially determined as described by Köhle et al. (1985). Leaf area (1.6 cm^2) of nonnecrotized leaves and of tissue surrounding necroses was cleared from chlorophyll with 80% ethanol–10 mM EDTA. Thereafter, callose was solubilized with 1 M NaOH at 80°C. One-fifth of the sample was assayed for callose with aniline blue (0.1% in water). Relative fluorescence was determined using a luminescence spectrometer (model LS 50B; Perkin-Elmer) (excitation of 400 nm and emission of 510 nm).

Visualizing Peroxidase Activities on SDS-Polyacrylamide Gels

Total proteins were homogenized in 50 mM Tris-HCl, 10% glycerol, 5% β-mercaptoethanol, 250 mM NaCl, pH 6.8. After incubation on ice for 30 min, debris was centrifuged for 3 min in a microcentrifuge, and the supernatant was run on a 10% SDS-polyacrylamide gel at 4°C without prior heat denaturation of the proteins. After separation of the proteins, the gel was washed twice with precooled 50 mM sodium acetate, pH 5.2, to remove SDS, and stained with 2 mM guaiacol (Sigma) in 50 mM sodium acetate, pH 5.2. The reaction was started by adding 50 μL of H_2O_2 per 100 mL.

Determination of Neutral Invertase Activity, Soluble Sugars, and Starch

Neutral invertase activity was determined as described by Sonnewald et al. (1991), and soluble sugars and starch were quantitated as described by Sonnewald (1992).

RNA Gel Blot Analysis

RNA gels and RNA gel blotting were performed as described by Herbers et al. (1995). Hybridization signals were quantified by means of an imaging analyzer (Fuji Bas 2000; Fuji, Tokyo, Japan).

Determination of Salicylic Acid

Samples of defined leaf areas from nonnecrotized leaf areas were harvested and lyophilized. Free and bound salicylic acid (SA) was extracted and quantitated using *ortho*-anisic acid as an internal standard as described previously (Meuwly and Métraux, 1993).

Infection of Tobacco Plants with Potato Virus Y (PVY^N)

PVY^N was obtained from the Bundesanstalt für Züchtungsforschung an Kulturpflanzen (Aschersleben, Germany). Leaves of infected tobacco plants were homogenized in 100 mM K-phosphate buffer, pH 7.0 (~1 g of leaf material in 20 mL of buffer), to obtain viral extract. Leaves to be infected were dusted with carborundum (SiC), and the viral extract was applied by gently rubbing the upper face of the respective leaves with a pistil. A few minutes later, the treated leaves were rinsed with water. Eight to 11 days after infection, symptoms appeared on the plants.

Immunological Detection of Virus Coat Protein

Plant material was homogenized with PBS buffer containing 0.05% Tween 20, 2% PVP 25,000, 0.2% BSA (1 g of plant material per 20 mL of buffer). Serial dilutions of homogenized extract were analyzed by means of the double-antibody sandwich test, using monoclonal antibodies raised against PVY (BIOREBA, Reinach, Switzerland). The ELISA procedure was performed according to the protocol by BIOREBA. Optical densities at 405 nm were measured using reader 340 ATTC (SLT, Crailsheim, Germany).

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