The Plant Cell, Vol. 9, 1825–1841, October 1997 © 1997 American Society of Plant Physiologists

Glucose and Stress Independently Regulate Source and Sink Metabolism and Defense Mechanisms via Signal Transduction Pathways Involving Protein Phosphorylation

Rainer Ehness, Margit Ecker, Dietmute E. Godt, and Thomas Roitsch[1]

Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, Universitätsstrasse 31, D-93053 Regensburg, Germany

In higher plants, sugars are required not only to sustain heterotrophic growth but also to regulate the expression of a variety of genes. Environmental stresses, such as pathogen infection and wounding, activate a cascade of defense responses and may also affect carbohydrate metabolism. In this study, the relationship between sugar- and stress-activated signal transduction pathways and the underlying regulatory mechanism was analyzed. Photoautotrophically growing suspension culture cells of *Chenopodium rubrum* were used as a model system to study the effects of the metabolic regulator D-glucose and of different stress-related stimuli on photosynthesis, sink metabolism, and defense response by analyzing the regulation of mRNAs for representative enzymes of these pathways. Glucose as well as the fungal elicitor chitosan, the phosphatase inhibitor endothall, and benzoic acid were shown to result in a coordinated regulatory mechanism. The mRNAs for phenylalanine ammonia–lyase, a key enzyme of defense response, and for the sink-specific extracellular invertase were induced. In contrast, the mRNA for the Calvin cycle enzyme ribulose bisphosphate carboxylase was repressed. This inverse regulatory pattern was also observed in experiments with wounded leaves of *C. rubrum* plants. The differential effect of the protein kinase inhibitor staurosporine on mRNA regulation demonstrates that the carbohydrate signal and the stress-related stimuli independently activate different intracellular signaling pathways that ultimately are integrated to coordinately regulate source and sink metabolism and activate defense responses. The various stimuli triggered the transient and rapid activation of protein kinases that phosphorylate the myelin basic protein. The involvement of phosphorylation in signal transduction is further supported by the effect of the protein kinase inhibitor staurosporine on mRNA levels.

INTRODUCTION

In plants, sugars are not only substrate for the growth of sink tissues, which depend on the import of carbohydrates, but they also regulate the expression of a variety of genes (Koch, 1996). It has been shown that genes encoding sink-specific enzymes, such as sucrose synthase (Salanoubat and Belliard, 1989), granule-bound starch synthase (Visser et al., 1991), and extracellular invertase (Roitsch et al., 1995), are induced by glucose or sucrose. Sugar-induced gene expression has also been demonstrated for enzymes involved in pathogen and stress response, such as proteinase inhibitor II of potato (Johnson and Ryan, 1990) and chalcone synthase (Tsukaya et al., 1991). In contrast, different genes encoding photosynthetic proteins, such as the small subunit of the Calvin cycle enzyme ribulose bisphosphate carboxylase (RbcS) and the chlorophyll *a* binding protein, were shown to be repressed by carbohydrates (Sheen, 1990; Krapp et al., 1993). It is not known whether a common mechanism is responsible for the differential metabolic regulation of these genes.

Expression of genes regulated by carbohydrates is also known to be affected by various other factors. It has been shown that sugar-responsive genes may also be regulated by light (Harter et al., 1993), phosphate (Sadka et al., 1994), hormones (DeWald et al., 1994), as well as wounding and anaerobiosis (Salanoubat and Belliard,1989). Whether there is any connection between the signal transduction pathways of sugar-regulated genes and other signal-response mechanisms remains to be determined. In particular, it will be interesting to determine whether the different internal and external signals are integrated to result in the coordinate regulation of defense response and source/sink relations, as is suggested by the metabolic regulation of the corresponding genes by sugars (Sheen, 1994). To date, very little is known about intracellular signaling in plants, the molecular processes that link different stimuli, and the coordination of gene regulation that ultimately leads to the physiological responses. Signal pathways known as mitogen-activated

[1] To whom correspondence should be addressed. E-mail thomas.roitsch@biologie.uni-regensburg.de; fax 49-941-943-3352.

protein (MAP) kinase cascades have been established in animals and yeast (Pelech and Sanghera, 1992; Nishida and Gotoh, 1993). It has been shown that the corresponding kinases play a key role in the transduction of external signals. Recently, it has been shown in plants that stress-related stimuli, such as wounding and elicitors, activate specific kinases that are homologous to MAP kinases of signal transduction pathways characterized in other organisms (Seo et al., 1995; Suzuki and Shinshi, 1995; Usami et al., 1995; Bögre et al., 1997).

Pathogen infection and wounding are the most dramatic environmental stresses to which plants may be subjected. A spectrum of reactions that are sequentially activated in response to pathogens has been elucidated (Bowles, 1990; Dixon and Lamb, 1990). An important component of plant defense response is the transcriptional activation of phenylpropanoid metabolism, because the reactions are common to a range of pathways leading to functionally diverse defense-related products. A key enzyme of this pathway is phenylalanine ammonia–lyase (PAL), which catalyzes the deamination of L-phenylalanine. Elicitors, like chitosan or specific oligosaccharides, as well as phosphatase inhibitors have been shown to mimic the effect of pathogen infection in different species (Chappell and Hahlbrock, 1984; MacKintosh et al., 1994). Elicitors usually induce acidification of the cytoplasm, and it has been shown that lowering the intracellular pH with weak lipophilic acids results in higher activities of defense-related enzymes (Mathieu et al., 1994). Several lines of evidence suggest a physiological role for the weakly acidic benzoic acid in the activation of defense responses (León et al., 1993). Benzoic acid is the precursor of salicylic acid, which is assumed to be important for inducing systemic acquired resistance (Lee et al., 1995).

Activation of the defense machinery requires energy and thus the induction of sink metabolism. Accordingly, physiological studies indicate that both photosynthetic capacity and carbohydrate metabolism are altered in response to pathogens (Técsi et al., 1994; Wright et al., 1995). In most plant species, sucrose is the main transport sugar, and cleavage of this disaccharide is the initial reaction for metabolizing sucrose in sink tissues. Invertases catalyze the irreversible hydrolysis of sucrose to glucose and fructose (Avigad, 1982). Based on physiological studies, which indicated the importance of a cell wall–bound invertase in rapidly growing tissues, a model for supplying carbohydrates via apoplastic cleavage of sucrose by an extracellular invertase has been proposed (Glasziou and Gayler, 1972; Eschrich, 1980). The importance of extracellular invertase is further supported by recent studies with maize invertase mutants (Miller and Chourey, 1992; Cheng et al., 1996) and bean embryos (Weber et al., 1995). It has been shown that the mRNA for extracellular invertase of carrot is induced in response to pathogen infection and wounding (Sturm and Chrispeels, 1990), and a physiological role for this enzyme in sink/source regulation is suggested by studies with *Chenopodium rubrum* (Roitsch et al., 1995).

The reaction of suspension-cultured plant cells to fungal elicitors has been studied as a model for plant defense responses (Chappell and Hahlbrock, 1984; Dixon and Lamb, 1990; Boller, 1995). However, previous studies have been conducted with heterotrophic cultures that depended on high concentrations of sugars, which excluded the possibility of analyzing the effect of pathogens on photosynthesis and source/sink relations. This limitation was overcome in this study by using photoautotrophically growing suspension culture cells of *C. rubrum*. These cultures already proved to be an appropriate experimental system to study both the regulation of photosynthetic (Krapp et al., 1993) and sink-specific genes (Godt et al., 1995; Roitsch et al., 1995) in response to the induction of heterotrophic metabolism.

To gain insight into the possible coordinated regulation of defense reactions, photosynthetic capacity, and carbohydrate partitioning, the regulation of mRNAs for representative enzymes was analyzed. *C. rubrum* cell cultures were treated with the metabolic regulator D-glucose as well as different stress-related stimuli, including the fungal elicitor chitosan, the phosphatase inhibitor endothall, and benzoic acid. The data were complemented by analyzing wounded leaves of *C. rubrum* plants. All stimuli tested resulted in coordinated regulation of mRNAs for the three classes of enzymes analyzed. The mRNAs for both extracellular invertase and PAL were induced. In contrast, the mRNA for RbcS was repressed. Different lines of experimental evidence indicate that the carbohydrate signal and the stress-related stimuli independently activate intracellular signaling pathways. Based on the effect of inhibitors of protein phosphorylation and protein biosynthesis on the regulation of mRNA levels and on the rapid and transient activation of kinases that phosphorylate the myelin basic protein, a model for the integration of the different stimuli via signal transduction pathways involving protein phosphorylation is proposed.

RESULTS

Cloning of cDNA Fragments of PAL and RbcS from *C. rubrum*

Polymerase chain reaction (PCR)–amplified cDNA fragments of PAL and RbcS from *C. rubrum* were cloned to be used as homologous probes for the determination of mRNA levels by RNA gel blotting. Degenerate oligonucleotide primers were designed based on nucleotide sequences of the corresponding enzymes from other plants available in the EMBL data bank. They were used for reverse transcriptase–PCR by using poly(A) RNA as substrate, as described in Methods. The identity of the resulting fragments of the expected size, subcloned into pUC19, was verified by sequence analysis (data not shown).

A 700-bp fragment encoding a putative PAL from *C. rubrum* showed 73 to 82% amino acid identity to PAL sequences of

six different species. The cloned fragment represents ~60% of the full-length PAL clones. Oligonucleotide primers based on highly conserved RbcS sequences resulted in amplification of a 380-bp fragment. The derived amino acid sequence from the putative RbcS cDNA fragment from *C. rubrum* shows 76 to 84% identity to RbcS sequences from five different species. The cloned fragment represents ~30% of the full-length RbcS sequences.

Metabolic Regulation of mRNAs by D-Glucose

To address the metabolic regulation by sugars, we added 40 mM D-glucose to an autotrophically growing *C. rubrum* cell culture for 8 hr. Total RNA was isolated from the corresponding culture and from an autotrophically grown control culture. mRNA levels were determined by RNA gel blot analysis, using the homologous cDNA fragments of PAL and RbcS described above and the full-length cDNA of extracellular invertase (Roitsch et al., 1995).

As shown in Figure 1A, the low levels of mRNAs for both PAL and extracellular invertase were highly elevated by D-glucose, whereas the high steady state level of the mRNAs for RbcS was reduced. The control incubation with mannitol supports a specific effect of the sugar applied. Whereas this experiment rules out a nonspecific osmotic effect on CIN1 and RbcS mRNAs, the PAL mRNA level was induced by mannitol, although to a much lesser extent than by glucose. To analyze

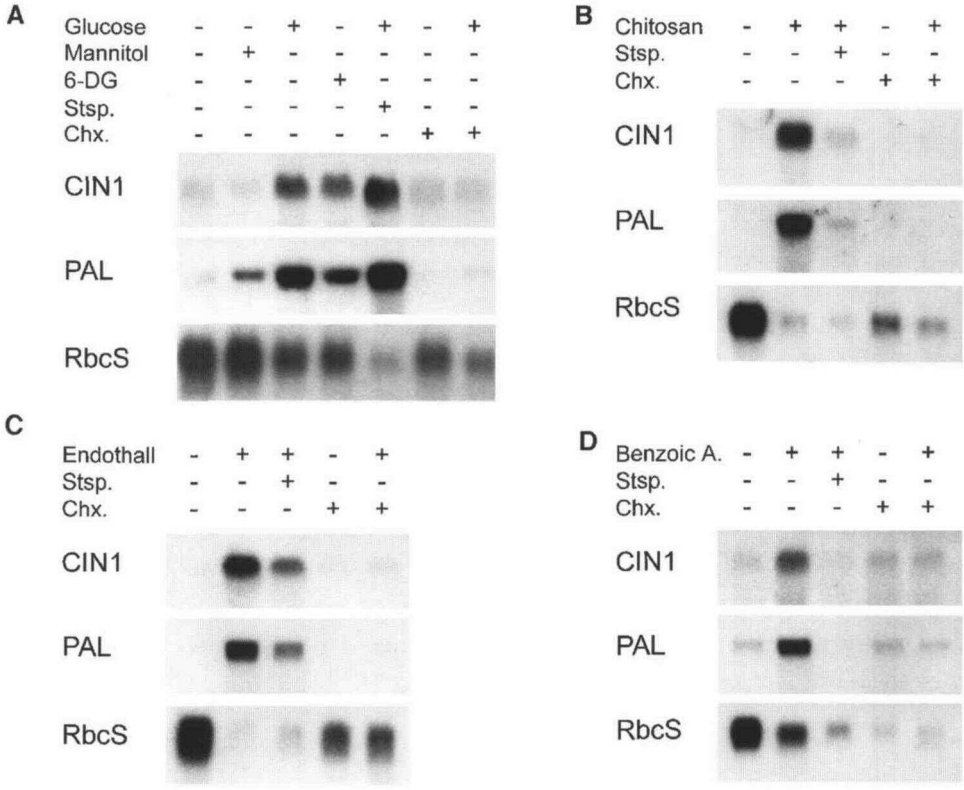

Figure 1. Coordinated Changes in Concentrations of mRNAs for Extracellular Invertase CIN1, PAL, and RbcS in Response to D-Glucose and Stress-Related Stimuli.

Shown is the effect of the kinase inhibitor staurosporine (Stsp.) and the protein biosynthesis inhibitor cycloheximide (Chx.) on regulation by the different stimuli. Total RNA was isolated and used for RNA gel blots hybridized with CIN1, PAL, and RbcS cDNA probes. (+) and (−) indicate whether the compound was or was not added, respectively. 6-DG, 6-deoxyglucose; benzoic A., benzoic acid.

(A) Suspension culture cells were treated for 8 hr with 40 mM D-glucose, 6-deoxyglucose, or mannitol in the presence or absence of 2 μM staurosporine and 5 μg/mL cycloheximide, as indicated.

(B) Suspension culture cells were treated for 8 hr with 0.1% chitosan in the presence or absence of 2 μM staurosporine and 5 μg/mL cycloheximide, as indicated.

(C) Suspension culture cells were treated for 8 hr with 50 μM endothall in the presence or absence of 2 μM staurosporine and 5 μg/mL cycloheximide, as indicated.

(D) Suspension culture cells were treated for 8 hr with 500 μM benzoic acid in the presence or absence of 2 μM staurosporine and 5 μg/mL cycloheximide, as indicated.

whether glucose or some metabolite thereof is responsible for the observed effect on the different mRNAs, the suspension culture cells were incubated with the nonmetabolizable 6-deoxyglucose. Figure 1A shows that the glucose analog, which cannot be phosphorylated, mimicked the differential effect of glucose on the level of all mRNAs analyzed.

Regulation of mRNAs by the Fungal Elicitor Chitosan

Elicitor-treated cell cultures have been successfully used to study plant defense reactions. A rise in pH of the culture media is one of the most easily measured indicators of rapid plasma membrane effects induced in plant cell cultures in response to elicitors derived from plant pathogens (Felix et al., 1991; Mathieu et al., 1994). Growth of *C. rubrum* suspension culture cells resulted in acidification of the unbuffered medium; the pH dropped from 5.7 to reach 3.5 at late-log phase. The addition of 0.1% of the fungal elicitor chitosan resulted in rapid alkalization of the culture medium by 1.9 pH units, indicating that chitosan is active as an elicitor of *C. rubrum* cells. The intracellular pH was increased by 0.75 pH units, as measured by the distribution of ^{14}C-labeled 5,5-dimethyl-2,4-oxazolidinedione (DMO; L'Allemain et al., 1984) (data not shown).

Figure 1B shows that incubation for 8 hr in the presence of chitosan resulted in highly elevated concentrations of mRNAs for extracellular invertase and the defense-related enzyme compared with the control culture. Whereas a high RbcS mRNA level was present in photoautotrophically growing cultures, the corresponding mRNA was strongly repressed in elicitor-treated cells.

Regulation of mRNAs by the Phosphatase Inhibitor Endothall

Different protein phosphatase inhibitors were shown to activate antifungal defense responses in cell cultures and tissues (Felix et al., 1994; MacKintosh et al., 1994). The herbicide endothall was shown to be a potent inhibitor of protein phosphatase PP2A. It proved to be particularly useful for intact plant cells, because it is water soluble and known to be taken up readily both by suspension culture cells and by different tissues of intact plants, whereas other phosphatase inhibitors do not appear to penetrate all types of cells (Matsuzawa et al., 1987; MacKintosh et al., 1994).

As shown in Figure 2, the incubation of the suspension culture cells in the presence of 50 μM endothall resulted in highly induced mRNA concentrations of extracellular invertase and PAL. In contrast, the mRNA for RbcS was completely repressed. Because endothall was originally used as an herbicide, we tested the effect of three additional phosphatase inhibitors and two herbicides with known mode of action to substantiate the results obtained with endothall. Figure 2 demonstrates that the phosphatase inhibitors cantharidin, okadaic acid, and calyculin resulted in the same changes in mRNA levels of extracellular invertase from *C. rubrum* (CIN1), PAL, and RbcS as occurred when endothall was used. In contrast, glufosinate ammonium and glyphosate, the active components of the herbicides Basta and Roundup, respectively, did not affect the concentrations of the mRNA levels for the enzymes analyzed (Figure 2), demonstrating that the effects of endothall on mRNA levels are related to its property as a phosphatase inhibitor.

Regulation of mRNAs by Benzoic Acid

Short chain fatty acids, such as acetate and butyrate, were shown to result in an elevated enzyme activity of PAL (Mathieu et al., 1994) and to repress the transcription of genes encoding photosynthetic proteins (Sheen, 1990, 1994). A physiological role for the weakly acidic benzoic acid as primary signal for salicylic acid synthesis and accumulation, and thus for the induction of defense reactions, such as systemic acquired resistance, has been suggested (Léon et al., 1993; Lee et al., 1995).

To analyze the response of *C. rubrum* cells to weak acids, we incubated the cells in the presence of 500 μM benzoic acid for 8 hr. Submitting the suspension culture cells to such an acid load resulted in reduction of the intracellular pH by 0.3 pH units. This intracellular acidification was accompanied by a rise of the pH of the incubation medium by 1.3 pH units (data not shown). Figure 1D shows that benzoic acid

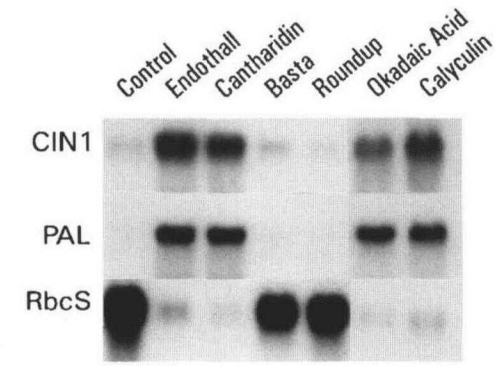

Figure 2. Coordinated Changes in Concentrations of mRNAs for Extracellular Invertase CIN1, PAL, and RbcS in Response to Endothall and Other Phosphatase Inhibitors.

Suspension culture cells were treated for 8 hr with the phosphatase inhibitors endothall (50 μM), cantharidin (50 μM), okadaic acid (0.5 μM), and calyculin (0.5 μM) and, as controls, with the active components of the herbicides Basta and Roundup, glufosinate ammonium (50 μM) and glyphosate (50 μM), respectively. Total RNA was isolated and used for RNA gel blots hybridized with CIN1, PAL, and RbcS cDNA probes.

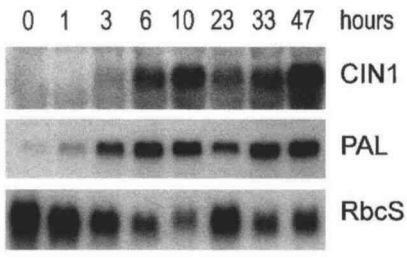

Figure 3. Coordinated Changes in Concentrations of mRNAs for Extracellular Invertase CIN1, PAL, and RbcS in Response to Wounding.

Source leaves were wounded by cutting into strips and shaken in MS medium. Samples were removed at the times indicated. Total RNA was isolated and used for RNA gel blots hybridized with CIN1, PAL, and RbcS cDNA probes.

resulted in the induction of mRNAs for CIN1 and PAL, whereas the mRNA for RbcS was reduced.

Regulation of mRNAs by Wounding

Wounding is one of the severest environmental stresses to which plants may be subjected, and it may come about through such diverse causes as mechanical injury and herbivore attack. To determine the effect of wounding on mRNA levels of the three classes of proteins analyzed, source leaves of *C. rubrum* plants were cut into strips and shaken in sugar-free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Samples were taken at various times after wounding, and total RNAs were isolated and used for RNA gel blot hybridizations. As shown in Figure 3, unwounded source leaves had no detectable mRNA for extracellular invertase and a low PAL transcript level. PAL mRNA was fully induced after 3 hr, and the level remained high throughout the experiment. After a lag time of 3 hr, the mRNA for extracellular invertase started to accumulate. The high level of RbcS mRNA in unwounded leaves was reduced after 1 hr and decreased further with longer incubation times. In the particular experiment shown in Figure 3, a transient decrease of the mRNA level for PAL and CIN1 after 23 hr was accompanied by a transient increase of the mRNA for RbcS. Although the reason for this change in mRNA levels is not clear, this observation further supports a coordinated regulation of the three classes of genes analyzed.

Concentration Dependence of mRNA Regulation

The data presented above indicate a coordinated regulation of the mRNAs for enzymes involved in source/sink relations and pathogen defense. Whereas the mRNAs for CIN1 and PAL were induced by D-glucose, chitosan, endothall, and benzoic acid, the mRNA for RbcS was repressed. To further prove the coordinated regulatory mechanism, we determined the concentration dependence of mRNA regulation in response to the various stimuli by using RNA gel blot analysis.

Figure 4 demonstrates that both the induction of mRNAs for CIN1 and PAL and the repression of RbcS mRNA showed the same concentration dependence in response to the metabolic stimulus and the three stress-related stimuli. We determined that 20 mM glucose (Figure 4A), 0.05% chitosan (Figure 4B), 25 μM endothall (Figure 4C), and 500 μM benzoic acid (Figure 4D) were required for mRNA regulation. Concentrations of >750 μM benzoic acid were found to be toxic to the cells.

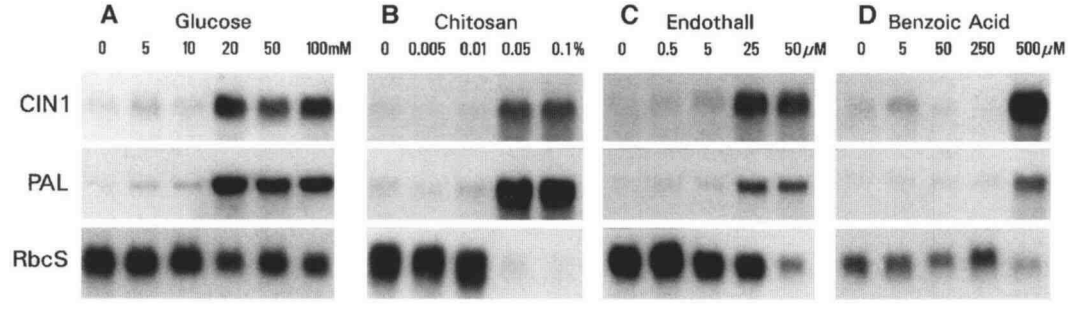

Figure 4. Concentration Dependence of Changes in Levels of mRNAs for Extracellular Invertase CIN1, PAL, and RbcS in Response to D-Glucose and Stress-Related Stimuli.

Total RNA was isolated and used for RNA gel blots hybridized with CIN1, PAL, and RbcS cDNA probes.
(A) Suspension culture cells were treated for 8 hr with glucose at the concentrations indicated.
(B) Suspension culture cells were treated for 8 hr with chitosan at the concentrations indicated.
(C) Suspension culture cells were treated for 8 hr with endothall at the concentrations indicated.
(D) Suspension culture cells were treated for 8 hr with benzoic acid at the concentrations indicated.

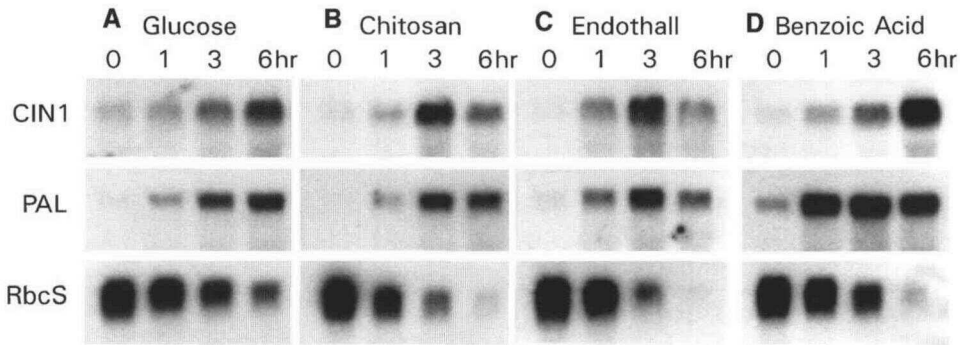

Figure 5. Time Course of Changes in Concentrations of mRNA for Extracellular Invertase CIN1, PAL, and RbcS in Response to D-Glucose and Stress-Related Stimuli.

Total RNA was isolated and used for RNA gel blots hybridized with CIN1, PAL, and RbcS cDNA probes.
(A) Suspension culture cells were treated with 40 mM glucose, and samples were removed at the times indicated.
(B) Suspension culture cells were treated with 0.1% chitosan, and samples were removed at the times indicated.
(C) Suspension culture cells were treated with 50 μM endothall, and samples were removed at the times indicated.
(D) Suspension culture cells were treated with 500 μM benzoic acid, and samples were removed at the times indicated.

Time Course of mRNA Regulation

To gain further insight into the mechanism of the coordinated regulation of CIN1, PAL, and RbcS, the time course of the effect of the various stimuli on the expression of three classes of genes was addressed by using RNA gel blot analysis. Figure 5 shows that both the metabolic regulator D-glucose (Figure 5A) and the stress-related stimuli chitosan (Figure 5B), endothall (Figure 5C), and benzoic acid (Figure 5D) affected mRNA levels for CIN1, PAL, and RbcS with comparable time courses. The mRNA concentrations of extracellular invertase and the defense-related protein increased after 1 hr and were fully induced after 3 to 6 hr. In contrast, the mRNA level of the photosynthetic protein was inversely regulated. Repression of RbcS mRNA was detectable after 1 hr, and the mRNA level further declined for up to 6 hr.

Effect of Inhibition of Protein Biosynthesis on the Regulation of mRNAs

To further characterize the regulation of mRNA levels by the various stimuli, we analyzed whether protein synthesis is involved in signal transduction. To analyze the effect of the protein biosynthesis inhibitor cycloheximide on *C. rubrum* suspension culture cells, we measured protein biosynthesis and quantified the inhibition by cycloheximide. Table 1 shows that cycloheximide resulted in a concentration-dependent inhibition of incorporation of ^{35}S-methionine into trichloroacetic acid (TCA)–precipitable material. Cycloheximide (5 μg/mL) resulted in 89% inhibition, which was not significantly increased at a 10-fold higher cycloheximide concentration.

Cells were treated with the different stimuli in the presence or absence of 5 μg/mL cycloheximide for 6 hr. Cycloheximide inhibited the induction of CIN1 and PAL by D-glucose, chitosan, endothall, and benzoic acid (Figures 1A to 1D, respectively). Control incubations show that cycloheximide alone had no effect on the low levels of mRNAs for CIN1 and PAL. These results indicate that de novo protein biosynthesis is required for transcriptional activation of the corresponding genes by the different stimuli. Cycloheximide alone reduced the mRNA level of RbcS; therefore, whether the repression of mRNA for RbcS by the different stimuli depended on protein biosynthesis could not be addressed.

Because cycloheximide repressed RbcS mRNA levels in control cells and inhibited induction of CIN1 and PAL by the different stimuli, experiments were conducted to rule out the possibility that cycloheximide results in general inhibition of mRNA synthesis. Nuclei were isolated from cycloheximide-treated samples, and control incubations and transcriptional

Table 1. Concentration-Dependent Effect of Cycloheximide on Protein Synthesis[a]

Cycloheximide (μg/mL)	*Inhibition of Protein Biosynthesis* (% Inhibition Compared with Control Cells)
0.005	20
0.05	45
0.5	83
5	89
50	91

[a] Cells were incubated for 15 min in the presence of cycloheximide, at the concentration indicated. After pulse labeling with ^{35}S-methionine for 5 min, the incorporation of label into TCA-precipitable material was determined in a scintillation counter. The results shown are the mean values of two independent experiments, and the deviations found were <10%.

activities were measured by run-off transcription assays. Total transcriptional activity was inhibited by 2, 4, and 11% in cells incubated for 1, 3, and 6 hr with cycloheximide, respectively. Control incubations with α-amanitin, a specific inhibitor of RNA polymerase II required for mRNA synthesis, revealed that cycloheximide inhibition does not specifically affect mRNA synthesis. Whereas 51% of the transcriptional activity of control cells was α-amanitin sensitive, the corresponding value for cells incubated for 1, 3, and 6 hr with cycloheximide was 48, 42, and 53%, respectively. The results are the mean values of two independent experiments, and deviations found were <15% for the transcriptional activity and <10% for the α-amanitin inhibition. The finding that the mRNA level for actin, chosen as a representative of a housekeeping gene, was not repressed by cyclohexmimide (data not shown) further proves that treatment with cycloheximide does not result in general inhibition of transcription.

Differential Effect of the Protein Kinase Inhibitor Staurosporine on the Regulation of mRNAs

Protein kinase inhibitors were shown to inhibit specific responses of suspension culture cells to elicitors. These responses include induction of mRNA levels of pathogenesis-related enzymes (Suzuki et al., 1995), protein phosphorylation (Felix et al., 1991), and extracellular alkalization (Mathieu et al., 1994). This indicates that the corresponding signal transduction pathways involve protein phosphorylation. To gain insight into corresponding signal transduction pathways, we tested whether the protein kinase inhibitor staurosporine affects the regulation of mRNA levels by D-glucose and the three stress-related stimuli tested.

It has been shown previously that staurosporine inhibits plant protein kinases in vitro and phosphorylation of proteins of suspension culture cells in vivo (MacKintosh et al., 1994). To characterize the effect of this kinase inhibitor on *C. rubrum* suspension culture cells, the effect of 2 μM staurosporine on total kinase activity in crude extracts was measured using three different substrates. The products of in vitro phosphorylation assays were separated by SDS-PAGE, and the inhibition of kinase activities was evident from a reduced phosphorylation of the myelin basic protein, casein, and histone (Figure 6A). The results of the experiment shown in Figure 6A have been quantified; inhibition of total phosphorylating activities in crude extracts was 66% for casein, 87% for histone, and 75% for the myelin basic protein. The inhibition of protein kinase activities was further supported by the finding that staurosporine resulted in a concentration-dependent inhibition of incorporation of ^{32}P-orthophosphate into proteins. Figure 6B shows that maximum inhibition was found at 2 μM staurosporine. Figure 6C demonstrates that staurosporine did not affect the concentration of mRNAs for CIN1, PAL, and RbcS in control cells.

Figure 1 shows that staurosporine inhibited the induction of the genes encoding extracellular invertase and PAL by

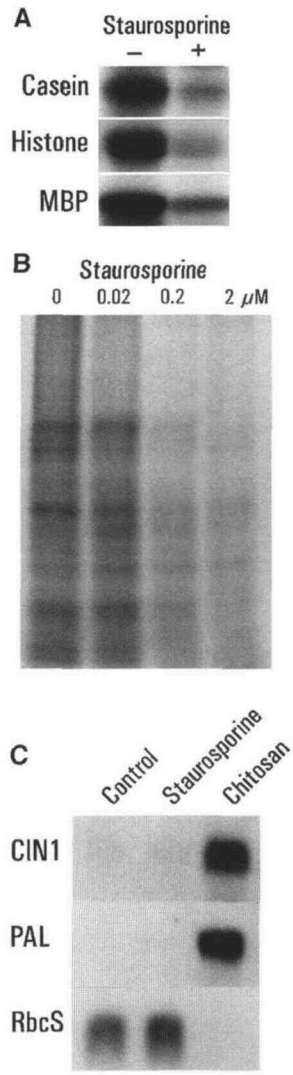

Figure 6. Controls to Demonstrate That Staurosporine Specifically Acts as Kinase Inhibitor under the Experimental Conditions Used.

(A) Effect of staurosporine on total kinase activity. Cells were incubated for 15 min in the absence (–) or presence (+) of 2 μM staurosporine. Crude extracts were prepared, and total kinase activity was tested with casein, histone, or myelin basic protein (MBP), respectively. The reaction mixtures were separated by SDS-PAGE, and the phosphorylated substrates were visualized by autoradiography.

(B) Effect of staurosporine on protein phosphorylation. Cells were incubated for 15 min in the absence or presence of 0.02, 0.2, and 2 μM staurosporine. After pulse labeling with ^{32}P-orthophosphate for 12 min, TCA-precipitable material was separated by SDS-PAGE, and phosphorylated proteins were visualized by autoradiography.

(C) Effect of staurosporine on mRNA levels of CIN1, PAL, and RbcS. Suspension culture cells were treated for 8 hr with 2 μM staurosporine or 0.1% chitosan as positive control. Total RNA was isolated and used for RNA gel blots hybridized with CIN1, PAL, and RbcS cDNA probes.

chitosan, endothall, and benzoic acid (Figures 1B to 1D, respectively). In contrast, in the presence of the kinase inhibitor, the glucose induction of mRNAs for the two enzymes was enhanced (Figure 1A). The differential effect of the kinase inhibitor demonstrates that D-glucose and the stress-related stimuli activate independent signal transduction pathways. This result also provides additional evidence that CIN1 and PAL are coordinately regulated and that the metabolic and stress-related signals are ultimately integrated. Figures 1 and 6 show that staurosporine alone did not affect RbcS mRNA but synergistically enhanced the repression by the different stimuli, which was particularly evident in the presence of D-glucose or benzoic acid.

Both D-Glucose and Stress-Related Stimuli Induce Rapid and Transient Activation of Protein Kinases

MAP kinases play a key role in signal transduction cascades of animals and yeast. They are rapidly and transiently activated and are characterized by phosphorylating the myelin basic protein. Protein phosphorylation was shown to be involved in the response of plant cells to elicitors (Dietrich et al., 1990), and only recently has the activation of specific plant MAP kinases in response to different stress-related stimuli, such as wounding and elicitor treatment, been demonstrated (Seo et al., 1995; Suzuki and Shinshi, 1995; Usami et al., 1995; Bögre et al., 1997).

An in-gel kinase assay was used to determine whether the different stimuli used throughout this study activate MAP kinases. *C. rubrum* suspension culture cells were treated with D-glucose, and the three stress-related stimuli and samples were removed after various incubation times. Protein extracts were prepared, and proteins were subjected to electrophoresis in SDS–polyacrylamide gels that contained the myelin basic protein. After denaturation of the proteins in the gels with guanidine hydrochloride and renaturation, we determined the kinase activities of the different extracts. D-Glucose (Figure 7C), chitosan (Figure 7D), and endothall (Figure 7E) resulted in rapid and transient activation of protein kinases with apparent molecular masses of 44 and 46 kD. Maximum activity was observed after 10 min, and the activity fell to background levels within 60 min. No kinases were activated in response to benzoic acid (Figure 7F). Control incubations were performed with water (Figure 7A) and mannitol (Figure 7B). A number of other faint bands with different mobilities were also detected, but their activities remained substantially unchanged by treatment with the different stimuli, and their activities varied between different experiments.

To gain insight into the specificity of the kinases activated by glucose, chitosan, and endothall, we performed in-gel kinase assays with two additional protein kinase substrates. No kinases were found to be activated in response to glucose, chitosan, and endothall when SDS–polyacrylamide gels were used that contained casein or histone as substrates, supporting the specificity of the activated kinases for the myelin basic protein (data not shown). Further control experiments using polyacrylamide gels without myelin basic protein revealed that the kinases activated by the different stimuli phosphorylate the myelin basic protein. In these gels, a nonregulated phosphorylating activity with a molecular mass of 35 kD could be identified as an autokinase (data not shown).

DISCUSSION

D-Glucose and Defense-Related Stimuli Activate Different Signal Transduction Pathways

There is increasing evidence that sugars not only function as carbon source for heterotrophic growth of plant tissues but affect sugar-sensing systems that initiate changes in gene expression (Koch, 1996). Jang and Sheen (1994) have proposed a model in which they postulate a sugar regulation mechanism that acts as a gene expression switch to facilitate cell defense responses. This model was supported by a recent study with sugar-accumulating transgenic plants (Herbers et al., 1996a). The essential assumption of this model is that the initial induction of an extracellular invertase by wounding or pathogen infection results in elevated sugar concentrations, which in turn are responsible for transcriptional repression of photosynthetic genes and for activation of defense-related genes. Thus, stress induction of extracellular invertase has to precede the sugar regulation of the other two classes of enzymes, which is a secondary effect according to this model.

It has been speculated that glucose functions as an extracellular indicator for pathogen infection. However, several lines of evidence obtained in this study indicate that D-glucose and stress-related stimuli, such as elicitors or wounding, activate independent signal transduction pathways.

The time course experiments with stress-related stimuli clearly demonstrate that induction of extracellular invertase does not precede the regulation of PAL and RbcS. In suspension cultures of *C. rubrum*, the expression of extracellular invertase was coordinately regulated with the expression of PAL and RbcS by the fungal elicitor chitosan. The same regulatory pattern was obtained with benzoic acid and the phosphatase inhibitor endothall. In wounded leaves of *C. rubrum* plants, the regulation of RbcS and PAL was even faster than was the induction of extracellular invertase. The latter result is supported by the finding that in carrot tissues, PAL induction also preceded the induction of extracellular invertase in response to wounding (Sturm and Chrispeels, 1990).

Because the experiments were performed with photoautotrophic suspension culture cells, an elevated level of extracellular invertase cannot result in an increased supply of sugars into the cells and thus lead to elevated intracellular sugar concentrations. In contrast to plant tissues, which may increase their sink strength and thus the import of sugar from the apoplast into the cytosol by an elevated level of extracellular invertase, there is no sucrose exogenously

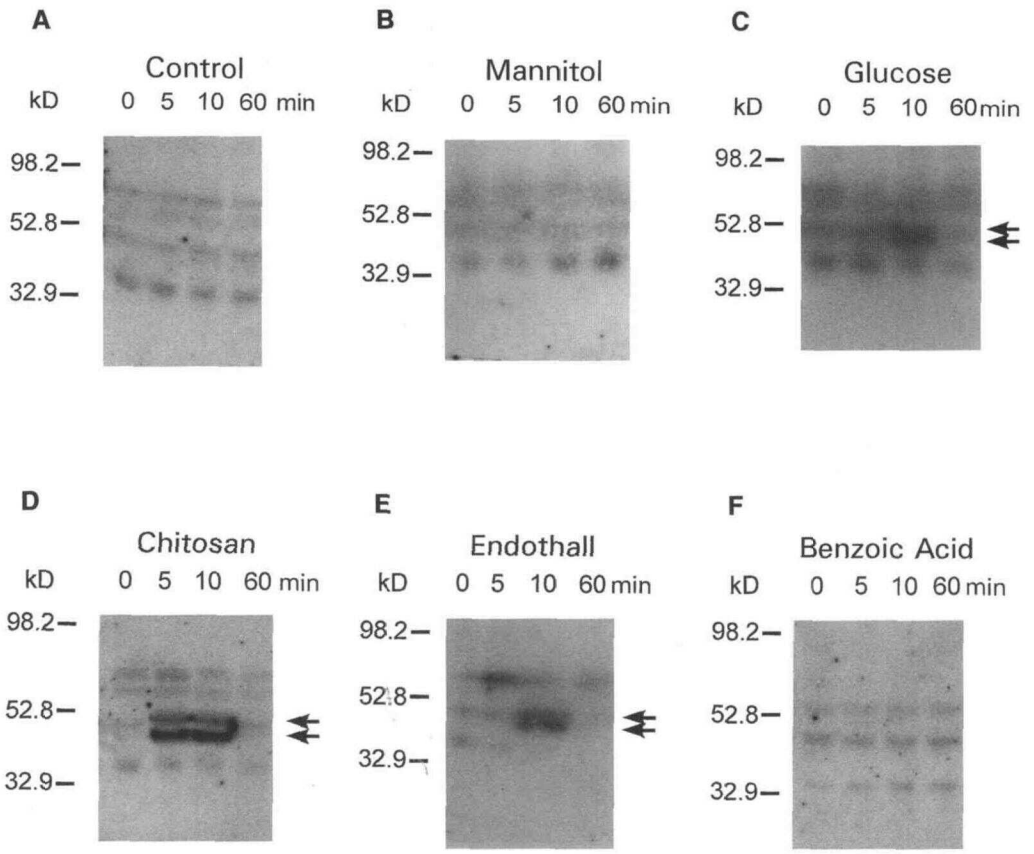

Figure 7. *Activation of Kinases That Phosphorylate the Myelin Basic Protein.*

Samples were removed at the times indicated, and crude extracts were prepared for in-gel kinase assays, as described in Methods. The positions of activated kinases are marked by arrows.

(A) Suspension culture cells were treated with water.

(B) Suspension culture cells were treated with 40 mM mannitol.

(C) Suspension culture cells were treated with 40 mM glucose.

(D) Suspension culture cells were treated with 0.1% chitosan.

(E) Suspension culture cells were treated with 50 μM endothall.

(F) Suspension culture cells were treated with 500 μM benzoic acid.

available in the suspension culture. If glucose alone were responsible for induction of defense reactions and repression of photosynthetic genes, then stress-related signals should have no effect on the corresponding mRNA levels in the autotrophic cultures. This is in contrast to the results obtained.

The protein kinase inhibitor staurosporine differentially affected the regulation by glucose and by stress-related signals. Whereas staurosporine inhibited the inducing effect of chitosan, endothall, and benzoic acid on CIN1 and PAL mRNA levels, the metabolic induction by glucose was enhanced. These results indicate that the metabolic signal D-glucose and the stress-related stimuli activate different and independent signal transduction pathways.

These data strongly suggest that an elevated glucose concentration may not be the extracellular signal in response to pathogen infection that activates defense reactions and represses photosynthetic genes, as suggested before by Jang and Sheen (1994). Although all data obtained with *C. rubrum* support previous results obtained with maize protoplasts (Jang and Sheen, 1994; repression of photosynthetic genes by sugars) and transgenic plants overexpressing a heterologous invertase (Herbers et al., 1996a; inverse regulation of photosynthetic genes and defense-related genes by sugars, repression, and induction, respectively), this study shows that sugar regulation is only one part of a more complex regulatory pattern. Two different signaling pathways are activated independently by the metabolic regulator glucose and by stress-related stimuli.

Coordinated Regulation of mRNAs for Enzymes of Sink Metabolism, Photosynthesis, and Defense Response by D-Glucose and Defense-Related Stimuli

Although the metabolic signal D-glucose and stress-related stimuli activate independent signal transduction pathways, as discussed above, the different stimuli coordinately regulate mRNAs for representative reporters for defense response as well as source and sink metabolism. The results obtained with suspension culture cells that were treated with D-glucose, chitosan, endothall, and benzoic acid are supported by analyzing wounded leaves of *C. rubrum* plants. The mRNA for PAL, a key enzyme of plant defense, and the mRNA for the sink-specific extracellular invertase were induced. In contrast, the mRNA for RbcS was repressed. The coordinated regulation mechanism is strongly supported by the findings that the regulation of mRNAs for CIN1, PAL, and RbcS in suspension culture cells showed the same concentration dependence and time course in response to both the metabolic stimulus D-glucose and the three stress-related stimuli tested.

There is increasing evidence for the importance of extracellular cleavage of sucrose by extracellular invertase to supply carbohydrates to sink tissues via an apoplastic pathway (Miller and Chourey, 1992; Weber et al., 1995). Although a number of cDNAs and genes for invertases have been cloned in recent years, little is known about the possible regulation by external or internal stimuli. The data presented here demonstrate that the mRNA for extracellular invertase of *C. rubrum* is induced by different stress-related signals. We found that the fungal elicitor chitosan, the phosphatase inhibitor endothall, and benzoic acid resulted in highly elevated mRNA levels. The observation that extracellular invertase of *C. rubrum* is wound inducible is supported by a previous study on the corresponding carrot isoenzyme (Sturm and Chrispeels, 1990). The finding that the mRNA for the cell wall invertase of *C. rubrum* was metabolically induced by glucose or a glucose analog has been reported before from our laboratory (Roitsch et al., 1995).

To address the regulation of photosynthesis, we analyzed the regulation of mRNA for RbcS. This study demonstrates that stress-related signals affect photosynthetic gene expression. RbcS mRNA levels were repressed in cell cultures treated with chitosan, benzoic acid, and all tested phosphatase inhibitors. In addition, we showed that RbcS transcripts were also reduced in wounded leaves of *C. rubrum* plants. The inhibitory effect of the protein phosphatase inhibitors okadaic acid and calyculin on RbcS expression has been observed before, leading to the suggestion that protein phosphatase activity is required for light-inducible gene expression (Sheen, 1993). However, our study indicates that the effect of the phosphatase inhibitors may be related to a general stress effect. Repression of photosynthetic genes by acetate and other short chain fatty acids has been reported and has been interpreted as the result of a metabolic regulation (Sheen, 1994). However, the data obtained with benzoic acid indicate that this regulatory pattern is more likely the effect of weak acids. This interpretation is supported by the finding that high concentrations of the auxin 2,4-D as well as the corresponding, biologically inactive analog 3,5-D have the same effect on RbcS mRNA levels (data not shown). Whereas the results presented here showed that the nonmetabolizable glucose analog 6-deoxyglucose resulted in repression of mRNA for RbcS, this nonphosphorylatable glucose analog was shown to have no effect on RbcS transcript levels in a previous study with *C. rubrum* suspension culture cells (Krapp et al., 1993) and on promotor activity of another photosynthetic gene in electroporated maize protoplasts (Sheen, 1990). Because the concentrations and incubation times of the different studies were comparable, the reason for these contradictory results remains to be elucidated and may be related to the particular experimental design. Figure 1A demonstrates that in our study, the repression of RbcS mRNA level by 6-deoxyglucose is consistent with the induction of CIN1 and PAL mRNAs by this glucose analog.

The expression of PAL has been analyzed as a representative of genes that are involved in pathogen defense. The induction of PAL mRNA by D-glucose is a novel finding. Because the mRNAs for other enzymes involved in defense responses, such as chalcone synthase (Tsukaya et al., 1991), proteinase inhibitor II (Johnson and Ryan, 1990), and pathogenesis-related proteins (Herbers et al., 1996b), are also inducible by sugar, this may be a general property of such enzymes. PAL mRNA was also induced by a weak acid and a phosphatase inhibitor in *C. rubrum*. This finding demonstrates that the observed higher PAL enzyme activity in response to these stimuli (Felix et al., 1994; MacKintosh et al., 1994) is due to increased transcript concentrations. The inducing effect of chitosan and wounding on PAL mRNA supports previous findings from other plants (Chappell and Hahlbrock, 1984; Schmelzer et al., 1989).

As summarized in Table 2, new data on the regulation of mRNA for enzymes involved in sink metabolism, photosynthesis, and defense reactions are presented in this study. In particular, so far only limited information was available on the regulation of extracellular invertase and photosynthetic genes in response to stress-related signals. Previous studies involved both a variety of experimental systems as well as different experimental approaches. This situation made comparisons and general conclusions difficult. In addition, heterotrophic cultures were used to study the effect of elicitors (Chappell and Hahlbrock, 1984; Dixon and Lamb, 1990; Boller, 1995), excluding the possibility to address simultaneously the regulation of sink and source metabolism and to test the effect of the metabolic regulator glucose. This limitation was overcome in this study by the use of photoautotrophically grown suspension culture cells of *C. rubrum;* a comprehensive set of data could be obtained with a single experimental approach. The fact that all data obtained with *C. rubrum* are compatible with available literature data strongly supports the proposed coordinated regulation.

Table 2. Regulation of mRNAs for Enzymes Involved in Sink Metabolism, Defense Response, and Photosynthesis by D-Glucose and Stress-Related Stimuli

Stimulus	Gene	Reference
Induction of sink metabolism		
D-Glucose	Susy[a]	Salanoubat and Belliard (1989)
	CWI[b]	Roitsch et al. (1995)
Glucose analog	CWI	Roitsch et al. (1995)
Elicitor	CWI	This work
Weak acid	CWI	This work
Phosphatase inhibitor	CWI	This work
Wounding	CWI	Sturm and Chrispeels (1990)
Pathogen infection	CWI	Sturm and Chrispeels (1990)
Induction of defense response		
D-Glucose	PI[c]	Johnson and Ryan (1990)
	PAL	This work
Glucose analog	PAL	This work
Elicitor	PAL	Chappell and Hahlbrock (1984)
Weak acid	PAL	This work
Phosphatase inhibitor	PAL	This work
Wounding	PAL	Lawton and Lamb (1987)
Pathogen infection	PAL	Schmelzer et al. (1989)
Repression of photosynthesis		
D-Glucose	RbcS	Sheen (1990)
Glucose analog	RbcS	This work
Elicitor	RbcS	This work
Weak acid	RbcS	Sheen (1990; acetate) This work (benzoic acid)
Phosphatase inhibitor	RbcS	Sheen (1993; okadaic acid and calyculin) This work (endothall and cantharidin)
Wounding	RbcS	This work
Pathogen infection	RbcS	Mouly and Roby (1988)

[a] Susy, sucrose synthase.
[b] CWI, cell wall invertase.
[c] PI, proteinase inhibitor.

The observed inverse regulation of transcripts for a photosynthetic enzyme and for a pathogenesis-related protein by D-glucose supports results obtained with sugar-accumulating transgenic plants (Herbers et al., 1996a). Our study shows that extracellular invertase, chosen as a representative of sink-specific enzymes, is also coordinately regulated by sugar with the other two classes of enzymes. Thus, the data indicate that repression of photosynthesis is accompanied by an induction of heterotrophic metabolism, which shows that the corresponding regulatory pattern may affect source/sink relations. However, as discussed above, stress signals also independently regulate the corresponding genes.

A common mechanism for the coordinated induction of mRNAs for extracellular invertase and defense-related enzymes is further supported by the identification of common *cis*-acting regulatory sequences. The promoter of the extracellular invertase gene of carrot contains specific sequences that are highly homologous to elicitor-, wound-, and stress-responsive elements in the promoter of defense-related enzymes such as PAL and proteinase inhibitor (Ramloch-Lorenz et al., 1993).

Both benzoic acid and the phosphatase inhibitor endothall were shown to mimic the effects of the fungal elicitor chitosan and wounding. Elicitor treatment of *C. rubrum* cells resulted in extracellular alkalization and intracellular acidification, and it has been proposed that cytosolic protons may function as secondary messengers in elicitor-induced defense responses (Mathieu et al., 1994), although an independent function in secondary metabolism was also proposed (Hagendoorn et al., 1994). The pH changes could be simulated by subjecting the *C. rubrum* cells to an acid load by treatment with benzoic acid, resulting in intracellular acidification. In addition, a direct physiological role for benzoic acid, a precursor of salicylic acid, has been suggested for the induction of pathogenicity-related proteins and disease resistance (León et al., 1993; Lee et al., 1995). The results presented here not only further support the possible function of benzoic acid in defense responses but also indicate that benzoic acid may act directly as an intracellular regulator. The latter suggestion is supported by the observation that, in contrast to the other stress-related stimuli tested, benzoic acid did not activate a MAP kinase and thus may bypass initial steps of the stress signal transduction pathway. At present, it cannot be distinguished whether the phosphatase inhibitor endothall unspecifically functions as elicitor or specifically interferes with a component of the signal transduction chain that is regulated by protein phosphorylation/dephosphorylation.

The effect of D-glucose on mRNA levels for the three different classes of enzymes analyzed could be mimicked by the glucose analog 6-deoxyglucose, which cannot be phosphorylated. This observation indicates that glucose, and not one of its metabolites, may be the primary signal that interacts with a putative receptor involved in transduction of the carbohydrate signal. In addition, these findings indicate that hexokinase may not be involved in sugar signal transduction, which is supported by other studies involving nonmetabolizable glucose analogs (Godt et al., 1995; Roitsch et al., 1995; Martin et al., 1997) or targeted expression of yeast invertase in transgenic tobacco plants (Herbers et al., 1996a). However, it has been reported in two previous studies that the expression of photosynthetic genes is not affected by 6-deoxyglucose (Krapp et al., 1993; Jang and Sheen, 1994) and that hexokinase functions as a sugar sensor in higher plants (Jang et al., 1997). With respect to this conflicting data, the possible role of hexokinase in sugar signaling remains to be elucidated.

Induction by Glucose and Stress-Related Stimuli Involves Protein Phosphorylation and de Novo Protein Biosynthesis

MAP kinases are important transducers of intracellular signals via protein phosphorylation that is initiated by various extracellular stimuli. Our study provides new evidence that MAP kinases exist and function in different intracellular signal transduction pathways of plants. This contributes to the increasing evidence that MAP kinases of plants play the same key roles in a wide variety of responses to extracellular signals, as has been shown in animals and yeast (Hirt, 1997). Although many putative kinases have been cloned from different plants (Jonak et al., 1994) and the importance of protein phosphorylation in conjunction with the activation of defined defense responses has been shown (Dietrich et al., 1990; Grosskopf et al., 1990; Felix et al., 1991), only a few examples have so far demonstrated the involvement of specific kinases in plant signal transduction (Seo et al., 1995; Suzuki and Shinshi, 1995; Usami et al., 1995; Bögre et al., 1997).

Protein kinase activities can be distinguished on the basis of molecular mass by an in-gel kinase assay using myelin basic protein–containing polyacrylamide gels for SDS-PAGE (Mizoguchi et al., 1994). A rapid and transient activation of myelin basic protein kinase activity was observed in cell cultures in response to D-glucose, chitosan, and endothall. Within 5 to 10 min, each of these stimuli activates proteins with apparent molecular masses of ~44 and 46 kD. These data support and extend previous studies demonstrating that plant protein kinases of 46 or 47 kD that phosphorylate the myelin basic protein are rapidly and transiently activated by wounding and elicitor treatment (Seo et al., 1995; Suzuki and Shinshi, 1995; Usami et al., 1995; Bögre et al., 1997). The data obtained with *C. rubrum* suspension cultures show that not only stress-related signals, such as chitosan and endothall, but also the metabolic regulator D-glucose result in activation of MAP kinases with similar time course. Because D-glucose and the stress-related signals activate different signal transduction pathways, *C. rubrum* may contain different isoforms of MAP kinases with similar molecular masses, which was indeed shown for tobacco (Usami et al., 1995). A sugar-induced increase of a membrane-associated autokinase with an apparent molecular mass of 54 kD has been reported from tobacco (Ohto and Nakamura, 1995). However, the time course of activation indicates that this protein may not be involved in signal transduction pathways leading to elevated mRNA levels within 1 hr. Although the RNA gel blot data obtained with benzoic acid correspond to data obtained with chitosan and endothall, this weak acid did not result in activation of a protein kinase. This finding may be related to the possible function of benzoic acid as an intracellular inducer for the activation of a defense-related pathway, as discussed above.

The critical function of protein phosphorylation in transduction of stress-related signals is further supported by the finding that the kinase inhibitor staurosporine inhibited induction of extracellular invertase and of PAL by chitosan, benzoic acid, and endothall. Because induction by glucose was enhanced, the kinases activated by this stimulus seem to be insensitive to the kinase inhibitor used.

Experiments involving cycloheximide indicate that protein biosynthesis is required for the signal transduction chain that ultimately leads to transcriptional regulation of the different genes. This is indirectly supported by the time course experiments demonstrating that only after a lag of 1 hr may an influence on mRNA levels be observed.

Based on the data on the regulation of mRNAs for representative enzymes presented here, the model shown in Figure 8 for the coordinated regulation of sink metabolism, defense-related genes, and photosynthetic genes is proposed. D-Glucose and stress-related signals activate different signal transduction pathways, which are distinguished by the effect of staurosporine. The signals are ultimately integrated to result in coordinated regulation of sink metabolism, photosynthesis, and pathogen defense. Both pathways include steps that require de novo protein biosynthesis. It is suggested that the transduction chains are further split into two pathways: one induces sink metabolism and defense response, and the other represses photosynthesis. Although we have no experimental evidence that the stimulating and the inhibitory pathways diverge from the same signal transduction chains, this regulatory mechanism would be the most economical way to integrate the different signals. This model is compatible with the available literature data on the regulation of extracellular invertases and defense-related and photosynthetic genes, including transgenic plants overexpressing yeast invertase (Herbers et al., 1996a) or *Escherichia coli* pyrophosphatase (Herbers et al., 1995). According to our model, extracellular invertase performs a dual function. It provides carbohydrates to establish and maintain sink metabolism, and it keeps the system induced by elevated sugar concentrations.

Because it is assumed that different pathways may lead to the activation of plant defense reactions (Dixon and Lamb, 1990; Suzuki et al., 1995), the proposed mechanism may be only part of an even more complex regulatory network. The differences of the time course of mRNA regulation in cell cultures and wounded plant tissues indicate that additional factors may specifically modulate the expression of individual genes. Such regulatory mechanisms may involve plant hormones, because it has been shown that auxin levels are influenced by wounding (Thornburg and Li, 1991) and that auxin modulates sugar-induced gene expression (DeWald et al., 1994). Additional work is necessary to further elucidate the complex signal transduction network involved in regulating source/sink relations and defense reactions and to identify individual components of the corresponding signal transduction chains. Our study provides the basis for future studies and shows that photoautotrophic suspension culture cells are an appropriate model system in which to

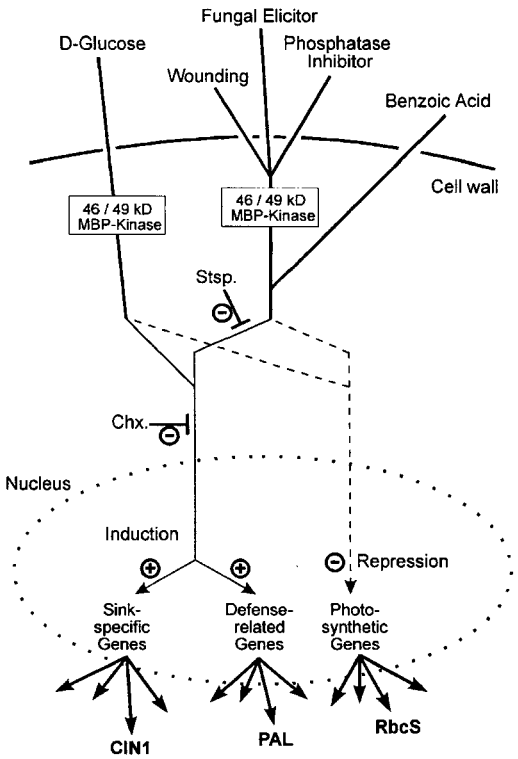

Figure 8. Model for the Regulation of Sink Metabolism, Photosynthesis, and Defense Response by D-Glucose and Stress-Related Stimuli.

The different signal transduction pathways are ultimately integrated to coordinately regulate gene expression. Intracellular signaling involves the activation of protein kinases, protein biosynthesis, and staurosporine-sensitive kinases, as indicated. Chx., cycloheximide; MBP, myelin basic protein; Stsp., staurosporine.

analyze the effects of various stimuli on carbohydrate metabolism and defense reactions.

METHODS

Plant Materials and Cell Culture

The photoautotrophic hormone and vitamin-independent suspension culture of *Chenopodium rubrum* (fat hen or goosefoot; Hüsemann, 1981) is a derivative of the culture established by Hüsemann and Barz (1977) and has been subcultured in our laboratory since 1991 (Roitsch and Tanner, 1994). The cells were grown in Murashige and Skoog (MS) medium without any organic constituents (Murashige and Skoog, 1962) in constant light (10 W m^{-2}) in two-tier Erlenmeyer flasks and at atmospheric CO_2 concentration of 2%, according to Hüsemann (1981). The cells were subcultured every 2 weeks and used for the experiments in the late-logarithmic growth phase at days 10 to 13 after subculturing. *C. rubrum* plants were grown in the greenhouse with additional illumination for 14 hr.

Treatment with Various Stimuli and Inhibitors

Suspension culture cells were treated with 40 mM D-glucose, 0.1% chitosan (Roth, Karlsruhe, Germany), 50 μM endothall (Biomol, Hamburg, Germany), or 500 μM benzoic acid, unless otherwise stated. To rule out variations between individual cultures, before the experiment we pooled the number of cultures required for a specific experiment and redistributed them into individual culture flasks. Thus, the populations of suspension culture cells in the control flask and the variously treated flasks were identical. For experiments involving staurosporine (Boehringer Mannheim) or cycloheximide, the inhibitors were added first, and the cultures were incubated for 15 min before the stimuli were added. Control cultures contained the same volume of the corresponding solutes ethanol (benzoic acid), DMSO (staurosporine), or water (all other stimuli). For wounding experiments, we cut leaves into small strips and shook them in MS medium without organic compounds and sugar at room temperature.

Pulse Labeling of Proteins with ^{35}S-Methionine

Aliquots (200 μL) of suspension culture cells in 1.5-mL reaction tubes (50 mg of cells) were pulse labeled with 0.4 MBq ^{35}S-methionine (Amersham) for 25 min. Incorporation of radioactive label in total cellular proteins was determined by precipitation with trichloroacetic acid (TCA), with bovine serum protein used as a carrier. Reactions were terminated by adding 75-μL aliquots of the reactions to 500 μL of ice-cold 0.1 mg/mL BSA containing 0.02% NaN_3, and incubation continued for 30 min on ice. Cells were transferred into 15-mL conical tubes, 1000 μL of 10% TCA was added to increase the volume, and cells were disrupted by sonicating two times for 20 sec at 20 W with a sonifier (Branson model B-12; G. Heinemann, Schwäbisch Gmünd, Germany). The suspension was filtered through a filtration apparatus under vacuum onto glass microfiber filter disks (Whatman GF/A, Maidstone, UK). The discs were washed twice with 5 mL of ice-cold 10% TCA and once with 5 mL of ethanol and air dried. Radioactivity was determined by scintillation counting.

Pulse Labeling of Phosphoproteins

Aliquots (300 μL) of suspension culture cells in 1.5-mL reaction tubes (70 mg of cells) were pulse labeled with 0.4 MBq carrier-free inorganic ^{32}P-phosphate (Amersham) for 12 min. Reactions were stopped by the addition of 300 μL of 10% TCA containing 10 mM ATP. Cell suspensions were transferred into 5-mL glass reaction tubes, and cells were disrupted by incubation in a sonicator bath (Branson model B-220; Ultrasonics, New York, NY) for 15 min. Samples were centrifuged for 5 min at 12,000*g* in a microcentrifuge. Pellets were washed with 600 μL of 80% acetone and extracted with 80 μL of SDS-PAGE sample buffer (Laemmli, 1970) at 56°C for 15 min and 95°C for 2 min. After centrifugation, 30-μL aliquots of the supernatants were subjected to SDS-PAGE (Laemmli, 1970) and autoradiography.

Reverse Transcriptase–Polymerase Chain Reaction Amplification, Cloning, and Sequence Analysis

Homologous cDNA fragments encoding phenylalanine ammonia–lyase (PAL) and the small subunit of ribulose bisphosphate carboxylase (RbcS) from *C. rubrum* were amplified by reverse transcriptase–polymerase chain reaction. Nucleotide sequences of the degenerate

oligonucleotides were based on available amino acid sequences of the corresponding proteins from other plant species. Primers used for amplification were ORB1 (CAAT/CGGA/TGGA/CA/CGA/TGTA/T/CA/CA/GA/GTGCATG) and ORB2 (GGC/TTTT/GT/GAG/AGCG/AATGAAG/ACTGA) for RbcS, and PAL1 (GGC/AATC/TAGATTTGAAATC/TTTA/GGAAGC) and PAL2 (GCCTTGTTT/CCTT/CGAAACATCG/AAT) for PAL. Synthesis of cDNA first strand using mRNA as substrate and amplification of cDNA sequences by polymerase chain reaction (PCR) were performed as described previously (Roitsch et al., 1995). For amplification of cDNA first strands, denaturation of DNA was conducted at 94°C for 6 min for the first cycle and then for 1 min. Primer annealing was conducted at 37°C for 1 min for five cycles and at 48°C for the following 32 cycles. Primer extension was conducted at 72°C for 1 min. Amplified sequences were purified from agarose gels by phenol extraction (Sambrook et al., 1989) and subcloned into pUC18 by using the SureClone ligation kit (Pharmacia Biotech, Freiburg, Germany). Nucleotide sequencing using the dideoxy chain termination method (Sanger et al., 1977) was performed by using the Sequenase 2.0 kit (Amersham). Sequence analysis was performed with the sequence analysis software package of the Genetics Computer Group (Madison, WI; Devereux et al., 1984) on a VAX microcomputer.

RNA Extraction and RNA Gel Blot Analysis

For the isolation of RNA, cells were harvested by centrifugation, frozen in liquid nitrogen, and ground with a mortar and pestle in the presence of liquid nitrogen. Total nucleic acids were isolated by phenol extraction, according to the method of Bell et al. (1986), and total RNA was isolated by using Nucleobond AX columns (Macherey-Nagel, Düren, Germany), according to the instructions of the supplier. Polyadenylated mRNA was isolated by affinity chromatography on two successive oligo(dT) cellulose columns (Sambrook et al., 1989).

Isolation of Nuclei and Run-Off Transcription

Cells (5 g) were harvested by centrifugation, frozen in liquid nitrogen, and ground with a mortar and pestle in the presence of liquid nitrogen. The material was homogenized with a mechanical homogenizer (polytron) in 15 mL of isolation buffer (20 mM Tris-HCl, pH 7.8, 250 mM sucrose, 5 mM $MgCl_2$, 5 mM KCl, 40% [v/v] glycerol, 0.25% [v/v] Triton X-100, and 0.1% [v/v] β-mercaptoethanol) with 0.1% octanol, the slurry was filtered through two layers of Miracloth (Calbiochem, Bad Soden, Germany), and the filtrate was subjected to a second filtration step through two layers of Miracloth. The nuclei were recovered by centrifugation, washed twice with isolation buffer, and resuspended in 700 μL of isolation buffer. The quantity and quality of the nuclei preparation were determined by microscopy after staining with 4′,4-diamidino-2-phenylindole, and DNA content was determined with indole-$CuSO_4$ (Somssich, 1994).

For run-off transcription assays, nuclei corresponding to 50 μg of DNA were washed twice with 700 μL of TS1 buffer (50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 25% [v/v] glycerol, and 10 mM β-mercaptoethanol) and resuspended in 37.5 μL of TS1 buffer. After the addition of 90 units of human placental RNase inhibitor, the assays were incubated for 15 min on ice and supplemented with 18.75 μL of TS2 buffer (100 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 8 mM $MnCl_2$, 200 mM NH_4SO_4, and 3.75 μL of an ATP, GTP, and CTP mixture [10 mM each]) and 3.75 mL of H_2O. Reactions were started by the addition of 5 μL of diluted ^{32}P-UTP (0.4 MBq; Amersham) and incubated at 30°C for 40 min. Reactions were terminated by the addition of 150 μL of stop solution (500 mM $NaPO_4$ buffer, pH 7.4, 0.2% SDS, 5% sodium pyrophosphate, and 1 mM UTP). To determine how much of the synthesis was due to RNA polymerase II activity, a similar incubation was performed simultaneously in the presence of 2 μg/mL α-amanitin. Incorporation of radioactive label in synthesized RNA was determined by precipitation with TCA. Aliquots (5 μL) of the samples were spotted onto glass fiber filter disks (Whatman GF/C). The disks were washed four times with 5% TCA containing 20 mM sodium pyrophosphate and once with 5 mL of ethanol and air dried. Radioactivity was determined by scintillation counting.

Measurement of Intracellular pH

Intracellular pH was measured by equilibration of ^{14}C-labeled 5,5-dimethyl-2,4-oxazolidinedione (DMO; L'Allemain et al., 1984). One milliliter of cell culture was washed in 500 μL of 0.1 M KPO_4 buffer, pH 6.0, and resuspended in 760 μL of the same buffer. Samples were incubated with 1 mM unlabeled DMO and 10^5 cpm ^{14}C-labeled DMO at 28°C for 10 min. Cells were separated from the supernatant by filtration through 0.8-μm nitrocellulose filters. The radioactivity of the filtrate and of the filters with the cells was determined in a liquid scintillation counter. The intracellular pH value was calculated according to the Henderson–Hasselbalch equation. The formula $6.3 + \log_{10}[(cpm_{cells} \times 760)(cpm_{filtrate} \times 166)^{-1}]$ is based on the pKa of DMO (6.3), the volume of the cells (166 μL), and the volume of the filtrate (760 μL).

Preparation of Crude Extracts

Cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at −80°C. The cells were thawed and homogenized in homogenization buffer consisting of 50 mM Hepes-KOH, pH 7.5, 1 mM EDTA, 2 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 100 μM benzamidine, 20 mM β-glycerophosphate, 20% glycerol, 1 μg/mL leupeptin, 1 μg/mL antipain, 5 μg/mL pepstatin, 1 μM microcystin, 10 μM endothall, 10 mM NaF, 1 mM Na_2VO_4, and 1 mM Na-molybdate. The extract was centrifuged at 14,000 rpm for 20 min in a microcentrifuge at 4°C, and the obtained supernatant is referred to herein as crude extract. The concentration of protein in the extract was determined by a dye binding assay (Bradford, 1976) with BSA as the standard.

Kinase Assays

To assay total kinase activity in crude extracts in vitro, kinase assays were performed according to the method of Zarzov et al. (1996), except that reactions were incubated for 30 min and 10 μg of the myelin basic protein, dephosphorylated casein, or histone III-S was used as substrate. The reaction products were run on 15% SDS–polyacrylamide gels, according to the method of Laemmli (1970).

The in-gel kinase assay using the myelin basic protein as substrate was performed essentially as described by Mizoguchi et al. (1994). Crude extracts containing 50 μg of total protein per lane were separated by SDS-PAGE (Laemmli, 1970). The myelin basic protein (0.5 mg/mL) was used as substrate that was polymerized in the gel. Separated proteins were denatured in the presence 6 M guanidine–hydrochloride and renatured. Kinase assays were performed in the presence of 40 mM Hepes, pH 7.5, 0.1 mM EGTA, 20 mM $MgCl_2$, 2 mM DTT, 50 μM ATP, and 4 MBq γ-^{32}P-ATP for 1 hr at room temperature. The gels were washed extensively with a solution of 5% TCA

and 1% sodium pyrophosphate. To distinguish the phosphorylation of an exogenous substrate from a possible autophosphorylation of the kinases, control kinase assays were performed using polyacrylamide gels in which the myelin basic protein was omitted or substituted by 0.5 mg/mL dephosphorylated casein or histone III-S. Phosphorylated proteins were detected by autoradiography of the dried gels on Kodak Biomax RS films.

ACKNOWLEDGMENTS

We thank Dr. Widmar Tanner for his interest in this work, stimulating discussions, and helpful comments on the manuscript. The skillful technical assistance of Edelgard Herold and Markus Hoffmann is gratefully acknowledged. We also thank Monika Kammerer for expert tissue culture work, Günther Peissig for taking care of the *C. rubrum* plants, and Dr. Ludwig Lehle for reading the manuscript.

Received May 28, 1997; accepted August 15, 1997.

REFERENCES

Avigad, G. (1982). Sucrose and disaccharides. In Encyclopedia of Plant Physiology, F.A. Lowus and W. Tanner, eds (Berlin: Springer-Verlag), pp. 217–347.

Bell, J.N., Ryder, T.B., Wingate, U.P.M., Bailey, J.A., and Lamb, C.J. (1986). Differential accumulation of plant defense gene transcripts in a compatible and incompatible plant–pathogen interaction. Mol. Cell. Biol. **6,** 1615–1623.

Bögre, L., Ligterink, W., Meskiene, I., Barker, P.J., Heberele-Bors, E., Nuskisson, N.S., and Hirt, H. (1997). Wounding induces the rapid and transient activation of a specific MAP kinase pathway. Plant Cell **9,** 75–83.

Boller, T. (1995). Chemoperception of microbial signals in plant cells. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46,** 189–214.

Bowles, D.J. (1990). Defense-related proteins in higher plants. Annu. Rev. Biochem. **59,** 873–907.

Bradford, M.M. (1976). A rapid and sensitive method for the determination of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. **72,** 248–254.

Chappell, J., and Hahlbrock, K. (1984). Transcription of plant defense genes in response to UV light or fungal elicitor. Nature **311,** 76–78.

Cheng, W.-H., Taliercio, E.W., and Chourey, P.S. (1996). The *Miniature1* seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. Plant Cell **8,** 971–983.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12,** 387–395.

DeWald, D.B., Sadka, A., and Mullet, J.E. (1994). Sucrose modulation of soybean *Vsp* gene expression is inhibited by auxin. Plant Physiol. **104,** 439–444.

Dietrich, A., Mayer, J.E., and Hahlbrock, K. (1990). Fungal elicitor triggers rapid, transient, and specific protein phosphorylation in parsley cell suspension cultures. J. Biol. Chem. **265,** 6360–6368.

Dixon, R.A., and Lamb, C.J. (1990). Molecular communication in interactions between plants and microbial pathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41,** 339–367.

Eschrich, W. (1980). Free space invertase, its possible role in phloem unloading. Ber. Dtsch. Bot. Ges. **93,** 363–378.

Felix, G., Grosskopf, D.G., Regenass, M., Basse, C.B., and Boller, T. (1991). Elicitor-induced ethylene biosynthesis in tomato cells. Plant Physiol. **97,** 19–25.

Felix, G., Regenass, M., Spanu, P., and Boller, T. (1994). The protein phosphatase inhibitor calyculin A mimics elicitor action in plant cells and induces rapid hyperphosphorylation of specific proteins as revealed by pulse labeling with [^{33}P]phosphate. Proc. Natl. Acad. Sci. USA **91,** 952–956.

Glasziou, K.T., and Gayler, K.R. (1972). Storage of sugars in stalks of sugar cane. Bot. Rev. **38,** 471–490.

Godt, D.E., Riegel, A., and Roitsch, T. (1995). Regulation of sucrose synthase expression in *Chenopodium rubrum*: Characterization of sugar induced expression in photoautotrophic suspension cultures and sink tissue specific expression in plants. J. Plant Physiol. **146,** 231–238.

Grosskopf, D.G., Felix, G., and Boller, T. (1990). K-252a inhibits the response of tomato cells to fungal elicitors in vivo and their microsomal protein kinase in vitro. FEBS Lett. **275,** 177–180.

Hagendoorn, M.J.M., Wagner, A.M., Segers, G., Van der Plas, L.H.W., Oostdam, A., and Van Walraven, H.S. (1994). Cytoplasmatic acidification and secondary metabolite production in different plant cell suspensions. Plant Physiol. **106,** 723–730.

Harter, K., Talke-Messerer, C., Barz, W., and Schäfer, E. (1993). Light- and sucrose-dependent gene expression in photomixotrophic cell suspension cultures and protoplasts of rape (*Brassica napus* L.). Plant J. **4,** 507–516.

Herbers, K., Mönke, G., Badur, R., and Sonnewald, U. (1995). A simplified procedure for the subtractive cDNA cloning of photoassimilate-responding genes: Isolation of cDNAs encoding a new class of pathogenesis-related proteins. Plant Mol. Biol. **29,** 1027–1038.

Herbers, K., Meuwly, P., Frommer, W.B., Métraux, J.-P., and Sonnewald, U. (1996a). Systemic acquired resistance mediated by the ectopic expression of invertase: Possible hexose sensing in the secretory pathway. Plant Cell **8,** 793–803.

Herbers, K., Meuwly, P., Métraux, J.-P., and Sonnewald, U. (1996b). Salicylic acid–independent induction of pathogenesis-related protein transcripts by sugars is dependent on leaf developmental stage. FEBS Lett. **397,** 239–244.

Hirt, H. (1997). Multiple roles of MAP kinases in plant signal transduction. Trends Plant Sci. **2,** 11–15.

Hüsemann, W. (1981). Growth characteristics of hormone and vitamin independent photoautotrophic cell suspension cultures from *Chenopodium rubrum.* Protoplasma **109,** 415–431.

Hüsemann, W., and Barz, W. (1977). Photoautotrophic growth and photosynthesis in cell suspension cultures of *Chenopodium rubrum.* Physiol. Plant. **40,** 77–81.

Jang, J.-C., and Sheen, J. (1994). Sugar sensing in higher plants. Plant Cell **6,** 1665–1679.

Jang, J.-C., León, P., Zhou, L., and Sheen, J. (1997). Hexokinase as a sugar sensor in higher plants. Plant Cell **9,** 5–19.

Johnson, R., and Ryan, C.A. (1990). Wound-inducible potato inhibitor II genes: Enhancement of expression by sucrose. Plant Mol. Biol. **14,** 527–536.

Jonak, C., Heberle-Bors, E., and Hirt, H. (1994). MAP kinases: Universal multi-purpose signaling tools. Plant Mol. Biol. **24,** 407–416.

Koch, K.E. (1996). Carbohydrate-modulated gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47,** 509–540.

Krapp, A., Hofmann, B., Schäfer, C., and Stitt, M. (1993). Regulation of the expression of *rbcS* and other photosynthetic genes by carbohydrates: A mechanism for the "sink regulation" of photosynthesis? Plant J. **3,** 817–828.

Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature **227,** 680–685.

L'Allemain, G., Paris, S., and Pouysségur, J. (1984). Growth factor action and intracellular pH regulation in fibroblasts. Evidence for a major role of the Na^+/H^+ antiport. J. Biol. Chem. **259,** 5809–5815.

Lawton, M.A., and Lamb, C.J. (1987). Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. Mol. Cell. Biol. **7,** 335–341.

Lee, H.-I., Léon, J., and Raskin, I. (1995). Biosyntheis and metabolism of salicylic acid. Proc. Natl. Acad. Sci. USA **92,** 4076–4079.

León, J., Lawton, M., and Raskin, I. (1993). Phenolic acids and the biosynthesis of defense-related compounds. In Plant Signals in Interactions with Other Organisms, J.C. Schultz and I. Raskin, eds (Rockville, MD: American Society of Plant Physiologists), pp. 65–78.

MacKintosh, C., Lyon, G.D., and MacKintosh, R.W. (1994). Protein phosphatase inhibitors activate anti-fungal defense responses of soybean cotyledons and cell cultures. Plant J. **5,** 137–147.

Martin, T., Hellmann, H., Schmidt, R., Willmitzer, L., and Frommer, W.B. (1997). Identification of mutants in metabolically regulated gene expression. Plant J. **11,** 53–62.

Mathieu, Y., Jouanneau, J.-P., Thomine, S., Lapous, D., and Guern, J. (1994). Cytosolic protons as secondary messengers in elicitor-induced defence responses. Biochem. Soc. Symp. **60,** 113–130.

Matsuzawa, M., Graziano, M.J., and Casida, J.E. (1987). Endothall and cantharidin analogs: Relation of structure to herbicidal activity and mammalian toxicity. J. Agric. Food Chem. **35,** 823–829.

Miller, E.M., and Chourey, P.S. (1992). The maize invertase-deficient *miniature-1* seed mutation is associated with aberrant pedicel and endosperm development. Plant Cell **4,** 297–305.

Mizoguchi, T., Gotoh, Y., Nishida, E., Yamaguchi-Shinozaki, K., Hayashida, N., Iwasaki, T., Kamada, H., and Shinozaki, K. (1994). Characterization of two cDNAs that encode MAP kinase homologues in *Arabidopsis thaliana* and analysis of the possible role of auxin in activating such kinase activities in cultured cells. Plant J. **5,** 111–122.

Mouly, A., and Roby, D. (1988). Decrease in mRNA coding for the small subunit of ribulose-1,5-bisphosphate carboxylase in sunflower plants infected with *Sclerotinia sclerotiorum.* Plant Physiol. Biochem. **26,** 165–171.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 473–497.

Nishida, E., and Gotoh, Y. (1993). The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Genet. **18,** 128–131.

Ohto, M.-a., and Nakamura, K. (1995). Sugar-induced increase of calcium-dependent protein kinases associated with plasma membrane in leaf tissues of tobacco. Plant Physiol. **109,** 973–981.

Pelech, S.L., and Sanghera, J.S. (1992). MAP kinases: Charting the regulatory pathways. Science **257,** 1355–1356.

Ramloch-Lorenz, K., Knudsen, S., and Sturm, A. (1993). Molecular characterization of the gene for carrot cell wall β-fructosidase. Plant J. **4,** 545–554.

Roitsch, T., and Tanner, W. (1994). Expression of a sugar transporter gene family in photoautotrophic suspension cultures of *Chenopodium rubrum.* Planta **193,** 365–371.

Roitsch, T., Bittner, M., and Godt, D.E. (1995). Induction of apoplastic invertase of *Chenopodium rubrum* by D-glucose and a glucose analog and tissue specific expression suggest a role in sink source regulation. Plant Physiol. **108,** 285–294.

Sadka, A., DeWald, D.B., May, G.D., Park, W.D., and Mullet, J.E. (1994). Phosphate modulates transcription of soybean *VspB* and other sugar-inducible genes. Plant Cell **6,** 737–749.

Salanoubat, M., and Belliard, G. (1989). The steady-state level of potato sucrose synthase mRNA is dependent on wounding, anaerobiosis and sucrose concentration. Gene **84,** 181–185.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Schmelzer, E., Krüger-Lebus, S., and Hahlbrock, K. (1989). Temporal and spatial patterns of gene expression around sites of attempted fungal infection in parsley leaves. Plant Cell **1,** 993–1001.

Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H., and Ohashi, Y. (1995). Tobacco MAP kinase: A possible mediator in wound signal transduction pathways. Science **270,** 1988–1992.

Sheen, J. (1990). Metabolic repression of transcription in higher plants. Plant Cell **2,** 1027–1038.

Sheen, J. (1993). Protein phosphatase activity is required for light-inducible gene expression in maize. EMBO J. **12,** 3497–3505.

Sheen, J. (1994). Feedback control of gene expression. Photosynth. Res. **39,** 427–438.

Somssich, I. (1994). Assay for gene expression using run-on transcription in isolated nuclei. In Plant Molecular Biology Manual, S.B. Gelvin and R.A. Schilperoort, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 1–11.

Sturm, A., and Chrispeels, M.J. (1990). cDNA cloning of carrot extracellular β-fructosidase and its expression in response to wounding and bacterial infection. Plant Cell **2,** 1107–1119.

Suzuki, K., and Shinshi, H. (1995). Transient activation and tyrosine phosphorylation of a protein kinase in tobacco cells treated with a fungal elicitor. Plant Cell **7,** 639–647.

Suzuki, K., Fukuda, Y., and Shinshi, H. (1995). Studies on elicitor-signal transduction leading to differential expression of defense genes in cultured tobacco cells. Plant Cell Physiol. **36,** 281–289.

Técsi, L.I., Maule, A.J., Smith, A.M., and Leegood, R.C. (1994). Complex, localized changes in CO_2 assimilation and starch content associated with the susceptible interaction between cucumber mosaic virus and a cucurbit host. Plant J. **5,** 837–847.

Thornburg, R.W., and Li, X. (1991). Wounding *Nicotiana tabacum* leaves causes a decline in endogenous indole-3-acetic acid. Plant Physiol. **96,** 802–805.

Tsukaya, H., Ohshima, T., Naito, S., Chino, M., and Komeda, Y. (1991). Sugar-dependent expression of the *CHS-A* gene for chalcone synthase from petunia in transgenic *Arabidopsis.* Plant Physiol. **97,** 1414–1421.

Usami, S., Banno, H., Yukihiro, I., Nishihama, R., and Machida, Y. (1995). Cutting activates a 46-kilodalton protein kinase in plants. Proc. Natl. Acad. Sci. USA **92,** 8660–8664.

Visser, R.G.F., Stolte, A., and Jacobsen, E. (1991). Expression of a chimeric granule-bound starch synthase *GUS* gene in transgenic potato plants. Plant Mol. Biol. **17,** 691–699.

Weber, H., Borisjuk, L., Heim, U., Buchner, P., and Wobus, U. (1995). Seed coat–associated invertases of fava bean control both unloading and storage functions: Cloning of cDNAs and cell type–specific expression. Plant Cell **7,** 1835–1846.

Wright, D.P., Baldwin, B.C., Shephard, M.C., and Scholes, J.D. (1995). Source–sink relationships in wheat leaves infected with powdery mildew. I. Alterations in carbohydrate metabolism. Physiol. Mol. Plant Pathol. **47,** 237–253.

Zarzov, P., Mazzoni, C., and Mann, C. (1996). The SLT2(MPK1) MAP kinase is activated during periods of polarized cell growth in yeast. EMBO J. **15,** 83–91.