The Plant Cell, Vol. 12, 1939–1950, October 2000, www.plantcell.org © 2000 American Society of Plant Physiologists

Glutathione and a UV Light–Induced Glutathione *S*-Transferase Are Involved in Signaling to Chalcone Synthase in Cell Cultures

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UV irradiation stimulates expression of the gene encoding the key enzyme chalcone synthase (*CHS*), which leads to the generation of protective flavonoids in parsley cell cultures. *CHS* transcripts increase after 3 to 4 hr, and early genes are involved in the signal transduction to the *CHS* promoter. By using the fluorescent differential display technique in a large-scale screening, several early UV light–induced genes were isolated. Of these, a novel glutathione *S*-transferase (PcGST1) is induced within 2 hr and precedes *CHS* expression. Overexpression of *PcGST1* in transformed cell lines containing a *CHS* promoter/luciferase reporter (*CHS-LUC*) affected the onset of *LUC* transcription. Supplementing these cell lines with glutathione immediately stimulated *CHS-LUC* expression within 2 hr in dark-incubated cells and resulted in a biphasic induction profile in UV-irradiated cells. Our data indicate the involvement of glutathione and PcGST1 in early events of a UV light–dependent signal transduction pathway to *CHS*. In this context, the oxidative status of a cell acts as a central regulating element.

INTRODUCTION

During photomorphogenesis, the expression of numerous genes is controlled by several photoreceptors that absorb in the visible and UV range of sunlight. In addition to the family of photoreversible phytochromes, several photoreceptors absorbing blue and UV-A light have recently been isolated from higher plants (Furuya and Schaefer, 1996; Cashmore et al., 1999). Although phytochrome-mediated responses have been extensively studied, only a few elements involved in signal transduction have been isolated so far (Neff et al., 2000). UV-B irradiation of higher plants also specifically induces various responses that are attributed to the action of a hypothesized UV-B photoreceptor (Jordan, 1996). Of these responses, the induction of phenylpropanoid and flavonoid glycoside biosynthetic pathways and the consequential accumulation of UV-protective flavonoids in UV-irradiated cells of a parsley suspension culture have been analyzed in detail (Hahlbrock and Scheel, 1989). As a key enzyme of flavonoid biosynthesis, chalcone synthase (CHS) is transcriptionally stimulated by UV light in this cell culture but also in leaves of adult parsley plants (Chappell and Hahlbrock, 1984; Frohnmeyer et al., 1992). *CHS* mRNA is strongly and exclusively induced by irradiation with low fluences of short-wavelength light with an apparent lag phase of a few hours (Chappell and Hahlbrock, 1984). This CHS induction is independent of the formation of dimerized DNA produced by irradiation with high fluences of UV-B (Frohnmeyer et al., 1999). Other external signals are ineffective in stimulating *CHS* expression, rendering the parsley cell culture a suitable system for the study of UV-dependent signal transduction.

The parsley *CHS* promoter contains four *cis*-acting elements that mediate the light response (Schulze-Lefert et al., 1989). Several *trans*-acting factors binding to the *CHS* promoter have been isolated (Weisshaar et al., 1991; Feldbruegge et al., 1997; Kircher et al., 1998), but their involvement in the light-mediated activation of *CHS* expression has not been clarified. Using pharmacological effectors, investigators have characterized several upstream components such as calcium, calmodulin, and serine/threonine kinases as elements mediating the UV-dependent *CHS* expression in parsley (Frohnmeyer et al., 1997, 1999). These components also affect the UV-induced *CHS* expression in Arabidopsis and soybean cell cultures, pointing to the existence of a conserved signaling cascade to the *CHS* promoter (Christie and Jenkins, 1996; Frohnmeyer et al., 1998; Long and Jenkins, 1998). In these cell cultures, the UV-induced *CHS* transcription additionally depends on intact protein synthesis, indicating the participation of early UV-induced gene products in this signal transduction (Christie and Jenkins, 1996; Frohnmeyer et al., 1998; Kircher et al., 1998).

To isolate such light-induced early genes, we performed a

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large-scale analysis by using fluorescent differential display (FDD) (Liang and Pardee, 1992), which has been successfully adapted to plant tissues (Uchida et al., 1998; Kuno et al., 2000). We analyzed the composition of genes stimulated within the first 2 hr under light regimes specifically leading to *CHS* induction and identified a novel UV-induced glutathione *S*-transferase (*PcGST1*). To investigate the role of PcGST1 in processes leading to *CHS* activation in vivo, we established stable transformation of parsley cell cultures with an effector and a reporter plasmid. Constitutive expression of *PcGST1* in a parsley cell line harboring a *CHS* promoter–controlled luciferase reporter gene (*CHS-LUC*) caused an earlier increase of UV-induced reporter gene activity and was accompanied by a substantial time shift of maximal LUC activity. Supplementing these cell lines with reduced glutathione (GSH), a substrate of GSTs, led to the immediate onset of *LUC* expression even without a light stimulus. These results indicate that PcGST1 and GSH are functionally involved in UV-induced signal transduction in parsley.

RESULTS

Identification of Early UV-B–Stimulated Genes by Differential Display

FDD was chosen to screen for UV-induced genes, which are expressed before onset of *CHS* transcription. Being highly UV responsive, protoplasts isolated from a parsley cell culture were irradiated with a 4-min UV-B pulse (using a 305-nm cutoff filter) and then incubated in the dark or with continuous UV-A light, blue light, red light, or far-red light. Cells were harvested after 2 hr, and RNA was prepared from two independent experiments. All samples were subjected to FDD analysis, and the detected fragments were monitored in parallel to compare directly their expression pattern. By this approach, the concurrent determination of differentially expressed genes enabled us to distinguish between genes preceding *CHS* expression (after UV-A and UV-B irradiation) and genes that are stimulated by long-wavelength light (blue, red, and far red) but are not related to *CHS* expression.

A large-scale screening with 280 different combinations of primer pairs led to the detection of ~25,000 constitutive expressed bands. Three fragments were upregulated exclusively under conditions (UV-A and UV-B) stimulating *CHS* expression. Figure 1 illustrates the most pronounced increase of one fragment, which specifically accumulates after UV-B pulse treatment, whereas irradiation with longer wavelengths was ineffective. Besides these, an unexpectedly large number of fragments were induced by red and far-red light (12) or by red and UV-A light (6), confirming that independent phytochrome-mediated gene expression exists in parsley cell cultures (Poppe et al., 1994).

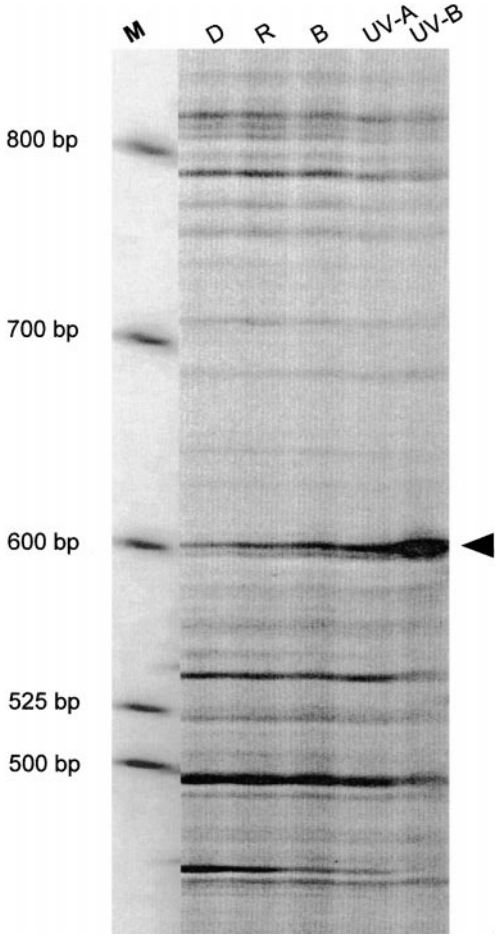

Figure 1. Representative Differential Display Gel with a UV-B–Induced Fragment Amplified Using 3′-dG(dT)$_{15}$dC as the Anchor Primer and CAGGCCCTTC as the Specific Primer.

Parsley cells were irradiated with continuous red (R), blue (B), or UV-A light or with a UV-B pulse or were kept in darkness (D) until harvest after 2 hr. The arrowhead indicates the position of the differentially expressed gene encoding PcGST1. M, molecular size marker, in base pairs.

PcGST1 Belongs to the Class of Type III GSTs and Has Substantial Enzymatic Activity

One UV-induced cDNA fragment detected by FDD (Figure 1) was further analyzed for single-strand cDNA polymorphism, and the inserts with the correct patterns were chosen for sequencing. Extension of the FDD fragment from a cDNA library of UV-irradiated parsley cells resulted in isolation of a 836-bp insert that contained a full-length cDNA with a 157-bp 3′ untranslated region (GenBank accession number AF177944). As shown in Figure 2, an open reading frame predicted a protein of 222 amino acids for which searches of the Swiss-Prot database revealed a 38 to 42% identity to

type III GSTs from higher plants. The deduced amino acid sequence from the isolated parsley cDNA, termed *PcGST1*, was conserved mainly in the N-terminal region encoding a glutathione binding site (Figure 2). Besides this homology, the appearance and position of one intron in the genomic sequence matched those of other type III GSTs (data not shown).

The enzymatic activity of recombinant PcGST1 was investigated after synthesis in *Escherichia coli*. The 25-kD protein revealed considerable transferase activity (114 units/mg protein) for 1-chloro-2,4-dinitrobenzene substrate, whereas the phenylpropanoid *p*-coumaric acid was not accepted as a substrate. In contrast to the transferase activity, the protein showed negligible glutathione peroxidase activity (0.4 unit/mg protein). These substrate specificities are comparable with those of other type III GSTs (Dixon et al., 1998) and provide evidence that PcGST1 can act as a transferase.

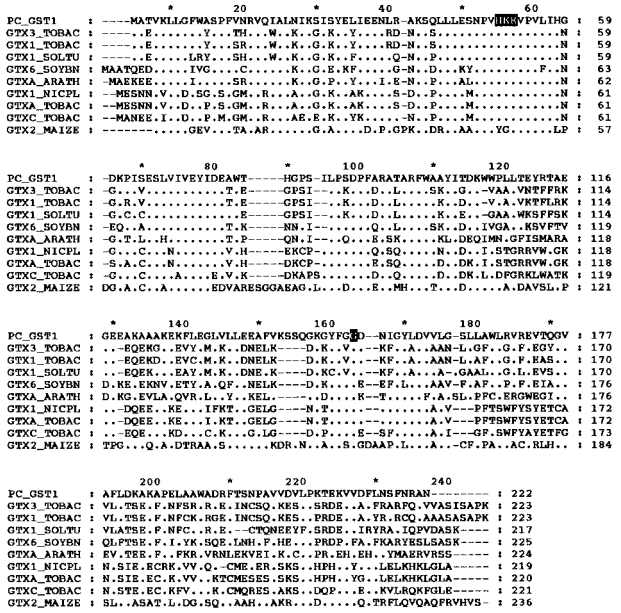

Figure 2. Amino Acid Alignment of PcGST1 with Type III GSTs from Various Plants.

Comparison of PcGST1 with the deduced amino acid sequences of GTX3_, GTX1_, GTXA_, and GTXC_TOBAC from tobacco (Takahashi et al., 1991; Takahashi and Nagata, 1992; Boot et al., 1993); GTX6_SOYBN from soybean (GH2/4, Hsp26; Hagen et al., 1988); GTX1_SOLTU from tomato (Taylor et al., 1990); GTXA_ARATH from Arabidopsis (GenBank accession number 46421); GTX1_NICPL from tobacco (Dominov et al., 1992); and GTX2_MAIZE from maize (Bz2; Nash et al., 1990). Sequence identities to *PcGST1* are between 38 and 42%. Conserved amino acids of type III GSTs (Droog, 1997) are presented as white letters. Amino acids conserved in type III GSTs and PcGST1 are indicated by dots. Dashes represent sequence gaps to allow for maximum alignment.

PcGST1 Expression Is Regulated by UV-B Light and Auxins

Most members of type III GSTs are transcriptionally induced by multiple stimuli, including fungal infection, heat shock, heavy metals, and hormone treatment (summarized in Marrs, 1996). In contrast, fast stimulation by UV light has not been reported. To determine whether other external signals also activate *PcGST1*, we performed an RNA gel blot analysis, as depicted in Figure 3. Additionally, *CHS* and *PcGST1* mRNAs were quantified by phosphoimaging analysis, and relative expression of *GST*/*UBI* and *CHS*/*UBI* (stimulated/nonstimulated) was calculated as *n*-fold induction values. Parsley cells were soaked on filter paper and subsequently treated with various stimuli such as UV-B light, elicitors, or the auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D). UV-B pulse irradiation and subsequent transfer to darkness resulted in a strong stimulation of *PcGST1* mRNA and 35-fold increased levels were detectable 2 hr after light treatment. The *PcGST1* mRNA accumulation was transient and returned to basal values (1.4-fold increase) within 6 hr, whereas *CHS* mRNA accumulated 39-fold at this time (Figure 3A). *PcGST1* was also increased fivefold after 2 hr in dark-incubated cells, possibly reflecting a partial activation by the mechanical stress of the handling procedure (Figure 3A). Irradiation of the cells with longer wavelengths did not activate *PcGST1* or *CHS* expression, thus confirming the data from FDD analysis (data not shown).

The fast transcriptional stimulation of several plant *GSTs* also occurs in the presence of protein synthesis inhibitors, defining these as early genes (Abel and Theologis, 1996). The rapid UV-induction of *PcGST1* was therefore monitored after inhibition of the translational machinery with cycloheximide. *PcGST1* mRNA accumulation under these conditions was similar to that found in untreated cells, and expression was maximal after 2 hr (Figure 3B). Again, a weak induction of the gene could be detected already in dark-incubated cells for the reasons discussed above. In contrast to *PcGST1*, however, accumulation of *CHS* mRNA was completely repressed after cycloheximide treatment (Figure 3B).

Preliminary experiments indicated that *PcGST1* expression was also induced after transfer of 5-day-old cultured cells to fresh medium containing 2,4-D as the only growth factor (data not shown). Treatment of parsley cells with 4.5 μM 2,4-D (representing the internal concentration of B5 medium) resulted in a stimulation of *PcGST1* mRNA irrespective of subsequent light treatment or dark incubation (Figure 3A). *CHS* mRNA accumulation was strictly dependent on UV treatment but was markedly higher (75-fold increase) in cells supplemented with 2,4-D (Figure 3A). The auxin homolog α-naphthylene acetic acid (α-NAA) was also capable of stimulating *PcGST1* expression, whereas a weaker auxin homolog (β-NAA) resulted in only slight induction (Figure 3C). To investigate whether fungal elicitors affect *PcGST1* expression, cells were treated with Pep25 polypeptide, an

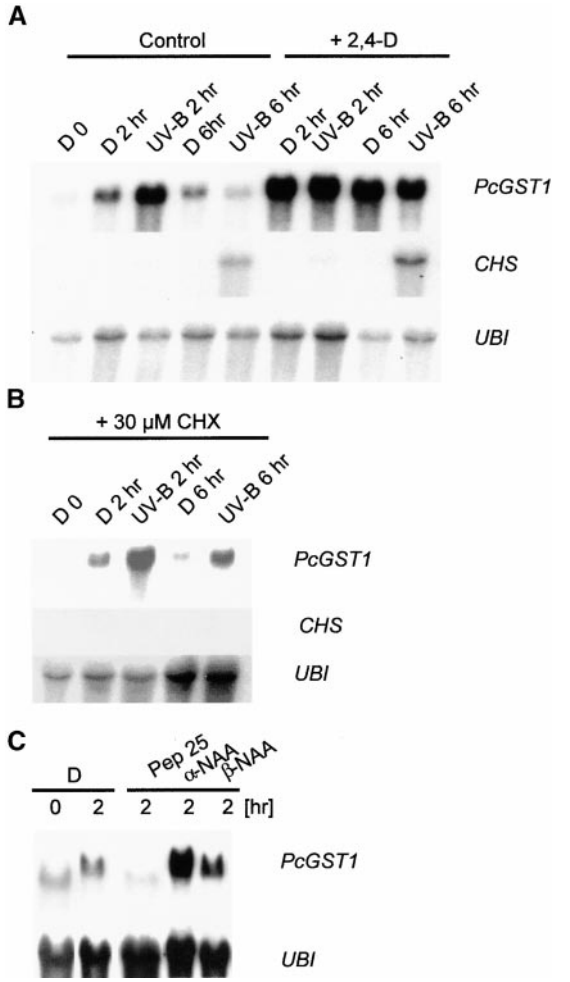

Figure 3. RNA Gel Blot Analysis of the Steady State Amounts of *PcGST1* and *CHS* Transcripts and Ubiquitin Concentrations in Response to UV-B Light, Hormone, and Elicitor Treatment.

(A) Detection of transcripts in response to exposure to UV-B light, treatment with 2,4-D, or both. Parsley cells were kept in darkness (D) or irradiated with a pulse of UV-B light through a 305-nm cutoff filter and kept in the dark until harvest after 2 hr (UV-B 2 hr) or 6 hr (UV-B 6 hr). Simultaneously, 4.5 μM 2,4-D was added to dark-incubated cells, which were then either irradiated with UV-B light or kept in darkness until harvest. RNA gel blot analysis was performed with 20 μg of total RNA, as described in Methods. Filters were probed with cDNA inserts for *PcGST1*, *CHS*, and ubiquitin (*UBI*) for loading control analysis.

(B) Detection of transcripts in the absence of intact protein synthesis. Shown is *PcGST1* and *CHS* mRNA accumulation of cells after 30-min pretreatment with 30 μM cycloheximide (CHX), inhibiting protein synthesis and subsequent dark incubation or UV-B irradiation, as described in **(A)**.

(C) Detection of transcripts in response to treatment with the synthetic elicitor Pep25 or the auxin analogs α-NAA and β-NAA. Shown is *PcGST1* mRNA accumulation after treatment of dark-incubated cells with 175 nM Pep25, 10 μM α-NAA, or 10 μM β-NAA. All cells were kept in darkness until harvest after 2 hr.

active elicitor component of *Phytophthera sojae* that causes a multicomponent defense response in parsley cell culture (Nuernberger et al., 1994). As shown in Figure 3C, *PcGST1* expression is not induced but repressed by Pep25, which therefore excludes the direct participation of *PcGST1* in the activation of the pathogen defense response.

CHS-LUC Expression Reflects Endogenous CHS Contents in Transgenic Parsley Lines

The early expression of *PcGST1* under light regimes that stimulate subsequent *CHS* transcription and flavonoid biosynthesis raises the question as to whether a functional link exists between these genes. As a prerequisite for such studies, we established the stable transformation of parsley cells and compared the UV responsiveness of reporter gene activity with the expression of endogenous *CHS*. Transformed lines were created by particle bombardment, and a *LUC* reporter gene under control of the parsley 615-bp full-length *CHS* promoter (*CHS-LUC*) was introduced into parsley cells. Hygromycin-resistant calli were selected, and stimulation of the reporter gene by UV-B was determined in vivo with a charge-coupled device camera (Frohnmeyer et al., 1999). One cell line revealed high responsiveness to UV light and was chosen as a background line for further experiments. As shown in Figure 4A, stimulation with UV light caused comparable expression of *LUC* and endogenous *CHS* mRNA after 6 hr, whereas neither transcript was detectable in dark-incubated cells. This pattern was verified at the protein level (Figure 4B), indicating that LUC activity in this line reflects endogenous CHS concentrations.

Coexpression of PcGST1 Causes Earlier UV-Dependent CHS-LUC Induction

The influence of PcGST1 on UV-induced *CHS* expression was further analyzed in double-transformed cell lines. For this, a cauliflower mosaic virus 35S promoter–driven *PcGST1* cDNA effector construct (GST_{ox}) was integrated by particle bombardment into the described background line containing *CHS-LUC* (Figure 4). As a control, the background line was transformed with the empty effector plasmid pRT99. The resulting double-transformed lines (GST_{ox} × CHS-LUC, hereafter referred to as $PcGST1_{ox}$ line and pRT99 × CHS-LUC, hereafter referred to as control or background line) were further selected as described in Methods. Seven resistant calli were analyzed, and their UV responsiveness with respect to CHS induction was compared with that of several control lines. A representative kinetic of *CHS-LUC* expression of one control line and of a $PcGST1_{ox}$ line is shown in Figure 5A. Luciferin was added to dark-incubated and UV-B–irradiated cells, and LUC activity was monitored every 30 min for 14 hr. The reporter enzyme activity reflects

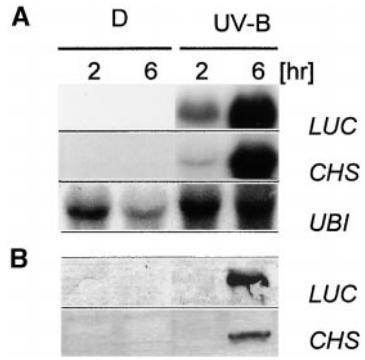

Figure 4. UV-B–Induced Steady State Transcript Amounts and Protein Accumulation of *LUC*, *CHS*, and Ubiquitin in Transformed Parsley Cell Lines.

(A) RNA gel blot analyses of transcript amounts of *LUC*, *CHS*, and ubiquitin (*UBI*) in response to exposure to UV-B light pulses. A stably transformed parsley line harboring a *CHS* promoter–*LUC* reporter fusion was kept in darkness (D) or irradiated with a UV-B light pulse through a 305-nm cutoff filter and kept in the dark until harvest after 2 or 6 hr. RNA gel blot analysis was performed as in Figure 3, except that one additional probe for *LUC* was used.

(B) Immunodetection of CHS and LUC proteins in response to UV-B light pulses. Cells were treated as described in **(A)**, and 15 μg of protein per lane was resolved by SDS-PAGE. CHS and LUC were detected on gel blots by using specific antibodies.

actual transcription rates under these conditions and allows detection of real-time gene expression events in vivo (Millar et al., 1992). LUC activity remained low in dark-incubated control and GST_{ox} cells. However, reporter gene activity started to increase 4.5 hr after UV-B pulse treatment in the pRT99-containing background line, reaching a maximum after 8 hr and returning to basal values within 14 hr (Figure 5A). This expression pattern could be reproduced with negligible deviation in three different control lines. GST_{ox} cells showed a similar expression pattern, but LUC activity was increasing already at 3.5 hr after stimulation with UV light (Figure 5B), which was manifested by an earlier maximal LUC activity (Figure 5A). The temporal shift of maximal LUC activity was verified in six of the seven transformed $PcGST1_{ox}$ lines, as determined in three independent experiments (Figure 5C). The maximal LUC activity in $PcGST1_{ox}$ lines appeared between 45 min and 2 hr earlier than in the background cell line (Figure 5C) and correlated with the amount of *PcGST1* expression for each $PcGST1_{ox}$ line (Figure 5D). One reason for this correlation might be that the PcGST1 protein is already present when $PcGST1_{ox}$ cell lines are stimulated with UV light, and this situation causes a shortcut in the signaling to *CHS*, which is reflected by a decrease of the initial lag phase. In wild-type cells, the synthesis of PcGST1 protein starts only after UV treatment and causes delayed *CHS* expression.

PcGST1 and GSH Are Sufficient for Immediate CHS Stimulation

Constitutive synthesis of PcGST1 affected the timing of UV-induced *CHS-LUC* expression, but an initial lag phase remained (Figures 5A and 5B). To address whether the GST substrate GSH is a limiting parameter, further kinetic studies were performed. In vivo LUC activity was determined in UV-irradiated control and $PcGST1_{ox}$ lines after the culture medium was supplemented with GSH. As shown in Figure 6A, *CHS-LUC* expression was immediately stimulated in $PcGST1_{ox}$ line 1 (described in Figures 5C and 5D) and reached a first maximum within 2 hr. Under these conditions LUC activity increased biphasically, with a second maximum appearing after 6 to 7 hr. A similar expression pattern was achieved with $PcGST1_{ox}$ lines 2 and 7 (Figures 5C and 5D). In the UV-irradiated control line pRT99, in contrast, *CHS-LUC* expression remained low during the early period, and the induction profile was not affected by GSH (Figure 6A). Supplementation with GSH caused the immediate increase of LUC activity even in dark-incubated cells of $PcGST1_{ox}$ line 1 (Figure 6B). Within 2 hr, a fourfold stimulation of reporter gene activity appeared under these conditions, whereas LUC activity did not increase in cells of the control line (Figures 6A and 6B). These data indicate that *CHS-LUC* expression can be immediately activated without the stimulus of UV light if GSH and PcGST1 concentrations are increased. In contrast, GSH supplementation negatively affected *CHS-LUC* expression 6 hr after UV irradiation in control and $PcGST1_{ox}$ lines and dampened the strong increase of reporter gene activity (Figure 6B). GSH obviously promotes *CHS-LUC* expression in the presence of PcGST1 at early stages but apparently generally inhibits an increase of reporter gene activity irrespective of PcGST1 at later stages. The influence of the antioxidant on early and late signaling events to *CHS* is thus controversial.

The influence of supplemented GSH on internal glutathione concentrations in dark-incubated cells was investigated by determining the concentrations of reduced and oxidized GSH (GSSG). In parallel, *CHS-LUC* expression was monitored under these same conditions. As shown in Tables 1 and 2, addition of GSH resulted in a strong, fivefold increase of intracellular GSH after 2 hr in the control and $PcGST1_{ox}$ lines. The total GSH amount was generally less in $PcGST1_{ox}$ lines, reflecting a greater consumption of this metabolite by the increased amounts of PcGST1. GSSG concentrations were notably greater in $PcGST1_{ox}$ lines but strongly decreased after supplementation with GSH (Table 2). Again, LUC activity increased only in dark-incubated, GSH-supplemented $PcGST1_{ox}$ lines (Table 2). Taken together, these findings show that increased LUC activity in the absence of a UV light stimulus can be induced if PcGST1 is expressed and if a high redox pool of GSH (corresponding to a low percentage of GSSG) is present in parsley cell cultures.

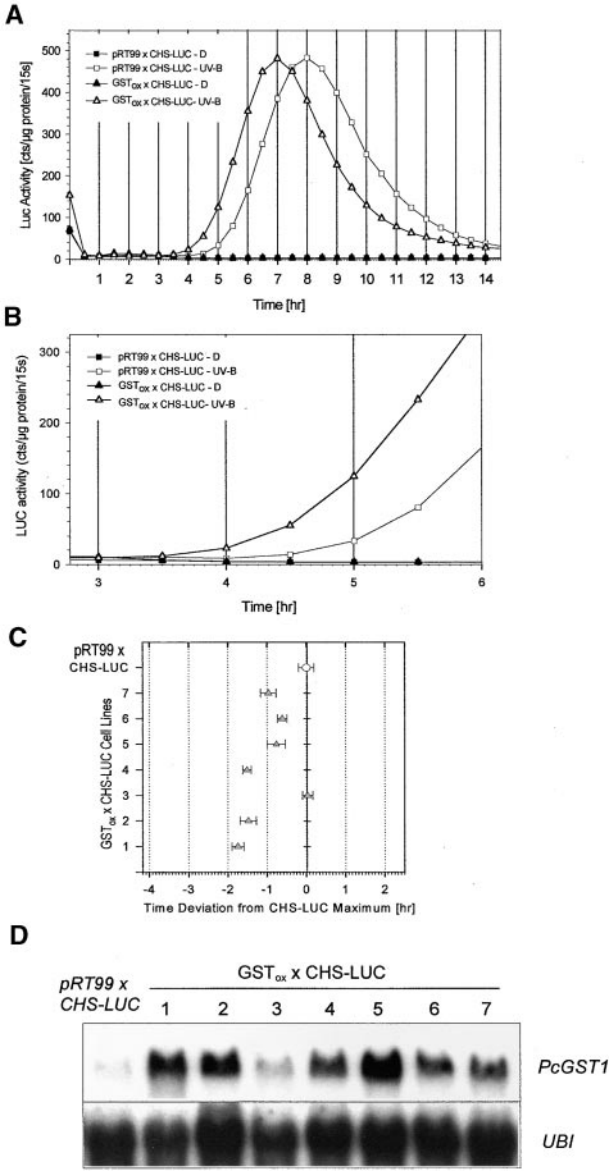

Figure 5. *CHS-LUC* Expression after UV-B Treatment and Effect of PcGST1.

(A) LUC activity of the *CHS-LUC* background line (pRT99 × CHS-LUC) compared with a double-transformed *PcGST1* overexpressing line (GST_{ox} × CHS-LUC). Cells of both lines were incubated in darkness (D) or irradiated with a 7-min pulse of UV-B light and subsequently transferred to darkness. Luciferin (1 mM) was added to the cells immediately after irradiation, and the actual counts for 15 sec (15s) were determined every 30 min during a 14-hr period as a measure of LUC activity. The protein concentration of each probe was determined after LUC measurement. Cts represent the amount of detected photons per time unit. LUC activity is expressed as the mean of three sample replicates from three independent experiments. Standard deviation was <15% in all cases.

(B) Section of **(A)** illustrating the early increase of LUC activity in pRT99 × CHS-LUC and GST_{ox} × CHS-LUC lines after UV stimulation.

(C) LUC activity of the *CHS-LUC* background line (pRT99 × CHS-LUC) compared with that of seven different double-transformed lines that in addition harbor cauliflower mosaic virus 35S promoter–controlled *PcGST1* cDNA (GST_{ox} × CHS-LUC 1 to 7). Cells were treated as described in **(A)**. The time of maximal *CHS-LUC* expression in the control cells was set as 0, and the deviation of maximal LUC activity from each line (1 to 7), determined in three independent experiments, was plotted in relation to it.

(D) RNA gel blot analysis of the steady state transcript amounts of *PcGST1* and ubiquitin (*UBI*) in the background line (pRT99 × CHS-LUC) and in dark-incubated, double-transformed lines expressing *PcGST1* cDNA (GST_{ox} × CHS-LUC 1 to 7). RNA gel blot analysis was performed as given for Figure 3.

DISCUSSION

UV-mediated signaling to *CHS* includes early genes, stimulated during the initial lag phase before onset of *CHS* transcription, as was found in parsley, soybean, and Arabidopsis (Christie and Jenkins, 1996; Frohnmeyer et al., 1998; Kircher et al., 1998). Using differential display, we isolated early UV-induced genes and analyzed the influence of PcGST1 on subsequent *CHS* expression. Four key observations suggest that GSH and PcGST1 are involved in a signal cascade that transmits the light stimulus to the *CHS* promoter: (1) *PcGST1* from parsley cell suspension cultures is expressed within 2 hr after a UV-B pulse. The transient accumulation of *PcGST1* transcripts needs no intact de novo protein synthesis and is stimulated only by wavelengths that also lead to subsequent *CHS* expression. (2) A functional analysis in transformed cell lines revealed that *CHS-LUC* reporter gene activity reflects endogenous *CHS* transcription. The onset of UV-induced *LUC* expression appeared after a lag phase of 4 to 5 hr, representing the time frame necessary to establish the complete signaling network to the *CHS* promoter. This initial period was chosen as a parameter to monitor the influence of GSH and PcGST1 on the signal transduction to *CHS*. (3) Coexpression of *PcGST1* in *CHS-LUC* lines caused a 1- to 2-hr earlier increase of LUC activity. In this situation, the maximal LUC activity appeared earlier than in control lines, although no kinetic differences of *LUC* expression were detected between these lines. The remaining lag phase in $PcGST1_{ox}$ lines indicates that PcGST1 influences the timing of *CHS-LUC* induction but is not sufficient for its light-independent stimulation. (4) Supplementing UV-stimulated and dark-incubated $PcGST1_{ox}$ lines with GSH removed the initial lag phase and caused immediate activation of *CHS-LUC* expression; meanwhile, the activity of the reporter gene remained low in pRT99-transformed control cells. The importance of these results for UV signaling to CHS and possible functions of GSH and GST action during these processes are discussed in the following sections.

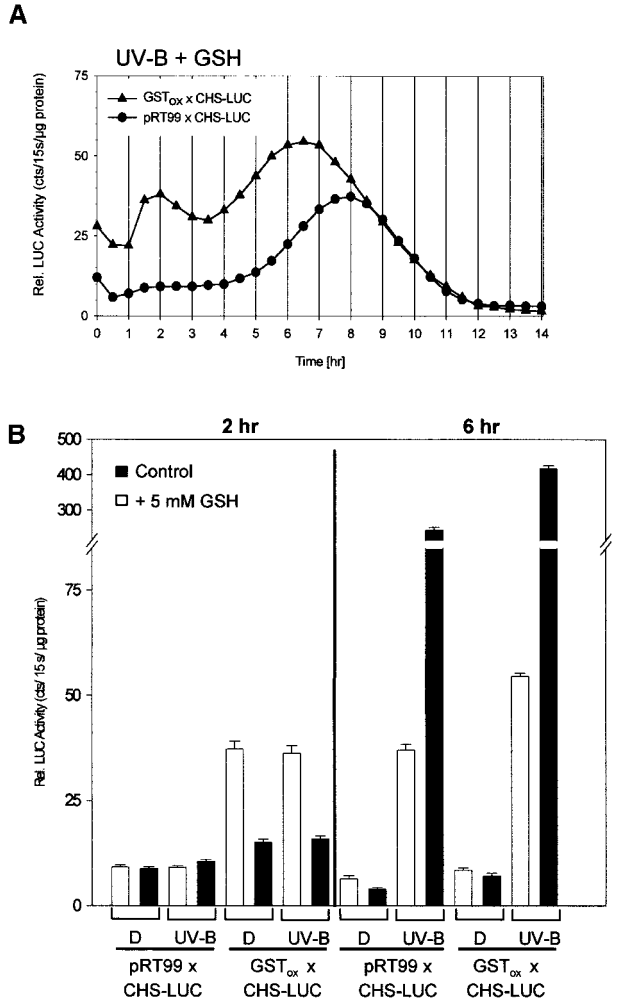

Figure 6. LUC Activity of pRT99 × CHS-LUC Background and GST_{ox} × CHS-LUC Lines and the Influence of GSH.

(A) Long-term kinetic *CHS-LUC* expression in UV-irradiated cells after addition of GSH (5 mM) to the cell culture medium (UV-B + GSH). Conditions were as described in Figure 5A.

(B) Influence of GSH on *CHS-LUC* expression in dark-incubated or UV-B light–irradiated lines. Cells of a background line and of a *PcGST1*-overexpressing line were incubated for 30 min in culture medium (Control) or in medium supplemented with GSH (+5 mM GSH). Cells were kept in darkness or irradiated with a 7-min pulse of UV-B light and further incubated in darkness in the presence of luciferin (1 mM). After 2 and 6 hr, LUC activity and protein concentration were determined from each probe. In each case, relative (Rel.) LUC activity was measured in three different cell lines (lines 1, 2, and 7) for a total of nine determinations for each data point. Bars represent the standard deviation.

Besides other stress factors, high fluences of light, including UV irradiation, can generate oxidative stress through reactive oxygen species (ROS) in plants (Green and Fluhr, 1995), leading to the production of oxidative damage. GSTs with GSH peroxidase activity participate in the detoxification of products created by oxidative damage and thereby protect cells against such stress (Dixon et al., 1998). This protection mechanism is often accelerated by increased amounts of GSH (May et al., 1998). Although these observations describe a possible scenario of GST and GSH action in cells that have been treated with high fluences of UV light, several findings disprove the possibility that such a mechanism mediates *CHS* induction. First, parsley and Arabidopsis cells already respond to low-fluence UV light by transcriptional stimulation of *CHS*, but the generation of ROS is not detectable under these light conditions (Christie and Jenkins, 1996; Frohnmeyer et al., 1997). Second, increased concentrations of PcGST1 and GSH already stimulate *CHS* expression in dark-incubated cells, whereas the generation of ROS is excluded under these conditions. Third, treatment of tobacco plants with ROS-promoting agents causes *PR1* stimulation, whereas flavonoid biosynthesis and *CHS* expression remain unaffected (Green and Fluhr, 1995). In addition, generation of ROS has been also reported after incubation of parsley cell cultures with fungal elicitors or synthetic peptides such as Pep25 (Nuernberger et al., 1994); however, this treatment represses UV-induced *CHS* (Lozoya et al., 1991) and also expression of *PcGST1*. From these data, the attractive hypothesis that GSH and PcGST1 act directly through the removal of oxidative stress products is unlikely and points to a different function during UV-dependent signal transduction.

PcGST1 belongs to plant type III GSTs, as revealed by sequence homology and genomic intron–exon patterns (Droog, 1997). Genes of this subclass were originally identified in a variety of species after induction by various different treatments—particularly by auxins, but also by pathogen infection, heavy metals, or heat shock (Marrs, 1996). *PcGST1* is the first member of this class found to be rapidly and transiently induced by UV-B. Strong but continuous stimulation of *PcGST1* was also found after treating parsley cells with auxins. Although the hormone-dependent PcGST1 stimulation points to the enzyme's additional function of conjugating 2,4-D and consequential detoxification besides its role in responding to UV light stimulation, interestingly, *CHS* expression is also increased after 2,4-D supplementation.

The function of PcGST1 during UV signaling, however, is unclear, and the identification of the substrate in vivo needs further investigation. Similarly, although many GSTs have been isolated from plants, their functions have been clarified in only a few cases. Coexpression of *GTXC* (ParC) (Takahashi and Nagata, 1992) increased the resistance of tobacco plants to low temperature and high salt concentrations (Roxas et al., 1997). These data provide direct evidence for a link between stress protection and GST activity, although

Table 1. Levels of Total GSH, Percentage of GSSG, and Corresponding LUC Activity in Control (pRT99) and GST_{ox} Lines after 2-hr Incubation in Darkness

	GSH + GSSG[a] (nmol/g fresh weight)	GSSG (%)	Relative LUC Activity[a] (cts/sec/μg protein)
CHS-LUC × pRT99	129	0.4	8
CHS-LUC × GST_{ox}	89	7.4	14

[a] The data represent the average results of two or three experiments. Variation between replicates was <10%.

the molecular reasons for this effect remain unknown. In contrast, the substrate of another type III GST has been identified in maize. Bz2 catalyzes the last step of anthocyanin biosynthesis and facilitates the transport of cyanidin-3-glucoside into the vacuole by an unknown mechanism (Marrs et al., 1995). Although low in sequence homology, *An9* from *Petunia hybrida* complements a maize *bz2* mutant and has a comparable function during anthocyanin biosynthesis (Alfenito et al., 1998). The involvement of PcGST1 during late steps of flavonoid biosynthesis in parsley is unlikely because its expression pattern does not match the late accumulation of flavonoid end products 24 to 48 hr after treatment with UV light (Hahlbrock, 1981; Dangl et al., 1987). In agreement with this, the enzyme accepts no phenylpropanoids, at least in vitro.

UV light irradiation also stimulates a supply pathway that provides substrates for subsequent flavonoid biosynthesis in parsley cell cultures. Members of this pathway are already synthesized in dark-incubated cells, and their expression is additionally stimulated in parallel to *PAL* and *CHS* by light treatment. One member of this pathway, acyl CoA oxidase, reveals a similar induction pattern as *PcGST1* but can also be stimulated by fungal elicitors (Logemann et al., 2000). As yet, no function of GSTs within this supply pathway has been described, and *PcGST1* expression is repressed after elicitor treatment of parsley cells. These findings argue against the involvement of GSTs in the supply pathway to flavonoid biosynthesis. Another early UV-induced gene encodes CPRF1, a transcription factor with the capacity for in vitro binding to the *CHS* promoter (Weisshaar et al., 1991). In contrast to *PcGST1*, coexpression of *CPRF1* does not stimulate but instead inhibits *CHS* expression (Feldbruegge et al., 1994), indicating a different role for CPRF1 during UV signal transduction.

Our data provide evidence for a novel function of GSTs involved in the UV-mediated signal transduction to *CHS*. In dark-incubated cells, increased amounts of the protein and of its substrate are essential for *CHS* stimulation. Interestingly, GSH and GST activity are already known as key elements during elicitor-stimulated *CHS* expression in cultured bean suspension cells. Although *CHS* is not UV-inducible in this system, it represents a key element during the hypersensitive response. Elicitor stimulation induces *GST* and *CHS* expression, and this effect can be partly mimicked by GSH supplementation (Dron et al., 1988; Wingate et al., 1988; Levine et al., 1994). Although UV light but not elicitors activates *PcGST1* or *CHS* in parsley, remarkable parallels are found in the signal transduction to *CHS* in both systems.

The molecular mechanisms underlying the activation of *CHS* expression in parsley include several possibilities. GSH and GSTs possibly affect *CHS* transcription by changing the redox state of antioxidant pools, as indicated by the increase of redox state of glutathione in $PcGST1_{ox}$ lines supplemented with external GSH. Such changes can alter the expression of nuclear genes (Creissen et al., 1999) and involve redox-modulated regulatory factors (Link, 1996). For example, binding of the FOS-JUN transcription factors to DNA in mammalian cells is regulated by the reduction and oxidation of conserved cystidine residues in the DNA binding domain (Abate et al., 1990). Besides these redox-modulated mechanisms, our working hypothesis for PcGST1 action during UV light signaling includes another possibility. Perhaps the protein can directly modify a repressor and thereby stimulate *CHS* expression. Further work is in

Table 2. Levels of Total GSH, Percentage of GSSG, and the Corresponding LUC Activity in Control (pRT99) and GST_{ox} Lines Incubated in 5 mM GSH-Containing Medium (as Described in Table 1)

	GSH + GSSG[a] (nmol/g fresh weight)	GSSG (%)	Relative LUC Activity[a] (cts/sec/μg protein)
CHS-LUC × pRT99	663	0.8	9
CHS-LUC × GST_{ox}	447	0.5	40

[a] The data represent the average results of two or three experiments. Variation between replicates was <10%.

progress to identify substrates and binding partners of PcGST1 in vivo.

METHODS

Plant Cell Culture, Protoplast Preparation, and Transient Transformation

Cell suspension cultures of parsley (*Petroselinum crispum*) were maintained in modified B5 medium in the dark, and one-tenth of its volume was subcultured weekly (Frohnmeyer et al., 1997). Protoplast preparation and transient transformation by electroporation were performed as described previously (Dangl et al., 1987; Renelt et al., 1993). Transgenic *chalcone synthase–luciferase* (*CHS-LUC*) cell lines were kept in a solid phase of modified B5 medium supplemented with 0.8% agarose and 30 mg/L hygromycin. Calli were grown for several months under these conditions, and stable integration of the plasmids was verified by DNA gel blot analysis. Every 2 weeks, one-tenth of the calli were transferred to new plates.

Light Sources and Experimental Conditions

Short-wavelength UV-B irradiation for periods ranging from 30 sec to 7 min was obtained from a Philips TL 40 W/12 fluorescent tube (λ_{max} 310 nm, half-bandwidth 40 nm, 17.6 μmol m^{-2} sec^{-1}). This light was filtered through transmission cutoff filters that reduced the fluence rate to 13 μmol m^{-2} sec^{-1} (>305 nm). UV-A light (15 μmol m^{-2} sec^{-1}), blue light (18 μmol m^{-2} sec^{-1}), and far-red light 13 μmol m^{-2} sec^{-1}) were as described previously (Schaefer, 1978; Frohnmeyer et al., 1992). For the isolation of early genes, protoplasts were freshly prepared and irradiated with continuous light (UV-A, blue, red, or far red) or with 4-min UV-B pulses and subsequent dark incubation until harvest after 2 hr. For experiments with growth factors (4.5 μM 2,4-D, 10 μM α-naphthylene acetic acid [α-NAA], 10 μM β-naphthylene acetic acid [β-NAA]), the elicitor Pep25 (175 nM), and reduced glutathione (5 mM), the substances were dissolved in culture medium.

Fluorescent Differential Display and Cloning of PcGST1

Total RNA from parsley suspension culture and derived protoplasts was prepared as previously described (Frohnmeyer et al., 1992) and treated with DNase (RQ1; Gibco BRL, Gaithersburg, MD). After phenol/chloroform extraction, the RNA was precipitated with ethanol, dissolved in Tris–EDTA buffer (10 mM Tris-HCL, pH 7.5; 1 mM EDTA), and subjected to differential display analysis (Uchida et al., 1998). For this, first-strand cDNAs were synthesized from 2.5 μg of total RNA with a Texas Red–labeled 3′-dG(dT)$_{15}$dG or 3′-dG(dT)$_{15}$dC primers by using a Superscript Preamplification System (Gibco BRL, Karlsruhe, Germany) and the reaction components were diluted to 200 μL by the 2.5 μM primer solution. Subsequent amplification was performed in a 96-well thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer, Norwalk, CT) containing 1 nmol of dNTP, a mixture of 0.5 units of Ampli-*Taq* polymerase (Perkin-Elmer) and 0.5 units of Taq polymerase (Nippon Gene, Toyama, Japan), 10 pmol of arbitrary primer (10 mer primer; OPERON Technologies, Alameda, CA), and 2 μL of cDNA solution that contained 5 pmol of Texas Red–labeled 3′-dG(dT)$_{15}$dG or 3′-dG(dT)$_{15}$dC primers in a 20-μL polymerase chain reaction (PCR) solution. The conditions for PCR were one cycle of 94°C for 3 min, 40°C for 5 min, and 72°C for 5 min, then 24 cycles of 94°C for 15 sec, 40°C for 2 min, and 72°C for 1 min, and a final extension for 5 min at 72°C. After addition of loading buffer, each PCR product was concentrated by evaporation at 60°C for 25 min and denatured at 80°C for 3 min. Aliquots of each sample were separated on a 6% Long Ranger Pharmacia gel system (FMC BioProducts, Rockland, ME) containing 6.1 M urea, and 1.2 × TBE (60 mM Tris-HCL, pH 8.3; 60 mM boric acid, 1.2 mM EDTA), and the fragments were visualized by an automated DNA sequencer (SQ-5500; Hitachi Ltd., Tokyo, Japan). To isolate the band of interest, the fingerprinting pattern was scanned by the fluorescent image analyzer (FMBIO-100; TaKaRa Shuzo, Tokyo, Japan). Positive bands were cut out from the gel and reamplified by using the same PCR conditions as were used for fluorescent differential display (FDD) (Ito et al., 1994). Amplified cDNA fragments were cloned into pT7 Blue T-vector (Novagen, Madison, WI) and reamplified by using Texas Red–labeled 3′-dG(dT)$_{15}$dG or 3′-dG(dT)$_{15}$dC and the appropriate arbitrary primer. The correct sizes of reamplified fragments were routinely verified on agarose gels and on gel systems used for FDD. Clones with identical sizes were sequenced with a sequencing kit (Amersham) and a DNA sequencer (SQ-5500; Hitachi Ltd.). The size and induction pattern of positive fragments was verified by RNA gel blot analysis. Cloning of full-length *PcGST1* cDNA was performed by using a cDNA library constructed from RNA of parsley cells that had been irradiated for 2 hr with UV light. This sequence has been submitted to the GenBank database under accession number AF177944.

Synthesis of *PcGST1* in *Escherichia coli*

The PcGST1 cDNA was introduced into the expression vector PQE30 (Qiagen, Hilden, Germany), and *E. coli* strain M15pREP4 was transformed with the resulting plasmid. Single-step purification of the fusion protein was performed as described in the manufacturer's protocol. The expression and all purification steps were monitored by protein staining of each fraction after SDS-PAGE. Assays for glutathione *S*-transferase (GST) were performed with the universal substrate 1-chloro-2,4-dinitrobenzene or *p*-coumaric acid, and activity was determined spectroscopically by the change in absorbance at 340 nm (Habig et al., 1974). Peroxidase activity was assayed with cumene hydroperoxide as the substrate. The reaction was measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm (Simmons et al., 1989).

RNA Gel Blot Analysis and Immunodetection

Total RNA (20 μg) extracted from cell suspension culture was separated in agarose–formaldehyde gels and blotted onto UV Duralon membranes (Stratagene, La Jolla, CA), as described (Frohnmeyer et al., 1997). A full-length cDNA fragment of *PcGST1* subcloned into Bluescript KSII was excised by EcoRI digestion. This fragment, together with *CHS* and *UBI4* cDNA (Reimold et al., 1983; Kawalleck et al., 1993), was used for preparing random-primed probes. After washing with 2 × SSC, 1 × SSC, and 0. 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 60 to 64°C, the filters were autoradiographed at −80°C for 6 to 24 hr by using an intensifying screen (BioMax TranScreen; Kodak, Rochester, NY). Blots from three experiments were quantitated with a Fuji BAS1000 bioimaging analyzer (Raytest, Paris, France) and using PCBASD software (Raytest,

Straubenhardt, Germany). Total protein determinations, separation of protein extracts by SDS-PAGE, protein blotting, and immunodetection were performed according to Frohnmeyer et al. (1992). Polyclonal antibodies raised against LUC were used at 1:1000 dilution and purchased from Rockland (Gilbertsville, PA).

Expression of PcGST1 in Parsley Cell Cultures and Detection of LUC Enzyme Activity

Construction of the *CHS-LUC* reporter in a plasmid conferring hygromycin resistance has been described (Frohnmeyer et al., 1999). The *PcGST1* open reading frame was introduced into the EcoRI site of pRT99neo (Toepfer et al., 1988). This vector contains a neomycin phosphotransferase gene conferring G418 resistance, which was used as a second selection for double-transformed cell lines with a uniform *CHS-LUC* background. *CHS-LUC* cells were continuously selected on hygromycin-containing solid medium. For transformation by particle inflow gun (Finer et al., 1992), calli were dissolved in liquid medium and used after 3 days. 35S/*PcGST1* was bound to gold particles by $CaCl_2$/ethanol precipitation (2 μg of DNA/0.5 mg of gold; 1.5 to 3 μm in diameter; Sigma) in the presence of spermidine, and cells were bombarded as described (Frohnmeyer et al., 1999). Double-transformed cells were selected on solid medium containing 7.5 mg/L G418 and 7.5 mg/L hygromycin and grown for 4 to 6 weeks until calli became visible. Further selection of these calli was performed on solid medium containing 15 mg/L G418 and 15 mg/L hygromycin for a few months. Calli were then dissolved in liquid medium and cultivated for several weeks, as described for wild-type cells (Frohnmeyer et al., 1997). Experiments were performed with cells 3 to 5 days after passage to new medium.

LUC assays were performed as described (Frohnmeyer et al., 1999) after pulverization of cell cultures or protoplasts in LucI buffer (100 mM KH_2PO_4, pH 7.8, and 1 mM DTE) and centrifugation for 10 min at 14,000*g*. The samples were normalized to the amount of soluble protein present, as determined by the Bradford assay (Bio-Rad). Steady state *LUC* expression was continuously monitored in vivo in a Berthold (Straubenhardt, Germany) Microlumat LB 96P by using the repeated mode after supplementing the cells with luciferin (1 mM).

Analysis of Glutathione

Parsley cell culture was ground in 2 mL of $HClO_4$ and centrifuged at 10,000*g* for 5 min; the supernatant used for determination of total glutathione (GSH) and oxidized GSH (GSSG) was as described by Griffith (1980).

ACKNOWLEDGMENTS

We thank Dierk Scheel for the generous gift of synthetic Pep25 elicitor and Karsten Jaekel for assistance during the determination of GSH concentrations and GST activity. This work was funded by Grant No. FR936/1 from Deutsche Forschungsgemeinschaft to H.F. and in part by a Hitachi Advanced Research Laboratory (HARL) grant to M.F. and H.F. L.L. was supported by a graduate fellowship from Landesgraduiertenfoerderung.

Received March 17, 2000; accepted July 4, 2000.

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