Plant Physiol. (1994) 104: 945–952

Involvement of Oxidative Processes in the Signaling Mechanisms Leading to the Activation of Glyceollin Synthesis in Soybean (*Glycine max*)

Norbert Degousée, Christian Triantaphylidès, and Jean-Luc Montillet*

Département de Physiologie Végétale et Ecosystèmes, Centre d'Etudes de Cadarache, Commissariat á l'Energie Atomique, F-13108 Saint Paul-Lez-Durance, Cedex, France

The efficiency of hydroperoxides (*tert*-butyl hydroperoxide, hydrogen peroxide) and sulfhydryl reagents (iodoacetamide, *p*-chloromercuribenzene sulfonic acid) as glyceollin elicitors was examined in relation to sulfhydryl oxidation, or alteration, and to lipid peroxidation, in 3-d-old soybean hypocotyl/radicle, *Glycine max*. These oxidative events were investigated as possible early steps in the transduction mechanisms leading to phytoalexin synthesis. Free protein sulfhydryl groups were not modified after any of the eliciting treatments, thus indicating that immediate massive protein oxidation or modification cannot be considered a signal transduction step. Unlike sulfhydryl reagents, which led to a decrease of the free nonprotein sulfhydryl group (free np-SH) pool under all of the eliciting conditions, the results obtained with hydroperoxides indicated that immediate oxidation of the np-SH is not required for the signal transduction. Moreover, elicitation with 10 mM *tert*-butyl hydroperoxide did not lead to further oxidation or to changes in np-SH level during the critical phase of phenylalanine ammonia-lyase activation (the first 20 h), suggesting that np-SH modifications are probably not involved in hydroperoxide-induced elicitation. On the other hand, all treatments leading to significant glyceollin accumulation were able to trigger a rapid (within 2 h) lipid peroxidation process, whereas noneliciting treatments did not. In addition, transition metals, such as Fe^{2+} and Cu^{+}, were shown to stimulate both hydrogen peroxide-induced lipid peroxidation and glyceollin accumulation, again emphasizing that the two processes are at least closely linked in soybean. Among the oxidative processes triggered by activated oxygen species, oxidation of sulfhydryl compounds, or lipid peroxidation, our results suggest that lipid peroxidation is sufficient to initiate glyceollin accumulation in soybean. This further supports the hypothesis that lipid peroxidation could be involved as a step in the signal cascade that leads to induction of plant defenses.

Phytoalexin accumulation in plant tissues is a regulated defense response to pathogen infections and also to biotic and abiotic elicitors. Although the transduction mechanisms leading to such a metabolic shift are poorly understood, there is much evidence that oxidative processes can play a crucial role during the very early steps following signal perception. Indeed, the production of powerful oxidizing species such as $O_2^{-\cdot}$ and H_2O_2 have been observed in plant cells soon after elicitor treatments (Apostol et al., 1989; Anderson et al., 1991; Arnott and Murphy, 1991; Vera-Estrella et al., 1992) or upon challenge with pathogens (Chai and Doke, 1987; Adam et al., 1989; Baker et al., 1991; Glazener et al., 1991; Devlin and Gustine, 1992). In addition, it has been shown that several cellular redox state-modifying compounds, such as sulfhydryl reagents, are able to elicit the accumulation of the isoflavonoid phytoalexins in clover callus (Gustine, 1981, 1987) and in soybean (*Glycine max*) hypocotyls (Stössel, 1984). It is difficult to assume that there is direct biological relevance to such sulfhydryl modified groups. Nevertheless, in so far as nonprotein sulfhydryl compounds (GSH or hGSH) may be concerned, this could bring about indirect consequences such as the depletion of the cellular antioxidant capacities. Consequently, transient production of AO species in cells is expected (Miccadei et al., 1988).

Such oxidative bursts could lead to the direct or indirect oxidation of some cellular sulfhydryl groups (protein or nonprotein), and these oxidized products could be themselves responsible for the transduction of the elicitor signal. Alternatively, AO species may be able to initiate a lipid peroxidation process in the plant tissues. This has already been described after elicitation (Rogers et al., 1988; Peever and Higgins, 1989; Vera-Estrella et al., 1992) or during incompatible plant/microorganism interactions (Keppler and Novacky, 1986; Adam et al., 1989; Popham and Novacky, 1991). The release of signals arising from this early peroxidative event has been postulated (Rogers et al., 1988) and has also been supported recently by studies demonstrating that peroxidized fatty acid metabolites may participate as intracellular messengers in the signaling system that activates various

* Corresponding author; fax 33–42–25–46–56.

Abbreviations: AO, activated oxygen; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); hGSH, homoglutathione; HODE, hydroxy octadecadienoic acid; 9-HODE, 9-hydroxy-,10,12(E,Z)octadecadienoic acid; 13(*S*)-HODE, 13(*S*)-hydroxy-9,11(Z,E)octadecadienoic acid; HPODE, hydroperoxy octadecadienoic acid; 13(*S*)-HPODE, 13(*S*)-hydroperoxy-9,11(Z,E)octadecadienoic acid; HOTE, hydroxy octadecatrienoic acid; 12-HOTE, 12-hydroxy-9,13,15(Z,E,Z)octadecatrienoic acid; 13(*S*)-HOTE, 13(*S*)-hydroxy-9,11,15(Z,E,Z)octadecatrienoic acid; 16-HOTE, 16-hydroxy-9,12,14(Z,Z,E)octadecatrienoic acid; HPOTE, hydroperoxy octadecatrienoic acid; 13(*S*)-HPOTE, 13(*S*)-hydroperoxy-9,11,15(Z,E,Z)octadecatrienoic acid; hyp/rad, hypocotyl/radicle; IA, iodoacetamide; LPI, lipid peroxidation index; np-SH, non-protein sulfhydryl group; PAL, phenylalanine ammonia-lyase; PCMBS, *p*-chloromercuribenzene sulfonic acid; p-SH, protein sulfhydryl group; $O_2^{-\cdot}$, superoxide anion; tBuOOH, *tert*-butyl hydroperoxide.

plant defense pathways (Li et al., 1991; Farmer and Ryan, 1992).

Considering that AO species production, lipid peroxidation, and sulfhydryl oxidation are interdependent phenomena belonging to an "oxidative cycle," their respective involvements as a source of endogenous signals of plant defense responses remain difficult to establish. To better answer this question, this work was undertaken to compare the efficiencies of two hydroperoxides, H_2O_2 and tBuOOH, and two sulfhydryl reagents, IA and PCMBS, as glyceollin elicitors in soybean, since these chemicals all have been described as powerful inducers of isoflavonoid phytoalexin accumulation (Gustine, 1981; Stössel, 1984; Montillet and Degousée, 1991). Next, we investigated their respective oxidative effects on sulfhydryl and lipid pools on a short-term basis. We also describe here a new sensitive and simple method that allows the quantification, in a single analysis, of all the fatty acid hydroperoxide stereoisomers present in a plant tissue. Finally, in light of our results, the possible involvement of the two oxidative processes in the signal cascade that leads to the soybean defense induction is discussed.

MATERIALS AND METHODS

Chemicals

Thirty percent H_2O_2 was purchased from Prolabo (Paris, France), and 70% tBuOOH was obtained from Aldrich Chemical Co. GSH, Cys, IA, PCMBS, and DTNB were purchased from Sigma, and sodium borohydride was from Fluka. Linoleic and linolenic acids (approximately 99% pure) and soybean lipoxygenase-1 (16 units mg^{-1} of protein) were from Fluka. HPLC grade solvents were from Rathburn (Walkerburn, Scotland).

Plant Material

Soybean seeds [*Glycine max* (L.) Merr, cv Soriano] (Northrup King Semences, Saint-Sauveur, France) were germinated under sterile conditions. The hyp/rad prepared by removing cotyledons from seedlings were used for elicitation treatments as previously described (Montillet and Degousée, 1991).

Glyceollin Extraction and Determination

Glyceollin analysis was essentially as previously reported (Montillet and Degousée, 1991) but slightly modified to simplify the extraction procedure. Briefly, sets of five hyp/rad were weighed and homogenized in 8 mL of absolute ethanol with an Ultra-Turrax at full speed for 20 s. The sample was then centrifuged at 3000*g* for 20 min, and the ethanolic supernatant was collected. The pellet was further washed with 2.5 mL of absolute ethanol, and after centrifugation, both supernatants were combined and dried under vacuum. The residue was taken up in 1 mL of methanol and filtered through a 0.45-μm filter (Millipore, Saint-Quentin en Yuelines, France) undergoing HPLC analysis (Montillet and Degousée, 1991).

Preparation of Tissue Homogenates for Sulfhydryl Group Determination

Three-day-old hyp/rad (2 g fresh weight) were homogenized at 4°C with an Ultra-Turrax at full speed for 20 s in 8 mL of an oxygen-free (obtained by helium bubbling) grinding solution (0.15% [w/v] sodium ascorbate, 20 mM EDTA). Two milliliters of 10% (w/v) SDS (prepared in the grinding solution) were immediately added to the sample to stabilize nonprotein free sulfhydryl groups and to improve yields in p-SH extraction. The homogenate was then filtered through one layer of Miracloth, and the filtrate was centrifuged at 30,000*g* for 15 min. The resulting supernatant was used to determine sulfhydryl group contents.

Protein and Nonprotein Free Sulfhydryl Group Determination

Free sulfhydryl group titration was based on the spectrophotometric method of Ellman (1959). Free total sulfhydryl group content was estimated by mixing an aliquot of 0.5 mL of the 30,000*g* supernatant with 1 mL of 1 M Tris buffer (pH 8.1) and 0.1 mL of a 10 mM DTNB solution in 0.2 M sodium phosphate buffer (pH 7.0). After 5 min A_{412} was measured with a Kontron 860 spectrophotometer. The molar extinction coefficient is 13,600 M^{-1} cm^{-1} (Ellman, 1959). A_{412} was corrected for the color of a sample blank prepared without DTNB, which was replaced by 0.2 M sodium phosphate buffer (pH 7.0), and for the color of a reagent blank prepared without plant extract, which was replaced by the grinding solution containing 2% (w/v) SDS.

For free np-SH assays, 5-mL aliquots of the 30,000*g* supernatant extract were mixed with 1.25 mL of 50% (w/v) TCA in the grinding solution. Protein precipitation was allowed to take place during 10 min at room temperature, and samples were centrifuged at 30,000*g* for 15 min. Then, 0.5 mL of the deproteinized supernatant was mixed with 1 mL of 1 M Tris buffer (pH 8.1) and 0.1 mL of the 10 mM DTNB solution. The A_{412} was determined and corrected as above. The free p-SH content was calculated by subtracting the free np-SH value from the free total sulfhydryl group value.

Estimation of Oxidized np-SH

Oxidized np-SH concentrations were estimated by subtracting the free np-SH value from the total np-SH value (free plus oxidized) obtained after a $NaBH_4$ reduction step. This last step was performed according to the slightly modified procedure of Davies et al. (1984). Briefly, an aliquot of the deproteinized supernatant was passed through a Sep-Pak C_{18} cartridge (Waters) to remove SDS prior to $NaBH_4$ reduction. Then, 1 mL of the SDS-free sample was slowly mixed with 1 mL of a solution composed of equal parts of 1.3 N NaOH and 4 M $NaBH_4$ in 2% (w/v) NaOH and 0.2 M EDTA at pH 9.0. After 5 min, excess borohydride was hydrolyzed by adding 0.2 mL of 5 N HCl. One milliliter of this mixture was added to 0.5 mL of 1 M Tris buffer (pH 8.1) and 0.1 mL of 10 mM DTNB solution. The A_{412} was measured and corrected for the colors of both reagents and sample blanks as above.

Hydroperoxy and Hydroxy Fatty Acid Extraction and Quantification

HPODE and HPOTE contents of the tissues were determined by HPLC quantification of their corresponding hydroxy derivatives, HODE and HOTE, obtained after $NaBH_4$ reduction and hydrolysis of a lipid extract. Briefly, 2.5 g fresh weight of soybean tissues were homogenized at 4°C with an Ultra-Turrax homogenizer for 1 min at 13,500 rpm in 5 mL of 5% (w/v) $NaBH_4$ prepared in 0.2 N NaOH. The homogenate was then slowly acidified to pH 4 with approximately 0.5 mL of $HClO_4$ (70%) and extracted with 2 volumes of chloroform:methanol (50:50, v/v); phase separation was effected by centrifugation at 700*g* for 5 min. The resulting aqueous phase was further extracted with 1 volume of chloroform, and both organic phases were pooled before vacuum evaporation of the solvent. The residue was then hydrolyzed by reflux for 15 min in 2.5 mL of 3.5 N NaOH containing 2.5 mL of absolute ethanol. After cooling, the sample was finally acidified to pH 4 with 2.5 mL of 3.5 N $HClO_4$ and extracted with 1.5 mL of hexane:diethyl ether (70:30, v/v). The organic phase was collected after centrifugation and used for HPLC analysis. Analytical HPLC was performed with an LKB instrument equipped with a stainless steel cartridge (4.6 × 250 mm) packed with 5 μm of Adsorbosphere HS silica (Alltech, Paris, France). Hydroxy fatty acid stereoisomers were separated by isocratic elution with hexane:diethyl ether:acetic acid (70:30:0.5, v/v/v) at a flow rate of 1 mL min^{-1}. A_{234} was monitored, and quantification was performed after calibration with authentic 13*S*-HODE (98% purity and 99% enantiomeric excess) using a molar extinction coefficient of 25,000 M^{-1} cm^{-1} (Iacazio et al., 1990). Chromatographic standards were prepared by $NaBH_4$ reduction of the peroxidized products (Iacazio et al., 1990). 13*S*-HODE and 13*S*-HOTE were obtained from authentic hydroperoxy fatty acids, 13*S*-HPODE and 13*S*-HPOTE (98% purity and 99% enantiomeric excess) following reaction with commercial soybean lipoxygenase-1, according to the method of Iacazio et al. (1990). Furthermore, all of the possible stereoisomers corresponding to the oxygenation products of the octadecapolyenoic acids were obtained by autoxidation of linoleic acid with hemoglobin at 20°C according to the method of Kühn et al. (1987) and by photooxidation of linolenic acid according to the method of Frankel et al. (1982).

PAL Assay

Soybean extracts were prepared according to the slightly modified procedure of Robbins et al. (1991). About 2 g fresh weight of soybean tissues were homogenized at 4°C with an Ultra-Turrax at full speed for 20 s in 5 mL of 0.1 M sodium borate buffer (pH 8.8), 15 mM 2-mercaptoethanol containing 5% (w/v) Dowex 1 × 2 and 2.5% (w/v) PVP. The extract was then wrung through two layers of nylon cloth, and the resulting filtrate was centrifuged at 20,000*g* for 10 min. PAL activity was spectrophotometrically assayed at 290 nm by diluting 0.1 mL of the enzyme extract to 1 mL with 0.1 M sodium borate buffer (pH 8.8) and then adding an equal volume of the same buffer containing 20 mM L-Phe. The mixture was incubated at 40°C for 1 h, and the change in *A* was measured against a blank containing no substrate. PAL activity was expressed in μkatal kg^{-1} of protein using a molar extinction coefficient for cinnamate of 10,000 M^{-1} cm^{-1}. Protein determination was carried out according to the method of Bradford (1976).

RESULTS

Effect of Sulfhydryl Reagents and Hydroperoxides on Glyceollin Accumulation in Soybean Tissues

The two sulfhydryl reagents IA and PCMBS, as well as the hydroperoxides tBuOOH and H_2O_2, were able to elicit glyceollin accumulation in soybean hyp/rad, whereas the phytoalexin levels in water-treated controls were not detectable (Fig. 1). The dose effects shown in Figure 1A indicated that in a concentration range of 1 to 10 mM the level of glyceollin in the sulfhydryl reagent-treated tissues did not exceed 60 μg g^{-1} fresh weight. IA was efficient at low concentrations, since a 1 mM treatment brought about significant phytoalexin levels (17 μg g^{-1} fresh weight) and a concentration of 3 mM nearly corresponded to the optimal dose. Higher IA concentrations (up to 10 mM) gave similar phytoalexin responses to those seen at 3 mM. On the other hand, PCMBS was ineffective at doses lower than 4 mM. Beyond this threshold, glyceollin levels linearly increased with the sulfhydryl reagent concentration, reaching 35 μg g^{-1} fresh weight for a 10 mM treatment.

Figure 1 shows that glyceollin accumulation peaked at 150 μg g^{-1} fresh weight with 10 mM tBuOOH and reached a plateau of 120 μg g^{-1} fresh weight at an H_2O_2 concentration of 0.75 M. No significant accumulation of glyceollin was observed at concentrations lower than 50 mM for an H_2O_2

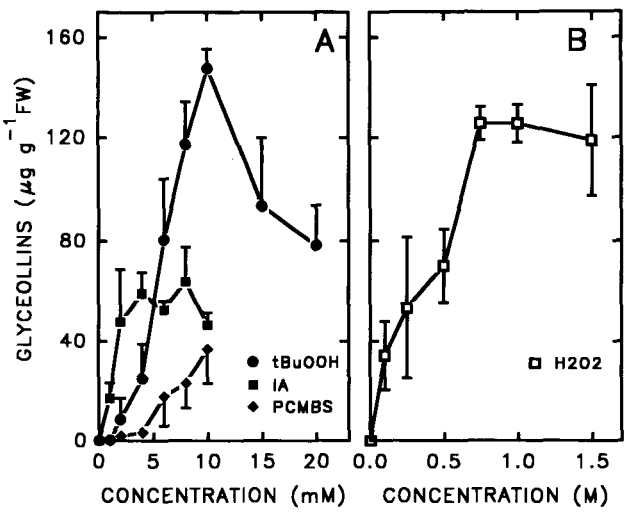

Figure 1. Production of glyceollins in hyp/rad of 3-d-old etiolated soybean seedlings immersed for 30 min in (A) aqueous solutions of tBuOOH (●), IA (■), or PCMBS (◆) and (B) aqueous solutions of H_2O_2 (□). Tissues were then rinsed with deionized water and incubated for 48 h at 25°C in the dark before glyceollin quantification. No glyceollin accumulation was detected in water-treated controls. Values are means ± SD for two experiments with three replicates each.

treatment and 2 mM for tBuOOH. To further compare the four elicitor treatments, the amount of accumulated glyceollin was followed over time after 1 M H_2O_2, 10 mM tBuOOH, 3 mM IA, and 8 mM PCMBS treatments. As shown in Figure 2, glyceollin levels in tissues were significant after a lag period of about 15 h and reached their maximum value by 39 h, whatever the treatment.

Effect on the Sulfhydryl Pools

Since the four abiotic elicitors, H_2O_2, tBuOOH, IA, and PCMBS, were all potentially able to modify the cellular sulfhydryl pools, we first investigated their dose effect on both free p-SH and np-SH (Fig. 3). The spectrophotometric quantifications were carried out immediately after the 30-min treatments. The p-SH levels were corrected for a recovery yield of 75% (determined with BSA), and the np-SH levels were similarly corrected for a recovery yield of 90% (determined with GSH). The results were expressed as a percentage of the water-treated control levels, which were 500 ± 40 nmol g^{-1} fresh weight for free p-SH and 260 ± 30 nmol g^{-1} fresh weight for free np-SH.

As shown in Figure 3, none of these compounds induced any significant modification of the free p-SH levels in a concentration range in which elicitor activity was observed. Under the same conditions, treatment with PCMBS, IA, and H_2O_2 caused an immediate concentration-dependent decrease in free np-SH (Fig. 3, A, B, and C, respectively). The lowest active doses were 5 mM for PCMBS, 0.5 mM for IA, and 0.5 M for H_2O_2, which led to free np-SH decreases of about 20 to 30% of the control in each case. In contrast, tBuOOH at eliciting concentrations (up to 10 mM) did not induce any comparable change (Fig. 3D). The same result was observed at 20 mM, whereas 40 and 100 mM treatments led to 40 and 65% free np-SH decreases, respectively (data not shown).

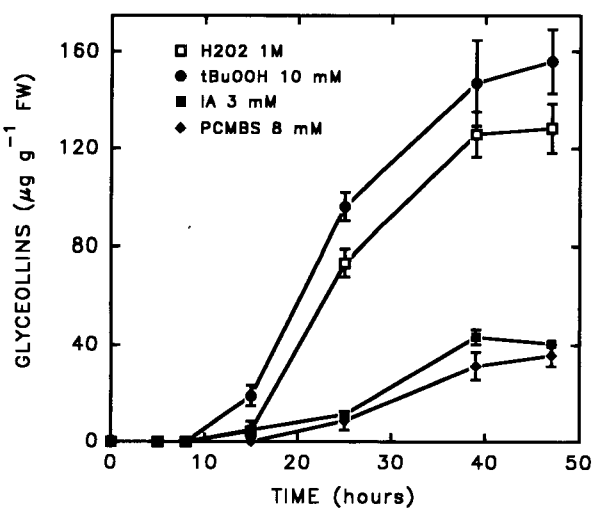

Figure 2. Time course of glyceollin accumulation in hyp/rad of 3-d-old etiolated soybean seedlings after treatments in aqueous solutions of 1 M H_2O_2 (□), 10 mM tBuOOH (●), 3 mM IA (■), and 8 mM PCMBS (♦) for 30 min. No glyceollin accumulation was detected in water-treated controls. Values are means ± SD for two experiments with three replicates each.

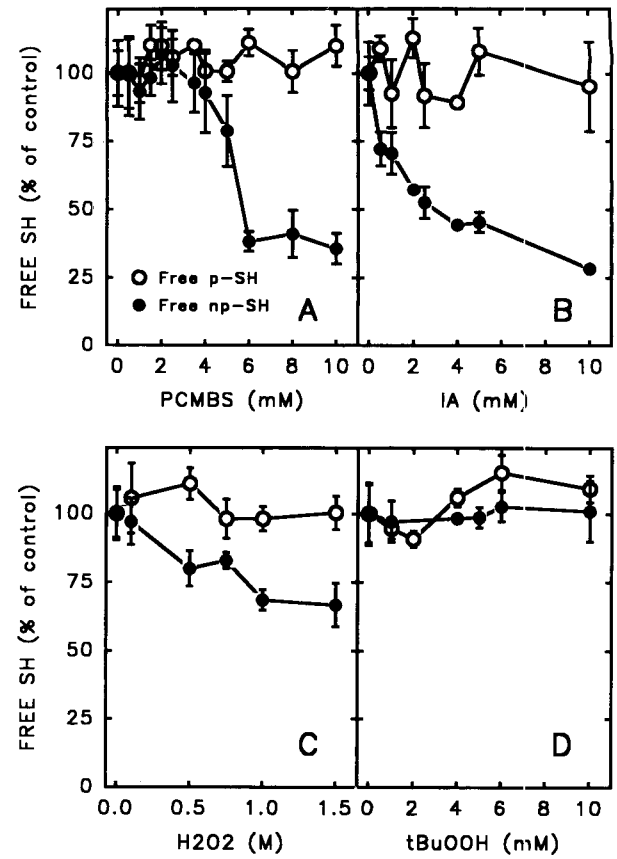

Figure 3. Dose effect of PCMBS (A), IA (B), H_2O_2 (C), and tBuOOH (D) on protein free sulfhydryl (free p-SH, open symbols) and non-protein free sulfhydryl groups (free np-SH, closed symbols). Sulfhydryl extractions and quantifications were performed immediately after a 30-min treatment of the hyp/rad of 3-d-old etiolated soybean seedlings as described in "Materials and Methods." Results are expressed as percentages of control and represent means ± SD for two experiments with three replicates each.

Since eliciting concentrations of tBuOOH did not lead to any immediate modification of the free np-SH pool, the evolution of both total and oxidized np-SH levels was followed during the critical phase of elicitation (Fig. 4). In this context, PAL activity was used as a marker for involvement of the phenylpropanoid pathway in glyceollin synthesis. As shown in Figure 4A, the PAL activity of soybean hyp/rad treated with 10 mM tBuOOH increased significantly after a 3-h lag period and peaked 15 h later at a value of 23 μkatal kg^{-1} of protein. In contrast, the activity of controls was fairly stable during this time at approximately 8 μkatal kg^{-1} of protein. Throughout this period (i.e. the first 20 h) no modification of either total np-SH (Fig. 4B) or oxidized np-SH (Fig. 4A) occurred in these tBuOOH-treated tissues. In addition, the mean of the total np-SH levels in both sets of samples remained constant at a value of 260 nmol g^{-1} fresh weight, and the oxidized form did not represent more than 10% of this pool.

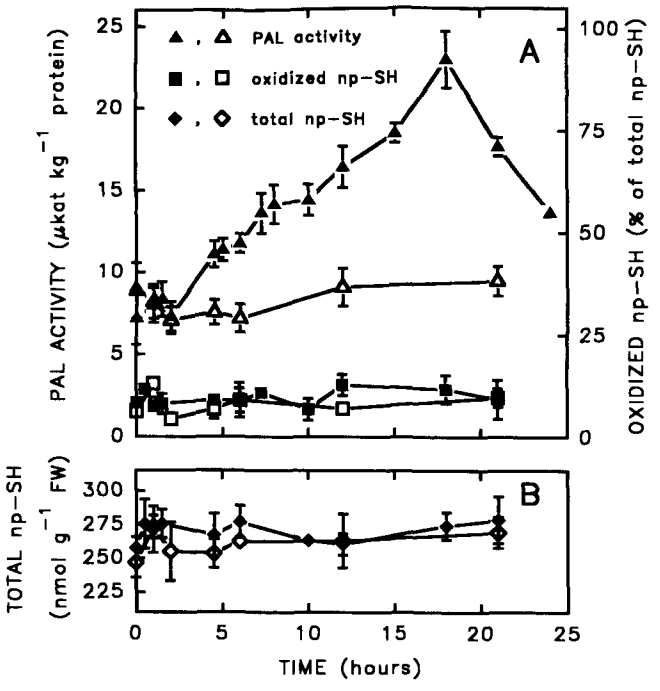

Figure 4. (A) Oxidized np-SH (■, □) and PAL activity (▲, △) and (B) total np-SH levels (♦, ◇) as a function of time after treatment of soybean hyp/rad with 10 mM tBuOOH (closed symbols) or in water-treated controls (open symbols). Values are means ± SD for two experiments with three replicates each.

The LPI

Lipid peroxides present in soybean tissues after hydroperoxide or sulfhydryl reagent treatments were quantified on lipid extracts after borohydride reduction and basic hydrolysis steps carried out to stabilize them as hydroxy derivatives. Under these conditions, free and esterified forms were all quantified. 13*S*-HPODE and 13*S*-HPOTE, enzymically prepared from linoleic and linolenic acids with 98% purity and 99% enantiomeric excess, were both recovered with a 65% yield when added to the soybean sample. Their corresponding reduced hydroxy derivatives, 13*S*-HODE and 13*S*-HOTE, were analyzed on a silica phase HPLC and were eluted with retention times of 16.3 and 16.9 min, respectively, under our chromatographic conditions (Fig. 5, A [black peak] and B [gray peak]). Furthermore, peak attribution to all the other regio-stereoisomers of hydroxy derivatives was carried out by analyzing the reduced autoxidation and photooxidation products of linoleic and linolenic acids, respectively.

As shown in Figure 5A, autoxidation of linoleic acid gave four peaks, two of which are major (peaks a and c); one of these (peak a) was analyzed as 13-HODE and represented 33% of the total, and the other (peak c, 21.2 min, 44%) was attributed to 9-HODE according to Kühn et al. (1987). The two minor peaks (peak b, 19.8 min; peak d, 24.9 min), assumed to be the all-*trans* isomers of 13- and 9-HODE (Kühn et al., 1987), did not represent more than 15% each. The photooxidation profile of linolenic acid (Fig. 5B) showed four major peaks, likely corresponding to the *cis*-conjugated *cis-trans* isomers of 9-, 12-, and 16-HOTE—peak f (17.4 min, 18%), peak h (22 min, 30%), and peak i (23 min, 25%)—in addition to 13-HOTE, peak e (16.9 min, 18%). Together, the three minor peaks, g, j, and k, did not exceed 10% and were tentatively attributed to the *cis*-conjugated *trans-trans* isomers. Finally, the profile in Figure 5C represented a typical soybean sample in which all of the above major isomers could be observed. The lipid peroxide level was quantified in a soybean sample by summing up the values corresponding to all of these isomers, and the LPI was determined by subtracting the lipid peroxide level of the control from the assay level.

Effect on Lipid Peroxidation

The time course evolution of the in vivo lipid peroxidation in soybean hyp/rad was followed by measuring the LPI in samples after five selected treatments: 1 M H_2O_2, 10 mM tBuOOH, 3 mM IA, and 3 and 8 mM PCMBS (Fig. 6). In three of these (H_2O_2, IA, and 8 mM PCMBS) the initial LPI was low (less than 8 nmol g^{-1} fresh weight) and progressively increased, reaching an approximately stable value of 20 nmol g^{-1} fresh weight after 2 h with both sulfhydryl reagents, whereas it continued to increase to a value of 60 nmol g^{-1} fresh weight at 4 h with H_2O_2. In contrast, a 10 mM tBuOOH treatment resulted in an immediate increase of the LPI, which remained fairly constant at approximately 25 nmol g^{-1} fresh

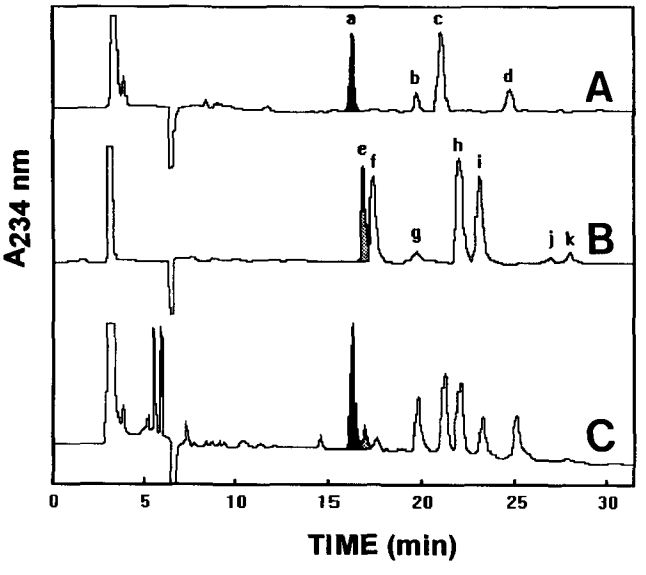

Figure 5. Silica-phase HPLC analysis of the reduced oxygenation products formed by autoxidation of linoleic acid with hemoglobin at 20°C (A), formed by methylene blue-mediated photooxidation of linolenic acid (B), or present in untreated 3-d-old soybean hyp/rad (C). Peak a corresponds to 13-HODE (co-eluted with the black peak: 13-*S* HODE), and peak e corresponds to 13-HOTE (co-eluted with the grey peak: 13-*S* HOTE). The other *cis-trans* isomers are peak c (9-HODE according to Kühn et al., 1987), f, h, and i (unidentified positional isomers of 9-, 12-, and 16-HOTE). The all-*trans* isomers of HODE have been tentatively identified as peaks b and d, whereas the *cis*-conjugated *trans-trans* isomers of HOTE are attributed to peaks g, j, and k.

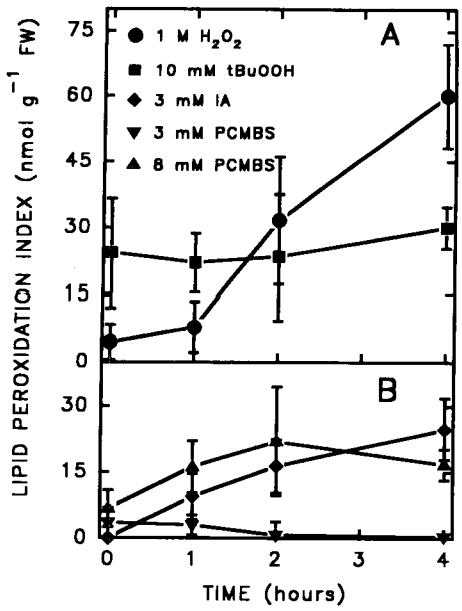

Figure 6. Changes of the LPI as a function of time in response to treatments with (A) 1 M H_2O_2 (●) and 10 mM tBuOOH (■); (B) 3 mM IA (◆), 3 mM PCMBS (▼), and 8 mM PCMBS (▲). The LPI was determined by subtracting the lipid peroxide level of the control (26 ± 3 nmol g^{-1} fresh weight) from the assay level. Values are means ± SD for two experiments with three replicates each.

weight for at least 4 h. During the same time, a 3 mM PCMBS treatment did not significantly increase the LPI.

Finally, the consequences of a transition metal modulation of the H_2O_2-induced lipid peroxidation were investigated by immersing soybean tissues in Fe^{2+} or Cu^{+} solutions prior to the peroxide treatment. As shown in Table I, neither Cu^{+} nor Fe^{2+} alone was able to induce any lipid peroxidation or glyceollin accumulation in tissues, whereas combined treatments with the transition metal plus H_2O_2 both enhanced glyceollin accumulation about 2-fold as compared to a 1 M H_2O_2 treatment.

DISCUSSION

In the present work we investigated the relationship between oxidative processes and glyceollin synthesis in soybean hyp/rad, cv Soriano. Initially, the elicitor efficiencies of two hydroperoxides, tBuOOH and H_2O_2, and of two sulfhydryl reagents, PCMBS and IA, were compared (Figs. 1 and 2). Both tBuOOH and H_2O_2 are able to elicit glyceollin accumulations at doses quite similar to those previously reported for cv Kingsoy (Montillet and Degousée, 1991). Indeed, the optimal tBuOOH concentration is about 10 mM (Fig. 1A), whereas that of H_2O_2 is approximately 1 M (Fig. 1B). PCMBS and IA show elicitor activity at millimolar levels as described elsewhere (Gustine, 1981, 1987; Stössel, 1984), but they are far less active than tBuOOH in concentrations ranging from 5 to 10 mM (Fig. 1A). Since the time course of glyceollin synthesis (Fig. 2) is similar regardless of the treatment (with an optimum glyceollin level in soybean tissues at about 48 h), we can speculate that such discrepancies in elicitor activity are likely due to differences in the elicitation signal intensity produced by the different agents.

All of these elicitors are able to oxidize or to alter the cellular sulfhydryl groups by the formation of a disulfide, or mercaptide, or -alkyl derivatives. The free p-SH pool of the 3-d-old soybean hyp/rad, (500 ± 40 pmol g^{-1} fresh weight, 65% of the total free SH) is clearly unaffected by any of the treatments (Fig. 3), thus indicating that immediate massive protein oxidation or modification cannot be considered as a signal transduction step. The level of free np-SH (260 ± 30 nmol g^{-1} fresh weight) was comparable to that reported in the soybean cv Maple Arrow (Klapheck, 1988). Considering that about 95% of this pool, in legumes and particularly in soybean, is represented by hGSH (γ-glutamyl-cysteinyl-β-Ala) (Klapheck, 1988), we can assume that changes in free np-SH (Fig. 3) are approximately equivalent to changes in the free hGSH pool.

The actions of the sulfhydryl reagents PCMBS and IA on the hGSH pool are quite different. IA concentrations as low as 0.5 to 1 mM induce significant glyceollin accumulations (Fig. 1A) and an immediate significant decrease (about 25%) in the level of the free hGSH (Fig. 3B), indicating that this reagent probably diffuses freely into the cells. A similar effect is observed only at 5 mM with PCMBS (Fig. 3A). The inability of PCMBS at lower concentrations to alter the hGSH pool is likely due to the presence of a negatively charged sulfonic acid group that reduces its lipid solubility and, consequently, its rate of penetration into the cells (Rothstein, 1970). In such conditions no eliciting property is observed (Fig. 1A). Higher PCMBS concentrations (greater than 5 mM) lead to both significant glyceollin elicitations and to a decrease in the free hGSH pool. Finally, we can conclude from the effects of the sulfhydryl reagents that there is a close relationship between

Table I. *Effect of transition metals on H_2O_2-induced lipid peroxidation and glyceollin accumulation*

Lipid peroxide levels in the 3-d-old soybean hyp/rad were estimated by quantification of the hydroxy fatty acid derivatives as described in "Materials and Methods" and glyceollin contents were determined at 48 h. ND, Not detectable. Lipid peroxidation and glyceollin levels are means ± SD for two experiments with three replicates.

Treatments	Lipid Peroxide Level		Glyceollin
	0 h	4 h	
	nmol g^{-1} fresh wt		*μg g^{-1} fresh wt*
Control[a]	26 ± 5	27 ± 2	ND
0.5 mM[b] Cu^{+}	27 ± 2	26 ± 3	ND
1 mM[b] Fe^{2+}	28 ± 3	30 ± 2	ND
1 M[c] H_2O_2	32 ± 4	85 ± 5	87 ± 10
0.5 mM Cu^{+} + 1 M[d] H_2O_2	65 ± 1	149 ± 2	185 ± 8
1 mM Fe^{2+} + 1 M[d] H_2O_2	59 ± 1	155 ± 2	206 ± 28

[a] Control was performed by immersing soybean tissues for 1 h in deionized water. Soybean tissues were immersed. [b] Thirty minutes in an aqueous solution of 0.5 mM CuCl or 1 mM $FeSO_4$, rinsed and immersed for 30 min in water. [c] Thirty minutes in water and 30 min in 1 M H_2O_2. [d] Thirty minutes in 0.5 mM CuCl or 1 mM $FeSO_4$, rinsed and immersed for 30 min in 1 M H_2O_2.

the immediate decrease of the free hGSH pool and glyceollin elicitation.

The hydroperoxide doses required to observe significant alterations of the free hGSH pool exceed 20 mM for tBuOOH (data not shown) and 0.1 M for H_2O_2 (Fig. 3C). However, from 1 to 10 mM tBuOOH or at 0.1 M H_2O_2, for which significant elicitations are observed, the free hGSH pool remains unchanged (Fig. 3, C and D). If we consider that tBuOOH easily penetrates the cells (Matsuo et al., 1989) its rather striking effects could be explained by the fact that we measured a very low affinity of soybean ascorbate peroxidase for this hydroperoxide (apparent K_m 175 mM). Consequently, if the ascorbate is not consumed, its recycling via dehydroascorbate reductase at the expense of hGSH does not operate (Foyer and Halliwell, 1976). On the contrary, the high affinity of soybean ascorbate peroxidase to H_2O_2 (apparent K_m 45 μM) hardly explains the high concentrations of this peroxide (more than 0.1 M) that are needed to obtain a significant effect on the sulfhydryl pool; however, catalase and extracellular peroxidase activities should also be taken into account. Nevertheless, unlike sulfhydryl reagents, which lead to a decrease of the free hGSH pool under all of the eliciting conditions, the results obtained with hydroperoxides indicate that immediate oxidation of hGSH is not required for signal transduction. Moreover, during the critical phase of PAL activation (the first 20 h), a 10 mM tBuOOH elicitation gave neither np-SH oxidation (Fig. 4A) nor modification of the total (free plus oxidized) np-SH pool (Fig. 4B). This latter result confirms previously reported data (Edwards et al., 1991), indicating that changes in intracellular np-SH levels do not participate in the transduction of the elicitation signal. Taken together, these results strongly suggest that an immediate decrease of the free hGSH level is not necessary (effect of the hydroperoxides) but could be sufficient (in the presence of sulfhydryl reagents) for signal transduction. Since the decrease of the free hGSH may actually correspond to the depletion of cellular antioxidant capacities, exogenously applied hydroperoxides or sulfhydryl reagents could bring about an increase in endogenous AO species, which in turn could trigger a common oxidizing process such as lipid peroxidation.

A new method designed for the analysis of the lipid peroxidation process in plants has been developed that combines a reduction step prior to the extraction and saponification of the total lipids. The stable hydroxy polyunsaturated fatty acid isomers thus obtained are analyzed by normal-phase HPLC (Fig. 5). This method allowed us not only to estimate the lipid peroxidation process through LPI determination in a single analysis but also to obtain information concerning the positional and *cis-trans* isomer distribution of the hydroxy linoleic and linolenic acids. As shown in Figure 6, all of the treatments inducing significant accumulations of glyceollin are able to trigger a lipid peroxidation process. On the contrary, a noneliciting concentration of PCMBS (i.e. 3 mM) does not modify the lipid peroxide level (Fig. 6B). Furthermore, change in the LPI as a function of time after treatment is compatible with its involvement as a step of the signal transduction chain, since the peroxidation of lipids is always induced within 2 h.

The low rate of tBuOOH metabolism discussed above likely explains the ability of this peroxide to immediately induce lipid peroxidation. For unknown reasons, H_2O_2 induces a time-dependent increase of the LPI (Fig. 6A). From the results of 3 mM IA or 8 mM PCMBS treatments, we can speculate that an immediate decrease in cellular antioxidant capacity (Fig. 3, A and B) may lead to a progressive increase of the endogenous levels of AO species, especially H_2O_2, which can then initiate the peroxidative process.

All of these results suggest that there is a close relationship between the lipid peroxidation process and glyceollin elicitation. Furthermore, transition metals are known to react with peroxides to produce oxygen-centered radicals able to initiate lipid peroxidation. The stimulatory effect of Fe^{2+} and Cu^{+} has been observed on both H_2O_2-induced lipid peroxidation and glyceollin accumulation (Table I), again providing evidence that the two processes could be linked by a cause and effect relationship. Finally, because the positional and *cis-trans* isomer distribution is always the same, regardless of the peroxide level in tissues, and in view of the abundance of the *cis-trans* forms (data not shown), the peroxidation process described here likely results from autoxidation rather than from lipoxygenase activity.

This work establishes that lipid peroxidation and glyceollin elicitation are closely linked phenomena in soybean. Sulfhydryl reagents should rapidly initiate a cellular antioxidant decrease by consuming a part of the free hGSH pool that likely leads to an increase of the cellular AO species and explains the further appearance of lipid peroxidation (Miccadei et al., 1988). On the other hand, hydroperoxides, which are more efficient glyceollin elicitors, are able to induce such a process directly (Fig. 7). Taking into account previously reported data (Apostol et al., 1989; Anderson et al., 1991; Arnott and Murphy, 1991; Vera-Estrella et al., 1992) indicating that biotic elicitors are able to rapidly increase cellular

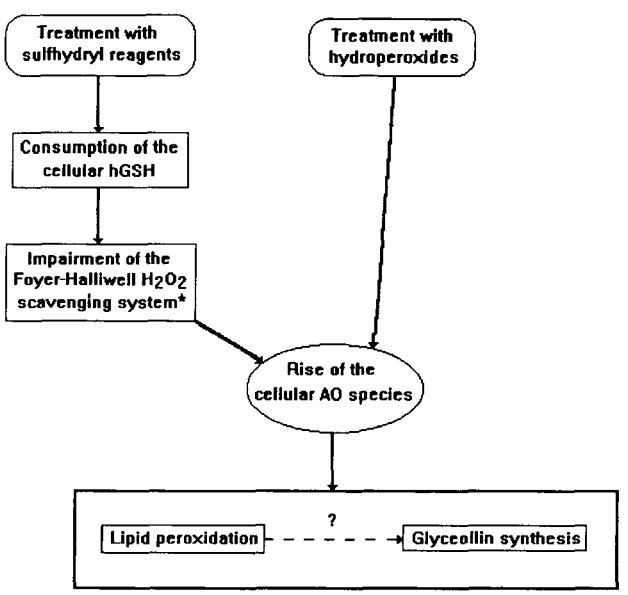

Figure 7. Hypothetical components of the chain transduction leading to glyceollin synthesis. *The H_2O_2 scavenging system (Foyer and Halliwell, 1976) is composed of ascorbate peroxidase, dehydrogenase reductase, and GSH reductase.

AO species levels, we can hypothesize that such a production should similarly lead to both lipid peroxidation and plant defense induction. We have not obtained definitive proof of the existence of a causal link between lipid peroxidation and glyceollin synthesis. However, in this work we provide strong evidence in favor of this assumption. Indeed, lipid peroxidation is rapidly induced in eliciting treatments, and its modulation by transition metals similarly influences the glyceollin production. Furthermore, it has been recently demonstrated that various oxygenated polyunsaturated fatty acid metabolites are able to act as messengers for plant defense metabolism induction (Li et al., 1991; Farmer and Ryan, 1992). This further supports the theory that lipid peroxidation is a step of the signal cascade that leads to plant defense induction. Finally, considering that about 65% of the total lipids are actually polar lipids in our plant material (Yoshida and Kajimoto, 1977), we assume that membranes are involved in the peroxidation process that takes place under eliciting treatments. This point is now under investigation, since such a membrane-linked process could explain some of the early biotic elicitor effects.

ACKNOWLEDGMENTS

The authors wish to thank Dr. G. Iacazio for providing lipid peroxide standards and for fruitful discussion and help.

Received August 4, 1993; accepted November 23, 1993.

LITERATURE CITED

Adam A, Farkas T, Somlyai G, Hevesi M, Kiraly Z (1989) Consequence of $O_2^{\cdot-}$ generation during a bacterially induced hypersensitive reaction in tobacco: deterioration of membrane lipids. Physiol Mol Plant Pathol **34:** 13–26

Anderson AJ, Rogers K, Tepper GS, Blee K, Cardon J (1991) Timing of molecular events following elicitor treatment of plant cells. Physiol Mol Plant Pathol **38:** 1–13

Apostol I, Heinstein PF, Low PS (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Plant Physiol **99:** 1208–1215

Arnott T, Murphy TM (1991) A comparison of the effects of a fungal elicitor and ultraviolet radiation on ion transport and hydrogen peroxide synthesis by rose cells. Environ Exp Bot **31:** 209–216

Baker CJ, O'Neill NR, Keppler LD, Orlandi EW (1991) Early responses during plant-bacteria interactions in tobacco cell suspensions. Phytopathology **81:** 1504–1507

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Chai HB, Doke N (1987) Superoxide anion generation: a response of potato leaves to infection with *Phytophtora infestans*. Phytopathology **77:** 645–649

Davies MH, Birt DF, Schnell RC (1984) Direct enzymatic assay for reduced and oxidized glutathione. J Pharmacol Methods **12:** 191–194

Devlin WS, Gustine DL (1992) Involvement of the oxidative burst in phytoalexin accumulation and the hypersensitive reaction. Plant Physiol **100:** 1189–1195

Edwards R, Blount JW, Dixon RA (1991) Glutathione and elicitation of the phytoalexin response in legume cell cultures. Planta **184:** 403–409

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys **82:** 70–77

Farmer EE, Ryan CA (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell **4:** 129–134

Foyer CH, Halliwell B (1976) Presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta **133:** 21–25

Frankel EN, Neff WE, Selke E, Weisleder D (1982) Photosensitized oxidation of methyl linoleate: secondary and volatile thermal decomposition products. Lipids **17:** 11–18

Glazener JA, Orlandi EW, Harmon GL, Baker CJ (1991) An improved method for monitoring active oxygen in bacteria-treated suspension cells using luminol-dependant chemiluminescence. Physiol Mol Plant Pathol **39:** 123–133

Gustine DL (1981) Evidence for sulfhydryl involvement in regulation of phytoalexin accumulation in *Trifolium repens* callus tissue cultures. Plant Physiol **68:** 1323–1326

Gustine DL (1987) Induction of medicarpin biosynthesis in ladino clover callus by *p*-chloromercuribenzoic acid is reversed by dithiothreitol. Plant Physiol **84:** 3–6

Iacazio G, Langrand G, Baratti J, Buono G, Triantaphylidès C (1990) Preparative, enzymatic synthesis of linoleic acid (13S)-hydroperoxide using soybean lipoxygenase-1. J Org Chem **55:** 1690–1691

Keppler LD, Novacky A (1986) Involvement of membrane lipid peroxidation in the development of a bacterially induced hypersensitive reaction. Phytopathology **76:** 104–108

Klapheck S (1988) Homoglutathione: isolation, quantification and occurrence in legumes. Physiol Plant **74:** 727–732

Kühn H, Wiesner R, Lankin VZ, Nekrasov A, Alder L, Schewe T (1987) Analysis of the stereochemistry of lipoxygenase-derived hydroxypolyenoic fatty acids by means of chiral phase high pressure liquid chromatography. Anal Biochem **160:** 24–34

Li WX, Kodama O, Akatsuka T (1991) Role of oxygenated fatty acids in rice phytoalexin production. Agric Biol Chem **55:** 1041–1047

Matsuo T, Kashinaki Y, Itoo S (1989) Membrane damage caused by exposure to *t*-butyl hydroperoxide. Phytochemistry **28:** 1003–1006

Miccadei S, Kyle ME, Gilfor D, Farber JL (1988) Toxic consequence of the abrupt depletion of glutathione in cultured rat hepatocytes. Arch Biochem Biophys **265:** 311–320

Montillet JL, Degousée N (1991) Hydroperoxydes induce glyceollin accumulation in soybean. Plant Physiol Biochem **29:** 689–694

Peever TL, Higgins VJ (1989) Electrolyte leakage, lipoxygenase, and lipid peroxidation induced in tomato leaf tissues by specific and nonspecific elicitors from *Cladosporium fulvum*. Plant Physiol **90:** 867–875

Popham PL, Novacky A (1991) Use of dimethyl sulfoxide to detect hydroxyl radical during bacteria-induced hypersensitive reaction. Plant Physiol **96:** 1157–1160

Robbins MP, Hartnoll J, Morris P (1991) Phenylpropanoid defense responses in transgenic *Lotus corniculatus*. 1. Glutathione elicitation of isoflavan phytoalexins in transformed root cultures. Plant Cell Rep **10:** 59–62

Rogers KR, Albert F, Anderson AJ (1988) Lipid peroxidation is a consequence of elicitor activity. Plant Physiol **86:** 547–553

Rothstein A (1970) Sulfhydryl groups in membrane structure and function. Curr Top Membr Transp **1:** 135–176

Stössel P (1984) Regulation by sulfhydryl groups of glyceollin accumulation in soybean hypocotyls. Planta **160:** 314–319

Vera-Estrella R, Blumwald E, Higgins VJ (1992) Effect of specific elicitors of *Cladosporium fulvum* on tomato suspension cells. Plant Physiol **99:** 1208–1215

Yoshida H, Kajimoto G (1977) Changes in lipid component and fatty acid composition of axis and root of germinating soybeans. Agric Biol Chem **41:** 2431–2436