Glutathione Causes a Massive and Selective Induction of Plant Defense Genes

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ABSTRACT

The reduced form of glutathione (GSH), when supplied to suspension cultured cells of bean (Phaseolus vulgaris L.) at concentrations in the range 0.01 to 1.0 millimolar, stimulates transcription of defense genes including those that encode cell wall hydroxyproline-rich glycoproteins and the phenylalanine biosynthetic enzymes phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) involved in lignin (PAL) and phytoalexin (PAL, CHS) production. Transcriptional activation of these genes leads to marked accumulation of the corresponding transcripts, contributing to a massive change in the overall pattern of protein synthesis which closely resembles that previously observed in response to fungal elicitor. GSH causes a marked increase in extractable PAL activity, whereas the oxidized form of glutathione, constituent amino acids, or other reducing agents are inactive. Possible roles of GSH in signaling biological stress are discussed.

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is a low mol wt thiol implicated in a wide range of metabolic processes (16). Functions proposed for glutathione in higher plants include: storage and transport of reduced sulfur; protein reductant; destruction of H2O2 in chloroplasts, and detoxification of xenobiotics including certain herbicides and pesticides (8, 20). Overall, glutathione appears to play a key role in protection against oxidative damage arising from a number of stresses such as irradiation (16), heat (18), and exposure to heavy metals (10).

Redox perturbations including generation of superoxide anions and lipid peroxidation appear to be a characteristic response to mechanical damage and microbial infection (4). Moreover, certain sulphydryl reagents stimulate the production of phytoalexins and the activation of other defense responses associated with the expression of disease resistance (11, 24). Taken together these observations suggest that glutathione may play a role in mediating the response of plant cells to biological as well as physical stresses.

In the present paper, we show that treatment of suspension-cultured cells of bean (Phaseolus vulgaris L.) with GSH causes a massive and selective induction of the transcription of defense genes encoding enzymes of phytoalexin and lignin biosynthesis, as well as stimulation of genes encoding cell wall HRGPs. The effects of GSH on the pattern of gene expression and protein synthesis closely resemble the response to fungal elicitor.

MATERIALS AND METHODS

Fungal Cultures and Elicitor Preparation. The source, maintenance and growth of cultures of Colletotrichum lindemuthianum were as previously described (1). Elicitor, at a final concentration of 60 μg glucose equivalents/ml, was the high mol wt fraction released by heat treatment of isolated mycelial cell walls (13).

Plant Material. French bean (Phaseolus vulgaris L. cv Canadian Wonder) cell cultures were grown as previously described, except the cultures were maintained in total darkness (13). Experiments were conducted with 7- to 10-d-old cell cultures, in which the growth medium exhibited a conductivity between 2.5 and 2.8 mho. This represents the period of maximum responsiveness to elicitor during the cell culture cycle (7).

Enzyme Extraction and Assay. Extraction and assay of PAL was as previously described (13). One unit of enzyme activity (1 kat) is defined as the amount of enzyme required for the formation of 1 mol of product in 1 sec under the assay conditions.

Extraction of RNA. Total cellular RNA was isolated from samples homogenized directly in a phenol/0.1 M Tris-HCl emulsion (pH 9.0), and purified as previously described (15). RNA was assayed spectrophotometrically at 260 nm. The yield of RNA was 150 to 250 μg/g fresh weight of tissue and the A260/A280 ratio varied between 1.8 and 2.1.

In Vitro Translation and Two-Dimensional Electrophoresis. Total RNA was translated in vitro in the presence of [35S]methionine (Amersham) using a message-dependent rabbit reticulocyte lysate (13). Translation products were fractionated by two-dimensional gel electrophoresis (9) with a pH range of 3.5 to 10 for isoelectric focusing in the first dimension, followed by 10% gel SDS-PAGE radiolabeled polypeptides were visualized by fluorography (3).

RNA Blot Hybridization. Total RNA was denatured by glyoxal and fractionated by electrophoresis in a 1.2% agarose gel in 10 mM phosphate buffer (pH 7.0) (15). Nitrocellulose blots (25) were hybridized with 32P-labeled cDNA sequences prepared by nick translation of the inserts of pPALS (7), pCHS (21), pHyp2.13, and pHyp4.1 (5). Following autoradiography, specific transcripts were quantitated by scanning densitometry. Several autoradiograms, exposed for different periods, were obtained for each blot to enable quantitation of each sample in the linear range of film response.

Nuclear Run-off Transcription. Isolation of nuclei and analysis of transcripts completed in vitro in the presence of [α-32P]UTP was as previously described (12). Immobilized sequences were PAL, cDNA pPALS (7); CHS, cDNA CHS1 (21); HRGP, equal amounts of cDNAs Hyp2.13 and Hyp4.1. H1 is a cDNA clone containing sequences from a constitutively transcribed gene.
RESULTS

Transcript Accumulation. PAL catalyzes the first reaction in the biosynthesis of L-phenylalanine to phenylpropanoid natural products including lignin and phytoalexins. CHS catalyzes the first reaction of a branch pathway of phenylpropanoid biosynthesis specific to the formation of flavonoid pigments and isoflavonoid phytoalexins. GSH caused a massive but transient, coordinate accumulation of PAL and CHS transcripts from low basal levels in suspension-cultured bean cells (Figs. 1 and 2). Maximum accumulation of these transcripts was observed about 6 h after addition of GSH, following which there was a decline to relatively low levels. GSH also caused the accumulation of HRGP transcripts Hyp4.1 and Hyp2.13, which had previously been shown to be induced by fungal elicitor (Fig. 1). As in elicitor treated cells, accumulation of these HRGP transcripts was less rapid but more prolonged than for PAL and CHS. GSH concentrations in the range 10 to 100 μM caused accumulation of PAL, CHS, Hyp2.13 and Hyp4.1 transcripts to levels comparable to, or greater than, those observed with optimal concentrations of fungal elicitor (Fig. 3).

Transcriptional Activation. Marked accumulation of defense gene transcripts from low basal levels suggested that GSH was stimulating transcription of these genes. The effects of GSH on PAL, CHS, and HRGP gene transcription were monitored by analysis of transcripts completed in vitro by nuclei isolated from cells at various times after GSH treatment. Isolation of nuclei...
FIG. 5. Effect of GSH on the pattern of protein synthesis. Two-dimensional gel electrophoretic analysis of [35S]methionine-labeled products synthesized by in vitro translation of total cellular RNA isolated from: A, control untreated cells; or cells 4 h after treatment with: B, GSH (1 mM); C, fungal elicitor (60 μg/ml); D, GSH plus fungal elicitor. Open arrows in panel B denote those species induced by both GSH and fungal elicitor; closed arrows denote those species induced by GSH but not fungal elicitor; p denotes PAL subunits; c denotes CHS subunits. IEF, Isoelectric focusing in the first dimension; SDS-PAGE: SDS-polyacrylamide gel electrophoresis in the second dimension.

and characterization of the runoff transcription assay have been described previously (12). cDNA clone H1 contains sequences complementary to an abundant transcript which is unaffected by elicitor treatment. Compared with the constitutive transcription of the H1 gene as an internal control, GSH caused a marked stimulation in the transcription of PAL, CHS, and HRGP genes (Fig. 4).

Pattern of Protein Synthesis. The impact on the overall pattern of protein synthesis was examined by two-dimensional gel electrophoretic analysis of the polypeptide products synthesized in vitro by translation of total cellular RNA (Fig. 5). By this criterion, GSH caused a major change in the pattern of protein synthesis compared to that in untreated control cells. Thus, GSH markedly stimulated the synthesis of a large number of polypeptides including sets of PAL and CHS isopolypeptides (Fig. 5). The effects of GSH on the pattern of protein synthesis closely resembled that observed following treatment of equivalent cells with fungal elicitor. However, in addition, GSH markedly stimulated the synthesis of four polypeptides whose levels of expression were little affected by fungal elicitor (Fig. 5). Simultaneous
addition of GSH and fungal elicitor altered the pattern of protein synthesis in a similar manner to GSH alone.

**Enzyme Activity and Product Accumulation.** GSH treatment caused a marked and prolonged increase in the level of extractable PAL activity (Fig. 2). The phase of most rapid increase in enzyme activity occurred between 3 and 8 h after GSH addition and hence was closely correlated with the timing of maximum accumulation of PAL transcripts. Likewise, the dose response for induction of PAL enzyme activity after 8 h resembled that for accumulation of PAL transcripts, with marked effects at concentrations of GSH as low as 10 μM (Table I). GSH stimulation of PAL enzyme activity led to increased flux through the pathway and appreciable accumulation of the phytoalexin end-product phaseollin (data not shown). GSH treatment also caused significant browning of the cells, which is characteristic of the accumulation of phenolic material.

**Specificity of GSH.** Induction of extractable PAL enzyme activity was used as a parameter to monitor the specificity of the effects of GSH. GSSG at a concentration of 1 mM caused only a weak stimulation of PAL activity, and at concentrations of 0.1 mM or lower, the oxidized form of glutathione had no significant effect (Fig. 6). Moreover, treatment of cells with ascorbate, cysteine, or a mixture of glutamate, glycine, and cysteine did not increase extractable PAL activity (Table II). Dithiothreitol likewise did not induce PAL activity (data not shown).

**DISCUSSION**

The present data demonstrate that exogenous GSH causes marked changes in the pattern of gene expression and protein synthesis in suspension cultured bean cells, including specific activation of defense genes and accumulation of the corresponding transcripts. While these effects qualitatively resemble those previously observed following treatment with fungal elicitor, a particularly striking feature is the massive quantitative effect of GSH. Thus, induction of PAL and CHS transcripts is several-fold greater and also more prolonged than with optimal concentrations of fungal elicitor. Moreover, PAL enzyme activities of about 200 μkat/kg protein obtained following GSH treatment are the highest we have observed in cell suspension cultures or other induction systems (13).

The effects of GSH are specific both in terms of the selective effects on gene activation, transcript accumulation, and protein synthesis, and also the lack of effect of other reducing agents, constituent amino acids, or the oxidized form of glutathione. In vivo, GSH is found at concentrations in the range of 0.05 to 1.5 mM (2, 6, 20, 22, 23) and hence the effects on defense genes occur at physiological concentrations of GSH. However, in some legumes, including bean and soybean, the major free low mol wt thiol is γ-L-glutamyl-L-cysteinyl-β-alanine (homoglutathione), with only trace amounts of glutathione (19). It will be of interest to determine whether exogenous GSH induces defense genes only in those species whose principal low mol wt thiol is homoglutathione.

Heavy metals such as cadmium perturb glutathione metabolism, leading to the synthesis of phytochelatins with the structure (γ-glutamyl-cysteinyl),-glycine where n = 3 to 7 (10). Heavy metals have also been shown to stimulate the synthesis of phytoalexins in soybean cotyledons (17), and the effects of GSH on plant defense gene expression suggest a possible mechanism. Moreover, GSH plays a protective role in cellular metabolism by acting as a reductant to remove free radicals (16, 20), and it has recently been shown that heat shock of maize roots elevates the cellular concentration of GSH (18). Oxidative reactions such as the release of superoxide anions and the peroxidation of lipids are early responses to mechanical damage and infection (4). Hence, GSH might function as a secondary signal of such redox perturbations, either as an intracellular second messenger mediating the effects of external stimuli such as fungal elicitors or as a subsequent intercellular signal of biological stress leading to activation of defense genes at a distance from the initial perturbation.

However, the close resemblance between the effects of exogenous GSH and fungal elicitor does not necessarily imply a physiological role in elicitor action. For example, recent evidence shows that a number of receptors at the surface of animal cells,

**Table I. Dose Response for the Effect of GSH on the Level of Extractable PAL Activity**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PAL Activity</th>
<th>0 h</th>
<th>4 h</th>
<th>8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>μkat/kg protein</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>GSH</td>
<td></td>
<td>25</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>0.01 mM</td>
<td></td>
<td>40</td>
<td>117</td>
<td></td>
</tr>
<tr>
<td>0.10 mM</td>
<td></td>
<td>30</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>1.00 mM</td>
<td></td>
<td>36</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Elicitor</td>
<td></td>
<td>61</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>Elicitor + GSH, 1.0 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table II. Effect of GSH, Constituent Amino Acids and Ascorbic Acid on the Level of Extractable PAL Activity**

All compounds were tested at a concentration of 0.1 mM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PAL Activity</th>
<th>0 h</th>
<th>4 h</th>
<th>8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cells</td>
<td>μkat/kg protein</td>
<td>18</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td>GSH</td>
<td></td>
<td>105</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td></td>
<td>7</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Cysteine, glycine, glutamate</td>
<td></td>
<td>19</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Ascorbate</td>
<td></td>
<td>13</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>
e.g., β-adrenergic receptors, possess intramolecular disulfide bridges, cleavage of which by thiol compounds activates the receptor in a manner similar to agonist binding (14). Thus, it is possible that in bean cells GSH may be able to cleave such linkages in an elicitor receptor and thus initiate defense gene activation in the absence of elicitor without implying a physiological role for GSH in this signal transduction pathway. Interestingly, a number of sulfhydryl reagents including p-chloromercuribenzoic acid and p-chloromercuribenzenzene sulfonic acid elicit synthesis of glyceollin in soybean hypocotyls and medicarpin in Ladin clover callus (11, 24).

Irrespective of the precise physiological role of GSH, the selective induction of plant defense genes by a small cellular metabolite of defined structure will provide an excellent experimental system for analysis of the molecular mechanisms underlying defense gene activation. Moreover, as a small, water-soluble, nontoxic cellular metabolite that strongly activates a specific set of plant genes, GSH treatment might prove useful for engineered regulation of chimeric transgenes driven by a responsive promoter.

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LITERATURE CITED