Physiological Roles of Glutathione S-Transferases in Soybean Root Nodules1[C][W][OA]


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Glutathione S-transferases (GSTs) are ubiquitous enzymes that catalyze the conjugation of toxic xenobiotics and oxidatively produced compounds to reduced glutathione, which facilitates their metabolism, sequestration, or removal. We report here that soybean (Glycine max) root nodules contain at least 14 forms of GST, with GST9 being most prevalent, as measured by both real-time reverse transcription-polymerase chain reaction and identification of peptides in glutathione-affinity purified extracts. GST8 was prevalent in stems and uninfected roots, whereas GST2/10 prevailed in leaves. Purified, recombinant GSTs were shown to have wide-ranging kinetic properties, suggesting that the suite of GSTs could provide physiological flexibility to deal with numerous stresses. Levels of GST9 increased with aging, suggesting a role related to senescence. RNA interference studies of nodules on composite plants showed that a down-regulation of GST9 led to a decrease in nitrogenase (acetylene reduction) activity and an increase in oxidatively damaged proteins. These findings indicate that GSTs are abundant in nodules and likely function to provide antioxidant defenses that are critical to support nitrogen fixation.

Nitrogen fixation in legume root nodules requires a careful balance of oxygen relations because of the high energy requirements and the conflicting risk for the generation of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide, and organic peroxides. This results in a perilous state of affairs referred to as the “oxygen paradox.” The mechanisms by which ROS are generated in nodules include the oxidation of leghemoglobin, the strong reducing conditions, the oxidation of enzymes such as nitrogenase, ferredoxin, and hydrogenase, and the usual suite of ROS-producing electron carriers in mitochondria (for review, see Dalton, 1995; Becana et al., 2000; Matamoros et al., 2003; Minchin et al., 2008). This high capacity for ROS generation requires strong antioxidant defenses. These defenses include high levels of ascorbate and glutathione as well as enzymes such as ascorbate peroxidase (APX), glutathione reductase, and superoxide dismutase (SOD). Elevated levels of antioxidants can increase rates of nitrogen fixation up to 4-fold in planta as well as in in vitro reconstitution systems (Bashor and Dalton, 1999; Ross et al., 1999).

We report here that legume root nodules also contain abundant glutathione S-transferases (GSTs), a ubiquitous class of enzymes with potential antioxidant properties. GSTs are best known for the detoxification of xenobiotics such as herbicides (e.g. atrazine; Marrs, 1996; Edwards et al., 2000), but they can also act as antioxidants by tagging oxidative degradation products (especially from fatty acids and nucleic acids) for removal or by acting as a glutathione peroxidase to directly scavenge peroxides (Dixon et al., 2002b; Frova, 2003). In addition to reducing lipid peroxides directly, GSTs may act to remove lipid peroxidation end products such as alkenals, 4-hydroxynonenal, ethacrynic acid (EA), and other α,β-unsaturated aldehydes. GSTs act by catalyzing the conjugation of reduced glutathione (GSH) with electrophilic, often hydrophobic toxic compounds to form derivatives that can be secreted from the cell, sequestered in the vacuole, or catabolized. GSTs are important in response to a range of abiotic and biotic stresses. Of special interest in nodules, GST can catalyze the conjugation of GSH to nitroso compounds (Eyer and Schneller, 1983), thus providing a likely means of controlling the damaging effects of nitrosyl leghemoglobin that forms in nodules exposed to nitrates.

GSTs comprise a large, complex gene family in plants. For instance, there are 25 GST genes in soybean (Glycine max), 42 in maize (Zea mays), and 47 in Arabidopsis (Arabidopsis thaliana; McGonigle et al., 2000; Wagner et al., 2002). Plant GST proteins are divided by sequence similarity into three categories (I, II, and III) or, alternatively, into six classes: tau, phi, zeta, theta, lambda, and DHAR (for glutathione-dependent dehydroascorbate...
Dalton et al.

RESULTS

Abundance of Different GSTs

Two-dimensional (2-D) gels of proteins recovered from GSH-affinity chromatography of nodule crude extracts were examined as a first screen to detect possible GSTs. These revealed a wide band of GSH-related proteins of the expected molecular mass of GSTs (approximately 25 kD), spanning a broad range of pI values from 5 to 6.5 (Supplemental Fig. S1). The banding pattern observed was in close agreement with the pattern based on predicted molecular mass and pI values. It was not practical to resolve individual GSTs on these gels, so the GSH-affinity eluates were analyzed directly by matrix-assisted laser-desorption ionization mass spectrometry (MALDI MS). The most abundant GSTs identified are shown in Table I. Although this list is ranked by the number of assigned spectra (i.e. the number of individual peptide fragments matched to each GST), it is only a crude indicator of relative abundance, since the number of fragments depends on the number of trypsin-cleavable sites on each protein as well as the abundance of the intact proteins. In summary, the proteins identified by these procedures indicate that GST9 and GST8 are most prevalent in nodules, but at least 13 other GSTs are present, including, in order of number of assigned spectra, GSTs 10, 13, 19, 14, 7, 16, 18, 13, 15, 12, 20, 22, and 16. DHAR was also abundant in our GSH-affinity purified samples. This enzyme catalyzes the GSH-dependent reduction of dehydroascorbate to ascorbate and thus is important in maintaining the function of the ascorbate-glutathione cycle, which is critical in plant redox homeostasis. DHAR has strong sequence similarity to GSTs and may be considered as a subclass of the GST superfamily (Dixon et al., 2002a).

The full list of peptides identified (Supplemental Table S1) also includes high frequencies of Suc synthase, lipoxygenases, and leghemoglobin. This is probably a reflection of the abundance of these proteins in nodules and the incomplete separation provided by one-step affinity chromatography rather than of some relationship to GSH metabolism. Protein samples processed in two consecutive passes through an affinity column contained few peptides that were not GSTs.

More definitive indications of the relative abundance of GSTs in nodules was provided by real-time quantitative reverse transcription (qRT)-PCR, which confirmed that GST9 was the prevalent form in 5-week-old soybean nodules at 33.3% of ubiquitin

Table I. MALDI MS identification of GSTs in GSH-affinity purified proteins from soybean nodules

<table>
<thead>
<tr>
<th>Column Type</th>
<th>Peptide Identity</th>
<th>Accession No.</th>
<th>No. of Assigned Spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH agarose</td>
<td>GST9</td>
<td>gi</td>
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<tr>
<td></td>
<td>GST8</td>
<td>gi</td>
<td>11385431</td>
</tr>
<tr>
<td></td>
<td>GST13</td>
<td>gi</td>
<td>11385441</td>
</tr>
<tr>
<td></td>
<td>GST10</td>
<td>gi</td>
<td>2920666</td>
</tr>
<tr>
<td></td>
<td>GST19</td>
<td>gi</td>
<td>11385453</td>
</tr>
<tr>
<td></td>
<td>GST14</td>
<td>gi</td>
<td>11385443</td>
</tr>
<tr>
<td></td>
<td>GST7</td>
<td>gi</td>
<td>417148</td>
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<tr>
<td></td>
<td>GST22</td>
<td>gi</td>
<td>11385459</td>
</tr>
<tr>
<td></td>
<td>GST16</td>
<td>gi</td>
<td>11385447</td>
</tr>
<tr>
<td>S-Hexyl GSH agarose</td>
<td>GST9</td>
<td>gi</td>
<td>11385433</td>
</tr>
<tr>
<td></td>
<td>GST8</td>
<td>gi</td>
<td>11385431</td>
</tr>
<tr>
<td></td>
<td>GST10</td>
<td>gi</td>
<td>2920666</td>
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<td>gi</td>
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<tr>
<td></td>
<td>GST13</td>
<td>gi</td>
<td>11385441</td>
</tr>
<tr>
<td></td>
<td>DHAR</td>
<td>gi</td>
<td>68131811</td>
</tr>
<tr>
<td></td>
<td>GST19</td>
<td>gi</td>
<td>11385453</td>
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<td>GST18</td>
<td>gi</td>
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<tr>
<td></td>
<td>GST2</td>
<td>gi</td>
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<tr>
<td></td>
<td>GST16</td>
<td>gi</td>
<td>11385447</td>
</tr>
</tbody>
</table>

Figure 1. Transcript levels of various GSTs and antioxidant genes in 5-week-old nontransformed soybean nodules. Each value is a mean ± s (n = 3), GR, Glutathione reductase.
levels, followed by GST2/10 at 22.5% and GST8 at 6.4% (Fig. 1). Lower levels of GST3, GST4, GST13, GST14, GST154, and GST18 were also detected. APX and SOD levels were generally high (about 50% of ubiquitin), as expected, considering their role as key antioxidants in nodules. Transcripts of leghemoglobin a (Leg a; data not shown) were very high: 12,400% of ubiquitin. The transcript levels of GST9 increased with time, reaching 54.7% of ubiquitin at 6 weeks and 78.0% at 9 weeks (n = 3, P < 0.05), suggesting a senescence-related increase, as would be expected from cumulative oxidative damage (Supplemental Fig. S2).

The pattern of relative abundance of the different GSTs was very different in other plant organs (Table II). Whereas GST9 was clearly the most abundant form in nodules, this GST was less abundant in other tissue types. GST2/10 (our primers did not distinguish between these two) was prevalent in leaves, GST4 was prevalent in stems, and GST8 was prevalent in uninfected roots.

### Heterologous Expression and Kinetic Properties of Recombinant GSTs

The major GSTs from nodules were successfully expressed in *Escherichia coli* (Fig. 2) and purified to near homogeneity by nickel-agarose affinity chromatography in order to study the differences in kinetic properties. The prominent bands around 31 kD in lanes 3 to 8 (Fig. 2) correspond to GSTs with a slightly increased (approximately 2.5 kD) mass due to the myc epitope and polyhistidine region, which are provided by the TrcHis vectors. As is typical of GSTs in general, the kinetic properties of different soybean recombinant GSTs varied widely. The $K_m$ values varied between 0.7 and 5.1 mM for 1-chloro-2,4-dinitrobenzene (CDNB) and 0.016 to 1.04 mM for EA (Supplemental Table S2). The turnover number and enzyme efficiency ($k_{cat}/K_m$) varied by a factor of over 100. GST9 was less efficient (high $k_{cat}$, low turnover number, low efficiency) than other GSTs, even though this GST is most abundant in nodules.

### GST RNAi Composite Plants

*A. rhizogenes* was used to introduce GST RNAi and control constructs to composite plants to examine the effect of GST silencing on nodule function. Since soybean is difficult to transform and regenerate, composite plants, consisting of wild-type shoots and RNAi-transformed nodulated roots, were produced. Two RNAi lines were utilized: CGT-5214, containing silencing elements targeted to GST9; and CGT-5215, containing silencing elements targeted to the entire family of GST genes. A third line of composite plants (AKK 1467B), containing a GFP reporter gene but no RNAi, was used for comparison. Callus and hairy roots were evident at shoot ends within 4 weeks of inoculation with *A. rhizogenes*. Approximately half of the shoots survived up to this stage. The resultant composite plants grew slowly and produced only three expanded, trifoliate leaves after an additional 4 to 6 weeks, at which time the plants were harvested. Plants left beyond this stage typically declined even more in vigor, although a few (less than 10%) of the GFP-composite controls (AKK 1467B) grew rapidly and produced numerous healthy, dark green leaves. There were no significant differences in the fresh weight of shoots, fresh weight of roots plus nodules, fresh weight of nodules only, root-to-shoot ratio, total protein extracted per gram of nodule fresh weight, or number of nodules per hairy root between the three types of composite plants. Nodules on hairy roots were visually indistinguishable from those on wild-type plants. Examination with light microscopy of numerous callus cross sections at 12 to 15 weeks after transformation showed a disorganized pattern of incomplete xylem differentiation, with partially formed xylem interspersed with thin-walled parenchyma cells (Fig. 3). The slow growth of the composite plants is likely a result of the poor vascular connection between the newly formed hairy roots and the stem, making it

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**Table II. Abundance of various GST transcripts in soybean tissues expressed as percentage of ubiquitin**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>GST No.</th>
<th>1</th>
<th>2/10</th>
<th>3</th>
<th>4</th>
<th>8</th>
<th>9</th>
<th>14</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>n.d.</td>
<td>53.2 ± 1.4</td>
<td>0.5 ± 0.1</td>
<td>16.8 ± 0.8</td>
<td>33.6 ± 5.5</td>
<td>4.6 ± 1.0</td>
<td>n.d.</td>
<td>0.1 ± 0</td>
<td></td>
</tr>
<tr>
<td>Stem</td>
<td>4.0 ± 1.8</td>
<td>12.8 ± 2.1</td>
<td>3.0 ± 0.2</td>
<td>23.7 ± 3.1</td>
<td>20.0 ± 6.5</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0</td>
<td>0.2 ± 0</td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>5.5 ± 2.7</td>
<td>3.8 ± 2.3</td>
<td>1.4 ± 0.1</td>
<td>0.9 ± 0</td>
<td>34.3 ± 8.3</td>
<td>n.d.</td>
<td>0.2 ± 0</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± se (n = 3), n.d., None detected.
questionable to compare the physiology of these plants with nontransformed plants. Therefore, our analyses focused on comparing control composite plants (AKK 1467B) with other composite plants containing RNAi constructs. Callus tissue, hairy roots, and nodules were found to consistently demonstrate GFP fluorescence (Fig. 4). Adventitious roots sometimes formed adjacent to the callus, but these could be distinguished by different morphology and lack of GFP fluorescence. These adventitious roots were removed and discarded.

Acetylene Reduction Activity

Nodule function was measured based on the ability to reduce acetylene to ethylene. Preliminary experiments were conducted with composite soybeans with constructs containing two types of promoters: either Cassava vein mosaic virus (CvMV), as described in “Materials and Methods,” or the superubiquitin promoter. In preliminary studies, nodules from CGT-5214 plants (CvMV promoter and GST9-specific RNAi) showed only 60% of the acetylene reduction activity of AKK 1467B (control) nodules. Activities from nodules from other constructs (CGT-5215 or either of the constructs with superubiquitin promoters) were not significantly different from those of the AKK 1467B controls. Therefore, we elected to proceed with more extensive studies focusing on constructs with CvMV promoters. In the expanded studies, acetylene reduction activity of CGT-5214 nodules was only 16% of the activity of AKK 1467B nodules (Fig. 5). There was no significant difference between the acetylene reduction activities of CGT-5215 and AKK 1467B nodules.

Oxidation Products in Nodules

Two common indicators of oxidative damage were used: thiobarbituric acid-reactive substances (TBARS), for lipid peroxidation, and Oxyblot, for protein oxidation. The values for lipid peroxide production in nodules, as measured by the TBARS assay, were 78 ± 7.1 nmol malondialdehyde (MDA) g⁻¹ fresh weight for AKK 1467B, 74 ± 3.7 nmol MDA g⁻¹ fresh weight for CGT-5214, and 71 ± 2.5 nmol MDA g⁻¹ fresh weight for CGT-5215 (means ± se, n = 5). These values were not significantly different between the treatments. Protein oxidation as measured by western blots with antibodies detecting the formation of protein carbonyls showed a higher degree of oxidation in nodules from CGT-5214 (Fig. 6). This result was consistent in four western blots, with Figure 6 being representative. The interpretation of these blots is based on the collective intensity of all bands regardless of molecular mass, with the relative intensity of any single band being of less interest. On this basis, lanes 2 and 3, corresponding to extracts from CGT-5214, clearly show a darker pattern, indicating protein oxidation.

GST Activity in Extracts from Nodules of Composite Plants

GST activities in crude extracts of nodules from composite plants were not significantly different for either substrate (CDNB or EA) between the different constructs. These are the two most commonly used substrates for measuring GST activity and can provide some insight into kinetic differences between the various GSTs as well as serve as a good basis for comparing nodule GSTs with those from a wide range of other organisms. The activities for all groups combined were 1.17 ± 0.13 nmol CDNB min⁻¹ mg⁻¹ protein and 0.17 ± 0.02 nmol EA min⁻¹ mg⁻¹ protein.

Real-Time RT-PCR Analysis of Nodules from Composite Plants

Real-time RT-PCR was used to compare transcript levels of key genes in nodules from composite plants (Fig. 7). These data are expressed in terms of the
percentage of transcript levels in the control (AKK 1467B) plants and thus indicate changes that can be ascribed to RNAi effects for either the GST9-specific construct (CGT-5214) or the broader target of all GSTs (CGT-5215). Both RNAi constructs resulted in about a 50% reduction in levels of GST9. Transcript levels of GST3 and GST4 were elevated in both RNAi lines, but the absolute levels of GST4 still remained low in terms of the percentage of ubiquitin (e.g. 8.1% ± 1.6% for CGT-5214 and 12.0% ± 5.2% for CGT-5215). GST8 was elevated only in construct CGT-5214. Transcript levels of other genes tested (GST14, APX, SOD, and Leg a) remained unchanged in the RNAi lines relative to the control. These results indicate that some GSTs (e.g. GST9) are down-regulated as expected and other GSTs (e.g. GST3) are up-regulated, perhaps in compensation for the decrease of other GSTs.

It is also instructive to view these data as transcript levels expressed as a percentage of ubiquitin levels within the same treatment type (Table III). When viewed in these terms, it is evident that there is a prominent shift in relative frequencies that is not related to GST silencing. Specifically, whereas GST9 is prevalent in nontransformed nodules, GST8 is prominent in all composite plants (including controls) regardless of silencing elements. GST14, barely present in nontransformed nodules, is also much increased in composite plants. Transcript levels of APX, SOD, and Leg a were consistent in nodules of all three types of composite plants. The specific values, again expressed as a percentage of ubiquitin for control plants, were as follows: APX, 61.9% ± 22.3%; SOD, 31.1% ± 9.8%; and Leg a, 11.700% ± 5.600%.

Transcript levels for GFP were also measured to confirm that the transgenic constructs were present and functioning in nodules of composite plants. GFP transcript levels, expressed as a percentage of ubiquitin control, were 0.9% ± 0.3% for AKK 1467B, 8.4% ± 4.2% for CGT-5214, and 1.5% ± 1.2% for CGT-5215. No GFP transcripts were detected in nontransformed nodules.

**DISCUSSION**

Our results indicate that GSTs should be added to the list of other proteins that are known antioxidants in nodules. These previously established antioxidants, most notably the enzymes of the ascorbate-GSH cycle and SOD, are recognized as critical in protecting nodules primarily because of the potential of leghemoglobin to produce ROS (Matamoros et al., 2003; Minchin et al., 2008). Recent studies by Gunther et al. (2007) have used RNAi technology to confirm that leghemoglobin is a major source of hydrogen peroxide. Heme oxygenase appears to be another antioxidant whose importance in nodules has only recently been recognized (Balestrasse et al., 2005). As the list of nodule antioxidants expands, it is becoming increasing clear that this aspect of nodule physiology is important in maintaining normal nodule function as well as enhancing resistance to environmental stresses such as drought (Naya et al., 2007).

Analysis of proteins and transcripts provides two independent lines of evidence that GST9 is the dominant form of GST in nodules. Although our data indicate that GST9 has low efficiency (high $K_m$) for EA, this form of GST is still capable of a high specific activity at saturating substrate concentrations (McGonigle et al., 2000). Activity with EA is correlated with activity with more physiologically relevant $\alpha,\beta$-unsaturated aldehydes such as 4-hydroxynonenal. 4-Hydroxynonenal is a lipid peroxidation by-product derived primarily from linoleic acid, a compound that has received a great deal of attention as a second signaling molecule regulating numerous cellular processes, including ROS-induced apoptosis (Awasthi, 2005). Linoleic acid makes up 25% of unsaturated fatty acids in nodules (Lynd and Ansman, 2005). Thus, the prevalence of GST9 is consistent with the putative role of GSTs as an antioxidant in nodules.

**Figure 5.** Acetylene reduction activity of nodules from composite soybean plants. CGT-5214 contains GST9-specific RNAi. Each value is the mean of five replicates ± se.

**Figure 6.** Western blot (Oxyblot) showing protein oxidation of nodule extracts from composite soybeans containing RNAi constructs designed to silence GSTs. Lane 1, AKK 1467B (control), 16.9 µg of protein; lane 2, CGT-5214, 15.7 µg of protein; lane 3, CGT-5214, 13.9 µg of protein; lane 4, CGT-5215, 14.7 µg of protein; lane 5, AKK 1467B, 15.7 µg of protein.
The presence of so many different GSTs in soybean raises questions concerning their potential physiological roles. Soybean GSTs vary widely with respect to their activity with different substrates (McGonigle et al., 2000), and this has been used as a rationale to suggest that multiple GSTs allow for flexibility in detoxifying a wide range of potential allochemicals and xenobiotics whose identities remain elusive. Furthermore, soybean nodules contain homoglutathione (γGlu-Cys-βAla) in addition to glutathione (Matamoros et al., 1999). Soybean GSTs discriminate between GSH and homoglutathione depending on the second substrate (McGonigle et al., 1998), thus imparting an extra dimension of potential physiological flexibility and perhaps some insight into why so many different GSTs are present.

Having a range of different GSTs may be especially critical in nodules because of the high potential for the production of α,β-unsaturated aldehyde genotoxins, such as 4-oxo-2-nonenal and 4-hydroxy-2-nonenal. Such DNA-reactive bifunctional electrophiles can be targeted for removal by GST. These compounds are derived from decomposition of lipid hydroperoxides, which in turn are formed nonenzymatically by the action of ROS on polyunsaturated fatty acids (e.g. linoleic acid) that are abundant in nodules. The presence of large amounts of ascorbic acid in nodules also contributes to this risk. Even though ascorbic acid is normally thought of as an antioxidant, it can also have deleterious properties by accelerating the decomposition of lipid hydroperoxides (Lee et al., 2001); thus, GSTs may be required to compensate.

While real-time qRT-PCR indicated a decrease in GST9 in response to RNAi silencing, there was a corresponding increase in other GSTs (Table III; Fig. 7) that may be compensatory. This could account for the observation that GST activity in crude extracts, a reflection of the total pool of GST proteins present, was unaltered in RNAi-silenced nodules. However, the decreased nitrogenase activity and increased levels of protein carbonyls suggest that this compensation was not entirely sufficient. This may suggest that GST9 has a distinct function in nodules that other GSTs cannot adequately fulfill.

The elevated levels of GSTs (especially GST9) in nodules compared with other plant tissues and the marked decline in acetylene reduction activity in nodules of the composite plant CGT-5214 (silenced for GST9) supports the conclusion that GSTs play an important role in nodule function. We also observed that an ortholog of GST9 was present at high levels in nodules of the model legume Medicago truncatula (data not shown), giving further support to this connection. The elevation in levels of protein oxidation observed in the Oxyblots of composite CGT-5214 nodules (silenced for GST9) supports the conclusion that GSTs are involved in antioxidant defense (Fig. 6). It also appears that GSTs play a wider role in plant-microbe interactions, as transcript levels of at least one GST are elevated by infection with various types of fungi, including mycorrhizal species (Strittmatter et al., 1996).

Composite plants are relatively easy to produce compared with stably transformed and regenerated plants and thus are useful tools for functional studies of a range of physiological processes in nodules (Govindarajulu et al., 2009). This is especially true in the case of soybean, which is difficult to regenerate in tissue culture. However, several caveats should be

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**Table III. Abundance of GST transcripts in wild-type and composite soybean nodules as measured by real time qRT-PCR**

<table>
<thead>
<tr>
<th>Sample</th>
<th>GST No.</th>
<th>1</th>
<th>3</th>
<th>4</th>
<th>6/7</th>
<th>8</th>
<th>9</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (not composite), 5–6 weeks old</td>
<td></td>
<td>0.1 ± 0</td>
<td>10.7 ± 2.1</td>
<td>7.6 ± 0.9</td>
<td>6.0 ± 2.6</td>
<td>1.1 ± 0.2</td>
<td>54.7 ± 6.7</td>
<td>1.6 ± 0.9</td>
<td>0.2 ± 0</td>
<td>5.9 ± 3.4</td>
<td>9.6 ± 5.6</td>
</tr>
<tr>
<td>AKK 1467B (control)</td>
<td></td>
<td>n.d.</td>
<td>n.d.</td>
<td>5.9 ± 1.1</td>
<td>n.d.</td>
<td>44.6 ± 6.7</td>
<td>35.6 ± 3.4</td>
<td>n.d.</td>
<td>3.6 ± 0.7</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CGT-5214 (control)</td>
<td></td>
<td>n.d.</td>
<td>n.d.</td>
<td>8.0 ± 5.8</td>
<td>n.d.</td>
<td>82.8 ± 7.6</td>
<td>19.3 ± 2.4</td>
<td>n.d.</td>
<td>3.9 ± 1.3</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CGT-5215 (control)</td>
<td></td>
<td>n.d.</td>
<td>n.d.</td>
<td>10.3 ± 6.7</td>
<td>n.d.</td>
<td>29.8 ± 2.5</td>
<td>20.2 ± 1.4</td>
<td>n.d.</td>
<td>2.7 ± 0.5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
CONCLUSION

root nodules. A valuable tool for studying gene function in symbiotic composite plants have been shown here to be that complicate the interpretation. Nevertheless, there may be unintended pleiotropic effects could spread from transformed roots to the shoots. can be systemic due to mobility in the phloem and pared with nontransformed plants. Although the responses arising from an incomplete vascular connection of hairy roots to the shoots, as shown in Figure 3. Composite plants have reduced vigor and are usually unable to maintain long-term growth compared with nontransformed plants. Although the shoots retain a wild-type genotype, the RNAi effects can be systemic due to mobility in the phloem and could spread from transformed roots to the shoots. Finally, there may be unintended pleiotropic effects that complicate the interpretation. Nevertheless, composite plants have been shown here to be a valuable tool for studying gene function in symbiotic root nodules.

Soybean Nodule Glutathione S-Transferringases

A theoretical 2-D “fingerprint” of soybean (Glycine max) GSTs was constructed from known M, values with a first-dimension pH range of 4 to 7 based on pl values predicted by Vector NTI 7 Software (Invitrogen). An extract of nodule total protein was prepared as described below, passed through a GSH-affinity chromatography column, concentrated to a volume of 25 μl (approximately 60 μg of protein), and combined with 300 μl of ReadyPrep Rehydration/Sample buffer (Bio-Rad). The sample was applied to IPC ReadyStrips (7 cm and pH 4-7; Bio-Rad) at 125 μl strip-1 and incubated at room temperature for 18 h. Samples were focused by electrophoresis along the IPC strips under the following conditions with the current not exceeding 50 μA IPC strip-1: 250 V for 15 min, rapid ramp to 4,000 V over 2 h, and constant 4,000 V for 5 h for a total of 20 kVh. Strips were equilibrated for 10 min in 50 mM Tris-HCl buffer (pH 8.8) containing 6 M urea, 30% glycerol, 2% SDS, 0.05% bromphenol blue, and 2% dithiothreitol. The solution was then replaced with the same buffer containing 2.5% iodoacetamide instead of dithiothreitol and incubated again for 10 min. The IPC strips were transferred to 10-cm 12% Tris-HCl Ready Gels (Bio-Rad). The second-dimension separation was carried out at 150 V for 1 h. Protein was visualized using Silver Stain Plus (Bio-Rad).

Analysis of the Total Pool of GSH-Related Proteins

Nodulated soybeans (‘Williams 82’) were grown in a greenhouse as described by Dalton et al. (1993). Four- to 6-week-old nodules (15 g) were ground in liquid N2 by mortar and pestle. After allowing the liquid N2 to boil off, the frozen powder was added to 4 g of insoluble polyvinylpolypyrrolidone and 45 ml of 50 mM HEPES, pH 7.0. The macerate was centrifuged at 10,000g for 20 min. The combined supernatant was added to affinity columns consisting of glutathione-agarose or S-hexylglutathione-agarose (Sigma-Aldrich) or GSTrap HP HiTrap affinity Sepharose (GE Healthcare; Supplemental Table S1) at a flow rate of 0.5 mL min-1 to ensure maximum binding. The column was washed with 5 bed volumes of phosphate-buffered saline (pH 7.3) and then eluted with 10 mL of 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0) at 1 mL min-1. Approximately 20 μg of protein was dried by vacuum centrifugation, digested with trypsin, and analyzed on a Thermo-Finnigan LTQ mass spectrometer. Each sample was loaded inline with a C18 trap column using the Agilent 1100 series autosampler. The samples were first passed through a 250 × 5-mm Zorbax C18 column. Samples were introduced using a ThermoFinnigan ion MA APL source. Peptides were identified using the National Center for Biotechnology Information (NCBI) nonredundant database (Viridiplantae, 387,441 proteins) with the Mascot search engine, with an average fragment tolerance of 0.8 D and Scaffold (Proteome Software) version 01_07_00 software.

CONCLUSION

Soybean nodules contain at least 14 of the 25 total GSTs in the soybean genome. GST9 is most abundant in nodules, followed by GST8 and GST10. Six different GSTs were expressed in E. coli to produce recombinant proteins that revealed a wide range of kinetic properties, suggesting that the range of GSTs may provide physiological flexibility to deal with various toxic products. RNAi silencing of GST9 in nodules of composite soybean plants resulted in decreased GST9 transcript levels, decreased nitrogenase (acetylene reduction) activity, and increased levels of oxidized protein products. This is consistent with the presumed role of GSTs as antioxidants. Several other GSTs were up-regulated in GST9-silenced plants, suggesting a compensatory response.

MATERIALS AND METHODS

2-D Gels

A theoretical 2-D “fingerprint” of soybean (Glycine max) GSTs was constructed from known M, values with a first-dimension pH range of 4 to 7 based on pl values predicted by Vector NTI 7 Software (Invitrogen). An extract of nodule total protein was prepared as described below, passed through a GSH-affinity chromatography column, concentrated to a volume of 25 μl (approximately 60 μg of protein), and combined with 300 μl of ReadyPrep Rehydration/Sample buffer (Bio-Rad). The sample was applied to IPC ReadyStrips (7 cm and pH 4-7; Bio-Rad) at 125 μl strip-1 and incubated at room temperature for 18 h. Samples were focused by electrophoresis along the IPC strips under the following conditions with the current not exceeding 50 μA IPC strip-1: 250 V for 15 min, rapid ramp to 4,000 V over 2 h, and constant 4,000 V for 5 h for a total of 20 kVh. Strips were equilibrated for 10 min in 50 mM Tris-HCl buffer (pH 8.8) containing 6 M urea, 30% glycerol, 2% SDS, 0.05% bromphenol blue, and 2% dithiothreitol. The solution was then replaced with the same buffer containing 2.5% iodoacetamide instead of dithiothreitol and incubated again for 10 min. The IPC strips were transferred to 10-cm 12% Tris-HCl Ready Gels (Bio-Rad). The second-dimension separation was carried out at 150 V for 1 h. Protein was visualized using Silver Stain Plus (Bio-Rad).

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qRT-PCR

Total RNA was isolated from 10 to 20 mg of nodules after homogenization using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer’s protocol. All samples were treated with Turbo DNA-free DNase (Ambion). RNA concentration was measured by UV spectrophotometry (A260 and A280) using a BioPhotometer (Eppendorf). cDNA was synthesized using approxi- mately 400 ng of RNA and oligo(dT) primers with the Script cDNA Synthesis Kit (Bio-Rad) as prescribed by the manufacturer. Specifically, samples were incubated at 65°C for 5 min without reverse transcriptase and snap chilled on ice for 1 min. Reverse transcriptase was then added, and the samples were incubated at 42°C for 90 min followed by 55°C for 5 min. Duplicate samples received nanopure water in place of reverse transcriptase to check for genomic DNA contamination by qPCR. qRT-PCR was used to determine the relative expression of genes normalized against ubiquitin. Primers (Supplemental Table S3) were designed to amplify 3’-end products between 150 and 200 bp with annealing temperatures between 54°C and 56°C using Primer3 (frodos.

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were analyzed for qPCR efficiency (E) using serial dilutions of cDNA acquired raw fluorescence between 60
water (www.growcells.com) in a 96-well DNA Engine Opticon 2 (MJ Re-
nanopure water, and 2.65
between the
following formula (Livak and Schmittgen, 2001):
Relative expression for each gene of interest (GOI) was determined with the
using 0.2
dfci.harvard.edu/tgi/plant.html) databases. All reactions were carried out
m and m = the slope of the line of least-squares fit of the log of the quantity versus cycle threshold (Ct; results ranged from E = 1.8 to E = 1.99). Relative expression for each gene of interest (GDI) was determined with the following formula (Livak and Schmittgen, 2001):
If \( E_{\text{dilution}} = E_{\text{control}} \), then the calculation 2 \( ^{-\Delta\Delta C T} \) (the \( \Delta\Delta C T \) method) was used for the sake of simplicity.

**Heterologous Expression of GSTs**

Full-length EST clones of soybean GSTs 3, 4, 8, 9,14, and 18 were provided by Daniel O’Keefe (DuPont; McGonigle et al., 2000). These were amplified with PCR, subcloned into the expression vector pTrehIS-TOPO, and transformed into TOP 10 cells (Invitrogen). Sequencing confirmed the correct in-frame construction to allow for isopropyl \( \beta \)-1-thiogalactopyranoside (IPTG) induction of His-tagged protein. After a 4-h induction with 2 mM IPTG, cells were lysed under denaturing conditions (8 m urea), passed over a nickel-
agarose affinity column (Invitrogen), rinsed, and then eluted with 100 mM imidazole. SDS-PAGE confirmed the presence of an approximately 30-kD protein that was not present in nontransformed TOP 10 cells. GST activity in affinity-purified extracts was determined as described below for nodule extracts ("Protein Extraction and Measurement of GST Activity").

**Construction of RNAi Plasmids**

A 223-bp GST9 gene-specific gene fragment and a 166-bp GST family-specific gene fragment were amplified by PCR from soybean genomic DNA using GST9 gene-specific forward primer (5' -CGAGGGAAGAGGATTATC-3') and GST9 gene-specific reverse primer (5' -TGACACACGCTCTTTCG-3'), GST family-specific forward primer (5' -TGGCTGGAGTTACTTACATG-3') and GST family-specific reverse primer (5'-CAAACATTACAA-3').

**Production of Composite Plants**

Composite plants (wild-type shoots and transgenic roots) were generated as described by Collier et al. (2005). Soybean seeds were sterilized with chloride gas, planted in 25-cm-diameter pots containing vermiculite, and maintained in a greenhouse under the conditions described by Dalton et al. (1993), except that the nutrient solution used also contained nitrogen (1.3 mM \( \text{NH}_4\text{NO}_3 \)), since it was not necessary for plants at this stage to rely on symbiotic nitrogen fixation. Plants were grown until the first trifoliate leaves were partially expanded, about 18 to 23 d after planting, at which time shoots were excised to generate hairy roots. \( \text{A. rhizogenes} \) K599 containing binary plasmid AKK 1467B, C5T-5214, or C5T-5215 was grown overnight at 30°C in 50 ml of Luria-Bertani broth containing 50 \( \mu \)g ml\(^{-1}\) kanamycin to an optical density at 600 nm of 0.1. In a laminar flow hood, sterile 1-cm\(^3\) fiber-glass cubes (Humann International) were punctured with a 1-ml pipet tip and saturated with 2 ml of resuspended bacteria in petri dishes. Apical shoots from greenhouse-grown soybeans were excised with a diagonal cut using a sterile scalpel and inserted into the cubes. Petri dishes with four explants per dish were placed in clean plastic trays, covered tightly with plastic domes, and kept in the greenhouse. Explants were allowed to recover for 24 h, and then the domes were halfway removed overnight, allowing the explants to partially wilt. They were then watered well with deionized water, and wet paper towels were placed in the trays before replacing the domes tightly. The explants were provided with excess water daily in order to maintain high humidity for the duration of the induction period. New roots were visible 2 weeks after inoculation. After 20 d, composite plants were inoculated with \( \text{Bradyrhizobium japonicum} \) by dipping the roots and cube in a slurry of commercial pea inoculant (Inoculant S; Nitragin). Composites were then planted two per 20-cm-diameter pot in vermiculite and grown in a greenhouse under the conditions described by Dalton et al. (1993), which included a daily supply of a nitrogen-free nutrient solution.

**Microscopy**

Callus tissue, which had formed at the site of inoculation with \( \text{A. rhizogenes} \), was separated from hairy roots and stems. The callus was fixed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer for 1 h. The tissue was then dehydrated through graded ethanol, embedded in LR White resin, cut into 1-µm sections, and stained with toluidine blue.

**Plant Tissue Harvest and Acetylene Reduction**

Nodules were harvested from 87 composite plants (32 CGT-5215, 31 CGT-5214, and 25 AKK 1467B) at 32 to 34 d after inoculation. Each plant was separated into shoots, lateral roots emerging above the cut site, and hairy roots emerging from callus tissue at the cut site. Each portion was weighed, and the number of roots was counted. Nodules from hairy roots were then carefully removed with forceps. Nodules from six to seven plants were pooled to obtain enough material to form five samples from each treatment type. Acetylene reduction (as described by Dalton et al., 1991) was performed immediately on a portion of these nodules, and another portion was stored at –80°C for later use in protein and RNA extraction. Additionally, nodules were harvested from six CGT-5214 and six AKK 1467B plants at 45 d after inoculation, and the same morphological data were collected.
Measurements of Oxidative Damage

The TBARS assay was modified from Puppo et al. (1991). About 50 mg of nodules was frozen in liquid nitrogen and ground to a powder in Eppendorf tubes with a small plastic pestle. To this was added 1 mL of ice-cold 0.1% trichloroacetic acid in deionized water. Extracts were spun down at 10,000g for 1 min, and two 450-μL aliquots of the supernatant were removed. To each was added 225 μL of 1% thiobarbituric acid in 50 mM NaOH and 45 μL of 2% butylated hydroxytoluene in 100% ethanol. Each sample was vortexed, supplemented with 225 μL of 25% HCl, and vortexed again. Samples were then heated to 100°C for 10 min, cooled on ice, extracted with 450 μL of 1-butanol, and centrifuged at 1,000g for 1 min. The upper organic layer was removed to a cuvette and measured at 532 nm against a butanol blank. MDA content was calculated using an extinction coefficient of 1.56 × 10^5 M^–1 cm^–1.

Oxidative damage to proteins was assessed by derivatization of protein carboxyls to 2,4-dinitrophenylhydrazone using the Oxyblot Protein Oxidation Detection Kit (Millipore) followed by separation on 12% SDS-PAGE precast gels (Bio-Rad). Separated proteins were electrophoretically transferred to nitrocellulose and immunoblotted following the Oxyblot instructions with a rabbit anti-2,4-dinitrophenylhydrazone primary antibody and secondarily with alkaline phosphatase conjugated to goat anti-rabbit IgG antibody (Immune Blot Assay Kit; Bio-Rad) and developed according to the manufacturer’s instructions.

Protein Extraction and Measurement of GST Activity

Protein extraction from nodules was performed as described by Dalton et al. (1993). The extraction buffer consisted of 250 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM β-mercaptoethanol, and 0.5% Triton X-100. After centrifuging at 13,000g for 10 min, the supernatant was measured for GST activity with either CDNB or EA as substrate using modifications of the procedures described by Habig and Jakoby (1981). Activity with CDNB was measured in a 1-mL reaction mixture containing 20 μL of root nodule extract, 1.0 mM GSH, 1.0 mM CDNB, and 0.1 mM potassium phosphate buffer (pH 7.5). The protein extract was added last, and the change in A_340 was measured for 300 s using a DU-640 Beckman spectrophotometer. Activity with EA was assayed in a 400-μL reaction in a quartz cuvette containing 15 μL of protein extract, 0.2 mM EA, 0.25 mM GSH, and 0.1 mM potassium phosphate buffer (pH 6.5). Protein was added last, and the change in A_410 was immediately measured for 20 s. For measurements of GST activity from Escherichia coli, cells were lysed by sonication and then purified by nickel-affinity chromatography using a Probond nickel-chelating resin with nondenaturing conditions as described by the manufacturer (Invitrogen). Activity assays were performed as described above with 50 μL (3–5 μg) of eluted protein. Concentrations of conjugates were calculated using the equation described by Griswold et al. (2005), which allows for subtraction of absorbance by the unconjugated substrates. Corrections were made for the slight nonenzymatic activity, and all extracts were measured in triplicate. Protein concentration was determined by Coomassie Brilliant Blue binding with bovine serum albumin as the standard (Bradford, 1976).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers M20363 (GST1), AF243365 (GST2/10), X68819 (GST4), AF048978 (GST4), AF243361 (GST6/7), AF243363 (GST8), AF243364 (GST9), AF243368 (GST13), AF243369 (GST14), AF243370 (GST15), AF243373 (GST18), L10292 (APX), L11632 (glutathione reductase), and V00453 (Leg a).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Predicted pattern of GST (top) on a 2-D gel based on calculated values for pI and Mr, and the actual pattern observed for GSH-affinity purified proteins from nodules (bottom; staining was with Coomassie Brilliant Blue).

Supplemental Figure S2. Transcript levels of various GSTs in soybean nodules at 4, 6, and 9 weeks after planting (data are means ± s.e., n = 3).

Supplemental Table S1. Complete list of proteins identified by MALDI MS from nodule extracts purified by glutathione-affinity chromatography and matched to the NCBI nonredundant database.

Supplemental Table S2. Kinetic properties of soybean GSTs.

Supplemental Table S3. Genes and primers used in this study.

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LITERATURE CITED


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