Cytosolic γ-Glutamyl Peptidases Process Glutathione Conjugates in the Biosynthesis of Glucosinolates and Camalexin in Arabidopsis

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The defense-related plant metabolites known as glucosinolates play important roles in agriculture, ecology, and human health. Despite an advanced biochemical understanding of the glucosinolate pathway, the source of the reduced sulfur atom in the core glucosinolate structure remains unknown. Recent evidence has pointed toward GSH, which would require further involvement of a GSH conjugate processing enzyme. In this article, we show that an Arabidopsis thaliana mutant impaired in the production of the γ-glutamyl peptidases GGP1 and GGP3 has altered glucosinolate levels and accumulates up to 10 related GSH conjugates. We also show that the double mutant is impaired in the production of camalexin and accumulates high amounts of the camalexin intermediate GS-IAN upon induction. In addition, we demonstrate that the cellular and subcellular localization of GGP1 and GGP3 matches that of known glucosinolate and camalexin enzymes. Finally, we show that the purified recombinant GGP enzymes can metabolize at least nine of the 10 glucosinolate-related GSH conjugates as well as GS-IAN. Our results demonstrate that GSH is the sulfur donor in the biosynthesis of glucosinolates and establish an in vivo function for the only known cytosolic plant γ-glutamyl peptidases, namely, the processing of GSH conjugates in the glucosinolate and camalexin pathways.

INTRODUCTION

Among the most well-studied defense-related compounds in plants is the group of metabolites known as glucosinolates. Glucosinolates are sulfur-containing secondary metabolites characteristic of the order Brassicales, which includes the agriculturally important oilseed rape (Brassica napus), the cruciferous vegetables, and the model plant Arabidopsis thaliana (Fahey et al., 2001). Together with the enzyme myrosinase, glucosinolates constitute the so-called mustard oil bomb, which is a binary defense system against generalist insects (Hopkins et al., 2009) and has also been implicated in defense against nonadapted pathogens (Bednarek et al., 2009; Clay et al., 2009). Apart from their ecological and agricultural importance, glucosinolates have been proposed to have cancer-preventive properties (Higdon et al., 2007; Hayes et al., 2008), which has sparked great interest in their metabolic engineering and heterologous production (Gasper et al., 2005; Geu-Flores et al., 2009). After a decade of glucosinolate research in the Arabidopsis postgenomic era, the biosynthetic pathway is well understood and most biosynthetic genes are known (Senderby et al., 2010). A notable exception is the step involving the incorporation of reduced sulfur, where the identity of the donating thiol has not been established (Senderby et al., 2010).

The tripeptide GSH (γ-Glu-Cys-Gly) is the most abundant low-molecular-weight thiol in the cell and is involved in numerous cellular processes, such as redox homeostasis, redox sensing, and detoxification of xenobiotics and heavy metals (Noctor and Foyer, 1998; Cobbett, 2000; Rea, 2007; Meyer, 2008; Rouhier et al., 2008; Pal and Rai, 2010; Cummins et al., 2011). By virtue of the reducing properties of thiols, GSH can detoxify reactive oxygen species either directly (e.g., by quenching free radicals) or indirectly (e.g., via the GSH-ascorbate cycle, which detoxifies H2O2 enzymatically) (Noctor and Foyer, 1998; Meyer, 2008). GSH is also involved in the detoxification of heavy metals and xenobiotics, where the strong nucleophilic properties of thiols are exploited biologically. Heavy metals are detoxified by chelation with GSH oligomers called phytochelatins (n = 2 to 11) and the apparently nontoxic organometallic complexes are stored in the vacuole (Cobbett, 2000; Pal and Rai, 2010). In turn, xenobiotics...
with electrophilic centers are conjugated to GSH either non-
enzymatically or by the action of glutathione S-transferases
(GSTs) (Cummins et al., 2011). The GSH conjugates are shuttled
into the vacuole by transporters of the ABC family (Rea, 2007).
Once in the vacuole, degradation to the Cys conjugates pro-
cceeds via the sequential action of a γ-glutamyl transpeptidase
(GGT) and a yet unidentified carboxypeptidase (Grzam et al.,
2006; Ohkama-Ohtsu et al., 2007b).
In addition to the proven biological roles of GSH, a role as
sulfur donor in the biosynthesis of glucosinolates has been
proposed but remains to be proven (Schlaeppi et al., 2008; Geu-
Flores et al., 2009; Dixon et al., 2010). Glucosinolates are
synthesized from different amino acids (Trp, Phe, or side
chain–elongated Met) through the action of at least five enzymes.
The two first are cytochromes P450 of the families CYP79 and
CYP83, respectively. These convert the amino acids to activated
aldoximes that react spontaneously with thiols to form thiol
conjugates. For decades, Cys was thought to be the in vivo sulfur
donor; therefore, a GST-like enzyme was proposed to catalyze
the specific conjugation to Cys. The Cys conjugate is further
processed to a glucosinolate by the sequential action of a C-S
lyase (SUR1), a glucosyltransferase (at least UGT74B1), and a
sulfotransferase (SOT16, 17, or 18) (Sonderby et al., 2010).

The first indication of the involvement of GSH in the biosynthesis
of glucosinolates came from the analysis of the phytoalexin
deficient2 (pad2) mutant plants. The pad2 mutation was mapped
to the GSH1 gene, which codes for γ-Glu-Cys synthetase, the
enzyme that catalyzes the first of two committed steps in the
biosynthesis of GSH from its amino acid constituents. Accord-
ingly, leaves of pad2 plants were shown to accumulate only
around 20% of wild-type GSH levels, while the accumulation of
Cys was increased 5-fold (Pariszy et al., 2007). With respect to
glucosinolates, the uninduced foliar levels were unchanged in
pad2 mutants when compared with wild-type plants, but upon
elicitation by a generalist insect, the mutants accumulated less
glucosinolates (50% of the wild-type levels of both I3M and 4MSB
after 24 h of constant challenge by Spodoptera littoralis; Schlaeppi
et al., 2008). This suggested a link between GSH and glucosinolate
biosynthesis; however, given the multifunctionality of GSH, the
exact nature of the link remained unclear. A connection implicating
redox regulation via the GSH-ascorbate cycle has been regarded
as unlikely, since the ascorbate-deficient mutant vtc1-1 (which has
25% of wild-type ascorbic acid levels) was not affected in
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The phytoalexin camalexin is another well-studied sulfur-
containing plant defense compound in Arabidopsis, and the
sulfur donor in its biosynthesis was recently shown to be GSH (Su
et al., 2011). The first step in the biosynthesis of camalexin is
shared with that of Trp-derived glucosinolates and involves the
conversion of Trp to its aldoxime by CYP79B2 or its homolog
CYP79B3 (Glawischning et al., 2004). The camalexin pathway
then branches off with the action of CYP71A13, which converts
the aldoxime to a nitrile (indo-3-acetonitrile [IAN]) (Nafisi et al.,
2007). After an unknown activation step, GSTF6 catalyzes the
conjugation to GSH to give GS-IAN (Su et al., 2011). Although the BGLS study
of GS-B in vitro (Geu-Flores et al., 2009). Although the BGLS study
in N. benthamiana suggests that GSH is the sulfur donor and that
GGP1 is the GSH conjugate-processing enzyme, experimental
evidence is needed to demonstrate this in naturally occurring
with the discovery of GS-B–processing enzyme. The latter hypothesis led
to the discovery of GS-B–processing enzyme. The latter hypothesis led
to the discovery of

RESULTS

The Arabidopsis GGP Gene Family

GGP1 has four homologous genes in Arabidopsis. Two of them,
which we have named GGP2 and GGP3, are arranged in tandem
with GGP1 on chromosome IV. The other two, which we have
named GGP4 and GGP5, are arranged in tandem on chromosome
II (see Supplemental Figure 1 online). Sequence analysis revealed that the encoded GGP proteins have amino acid identities ranging from 61 to 76% (see Supplemental Table 1 online). Analysis of public microarray-based expression data using Genevestigator (https://www.genevestigator.com/) showed that GGP1 and GGP3 exhibited high expression levels across all tissues. By contrast, the expression of GGP4 was very low and confined to root tissues, and the expression of GGP5 seemed to be restricted to pollen. Finally, GGP2 seemed not to be expressed at all (see Supplemental Figure 2 online). Coexpression analyses using ATTED-II (http://atted.jp) showed that GGP1 was the only GGP gene that was coregulated with known glucosinolate-related genes throughout all the available tissues and treatments.

**Analysis of ggp1 Mutants**

The following publicly available *Arabidopsis* T-DNA mutants were analyzed by PCR for insertions in the GGP1 gene: SALK_02930, SAIL_225_G01, GK-960B11, and GK-319F10. For the SALK and the SAIL lines, the T-DNA insertion could not be confirmed. From the two remaining lines, both with confirmed insertions in the first intron, only line GK-319F10 lacked the GGP1 transcript in the homozygous state as seen by RT-PCR. We named this line ggp1-1. Relative transcript quantification by quantitative RT-PCR revealed that the homozygous ggp1-1 mutant retained 0.7 to 1.0% of wild-type GGP1 transcript levels.

We searched for a glucosinolate phenotype in leaves of the ggp1-1 homozygous knockdown, but the levels of the different glucosinolates were unaltered in 3-week-old rosette leaves (data not shown). Based on publicly available microarray data, GGP1 was the only GGP gene that was coregulated with known glucosinolate genes; however, the GGP3 gene was also expressed throughout all tissues (see previous section and Supplemental Figure 2 online). We therefore hypothesized that the GGP3 gene was able to substitute for the impaired GGP1 function in the ggp1-1 knockdown mutant. This would require three conditions to be met simultaneously: first, both genes should have overlapping expression patterns; second, both proteins must localize to the same subcellular compartment; and third, both proteins must catalyze the same enzymatic reactions.

![Figure 1](image.jpg)

**Figure 1.** Cellular and Subcellular Localization of GGP1 and GGP3.

(A) GUS staining of rosette leaves of representative transgenic lines transformed with GGP1pro::GUS, GGP3pro::GUS, or empty vector as negative control. GGP1pro::GUS plants were stained for 3 h, whereas GGP3pro::GUS and control plants were stained for 6 h. 

(B) CLSM analysis of intact epidermal cells and mesophyll protoplasts of *N. benthamiana* leaves transiently transformed with constructs coding for GGP1-YFP or GGP3-YFP (C-terminal fusions). Pictures were taken 7 d after infiltration with *Agrobacterium.*
Comparison of GGP1 and GGP3 Expression, Subcellular Localization, and Activity

The 2-kb promoter regions of GGP1 and GGP3 (GGP1pro or GGP3pro, respectively) were cloned upstream of an open reading frame encoding β-glucuronidase (GUS). Along with an empty vector control, the resulting constructs were introduced separately into wild-type Arabidopsis Columbia-0 (Col-0) using Agrobacterium tumefaciens–mediated transformation. Leaf GUS analysis of at least three different lines per construct showed that both promoters conferred expression to (or in the vicinity of) vascular tissue (Figure 1A). The subcellular localization of GGP1 and GGP3 was investigated using C-terminal yellow fluorescent protein (YFP) fusions transiently expressed in leaves of N. benthamiana using Agrobacterium. The YFP fluorescence was visualized 7 d after infiltration using confocal laser scanning microscopy (CLSM), which showed that both protein fusions were cytosolic (Figure 1B).

We previously reported that GGP1 is able to enhance the production of BGLS in N. benthamiana by metabolizing the GSH conjugate that accumulates in its absence (GS-B) (Geu-Flores et al., 2009). We investigated whether GGP3 had the same effect as GGP1 in this heterologous system. The APK2 gene was included in these experiments, as we have recently found that it ensures complete conversion of desulfoBGLS (the last intermediate in the pathway) to BGLS by siphoning off the increased demand for activated sulfate (Møldrup et al., 2011). The experiments showed that GGP3 was able to enhance the accumulation of BGLS and suppress the accumulation of GS-B to almost the same extent as GGP1 (1.9-fold mean increase in BGLS compared with 2.4-fold for GGP1; 96.4% mean decrease in GS-B compared with 99.5% for GGP1) (Figure 2).

Downregulation of GGP3 Using Artificial MicroRNAs and Construction of Double Knockdown Mutants

Since GGP1 and GGP3 are located ~2.5 kb apart on chromosome IV, the construction of a double T-DNA insertion mutant was not practically feasible. Instead, we decided to downregulate GGP3 using artificial microRNA (amiRNA) (Ossowski et al., 2008) and to cross the resulting plants to the ggp1-1 mutant. We investigated whether this system was suitable for knockdown experiments. We used the Web MicroRNA Designer (http://wmd3.weigelworld.org/) for the design of two amiRNA constructs targeting GGP3 and we assembled them using the USER fusion strategy (Nour-Eldin et al., 2010). The constructs were introduced separately into wild-type Arabidopsis Col-0 by Agrobacterium-mediated transformation. Using agarose gel-based RT-PCR, we screened all T1 transformants and selected the line with the lowest apparent GGP3 transcript level. We called this line ggp3-1.

We then crossed the T1 ggp3-1 amiRNA plant to a homozygous ggp1-1 plant and used PCR to select F1 plants with both the amiRNA construct and the T-DNA insertion in the GGP1 gene. Using a combination of PCR and segregation analysis, we generated comparable batches of T3 seeds coming from homozygous plants of each of the four following four genotypes: the wild type, ggp1-1, ggp3-1, and ggp1-1/ggp3-1. Comparative transcript analysis by real-time RT-PCR (quantitative RT-PCR) showed that the amiRNA construct was specific for GGP3 and remained functional throughout several generations. Accordingly, transcript levels of both GGP1 and GGP3 were reduced in leaves of ggp1-1/ggp3-1 plants and represented ~2 and ~18% of wild-type levels, respectively (Table 1).

ggp1-1 ggp3-1 Plants Have Altered GLS Levels and Accumulate Substantial Amounts of Glucosinolate-Related GSH Conjugates

Analysis of 3-week-old rosette leaves revealed a perturbed glucosinolate profile of ggp1-1 ggp3-1 compared with wild-type

![Figure 2. GGP-Aided Production of BGLS in N. benthamiana.](image)

Leaves of N. benthamiana were infiltrated with a mixture of Agrobacterium strains carrying constructs that coded for P19 (silencing suppressor), CYP79A2, CYP83B1, SUR1, UGT74B1, SOT16, APK2, and GGP1, GGP3, or GFP (negative control). Each data point represents the mean of six biological replicates with error bars representing SE. Double asterisks indicate data points with highly significant differences compared with the GFP controls (P < 0.01 in unpaired Student’s t tests).

(A) Accumulation of BGLS.
(B) Accumulation of GS-B.
FW, fresh weight.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GGP1 Transcript</th>
<th>GGP3 Transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td>ggp1-1</td>
<td>0.7–1.0%</td>
<td>Unaltered</td>
</tr>
<tr>
<td>ggp3-1</td>
<td>Unaltered</td>
<td>3–13%</td>
</tr>
<tr>
<td>ggp1-1 ggp3-1</td>
<td>1–4%</td>
<td>6–30%</td>
</tr>
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</table>
plants (Figure 3, see legend for glucosinolate abbreviations). Whereas the levels of short-chained Met-derived glucosinolates (3msp, 4msb, and 4mtb) were not significantly altered, the levels of the long-chained ones were considerably reduced (5msp, 60% left; 7msh, 43% left; and 8mso, 50% left). Regarding Trp-derived glucosinolates, the levels of the most abundant one were increased (i3m, 1.7-fold higher) and the levels of its methoxylated derivatives were unchanged (1M-i3m and 4M-i3m) (Figure 3). Induction experiments using methyl jasmonate did not result in further significant reduction of glucosinolate accumulation in induced ggp1-1 ggp3-1 plants compared with induced wild-type controls (see Supplemental Figure 3 online). Although the fold induction for several glucosinolates appeared to be lower for ggp1-1 ggp3-1 plants than for wild-type plants, these differences

![Figure 3](image)

**Figure 3.** Glucosinolate Analysis of 3-Week-Old Rosette Leaves of Wild-Type, ggp1-1, ggp3-1, and ggp1-1 ggp3-1 Plants.

Each data point represents the mean of eight biological replicates with error bars representing SE. Single and double asterisks indicate data points with significant or highly significant differences compared with their corresponding wild-type (WT) control (P < 0.05 or P < 0.01 in unpaired Student’s t tests, respectively). 3msp, 3-(methylsulfinyl)propylglucosinolate; 4mtb, 4-(methylthio)butylglucosinolate; 4msb, 4-(methylsulfinyl)butylglucosinolate; 5msp, 5-(methylsulfinyl)pentyllglucosinolate; 7msh, 7-(methylsulfinyl)heptylglucosinolate; 8mso, 8-(methylsulfinyl)octylglucosinolate; i3m, indole-3-yl-methylglucosinolate; 4M-i3m, 4-methoxyindole-3-yl-methylglucosinolate; 1M-i3m, 1-methoxyindole-3-yl-methylglucosinolate. FW, fresh weight.

![Figure 4](image)

**Figure 4.** LC-MS Analysis of 3-Week-Old Rosette Leaves of Wild-Type, ggp1-1, ggp3-1, and ggp1-1 ggp3-1 Plants.

(A) TICs. WT, wild type.

(B) EICs using the m/z ([M+H]^+) of all possible glucosinolate-related GSH conjugates. The identity of peaks 1 to 9 is specified in Table 2.
were not significant in two-way analysis of variance tests (e.g., \( P = 0.053 \) for Ggp3) (see Supplemental Figure 3 online).

To investigate the accumulation of intermediates in leaves of the single and double mutants, we performed an untargeted metabolite analysis by liquid chromatography–mass spectrometry (LC–MS). As seen in total ion chromatograms (TICs), the metabolite profile of leaf extracts did not vary visibly in the single ggp1-1 and ggp3-1 mutants when compared with the wild type. However, the profile of the ggp1-1 ggp3-1 leaves was dramatically different because of the appearance of several abundant peaks (Figure 4A).

Inspection of the mass spectrum of these peaks showed that all of them corresponded to GSH conjugates that could potentially be intermediates in the biosynthesis of glucosinolates. Extracted ion chromatograms (EICs) using the masses of all theoretically possible glucosinolate-related GSH conjugates showed that ggp1-1 ggp3-1 plants accumulated nine different GSH conjugates (Figure 4B; Table 2) and that most, if not all, of the variation seen in the TICs was due to these accumulating compounds. The EICs also showed that the ggp1-1 mutant accumulated traces of these compounds, whereas the ggp3-1 mutant and the wild type did not accumulate them at all. The identity of the GSH conjugates was confirmed by accurate mass determination (Table 2) and fragmentation analysis (see Supplemental Table 2 online).

The ggp Mutants Produce Less Camalexin and Accumulate the Related GSH Conjugate upon Induction

We investigated whether the ggp mutants had a camalexin phenotype by inducing camalexin production in 3-week-old rosette leaves with AgNO\(_3\) and analyzing leaf extracts 24 h later using HPLC coupled to a fluorescence detector. Whereas the ggp3-1 mutant did not accumulate significantly less camalexin than the wild type, the ggp1-3 mutant accumulated \( \sim 40\% \), and the ggp1-1 ggp3-1 double mutant accumulated \( \sim 11\% \) of wild-type camalexin levels (Figure 5A).

We used LC–MS to search for the GSH conjugate of the camalexin intermediate IAN (GS-IAN) in the same leaf extracts. Whereas both the wild type and the ggp3-1 mutant accumulated trace amounts of a compound with the mass of GS-IAN, both ggp1-1 and the double ggp1-1 ggp3-1 mutant accumulated very high amounts of this compound. We confirmed that this compound was GS-IAN by chemically synthesizing a standard and comparing retention times and fragmentation patterns (Figures 5B and 5C). Quantification of GS-IAN in the leaf extracts showed that the ggp1-1 mutant and the ggp1-1 ggp3-1 double mutant produced a similar amount of GS-IAN (200 to 300 pmol/mg fresh weight), which was comparable to the amount of camalexin produced by wild-type plants (Figure 5D).

Recombinant GGP1 and GGP3 Can Metabolize Glucosinolate-Related GSH Conjugates and GS-IAN

As final proof of the multiple and overlapping enzymatic activities of GGP1 and GGP3, we expressed their His-tagged versions in Escherichia coli and analyzed the activity of the purified proteins in vitro. As substrates, we used either synthetic GS-IAN (camalexin intermediate) or extracts of ggp1-1 ggp3-1 leaves containing the different glucosinolate-related conjugates. In the extracts, an additional glucosinolate-related conjugate was detected, which had a side chain corresponding to the glucosinolate 5mtp (compound ID# 10; accurate mass error of +1.6 ppm) compared with theoretical mass; see Supplemental Table 2 online). The assays with the extracts showed that both GGP1 and GGP3 were able to metabolize nine out of 10 GSH conjugates to the corresponding enzymatic products (Cys-Gly conjugates), all of which spontaneously rearranged to their cyclized forms as previously seen for GS-B (Geu-Flores et al., 2009). The cyclized Cys-Gly conjugates were readily detectable in GGP1 and GGP3 reaction mixtures and virtually absent in control mixtures, where only trace amounts near detection limits were observed (Figure 6). A notorious exception was the cyclized Cys-Gly conjugate related to the glucosinolate 4msb, which was also present in control mixtures (Figure 6). The identity of all detectable cyclized Cys-Gly conjugates was confirmed by accurate mass determination (Table 3) and fragmentation analysis (see Supplemental Table 3 online). Finally, the assays with GS-IAN showed that both GGP1 and GGP3 were able to yield a compound with mass and fragmentation patterns consistent with the Cys-Gly conjugate of IAN (Cys-Gly-IAN) (Figure 7).

**DISCUSSION**

This article provides direct genetic evidence of the involvement of GSH as sulfur donor in the biosynthesis of glucosinolates.

<table>
<thead>
<tr>
<th>Compound ID No.</th>
<th>Side Chain</th>
<th>Elemental Composition</th>
<th>Protonated Molecular Ion [M+H]+</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Theoretical m/z</td>
</tr>
<tr>
<td>1</td>
<td>-(CH(_2))(_2)(S=O)CH(_3)</td>
<td>C(<em>{16})H(</em>{20})N(_4)O(_6)S(_2)</td>
<td>455.1265</td>
</tr>
<tr>
<td>2</td>
<td>-(CH(_2))(_3)(S=O)CH(_3)</td>
<td>C(<em>{17})H(</em>{22})N(_4)O(_7)S(_3)</td>
<td>469.1421</td>
</tr>
<tr>
<td>3</td>
<td>-(CH(_2))(_4)(S=O)CH(_3)</td>
<td>C(<em>{18})H(</em>{24})N(_4)O(_8)S(_4)</td>
<td>483.1578</td>
</tr>
<tr>
<td>4</td>
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<td>C(<em>{19})H(</em>{26})N(_4)O(_9)S(_5)</td>
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</tr>
<tr>
<td>5</td>
<td>-(CH(_2))(_6)(S=O)CH(_3)</td>
<td>C(<em>{20})H(</em>{28})N(_4)O(_10)S(_6)</td>
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<tr>
<td>6</td>
<td>-(CH(_2))(_7)(S=O)CH(_3)</td>
<td>C(<em>{21})H(</em>{30})N(_4)O(_11)S(_7)</td>
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<tr>
<td>7</td>
<td>-(CH(_2))(_8)(S=O)CH(_3)</td>
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<td>439.1316</td>
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<tr>
<td>8</td>
<td>-(CH(_3))(_8)SCH(_3)</td>
<td>C(<em>{15})H(</em>{38})N(_4)O(_13)S(_9)</td>
<td>453.1472</td>
</tr>
<tr>
<td>9</td>
<td>Indol-3-ylmethyl-</td>
<td>C(<em>{20})H(</em>{36})N(_4)O(_1)S</td>
<td>480.1548</td>
</tr>
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</table>
evidence surfaced from the analysis of Arabidopsis mutants impaired in the production of GGPs. Specifically, the ggp1-1 ggp3-1 double knockdown mutant showed an altered foliar glucosinolate profile and accumulated up to 10 different GSH conjugates related to glucosinolate biosynthesis. Our data on cellular and subcellular localization of GGP1 and GGP3 are consistent with a glucosinolate biosynthetic role. Indeed, published promotor-GUS fusion experiments performed with core glucosinolate biosynthetic genes have all shown a similar expression in leaf vascular tissues (Mikkelsen et al., 2000; Reintanz et al., 2001; Tantikanjana et al., 2001, 2004; Chen et al., 2003; Grubb et al., 2004; Kusnierczyk et al., 2007). Furthermore, the cytosolic localization of the GGP1- and GGP3-GFP (green fluorescent protein) fusions is in agreement with the proposed cytosolic localization of the core glucosinolate pathway. The latter is based on the association of cytochromes P450 with the endoplasmic reticulum, with their catalytic domain facing the cytosol (relevant for CYP79s and CYP83s) (Schuler and Werck-Reichhart, 2003), and on the lack of predicted targeting peptides of the known soluble enzymes (SUR1, UGT74B1, and SOT16, 17, and 18), experimentally shown for the SOTs (Klein et al., 2006). Combined with biochemical in vitro data showing the ability of both GGPs to use at least nine out of 10 of the accumulating GSH conjugates as substrates, we conclude that GGP1 and GGP3 are enzymes metabolizing GSH conjugates in the glucosinolate pathway in Arabidopsis. In accordance with this, we conclude that the reduced sulfur atom in the core glucosinolate structure is derived from GSH.

The fact that the levels of only long-chained Met-derived glucosinolates were reduced in ggp1-1 ggp3-1 plants (and not the levels of all glucosinolates) may reflect that the mutant was a double knockdown mutant and not a double knockout mutant. In other words, the residual GGP1 and GGP3 transcripts (up to 4 and 30% of wild-type levels, respectively) are likely to have generated sufficient protein so as to prevent a more marked phenotype. Furthermore, the increase in levels of the main Trp-derived glucosinolate (i3m) in ggp1-1 ggp3-1 resembles a similar increase in i3m observed in another mutant deficient in Met-derived glucosinolates, namely, cyp83a1 (Hemm et al., 2003). This provides additional evidence of a complex interplay between the productions of Met- and Trp-derived glucosinolates in Arabidopsis. Regardless of the subtle glucosinolate end-product phenotype, the ggp1-1 ggp3-1 double mutant accumulated substantial amounts of GSH conjugates not only related to the glucosinolates with decreased levels, but also related to all other glucosinolates normally present in wild-type leaves (Kliebenstein et al., 2001).

The accumulation of GSH conjugates with sulfinyl side chains (compounds 1 to 6) is unexpected as side chain modifications such as the oxidation of thio to sulfinyl glucosinolates (e.g., the oxidation of 4-mtb to 4-msb) are believed to happen after the GGP step. However, it was shown for FMOGS-OX1 (catalyzing the mentioned oxidation) that the reaction can occur not only at the glucosinolate level, but also at the desulfoglucosinolate level (Hansen et al., 2007). A possible explanation could be that FMOs can also act at the GSH conjugate level, converting the accumulating thio GSH conjugates to sulfinyl GSH conjugates. An alternative explanation could be that the thio and sulfinyl side chains
chains are subject to nonenzymatic interconversions dependent on redox levels of the surrounding medium. Either proposition could explain not only the accumulation of compounds 1 to 6, but also the absence of the thio GSH conjugates related to 7-mtp and 8-mto, whose sulfinyl counterparts were abundant in the double mutant (compounds 5 and 6). The accumulation of cyclized Cys-Gly conjugate related to the glucosinolate 4-msb (Figure 6, compound 12) is also worth discussing, since the mentioned compound accumulated in 

\[ \text{ggp1-1 ggp3-1} \]

mutants, but not in wild-type plants (see Supplemental Figure 4 online). Considering that 4-msb is the most abundant glucosinolate in Arabidopsis leaves, a possible explanation is that the related GSH conjugate (compound 2) accumulated to such extent that it entered the default xenobiotic detoxification pathway. As mentioned in the Introduction, GSH conjugates of xenobiotics are transported to the vacuole, where they are initially cleaved by a GGT. Vacuolar cleavage of compound 2 in the absence of the rest of the glucosinolate biosynthetic machinery could lead to cyclization and, thus, to a metabolic dead end.

Our results also demonstrate that GGPs are involved in camalexin biosynthesis, since leaves of the single 

\[ \text{ggp1-1} \]

mutant were impaired in the accumulation of the phytoalexin when compared with wild-type leaves, and the double mutant 

\[ \text{ggp1-1 ggp3-1} \]

accumulated even less camalexin. In addition, both of

![Figure 6. LC-MS Analysis of Enzymatic Assays Using His-Tagged GGP1 or GGP3 with an Extract from ggp1-1 ggp3-1 Plants.](image)

Protein from an expression strain carrying the empty vector was used as negative control. Four different EICs (extracted masses) accounting for nine cyclized Cys-Gly conjugates are presented. The identity of each marked peak is specified in Table 3.

**Table 3.** Accurate Mass Measurements of the Cyclized Cys-Gly Conjugates Found in Enzymatic Assays Using Extracts of 

\[ \text{ggp1-1 ggp3-1} \]

Leaves and Recombinant GGP1 or GGP3

<table>
<thead>
<tr>
<th>Compound ID No.</th>
<th>Side Chain</th>
<th>Elemental Composition</th>
<th>Protonated Molecular Ion [M+H]+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Theoretical m/z</td>
</tr>
<tr>
<td>11</td>
<td>-(CH(_2)_3)(S=O)CH(_3)</td>
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<td>293.0624</td>
</tr>
<tr>
<td>12</td>
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<tr>
<td>14</td>
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<tr>
<td>16</td>
<td>-(CH(_2)_3)(S=O)CH(_3)</td>
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</tr>
<tr>
<td>17</td>
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<td>C(<em>{10})H(</em>{16})N(_2)O(_3)S(_2)</td>
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</tr>
<tr>
<td>18</td>
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<tr>
<td>19</td>
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</tr>
<tr>
<td>20</td>
<td>Indol-3-ylmethyl-</td>
<td>C(<em>{12})H(</em>{15})N(_3)O(_3)S</td>
<td>318.0907</td>
</tr>
</tbody>
</table>

n.d., not determined.
these mutants accumulated high amounts of the related GSH conjugate GS-IAN. Our results on the cytosolic localization of GG1 and GG3 are consistent with the likely cytosolic localization of the camalexin pathway. Indeed, three of the four known enzymes in the pathway (including the first and the last one) are cytochromes P450, which are associated with the endoplasmic reticulum, having their catalytic domain facing the cytosol (Schuler and Werck-Reichhart, 2003). The fourth enzyme, GSTF6, lacks predicted signaling peptides and is therefore most likely cytosolic. In combination with the in vitro biochemical evidence showing that both GGPs were able to use synthetic GS-IAN as a substrate, we conclude that GG1 and GG3 are enzymes metabolizing GS-IAN in the camalexin pathway.

Figure 7. LC-MS Analysis of Enzymatic Assays Using His-Tagged GG1 or GG3 with GS-IAN as Substrate.

Protein from an expression strain carrying the empty vector was used as negative control. 
(A) EICs using masses corresponding to the substrate ([M+H]+ of 462) and the product ([M+Na]+ of 355).
(B) MS spectrum of the product peak at 7.5 min. The structure of the product, Cys-Gly-IAN, is shown.
(C) MS2 fragmentation of the m/z = 354.9 ion corresponding to the [M+Na]+ adduct of Cys-Gly-IAN. The diamond indicates the mass of the mentioned ion. The arrows represent possible relations between parent and daughter ions.

Figure 8. Proposed Model for the Biosynthesis of Trp-Derived Glucosinolates and Camalexin in Arabidopsis.

The pathway leading to Trp-derived glucosinolates is represented in the left branch, and the pathway leading to camalexin is represented in the right branch. As indicated in the boxed insert, R represents an indole-3-yl group. Since it is not currently known whether the substrates of SUR1 are the Cys-Gly conjugates or the Cys conjugates (see Discussion), both possibilities have been depicted.
GGP1 and GGP3 are the only known plant enzymes capable of hydrolyzing the γ-glutamyl residue of GSH conjugates in the cytosol, since the only other enzymes with similar activities, the GGTs, are located either in the extracellular space (GGT1 and GGT2) (Martin et al., 2007; Ohkama-Ohtsu et al., 2007a) or in the vacuole (GGT4) (Grzam et al., 2006; Ohkama-Ohtsu et al., 2007b). Indirect evidence for cytosolic γ-glutamyl peptidase activity was presented by Grzam et al. (2006), who showed that the vacuolar sequestration of GS-bimane in wild-type Arabidopsis leaves could be efficiently inhibited by azide (N$_3^-$); however, its degradation to the Cys conjugate proceeded normally, perhaps even at a faster rate. This observation pointed to the presence of an efficient cytosolic γ-glutamyl peptidase and can now possibly be explained by the existence of GGP1 and GGP3.

Recently, Su et al. (2011) suggested a role for GGT1 and GGT2 in the processing of GS-IAN in camalexin biosynthesis. Their suggestion is based on the lower camalexin content of ggt1 and ggt2 mutants when compared with a wild-type plant and on the lower camalexin content of wild-type plants when treated with the GGT inhibitor aevicin. The camalexin phenotype in the mutants is surprising given that there are two extracellular localization of the GGTs (see above). The inhibition experiments cannot be taken as conclusive in favor of the involvement of GGTs, since aevicin is likely to inhibit GGTs as well. Moreover, GGP1 and GSTF6 were found among the 20 proteins whose levels were increased upon MKK9-induced camalexin production, whereas GGT1 and GGT2 were not (Su et al., 2011). Future studies are needed to explain the interesting camalexin phenotype seen upon knockout of the extracellular GGTs, including the search for accumulating intermediates in the knockouts.

The glucosinolate and camalexin pathways have been suggested to have a biogenetic relationship (Rauhut and Glawischnig, 2009), and the involvement of GGPs in both pathways supports this hypothesis. In the camalexin pathway, another peptidase, a carboxypeptidase, is required to hydrolyze the Cys-Gly peptide bond to give the established intermediate Cys-IAN. Su et al. suggested PCS1 as a candidate; however, their own data showed that the pcs7-1 mutant was not significantly reduced in camalexin accumulation upon biotic treatment in comparison to wild-type plants (Su et al., 2011). In the absence of further evidence, we consider the carboxypeptidase as unknown. This second peptidase may or may not be part of the glucosinolate pathway, as it is currently unknown whether the Cys-Gly conjugates or the Cys-conjugates are the substrates of the next known enzyme in the pathway, SUR1. Like any other C-S lyase, SUR1 requires its substrates to have a free amino group in the Cys moiety (Schwimmer and Kjaer, 1960), and both types of conjugates (but not GSH conjugates) fulfill this requirement. However, given the likely biogenetic relationship between the two pathways and the broader distribution of glucosinolates throughout the Brassicaceae order (Rauhut and Glawischnig, 2009), it is possible that the GGPs and the unknown carboxypeptidase were recruited together to the camalexin pathway from the glucosinolate pathway. Our current model for glucosinolate and camalexin biosynthesis in Arabidopsis is outlined in Figure 8.

Until the discovery of GGPs, GGTs were the only known plant enzymes capable of hydrolyzing GSH conjugates (Martin et al., 2007). It is noteworthy that GGTs are not related to GGPs but are related to Gln amidotransferases, enzymes that transfer the amido nitrogen of Gln to different acceptor substrates (Mouilleron and Golinelli-Pimpanneau, 2007). Two other enzymes related to Gln amidotransferases have been linked to unexpected biochemical reactions. The first one is puuD from E. coli, which has been shown to catalyze the hydrolysis of γ-glutamyl-γ-aminobutyrate in the utilization pathway of putrescine (Kurihara et al., 2005, 2006). The second one is DUG3 from the yeast Saccharomyces cerevisiae, which has been linked to the hydrolysis of the γ-Glu-Cys peptide bond of GSH in a previously unknown GSH degradation pathway (Ganguly et al., 2007), although proof of its direct catalysis is missing. The structural resemblance between Gln, GSH conjugates, γ-glutamyl-γ-aminobutyrate, and GSH, namely, the shared γ-glutamyl moiety, provides an explanation for the unexpected activities of GGPs, puuD and DUG3, and suggest that only the γ-glutamyl moiety is crucial for substrate recognition. This is supported by the fact that both GGP1 and GGP3 are able to use nine different glucosinolate-related GSH conjugates and the camalexin-related GS-IAN as substrates. Further studies need to be performed to determine the extent of this partial promiscuity. Particularly interesting is whether GGP1 and GGP3 can hydrolyze GSH, since their ubiquitous expression and cytosolic localization (where GSH is abundant) suggest that they cannot.

In summary, our results demonstrate that GSH is the sulfur donor in the biosynthesis of glucosinolates and that GGP1 and GGP3 are cytosolic enzymes metabolizing GSH conjugates in the biosynthesis of both glucosinolates and camalexin in Arabidopsis.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana lines SALK_02930 and SAIL_225_G01 were obtained from the ABRC (Alonso et al., 2003), Arabidopsis lines Gk-960B11 and Gk-319F10 (renamed ggp1-1) were obtained from the University of Bielefeld (Bielefeld, Germany) (Rosso et al., 2003). All Arabidopsis plants were grown in growth chambers at 20°C and 70% relative humidity with 16-h photo-periods at 100 μE m$^{-2}$ s$^{-1}$. All Nicotiana benthamiana plants were grown in a greenhouse with a day/night regime of 28/25°C and 16-h-long days.

Identification of GGP Family Members and Sequence Analysis

The annotated amino acid sequence of GGP1 (NP_194782) was used as input in a position-specific iterated BLAST search using the Reference Protein database (refseq_protein) at the National Center for Biotechnology Information limited to Arabidopsis sequences. The search was terminated after the third iteration. Four homologs were identified. Sequence identities were determined by pairwise alignment using ClustalW (default settings).

Genotyping of Potential ggp1 Mutants

Line SALK_02930 was genotyped using primers 5'-TTGAGCCATAGA-GGGAAAAATG-3' and 5'-TTGCTTGTGTAATAACTTATG-3', together with the SALK left-border primer 5'-TGTTTCAGTGTAATGCGCATG-3'. Line SALK_225_G01 was similarly genotyped using primers 5'-GGAT-ACGCAGCTTATG-3', 5'-TTTGTCACTGGAACTTATG-3', and the SAIL left-border primer 5'-ATTTGCGGATTGCAAC-3'. Lines

Cytosolic γ-Glutamyl Peptidases 2465
Cellular Localization Using Promoter-GUS Fusions

The 2-kb promoter regions of GGP1 and GGP3 were amplified from wild-type (Col-0) Arabidopsis genomic DNA using primer pairs 5'-GGCTTAAUATGGTGGAG-3' and 5'-GGTTTAAUGTGGATCCCCCCATGGCGATGC-3'. All PCR fragments were amplified from pRS300 (Ossowski et al., 2010). The coding regions of GGP1 and GGP3 were amplified without stop codons from cDNA clones RALF06-16-J02 (RIKEN BioResource Center) and U21128 (ABRC) using primer pairs 5'-GGGTTCAATGTTGGGAG-CAAAGAGATACGC-3' and 5'-GGTTTAAUGTGGATCCCCCCATGGCGATGC-3'. The constructs were transformed into Agrobacterium, transformed into Arabidopsis, and transformed into leaves of N. benthamiana. The selected T1 transformants were either homozygous or lacking the amiRNA construct. For the phenotypic analysis, F3 seed batches were obtained from each selected F2 plant. For the genotypes having the amiRNA construct, 40 seedlings from each seed batch were sprayed with the herbicide Basta to select batches where the amiRNA construct (linked to the Basta resistance gene) did not segregate out. Only the seed batches where no seedlings died were subjected to phenotypic analysis.

Transcript Analysis of ggp Mutant Plants

Total RNA was extracted from 3-week-old rosette leaves using the NucleoSpin RNA II kit (Macherey-Nagel), including on-column DNase treatment. Real-time RT-PCR (quantitative PCR) was performed using the intron-spanning primer pairs 5'-TCACGATGCTTCTGAGAATATG-3' and 5'-AATCCATGGCGACCC-3'. The primers used were the same as specified for the screening of T1 amiRNA transformants. For the quantification of GGP1 and GGP3 transcripts in the single and double mutants, real-time RT-PCR (quantitative PCR) was performed using a Rotor-Gene 6000 (Corbett Life Science/Giaglar) and the DyNamo Flash SYBR Green qPCR kit (Finzymes) in total reaction volumes of 20 μL. The primers used were the same as specified for the screening of T1 amiRNA transformants. For all primer pairs, efficiency and linear amplification ranges were determined. For each genotype, three biological replicates were used, each measured in triplicate.

Routine Metabolite Analyses of ggp Mutant Plants

All metabolite analyses were performed using rosette leaves of 3-week-old plants. Glucosinolate analysis was performed using the plant transformation constructs were assembled into pcAMBIA3300-3Ssu using the USER fusion strategy (Ossowski et al., 2008; Nour-Eldin et al., 2010). All PCR fragments were amplified from pRS300 (Ossowski et al., 2008). The fragment carrying the microRNA loop was amplified using the primer pair 5'-AAAGAGAAUCATGTCAATTTGCT-3' and the left-border primer 5'- ATATTGACCATGACATCTACCTG-3'.

Construction of the ggp1-1 ggp3-1 Double Mutant

The selected T1 ggp3-1 amiRNA plant, which was derived from amiRNA construct 1, was crossed to homozygous ggp1-1 plants, and F1 plants with both the amiRNA construct and the T-DNA insertion in the GGP1 gene were selected by PCR as described for the parental lines. We then used multiplex PCR to select F2 plants that were either wild-type for the GGP1 locus or ggp1-1 homozygous, each selected plant either having or lacking the amiRNA construct. For the phenotypic analysis, F3 seed batches were obtained from each selected F2 plant. For the genotypes having the amiRNA construct, 40 seedlings from each seed batch were sprayed with the Basta to select batches where the amiRNA construct was segregated out. Only the seed batches where no seedlings died were subjected to phenotypic analysis.

Subcellular Localization Using YFP Fusions

The coding regions of GGP1 and GGP3 were amplified from cDNA clones RALF06-16-J02 (RIKEN BioResource Center) and U21128 (ABRC) using primer pairs 5'-GGCTTAAUATGGTGGAG-CAAAGAGATACGC-3' and 5'-GGTTTAAUGTGGATCCCCCCATGGCGATGC-3’. The constructs were transformed into Agrobacterium and infiltrated into leaves of N. benthamiana. The selected T1 transformants were either homozygous or lacking the amiRNA construct. For the phenotypic analysis, F3 seed batches were obtained from each selected F2 plant. For the genotypes having the amiRNA construct, 40 seedlings from each seed batch were sprayed with the Basta to select batches where the amiRNA construct (linked to the Basta resistance gene) did not segregate out. Only the seed batches where no seedlings died were subjected to phenotypic analysis.

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covering the adaxial side with small droplets of 5 mM AgNO₃. The
Geu-Flores et al., 2009). Untargeted analysis by LC-MS was performed on a
together with published response factors for the individual glucosinolates
Brown et al., 2003). As a control, the same
sprayed with either 250 μM methyl jasmonate in 0.25% ethanol or with
0.25% ethanol (mock solution) 24 h prior to harvesting. The glucosinolate
singinir (not present in Arabidopsis) was used as an internal standard
together with published response factors for the individual glucosinolates
Brown et al., 2003). Untargeted analysis by LC-MS was performed on a
fraction of the spun-down methanolic extracts as previously described
(Geu-Flores et al., 2009).

For the analysis of camalexin, nondetached leaves were induced by
covering the adaxial side with small droplets of 5 mM AgNO₃. The
induction procedure was repeated 12 h later. Twenty-four hours after the initial
induction, entire leaves were harvested and crushed in methanol.

Accurate Mass Determination and Fragmentation Analysis
Chromatographic separations were performed on an Acquity UPLC
system (Waters) equipped with a HSS T3 column (100 × 1.0 mm, particle
size 1.8 μm; Waters) applying the following binary gradient at a flow rate
of 150 μL min⁻¹: 0 to 1 min, isocratic 95% A (water, 0.1% formic acid), 5% B
(acetonitrile, 0.1% formic acid); 1 to 6 min, linear from 5 to 30% B; 6 to
10 min, linear from 30 to 95% B; 10 to 12 min, isocratic 95% B; 12 to 14
min, isocratic 5% B. Eluted compounds were detected from mass-to-
charge ratio (m/z) 100 to 1000 using a MicrOTOF-Q II hybrid quadrupole
time-of-flight mass spectrometer (Bruker Daltonics) equipped with an
Apollo II electrospray ion source in positive ion mode using the following
instrument settings: nebulizer gas N₂, 1.4 bar; dry gas N₂, 8 L/min, 190°C;
capillary, -5000 V; end plate offset, −500 V; funnel 1 RF, 200 Vpp, funnel
2 RF, 200 Vpp; in-source collision-induced dissociation energy, 0 V;
hexapole RF, 100 Vpp; quadrupole ion energy, 3 eV; collision gas, N₂;
collision energy, 7 eV; collision RF 150/350 Vpp (timing 50/50); transfer
time, 70 μs; pulse frequency, 5 μs; pulse frequency, 10 kHz; spectra rate,
3 Hz. Internal mass calibration of each analysis was performed by
infusion of 20 μL 10 mM lithium formiate in isopropanol/water, 1/1 (v/v), at
a gradient time of 10 min using a diverter valve. For fragmentation analyses,
precursor ions were selected in Q1 with an isolation width of ± 6 D
and fragmented in the collision cell applying collision energy of 30 eV.
N₂ was used as collision gas. Collision-induced dissociation mass
spectra were recorded using the following parameter settings: collision
RF 150/350 Vpp (timing 50/50); transfer time, 70 μs; pulse frequency, 5 μs;
pulse frequency, 10 kHz; spectra rate, 1.5 Hz.

Synthesis of GS-IAN
See Supplemental Protocol online.

Heterologous Expression of GGP1 and GGP3 and Enzymatic Assays
The coding sequence of GGP3 was amplified from clone U21128 (ABRC)
using primers 5’-AAATACCTGGATGTTATGACGAGAAC-3’
and 5’-AAATACAGATTCTCAACCTTCAAGATTTTGG-3’ and cloned
into the Escherichia coli expression vector pRSET-A (Invitrogen) using Xhol
and EcoR restriction sites. GGP1 and GGP3 were then expressed in E. coli
BL21(DE3)pLysS and purified as previously described for GGP1
(Geu-Flores et al., 2009). As a control, the same E. coli strain carrying the empty
pRSET-A was used. For assays with ggp7-1 ggp3-1 leaf extracts, 200 mg of
leaf material were homogenized in 600 μL 85% methanol and spun down for
20 min at 20,000g. The supernatant was passed through a methanol-
ashed SepPak Light C18 cartridge (Waters), and the cleared extract was
evaporated and redissolved in 200 μL water. Enzymatic assays were
performed in a final volume of 100 μL at room temperature for 1 h in 20
mM Tris buffer, pH 7.5, supplemented 15 μL redissolved extract and 1 μg of
purified His-GGP1, His-GGP3, or protein from the empty vector control.
Assays with GS-IAN were performed under similar conditions, except that
the reaction mixtures were supplemented with 200 μM GS-IAN instead of
leaf extracts. Analysis of the products by LC-MS was performed as
previously described for GS-B (Geu-Flores et al., 2009). Accurate mass
measurements and fragmentation analysis of the products were performed
as described above.

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome
Initiative or GenBank/EMBL databases under the following accession numbers:
At4g30560 (GGP1), At4g30540 (GGP2), At4g30550 (GGP3),
At2g23960 (GGP4), At2g23970 (GGP5), At5g05260 (CYP79A2),
At4g39950 (CYP79B2), At2g22330 (CYP79B3), At4g31500 (CYP83B1),
At2g0610 (SUR1), At1g24100 (UGT74B1), At1g74100 (SOT16),
At1g18590 (SOT17), At1g74090 (SOT18), At3g39940 (APK2),
At4g23100 (PAD2/GSH1), At2g30770 (CYP71A13), At1g02930 (GSTF6),
At4g3640 (GGT1), At4g39650 (GGT2), At4g29210 (GGT4), At5g44070
(PCS1), and At3g26830 (PAD3/CYP71B15).

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

Supplemental Figure 1. Approximate Location of GGP Genes Relative
to Each Other in the Arabidopsis Genome.

Supplemental Figure 2. Microarray-Based Expression Analysis of the Different
GGP Family Members in Different Tissues of Arabidopsis as
Visualized Using Genevestigator.

Supplemental Figure 3. Glucosinolate Analysis of Wild-Type and
ggp7-1 ggp3-1 Plants 24 h after Treatment with Either a Mock
Solution or a Solution of 250 μM Methyl Jasmonate.

Supplemental Figure 4. Extracted Ion Chromatogram from LC-MS
Analysis of Wild-Type and ggp7-1 ggp3-1 Plants Accounting for the
Cyclized Cys-Gly Conjugates Related to the Glucosinolate 4-msb.

Supplemental Table 1. Amino Acid Identities between the Different
GGP Family Members.

Supplemental Table 2. Fragmentation Analysis of [M+H]+ Ions of
Compounds 1 to 10 Found in Extracts of ggp7-1/ggp3-1 Plants.

Supplemental Table 3. Fragmentation Analysis of [M+H]+ Ions of
Compounds 12 to 20 Found in Enzymatic Assays with Extracts of
ggp7-1/ggp3-1-1 Plants and Recombinant GGP1 or GGP3.

Supplemental Protocol. Synthesis of GS-IAN [H-Glu(Cys)[AN]-Gly-OH]-OH.

ACKNOWLEDGMENTS
We thank David P. Dixon for the kind donation of synthetic camalexin.
This work was funded by the Danish Council for Independent Research
–Technology and Production Sciences and by the Villum Kann Rasmussen
Fond through its support to the Villum Kann Rasmussen Research
Centre for Pro-Active Plants.
AUTHOR CONTRIBUTIONS

F.G.-F., M.E.M., and B.A.H. designed the study. Most of the experimental work was performed by F.G.-F. and M.E.M. GS-IAN was synthesized by C.B., who also performed accurate mass measurements and fragmentation analyses. All other LC-MS analyses were performed by C.E.O. D.S. obtained the funding for C.B., and B.A.H. obtained the funding for F.G.-F. B.A.H. also performed the function of overall study director and supervisor.

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REFERENCES


Suppl. Figure 1. Approximate location of GGP genes relative to each other in the Arabidopsis genome.
Suppl. Figure 2. Micro-array-based expression analysis of the different GGP family members in different tissues of Arabidopsis as visualized using Genevestigator. Error bars represent standard errors.
Suppl. Figure 3. Glucosinolate analysis of WT and ggp1-1 ggp3-1 plants 24 hours after treatment with either a mock solution or a solution of 250 µM methyl jasmonate (MeJA). All data points represent the means of 11 biological replicates with error bars representing standard errors. Single and double asterisks indicate data points with significant or highly significant differences compared to their corresponding WT control (p < 0.05 or p < 0.01 in unpaired Student’s T-tests, respectively). 3msp, 3-(methylsulfinyl)propylglucosinolate; 4mtb, 4-(methylthio)butylglucosinolate; 4msb, 4-(methylsulfinyl)butylglucosinolate; 5msp, 5-(methylsulfinyl)pentylglucosinolate; 7msh, 7-(methylsulfinyl)heptylglucosinolate; 8mso, 8-(methylsulfinyl)octylglucosinolate; i3m, indole-3-yl-methylglucosinolate; 4M-i3m, 4-methoxyindole-3-yl-methylglucosinolate; 1M-i3m, 1-methoxyindole-3-yl-methylglucosinolate.
**Suppl. Table 1.** Amino acid identities between the different GGP family members (ClustalW).

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<th></th>
<th>GGP1</th>
<th>GGP2</th>
<th>GGP3</th>
<th>GGP4</th>
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<tr>
<td>GGP1</td>
<td></td>
<td></td>
<td>68%</td>
<td>72%</td>
<td>68%</td>
</tr>
<tr>
<td>GGP2</td>
<td>61%</td>
<td></td>
<td>58%</td>
<td>64%</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>64%</td>
<td>76%</td>
<td></td>
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</tr>
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<tr>
<td>GGP5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>64%</td>
</tr>
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</table>
Table 2. Fragmentation analysis of [M+H]^+ ions of compounds 1-10 found in extracts of *ggp1-1/ggp3-1* plants. Compound 10 was not detected in the experiment presented in Figure 6, but was found in extracts prepared for the enzymatic assays. Numbers indicate m/z values with their respective abundances in parenthesis. Structural proposals for observed fragment ions are supported by accurate mass measurements (± 10 ppm).

<table>
<thead>
<tr>
<th>Comp. #</th>
<th>R group</th>
<th>[M+H]^+</th>
<th>[M-Gly+H]^+</th>
<th>[M-Gly-NH2OH+H]^+</th>
<th>[a+H]^+</th>
<th>[a-H2O+H]^+</th>
<th>[a-CHS+H]^+</th>
<th>[b+H]^+</th>
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<th>[d1+H]^+</th>
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Suppl. Table 3. Fragmentation analysis of [M+H]+ ions of compounds 12-20 found in enzymatic assays with extracts of ggp1-1/ggp3-1 plants and recombinant GGP1 or GGP3. Numbers indicate m/z values with their respective abundances in parenthesis. Structural proposals for observed fragment ions are supported by accurate mass measurements (± 10 ppm).

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**Suppl. Protocol: Synthesis of GS-IAN (H-Glu(Cys(IAN)-Gly-OH)-OH)**

**Reagents:**
1. HCl·H-Gly-OBu, TBTU, NMM
2. DBU
3. Boc-Glu(OH)-O\text{Bu}, DIC, HOBt
4. I\text{2}
5. DTT, Et\text{3}N
6. (Boc)\text{2}O, DMAP
7. NBS, AIBN
8. DIPEA
9. TFA/H\text{2}O

**Abbreviations:**
- Acm, acetamidomethyl
- AIBN, azobisisobutyronitrile
- Boc, tert-butyloxycarbonyl
- DIPEA, N,N'-diisopropylethylamine
- HOBt, hydroxybenzotriazole
- Fmoc, 9H-fluoren-9-ylmethoxycarbonyl
- NBS, N-bromosuccinimide
- TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium tetrafluoroborate

**General**
Solvents were purified and dried prior to use using standard procedures. Protected amino acids were purchased from Bachem AG. TLC was performed on silica gel 60 F254 (Merck) with detection by UV light or phosphomolybdic acid/ceric sulphate in 5% aqueous sulfuric acid followed by heating. Flash chromatography was performed using silica gel (32–63 μm) with solvent systems given in the text. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on a Varian spectrometer operating at 300 and 75 MHz, respectively. LC/MS analyses were performed on an Waters Acuity UPLC system equipped with a HSS T3 column (100 • 1.0 mm, particle size 1.8 μm, Waters) applying the following binary gradient at a flow rate of 150 μL min\textsuperscript{-1}: 0-1 min, isocratic 95% A (water/0.1% formic acid), 5% B (acetonitrile / 0.1% formic acid); 1-16 min, linear from 5 to 95% B; 16-18 min, isocratic 95% B; 18-20 min, isocratic 5% B. Eluted compounds were detected using a MicrOTOF-Q hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an Apollo II electrospray ion source in positive ion mode. The synthesis of 4 was adapted from protocols described by Burg et al. (2002).

**Fmoc-Cys(Acm)-Gly-O\text{Bu} (1).** To a solution of Fmoc-Cys(Acm)-OH (1.24 g, 3.0 mmol) in DMF (15 mL) N-methylmorpholine (NMM, 0.30 g, 3.0 mmol) and TBTU (0.96 g, 3.0 mmol) were added. After stirring for 2 min a solution of HCl·H-Gly-O\text{Bu} (0.55 g, 3.3 mmol) and NMM (0.64 g, 6.3 mmol) in DMF (5 mL) was added and the reaction mixture stirred for 18 h at room temperature. DMF was removed in vacuo and the residue dissolved in ethyl acetate (150 mL), washed with 0.1 M HCl (30 mL), saturated aqueous NaHCO\text{3} (30 mL), water (30 mL)
and brine (30 mL). After drying over Na₂SO₄ and removal of the solvent I was purified by flash chromatography (Ø 40, L 300, ethyl acetate, Rₜ 0.40). Yield: 1.25 g (79%). UPLC/ESI(+)-TOF-MS: tᵣ = 598 s; m/z (rel. int. (%)) = 550.1989 (61) [M+Na]⁺, 528.2174 (73) [M+H]⁺ (calc. for C₁₇H₂₅N₁₆O₅S²⁻: 528.21628), 472.1546 (39) [M+H-C₄H₈]⁺, 401.1184 (100) [Fmoc-Cys-Gly-OH+H]⁺.

**Boc-Glu(Cys(Acm)-Gly-O'Bu)-O'Bu (2).** DBU (0.38 g, 2.5 mmol) was added to a solution of 1 (1.24 g, 2.4 mmol) in CH₂Cl₂ (20 mL). The mixture was stirred at room temperature until TLC analysis indicated complete consumption of the starting material (20 min). The volatiles were removed in vacuo and the remaining residue was purified by flash chromatography (Ø 40, L 300, silica gel, CH₂Cl₂:MeOH 20:1, Rₜ 0.31). Yield: 1.16 g (82%). UPLC/ESI(+)-TOF-MS: tᵣ = 546 s; m/z (rel. int. (%)) = 613.2918 (47) [M+Na]⁺, 591.3101 (65) [M+H]⁺ (calc. for C₂₀H₂₅N₁₈O₅S²⁻: 591.30853), 491.2590 (100) [M+H-CO₂-C₄H₈]⁺, 435.1933 (33) [M+H-CO₂-2C₄H₈]⁺, 379.1288 (4) [M+H-CO₂-3C₄H₈]⁺.

**[Boc-Glu(Cys-Gly-O'Bu)-O'Bu]₂ (3).** 2 (1.16 g, 2.0 mmol) was dissolved in MeOH (20 mL). A solution of I₂ (1.02 g, 4.0 mmol) in MeOH (15 mL) was added dropwise over a period of 30 min. The reaction mixture was stirred until TLC analysis indicated complete consumption of the starting material (10 min). The solution was decolorized by addition of 1 M aqueous Na₂S₂O₃. Ethyl acetate (200 mL) was added and the organic phase was washed with 0.5 M aqueous Na₂S₂O₃ (50 mL), water (2 × 50 mL) and brine (50 mL). After drying over Na₂SO₄ and evaporation of the solvent 3 was obtained as colorless oil which was homogeneous by TLC analysis (ethyl acetate, Rₜ 0.70) and used without further purification. Yield: 1.02 g (98%). UPLC/ESI(+)-TOF-MS: tᵣ = 808 s; m/z (rel. int. (%)) = 1059.4995 (67) [M+Na]⁺, 1037.5178 (55) [M+H]⁺ (calc. for C₄₃H₄₇N₂₀O₅S²⁻: 1037.51450), 937.4660 (16) [M+H-CO₂-C₄H₈]⁺, 541.2364 (19) [M+2Na]²⁺, 307.0808 (100) [H-Glu(Cys-Gly-OH)-OH]₂+2H]²⁺.

**Boc-Glu(Cys-Gly-O'Bu)-O'Bu (4).** To a solution of 3 (0.52 g, 0.5 mmol) and triethylamine (0.16 g, 1.6 mmol) in CH₂Cl₂ (30 mL) dithiothreitol (0.32 g, 2.1 mmol) was added and the reaction mixture stirred at room temperature under an argon atmosphere until TLC analysis indicated complete consumption of the starting material (3 h). The solution was diluted with CH₂Cl₂ (70 mL) and extracted with 0.1 M HCl (50 mL) and water (2 × 50 mL). After drying over Na₂SO₄ and evaporation of the solvent 4 was obtained as white solid which was homogeneous by TLC analysis (ethyl acetate, Rₜ 0.63). 4 was used without further purification and stored under argon at 4°C until further use. Yield: 0.49 g (94%). UPLC/ESI(+)-TOF-MS: tᵣ = 602 s; m/z (rel. int. (%)) = 542.2546 (13) [M+Na]⁺, 520.2741 (100) [M+H]⁺ (calc. for C₂₃H₄₉N₁₈O₈S⁻: 520.26871), 420.2221 (71) [M+H-CO₂-C₄H₈]⁺, 364.1548 (57) [M+H-CO₂-2C₄H₈]⁺, 308.0939 (27) [M+H-CO₂-3C₄H₈]⁺.
1-(tert-Butyloxy carbonyl)-3-(cyanomethyl)indole (5). A solution of 2-(indol-3-yl)acetonitrile (4.69 g, 30 mmol) and 4-(dimethylamino)pyridine (0.13 g, 1 mmol) in THF (150 mL) was treated with di-tert-butyl dicarbonate (6.99 g, 32 mmol). The mixture was stirred for 4 h at room temperature and the solvent removed under reduced pressure. The residue was dissolved in ethyl acetate (150 mL) and washed with 1 M HCl (2·50 mL) and brine (50 mL) and dried over Na2SO4. After removal of the solvent I was obtained as solid which was homogeneous by TLC analysis (hexanes/ethyl acetate 1/1, Rf 0.64). Yield: 7.42 g, (97%). UPLC/ESI(+)-TOF-MS: t_r = 662 s, m/z (rel. int. (%)) = 279.1079 (100) [M+Na]^+ (calc. for C_13H_16N_2O_2Na+: 279.11040), 257.1259 (7) [M+H]^+, 223.0460 (19) [M+Na-C_4H_4]^+, 201.0641 (3) [M+H-C_4H_4]^+, 174.0535 [M+H-C_4H_4-HCN]^+ (6), 130.0644 (15) [M+H-C_4H_4-HCN-CO_2]^+.

H-Glu(Cys(IAN))-Gly-OH)-OH (6). A solution of 5 (2.56 g, 10.0 mmol) in CCl_4 (30 mL) was heated to reflux. NBS (1.87 g, 10.5 mmol) and AIBN (0.05 g, 0.3 mmol) were added in three portions within 5 min. Afterwards, three portions of AIBN (3·0.01 g) were added, one each 30 min and the mixture refluxed for an additional 2 h. The mixture was allowed to cool to room temperature. Precipitated succinimide was filtered off and washed with CCl_4 (2·10 mL). To a solution of 4 (0.49 g, 0.9 mmol) and ethyldiisopropylamine (0.12 g, 0.9 mmol) in CH_2Cl_2 (50 mL) one-fourth of the crude bromination mixture was added. The solution was stirred for 16 h at room temperature and solvents were evaporated under reduced pressure. The remaining residue was taken up in ethyl acetate (100 mL) and washed with 0.1 M HCl (2·20 mL) and brine (20 mL). After drying over Na_2SO_4 and removal of the solvent the resulting complex mixture was fractionated by flash chromatography (20, L 300, hexanes/ethyl acetate 5/4). Fractions containing components with R_f 0.2-0.3 were pooled, evaporated to dryness and subjected to deprotection by treatment with TFA/H_2O 95/5 (5 mL) for 2 h. The mixture was evaporated to dryness, reconstituted in methanol/water 1/5 (5 mL). 6 was isolated by preparative HPLC (20, L 150, YMC ODS-A, pore size 120 Å, particle size 5 μm, flow rate 10 mL/min, isocratic: 80% H_2O / 0.1% formic acid, 20% CH_3CN / 0.1% formic acid, detection at 270 nm, two diastereomers (t_r = 8.1 / 9.4 min)). Yield: 0.03 g (7%). ^1H-NMR (300 MHz, D_2O): δ (ppm) 2.04-2.11 (m, 2H), 2.34-2.39 (m, 2H), 2.88 (dd, 1H, J = 8.5, 14.4 Hz), 3.22 (dd, 1H, J = 5.0, 14.4 Hz), 3.74 (m, 1H), 3.76 (d, 1H, J = 17.9 Hz), 3.85 (d, 1H, J = 17.9 Hz), 4.49 (m, 1H), 5.52 (s, 1H), 7.20-7.25 (m, 1H), 7.29-7.34 (m, 1H), 7.53 (s, 1H), 7.55 (d, 1H, J = 8.5 Hz), 7.80 (d, 1H, J = 7.9 Hz). ^13C-NMR (75 MHz, D_2O): δ (ppm) 27.0, 30.3, 32.1, 33.3, 42.6, 53.5, 54.8, 106.1, 113.5, 119.7, 120.3, 121.2, 124.0, 125.0, 125.6, 133.7, 173.0, 174.6, 174.7, 175.6. UPLC/ESI(+)-TOF-MS: t_r = 276 s, m/z (rel. int. (%)) = 462.1427 (100) [M+H]^+ (calc. for C_20H_23N_2O_2S^+: 462.14418), 308.0904 (91) [H-Glu(Cys-Gly-OH)-OH]^+, 179.0482 (21) [H-Cys-Gly-OH]^+, 155.0609 (10) [C_10H_12N_2]^+.

References
Cytosolic γ-Glutamyl Peptidases Process Glutathione Conjugates in the Biosynthesis of Glucosinolates and Camalexin in Arabidopsis

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