Integration of Biosynthesis and Long-Distance Transport Establish Organ-Specific Glucosinolate Profiles in Vegetative Arabidopsis

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Although it is essential for plant survival to synthesize and transport defense compounds, little is known about the coordination of these processes. Here, we investigate the above- and belowground source-sink relationship of the defense compounds glucosinolates in vegetative Arabidopsis thaliana. In vivo feeding experiments demonstrate that the glucosinolate transporters 1 and 2 (GTR1 and GTR2), which are essential for accumulation of glucosinolates in seeds, are likely to also be involved in bidirectional distribution of glucosinolates between the roots and rosettes, indicating phloem and xylem as their transport pathways. Grafting of wild-type, biosynthetic, and transport mutants show that both the rosette and roots are able to synthesize aliphatic and indole glucosinolates. While rosettes constitute the major source and storage site for short-chained aliphatic glucosinolates, long-chained aliphatic glucosinolates are synthesized both in roots and rosettes with roots as the major storage site. Our grafting experiments thus indicate that in vegetative Arabidopsis, GTR1 and GTR2 are involved in bidirectional long-distance transport of aliphatic but not indole glucosinolates. Our data further suggest that the distinct rosette and root glucosinolate profiles in Arabidopsis are shaped by long-distance transport and spatially separated biosynthesis, suggesting that integration of these processes is critical for plant fitness in complex natural environments.

INTRODUCTION

Plants are highly skilled organic chemists capable of synthesizing a vast number of defense compounds. Typically, these compounds accumulate to their highest levels in those tissues that are most likely to be attacked, which is assumed vital for plant survival by enabling them to adapt to their surroundings (McKey, 1974). Specific distribution patterns of these important compounds may be obtained by in situ synthesis and/or long-distance transport (Matsuda et al., 2010). The alkaloid nicotine in tobacco (Nicotiana tabacum) is an example of long-distance transport of a defense compound to organs distant from sites of synthesis, as it is synthesized in roots but stored in leaf tissues (Shoji et al., 2000; Yazaki, 2005; Morita et al., 2009). Hitherto, research on plant defense compounds has mostly focused on elucidation of their biosynthetic pathways, with much less attention paid to the contribution of transport to the defense strategies.

The long-distance transport system in plants consists of xylem and phloem. Translocation in xylem is mainly driven by an upward pull generated by transpiration from the aboveground organs and capillary forces. Hence, flow of water and solutes through the xylem is unidirectional. As xylem is part of the apoplastic conduit, the sole requirement for entry of compounds to the ascending xylem is export across the plasma membrane. By contrast, flow through the symplasmic phloem is driven by an osmosis-regulated hydrostatic pressure difference between source and sink tissues, primarily generated by the bulk flow of sucrose. Depending on this relationship, transport of compounds through the phloem can be considered as bidirectional (Thompson, 2006). Compared with xylem, an additional prerequisite for transport via phloem is the import of compounds from the apoplast into the symplasmic continuum of companion cells and their adjacent sieve elements. In apoplastic loaders, such as Arabidopsis thaliana, this import is predominantly facilitated by active transport through transport proteins (Rennie and Turgeon, 2009).

In Arabidopsis, major components of the chemical defense system are Met-derived and Trp-derived glucosinolates (aliphatic and indole GLS, respectively) (Kliebenstein et al., 2001a; Halkier and Gershenzon, 2006). Among the aliphatic GLS, a diversification based on chain elongation of Met has occurred, resulting in up to eight methylene groups in the side chain (Grubb and Abel, 2006). Throughout leaf development, the highest GLS concentrations are found in young leaves with the levels decreasing with leaf age until GLS are virtually absent upon senescence, when accumulation of GLS occurs in seeds (Brown et al., 2003). In comparison, in roots, GLS content has been shown to increase at least until the bolting stage (Petersen et al., 2002). The GLS profile of leaves is mostly dominated by aliphatic GLS, whereas roots contain higher amount of indole GLS (Petersen et al., 2002; Brown et al., 2003). These observations indicate that accumulation of GLS in
the various organs of Arabidopsis is under spatiotemporal regulation, although little is known about how these distribution patterns are obtained. Recently, we identified two proton-driven transport proteins of the nitrate/peptide transporter family in Arabidopsis, glucosinolate transporter 1 and 2 (GTR1 and GTR2), as high-affinity transporters of GLS (Nour-Eldin et al., 2012). Trace levels of GLS in seeds of a gtr1 gtr2 mutant and a concomitant increase in rosettes and siliques walls showed that these transporters are essential for long-distance GLS transport to seeds. Based on their plasma membrane localization, we suggested a role of GTR1 and GTR2 in importing GLS into companion cells for subsequent long-distance phloem transport. Noticeably, in the gtr1 gtr2 mutant, only aliphatic GLS and not indole GLS levels were increased in wilted rosettes (Nour-Eldin et al., 2012), suggesting that different processes underlie aliphatic and indole GLS distribution.

In this work, we investigate organ-specific distribution as well as long-distance transport processes of GLS in Arabidopsis at the vegetative growth stage. We use in vivo feeding of GLS and micrografting of biosynthetic and transport knockout mutants to examine the vascular mobility and the source-sink relationship of GLS between above- and belowground organs. Our results indicate that aliphatic GLS of different chain lengths differ with respect to site of synthesis and storage and that GTR1 and GTR2 are essential for shaping their rosette/root distribution through long-distance transport.

RESULTS

GLS Distribution between the Rosette and Roots Is Dependent on GTR1 and GTR2

We initiated our investigations by analyzing GLS in leaves of 3-week-old soil-grown gtr1 and gtr2 single mutants as well as the gtr1 gtr2 double mutant. No significant changes were observed in the GLS profiles of leaves from gtr1 and gtr2 mutants when compared with the wild type (Table 1). However, leaves of the gtr1 gtr2 double mutant contained approximately fivefold higher concentrations of aliphatic GLS with no significant changes observed for indole GLS (Table 1). Interestingly, this buildup could mainly be ascribed to 8-methylsulphonyloctyl GLS (see Supplemental Figure 1 online). Based on the proposed role of GTR1 and GTR2 in phloem loading of GLS (Nour-Eldin et al., 2012), this over-accumulation of aliphatic GLS in rosette leaves at the vegetative stage suggested that GTR1 and GTR2 are involved in transport of GLS between the rosette and roots. To test this, we measured the aliphatic GLS concentrations in roots using a hydroponic growth system. Similar to those of soil-grown plants (Table 1), the entire rosette of 3-week-old hydroponically grown gtr1 gtr2 mutants accumulated significantly higher concentrations of aliphatic GLS when compared with the wild type (P ≤ 0.01) (Figure 1A; see Supplemental Figure 2 online). When analyzing the roots of gtr1 gtr2 mutants, these were found to contain strongly reduced concentrations of aliphatic GLS compared with the wild type (P ≤ 0.01) (Figure 1B). This reduction was mainly attributed to 8-methylthiooctyl GLS (MTO) (see Supplemental Figure 2 online).

<table>
<thead>
<tr>
<th>Table 1. GLS Content in 3-Week-Old Soil-Grown gtr Mutants</th>
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<tr>
<td>Amount GLS per Leaf (nmol/mg Fresh Weight)</td>
</tr>
<tr>
<td>Wild type</td>
</tr>
<tr>
<td>gtr1</td>
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<tr>
<td>gtr2</td>
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<td>gtr1 gtr2</td>
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<td>gtr1 gtr2</td>
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Aliphatic and indole GLS in leaves of 4-week-old soil-grown Col-0 wild type, gtr1, gtr2, and gtr1 gtr2 mutants. Numbers are average ± sd; *two-tailed t test (P < 0.01) versus the wild type (n = 12).

Both the Rosette and Roots Are Capable of Synthesizing Aliphatic and Indole GLS

As a prerequisite for understanding the apparent role of GTR1 and GTR2 in regulating the distribution of aliphatic GLS, the ability of the above- and belowground organs to synthesize GLS was investigated. We utilized micrografting to reciprocally combine rosette and roots from the wild type and a mutant impaired in GLS biosynthesis. The latter was a quadruple mutant (hereafter qko) carrying T-DNA insertions in genes encoding the transcription factors MYB28 and MYB29, which regulate the production of aliphatic GLS, as well as in genes encoding the P450 enzymes CYP79B2 and CYP79B3, which are essential for synthesis of indole GLS (Müller et al., 2010). Scions and rootstocks of 4-d-old wild-type and qko seedlings were reciprocally grafted and analyzed for GLS in the rosette and roots when 3 weeks old (i.e., before the onset of bolting). With rosette/root homografts of wild-type plants (wt/wt) set to 100%, qko homografts (qko/qko) were found to contain only 1.8% ± 1.3% total GLS (Table 2), demonstrating that the grafting procedure did not induce GLS production in the qko mutant. In heterografts with impaired GLS synthesis in roots (wt/qko), the total content of aliphatic and indole GLS was reduced to 28.8% ± 5.1% and 20.7% ± 3.8% of wild-type homografts levels, respectively (P ≤ 0.05), constituting ~25% of the total GLS content in wild-type homografts (Table 2). In grafts with impaired GLS synthesis in the rosette (qko/wt), aliphatic and indole GLS were reduced to 50.7% ± 4.4% and 56.6% ± 23.3%, respectively (P ≤ 0.05), constituting ~50% of total GLS content in wild-type homografts (Table 2). These observations demonstrate that both the rosette and roots of vegetative Arabidopsis are able to de novo synthesize aliphatic as well as indole GLS. The content of individual GLS in all grafts is shown in Supplemental Table 1 online.

Bidirectional Transport of Exogenously Applied GLS between the Rosette and Roots Is GTR1 and GTR2 Dependent

With the apparent role of both the rosette and roots as sources for GLS, interorgan transport might occur in a bidirectional
manner. Accordingly, we tested the GTR1- and GTR2-dependent vascular mobility of GLS between the rosette and roots. We performed in vivo feeding of 2-propenyl GLS, which although not present in the ecotype Columbia-0 (Col-0), represents an endogenous aliphatic GLS found in other Arabidopsis accessions (Kliebenstein et al., 2001b). 2-Propenyl GLS was infiltrated into a leaf of 3-week-old hydroponically grown plants. After 72 h, wild-type plants contained ~8% of the total detected 2-propenyl GLS in roots, whereas ~59 and 33% were found in the remaining rosette and the fed leaf, respectively (Figure 2A). In contrast with the wild type, leaf infiltration of 2-propenyl GLS into gtr1 gtr2 mutants led to no detectable accumulation in roots, while ~45 and 55% of the total detected 2-propenyl GLS was found in the remaining rosette and the fed leaf, respectively (Figure 2A). Noticeably, although the fed amount of 2-propenyl GLS was ~10 nmol, we recovered only around 8 nmol in either genotype (Figure 2A), which is likely due to degradation. These data support previous observations of long-distance transport of GLS from the rosette to roots (Brudenell et al., 1999; Chen et al., 2001) and demonstrate that GTR1 and GTR2 are key facilitators of this transport. Based on the ability of roots to synthesize GLS, we further examined if GLS can be transported from roots to rosette and whether this involves GTR1 and GTR2 by incubating roots of hydroponically grown plants in medium containing 2-propenyl GLS. After 72 h incubation, ~3% of the recovered 2-propenyl GLS was found in roots of wild-type plants. Interestingly, in gtr1 gtr2 mutants, virtually no 2-propenyl GLS was detected in roots, although the total amount recovered from the plants was similar to that of the wild type (Figure 2B). A similar GTR1- and GTR2-dependent distribution was observed when feeding the aromatic p-hydroxybenzyl GLS (p-OHB) (see Supplemental Figure 4 online), which although being exogenous to Arabidopsis, has been demonstrated to undergo long-distance transport (Chen et al., 2001) and to be an in vitro substrate for GTR1 and GTR2 (Nour-Eldin et al., 2012). These observations show that GLS has the ability to move from roots to the rosette and suggest that GLS accumulation in roots is dependent on GTR1 and GTR2.

**GTR1 and GTR2 Are Expressed in the Root Vasculature with Highest Expression in Lateral Branching Points**

The possibility that GTR1 and GTR2 are involved in regulating the accumulation of GLS in roots primed us to investigate their expression in this organ. According to publically available cell type–specific expression data (Mustroph et al., 2009; http://efp.ucr.edu/), both transporters are translated in the vasculature and cortex (see Supplemental Figure 5 online). To validate their expression in the root vasculature, we analyzed transgenic plants expressing nuclear localization signal (NLS)–linked green fluorescent protein (GFP)–β-glucuronidase (GUS) fusion proteins driven by 2-kb promoter sequences upstream of either GTR1 or GTR2 (Nour-Eldin et al., 2012). GUS staining of 3-week-old plants and confocal laser

![Figure 1](https://example.com/figure1.png)

**Figure 1.** GLS Content in 3-Week-Old Hydroponically Grown Wild-Type and gtr1 gtr2 Mutants.

HPLC analysis of GLS content in the rosette (A), roots (B), and in the entire plant (C) of 3-week-old hydroponically grown Col-0 wild-type and gtr1 gtr2 mutants (black bars are the wild type; gray bars are gtr1 gtr2). Bars indicate so. * Two-tailed t test (P < 0.01) versus the wild type (n = 12 to 16).
scanning microscopy on the nuclear-targeted GFP-GUS fusions confirmed expression of both GTR1 and GTR2 in root vasculature with the highest transcriptional activity observed in the lateral root branching point areas (Figure 3).

### Rosettes Are the Major Site for Short-Chain Aliphatic GLS Synthesis and Storage

The finding that in vivo–fed 2-propenyl GLS could move bidirectionally and that this was influenced by GTR1 and GTR2, supports that these transporters are involved in regulating above- and belowground distribution of endogenous GLS. The expression levels of GTR1 and GTR2 have previously been shown to be unaffected by the absence of MYB28 and MYB29 (Sønderby et al., 2010). This enabled us to use qko organs to examine GTR1- and GTR2-dependent transport of endogenous GLS by micrografting wild-type, qko, and/or gtr1 gtr2 organs. For simplicity, in the following, aliphatic GLS containing three to five methane groups (C3 to C5) and six to eight methylene groups (C6 to C8) in their side chain will be referred to as short-chain (SC) and long-chain (LC) aliphatic GLS, respectively. When analyzing the levels of SC aliphatic GLS in homografts, none were detected in any qko organs, while gtr1 gtr2 contained significantly reduced levels only in the roots compared with wild-type homografts (P ≤ 0.05) (Figure 4A). For heterografts consisting of a wild-type rosette grafted onto qko roots (wt/qko), the rosette accumulated SC aliphatic GLS levels similar to those of wild-type homografts, whereas a trend toward reduction was observed in the roots (P > 0.05) (Figure 4A). In a similar manner, heterografts consisting of a gtr1 gtr2 rosette grafted onto qko roots (gtr1 gtr2/qko) contained SC aliphatic GLS levels in the rosette indistinguishable from those found in wild-type homografts (P > 0.05). While the gtr1 gtr2/qko grafts contained significantly reduced SC aliphatic GLS levels in roots when compared with wild-type homografts, these were in the same range as those detected in roots of the wt/qko grafts (P > 0.05) (Figure 4A). Heterografts consisting of a qko rosette grafted onto either wild-type or gtr1 gtr2 roots (qko/wt or qko/gtr1 gtr2, respectively) contained strongly reduced total amounts of SC aliphatic GLS, which was mainly due to reduced levels in the rosette (Figure 4A). Thus, bidirectional transport of SC aliphatic GLS can occur but may not be dependent on GTR1 and GTR2. Noticeably, heterografts consisting of a wild-type rosette grafted onto gtr1 gtr2 roots (wt/gtr1 gtr2), or the reciprocal combination (gtr1 gtr2/wt), showed similar levels of SC aliphatic GLS to those of wild-type homografts in the rosette and roots (P > 0.05) (Figure 4A). In summary, these observations demonstrate that the rosette is the major site for synthesis and storage of SC aliphatic GLS.

### LC Aliphatic GLS Are Synthesized in Above- and Belowground Organs and Retained in Roots by GTR1 and GTR2

When analyzing LC aliphatic GLS in homografts, qko rosettes contained levels below the detection limit, while roots had strongly increased levels of LC aliphatic GLS compared with wild-type roots (P < 0.05) (Figure 4A). In a similar manner, roots of qko grafts (wt/qko) contained LC aliphatic GLS levels similar to those of wild-type homografts (P > 0.05), whereas roots grafted onto gtr1 gtr2 (wt/gtr1 gtr2) contained significantly reduced levels of LC aliphatic GLS (P < 0.05) (Figure 4A). While gtr1 gtr2/qko grafts contained significantly reduced LC aliphatic GLS levels in the roots compared with wild-type homografts, these were in the same range as those detected in roots of the wt/qko grafts (P > 0.05) (Figure 4A). Heterografts consisting of a qko rosette grafted onto either wild-type or gtr1 gtr2 roots (qko/wt or qko/gtr1 gtr2, respectively) contained strongly reduced total amounts of LC aliphatic GLS, which was mainly due to reduced levels in the roots (Figure 4A). Thus, bidirectional transport of LC aliphatic GLS can occur but may not be dependent on GTR1 and GTR2. Noticeably, heterografts consisting of a wild-type rosette grafted onto gtr1 gtr2 roots (wt/gtr1 gtr2), or the reciprocal combination (gtr1 gtr2/wt), showed similar levels of LC aliphatic GLS to those of wild-type homografts in the rosette and roots (P > 0.05) (Figure 4A). In summary, these observations demonstrate that the rosette is the major site for synthesis and storage of SC aliphatic GLS.

### Table 2. GLS Content of Micrografted Plants with Impaired Biosynthesis in the Rosette or Roots

<table>
<thead>
<tr>
<th>Genotype (Rosette Roots)</th>
<th>GLS Biosynthesis</th>
<th>GLS Content of Entire Plant (% of the Wild Type)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aliphatic GLS</td>
<td>Indole GLS</td>
</tr>
<tr>
<td>wt/wt</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>qko/qko</td>
<td>3.5 (±2.6)**</td>
<td>nd</td>
</tr>
<tr>
<td>wt/qko</td>
<td>28.8 (±5.1)**</td>
<td>20.7 (±3.8)**</td>
</tr>
<tr>
<td>gtr1/gtr2</td>
<td>50.7 (±4.4)**</td>
<td>56.6 (±23.3)**</td>
</tr>
</tbody>
</table>

Plants with impaired GLS synthesis in roots or the rosette were constructed by reciprocal micrografting of the myb28 myb29 cyp79b2 cyp79b3 quadruple mutant (qko) and Col-0 wild type (wt). GLS content of the entire 3-week-old grafted plants was analyzed by LC-MS and related to levels found in the entire wild type. Numbers are averages, and SD is shown in parentheses. Two-tailed t test, *P < 0.05 and **P < 0.01 versus the wild type (n = 5 to 7). nd, none detected.
reduced levels when compared with wild-type homografts (Figure 4B). gtr1 gtr2 homografts overaccumulated LC aliphatic GLS in the rosette and showed a strong reduction in root levels when compared with the wild type (Figure 4B). This distribution was also observed in identically treated but nongrafted gtr1 gtr2 mutants (see Supplemental Figure 2 online). When analyzing heterografts, those consisting of a qko rosette grafted onto wild-type roots (wt/qko) or the reciprocal combination (qko/wt) contained reduced total amounts of LC aliphatic GLS ($P \leq 0.05$) (Figure 4A). This shows that, in contrast with SC aliphatic GLS, both the rosette and roots can contribute significantly to the synthesis of LC aliphatic GLS.

Furthermore, when comparing LC aliphatic GLS levels in heterografts consisting of wild-type or gtr1 gtr2 rosettes grafted onto qko roots (wt/qko and gtr1 gtr2/qko), LC aliphatic GLS accumulation in the roots was significantly higher in the wt/qko graft, demonstrating that endogenous GLS can move downward from rosette to roots in a GTR1- and GTR2-dependent manner (Figure 4B). Interestingly, in both wt/qko and qko/wt heterografts, LC aliphatic GLS accumulated to levels indistinguishable from wild-type homografts in the rosette, while a significant reduction was found in roots of both combinations ($P \leq 0.05$). This emphasizes that both the rosette and roots represent sinks for LC aliphatic GLS (Figure 4B). Strikingly, when qko or wild-type rosettes were grafted onto gtr1 gtr2 roots (qko/gtr1 gtr2 or wt/gtr1 gtr2), LC aliphatic GLS were found to overaccumulate in rosettes to a similar extent to that found in gtr1 gtr2 homografts ($P < 0.05$) (Figure 4B). This shows that LC aliphatic GLS can be transported upwards from roots to rosette but are retained in roots by a process that is dependent on the presence of GTR1 and GTR2 in the roots. Noticeably, in the qko/gtr1 gtr2 grafts, the total LC aliphatic GLS content was similar to that in wild-type homografts, in contrast with the significantly reduced total contents in all other heterografts containing qko tissues (Figure 4B).

**LC Aliphatic GLS Are in Vitro Substrates of GTR1 and GTR2**

To biochemically validate LC aliphatic GLS as substrates of GTR1 and GTR2, we compared uptake of 8MTO with that of the previously characterized in vitro substrate 4-methylthiobutyl GLS (4MTB) into Xenopus laevis oocytes. When accumulation of 4MTB in oocytes expressing GTR1 or GTR2 was set to 100%, both showed $\sim40\%$ uptake of 8MTO, while no accumulation was observed in uninjected oocytes (Figure 5). This ability of GTR1- or GTR2-expressing oocytes to take up 8MTO supports the in planta evidence for GTR1 and GTR2 as being involved in transport of LC aliphatic GLS.
Figure 4. Relative Content of GLS in Micrografted Arabidopsis Plants in the Vegetative Growth Phase.
Indole GLS Can Be Synthesized in Both Rosette and Roots and Can Be Transported Bidirectionally

When analyzing homografts for indole GLS, levels in both the rosettes and roots were below the detection limit in qko homografts, whereas homografted gtr1 gtr2 was found to contain levels similar to those found in the homografted wild type (P > 0.05) (Figure 4C). Indole GLS accumulated in all grafted qko organs, suggesting that these GLS can move bidirectionally between the rosette and roots (Figure 4C). Intriguingly, qko rosettes grafted to gtr1 gtr2 roots or the reciprocal combination (i.e., qko/gtr1 gtr2 or gtr1 gtr2/qko, respectively) showed no reduction in indole GLS accumulation when compared with qko organs grafted to the corresponding wild type (i.e., qko/wt or wt/qko, respectively) (P > 0.05) (Figure 4C). Furthermore, heterografts consisting of a wild-type rosette and qko roots (wt/qko) or the reciprocal combination (qko/wt) accumulated indole GLS in the respective qko organ (Figures 4A to 4C). In summary, this indicates that, in contrast with LC aliphatic GLS, distribution of indole GLS is not regulated by GTR1 and GTR2 at the vegetative stage. An overview of the individual GLS in all grafted and nongrafted rosettes and roots can be found in Supplemental Tables 1 to 4 online.

GTR1 and GTR2 Show Highest Expression in Cells Surrounding the Lateral Root Branching Points

To further investigate how GTR1 and GTR2 could be involved in retaining GLS in the roots, localization of GTR1-YFP (for yellow fluorescent protein) and GTR2-mOrange fusion proteins was investigated by confocal laser scanning microscopy. Root cross sections ~2 cm below the hypocotyl of 3-week-old plants showed GTR1-YFP and GTR2-mOrange to be expressed inside the stele (Figures 6F and 6I). As both GTR1 and GTR2 were found to have highest transcriptional activity in lateral root branching points (Figure 3), we further investigated the presence of GTR1-YFP and GTR2-mOrange in this area by cross-sectioning the main root in areas containing lateral root branching points. In these sections, both GTR1- and GTR2-specific signals were mainly detected in areas that appeared to be cortex cells surrounding the lateral root branching points (Figures 6E and 6H).

DISCUSSION

Arabidopsis Roots Contribute to GLS Production in the Vegetative Growth Phase

Investigations of GLS in various plant species have to a large extent focused on aboveground organs (Dam et al., 2008). Consequently, the ability of roots to synthesize GLS has hitherto received little attention. In Arabidopsis, an apparent low expression level of key biosynthetic genes in roots compared with leaves (Brady et al., 2007) has led to the general assumption that GLS are primarily made aboveground. Initially, and based on the proposed role of GTR1 and GTR2 in phloem loading of GLS in leaves (Nour-Eldin et al., 2012), we hypothesized that an overaccumulation of aliphatic GLS in the rosette of gtr1 gtr2 mutants at the vegetative stage (Figure 1) was caused by a decreased ability of this mutant to facilitate downward GLS transport. With the assumption that roots of Arabidopsis are incapable of GLS biosynthesis, this suggested that roots constitute a sink for aliphatic GLS transported through the phloem. This was strengthened by the observation that, when fed to a leaf, the aliphatic 2-propenyl GLS accumulated in roots of the wild type, but not of gtr1 gtr2 (Figure 2). However, the finding that Arabidopsis roots are capable of synthesizing both aliphatic and indole GLS (Table 2) contradicted the initial hypothesis and suggested that the source-sink relationship of the rosette and roots for GLS in vegetative Arabidopsis is more complex than originally assumed.

Phloem and Xylem as Transport Pathways for GLS

GLS are organic anions with a very low permeability coefficient (Brudenell et al., 1999). According to the Kleier model (Kleier, 1988), they have physicochemical properties consistent with mobility in both phloem and xylem (Brudenell et al., 1999). In previous experiments, radiolabeled p-OHB fed to leaves of Arabidopsis and rape (Brassica napus) was shown to follow the phloem pathway and accumulate in seeds and roots (Brudenell et al., 1999; Chen et al., 2001). We previously showed transport of GLS to seeds to be facilitated by GTR1 and GTR2 (Nour-Eldin et al., 2012). In this study, feeding of both an aliphatic (2-propenyl) and an aromatic (p-OHB) GLS to leaves of the gtr1 gtr2 mutant (Figure 2A; see Supplemental Figure 4 online) demonstrated that GTR1 and GTR2 are also essential for transport of GLS from rosette to roots in the phloem. Interestingly, feeding of GLS to roots of the wild type and gtr1 gtr2 resulted in upward transport to the rosette (Figure 2B; see Supplemental Figure 4 online). Given the directional flow of the vascular transport pathways, and the physiochemical properties of GLS, this transport is expected to occur either in the apoplastic matrix or in the ascending xylem sap. Regardless of the route, upwards translocation in the gtr1 gtr2 mutant suggests the presence of a GTR1- and GTR2-independent ability to translocate GLS from roots to the rosette. In wild-type plants, part of the root fed GLS accumulated in the roots, whereas in the gtr1 gtr2 mutant, this was only observed to a very low degree (Figure 2B; see Supplemental Figure 4 online). This might...
reflect a GTR1- and GTR2-dependent redistribution of GLS from the rosette to roots following the upward transport. However, in light of expression of GTR1 and GTR2 in the root vasculature (Figures 3 and 6), we propose that GTR1 and GTR2 are involved in removal of GLS from the xylem in roots. In support of this, a study aimed at investigating transcriptional pro

distinct above- and belowground source-sink relationships exist for SC and LC aliphatic GLS in vegetative Arabidopsis

SC aliphatic GLS constitute the majority of GLS in the vegetative rosette of Arabidopsis (Col-0) (Brown et al., 2003). Our grafting experiments indicate that SC aliphatic GLS are primarily made and stored in the rosette while the roots do not appear to serve as a major source or sink at this growth stage (Figure 4A). In comparison to SC aliphatic GLS, both the rosette and roots were observed to contribute considerably to LC aliphatic GLS production (Figure 4B). In support of this, transcriptional analyses have shown that MAM3 and CYP79F2, which are involved in LC aliphatic GLS biosynthesis, are indeed expressed in both above- and belowground organs, with highest expression in the roots (Chen et al., 2003; Tantikanjana et al., 2004; Textor et al., 2007; Redovnikovic et al., 2012). In contrast with SC aliphatic GLS, the major storage site for LC aliphatic GLS in vegetative Arabidopsis appears to be in the roots (Figure 4B; see Supplemental Figure 2 online), indicating that LC aliphatic GLS produced in rosettes might be transported to roots. In support of this, a similar GTR1 and GTR2 dependency as for downward 2-propieny GLS transport was observed for the LC aliphatic GLS (Figure 2A), which indicates that the movement of endogenous GLS from rosette to roots occurs through the phloem. In the presence of GTR1 and GTR2, LC aliphatic GLS are retained in the roots, whereas in the absence of functional GTR1 and GTR2 in the roots, the majority of these GLS are translocated via the xylem to the rosette (Figure 4B). This suggests that for upward movement of GLS from roots to the rosette, GTR1 and GTR2 activity in roots may represent a mechanism by which Arabidopsis regulates their distribution.

Integration of Biosynthesis and Transport in Plant Defenses?

It has previously been suggested that differences in above- and belowground GLS profiles are due to in situ biosynthesis rather than transport (van Dam, 2009). In this study, plants with impaired GLS synthesis in rosettes accumulated LC aliphatic GLS to only 35% of wild-type levels (Figure 4B, qko/wt). However, impaired GLS biosynthesis in the rosette combined with abolishment of GTR1 and GTR2 in roots (qko/gtr1 gtr2) led to LC aliphatic GLS levels in the entire plant indistinguishable from those of wild-type homografts. Thus, the GTR1- and GTR2-dependent retention of LC aliphatic GLS appears to inhibit their biosynthesis in roots. This is supported by the increased total level of aliphatic GLS in the ungrafted gtr1 gtr2 mutant (Figure 1C; see Supplemental Figure 1 online). These observations indicate that an unknown crosstalk between aliphatic GLS synthesis, transport, and storage exists in Arabidopsis.

In the example of nicotine, which is synthesized in roots and transported to leaves in the xylem (Shoji et al., 2000; Yazaki, 2005; Morita et al., 2009), root nicotine synthesis has been shown to increase in response to insect attack of the leaves. Several other compounds, including tropane alkaloids in Solanaceae species (Ziegler and Facchini, 2008) and pyrrolizidine alkaloids in Asteraceae (Hartmann and Ober, 2000), have been shown to follow a similar pattern of synthesis in roots followed by translocation to leaves. These examples have led to the appreciation that roots play important roles in the aboveground defensive mechanisms (Erb et al., 2009). Only LC and not SC aliphatic GLS seem to be primarily made in roots and distributed to other parts of the plant. Differences in chain length of aliphatic GLS have also been shown to play a role in both resistance toward the diamondback moth (Plutella xylostella) (Kroymann et al., 2003) and the relative abundance of two specialist aphid species (Züst et al., 2012). This illustrates that GLS chain lengths can be critical for survival of plants. In a study with the green peach aphid (Myzus persicae) on Arabidopsis, the LC aliphatic GLS 8-methylsulphonyloctyl was
observed to increase significantly in aphid-infested leaves without any changes in the systemically connected leaves (Kim and Jander, 2007). As the roots are a major site of LC aliphatic GLS storage, this suggests that LC aliphatic GLS may be translocated from roots to leaves upon aphid attack. In another study, however, transcriptional analysis of leaves infected with the pathogenic ascomycete S. sclerotiorum showed an increased expression of LC aliphatic GLS-specific genes in comparison to mock-treated leaves (Stotz et al., 2011). This suggests that Arabidopsis may also respond with in situ synthesis of LC aliphatic GLS. In light of our data, these studies give rise to the hypothesis that Arabidopsis responds to different biotic stresses by integrating both long-distance transport and in situ synthesis of LC aliphatic GLS. Under the assumption that SC and LC aliphatic GLS differ in their biological functions, the differences in major storage site and source-sink relationships for SC and LC aliphatic GLS highlight the notion that several different strategies may underlie the general flexibility in tissue-specific defense compound accumulation in response to interacting organisms.

A Model for Spatial Organization of GLS Synthesis and Long-Distance Transport between the Rosette and Roots

Based on our data, we propose a model for transport of GLS between the rosette and roots in the vegetative growth phase of Arabidopsis (Figure 7). According to the model, SC aliphatic GLS are primarily produced and stored in the rosette, while both the rosette and roots contribute to the synthesis and accumulation

Figure 6. Localization of Fluorescent GTR1 and GTR2 Protein Fusions in Roots.

Roots of 3-week-old transgenic lines expressing full-length genomic regions of GTR1 or GTR2 C-terminally fused to YFP or mOrange, respectively, under control of the 2-kb endogenous promoters used in Figure 3. (A), (D), and (G) show longitudinal optical sections along the xy axis of intact roots, while subfigures (B), (E), and (H) show cross sections of the main root in lateral root branching points. Subfigures (C), (F), and (I) show cross sections of the main root alone. LR, lateral root. Bars = 50 μm. (A) to (C) Wild-type overlay of YFP and mOrange channels. (D) to (F) YFP signal from a representative plant expressing GTR1-YFP. (G) to (I) GTR2-mOrange signal from a representative plant expressing GTR2-mOrange. Arrows point to areas with expression outside the stele.
of LC aliphatic and indole GLS (Figure 7A). While the mechanism underlying transport of indole GLS is currently unknown, the model proposes a complex source-sink relationship for LC aliphatic GLS where GTR1 and GTR2 are involved in obtaining organ-specific accumulation through long-distance transport in phloem as well as xylem. In roots, a GTR1- and GTR2-dependent import of aliphatic GLS from the apoplast is likely to occur into cells adjacent to xylem (Figure 7B), possibly into phloem cells as well as storage cells in the lateral root branching points, thereby attenuating upward xylem transport. The proposed model for GLS transport further suggests an unknown mechanism for export of GLS into the apoplast in roots. Apo, apoplast; CC, companion cell; Pd, plasmodesmata; SE, sieve element; Sym, symplast.

**METHODS**

**GLS Analysis**

GLS were analyzed as desulfo GLS as previously described (Kliebenstein et al., 2001b). The 96-well filter plates were charged with 45 mg of DEAE Sephadex A25 and 300 μL of water per well and equilibrated at room temperature for 2 h. The water was removed using a vacuum manifold (Millipore). Plant material was harvested in either 300 μL or 5 mL 85% methanol containing 50 μM allyl GLS (sinigrin) or p-hydroxybenzyl GLS (p-OHB) added to each sample as an internal standard before sample aliquots (20 μL) were applied to the filter plates and absorbed on the ion exchanger by vacuum filtration for 2 to 4 s. Sephadex material was washed with 2 × 100 μL 70% methanol (v/v) and 2 × 100 μL water and briefly centrifuged before addition of 20 μL of sulfatase solution (1.25 mg mL−1, sulfatase type H1; Sigma-Aldrich) on each filter. For analysis of growth medium, 50 mL medium was run through filters charged with 150 mg DEAE Sephadex A25 and 2 mL water per filter and equilibrated at room temperature for 2 h before addition of 500 μL sulfatase solution. After
incubation at room temperature overnight, desulfo GLS were eluted with 80 µL water for 96-well filter plates and 250 µL for media samples. Media sample elute was lyophilized and resuspended in 50 µL water. All samples were analyzed by HPLC on an Agilent HP1200 Series instrument equipped with a C-18 reversed phase column (Zorbax SB-Aq, 25-3 (4.6 mm), 5-µm particle size, Agilent) using a water (solvent A)-acetonitrile (solvent B) gradient at a flow rate of 1 mL min⁻¹ at 25°C (injection volume 45 µL). The gradient applied was as follows: 1.5 to 7% B (5 min), 7 to 25% (6 min), 25 to 80% (4 min), 80% B (3 min), 80 to 35% B (2 min), and finally 1.5% B (5 min). The eluent was monitored by diode array detection between 200 and 400 nm (2-nm interval). Desulfo GLS were identified based on comparison of retention times and UV absorption spectra with those of known standards (Brown et al., 2003) and quantified in relation to the internal standard. For grafted plants, the GLS content was measured by liquid chromatography-mass spectrometry (LC-MS) using an Agilent 1100 Series liquid chromatograph coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics). A Zorbax SB-C18 column (Agilent: 1.8 µm, 2.1 mm i.d. x 50 mm) was used at a flow rate of 0.2 mL/min, and the mobile phases were as follows: A, water with 0.1% (v/v) formic acid and 50 µM NaCl; B, acetonitrile with 0.1% (v/v) HCOOH. The gradient program was as follows: 0 to 0.5 min, isotropic 2%; B 0.5 to 7.5 min, linear gradient 2% to 40%; B 7.5 to 8 min, linear gradient 40% to 90%; B 8.5 to 11.5 min isotropic 90%; B 11.6 to 15 min, isotropic 2%. The flow rate was increased to 0.3 mL/min in the interval 11.2 to 13.5 min. The mass spectrometer was run in positive electrospray mode. The relative GLS content was determined by relating all extracted peaks to the average wild-type content.

**Xenopus laevis Oocyte Uptake Assay**

Oocytes were kept in a Kuli-based solution containing 90 mM NaGlucosinate, 1 mM KGlucosinate, 1 mM MgGlucosinate, 1 mM LaCl₃, and 10 mM MES, pH 5. For uptake assays, 8MTB or 4MTB to a total concentration of 100 µM was added to the solution. Solutions were adjusted to 220 mOsmol kg⁻¹ using D-sorbitol. After 1 h of incubation in medium containing GLS, the oocytes were washed and homogenized individually in 100 µL 10% SDS and analyzed for GLS content by LC-MS as described above. The content of 8MTB was normalized to the content of similar oocytes subjected to 4MTB uptake.

**GLS Feeding**

p-OHB GLS was extracted from seeds of white mustard (Sinapis alba) (SeedCom) as previously described (Thies, 1979; Zrybko et al., 1997). The seeds were extracted with 80% (v/v) methanol, and the extract was loaded onto a DEAE Sephadex A25 column from which intact GLS were eluted with 0.5 M K₂SO₄ containing 3% (v/v) isopropanol. The samples were concentrated to dryness and washed with 100% (v/v) ethanol and then with 100% (v/v) ethanol before resuspension in water. 2-propenyl GLS was obtained from Sigma-Aldrich (lot 121K7063). Feeding of 3-week-old GLS was obtained from Sigma-Aldrich (lot 121K7063). Feeding of Arabidopsis lines harboring expression of a NLS and GUS-GFP under control of 2 kb of either GTR1 or GTR2 promoters including the 5’ untranslated regions (Nour-Eldin et al., 2012) were submerged in a solution containing 0.5 mg/mL X-Glc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), 0.05% Triton X-100, 20% (v/v) methanol, 50 mM NaH₂PO₄, pH 7.5, 10 mM K₃[Fe(CN)₆], 10 mM K₄[Fe(CN)₆], 1 M NaCl; B, acetonitrile with 0.1% (v/v) HCOOH. The gradient was as follows: 0 to 0.5 min, isotropic 2%; B 0.5 to 7.5 min, linear gradient 2% to 40%; B 7.5 to 8 min, linear gradient 40% to 90%; B 8.5 to 11.5 min isotropic 90%; B 11.6 to 15 min, isotropic 2%. The flow rate was increased to 0.3 mL/min in the interval 11.2 to 13.5 min. The mass spectrometer was run in positive electrospray mode. The relative GLS content was determined by relating all extracted peaks to the average wild-type content.

**Arabidopsis Lines**

**Cellular Localization**

**Plant Growth Conditions**

**Statistical Data Analysis**

To represent the change of GLS levels in grafted plants, levels in all grafted combinations were calculated as a percentage of the levels found in wild-type homografts grown under identical conditions. Significance levels of GLS differences were tested by performing a one-way analysis of variance (ANOVA). To achieve normal distributed data, a log₁₀ transformation was performed. The obtained data contain plants from at least three independent grafting experiments. Two-tailed Student's t tests were used to identify the variation in the GLS content between the wild type and mutants in all other analyses. One-way ANOVA analysis was done using Sigmaplot for Windows version 11 (www.systat.com), and Student’s t tests were performed using Microsoft Excel 2010.
Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At1g62500, Cortex specific transcript (pCO2); At1g39950 (CYP79B2); At2g22330 (CYP79B3); At1g09750 Endopeptidase (pPFP); At1g79840 GLABRA2 (pGL2); At3g47960 (GTR1); At5g62680 (GTR2); At5g91420 (MYB28); At5g07690 (MYB29); At3g54220 SCARECROW (pSCR); At4g37650 SHORTROOT (pSHR); At1g22710 Sucrose transporter2 (pSULTR2;2).

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

Supplemental Figure 1. Glucosinolate Content in Soil-Grown 3-Week-Old gtr1 gtr2 Mutants.

Supplemental Figure 2. Glucosinolate Content in Hydroponically Grown gtr1 gtr2 Mutants.

Supplemental Figure 3. Tissue Weights of Hydroponically Grown 3-Week-Old gtr1 gtr2 Mutants.

Supplemental Figure 4. In Silico Microarray-Based Expression Profile of GTR1 and GTR2 in Roots.

Supplemental Figure 5. Translocation of p-Hydroxybenzyl Glucosinolate Administered to Leaves and Roots of Arabidopsis in the Vegetative Growth Phase.

Supplemental Table 1. Glucosinolate Content in Rosettes of Micrografted Plants.

Supplemental Table 2. Glucosinolate Content in Roots of Micrografted Plants.

Supplemental Table 3. Glucosinolate Content in Rosettes of Micrografted and Ungrafted Plants.

Supplemental Table 4. Glucosinolate Content in Roots of Micrografted and Ungrafted Plants.

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