Elucidating the Role of Transport Processes in Leaf Glucosinolate Distribution[1][C][W][OPEN]

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In Arabidopsis (*Arabidopsis thaliana*), a strategy to defend its leaves against herbivores is to accumulate glucosinolates along the midrib and at the margin. Although it is generally assumed that glucosinolates are synthesized along the vasculature in an Arabidopsis leaf, thereby suggesting that the margin accumulation is established through transport, little is known about these transport processes. Here, we show through leaf apoplastic fluid analysis and glucosinolate feeding experiments that two glucosinolate transporters, GTR1 and GTR2, essential for long-distance transport of glucosinolates in Arabidopsis, also play key roles in glucosinolate allocation within a mature leaf by effectively importing apoplastically localized glucosinolates into appropriate cells. Detection of glucosinolates in root xylem sap unambiguously shows that this transport route is involved in root-to-shoot glucosinolate allocation. Detailed leaf dissections show that in the absence of GTR1 and GTR2 transport activity, glucosinolates accumulate predominantly in leaf margins and leaf tips. Furthermore, we show that glucosinolates accumulate in the leaf abaxial epidermis in a GTR-independent manner. Based on our results, we propose a model for how glucosinolates accumulate in the leaf margin and epidermis, which includes symplasmic movement through plasmodesmata, coupled with the activity of putative vacuolar glucosinolate importers in these peripheral cell layers.

Feeding behavior of herbivorous insects and distribution of defense compounds in plants have been suggested to be a result of an arms race between plants and insects that has spanned millions of years (Ehrlich and Raven, 1964). Whether insects adapted first to plants or the other way around is an ongoing debate in this research field (Schoonhoven et al., 2005; Ali and Agrawal, 2012). Leaf margin accumulation of defense compounds has been demonstrated in various plant species (Gutterman and Chauser-Volfson, 2000; Chauser-Volfson et al., 2002; Kester et al., 2002; Cooney et al., 2012). In the model plant Arabidopsis (*Arabidopsis thaliana*), higher concentration of glucosinolates, which constitute a major part of the chemical defense system in this plant (Kliebenstein et al., 2001a; Halkier and Gershenzon, 2006), was found at the leaf midrib and margins compared with the leaf lamina (Shroff et al., 2008; Sønderby et al., 2010). This nonuniform leaf distribution of glucosinolates appeared to explain the feeding pattern of a generalist herbivore (*Helicoverpa armigera*), as it avoided feeding at the leaf margin and midrib (Shroff et al., 2008). A similar feeding pattern on Arabidopsis was observed for a different generalist herbivore, *Spodoptera littoralis* (Schweizer et al., 2013). Interestingly, *S. littoralis* was shown to favor feeding from Arabidopsis leaf margins in glucosinolate-deficient mutants (Schweizer et al., 2013), which could indicate an inherent preference for margin feeding and that Arabidopsis adapted to such behavior by accumulating defense compounds here. A damaged leaf margin may be more critical for leaf stability than damage to inner leaf parts (Shroff et al., 2008), further motivating protection of this tissue. The margin-feeding preference of *S. littoralis* might be explained by better nutritional value of the leaf margin cells (Schweizer et al., 2013), which has been shown to consist of specialized elongated cell files (Koroleva et al., 2010; Nakata and Okada, 2013).

Other distribution patterns have been reported for glucosinolates in an Arabidopsis leaf. A study investigating spatiotemporal metabolic shifts during senescence in Arabidopsis reported that fully expanded mature leaves exhibited a glucosinolate gradient from base to tip, with highest level of glucosinolates at the leaf base (Watanabe et al., 2013). In contrast to the horizontal plane, less has been reported on distribution of glucosinolates in the vertical plane of a leaf. A localization study of cyanogenic glucosides, defense molecules related to glucosinolates (Halkier and Gershenzon, 2006), determined that these compounds primarily were located in the epidermis of sorghum (*Sorghum bicolor*; Kojima

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et al., 1979). Whereas epidermis-derived trichomes in Arabidopsis were recently demonstrated to contain glucosinolates and to express glucosinolate biosynthetic genes (Frerigmann et al., 2012), no studies have investigated glucosinolates in the epidermal cell layer.

Based on promoter-GUS studies, biosynthesis of glucosinolates in leaves of Arabidopsis has been associated with primarily major and minor veins in leaves and silique walls (Mikkelsen et al., 2000; Reintanz et al., 2001; Tantikanjana et al., 2001; Chen et al., 2003; Grubb et al., 2004; Schuster et al., 2006; Gigolashvili et al., 2007; Li et al., 2011; Redovniković et al., 2012). The discrepancy between vasculature-associated glucosinolate biosynthesis and margin accumulation of glucosinolates suggests that transport processes must be involved in establishing the distribution pattern of glucosinolates within a leaf.

Plant transport systems include the apoplastic xylem, the symplastic phloem, and plasmodesmata. Xylem transport is mainly driven by an upward pull generated by transpiration from aerial plant organs, thereby directing transport to sites of rapid evaporation (such as leaf margins; Sattelmacher, 2001). Phloem flow is facilitated by an osmosis-regulated hydrostatic pressure difference between source and sink tissue, primarily generated by Suc bulk flow (Lucas et al., 2013). Plasmodesmata are intercellular channels that establish symplasmic pathways between neighboring cells, and most cell types in a plant are symplastically connected via plasmodesmata (Roberts and Oparka, 2003). Translocation of small molecules in these channels is driven by diffusion and is regulated developmentally as well as spatially to form symplastically connected domains (Roberts and Oparka, 2003; Christensen et al., 2009). To what extent any of these transport processes are involved in establishing specific distribution patterns of glucosinolates within leaves is not known.

Recently, two plasma membrane-localized, glucosinolate-specific importers, GLUCOSINOLATE TRANSPORTER1 (GTR1) and GTR2, were identified in Arabidopsis (Nour-Eldin et al., 2012). In leaf, their expression patterns were shown to be in leaf veins (GTR1 and GTR2) and surrounding mesophyll cells (GTR1; Nour-Eldin et al., 2012). Absence of aliphatic and indole glucosinolates in seeds of the *gtr1gtr2* double knockout (dKO) mutant (*gtr1gtr2* dKO) demonstrated that these transporters are essential for long-distance glucosinolate transport to the seeds and indicates a role in phloem loading (Nour-Eldin et al., 2012). Another study investigating long-distance transport of glucosinolates in the 3-week-old wild type and *gtr1gtr2* dKO indicated that GTR1 and GTR2 were involved in bidirectional transport of aliphatic glucosinolates between root and shoot via both phloem and xylem pathways (Andersen et al., 2013). The authors suggested a role for GTR1 and GTR2 in the retention of long-chained aliphatic glucosinolates in roots by removing the compounds from the xylem (Andersen et al., 2013).

Identification of the glucosinolate transporters GTR1 and GTR2 has provided a molecular tool to investigate the role of transport processes in establishing leaf glucosinolate distribution. In this study, we have performed a detailed spatial investigation of the distribution of an exogenously fed glucosinolate (sinigrin) and endogenous glucosinolates within mature wild-type and *gtr1gtr2* dKO Arabidopsis leaves, achieved by collecting and analyzing leaf parts at the horizontal (y axis: petiole, base, and tip; x axis: midrib, lamina, and margin) as well as at the vertical leaf plane (z axis: abaxial epidermis). Furthermore, we analyze wild-type and *gtr1gtr2* dKO root xylem sap and leaf apoplastic fluids for glucosinolates. Based on our results, we propose a model where GTR1 and GTR2 import glucosinolates from the apoplast to the symplast and where the glucosinolate distribution pattern within an Arabidopsis leaf is established via symplasmic movement of glucosinolates through plasmodesmata, coupled with the activity of putative vacuolar glucosinolate importers in peripheral cell layers.

RESULTS

Glucosinolates are amino acid-derived compounds, and in Arabidopsis, they are mainly derived from Met (aliphatic glucosinolates) and Trp (indole glucosinolates). During biosynthesis, the side chain of Met undergoes a different number of rounds of chain elongation, leading to both short-chained (with three to five methylene groups added, C3–C5) and long-chained (with six to eight methylene groups added, C6–C8) aliphatic glucosinolates (Grubb and Abel, 2006). When describing leaf dissection results, we group glucosinolates into total, aliphatic, and indole glucosinolates. Additionally, aliphatic glucosinolates are further grouped into short-chained and long-chained glucosinolates, with 4-methylsulfinylbutyl (4-MSB) and 8-methylsulfinyloctyl (8-MSO) being the prominent aliphatic glucosinolates in the respective groups.

Are GTR1 and GTR2 Required for Leaf Margin Accumulation of Glucosinolates?

To investigate whether GTR1 and GTR2 are required to establish glucosinolate accumulation in the leaf margin, similar mature leaves were dissected into midrib, margins, and lamina, and each part was analyzed for glucosinolates (Fig. 1). In wild-type leaves, aliphatic glucosinolates were present in equal concentrations in midrib and lamina, whereas accumulation in the margin was significantly higher (approximately 2-fold). The concentration of aliphatic glucosinolates in leaf margins was not reduced in leaves from *gtr1gtr2* dKO plants compared with leaves from wild-type plants. On the contrary, the concentration of aliphatic glucosinolates in *grt1gtr2* dKO leaf margins was increased by a factor of six compared with wild-type leaf margins (Fig. 1). This increase was due to short-chain but mainly highly elevated levels of long-chain aliphatic glucosinolates in *gtr1gtr2* dKO margins. Within *gtr1gtr2* dKO leaves, aliphatic

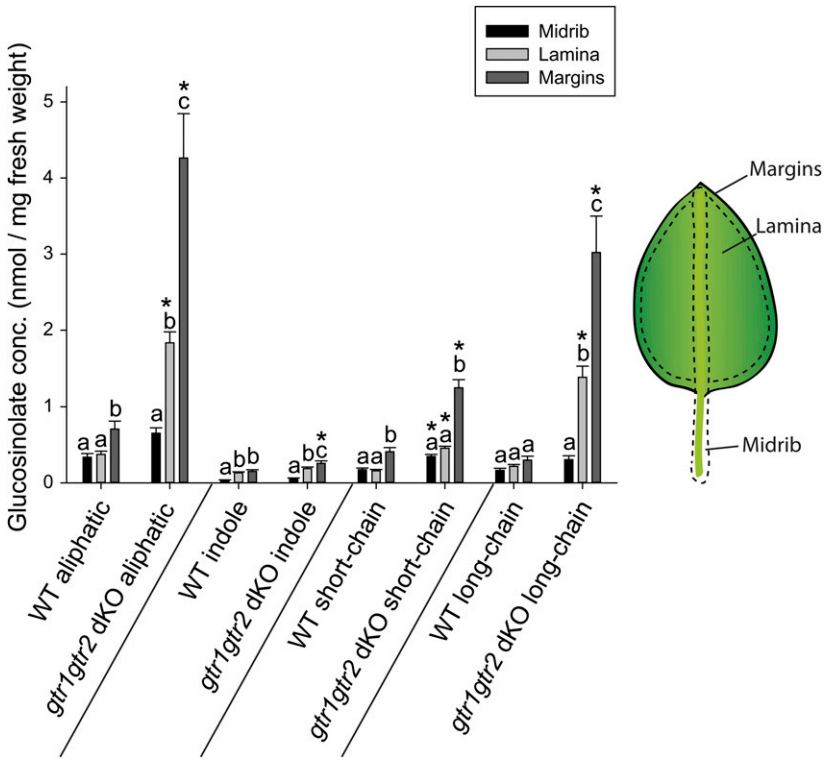

Figure 1. Glucosinolate concentration in mature Arabidopsis wild-type (WT) and *gtr1gtr2* dKO mutant leaves dissected into midrib, lamina, and margins. Glucosinolates are grouped into total, aliphatic, indole, short-chained aliphatic (C3–C5), and long-chained aliphatic (C6–C8) glucosinolates. Dashed lines on the leaf cartoon to the right depict dissected leaf parts. For individual glucosinolate data, see Supplemental Figure S1. Error bars are SE ($n = 5$). Letters above grouped columns indicate statistically significant differences within dissected leaves (one-way ANOVA, $P < 0.05$). Asterisk indicates statistically significant differences of *gtr1gtr2* dKO leaf parts compared with equivalent wild-type leaf parts (two-tailed Student's *t* test, $P < 0.05$). [See online article for color version of this figure.]

glucosinolates were strongly allocated to the margin compared with lamina (approximately 2.5-fold increase) and midrib (approximately 6.5-fold increase). The concentration of indole glucosinolates was similar between wild-type and *gtr1gtr2* dKO leaves (Fig. 1). In wild-type leaves, indole glucosinolate concentration was significantly lower in the midrib compared with lamina and margins, which accumulated indole glucosinolates equally. In *gtr1gtr2* dKO leaves, indole glucosinolates accumulated to a significantly higher concentration in the leaf margins compared with midrib and lamina. Analyses of individual glucosinolates showed that leaf margin accumulation in wild-type and *gtr1gtr2* dKO leaves was reflected in all glucosinolates (except for 5-methylsulfinylpentyl in the wild type), with 4-MSB and 8-MSO being the dominant glucosinolates at the margin (Supplemental Fig. S1). Interestingly, one glucosinolate, 4-methylthiobutyl glucosinolate (4-MTB), showed a very strong margin allocation in both wild-type and *gtr1gtr2* dKO leaves. The accumulation of glucosinolates at leaf margins in the *gtr1gtr2* mutant clearly showed that GTR1 and GTR2 are not required for allocation of glucosinolates to the leaf margin.

Higher Glucosinolate Levels in Leaf Base Is Dependent on GTR1 and GTR2

Next, we investigated whether GTR1 and GTR2 play a role in the longitudinal glucosinolate leaf distribution, where glucosinolate concentrations are reported to be higher at the base of the leaf lamina (Watanabe et al., 2013). Mature leaves of the wild type and *gtr1gtr2* dKO were dissected transversely into petiole, base, and tip sections and analyzed for glucosinolates (Fig. 2; Supplemental Fig. S2). In the wild type, aliphatic glucosinolates were evenly concentrated in the different leaf parts, with a tendency toward a higher concentration at the leaf base. In *gtr1gtr2* dKO leaves, a significant concentration gradient for aliphatic glucosinolates was seen, with highest concentration in leaf tip, intermediate concentration in base, and lowest concentration in petiole (Fig. 2). The concentration of aliphatic glucosinolates in *grt1gtr2* dKO leaf tip and base were increased by a factor of 11 and five compared with equivalent wild-type leaf parts, respectively. For indole glucosinolates in both genotypes, the concentration was not different between base and tip but was significantly lower in the petiole (Fig. 2). These observations suggest that GTR1 and GTR2 are involved in retention of aliphatic glucosinolates at the base of mature Arabidopsis leaves.

Glucosinolates Accumulate in Abaxial Epidermis Cells of Leaves

Epidermis-derived trichomes have been shown to contain glucosinolates and supposedly also the glucosinolate biosynthetic machinery (Frerigmann et al., 2012), but it is currently not known whether the Arabidopsis leaf epidermis contains glucosinolates. As GTR1 has been localized to the epidermis (Nour-Eldin et al., 2012), it is plausible that this tissue could represent a GTR1-dependent glucosinolate sink within an Arabidopsis leaf. By using a tape sandwich method, we

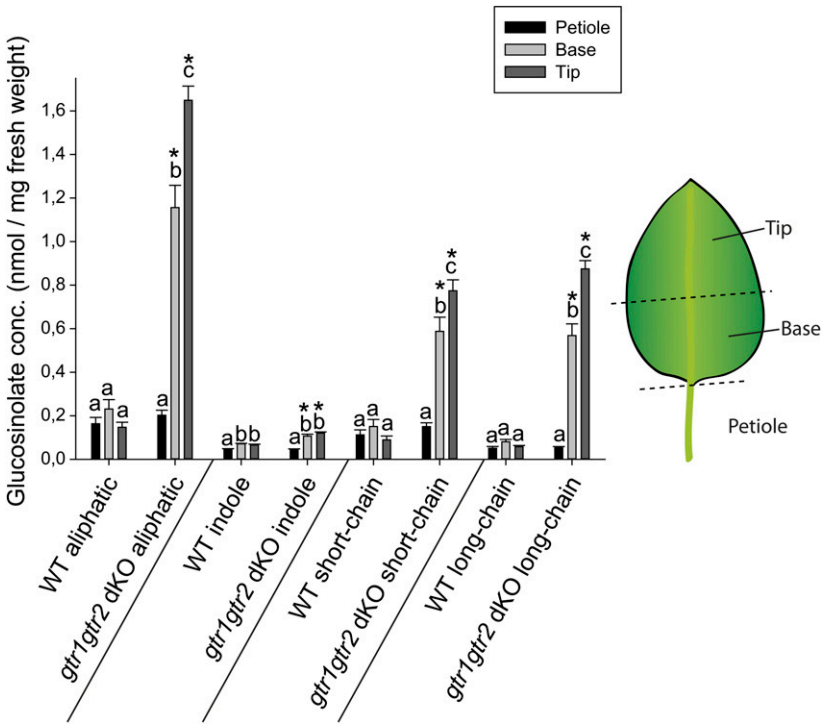

Figure 2. Glucosinolate concentration in mature Arabidopsis wild-type (WT) and *gtr1gtr2* dKO mutant leaves dissected into petiole, base, and tip. Glucosinolates are grouped into total, aliphatic, indole, short-chained aliphatic (C3–C5), and long-chained aliphatic (C6–C8) glucosinolates. Dashed lines on the leaf cartoon to the right depict dissected leaf parts. For individual glucosinolate data, see Supplemental Figure S2. Error bars are SE ($n = 5$). Letters above grouped columns indicate statistically significant differences within dissected leaves (one-way ANOVA, $P < 0.05$). Asterisk indicates statistically significant differences of *gtr1gtr2* dKO leaf parts compared with equivalent wild-type leaf parts (two-tailed Student's *t* test, $P < 0.05$). [See online article for color version of this figure.]

separated the abaxial (lower) epidermis from the remaining mature leaf and analyzed the two parts for glucosinolates (Fig. 3; Supplemental Fig. S3). Epidermis peels from both wild-type and *gtr1gtr2* dKO leaves contained glucosinolates, with an approximated 2.5-fold increase in aliphatic glucosinolate concentration in abaxial epidermis from *gtr1gtr2* dKO compared with the wild type. Short-chained aliphatic glucosinolate concentration was higher (only significant for *gtr1gtr2* dKO) in the epidermis compared with the remaining leaf part for both genotypes, and particularly, the 4-MTB concentration was increased in the epidermis compared with remaining leaf in the wild type (approximately 8-fold) and especially in the *gtr1gtr2* dKO (approximately 38-fold; Supplemental Fig. S3). For indole glucosinolates, both genotypes exhibited approximately 3-fold higher concentration in the epidermis compared with remaining leaf (Fig. 3), primarily due to a 4- to 5-fold higher concentration of indol-3-ylmethyl glucosinolate (I3M) in the epidermis relative to remaining leaf (Supplemental Fig. S3). Our data suggest that GTR1 and GTR2 are not required for transporting glucosinolates into the abaxial leaf epidermis.

Aliphatic Glucosinolates Accumulate in Apoplastic Fluids of the *gtr1gtr2* dKO Mutant

It has previously been reported that glucosinolates have physical properties consistent with mobility in both phloem and xylem (Brudenell et al., 1999). In this study, we measured the levels of endogenous glucosinolates in the root xylem sap and investigated whether there were any differences in glucosinolate levels between the wild type and *gtr1gtr2* dKO. Analysis of collected xylem sap for glucosinolates showed that xylem from both the wild type and *gtr1gtr2* dKO contained aliphatic but no indole glucosinolates and that the total glucosinolate concentration was approximately 4-fold higher in the *gtr1gtr2* dKO (Fig. 4A). Interestingly, when grouping into short- and long-chained aliphatic glucosinolates, xylem sap from the *gtr1gtr2* dKO mutant contained approximately 6-fold more long-chained glucosinolates than wild-type xylem (especially 8-methylthiooctyl glucosinolate [8-MTO]), whereas short-chained glucosinolates were strongly reduced (approximately 20-fold) when compared with the wild type. The observation that root xylem sap of *gtr1gtr2* dKO contained a high content of long-chained glucosinolates compared with the wild type prompted us to analyze if the leaf apoplastic fluid would be composed of a similar profile. Hydathodes located at leaf margins secrete guttation drops believed to comprise contents from the xylem and the leaf apoplasm (Pilot et al., 2004). We collected guttation drops from wild-type and *gtr1gtr2* dKO rosettes and analyzed glucosinolate content (Fig. 4B). In wild-type leaves, very low levels of glucosinolates were detected in guttation drops, suggesting that glucosinolates are removed from the leaf apoplast in the wild type. By contrast, *gtr1gtr2* dKO guttation drops exhibited a strongly increased concentration of aliphatic glucosinolates compared with the wild type. Indole glucosinolates (I3M) could only be detected in trace amounts for both genotypes. Interestingly, *gtr1gtr2* dKO guttation drops included high levels of not only long-chained aliphatic glucosinolates (primarily 8-MSO), but also short-chained aliphatic glucosinolates (mainly 4-MSB; Fig. 4B). In addition, we collected wild-type and *gtr1gtr2* dKO leaf apoplastic fluid by vacuum infiltration

and analyzed for glucosinolates (Fig. 4C). As in guttation drops, the concentration of aliphatic glucosinolates (8-MSO and 4-MSB) was increased approximately 50-fold in the apoplastic fluid from *gtr1gtr2* dKO compared with the wild type.

To determine if the increase in absolute total aliphatic glucosinolate concentration in *gtr1gtr2* dKO mutant leaves compared with the wild type could be accounted for by the elevated content in the apoplast, we analyzed the remaining glucosinolates in leaves from which apoplastic fluid had been extracted. Glucosinolate content in the apoplastic fluid only accounted for about 1.5% and 0.1% of total leaf content in, respectively, the *gtr1gtr2* dKO and wild type (Table I). We dissected leaves from which apoplastic fluid had been extracted and analyzed leaf parts for glucosinolates (Supplemental Fig. S4). As expected, based on the low glucosinolate content in apoplastic fluid compared with total leaf content, glucosinolate distribution in leaves extracted for apoplastic fluid was similar to non-extracted leaves.

Petiole Feeding of an Exogenous Glucosinolate Shows Distinct Leaf Distribution Patterns in the Wild Type and *gtr1gtr2* dKO

To examine the role of transport processes in distribution of glucosinolates in mature leaves of the wild

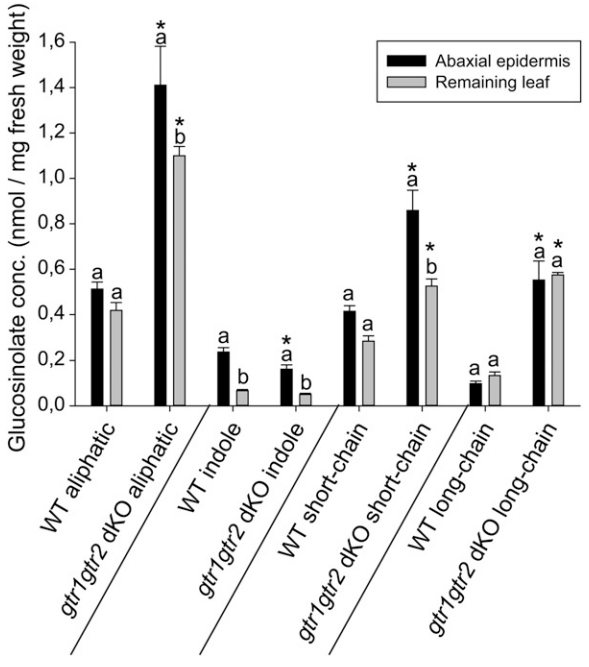

Figure 3. Glucosinolate concentration in mature Arabidopsis wild-type (WT) and *gtr1gtr2* dKO mutant leaves dissected into abaxial epidermis and remaining leaf parts. Glucosinolates are grouped into total, aliphatic, indole, short-chained aliphatic (C3–C5), and long-chained aliphatic (C6–C8) glucosinolates. For individual glucosinolate data, see Supplemental Figure S3. Error bars are SE ($n = 5$). Letters above pairwise columns indicate statistically significant differences within dissected leaves (two-tailed Student's *t* test, $P < 0.05$). Asterisk indicates statistically significant differences of *gtr1gtr2* dKO leaf parts compared with equivalent wild-type leaf parts (two-tailed Student's *t* test, $P < 0.05$).

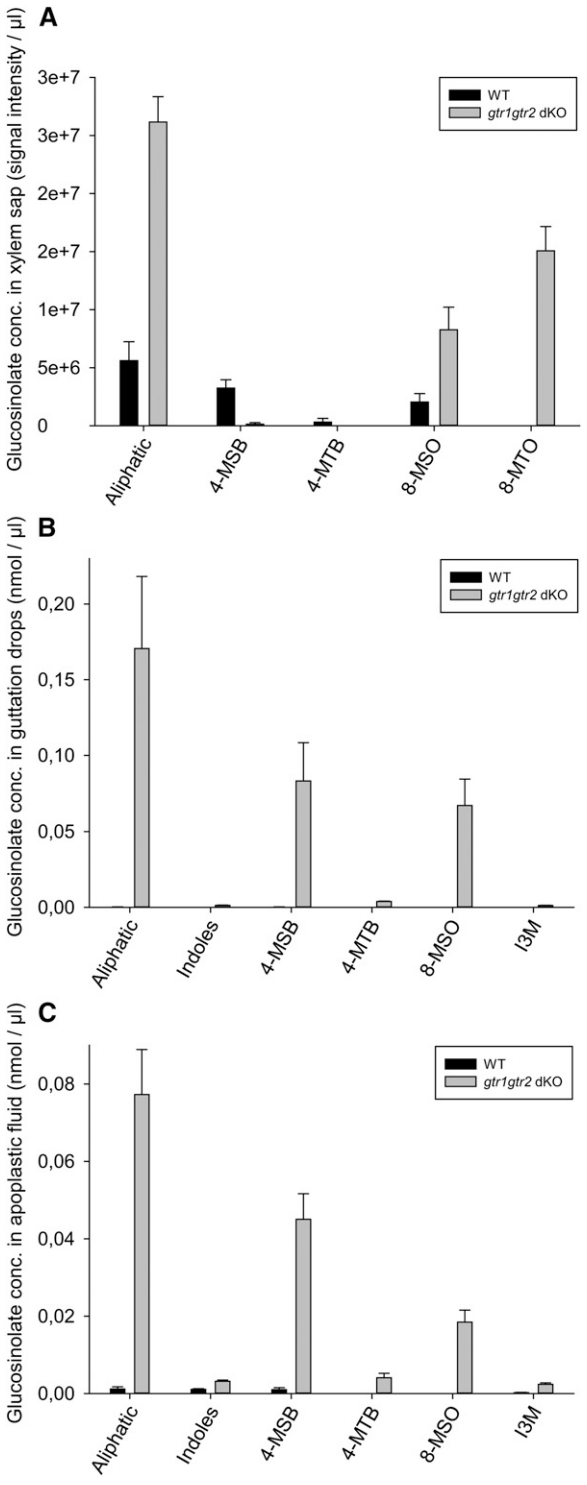

Figure 4. Glucosinolate concentration in root xylem sap, guttation drops, and leaf apoplastic fluid from Arabidopsis wild-type (WT) and *gtr1gtr2* dKO mutant plants. A, Liquid chromatography-mass spectrometry (LCMS) analysis of glucosinolates from root xylem sap. B, HPLC analysis of glucosinolates in guttation drops from mature (nonbolting) Arabidopsis rosettes. C, HPLC analysis of glucosinolates in leaf apoplastic fluid from mature leaves. Error bars are SE ($n = 4–8$).

Table I. *Glucosinolate content in wild-type and gtr1gtr2 dKO leaves and leaf apoplastic fluid*

Bold values emphasize the contribution of leaf apoplast glucosinolates to total leaf glucosinolates (glucosinolate content in extracted leaves plus glucosinolate content in leaf apoplastic fluid). Control leaves represent leaves not extracted for apoplastic fluid. SD are shown ($n = 5$).

Sample	Leaf Weight[a]	SD	Volume of Apoplastic Fluid	SD	Glucosinolate Content	SD	Total Leaf Glucosinolates	SD
	mg		*μl*		*nmol*		%	
Wild type extracted leaves	154.11	13.16	—	—	80.16	26.79	99.88	0.10
Wild type leaf apoplastic fluid	—	—	37.00	6.81	0.08	0.07	**0.12**	0.10
gtr1gtr2 dKO extracted leaves	161.58	22.33	—	—	181.76	27.97	98.52	0.52
gtr1gtr2 dKO leaf apoplastic fluid	—	—	35.38	8.45	2.74	1.09	**1.48**	0.52
Wild type control leaves	150.60	10.38	—	—	64.27	12.01	—	—
gtr1gtr2 dKO control leaves	135.92	14.06	—	—	176.51	45.59	—	—

[a]Values refer to the weight of nontreated leaves (not infiltrated or centrifuged; see "Materials and Methods").

type and *gtr1gtr2* dKO, we petiole fed leaves with the exogenous glucosinolate sinigrin. After 24 h of feeding, we dissected leaves and analyzed parts for sinigrin. Wild-type and *gtr1gtr2* dKO leaves accumulated similar amounts of sinigrin (approximately 500 nmol; Supplemental Fig. S5). In wild-type leaves, sinigrin mainly accumulated in petiole (approximately 20%), midrib (approximately 30%), and lamina (approximately 40%), whereas margins contained less than 10% (Fig. 5). By contrast, in *gtr1gtr2* dKO leaves, sinigrin accumulated primarily in tip margins (approximately 40%) and tip lamina (approximately 35%). The distribution of endogenous glucosinolates in sinigrin-fed leaves (data not shown) was similar to the distribution shown for leaves extracted for apoplastic fluid (Supplemental Fig. S4).

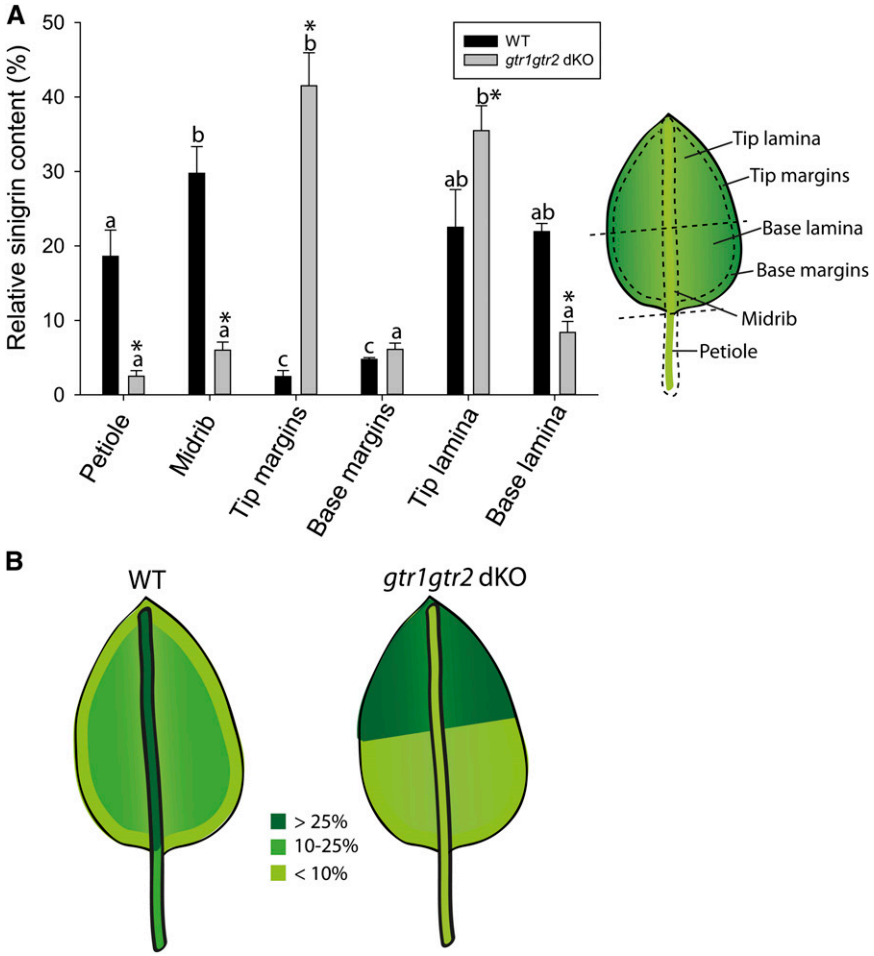

Figure 5. Distribution of petiole-fed sinigrin in mature Arabidopsis wild-type (WT) and *gtr1gtr2* dKO leaves. A, Detached leaves were petiole fed with sinigrin for 24 h and dissected into petiole, midrib, tip margins, base margins, tip lamina, and base lamina (depicted by dashed lines on the leaf cartoon to the right). No genotype difference in total sinigrin uptake was seen (Supplemental Fig. S5). Data are expressed as relative sinigrin content, i.e. as the percentage of sinigrin in dissected leaf parts relative to total leaf sinigrin. Error bars are SE ($n = 5$). Letters above genotype-specific columns indicate statistically significant differences within dissected leaves (one-way ANOVA, $P < 0.05$). Asterisk indicates statistically significant differences of *gtr1gtr2* dKO leaf parts compared with equivalent wild-type leaf parts (two-tailed Student's *t* test, $P < 0.05$). B, Simplified illustration of the relative sinigrin distribution from A. Dark green represents more than 25% of total leaf sinigrin content, green represents 10% to 25% of total leaf sinigrin content, and light green represents less than 10% of total leaf sinigrin content.

DISCUSSION

GTR1 and GTR2 Are Not Required for Allocation of Glucosinolates to the Leaf Margin

Within-leaf distribution of defense compounds is an important part of plants' strategy to fight against insect herbivory. In Arabidopsis, glucosinolates accumulate along the midrib and at the margin (Shroff et al., 2008; Sønderby et al., 2010). A given distribution pattern reflects a combination of biosynthesis and transport processes. As transcriptional evidence suggests that glucosinolates are synthesized along the vasculature (for review, see Grubb and Abel, 2006), it was initially hypothesized that loss of GTR1 and GTR2 would lead to a reduction in glucosinolates at the margin in the transporter mutant. By contrast, we observed a stronger margin accumulation in *gtr1gtr2* dKO leaves than in the wild type (Fig. 1), from which we conclude that the GTRs are not required for transport of glucosinolates to the leaf margin. This prompted us to investigate which process(es) and compartments were involved in distribution of glucosinolates within a mature Arabidopsis leaf.

Glucosinolates Are Transported from Root to Shoot via the Xylem

To understand how the leaf distribution of glucosinolates is established, it is important to identify the sources that contribute to the distribution profile. Recently, it was hypothesized that glucosinolates, in addition to being transported through the phloem, could move from root to shoot via the xylem (Andersen et al., 2013). Here, we measured the presence of glucosinolates directly in root xylem sap of both the wild type and the *gtr1gtr2* dKO and found that the levels and composition of glucosinolates were very different between the two genotypes (Fig. 4A). In xylem sap of the *gtr1gtr2* dKO, an approximately 12-fold increase in concentration of long-chained aliphatic glucosinolates, and in particular 8-MTO, was observed compared with wild-type xylem sap, which is also a group of glucosinolates accumulating to high levels in mature leaves of the *gtr1gtr2* dKO compared with the wild type (Figs. 1 and 2). This suggests that a xylem-mediated flow of long-chained aliphatic glucosinolates from root to shoot is responsible for the overaccumulation of these glucosinolates in the mature leaves of the *gtr1gtr2* dKO, as has been proposed in a previous study where a buildup of 8-MSO in rosettes of 3-week-old *gtr1gtr2* dKO was accompanied by a reduction of long-chained glucosinolates in roots of the transporter mutant (Andersen et al., 2013). The accumulation of 8-MSO rather than 8-MTO in leaves suggests that 8-MTO was oxidized to 8-MSO along the vascular route from root to shoot, which fits with the previously reported localization of FLAVIN MONOOXYGENASE GLUCOSINOLATE *S*-OXYGENASES ($FMO_{GS\text{-}OX1\text{-}5}$; Li et al., 2008, 2011).

The glucosinolates found in root xylem of the wild type were primarily the short-chained aliphatic glucosinolates, which were barely detectable in *gtr1gtr2* dKO. Assuming that short-chained aliphatic glucosinolates are mainly produced in the shoot, this difference in xylem glucosinolate composition suggests that in the wild type, short-chained aliphatic glucosinolates are loaded onto the phloem in a GTR-dependent manner in source shoot tissue and reach the root via the phloem, from where these can be recycled back to the shoot through the xylem pathway. Such recycling has been established for various nutrients in earlier studies (Marschner et al., 1996). In the *gtr1gtr2* dKO, shoot-produced short-chained glucosinolates may not be loaded onto the phloem destined for the root, and consequently, these are not present in the root for recycling to the shoot via the xylem.

Glucosinolates Accumulate in Apoplastic Fluid and Guttation Drops of *gtr1gtr2* dKO But Not Wild-Type Leaves

The plasma membrane localization of the glucosinolate importers GTR1 and GTR2 (Nour-Eldin et al., 2012) indicates that glucosinolates must occur in the apoplast. When leaf apoplastic fluids and guttation drops were analyzed for glucosinolates (Fig. 4, B and C), wild-type samples were practically devoid of these, which suggests that GTR1- and GTR2-mediated removal of glucosinolates from the apoplast is very effective and that glucosinolates are primarily stored inside cells within the leaf. For *gtr1gtr2* dKO, leaf apoplastic fluid and guttation drops contained high levels of both long-chained aliphatic and short-chained glucosinolates (primarily 4-MSB) compared with the wild type. As the *gtr1gtr2* dKO xylem was almost devoid of short-chained glucosinolates, this suggests that these glucosinolates originate from the shoot. This is in agreement with a previous study where the rosette was proposed to be the main synthesis and storage site for short-chained aliphatic glucosinolates (Andersen et al., 2013). It appears likely that 4-MSB accumulates in the leaf apoplastic space due to lack of GTR-dependent uptake in a similar manner as suggested for 8-MTO/8-MSO in roots of the *gtr1gtr2* dKO (Andersen et al., 2013). These authors proposed that absence of GTR1 and GTR2 uptake of long-chained glucosinolates from the root apoplast into cells adjacent to the xylem resulted in xylem sap containing high amounts of long-chained glucosinolates that followed the transpiration stream to the shoot. The accumulation of 4-MSB in the leaf apoplastic space of the *gtr1gtr2* dKO shows that at least some 4-MSB upon synthesis is exported out of cells and into the apoplast, from where it is imported into cells by GTR1 or GTR2 located at the plasma membrane.

Exogenous Glucosinolates Delivered to Apoplastic Space Reach the Leaf Margin through a Symplasmic Route

The severalfold higher aliphatic glucosinolate concentrations in guttation drops (collected at leaf margins),

apoplastic fluids, and root xylem sap in *gtr1gtr2* dKO compared with the wild type suggest that the increased margin accumulation in *gtr1gtr2* dKO leaves could be due to an increased flow of glucosinolates toward the leaf margin in the transpiration stream. This increased glucosinolate flow in *gtr1gtr2* dKO leaves would be caused by an inability to remove these from the apoplast by GTR1 and GTR2. As approximately 98% of the glucosinolates in *gtr1gtr2* dKO leaves are intracellularly stored (Table I) and as *gtr1gtr2* dKO leaves showed a glucosinolate allocation in the margin even after apoplastic fluids had been extracted (Supplemental Fig. S4), these observations suggest that a GTR1- and GTR2-independent mechanism exists for translocation of glucosinolates into leaf margin cells.

How do glucosinolates reach the margins in a wild-type leaf? The observations made in the *gtr1gtr2* dKO suggested that an apoplastic route is possible via the transpiration stream (Fig. 4). However, as the glucosinolate concentration in apoplastic fluid from wild-type leaves was only 8% of the concentration found in apoplastic fluids from *gtr1gtr2* dKO leaves, this hypothesis appears unlikely. Alternatively, it can be envisioned that glucosinolates delivered to leaf apoplast either via the xylem (Fig. 4A) or through export from glucosinolate-producing cells within the leaf (Fig. 4, B and C) are immediately imported by GTR1 and GTR2 into cells from where the glucosinolates follow a symplasmic route via plasmodesmata toward leaf margins. We therefore investigated the destiny of a glucosinolate that was artificially delivered to the apoplast by feeding detached leaves with the exogenous glucosinolate sinigrin for 24 h and subsequently analyzing the glucosinolate content in dissected leaf parts (Fig. 5). Sinigrin fed into mature leaves can be expected to be taken up and follow the xylem stream (Shatil-Cohen et al., 2011). As wild-type leaves accumulated a large proportion of sinigrin in the petiole and midrib (approximately 50%) compared with *gtr1gtr2* dKO leaves (approximately 10%; Fig. 5), it appears that sinigrin present in the xylem sap in a wild-type leaf is GTR-dependently imported into phloem or storage cells along the midrib. In comparison, only a small proportion of sinigrin was found in wild-type leaf margins (approximately 7%), whereas *gtr1gtr2* dKO leaf margins contained approximately 45% of total sinigrin. This shows that glucosinolates delivered to the apoplastic space in a wild-type leaf are transported to leaf margins, but that this transport process appears to occur in a slower and more restricted manner than in the *gtr1gtr2* dKO. Combining this observation with the difference in leaf margin accumulation of sinigrin in the wild type (equal distribution at margin) and *gtr1gtr2* dKO (mainly distributed to tip margins; Fig. 5), this indicates that apoplastically delivered sinigrin follows different routes to the leaf margin in wild-type leaves compared with *gtr1gtr2* dKO. Based on our observations, we suggest that sinigrin present in the wild-type leaf xylem is GTR-dependently imported into either storage or phloem cells along the vasculature or into vasculature-associated mesophyll cells, from where the glucosinolates can follow a symplasmic intracellular route toward the leaf margin through plasmodesmata. We further suggest that compartmentalization into vacuoles at the leaf margin cells by a yet unidentified vacuolar importer creates a cytosolic concentration gradient across this symplasmic continuum with minimum concentration at the leaf margins, which ensures a flow of glucosinolates toward leaf margins and enables the buildup of a local, high concentration (Fig. 6A).

Glucosinolates Accumulate at the Leaf Lamina Base in a GTR-Dependent Manner

When dissecting mature wild-type and *gtr1gtr2* dKO leaves transversely along the leaf axis, major differences were observed in distribution of aliphatic but not indole glucosinolates in the different leaf sections (Fig. 2; Supplemental Fig. S2). The GTR-dependent accumulation of aliphatic glucosinolates mainly at the lamina base in the wild type is in agreement with what has been previously reported (Watanabe et al., 2013). Buildup of aliphatic glucosinolates at the tip of *gtr1gtr2* dKO leaves corresponded to the accumulation of the petiole-fed sinigrin in *gtr1gtr2* dKO leaves (Fig. 5), suggesting that endogenous aliphatic glucosinolates flowing through the xylem contribute to the leaf tip (both lamina and margin) allocation of glucosinolates in the *gtr1gtr2* dKO mutant. This is, however, in contradiction with the observation that apoplastic glucosinolates contribute only approximately 1.5% to total *gtr1gtr2* dKO leaf glucosinolates (Table I). This may reflect that glucosinolates unloaded from the xylem in *gtr1gtr2* dKO leaves (mainly at tip margin and tip lamina) are being transported into cells in a GTR-independent manner. This is supported by the fact that dissected *gtr1gtr2* dKO leaves extracted for apoplastic fluid also displayed an aliphatic glucosinolate allocation toward the tip (Supplemental Fig. S4).

For endogenous glucosinolates produced outside a leaf and arriving to the apoplastic leaf space (e.g. via the xylem), these data, in combination with data generated from sinigrin-fed leaves (Fig. 5), indicate that GTR1 and GTR2 will import these into the symplasmic continuum, either into storage or phloem or into cells where glucosinolates can move through plasmodesmata toward the leaf margins. The observation that leaf distribution of short-chained aliphatic glucosinolates (primarily produced in the shoot; see above) follows xylem transport to the leaf tip in *gtr1gtr2* dKO plants (Fig. 2; Supplemental Fig. S2) suggests that glucosinolates produced in the leaf are mainly exported from biosynthetic cells before being GTR-dependently imported into cells to create the glucosinolate distribution seen in wild-type leaves. Alternatively, a fraction of newly produced glucosinolates in the leaf may not be exported from biosynthetic cells and is instead directly channeled through the symplasmic plasmodesmata to, for example, the leaf margin (Fig. 6).

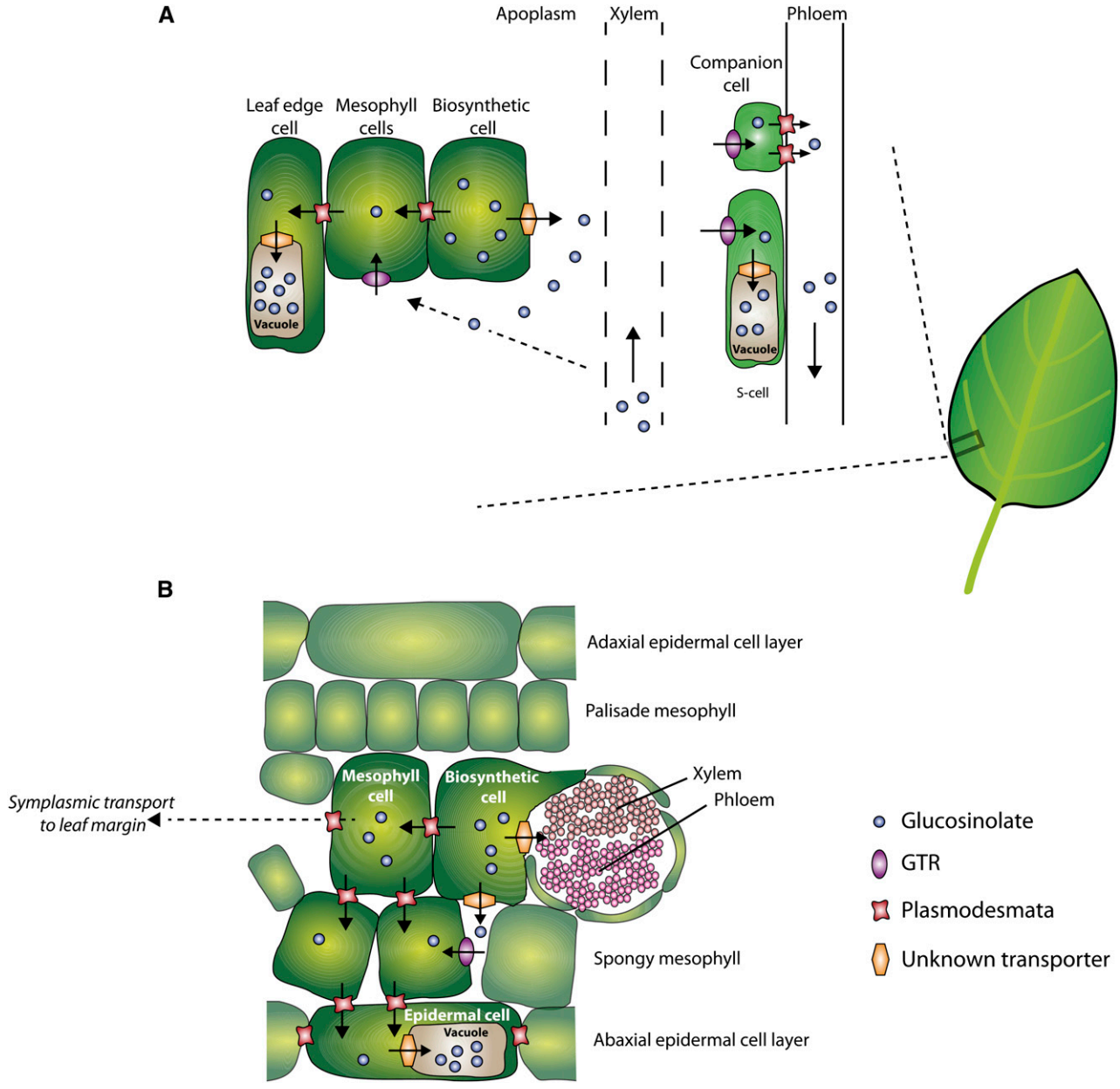

Figure 6. Model for glucosinolate distribution within a mature Arabidopsis leaf. A, When a glucosinolate is produced in a cell adjacent to the vasculature, different transport routes are possible. If the glucosinolate is being exported out of the biosynthetic cell, a GTR1- and GTR2-dependent uptake of the glucosinolate from the apoplast is likely to occur into cells adjacent to xylem, e.g. into phloem companion cells for long-distance transport out of the leaf, as well as into storage cells, such as S-cells, or adjacent mesophyll cells. Alternative to export from the biosynthetic cell, the newly produced glucosinolate can remain in the cytosol and enter a symplasmic route toward the leaf margin (mainly base margin) via plasmodesmata. Compartmentalization into vacuoles by an unknown vacuolar importer at the leaf margin enables the buildup of a high concentration, thereby ensuring a flow of glucosinolates toward the leaf periphery. Glucosinolates present in the leaf xylem sap will GTR dependently be transferred into phloem companion cells, storage cells (S-cells), or vascular-associated mesophyll cells, from where glucosinolates can flow via plasmodesmata toward the leaf margin. B, In a similar manner, we suggest that plasmodesmatal connections between biosynthetic and/or mesophyll cells to the abaxial epidermis cells along with compartmentalization into epidermal cell vacuoles by an unknown vacuolar importer ensure accumulation of glucosinolates in the outermost cell layer of the leaf. The figure is a schematic illustration of our model. Compartments, cell types, transporters, glucosinolates, and direction of transport are indicated by illustrations and named accordingly.

Glucosinolates Accumulate in the Abaxial Epidermis through a GTR-Independent Mechanism

The leaf epidermis represents a barrier with important roles in adapting to the surrounding environment (Ebert et al., 2010). This outermost cell layer has so far not been shown directly to contain glucosinolates in an Arabidopsis leaf. Here, we demonstrate the presence of glucosinolates in the abaxial epidermis in both the wild type and *gtr1gtr2* dKO and show that epidermal accumulation is not dependent on GTR1 or GTR2, as evidenced by a 2.5-fold higher aliphatic glucosinolate concentration in the transporter mutant compared with the wild type (Fig. 3). The increased glucosinolate concentration in epidermis samples compared with remaining leaf (for both the wild type and *gtr1gtr2* dKO) indicates specific roles for these defense compounds, for example, as a peripheral defense against generalist herbivores, as suggested for glucosinolates in leaf margin cells. The presence of glucosinolates in the wax layer covering the leaf surface has been suggested to stimulate oviposition of specialist insects (Griffiths et al., 2001; Badenes-Pérez et al., 2011). Other studies rejected the presence of glucosinolates on the leaf surface and suggested that specialist herbivores perceive the compounds from cells below the surface wax layer (Shroff et al., 2008; Stadler and Reifenrath, 2009). Thus, epidermis samples in this work potentially contained glucosinolates from abaxial epidermis cells as well as from the wax layer covering the abaxial leaf surface. Interestingly, the concentration of indole glucosinolates (mainly I3M) was biased more toward abaxial epidermis than was the case for other glucosinolates (Fig. 3; Supplemental Fig. S3). For some specialist insects, indole glucosinolates (I3M) have proved more potent as oviposition stimulants than aliphatic glucosinolates (Agerbirk et al., 2009), which could fit their epidermis accumulation reported here. Although the biosynthetic machinery has not been localized to the epidermis (for review, see Grubb and Abel, 2006), a glucosinolate hydrolysis promoting enzyme (epithiospecifier protein; Burow et al., 2007) and secondary modification enzymes of both aliphatic ($FMO_{GS\text{-}OX1}$; Li et al., 2011) and indole glucosinolates CYTOCHROME P450, FAMILY81, SUBFAMILY F, POLYPEPTIDE2 (CYP81F2; Supplemental Fig. S6) have been localized to this tissue in Arabidopsis. This suggests a specialized role for products of these enzymes in the epidermis, e.g. as part of leaf defense against microbial pathogens as suggested for 4-methoxy-indol-3-ylmethyl glucosinolate (Bednarek et al., 2009; Clay et al., 2009), or as compounds involved in stomatal closure after breakdown by myrosinases in guard cells (Zhao et al., 2008; Islam et al., 2009; Khokon et al., 2011). The strong allocation of 4-MTB toward the abaxial epidermis compared with remaining leaf sample (Supplemental Fig. S3) was unexpected considering the epidermis expression of $FMO_{GS\text{-}OX1}$ that would convert 4-MTB to 4-MSB. On the other hand, such strong allocation of 4-MTB was also seen for 4-MTB in the leaf margin (Supplemental Fig. S1). The biological significance of this specific distribution of 4-MTB in the periphery of the leaf is currently unknown.

Based on the general increase in glucosinolate concentration in epidermis samples compared with remaining leaf samples, we suggest a similar symplasmic route of glucosinolates through plasmodesmata to the abaxial epidermis as to the leaf margin, where compartmentalization into vacuoles in epidermis cells ensures a flow of glucosinolates and enables the buildup of a local, high concentration (Fig. 6B).

A recent study reported that isolated Arabidopsis trichomes contain glucosinolates, mainly 4-MSB, 8-MSO, and I3M (Frerigmann et al., 2012). As trichomes were not removed before abaxial epidermis peeling in this study, glucosinolates from these may have contributed to glucosinolate levels observed in the epidermis (Fig. 3; Supplemental Fig. S3). However, glucosinolate content in trichomes was reported to represent 1% of the glucosinolate content in the remaining leaf (Frerigmann et al., 2012), whereas glucosinolate content in the abaxial epidermis in this study represents approximately 30% of the glucosinolate content in the remaining leaf, for both the wild type and gtr1gtr2 dKO (data not shown). This indicates a minimal influence of glucosinolates present in trichomes on the reported glucosinolate levels in epidermis peels. 4-MTB, which is strongly allocated toward the abaxial epidermis (Supplemental Fig. S3), was not found in isolated trichomes (Frerigmann et al., 2012), indicating that this 4-MTB allocation is not due to a high concentration of this glucosinolate in trichomes.

A Model for Glucosinolate Distribution within a Mature Arabidopsis Leaf

Based on data presented here, we propose a model for how glucosinolates within a mature wild-type leaf are allocated to margins and the epidermis, and we suggest a mechanism to explain how glucosinolates accumulate in these peripheral cell layers (Fig. 6). The model is based on the assumption that biosynthesis of indole as well as long-chained and short-chained aliphatic glucosinolates primarily occurs at the vasculature within the leaf (Grubb and Abel, 2006; Andersen et al., 2013). Furthermore, as demonstrated in this study (Fig. 4A), the model assumes a contribution to leaf glucosinolates from the xylem sap.

When glucosinolates are produced in a cell adjacent to the vasculature, our data strongly indicate that these can be exported out of the biosynthetic cell (Fig. 4, B and C), mediated by an unknown exporter. When in the apoplast, different transport routes for glucosinolates are possible (Fig. 6A): GTR1- and GTR2-dependent uptake of glucosinolates from the apoplast is likely to occur into phloem cells for long-distance transport out of the leaf (Arabidopsis is an apoplastic phloem loader; Rennie and Turgeon, 2009), into storage cells such as S-cells (Koroleva et al., 2000, 2010), or into vasculature-associated mesophyll cells, from where a symplasmic route via plasmodesmata can be followed to the leaf

margin (Fig. 5). As an alternative to export from the biosynthetic cell, some of the newly produced glucosinolates may remain in the cytosol and enter a symplasmic route toward the leaf margin via plasmodesmata. Compartmentalization into vacuoles by an unknown vacuolar importer at the leaf margin enables the buildup of a high concentration by ensuring a flow of glucosinolates toward the leaf periphery. In a similar manner, we suggest that plasmodesmatal connections between biosynthetic and/or mesophyll cells to the abaxial epidermis cells along with compartmentalization into vacuoles by an unknown vacuolar importer ensure accumulation of glucosinolates in the outermost cell layer of the leaf (Fig. 6B).

CONCLUSION

This study has explored the role of transport processes in distribution of defense compounds within a mature Arabidopsis leaf. We show that GTR1 and GTR2 play a key role in glucosinolate allocation within a leaf by effectively importing apoplastically localized (in xylem sap and leaf apoplast) glucosinolates into appropriate cells. Our data suggest that glucosinolates accumulate in the leaf margin and abaxial epidermis via symplasmic movement through plasmodesmata, coupled with the activity of putative vacuolar glucosinolate importers in these peripheral cell layers. Knowledge gained here about distribution of leaf defense compounds in the model plant Arabidopsis may provide novel insights on universal mechanisms by which plants allocate their chemical defenses, thereby improving our understanding of the arms race between plants and their enemies, which ultimately might enable the production of crops more resistant against pests.

MATERIALS AND METHODS

Plant Growth Conditions

Seeds of the Arabidopsis (*Arabidopsis thaliana*) wild type (ecotype Columbia) and mutants with transfer DNA insertions in both GTR1 (At3g47960) and GTR2 (At5g62680; *gtr1gtr2* dKO) were sown on plant substrate (Pindstrup Mosebrug A/S) in 4-cm pots. Seeds were cold stratified at 4°C for 2 d and transferred to a growth chamber with 16-h daylength at 20°C, 70% relative humidity, and a light intensity of 100 μE. Leaves/plants used for sinigrin feeding and apoplastic fluid extractions (xylem sap, guttation drops, and leaf apoplastic fluid) were cultivated at 12 h of daylength but otherwise with identical settings. Mature, nonbolting Arabidopsis plants were used for experiments.

Glucosinolate Analysis

Glucosinolates were analyzed as desulfo-glucosinolates through a modified version of a previously described method (Kliebenstein et al., 2001b). Leaf material was homogenized in 85% (v/v) methanol containing 0.02 mM *para*-hydroxybenzyl glucosinolate (pOHB) as internal standard. Samples were centrifuged at 2500*g* for 10 min, and the supernatant was transferred to a 96-well filter plate (Millipore) loaded with 45 μL of DEAE Sephadex A-25 column material (GE Healthcare Biosciences). Column material had been equilibrated for a minimum of 2 h in 300 μL of water before samples were applied. Glucosinolates were bound to the column material, while samples were sucked through the filter plate by applying brief vacuum. Afterward, columns were washed with 2×100 μL of 70% (v/v) methanol and 2×100 μL of water, respectively. Twenty microliters of sulfatase (Sigma E.C. 3.1.6.) solution (2 mg mL^{-1}) was added to the columns and allowed to incubate at room temperature overnight. One hundred microliters of water was applied to the columns, and a short spin eluted the desulfo-glucosinolates into a 96-well format plate. The samples were analyzed on an Agilent Technologies 1200 series HPLC-DAD system and separated on a Zorbax SB-AQ column (4.6 mm × 25 cm, 5-μm particle size) at a flow rate of 1 mL min^{-1}. Compounds were detected at 229 nm using a diode array UV and separated utilizing eluents (A: water; B: 100% [v/v] acetonitrile) using the following program: 5-min gradient from 1.5% to 7% eluent B; 5-min gradient from 7% to 25% eluent B; 4-min gradient from 25% to 80% eluent B; 3 min at 80% eluent B; 2-min gradient from 80% eluent B to 35% eluent B; 2-min gradient from 35% to 1.5% eluent B; and a final 3 min at 1.5% eluent B. Response factors for A_{229} were used to quantify the desulfo-glucosinolates (Fiebig and Arens, 1992; Reichelt et al., 2002; Brown et al., 2003).

For xylem samples, intact glucosinolates were measured by LCMS using an Agilent 1100 Series liquid chromatograph coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics). A Zorbax SB-C18 column (1.8 mm, 2.1 mm i.d., 350 mm; Agilent) was used at a flow rate of 0.2 mL min^{-1}, and the mobile phases were as follows: A, water with 0.1% (v/v) formic acid and 50 mM NaCl, and B, acetonitrile with 0.1% (v/v) HCOOH. The gradient program was as follows: 0 to 0.5 min, isocratic 2% B; 0.5 to 7.5 min, linear gradient 2% to 40% B; 7.5 to 8.5 min, linear gradient 40% to 90% B; 8.5 to 11.5 min, isocratic 90% B; and 11.6 to 15 min, isocratic 2% B. The flow rate was increased to 0.3 mL min^{-1} in the interval 11.2 to 13.5 min. The mass spectrometer was run in positive electrospray mode.

In Silico Analysis

Cell type-specific expression for CYP81F2 (AT5G57220) was derived from microarray studies of RNA bound to ribosomes that were immunoprecipitated by use of epitope-tagged ribosomal protein from seedlings (Mustroph et al., 2009; http://efp.ucr.edu/).

Dissection Experiments

Mature (fifth) leaves (same size and appearance for both genotypes) collected with petioles were dissected with a scalpel in three different ways: (1) midrib/margins/lamina, two cuts approximately 1 mm on either side of the midrib and two cuts along margins from each leaf half about 1 mm from each margin; (2) petiole/base/tip, the petiole was dissected and the leaf lamina was divided in two equal parts; and (3), a combination of (1) and (2): petiole/midrib/tip margins/base margins/tip lamina/base lamina. Sections were weighed and immediately submerged and homogenized in 85% (v/v) methanol containing 0.02 mM pOHB. Glucosinolates were analyzed as described above.

Mature (fifth) leaves were weighed and put on a piece of transparent tape with the adaxial (upper) epidermis facing the tape. The leaf attached to the tape was weighed. After this, a new piece of tape was placed on the leaf abaxial (lower) epidermis to create a sandwich. By carefully peeling the pieces of tape apart, the lower epidermis was isolated. Next, remaining leaf attached to the tape without lower epidermis was weighed. The epidermis plus tape fractions were dipped in water to remove apoplastic glucosinolates. Samples were placed in separate tubes containing metal balls and placed in liquid nitrogen. Next, samples were homogenized using a mixer mill (Retsch), followed by the addition of 85% (v/v) methanol containing 0.02 mM pOHB as internal standard. Methanol extracts were analyzed for glucosinolates.

Apoplastic Fluid Isolation

Cut mature (fifth) leaves were dipped in deionized water to remove any surface contaminants and vacuum infiltrated with water containing 0.004% (v/v) Triton-X1000. Vacuum was released and reapplied successively to reach 100% infiltration, which was estimated by the dark-green color of the saturated tissue (3×1 min). Following infiltration, the tissue was again dipped in deionized water. Tissue was dried lightly with tissue. To collect apoplastic fluid, leaves were stacked between pieces of Parafilm and taped onto the outside of a 15-mL falcon tube (leaf tip down). This aggregate was placed into a cut 50-mL falcon tube and firmly taped so that leaves would not reach the bottom of the 50-mL falcon tube during centrifugation. Leaves were centrifuged at 900*g* for 15 min, after which leaves were weighed and analyzed for glucosinolates (see above). Control leaves not extracted for apoplastic fluid were analyzed for glucosinolates in parallel. The volume of apoplastic fluid

(present in the bottom of the 50-mL falcon tube) was measured with a pipette. Cytoplasmic contamination was tested by adding 50 μL of water containing 0.02 mM sinigrin to each sample followed by incubation for 15 min at room temperature to allow for myrosinase activity to break down glucosinolates. Next, 100% (v/v) MeOH containing 0.01 mM pOHB (internal standard) was added. Samples were analyzed for glucosinolates by HPLC (see above). Apoplastic fluid samples from leaves exposed to intentional leaf damage did not contain any glucosinolates (data not shown).

Collection of Guttation Drops

Five- to six-week-old Arabidopsis plants (nonbolting) were placed in high humidity overnight, and the next morning, guttation drops from hydathodes were collected from rosette leaves with a pipette. To each sample, 25 μL of water plus 0.02 mM sinigrin was added to test myrosinase breakdown of glucosinolates. Samples were analyzed for glucosinolates by HPLC.

Collection of Xylem Sap

Inspired by Alexou and Peuke (2013), 5- to 6-week-old Arabidopsis plants (nonbolting) were watered plenty and left for a couple of hours to generate high water pressure. Rosettes were then cut with a sharp scalpel, and the remaining root stocks were rinsed with water two times to avoid myrosinase-mediated glucosinolate breakdown and phloem contamination. Next, plastic tubes were tightly sealed around root stocks, and xylem sap was collected as it appeared in tubes. To each sample, 25 μL of water plus 0.02 mM sinigrin was added to test myrosinase breakdown of glucosinolates. Xylem sap was analyzed for glucosinolates by LCMS (see above).

Sinigrin Feeding

Mature (fifth) leaves were cut, and petioles were briefly rinsed in water before being placed in separate tubes containing water with 0.2 mM sinigrin. After 24 h of feeding, petioles were rinsed in water, and leaves were dissected according to the description above before glucosinolate analysis.

Statistical Data Analysis

To represent the changes in glucosinolate levels, one-way ANOVA or two-tailed Student's *t* tests were performed using either SigmaPlot (http://www.systat.com) or R (http://www.r-project.org).

Sequence data from this article can be found in the Arabidopsis Genome Initiative or EMBL/GenBank data libraries under accession numbers At3g47960 (GTR1), At5g62680 (GTR2), At5g57220 (CYP81F2), and At1g65860 ($FMO_{GS\text{-}OX1}$).

Supplemental Data

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

Supplemental Figure S1. Individual glucosinolate concentration in mature Arabidopsis wild-type and *gtr1gtr2* dKO mutant leaves dissected into midrib, lamina, and margins.

Supplemental Figure S2. Individual glucosinolate concentration in mature Arabidopsis wild-type and *gtr1gtr2* dKO mutant leaves dissected into petiole, base, and tip.

Supplemental Figure S3. Individual glucosinolate concentration in mature Arabidopsis wild-type and *gtr1gtr2* dKO mutant leaves dissected into abaxial epidermis and remaining leaf parts.

Supplemental Figure S4. Glucosinolate distribution in mature Arabidopsis wild-type and *gtr1gtr2* dKO leaves extracted for apoplastic fluid.

Supplemental Figure S5. Sinigrin uptake of mature Arabidopsis wild-type and *gtr1gtr2* dKO leaves.

Supplemental Figure S6. Cell type-specific expression for CYP81F2.

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