RESEARCH ARTICLE

The cyclophilin A DIAGEOTROPICA gene affects auxin transport in both root and shoot to control lateral root formation

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ABSTRACT

Cyclophilin A is a conserved peptidyl-prolyl cis-trans isomerase (PPIase) best known as the cellular receptor of the immunosuppressant cyclosporine A. Despite significant effort, evidence of developmental functions of cyclophilin A in non-plant systems has remained obscure. Mutations in a tomato (Solanum lycopersicum) cyclophilin A ortholog, DIAGEOTROPICA (DGT), have been shown to abolish the organogenesis of lateral roots; however, a mechanistic explanation of the phenotype is lacking. Here, we show that the dgt mutant lacks auxin maxima relevant to priming and specification of lateral root founder cells. DGT is expressed in shoot and root, and localizes to both the nucleus and cytoplasm during lateral root organogenesis. Mutation of ENTIRE/IAA9, a member of the auxin-responsive Aux/IAA protein family of transcriptional repressors, partially restores the inability of dgt to initiate lateral root primordia but not the primordia outgrowth. By comparison, grafting of a wild-type scion restores the process of lateral root formation, consistent with participation of a mobile signal. Antibodies do not detect movement of the DGT protein into the dgt rootstock; however, experiments with radiolabeled auxin and an auxin-specific microelectrode demonstrate abnormal auxin fluxes. Functional studies of DGT in heterologous yeast and tobacco-leaf auxin-transport systems demonstrate that DGT negatively regulates PIN-FORMED (PIN) auxin efflux transporters by affecting their plasma membrane localization. Studies in tomato support complex effects of the dgt mutation on PIN expression level, expression domain and plasma membrane localization. Our data demonstrate that DGT regulates auxin transport in lateral root formation.

KEY WORDS: Auxin response, Auxin transport, Lateral root initiation, Cyclophilin A, DIAGEOTROPICA

INTRODUCTION

The formation of root branches, known as ‘lateral roots’, continues throughout the entire lifespan of a plant. In most eudicot plants such as Arabidopsis and tomato, lateral root meristems form de novo from cells in the pericycle cell layer of the parent root (for a recent review, see Lavenus et al., 2013a). This process comprises several distinct phases. First, some of the pericycle cells adjacent to a protoxylem pole in the basal region of the root apical meristem (also referred to as the transition zone) undergo pre-selection or ‘priming’ (De Smet et al., 2007; Moreno-Risueno et al., 2010). In the differentiation zone of the root, selected pericycle cells become specified as lateral root founder cells; these cells undergo asymmetric anticlinal (perpendicular to the root surface) division, giving rise to a file of short cells referred to as the stage I primordium (Malamy and Benfey, 1997). Cells in the stage I primordium divide periclinically (parallel to the root surface) to form a two-cell layered primordium (stage II). Further development generates a dome-shaped advanced primordium, then a recognizable meristem forms and the new lateral root emerges through the overlying tissues of the parent root. A number of studies have highlighted the organogenetic power of the plant hormone auxin. Auxin biosynthesis, perception, signaling and polar transport (PAT) are all required for normal lateral root formation (reviewed by Benková et al., 2009; Lavenus et al., 2013b; Overvoorde et al., 2010; Vanneste and Friml, 2009). PAT and auxin responses are tightly interlinked and difficult to resolve in planta (Vieten et al., 2005). Expression of the auxin-responsive reporter gene DR5 in vascular cells in the Arabidopsis root apical meristem associates with priming of adjacent pericycle cells (De Smet et al., 2007; Moreno-Risueno et al., 2010), whereas DR5 expression in pericycle cells in the root differentiation zone of Arabidopsis and tomato marks their specification as lateral root founder cells (Benková et al., 2003; Dubrovsky et al., 2008; Himanen et al., 2002). Both types of DR5 expression patterns are abolished upon application of the auxin efflux inhibitor 1-N-naphthylphthalamic acid (NPA), indicating that these auxin responses depend on PAT (De Smet et al., 2007; Himanen et al., 2002). In Arabidopsis, a gain-of-function mutation solitary root (slr-1/iaa14) leads to accumulation of a stabilized form of SLR/IAA14, a member of the Aux/IAA protein family of transcriptional repressors, and expression of mIAA14 in wild-type plants inhibits lateral root formation (Fukaki et al., 2002). A close tomato SLR ortholog is ENTIRE (E)/SI-IAA9 (Wu et al., 2012). RNAi lines with decreased SI-IAA9 gene expression (Wang et al., 2005) and loss-of-function e/iaa9 mutants (Zhang et al., 2007) show shoot morphological defects but normal root development.

In Arabidopsis, PAT relies on two major families of membrane-localized auxin efflux proteins, PIN and ABCB, and a family of auxin influx proteins, AUXI/ LAX (reviewed by Vanneste and Friml, 2009). Dynamic recycling of PINs to and from the plasma membrane is essential for PIN functionality (Geldner et al., 2001; Gruenewald and Friml, 2010; Kleine-Vehn et al., 2008). In the root, auxin runs from the base towards the tip (acropetal stream) and from the tip towards the base (basipetal stream). Using a self-referencing IAA-specific microelectrode that permits noninvasive continuous recordings of auxin flux rate along the root, it is possible to detect...
a distinct peak at the root transition zone (Mancuso et al., 2005; Santelia et al., 2005), correlating with a PIN-dependent auxin ‘reflux loop’ from peripheral towards central vascular cells (Blilou et al., 2005). Less is known about how auxin transporters are regulated by protein interactions that may influence their conformation and thus affect trafficking, stability or activity. Trafficking of ABCBs from the ER to the plasma membrane and their functionality on the membrane is maintained by the peptidyl-prolyl cis-trans isomerase (PPIase) FKBP42/TWD1, which does not interact with PIN auxin transporters (Bouchard et al., 2006; Wang et al., 2013; Wu et al., 2010).

Similar to FKBP5s, cyclophilins (Cyps) display a PPIase activity in vitro, suggesting they act in protein folding (Schiene-Fischer and Yu, 2001). FKBP5s and Cyps are commonly referred to as immunophilins due to their high affinity for the immunosuppressive drugs FK506 and cyclosporine A, respectively. Cyclophilin A consists of only the core PPIase domain, localizes primarily in the cytosol and nucleus, and is highly conserved from yeast to humans [reviewed by Wang and Heitman (2005)]. In higher plants, cyclophilin A has been linked to auxin-regulated development through the cloning of the diageotropica (dgt) mutation in tomato (Oh et al., 2006). DGT possesses PPIase activity and might affect plant development through physiological refolding of target proteins (Oh et al., 2006). One of the most remarkable phenotypes of dgt is the lack of lateral root primordium organogenesis (Ivanchenko et al., 2006). In dgt, the expression of members of the auxin-regulated Aux/IAA gene family is abnormal to a different degree, depending on organ and developmental stage (Balbi and Lomax, 2003; Mignolli et al., 2012; Mito and Bennett, 1995; Nebenführ et al., 2000). Protoplasts from dgt hypocotyls do not swell but instead decrease in volume when treated with auxin or antibodies against AUXIN BINDING PROTEIN 1 (ABP1), further suggesting an abnormal auxin response (Christian et al., 2003). Previous work has reported unchanged auxin transport in dgt root and shoot, which has led to the hypothesis that DGT regulates auxin perception or signaling but plays no role in PAT (Daniel et al., 1989; Muday et al., 1995). Here, we show that DGT is required for effective auxin transport in planta and in heterologous auxin-transport systems that lack plant-specific components of auxin perception and signaling. In contrast to TWD1 involved in regulation of ABCB auxin transporters, DGT appears to regulate PIN transporters.

RESULTS
The dgt mutant lacks auxin maxima related to pericycle cell priming and founder cell specification

We analyzed auxin signals in vascular cells in the root apical meristem related to pericycle cell priming using DR5:GUS and IAA2:GUS reporters. DR5 was expressed in vascular cells in wild-type root tips (Fig. 1A), similar to the pattern reported in Arabidopsis (De Smet et al., 2007). Remarkably, vascular DR5 signals were completely absent in dgt, although expression in the quiescent center region (QC) and the central root cap (columella) was present and even appeared increased compared with wild type. Treatment with the auxin transport inhibitor NPA abolished DR5 expression in the vascular cells of wild-type roots, causing them to resemble untreated dgt roots. Upon a pulse treatment with IAA (5 µM for 3 h), DR5 activity increased in the wild-type vascular cells but no expression was induced in dgt. On comparing the effect of a longer IAA treatment (5 µM for 40 h) with that of the synthetic auxins NAA and 2,4-D, postulated to be inefficiently transported by the auxin efflux and influx transporter, respectively (Marchant et al., 1999), we found that IAA and NAA induced multiple lateral root primordia in the wild-type root tip, whereas 2,4-D increased DR5 expression without primordium induction. None of these treatments

Fig. 1. The dgt mutant lacks auxin signals related to pericycle cell priming and lateral root organogenesis. (A) DR5:GUS auxin reporter expression in wild-type and dgt root tips after treatments as indicated. Note reporter expression in vascular cells in the wild-type root apical meristem and its absence in dgt (arrowhead). The dgt root exhibits DR5 expression only in the QC region and central root cap (arrow). Treatment with auxin transport inhibitor NPA (10 µM for 20 h) abolishes DR5 expression in the vascular region of wild type. Pulse treatment with IAA (5 µM for 3 h) increases DR5 expression in stele of the wild-type root tip but has no effect on dgt. Treatment with IAA or NAA for 40 h induces lateral root primordia in the wild-type root tip but not in dgt. 2,4-D increases DR5 expression at the root tip in wild type but not in dgt. (B) IAA2:GUS reporter expression in newly emerged lateral roots induced through meristem decapitation shows strong expression in stele of wild type and more peripheral expression in dgt. (C) Tomato root anatomy (one half of the root is shown). (D) Wild-type lateral root primordia showing DR5 expression at the primordia tips. (E) The differentiation zone of wild-type and dgt roots after treatment with 5 µM IAA for 3 h. Arrows in D,E denote divided pericycle cells that do not show DR5 expression. c, cortex; cc, central cylinder; e, endodermis; ep, epidermis; p, pericycle; x, xylem. Scale bars: 100 µm in A;B; 30 µm in D,E.
induced DR5 expression in the vascular cells of dgt or primordium formation (Fig. 1A). Although dgt is unable to form lateral roots, these can be induced in both wild type and dgt following meristem decapitation. In induced lateral roots, which are thinner and allow for more precise tissue assessment in tomato, IAA2 reporter expression was strong in wild-type vascular cells, whereas in dgt it was decreased in vascular and increased in peripheral tissues (Fig. 1B). Furthermore, upon gravitropic stimulation wild-type roots reoriented their growth direction and demonstrated asymmetric IAA2:GUS reporter expression on the lower side of the root, whereas dgt roots did not reorient, and did not show asymmetric IAA2:GUS expression (supplementary material Fig. S1). Thus, dgt root tips displayed spatial abnormalities in auxin reporter expression that could be justifiably interpreted as resulting from a defect in PAT.

We next analyzed auxin signals in pericycle cells in the dgt root differentiation zone. The tomato root anatomy is similar to that of Arabidopsis except that the cortex has three cell layers instead of one (Fig. 1C). In the differentiation zone of the wild-type root, DR5 was expressed in lateral root primordia and increased at the primordia tips (Fig. 1D). It appears that in tomato, some primordia are initiated by relatively longer stretches of divided pericycle cells but those extra cells do not show DR5 expression and do not participate in further primordium development (Fig. 1D, arrows). Because, in tomato, DR5 expression was insufficiently strong at early stages of primordium organogenesis, we analyzed roots that were pulse-treated with IAA for 3 h (Fig. 1E). In these roots, DR5 expression/signal was clearly seen in founder cells and stage I primordia in wild type, apparently associated with primordia centers and absent from short pericycle cells at the primordia periphery. In dgt, no DR5 expression was observed in this zone. Although some dgt roots exhibited short pericycle cells apparently resulting from anticlinal pericycle cell division, these cells did not show DR5 expression (Fig. 1E). We conclude that, in tomato, DR5 marks auxin signals associated with primordium initiation and growth; however, such signals are absent in dgt.

To confirm that DR5 expression in vascular cells at the wild-type tomato root tip is indeed related to the process of pericycle cell priming, as has been shown in Arabidopsis, we treated seedlings with a 3 h pulse of IAA, transferred them onto fresh agar plates, and marked the position of the root tips. As roots elongated below the mark, the DR5-positive vascular tissues remained just above the mark. Analysis of the root zone above the mark in a time course revealed DR5 activity in pericycle cells 20 h after the treatment, and a few hours later a primordium formed, then a lateral root emerged (Fig. 2). However, no primordium organogenesis or lateral root formation was observed in dgt, consistent with the lack of DR5 response in vascular cells of the root apical meristem (Fig. 2).

**DGT tissue-specific expression and subcellular localization**

*DGT:GUS* expression was observed in roots, cotyledons and leaves, and is apparently associated with the vasculature in these tissues (Fig. 3A-C). *DGT* activity was broader in cross-sections through the middle part of the meristem (Fig. 3D), but was restricted to the central cylinder and pericycle and endodermis near the transition and elongation zone, where it was observed predominantly at the phloem poles (Fig. 3E). At the beginning of the differentiation zone, some *DGT* expression was also detected in early-stage lateral root primordia (Fig. 3F). To observe the subcellular localization of *DGT* in pericycle cells, we stably expressed a *DGT:mCherry-DGT* fusion in Arabidopsis, which has thinner roots that are amenable to confocal microscope imaging of inner tissues. Consistent with the *DGT:GUS* expression in tomato, mCherry-DGT fluorescence was observed in lateral root founder cells and lateral root primordia in Arabidopsis roots (Fig. 3G). At the subcellular level, DGT was observed in the nucleus and the cytoplasm in early-primordia cells, consistent with the subcellular localization of yeast and mammalian cyclophilin A (reviewed by Wang and Heitman, 2005). Transformation the DGT:mCherry-DGT construct into dgt restored the lateral root formation defect, demonstrating the functionality of the mCherry-DGT fusion (supplementary material Fig. S2). The DGT expression pattern supports participation in lateral root primordium organogenesis and suggests a nuclear and a cytoplasmic function of DGT.

**DGT and E/SI-IAA9 pathways overlap partially**

Transformation of the Arabidopsis IAA14:mIAA14-GFP construct in tomato resulted in a dramatic reduction in lateral root formation (Fig. 4A) similar to that observed in Arabidopsis (Fukaki et al., 2002), indicating conservation of the SLR-governed pathway in tomato. To test whether DGT genetically interacts with the tomato SLR ortholog E, we generated a dgt e double mutant. Lateral root formation was partially restored in the dgt e background, although the appearance of root branches was much delayed (Fig. 4B). Comparing the primordium development, we found that wild-type and the e single mutant roots exhibited primordia of all stages, dgt roots rarely exhibited primordium initiation, and dgt e roots predominantly exhibited structures resembling stage I primordia (Fig. 4C). On exposure to 5 µM IAA for 40 h, the wild type and the e mutant roots formed lateral root primordia close to the root apex as expected, whereas dgt and the dgt e double mutant were equally insensitive to the treatment (Fig. 4D). Thus, downregulation of E partially restored the primordium initiation in dgt but not the primordium outgrowth and the ability to respond to exogenously applied auxin.

**Grafting of a wild-type shoot partially restores the lateral root defect in dgt**

Because shoot-derived auxin is known to stimulate the outgrowth of lateral root primordia in Arabidopsis (Bhalerao et al., 2002), and application of the auxin transport inhibitor NPA was shown to
inhibit lateral root formation (Reed et al., 1998), we tested whether grafting of a wild-type scion would improve the lateral root formation in the dgt rootstock. Seedlings were grafted at the middle of the hypocotyl as soon as they germinated. At this stage, neither wild type nor dgt had root branches, and the primary wild-type root had on average seven primordia, whereas primordia were very rare in dgt. Older soil-grown dgt plants also had root systems much smaller than those in wild type. Grafted seedlings were analyzed for root development at 12 days post-grafting (Fig. 5). As expected, root development was minimal in self-grafted dgt seedlings compared with self-grafted wild-type plants (Fig. 5A). The grafting of a dgt scion onto a wild-type rootstock did not affect the root development. However, when a wild-type scion was grafted onto a dgt rootstock, the development of the dgt rootstock was significantly improved, confirming an earlier report (Zobel, 1973). Histological markers in the differentiation zone were improved, including asymmetric pericycle cell division, early-stage primordium formation and DR5 expression in primordia (Fig. 5B). At the root tip, DR5 expression was missing in vascular cells of self-grafted dgt plants, and was restored in some of the plants with a dgt rootstock grafted on a wild-type shoot (Fig. 5C). Furthermore, root growth in a dgt1-1 (AC background) rootstock was restored upon grafting of an ethylene overproducing Epinastic (Epi) mutant scion (VFN8 background) and e mutant scion (AC background) but not dgt-dp (Chatham background), indicating that the effect was a property of the DGT protein and not the genetic background used for grafting (supplementary material Fig. S3). Thus, grafting improved the auxin responses and lateral root formation in the dgt rootstock, consistent with participation of a mobile signal. We therefore tested whether DGT could move from the shoot into the root in grafted plants. An Arabidopsis cyclophilin A antibody (Lippuner et al., 1994) detects DGT in wild-type tissues but not in dgt tissues in western blots (Oh et al., 2006). Using this antibody, we could not detect any DGT signal in dgt rootstocks grafted on wild-type scions (n=5 plants) (supplementary material Fig. S4), ruling out the possibility that DGT movement restored the lateral root formation in grafted dgt rootstocks.

**Measurements of auxin transport detect abnormal PAT fluxes in dgt**
To investigate defects in PAT in dgt, transport of radiolabelled IAA was assayed. Root IAA transport from the root-shoot junction to the root tip (root-ward) was increased in dgt (Fig. 6A), whereas transport from the root tip toward its base (shoot-ward) was decreased (Fig. 6B). By comparison, movement of benzoic acid (BA), assayed as a diffusion control, was unchanged between dgt and wild type (Fig. 6A,B). Using an IAA-specific microelectrode, we then analyzed the IAA influx velocity along the root tip. In wild type, the transition between the meristem and elongation zone was at 0.85±0.06 mm from the root apex; in dgt it was at 0.58±0.04 mm (Fig. 6C). An IAA influx peak averaging 188 fmoles cm⁻² s⁻¹ was recorded in this zone in wild type that was dramatically reduced to 106 fmoles cm⁻² s⁻¹ in presence of NPA, as expected (Fig. 6C,D). In dgt, the IAA influx peak averaged only 98 fmoles cm⁻² s⁻¹, comparable to that in NPA-treated wild-type roots, and even more strikingly was completely unaffected by the presence of NPA (Fig. 6C,D). Thus, the dgt root tip seems to be inefficient in generating an IAA reflux loop at the transition zone and supplying auxin into vascular cells involved in lateral root formation.

**Modulating the DGT level results in changes in cellular IAA efflux, and subcellular localization and functionality of PIN auxin transporters**
Protoplasts prepared from dgt leaves had an increased IAA efflux compared with wild type, indicating that DGT is a negative PAT regulator at the cellular level (Fig. 7A). To separate the effect of DGT on PAT from that on auxin signaling, we then used a yeast (Saccharomyces cerevisiae) auxin-transport system. HA-DGT had no effect on its own, but reduced Arabidopsis PIN2-driven and synergistic ABCB1/PIN1-mediated IAA efflux, apparently acting
in line with the above-described function as a negative regulator of auxin efflux (Fig. 7B). HA-DGT had no significant effect on ABCB1 alone (Fig. 7B), indicating that DGT might act preferably as a negative regulator of PIN transporters. Because PIN1 is not functional in S. cerevisiae without ABCB1 (Blakeslee et al., 2007; Kim et al., 2010), we re-tested the effect of DGT on PIN1 in a tobacco (Nicotiana benthamiana) leaf transport system (Henrichs et al., 2012). Analogous to the yeast system, a mCherry-DGT fusion had a negative effect on PIN1-driven IAA efflux but no significant effect on ABCB1-driven IAA efflux, demonstrating a preferential regulation of PIN transporters (Fig. 7C).

**Fig. 4.** Partial overlap between DGT and ENTIRE (E)/SI-IAA9 pathways. (A) Inhibition of lateral root formation in tomato upon expression of Arabidopsis IAA14:mIAA14-GFP construct. (B) Comparison of wild-type and mutant phenotypes. Lateral root formation is partially restored in dgt e mutant compared with dgt. (C) Quantification of primordia stages: ~3-cm-long root apices were excised from each genotype and primordia quantified after root clearing. The percentages of stage I primordia are indicated. Only two primordia, both from stage I, were found in 20 dgt roots. Data are mean±s.e.m. (D) Inability of dgt and dgt e mutants to form primordia upon treatment with IAA (5 µM for 40 h). Morphogenesis of primordia in the transition zone in wild type and e is marked with asterisks. The larger cell size in dgt and dgt e indicates that the IAA treatment did not suppress cell elongation in these genotypes. Scale bar: 30 µm in D.

**Fig. 5.** Grafting of a wild-type shoot partially restores development in the dgt rootstock. (A) Root system phenotypes in grafted tomato seedlings. Arrowheads indicate the sites of the graft junctions. The tissue identity is indicated. (B) Improvement of primordium organogenesis in dgt roots upon grafting of a wild-type scion. (C) DR5 expression in root tips of grafted plants. n (dgt/dgt)=10 roots from eight plants, n (dgt/wt)=11 roots from three plants, n (wt/dgt)=14 roots from five plants, n (wt/wt)=11 roots from three plants. The number of roots with DR5 expression in vascular tissues is indicated. Scale bars: 10 mm in A; 30 µm in B; 50 µm in C.

TargetP searches (at http://www.cbs.dtu.dk/services/TargetP/) did not reveal any canonical subcellular localization signals in DGT, with the exception of a potential palmitoylation signal at the C terminus; such signals are important for protein targeting to the plasma membrane and/or interactions with membrane proteins. To explore how DGT could functionally affect auxin transporters, we analyzed the colocalization of DGT with Arabidopsis PIN1 and ABCB1 upon co-expression in N. benthamiana leaves. When expressed alone, DGT localized predominately in the nucleus, in addition to signals in the cytoplasm and the cell periphery as expected; PIN1 and ABCB1 localized predominately to the plasma membrane, consistent with previous results (Henrichs et al., 2012) (Fig. 7D). When DGT was co-expressed with ABCB1, the localization of ABCB1 did not change significantly, but most of the DGT signal disappeared from the nucleus and appeared on the cell periphery (Fig. 7E), suggesting that ABCB1 may directly or indirectly affect localization and putative nuclear and cytoplasmic function of DGT. When DGT was co-expressed with PIN1, the localization of both proteins was modified:
a significant proportion of PIN1 shifted from the plasma membrane to the nuclear periphery, whereas DGT increased on the cell periphery (Fig. 7F). The PIN1 internalization following DGT co-expression explains the negative effect of DGT on PIN-driven auxin efflux, and the lack of DGT effect on ABCB1 localization is in line with unchanged ABCB1-driven auxin efflux (Fig. 7B,C). Together, the data supported a function of DGT in PAT that was independent of auxin signaling, and identified distinct interactions of DGT with different types of auxin transporters.

**DGT affects PIN expression and localization to the plasma membrane at the root tip**

We analyzed the expression of PIN mRNAs in the apical 1 cm region of the root. We found no significant change in tomato PIN1, b levels, whereas the expression of PIN2 was reduced (Fig. 8A). We also analyzed the PIN protein behavior using Arabidopsis PIN1 and PIN2 antibodies. Tomato PIN1 and PIN2 showed a typical...
localization known from Arabidopsis, with PIN1 localizing on the lower/rootward face of cells in central tissues, and PIN2 on the upper/shootward face of cells in more peripheral tissues (Fig. 8B,C). Notably, PIN1 signals were essentially missing in stele tissues at the dgt root tip (Fig. 8B). We could not assess the PIN1 plasma membrane localization in those cell files due to the low expression level. In the cell files where PIN1 was normally present its plasma membrane localization appeared normal (Fig. 8B, inset). By contrast, PIN2 plasma membrane localization was modified showing a notably broader localization domain with ‘fuzzy’ appearance in the wild type when compared with a narrow more-compact signal in dgt (Fig. 8C, inset). The PIN2 signal distribution along the membrane (measured in pixels) was similar in wild type and dgt (17.2±2.7 vs. 16.2±3.3, respectively) but the distribution of the signal across the plasma membrane reached 5.0±1.4 in wild type and only 2.4±0.9 in dgt (P=6.17519E-36; mean±s.d.; n=266 cells from 13 roots in wild type and 191 cells from eight roots in dgt). In the wild type, the mean PIN2 signal intensity at the plasma membrane was 60.64±0.9043 and inside the cell it was 51.12±0.8276 (ratio inside/PM: 0.7646), whereas in the dgt roots the PIN2 signal at the membrane was 54.55±1.099, and inside the cell it was 42.22±0.9123 (ratio inside/PM: 0.7663). Thus, the PIN2 signal in dgt roots was overall lower but more sharply defined at the plasma membrane, and the proportion of PIN2 allocated to the plasma membrane was significantly higher (P=0.05).

In an attempt to analyze the subcellular trafficking of PIN2, we also tried treatments with the trafficking inhibitor BFA that in Arabidopsis interferes with the constitutive endocytic recycling of PIN proteins to the plasma membrane and leads to PIN internalization (Geldner et al., 2001; Kleine-Vehn et al., 2008). The BFA treatment in tomato roots was ineffective as we did not see the typical ‘BFA compartments’ with internalized PIN proteins, as observed in Arabidopsis, presumably due to different arrangements of BFA-sensitive and -insensitive ARF GEFs in tomato when compared with Arabidopsis. Altogether, the results show that the dgt mutation affects the PIN expression domain and expression level, as well as the plasma membrane localization of PIN proteins.

**DISCUSSION**

Our results show that DGT is required for the generation of PAT-driven auxin maxima that are essential for lateral root formation. Two earlier works reported unchanged PAT in dgt (Daniel et al., 1989; Muday et al., 1995). However, it is important to note that both studies detected increased transport of radiolabelled IAA in dgt hypocotyls (Daniel et al., 1989, Fig. 2) and from the root base towards the root tip (Muday et al., 1995, Fig. 8) but interpreted this as ‘normal’. We observed increased root transport from the root-shoot junction to the root tip and decreased transport from the root tip toward the root base, demonstrating clearly abnormal PAT fluxes and explaining our earlier findings of increased auxin level and abnormal distribution along the dgt root tip (Ivanchenko et al., 2006). Although more auxin moves from the aerial parts of dgt into the root, it is abnormally distributed and no response maxima occur in stele tissues related to lateral root initiation. Low auxin supply into the stele of dgt is evident from low expression of auxin-responsive DR5, IAA2 and PIN1 signals, and inability of the e/sl-iaa9 mutation to restore the outgrowth of lateral root primordia. The increased PAT in the dgt shoot might result from increased cellular efflux, occur in response to PAT deficiencies in the root, or be related to a putative yet unknown function of DGT in leaves, whereas the decreased haispetal PAT at the root tip correlated with decreased expression of PIN2.

The root tip is the most dynamic root region with respect to PAT. In the tip, auxin is moved down the vascular tissues mainly by PIN1, and redirected at the transition zone from peripheral into vascular tissues in a ‘reflux loop’ by PIN2, PIN3 and PIN7, providing stable auxin circulation through the meristem (Bilou et al., 2005). The IAA influx peak recorded at the dgt transition zone with an IAA-specific microelectrode was reduced to 50% and was insensitive to NPA, a potent auxin-efflux inhibitor, consistent with inefficient IAA supply into vascular cells. This defect was much greater than those reported in loss-of-function Arabidopsis twd1 mutant (Bouchard et al., 2006; Wang et al., 2013) and pin2 mutant in blue light conditions (Wan et al., 2012), the peaks of which average at ~80% of wild type. The more severe dgt phenotype argues that multiple transporters, PIN1, PIN2, and potentially also PIN3 and PIN7, might be regulated by DGT.

We also observed that grafting of a wild-type scion partially rescued the auxin response in the root tip vasculature of the dgt rootstock, leading to primordium initiation, and antibodies did not detect DGT movement into the rootstock. We therefore hypothesize that the rescue was achieved through improving auxin transport from the wild-type scion. A recent study in Arabidopsis has shown
that radiolabelled auxin moves down the vasculature from the shoot through plasmodesmal connections in the phloem and accumulates at the root tip, but that the signal is barely perceptible in NPA-treated plants, demonstrating a strong dependence on PAT (Bishopp et al., 2011). Thus, the simplest explanation of our grafting results is that in the grafted wild-type scion, auxin is more successfully channeled into the vasculature, allowing for movement into the vasculature of the dgt rootstock. Auxin transport from developing true leaves has been reported to stimulate the emergence of lateral root primordia (Bhalerao et al., 2002), whereas basipetal PAT from the root tip has been proposed to stimulate the primordium initiation (Casimiro et al., 2001). Our results show that, at least in tomato, the shoot is important for root primordium initiation.

In contrast to the Arabidopsis immunophilin TWD1, which has been demonstrated to act as a positive regulator of ABCB-driven auxin efflux (Bouchard et al., 2006; Wang et al., 2013; Wu et al., 2010), our functional studies implicate DGT as a negative regulator of auxin efflux that preferentially affects PIN transporters at the cellular level. First, protoplasts from dgt leaves displayed an increased IAA efflux, whereas overexpression of DGT in tobacco leaves reduced the PIN-mediated IAA efflux. Second, in a yeast-based auxin-transport system, which lacks plant-specific auxin responses, DGT co-expression still reduced the PIN-mediated IAA efflux, providing strong evidence that DGT affects PIN functionality independently of auxin signaling. As to how DGT could affect auxin transporters at the protein level, DGT reduced the PIN1 plasma membrane localization simultaneously with reducing the PIN1-mediated auxin efflux upon co-expression in tobacco leaves. This result agrees with previous observations in Arabidopsis showing that increasing PIN levels at the plasma membrane leads to elevated auxin efflux (Paciorek et al., 2005; Robert et al., 2010). Furthermore, the proportion of PIN2 on the plasma membrane versus inside the cell was also increased at the dgt root tip and displayed a sharper signal when compared with wild type. Together, the data demonstrate that DGT is implicated in a mechanism related to membrane localization of PINs. However, both PIN1 and ABCB1 were also able to modify the subcellular localization of DGT upon co-expression in tobacco leaves, suggesting complex inter-relationships among all three types of proteins. Whether DGT interacts with PINs (and ABCBs) directly or via other proteins or molecules remains to be determined. Given that the gene transcription of DGT is downregulated by auxin at the root tip (Ivanchenko et al., 2013), that PIN expression is upregulated in Arabidopsis (Vieten et al., 2005), and PIN and ABCB activities interact synergistically (Blakeslee et al., 2007), one can envision an extremely complex functional feedback between DGT level, auxin level, functionality and membrane localization of PINs, and maintenance of PAT fluxes at the plant level. This complexity is evident in the observed complex effect of the dgt mutation on the expression levels of the PIN genes and their expression domain, and on PIN protein localization to the plasma membrane at root tip.

It has been shown that mutations in the rice cyclophilin gene OsCYP2 cause a similar inability to form lateral roots (Kang et al., 2013; Zheng et al., 2013) and mutations in the DGT ortholog in the moss Physcomitrella patens cause auxin-resistant phenotypes (Lavy et al., 2012), demonstrating a conservation of DGT-like function in auxin-regulated development. Several aspects of cyclophilin A function have also emerged in non-plant systems, suggesting it to be a multifunctional protein. Cyclophilin A has been linked to regulation of protein activity (Brazin et al., 2002; Colgan et al., 2004), protein interactions (Zander et al., 2003; Sorin and Kalpana, 2006) and protein trafficking (Ansari et al., 2002; Galigniana et al., 2004). Another suggested function of yeast and mammalian cyclophilin A is regulation of gene expression at the level of chromatin folding (Arévalo-Rodríguez et al., 2000; Pijnappel et al., 2001; Arévalo-Rodríguez and Heitman, 2005; Lu et al., 2006). Our findings demonstrate a role of a plant cyclophilin A in polar auxin transport. Some phenotypes of the dgt mutant cannot be directly explained by a defect in auxin transport, e.g. the inability of dgt to respond to exogenously applied auxin with increased DR5 auxin reporter expression at the root tip, and the partial restoration of lateral root formation by a loss of ENTIRE (IAA9). Therefore, in addition to affecting auxin-regulated gene expression via regulating PAT, DGT could also have a direct effect on auxin signaling, or even act more broadly on gene expression, possibilities suggested by the nuclear expression of DGT. In addition, the dgt root tip shows an increased level of hydrogen peroxide (H2O2) (Ivanchenko et al., 2013), and this oxidative environment could contribute to decreasing the auxin sensitivity of dgt due to auxin oxidation, to which grafted plants might be less susceptible because of the direct auxin delivery into vascular tissues.

MATERIALS AND METHODS
Plant material and growth conditions
Wild-type tomato (Solanum lycopersicum), dgt-1 and entire (e) mutants in the Ailsa Craig (AC) background, and Arabidopsis seedlings in the Columbia 0 (Col) background were used unless otherwise stated. The dgt-1 and dgt-dp tomato mutant alleles (Oh et al., 2006), the e mutant (Zhang et al., 2007), Epi mutant (Fujino et al., 1988), transgenic tomato DR5::GUS line (Dubrovsky et al., 2008), tomato IAA2::GUS line (Dubrovsky et al., 2011) and IAA14-mIAA14::GFP construct (Fukaki et al., 2002) have been reported. Tomato and Arabidopsis seedlings were grown in 0.2× MS agar medium with vitamins (PhytoTechnology). Indole-3-acetic acid (IAA) (Sigma), N-naphthalene-3-acetic acid (NAA) (Sigma), 2,4-dichloro phenoxyacetic acid (2,4-D) (Sigma) and NPA (Chem Service) were used at concentrations and exposure times as indicated. For grafting, tomato seedlings were germinated in vermiculite moistened with 0.2× MS liquid medium and grafted as described in Arabidopsis (Turnbull et al., 2002).

Cloning procedures and plant transformation
For the DGT::GUS construct, the 5′ flanking region of the DGT gene from 1389 to +36 bp was cloned between the KpnI and BamHI sites in a pCAMBIA1300 vector (http://www.cambia.org), and the GUS-coding sequence between the BamHI and SalI sites. A TGA stop codon was introduced at the end of GUS. For the DGT::mCherry-DGT construct, the 5′ region of the DGT gene was cloned between the HindIII and SalI sites of pCAMBIA1300, and an mCherry-DGT in-frame fusion was introduced between the SalI and BamHI sites. For expression in N. benthamiana leaves, the mCherry-DGT fusion was amplified by PCR and cloned under 35S constitutive promoter promoter between BamHI and SpeI sites in pCB302-3. For yeast expression, HA-DGT fusion was generated by PCR and cloned between BamHI and SalI sites in pRS314CUP. Arabidopsis transformation was performed by the floral dip method, and tomato transformation as described previously (Ivanchenko et al., 2006).

Histological analyses and microscopy
GUS staining was performed as described previously (Ivanchenko et al., 2006) and western blot as described previously (Oh et al., 2006). For meristem and lateral root primordium analyses, roots were cleared as described previously (Malamy and Benfey, 1997), and mounted in saturated chloral hydrate solution in 10% glycerol. For tissue sectioning, roots stained for GUS were imbedded in Technovit 7100 (Electron Microscopy Sciences). Root samples were analyzed under a Zeiss Axiovert microscope with differential interference contrast (DIC) optics. Confocal microscopy in Arabidopsis roots and N. benthamiana leaves was performed using an inverted Zeiss LSM 510 Meta (Carl Zeiss) microscope with ×63 (NA 1.2, C-Apochromat) objective with water immersion.
Auxin transport assays
Root acropetal (root-shoot junction to root tip) and basipetal (root tip to root base) PAT measurements were performed as described previously (Lewis and Muday, 2009). Continuous recordings of IAA fluxes at the root apex with a self-referencing IAA-specific microelectrode were performed as described previously (Mancuso et al., 2005). For NPA response, plants were treated with or without 5 μM NPA for 2 h. Yeast IAA transport was performed as described previously (Kim et al., 2010). Relative export from yeast is calculated from retained radioactivity as follows: (radioactivity in the yeast at time t=10 min) − (radioactivity in the yeast at time t=0)/(100%) (radioactivity in the yeast at t=0 min); mean values from four independent experiments are presented. IAA export from N. benthamiana leaf tissue was analyzed as described previously (Mračev et al., 2009; Henrichs et al., 2012). Tomato protoplast assays were conducted as for tobacco, except that enzyme digestion was performed overnight at room temperature. Relative export from protoplasts is calculated from exported radioactivity as follows: (radioactivity in the protoplasts at time t=x) − (radioactivity in the protoplasts at t=0)/(100%) (radioactivity in the protoplasts at t=0 min); mean values from four independent experiments are presented.

Quantification of PIN mRNA expression
RT-qPCR was performed as described previously (Ivanchenko et al., 2013). Primers for tomato PIN1a (Bayer et al., 2009), PIN1b (Acc. HX127074) and PIN2 (Acc. HX127077) were designed to include part of the 3′ UTRs: PIN1a F 5′-AGGACAGGGTCATATTTGG, R 5′-TCTCCATGCCAATTGCT; PIN2 F 5′-TCTCGACATTCTTAGCACAGC, R 5′-TTTACCTGACATTCTTAGCACAGC, R 5′-TTA-AGCACAGGGTTCATATTTGG, R 5′-ACCTCACATCTACACAGAAGG. RT-qPCR was performed as described previously (Ivanchenko et al., 2013). Mean values from four independent experiments are presented.

Analyses of PIN expression at the root tip
Roots from 8-day-old tomato seedlings were probed with Arabidopsis anti-PIN1 or -PIN2 primary antibody (1:1000) and Cyanine Dye3 (Cy3)-conjugated anti-rabbit secondary antibody (1:600) (Sigma) following a whole-mount procedure as described for Arabidopsis (Sauer et al., 2006). Images were acquired using a Zeiss LSM 700 upright confocal microscope. To quantify PIN2 distribution in root epidermal cells of wild type and mutants, and performed grafting experiments. J.Z. and B.W. performed PAT measurements in tomato, N. benthamiana and S. cerevisiae auxin transport assays, and confocal microscope imaging of protein localization. E.M. analyzed PIN1 and PIN2 expression in tomato root tips. Y.D. analyzed PIN expression in response to BFA treatment. E.A. and S.M. performed measurements of IAA fluxes with an IAA-specific microelectrode. M.M. performed statistical analysis of PIN expression in BFA-treated roots. S.F. performed RT-qPCR of PIN mRNA expression. J.G.D. performed confocal microscope analyses of DGT:mCherry expression in Arabidopsis roots. M.G.I. wrote the paper with inputs from M.G. and J.F. All authors participated with data analysis and interpretation.

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Supplementary material
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AtPGP4 is involved in auxin-mediated lateral root and root hair development.


Supplementary Figure S1. Lack of gravitropic response and asymmetric expression of *IAA2:GUS* auxin reporter expression in *dgt*. (A) Images of seedlings gravistimulated at 135° for 16 h. (B) Images of roots stained for GUS. Scale bars: (A) 1 cm, (B) 100 µm.
Supplementary Figure S2. Restoration of lateral root formation in dgt upon transformation of DGT:mCherry-DGT construct. (A) Image of a transformed seedling. (B) Restoration of root branching correlates with red fluorescence confirming presence of the mCherry-DGT protein. Scale bar: (B) 100 µm.
**Supplementary Figure S3. Restoration of lateral root formation in dgt upon grafting is not affected by the genetic background.** Root growth in dgt1-1 (AC background) rootstock was restored upon grafting of Epi mutant scion (VFN8 background) and e mutant scion (AC background) but not dgt-dp (Chatham background).
Supplementary Figure S4. Western blot of DGT with anti-cyclophilin A antibody with root tips of grafted plants. The tissue identity in the different combinations is indicated. An arrowhead denotes the position of DGT. No DGT protein was detected in *dgt* rootstock grafted on *dgt* scion or wild type scion.