Accumulation of glycolipids and other non-phosphorous lipids in Agrobacterium tumefaciens grown under phosphate deprivation

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Phosphate deficiency is characteristic for many natural habitats, resulting in different physiological responses in plants and bacteria including the replacement of phospholipids by glycolipids and other phosphorous-free lipids. The plant pathogenic bacterium Agrobacterium tumefaciens, which is free of glycolipids under full nutrition, harbors an open reading frame (ORF) coding for a processive glycosyltransferase (named as Pgt). This glycosyltransferase was previously shown to synthesize glucosylgalactosyldiacylglycerol (GGD) and digalactosyldiacylglycerol (DGD) after heterologous expression. The native function of this enzyme and the conditions for its activation remained unknown. We show here that Pgt is active under phosphate deprivation synthesizing GGD and DGD in Agrobacterium. A corresponding deletion mutant (Δpgt) is free of these two glycolipids. Glycolipid accumulation is mainly regulated by substrate (diacylglycerol) availability. Diacylglycerol and the total fatty acid pool are characterized by an altered acyl composition in dependence of the phosphate status with a strong decrease of 18:1 and concomitant increase of 19:0 cyco during phosphate deprivation. Furthermore, Agrobacterium accumulates two additional unknown glycolipids and digalactosyldiacylglycerol (DGTs) during phosphate deprivation. Accumulation of all these lipids is accompanied by a reduction in phospholipids from 75 to 45% in the wild type. A further non-phosphorous lipid, ornithine lipid, was not increased but its degree of hydroxylation was elevated under phosphate deprivation. The lack of GGD and DGD in the Δpgt mutant has no effect on growth and virulence of Agrobacterium, suggesting that these two lipids are functionally replaced by DGTS and the two unknown glycolipids under phosphate deprivation.

Keywords: cyclopropane fatty acid / diacylglycerol trimethylhomoserine / digalactosyldiacylglycerol / glycosyltransferase / ornithine lipid

Introduction
The availability of phosphate is often a limiting factor for growth in natural habitats. Plants, algae and different bacteria have developed different mechanisms to cope with phosphate limitation, e.g. by replacement of phospholipids with glycolipids and other non-phosphorous lipids to save phosphate required for nucleotide synthesis and maintenance of other important physiological processes. The lipid changes in response to phosphate deprivation have been well characterized in plants, algae and cyanobacteria, all of which show an increased synthesis of the two glycolipids digalactosyldiacylglycerol (DGD) and sulfuroinosyldiacylglycerol (SQD) (see review Hözl and Dörmann 2007). The impact of phosphate deprivation on the lipid composition of bacteria has been studied for only a few species. In contrast to plants and cyanobacteria, other bacteria are characterized by a higher variety of glyco- and other non-phosphorous lipids (Hözl and Dörmann 2007). In the two Gram-negative bacteria Rhodobacter sphaeroides and Sinorhizobium melloti (Benning et al. 1993, 1995; López-Lara et al. 2005) the synthesis of SQD and ornithine lipid (OL) is strongly increased during phosphate limitation. OL is a glycerol-free membrane lipid with ornithine linked to a hydroxy fatty acid via an amide bond and a second fatty acid esterified to the hydroxy group of the hydroxy fatty acid. The two organisms also synthesize a further non-phosphorous lipid, i.e. diacylglycerol trimethylhomoserine (DGTs), which is only present under phosphate deprivation. Additionally, Rhodobacter accumulates a unique glycolipid, glucosylgalactosyldiacylglycerol (GGD), with α-glucose (1→4)-linked to β-galactose. Recently, the lipid response of the gram-negative bacterium Mesorhizobium loti was investigated in detail (Devers et al. 2011). Mesorhizobium loti is a nitrogen-fixing bacterium with Lotus japonicus as its host. It shows a very complex lipid composition when grown under phosphate deprivation by accumulating a series of di-
triglycosyldiacylglycerols with different combinations of glucose and galactose in their head groups. The DGD and GGD from *Mesorhizobium* differ from DGD and GGD from plants and *Rhodobacter*, respectively, by their glycosidic linkages and anomeric configurations. The sugars of the *Mesorhizobium* glycolipids are bound in β-anomeric configuration and (1→6)-linked to each other (Hölzl et al. 2005). They are synthesized by one enzyme, characterized as a processive glycosyltransferase. Besides, two further glycolipids with unknown head group structures were detected in *Mesorhizobium*, which are synthesized by unknown glycosyltransferases. A further important surrogate lipid for phospholipids is DGTS. OL may also play a role in the lipid response to phosphate deprivation. It was speculated that DGTS and glycolipids are mutually interchangeable in *Mesorhizobium*.

The Gram-negative bacterium *Agrobacterium tumefaciens* is a soil-borne plant pathogen and represents one of the most important tools for the production of transgenic plants in plant biotechnology. A processive glycosyltransferase (named as Pgt) with homology to the glycosyltransferase from *Mesorhizobium* was also described for *Agrobacterium* (open reading frame, ORF, Atu1808). This enzyme which was analyzed by heterologous expression and by overexpression in *Agrobacterium*, synthesizes DGD and GGD with the same head group configuration as shown for *Mesorhizobium* (Hölzl et al. 2005). The formation of higher glycosylated glycolipids was not reported. As *Agrobacterium* is free of glycolipids when grown under optimal conditions, the in vivo function of this glycosyltransferase remained unknown, and lipid analyses of phosphate-deprived cells were never reported.

*Agrobacterium* contains the phospholipids phosphatidylethanolamine (PE), monomethyl-PE (MMPE), phosphatidyglycerol (PG) and di-PG or cardiolipin (CL) as main membrane lipids (Das et al. 1979). There were no studies on the occurrence or quantification of OL or DGTS, but different publications report on the existence of genes coding for enzymes involved in OL and DGTS synthesis in *A. tumefaciens* (López-Lara et al. 2003; Gao et al. 2004; Riekhof et al. 2005; Vences-Guzmán et al. 2011).

To investigate the in vivo function of Pgt encoded by the ORF Atu1808, we analyzed the lipid composition of the wild type and the corresponding deletion mutant grown under phosphate-replete and phosphate-depleted conditions, including the changes in fatty acid profiles during growth. Further, we investigated the regulation of Pgt activity with enzyme assays and determined the DAG content in the cells. Finally, we tested the capacities of the wild type and the deletion mutant to infect tobacco leaf discs.

**Results**

**Pgt from Agrobacterium is active under phosphate starvation and synthesizes DGD and GGD**

It was previously shown that *Mesorhizobium* can synthesize different glycolipids under phosphate starvation (Devers et al. 2011). Therefore, we tested whether *Agrobacterium* displays an analogous phosphate deprivation response. To this end, wild type and Pgt deletion mutant (Δpgt) cells (created in this study) were grown in a liquid medium with or without phosphate and lipids analyzed via thin-layer chromatography (TLC). Phosphate starvation led to the accumulation of several glycolipids (Figure 1). The glycolipid pattern was more complex when compared with the previous study where the *Agrobacterium* Pgt was heterologously expressed in *E. coli* or overexpressed in *Agrobacterium* (Hölzl et al. 2005). Two of the lipids, only present in phosphate-deprived wild-type cells, were identified as DGD and GGD (Figure 1). Two further, but unknown glycolipids (U1 and U2) were detected both in the wild type and the Δpgt mutant. Phosphate starvation led to reduced growth of *Agrobacterium* cells, but the extent of growth reduction was similar for wild type and Δpgt (Figure 2). Therefore, the two
glycolipids GGD and DGD are not essential for growth under phosphate starvation.

**Glycolipids and DGTS replace phospholipids in Agrobacterium grown under phosphate starvation**

Agrobacterium wild-type cells grown under full nutrition synthesize different phospholipids PE, MMPE, DMPE, PC, PG and CL and two forms of OL, denoted as OL1 and OL2 (Figure 3A). DMPE was only present in traces. The two OL forms were analyzed via quadrupole time-of-flight (Q-TOF) mass spectrometry (MS; Supplementary data, Figure S1). OL2 was identified as the hydroxylated form of OL1. Our results are consistent with the hydroxy group linked to the ornithine head group. The glycolipids GGD, DGD, U1 and U2 and a further non-phosphorous lipid identified as DGTS (via Q-TOF MS, Supplementary data, Figure S2) accumulate only under phosphate starvation (Figure 3B).

The synthesis of different glycolipids and other non-phosphorous lipids in Agrobacterium suggests a role of these lipids during phosphate starvation as surrogates for phospholipids as shown for several other organisms (Hölzl and Dörmann 2007; Devers et al. 2011). We quantified all polar lipids from *Agrobacterium* wild type and Δpgt as described in Devers et al. (2011). As GGD and DGD were poorly separated and their ratio varied in the lipid extracts from different cultures, these two lipids were quantified together. Under full nutrition, the wild type accumulates about 75% phospholipids and 25% OL, with OL1 being the predominant form. OL2 was below 1% (Figure 4A). The same lipid composition was observed for Δpgt. Under phosphate starvation, the Pgt-dependent glycolipids GGD and DGD amount to 5–6% in the wild type. The Pgt-independent glycolipids U1, U2 and DGTS were found in the wild type and Δpgt comprising 2, 8 and 20%, respectively. The sum of all glycolipids and DGTS is in the range of 35% in the wild type. The accumulation of these lipids is accompanied by a reduction in phospholipids to 45%. An intriguing alteration was observed for OL. OL2 is strongly increased by a factor of 9, whereas OL1 is reduced during phosphate deprivation in wild type and Δpgt.

**Pgt overexpression has almost no effect under full nutrition but leads to a strong increase in DGD/GGD and a decrease in DGTS, U1 and U2 under phosphate deprivation**

The loss of Pgt-dependent glycolipids has no major effect on the composition of the remaining lipids in the Δpgt mutant. The mutant lipid pattern is, apart from the lack of DGD and GGD, similar to the wild-type lipid composition. Therefore, we overexpressed Pgt in *Agrobacterium* to study the effect of an increase in DGD and GGD on the regulation of the amounts of the remaining membrane lipids. For this purpose, wild type and Δpgt were transformed with the inducible Pgt expression vector pTnVagro (Hölzl et al. 2005). Pgt overexpression is induced with IPTG and thus independent from phosphate supply. Therefore, we would expect similar Pgt activities in Pgt overexpression cultures grown under phosphate-replete and phosphate-depleted conditions. However, lipid measurements showed remarkable differences in the accumulation of GGD and DGD. Under full nutrition, Pgt overexpression had almost no effect. The overexpressers accumulate very low amounts of GGD and DGD (1–2%, Figure 4B). In contrast, phosphate starvation led to the accumulation of high amounts of GGD and DDG (~25%, Figure 4B), which is 4–5 times more than in the non-transformed cells (Figure 4A). Furthermore, DGTS seems to be the counterpart of GGD/DGD, because this lipid is most affected. It is reduced from 20% (Figure 4A) to ~5% (Figure 4B). U1 and U2 are reduced by a factor of 2. As observed before in non-transformed cells, the sum of all glycolipids and DGTS does not exceed 35% in the overexpressers (Figure 4A and B). Therefore, the strong accumulation of GGD/DGD has no profound effect on the amounts of phospholipids and of total OL.

In addition to Pgt overexpression, we also expressed a heterologous glucosyltransferase Ugt106B1 from *Staphylococcus aureus* in *Agrobacterium* wild type and Δpgt. The Ugt106B1 enzyme has been shown to synthesize diglucosyldiaclylglycerol (DGlcD) and higher glucosylated glycolipids (Jorasch et al. 2000). This expression led to similar results as observed for Pgt overexpression, i.e. a strong accumulation of glycolipids accompanied by the reduction in DGTS during phosphate deprivation, showing that other glycolipids like DGlcD can also participate in the phosphate starvation-dependent lipid response (Supplementary...
All these data indicate that the synthesis of glycolipids and DGTS is interconnected, and moreover, the synthesis of these lipids is regulated, probably on a substrate level.

Phosphate starvation leads to characteristic changes in the fatty acid profile of Agrobacterium wild type and Δpgt

To analyze the effect of phosphate starvation and the loss of glycolipids on the fatty acid profile of Agrobacterium, total lipids of wild type and Δpgt were analyzed. On the one hand, we investigated the fatty acid profiles of cultures previously used for lipid quantification; on the other hand, we determined the changes in the fatty acid compositions during the growth of the cultures used for growth curves (Figure 2).

Eight different fatty acids were identified in Agrobacterium. Table I shows the fatty acid composition of cultures as used

<table>
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<th>Fatty acid</th>
<th>Wild type</th>
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<tr>
<td></td>
<td>P+</td>
<td>P−</td>
</tr>
<tr>
<td>14:0</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>16:0</td>
<td>9.9 ± 0.6</td>
<td>16.1 ± 0.6</td>
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<tr>
<td>16:1</td>
<td>1.4 ± 0.5</td>
<td>tr</td>
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<tr>
<td>17:0 cylo</td>
<td>tr</td>
<td>3.5 ± 0.2</td>
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<tr>
<td>18:0</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>18:1</td>
<td>81.7 ± 1.4</td>
<td>27.7 ± 2.1</td>
</tr>
<tr>
<td>18:1-Me</td>
<td>tr</td>
<td>1.0 ± 0.2</td>
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<tr>
<td>19:0 cylo</td>
<td>6.6 ± 0.9</td>
<td>50.7 ± 3.1</td>
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Values are given in mol% and represent the mean of three independent measurements ± SD. Data of hydroxylated fatty acids are not included. tr, trace amounts (<1.0%).
for lipid quantification. The predominant fatty acids were 16:0, 18:1 and 19:0 cyclo, which altogether account for more than 95%. Minor fatty acids were 16:1 and 17:0 cyclo, which constitute ~4% or less; 14:0, 18:0 and 18:1-Me were only found in trace amounts. These results as shown in Table I represent a snapshot because during the time of growth there is a considerable alteration in the fatty acid profiles of the different cultures (Figure 5). At the beginning of the growth curves, all cultures were characterized by high amounts of 18:1 (~65%) and lower amounts of 19:0 cyclo (~15%) and 16:0 (~14%). The entry into the logarithmic phase (Figure 2) led to a clear increase of 18:1 and a concomitant reduction of 19:0 cyclo. This trend is continued during the logarithmic phase in cultures grown under phosphate-rich conditions with a maximum of 85% for 18:1 and a minimum of 3.5% for 19:0 cyclo in the wild type. In contrast, the transition into the stationary phase (after 1 day) led to a continuous decrease of 18:1 and increase of 19:0 cyclo. After 2 days of growth, 18:1 reached its minimum (~50%) and 19:0 cyclo its maximum (~30%). Cells grown under phosphate starvation showed already during the logarithmic phase a strong decrease of 18:1 and to a similar extent an increase of 19:0 cyclo (Figures 2 and 5). This trend is continued during the entire growth experiment leading to a minimum of 32% and a maximum of 45% for 18:1 and 19:0 cyclo, respectively, in the wild type. 16:0 and 17:0 cyclo showed a moderate change during the growth similar to the changes of 19:0 cyclo. This fatty acid redistribution as observed in the wild type was more pronounced in Δpgt both under phosphate-replete and phosphate-depleted conditions in all our experiments (Table I, Figure 5).

In addition to total fatty acids, we further analyzed fatty acid profiles of the different membrane lipids from Agrobacterium wild type. All phospholipids of cells grown under full nutrition were characterized by large proportions of 18:1 (50–60%) and low proportions of 19:0 cyclo (20%; 40% in PC; Figure 6). The amounts of 16:0 were between 10 and 20%. Phosphate starvation led to a marked redistribution of fatty acids in the phospholipids with a considerable reduction of 18:1 (10%) and a strong increase of 19:0 cyclo (60%). 16:0 was also increased in most phospholipids (20%). Glycolipids and DGTS differ from the phospholipids because they contain similar amounts of 18:1 and 19:0 cyclo. The hydroxy-bound fatty acids in OL lipids were almost exclusively composed of 19:0 cyclo especially under phosphate starvation (the amide bound fatty acids were not analyzed, see Material and methods). Altogether, the results are in agreement with the total fatty acid analysis, suggesting that subsets of phospholipids with high 18:1 content are converted into glycolipids during phosphate starvation.

The amount of DAG is not changed during phosphate starvation but there is an alteration in its fatty acid profile

The results of Pgt or Ugt106B1 overexpression indicated that glycolipid accumulation during phosphate deprivation is presumably regulated via substrate availability. To further our understanding of the regulation mechanism, we analyzed the fatty acid profiles and absolute amounts of DAG in cells grown with or without phosphate. We used wild type, Δpgt
and the Pgt overexpression line for these studies. The DAG content and composition were measured by Q-TOF MS and normalized to cell density [equal volumes of 50 mL of cells with the optical density (OD_{600}) of 1.0]. We observed no differences in the total amounts of DAG in the different cell lines or in cells grown under phosphate-replete or phosphate-depleted conditions. The amounts of DAG were in the range of 1.1–1.3 nmol (Figure 7). In contrast to the membrane lipids where 16:1, 17:0, 18:0 and 18:1-Me were detected in trace amounts, these fatty acids were enriched in DAG. We observed considerable differences in the molecular species composition of DAG depending on the growth conditions. Most DAG species containing 18:1 were decreased in cells grown under phosphate starvation. Most of the remaining DAG species were increased, especially those containing 19:0 cyclo. This shift from 18:1 to 19:0 cyclo in DAG is consistent with the data previously obtained for total fatty acids or for phospholipids (Table I, Figures 5 and 6). Our data suggest that phosphate starvation leads to the provision of DAG with a different fatty acid profile, which may serve as a substrate for the biosynthesis of glycolipids and DGTS.

Enzyme assays suggest a high transcription in Pgt overexpressers and a basal Pgt transcription under phosphate-replete conditions

To get further insights into the regulation of glycolipid accumulation, enzyme assays with protein extracts isolated from Agrobacterium cells grown with or without phosphate were performed. We tested wild type, ∆pgt and Pgt overexpressers. The fluorescent ceramide (N-erythro-N[6-amino-N′-7-nitrobenzo-2-oxa-1,3,5-triazolo]-hexanoyl]-ceramide) (NBD-Cer) was used as a sugar acceptor, which is an appropriate substrate for Pgt (Hölzl et al. 2005), and UDP-Gal as a sugar donor. The reaction products monogalactosyl-NBD-Cer (MG-Cer) and digalactosyl-NBD-Cer (DG-Cer) were separated via TLC and visualized under UV light. Their structures were confirmed by MS (Q-TOF). The in vitro activities were quantified densitometrically, and relative values were calculated. As expected, MG-Cer synthesis was detected in the assay with protein derived from wild-type cells grown under phosphate starvation (Figure 8). But we also observed a weak in vitro Pgt activity in wild-type cells grown under phosphate-replete conditions. The latter result suggests a basal transcription of Pgt under full phosphate conditions, even though we could never detect any glycolipids in cells grown with phosphate. For quantification, this basal activity was set to the relative value 1. Our measurements show that phosphate starvation led to an increase in the Pgt-activity by a factor of 7 (Figure 8). No Pgt activity was observed in ∆pgt. Compared with the wild type, the Pgt activities in the overexpresser were clearly higher (23 times under phosphate-replete and 17 times under phosphate-depleted conditions in relation to the basal Pgt activity, Figure 8). This highly increased Pgt activity led to the additional formation of DG-Cer, which was not detected in assays with protein from wild-type cells. All the same, the high Pgt transcription in these cells does not lead to remarkable accumulation of glycolipids in vivo under phosphate-replete conditions as shown in Figure 4, reinforcing our assumption of the regulation of glycolipid accumulation via substrate availability.

The role of Pgt-dependent glycolipids for plant–pathogen interactions

To investigate the role of Pgt-dependent glycolipids for the virulence of Agrobacterium, tobacco leaf discs were infected with wild type or ∆pgt containing binary plant expression vectors. The successful infection should lead to callus formation on the leaf discs after selection with the appropriate selection markers. In a semi-quantitative way, the numbers of calli on the leaf material can be taken as an indication for virulence of the respective Agrobacterium strains. The plants and bacteria were grown under phosphate-replete or phosphate-depleted conditions. In a first experiment, the leaf discs were separately infected with wild type or with the ∆pgt mutant. After 6 weeks of incubation, the formation of calli was analyzed. The two strains were able to cause callus formation under phosphate-replete or phosphate-depleted conditions. There were no significant differences in the number of calli formed by wild type or the ∆pgt mutant (data not shown). Phosphate deficiency led to a reduced number and decreased size of calli independent from the respective Agrobacterium strain. To investigate a more subtle role of
glycolipids during the infection process, a competitive experiment was performed between wild type and Δpgt. For this purpose, the two strains were mixed prior to leaf disc infection with equal numbers of cells. The use of plant expression vectors with different reporter genes (cyan fluorescent protein, CFP; or red fluorescent protein, DsRed) allowed the distinction of calli originating from the infection with Agrobacterium wild type or Δpgt. In the first experiment, wild type contained the CFP reporter and the Δpgt the DsRed reporter, while in the second experiment, the reporter constructs were exchanged between the two strains. The calli formed after infection were analyzed by fluorescence microscopy. Infection with Agrobacterium wild type or Δpgt under competitive conditions resulted in a comparable number of calli (data not shown). Therefore, Pgt-dependent glycolipids do not play an essential role for the virulence of Agrobacterium.

Fig. 7. DAG composition and content of Agrobacterium wild type, Δpgt and Pgt overexpresser (wild-type background) grown under phosphate-replete (+P) or phosphate-depleted (−P) conditions. Left panel, composition of molecular DAG species; right panel, total DAG content (nmol) in 50 mL of cells normalized to an OD600 of 1. Mean values of six independent measurements ±SD of one biological replicate each. We analyzed three biological replicates of each line with similar results. Arrows mark the decrease of 18:1 and increase of 19:0 cyclo containing DAG species.
Discussion

In a previous study, we identified a Pgt in Agrobacterium and Mesorhizobium, synthesizing different glycolipids after expression in different host organisms (Hölzl et al. 2005). The present study is the first report on the accumulation of glycolipids (GGD, DGD, U1 and U2) in Agrobacterium during the response to phosphate deprivation. The presence of neutral glycolipids was never before described for any members of the Rhizobiaceae. Mesorhizobium loti (Phylllobacteriaceae) responds to phosphate deprivation in a similar manner. Different related species (e.g. Rhodobacter and Sinorhizobium) accumulate the charged glycolipid SQD under phosphate starvation (Gage et al. 1992; Cedergren and Hollingsworth 1994; Benning et al. 1995), but SQD was not detected in Agrobacterium. The accumulation of U1 and U2 indicates the existence of further phosphate starvation-dependent glycosyltransferases in Agrobacterium. U1 and U2 will be characterized in a following study. Furthermore, the accumulation of DGTS in Agrobacterium is described here for the first time. DGTS is found in many organisms (Rhodobacter, Sinorhizobium, Mesorhizobium, Chlamydomonas) where it serves as a surrogate lipid for phospholipids (Giroud et al. 1988; Benning et al. 1995; Geiger et al. 1999; Vieler et al. 2007; Devers et al. 2011). OL, a further non-phosphorous lipid, was also shown to accumulate in different bacteria during phosphate starvation (Giroud et al. 1988; Geiger et al. 1999; López-Lara et al. 2005). In Agrobacterium, however, it seems to play a special role, because OL (in this study designated as OL1) is decreased, but its hydroxylated form OL2 is strongly increased during phosphate deprivation. Because the sum of OL1 and OL2 is not changed, the total OL (OL1 + OL2) does not contribute to the replacement of phospholipids in Agrobacterium during phosphate-deprived growth. The shift in the synthesis from OL1 to OL2 might rather represent a response to general stress. Hydroxylated forms of OL play a role under acid and temperature stress in Rhizobium tropici and are important for symbiosis (Rojas-Jiménez et al. 2005; Vences-Guzmán et al. 2011). We identified OL2 by Q-TOF MS. The genes encoding the OL hydroxylase involved in the synthesis of hydroxylated OL (OlsE) were recently isolated from Rhizobium and Agrobacterium (ORF Atu0318; Vences-Guzmán et al. 2011).

The OL2 MS/MS spectrum (Supplementary data, Figure S1B) is similar to the OL spectrum published by Zhang et al. (2009), with the exception of an additional hydroxy group presumably bound to the head group. The latter conclusion is based on the presence of the positive ion fragment m/z 131 presumably derived from ring closure via an amide bond of the OL head group carrying an extra hydroxy group (Supplementary data, Figure S1B). Q-TOF analysis does not allow the exact determination of the position of the hydroxyl group on the ornithine moiety. OL2 from Agrobacterium might be equivalent to the hydroxy OL previously described in Rhizobium which also carries the hydroxy group at the ornithine head group (Vences-Guzmán et al. 2011).

The loss of GGD and DGD in Δpgt or the enrichment of these lipids in Pgt overexpressers has no effect on the levels of phospholipids or OL. However, Pgt overexpression has a considerable influence on the levels of DGTS, suggesting a complementary function of DGTS for GGD/DGD. These lipids (including U1 and U2) can mutually replace each other as described for Mesorhizobium (Devers et al. 2011). This may be the reason why the loss of DGD and GGD had no effect on growth and virulence of Δpgt. The presence of different non-phosphorous lipids with redundant functions may increase the competitiveness in natural environments with changing conditions.

In Proteobacteria, the phosphate regulation of gene expression is often mediated by the PhoB protein. Under phosphate deprivation, the PhoB protein is phosphorylated and binds to a highly conserved motif of 18 nucleotides called a PhoB box in the promoter region of the regulated gene. Yuan et al. (2006) investigated the PhoB binding sites of the S. meliloti genome and compiled a PhoB box consensus sequence. They also included different other Gram-negative bacteria in their study. Thus, a PhoB box motif was predicted for the BtaA gene from Agrobacterium, which is probably responsible for DGTS synthesis, based on a match of 13 bases with the Sinorhizobium consensus (Table II). Therefore, DGTS synthesis may be regulated on a transcript level by PhoB. Our data indicate that DGTS synthesis is additionally regulated on a lipid substrate level, shown by the strong reduction in DGTS in the Pgt overexpressers (Figure 4B). A possible explanation may be that the substrate DAG is mainly consumed by the increased DGD/GGD synthesis rate, leading to a decreased availability for DGTS synthesis. The analysis of the Atu0318 (OlsE) promoter region of Agrobacterium revealed a weak similarity (Table II) with a match of 11 nucleotides to the PhoB box consensus (Yuan et al. 2006). Therefore, it remains

<table>
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<th>Gene</th>
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<th>Number of Matches</th>
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<tr>
<td>Atu1808 (Pgt)</td>
<td>ATGACATGTAGTCCATGTCAC</td>
<td>12</td>
</tr>
<tr>
<td>Atu0318 (OlsE)</td>
<td>CAGTTCACACCGTTCGC</td>
<td>11</td>
</tr>
<tr>
<td>Atu2119 (BtaA)</td>
<td>CTGTCACTCACTGTCAGC</td>
<td>13</td>
</tr>
<tr>
<td>PhoB box consensus</td>
<td>CTGTCAATAATCCCATGTCAT</td>
<td></td>
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</table>

Matching bases are underlined.
unclear whether OL2 synthesis by Atu0318 (OlsE) is regulated by PhoB.

According to Yuan et al. (2006), the promoter region of Pgt from Agrobacterium does not contain a PhoB box. However, we found a motif in the Agrobacterium Pgt promoter with a match of 12 nucleotides with the consensus (Table II), suggesting that Pgt might also be subject to regulation on a transcriptional level. The increased in vitro Pgt activity in our enzyme assays (Figure 7) of wild-type cells grown under phosphate starvation may be ascribed to an increased transcription rate. Observations of a basal Pgt enzyme activity (Figure 7) in Agrobacterium grown under phosphate-replete conditions are in accordance with results of a previous study where a reporter gene was inserted downstream of the Pgt promoter showing a weak transcriptional activity under full nutrition (Hölzl et al. 2005). Our results further suggest that Pgt is not regulated on an enzyme activity level, because Pgt activity (see enzyme assays) is not down-regulated in the overexresser grown under phosphate-rich conditions (Figure 8). Our results indicate that substrate availability plays a major role in the regulation of glycolipid accumulation in Agrobacterium. Pgt overexpression, under control of an inducible promoter, leads to high in vitro enzyme activities independent from phosphate supply (Figure 8). This is in marked contrast to the in vivo results, where the amounts of glycolipids in overexresser cells are considerably different with respect to phosphate supply (Figure 4B). The most obvious explanation for these results is that glycolipid synthesis is dependent on the availability of DAG, which might be limited under phosphate-replete conditions. Alternatively, there might be a restricted access of Pgt to DAG which would suggest the existence of different DAG pools. Limited availability of or restricted access to DAG would also explain why the basal Pgt expression under phosphate-replete conditions does not lead to the in vivo synthesis of detectable amounts of glycolipids.

The total amounts of DAG remain constant during phosphate deprivation. This observation does not exclude a regulation of glycolipid synthesis by substrate (DAG) availability, because under phosphate deprivation, the supply of DAG (by phospholipase breakdown of phospholipids) and the metabolism of DAG (by glycolipid and DGTS synthesis) might be in balance, leading to a low and constant steady-state level of the DAG pool. We could show that depending on phosphate supply, DAG is characterized by a change in its fatty acid composition. These results reflect the alterations in the total fatty acid composition (especially 16:0, 18:1 and 19:0 cyclo). Furthermore, DAG contains elevated levels of fatty acids found only in trace amounts in total lipid extracts. These observations suggest the possibility of different DAG pools, which may be accessible by different metabolic pathways or under certain stress conditions.

A special role during phosphate deprivation was shown for a phospholipase C (PLC), which is required for lipid remodeling in Sinorhizobium (Zavaleta-Pastor et al. 2010). A homologous gene sequence was also reported for Agrobacterium. Thus, a PLC might be involved in the degradation of phospholipids and release of DAG during phosphate deprivation in Agrobacterium. Phospholipid degradation by phospholipases and the release of DAG were also described for phosphate-deprived plants, where the released DAG was suggested to be the substrate for glycolipid synthesis (Jouhet et al. 2003; Gaude et al. 2008).

The considerable differences in the fatty acid profiles of DAG, phospholipids and glycolipids/DGTS in Agrobacterium with respect to phosphate supply indicate special regulatory roles of the enzymes participating in the conversion of phospho- into glycolipids/DGTS and of fatty acids (e.g. 18:1 into 19:0 cyclo). The conversion of mono-unsaturated fatty acids into cyclopropane fatty acids is catalyzed by the cyclopropane fatty acyl synthase (CFS) which works on a lipid level, with highest activities under stress conditions or during the stationary growth of cells (Grogan and Cronan 1997; Saborido Basconcillo et al. 2009). A CFS ortholog was also found in Agrobacterium (Saborido Basconcillo et al. 2009).

There are no studies on the specificities of this agrobacterial CFS. Therefore, it remains unknown whether GGD, DGD and DGTS are accepted as substrates. The increased conversion of 18:1 to 19:0 cyclo indicates that CFS activity in Agrobacterium is increased under phosphate deprivation and during the stationary growth phase.

A model for the changes in 18:1 and 19:0 cyclo fatty acids in the different lipid classes in Agrobacterium is presented in Figure 9. Under phosphate-replete conditions, the de novo

\[
\text{PL-18:1} \xrightarrow{\text{CFS}} \text{PL-19:0c} \quad \text{PLC} \quad \text{PLC} \\
\text{DAG-18:1} \quad \text{DAG-19:0c} \\
\text{PL-18:1} \xrightarrow{\text{CFS}} \text{PL-19:0c} \quad \text{PLC} \quad \text{PLC} \\
\text{DAG-18:1} \quad \text{DAG-19:0c} \\
\text{GL-18:1} \quad \text{GL-19:0c} \\
\]

\text{de novo synthesis} \\
\text{PL-18:1} \xrightarrow{\text{CFS}} \text{PL-19:0c} \quad \text{PLC} \quad \text{PLC} \\
\text{DAG-18:1} \quad \text{DAG-19:0c} \\
\text{PL-18:1} \xrightarrow{\text{CFS}} \text{PL-19:0c} \quad \text{PLC} \quad \text{PLC} \\
\text{DAG-18:1} \quad \text{DAG-19:0c} \\
\text{GL-18:1} \quad \text{GL-19:0c} \\

Fig. 9. Simplified model for the changes in the fatty acid compositions of DAG, phospholipids and glycolipids under phosphate-replete (+P, top) and phosphate-depleted (−P, bottom) conditions. Bold arrows indicate increased metabolic fluxes; black frames depict enrichment of the respective molecular species; grey frames indicate comparable similar amounts of molecular species in a given lipid.
Construction of an A. tumefaciens deletion mutant Δpgt by inactivation of the pgt+ locus using a Gm′ cassette

As the Agrobacterium wild-type strain used in the previous study (Hölzl et al. 2005) had lost its virulence and thus was not suitable for physiological studies, an independent mutant line (Δpgt) was generated using the Agrobacterium strain C58C1 (pGV2260). For cloning of the deletion construct, a fragment of 3.3 kb was amplified from genomic DNA from A. tumefaciens comprising the sequence upstream of the pgt+ locus of 1.2 kb, the pgt+ ORF itself and the region downstream of the pgt+ locus of 0.9 kb. The primers were bn241 (GCAATGGTTTTCGATTTTGTGAAC) and bn242 (ACTGTGGTCTTTTTCGATAATTG). The fragment was cloned into pUC18 and subsequently digested with Ncol/MluI to excise a major part (0.9 kb) of the pgt+ sequence. The remaining sequences served as flanking sequences for gene disruption with a Gm′ cassette by homologous recombination. The Gm′ cassette was amplified with the primers bn243 (TAGACACGGGTGAAACGGGATTCAAGGACG) and bn244 (ACAGGCCATGTTAGGCTGCCTTGGTGG), subcloned into pGEM-T Easy, released with MluI/Ncol and inserted into the flanking sequences in antisense orientation. The gene disruption was checked by PCR with the specific primer pairs: P1 (AGGACTTTCTGAGCGCACGTCG) and P2 (AGGTCGCTCTCCTATAAAAAGG); P3 (TTCGGTACTTTTCGACCGAC) and P4 (TTTGGTTTGTCTGTAAGGCC); P5 (CTGGTCACGAAACGTTATTACT) and P6 (GAGGGTCATCATCATAACGTTAC).
Therefore, hydroxylated fatty acids, which are only present in OL (Choma and Komaniecka 2002), were not included in the analyses. Isolated lipids were also analyzed by Q-TOF mass spectrometer (Agilent) by direct nanospray infusion in the positive mode (Devers et al. 2011). The fragmentation energy was 50 V for DGTS, 30 V for OL1 and OL2 and 20 V for DAG.

**Enzyme assays and DAG quantification**

*Agrobacterium* liquid cultures were harvested by centrifugation. The cell pellets were resuspended in equal volumes (as liquid cultures) of water and centrifuged again. After that, the cells were resuspended in water and the OD$_{600}$ was measured. The OD$_{600}$ was used as a reference for DAG quantification and for enzyme assays. Volumes of 50 mL of these cells with an OD$_{600}$ of 1.0 were used. The cells were harvested by centrifugation.

For enzyme assays, the cell pellets were resuspended in 1 mL of buffer 1 (Hölzl et al. 2005) and disrupted with glass beads with the Precellys homogenizer (Peqlab). Cell debris was removed by centrifugation at 70 × g for 1 min. The assays were performed in a final volume of 200 µL with 50 µL of buffer 2 (Hölzl et al. 2005), 50 µL of UDP-Gal (40 pmol/µL) and 100 µL of protein extract. NBD-Cer (1.3 nmol, dissolved in 5 µL of ethanol; Larodan) was added with a Hamilton syringe. The assays were incubated for 60 min at 28°C and terminated by the addition of chloroform/methanol (2:1) and 100 µL of protein extract. NBD-Cer (1.3 nmol, dissolved in 5 µL of ethanol; Larodan) was added with a Hamilton syringe. The assays were incubated for 60 min at 28°C and terminated by the addition of chloroform/methanol (2:1) and NaCl solution (0.9%). The lipids were extracted as described (Devers et al. 2011) and separated by TLC. NBD-Cer fluorescence on TLC plates was scanned with the E-Box 1000/26M Gel Documentation System (Peqlab) and the density of lipid bands evaluated.

For DAG isolation and quantification, the cell pellets (see above) were resuspended in 1–5 mL of water and boiled for 10 min to inactivate lipid degrading enzymes. After centrifugation, 50 µL of an internal DAG standard (see below) was added to each cell pellet followed by lipid extraction (Devers et al. 2011). The resulting total lipid extracts were fractionated using SPE SI-1 columns (Phenomenex, Torrance). The non-polar lipids including DAG were eluted with chloroform. The DAG content was analyzed by Q-TOF MS (vom Dorp et al. 2012). The internal DAG standard consisted of an equimolar mixture containing 1 nmol each of four molecular species: (i) 1,2/1,3-di-14:0-DAG, (ii) 1,2/1,3-di-20:0-DAG, (iii) 1,2/1,3-di-14:1-DAG and (iv) 1,2/1,3-di-20:1-DAG (Avanti Lipids, Alabaster, AL, USA). (i) and (ii) were used for the quantification of saturated and (iii) and (iv) for unsaturated DAG species. A trend line was calculated for the set of two saturated and two unsaturated internal standards to normalize for the differences in ionization due to the different masses of the DAG molecules.

**Tobacco leaf disc transformation with Agrobacterium**

Tobacco plants were grown under sterile conditions on MS medium (Murashige and Skoog 1962) containing 2% of sucrose with 16 h of light and 8 h of darkness at 22°C. For leaf disc inoculation, *Agrobacterium* wild type (strain C58C1, pGV2260) or Δpgt were first grown with or without phosphate as described above. After harvesting by centrifugation, the cells were resuspended in a 10-mM MgSO$_4$ solution with an OD$_{600}$ of 0.5 and the leaf discs incubated for 5 min. About 25 mL of this cell solution were sufficient for inoculation of 25 leaf discs each. The *Agrobacterium* strains used were transformed before with binary vectors, which are derivatives of pLH9000 (DNA Cloning Service, Hamburg, Germany). These vectors, which confer resistance for streptomycin and spectinomycin for selection in bacteria, were equipped with sequences for the expression of DsRed or CFP fluorescence markers driven by a 35S promoter. After inoculation, the leaf discs were transferred first to antibiotic-free minimal medium, which was prepared with or without phosphate (Estelle and Somerville 1987; Essigmann et al. 1998), depending on the experiment, and incubated for 2 days in the darkness to establish the infection. After that, the leaf discs were incubated on phosphate containing minimal medium with kanamycin (50 mg/L) and cefotixin (250 mg/L) for 5–6 weeks in the darkness at room temperature. Kanamycin was used for the selection of transformed tobacco cells; cefotixin was used to stop growth of *Agrobacterium*. The calli formed on the leaf discs were counted or analyzed by fluorescence microscopy.

**Supplementary data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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**Conflict of interest**

None declared.

**Abbreviations**

CFP, cyan fluorescent protein; CFS, cyclopropane fatty acyl synthase; CL, cardiolipin; DG-Cer, digalactosyl-NBD-Cer; DGD, digalactosyldiacylglycerol; DGlCd, diglucosyldiacylglycerol; DGTS, diacylglycerol trimethylhomoserine; DsRed, red fluorescent protein; GC-MS, gas chromatography–mass spectrometry; GGD, glucosylgalactosyldiacylglycerol; GL, glycolipid; MG-Cer, monogalactosyl-NBD-Cer; MMPE, monomethyl-PE; NBD-Cer, µ-erythro-[6-amino-N-4’(7-nitrobenzo-2-oxa-1,3-diazolo)-hexanoyl]-ceramide; OL, ornithine lipid; ORF, open reading frame; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; Pgt, processive glycosyltransferase; PLC, phospholipase C; Q-TOF, quadrupole time-of-flight; SQD, sulfoquinovosyldiacylglycerol; TLC, thin-layer chromatography.

**References**


