The molecular synthesis pathway of chrysos laminarin in the diatom *Phaeodactylum tricornutum*

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submitted by

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Zusammenfassung

Diatomeen stellen eine bedeutende Gruppe von Phytoplankton Organismen dar, die zu mehr als 20 % der globalen Primärproduktion beitragen, was den terrestrischen tropischen Regenwäldern und Wiesen gleichwertig ist. Chrysolaminarin, das einzigartige Speicher-Polysaccharid in Diatomeen, kann 10-30 % des organischen Trockengewicht in der exponentiellen Wachstumsphase ausmachen, aber es kann sich in der stationären Phase sogar bis zu 80 % anreichern. Chrysolaminarin trägt somit zu einem hohen Teil an der Partikelsubstanz im Ozean bei. Die Diatomeen spielen also eine wesentliche Rolle in den globalen und ozeanischen biogeochemischen Zyklen. Chrysolaminarin besteht aus einer linearen β-1,3-Glucan Kette, welche gelegentlich mit β-1,6-Glucose verzweigt ist. Allerdings bleibt der molekulare Syntheseweg von Chrysolaminarin immer noch ein Rätsel.

In dieser Arbeit beschreiben wir die Charakterisierung von zwei 1,6-β-Transglykosylasen (TGS) von Phaeodactylum tricornutum, welche möglicherweise am Hinzufügen der Seitenketten an die Hauptkette von Chrysolaminarin beteiligt sind. Sie enthalten zwei vorhergesagte Vakuolen Sortierungsmotive (DGRLV und YRLF) und sind offenbar tatsächlich auf die Vakuolen von Diatomeen ausgerichtet, was durch die Expression von GFP-Fusionsproteinen in P. tricornutum dargestellt wurde. Darüber hinaus haben wir die Funktionalität von TGS1 und TGS2 durch funktionelle Komplementierung in Saccharomyces cerevisiae Mutanten nachgewiesen, in denen eine Transglykosylase (kre6) deletiert worden war.

Die β-1,3-Glucan-Synthase (GS) katalysiert die Bildung von β-1,3-Glucanen durch das Übertragen des aktivierten Glucose-Donors, UDP-Glucose, um die β-1,3-Glucan-Bindung zu synthetisieren. Offensichtlich ist GS für die Bildung von Chrysolaminarin essentiell. Daher haben wir eine einzigartige GS in P. tricornutum (PtGS) identifiziert, die 2150 Aminosäurereste und 23 Transmembranhelices enthält. Durch die Expression des PtGS GFP-Fusionsproteins in P. tricornutum wurde PtGS in der Vakuolenmembran lokalisiert. Gemäß der Lokalisierung haben wir den putativen molekularen Chrysolaminarin Syntheseweg aufgedeckt, in dem die Vakuolenmembran-assoziierte PtGS auf UDP-Glucose aus dem Cytosol zugreift, und nicht aus Plastiden/Chloroplasten ER, um die Synthese des β-1,3-Glucan-Polymers zu initiieren.

Vergleich zum Wildtyp gezeigt. Dies ist der erste Nachweis über die erhöhte Lamellenzahl pro Thylakoid-Stapel in Diatomeen.

Abstract

Diatoms are a major group of phytoplanktonic organisms, which contribute to more than 20% global primary productivity, equivalent to terrestrial tropical rain forests and grasslands. Chrysolaminarin, the unique storage polysaccharide in diatoms, may constitute 10-80% of the organic dry weight. Hence, chrysolaminarin contributes highly to marine particulate matter, which indicates diatoms play an essential role in the global and oceanic biogeochemical cycles. Chrysolaminarin consists of a linear β-1,3-glucan chain branched occasionally with β-1,6-glucan units. However, the molecular synthesis pathway of chrysolaminarin still remains a mystery.

In this work, we described the characterization of two 1,6-β-transglycosylases (TGS) of *Phaeodactylum tricornutum*, potentially involved in adding side chains to the chrysolaminarin main chain. They do contain two predicted vacuolar sorting motifs (DGRLV and YRLF) and apparently indeed are targeted to the diatom vacuoles as demonstrated via expression of GFP fusion proteins in *P. tricornutum*. Furthermore, we demonstrated the functionality of TGS1 and TGS2 by achieving functional complementation of *Saccharomyces cerevisiae* mutants in which a transglycosylase (*kre6*) had been deleted.

β-1,3-glucan synthase (GS) catalyzes the formation of β-1,3-glucans, via transferring the activated glucose donor, UDP-glucose, to synthesize the β-1,3-glucan bond. Apparently, GS is essential for the formation of chrysolaminarin. Here, we identified a unique GS (*PtGS*) in *P. tricornutum*. By expressing *PtGS*-GFP fusion protein in *P. tricornutum*, *PtGS* was localized on the vacuolar membrane. Here, we created and characterized the *PtGS* knock-down strains. Lower expression of *PtGS* leads to a severe growth impairment, but an increase in the intracellular soluble sugars compared to the wild type. Furthermore, the *gs* mutants exhibit lower photosynthetic capacities as well as higher photoprotective abilities compared to the wild type. The correspondingly increased amounts of xanthophyll cycle pigments in the *gs* mutants were located within the fucoxanthin chlorophyll protein complexes. We observed an increase in the numbers of lamellae per thylakoid stack. Accompanied with the increased lamellar number, a reduction in the numbers of thylakoids per plastid was also shown in *gs* cell lines compared to wild type.
Blocking chrysolaminarin synthesis induces the increase of soluble sugar in gs cell lines, and causes strikingly physiological and morphological changes of the photosynthetic apparatus. These results may imply a regulation between the soluble sugars and photosystem in diatoms. Hence, the gs mutants might represent a valuable tool for investigating the sugars signaling in diatoms.
Chapter 1

General introduction
1. General Introduction

1.1. Diatoms

Diatoms (Bacillariophyceae) are unicellular photoautotrophic eukaryotes which can be found in any aquatic habitat. The number of diatom species is estimated in 100,000 with a remarkable variety of shapes (Norton et al. 1996). The diatom cell wall is made out of silica, which is one of the most characteristic feature of diatoms (Tesson and Hildebrand 2010). The cell wall is composed of two overlapping shells (valves), arranged like a petri dish (Tesson and Hildebrand 2010). According to the shape and structures of the valves, diatoms are classified into two major groups: i) centric diatoms equipped with radially symmetrical valves and ii) pennate diatoms with bilaterally symmetrical valves (Christensen 1991). When diatoms reproduce asexually, a parent cell divides into two daughter cells. Each of the daughter contains a valve from the parent cell, served as the larger half (Falciatore and Bowler 2002). Hence the successive mitotic divisions reduce the size of one of the daughter cell. However the original size is recovered via sexual reproduction followed by the combination of the male and female gametes (Falciatore and Bowler 2002) and (Mann 1993).

1.2. Diatom-plastid evolution

Diatoms have evolved via secondary endosymbiosis and belong to the phylum Stramenopiles (Kroth and Strotmann 1999; Keeling 2013). Cyanobacterium was engulfed by a non-photosynthetic host cell. This was referred to primary endosymbiosis, from which the plastids of land plants and green algae, red algae, and glaucocystophyte algae originated. This process resulted in a primary plastid surrounded with two membranes, which possibly derived from the inner and outer membranes of the gram-negative cyanobacterial cell, or the outer plastid membrane may derive from phagosomal membrane (Fig. 1.1) (Cavalier-Smith 1982; Howe et al. 2008; Kroth and Strotmann 1999). Secondary endosymbiosis is the uptake of a eukaryotic photosynthetic endosymbiont into a eukaryotic host cell. The involvement of a green algal endosymbiont gave rise to euglenids and chlorarachniophytes, whereas, the uptake of a red algae resulted in cryptomonads, haptophytes, stramenopiles, and alveolates (Keeling 2013). This process led to plastids with three or four surrounding membranes and a nuclear genome.
comprising genes of the former cyanobacterial endosymbiont, as well as those of the primary and the secondary host cells (Fig. 1.1) (Archibald 2009). The plastids of diatoms, haptophytes, chlorarachniophytes and chlorarachniophytes are surrounded by four membranes (Keeling 2013). The two inner membranes obviously originated from the inner and outer membranes of the primary symbiont. The third membrane from inside to outside, is proposed to be originated from the primary algal cytoplasmic membrane (Keeling 2004). The outermost plastid envelope possesses attached ribosomes, was suggested to represent the endoplasmic reticulum (ER) of the host, hence it was named “chloroplast ER” (CER) (Gibbs 1979; Kroth and Strotmann 1999). In euglenids and dinoflagellates, the plastid is bounded by three membranes, and the primary algal cytoplasmic membrane (second from outside) is assumed to have been lost.

1.3. The forms and intracellular localization of storage polysaccharide in different organisms

In cyanobacteria, carbohydrates are stored in the cytoplasm, either as soluble glycogen or as insoluble semi-amylopectin, which is closely analogous to amylopectin found in cereal plants (Suzuki and Suzuki 2013). After primary endosymbiosis, starch consisting of α-1,4-glucans branched with α-1,6-glucan, an insoluble and semi-crystalline form of storage polysaccharide, has evolved as the storage polysaccharide in Archaeplastida (glaucophytes, red algae, green algae and plants) (Ball et al. 2011) (Figure 1.1). Starch in glaucophytes and red algae is stored in the cytosol, whereas in plants or green algae it is located within the chloroplast (Ball et al. 2011) (Fig. 1.1 and Table 1.1). It has been proposed that the biosynthetic pathway of starch in Archaeplastida is derived from the merging of the glycogen synthesis pathway in the eukaryotic host cell with the synthesis pathway of cyanobacterial starch, which is termed semi-amylopectin (Ball and Morell 2003; Deschamps et al. 2008; Nakamura et al. 2005; Plancke et al. 2008).

However, starch is still retained in some secondary endosymbiosis derivatives, including dinoflagellates, apicomplexans and cryptophytes. In dinoflagellates and apicomplexans for instance, starch is found in the cytosol. In cryptophytes, starch is stored in the periplastidial space, a compartment derived from the cytosol of the archaeplastidal alga (Ball et al. 2011). Hence, chloroplasts serving as the storing compartment of starch seems to be an exception.
In some organisms derived from secondary endosymbiotic events, β-1,3-glucans has evolved as the major storage polysaccharide (Michel et al. 2010b) (Fig. 1.1). Available in four forms, chrysolaminarin, mycolaminarin, laminarin and paramylon, β-1,3-glucans occur in haptophytes (chrysolaminarin), stramenopiles (chrysolaminarin or laminarin), euglenids (Paramylon) and chlorarachniophytes. Except in euglenids, β-1,3-glucans are stored in vacuoles (Chang 1984; Chiovitti et al. 2004; McFadden et al. 1997; Parke et al. 1971). Euglenids store the paramylon which is highly crystalline in cytosol (Kondo et al. 1992).
General Introduction

Presentation of forms and intracellular localization of storage polysaccharide in different organisms, as the plastids evolve.

Uptake of a cyanobacteria by a eukaryotic cell led to primary endosymbiosis, which resulted in the primary plastid surrounded by two membranes. Land plants and green algae, red algae, and glaucocytophytes were originated from primary endosymbiosis (Ball et al. 2011). All of these organisms contain only starch consisting of $\alpha$-1,4-glucans. Secondary endosymbiosis is the uptake of a eukaryotic photosynthetic endosymbiont into a eukaryotic host cell. The involvement of a green algal endosymbiont gave rise to euglenids and chlorarachniophytes, whereas, the uptake of a red algae resulted in cryptomonads, haptophytes, stramenopiles, and alveolates (Keeling 2013). This process led to plastids with three or four surrounding membranes. In some organisms derived from secondary endosymbiotic events, $\beta$-1,3-glucans has evolved as the major storage polysaccharide (Michel et al. 2010b).

**Figure 1.1.** Presentation of forms and intracellular localization of storage polysaccharide in different organisms, as the plastids evolve.

Uptake of a cyanobacteria by a eukaryotic cell led to primary endosymbiosis, which resulted in the primary plastid surrounded by two membranes. Land plants and green algae, red algae, and glaucocytophytes were originated from primary endosymbiosis (Ball et al. 2011). All of these organisms contain only starch consisting of $\alpha$-1,4-glucans. Secondary endosymbiosis is the uptake of a eukaryotic photosynthetic endosymbiont into a eukaryotic host cell. The involvement of a green algal endosymbiont gave rise to euglenids and chlorarachniophytes, whereas, the uptake of a red algae resulted in cryptomonads, haptophytes, stramenopiles, and alveolates (Keeling 2013). This process led to plastids with three or four surrounding membranes. In some organisms derived from secondary endosymbiotic events, $\beta$-1,3-glucans has evolved as the major storage polysaccharide (Michel et al. 2010b).
### Table 1.1. Forms and intracellular localization of storage polysaccharide in different organisms

<table>
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<tr>
<th>Division Major classes</th>
<th>Storage polysaccharides</th>
<th>Location</th>
</tr>
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<tbody>
<tr>
<td><strong>Cyanobacteria</strong></td>
<td>Glycogen or Semi-amylpectin (α-1,4-glucan)</td>
<td>Cytosol</td>
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<tr>
<td><strong>Glaucophytes</strong></td>
<td>Starch (α-1,4-glucan)</td>
<td>Cytosol</td>
</tr>
<tr>
<td><strong>Red algae</strong></td>
<td>Starch (α-1,4-glucan)</td>
<td>Cytosol</td>
</tr>
<tr>
<td><strong>Green algae or land plants</strong></td>
<td>Starch (α-1,4-glucan)</td>
<td>Chloroplast</td>
</tr>
<tr>
<td><strong>Euglenids</strong></td>
<td>Paramylon (β-1,3-glucan)</td>
<td>Cytosol</td>
</tr>
<tr>
<td><strong>Chlorarachiophytes</strong></td>
<td>β-1,3-glucan</td>
<td>Vacuole</td>
</tr>
<tr>
<td><strong>Stramenopiles</strong></td>
<td>Chrysolaminarin (β-(1,3;1,6)-glucan)</td>
<td>Vacuole</td>
</tr>
<tr>
<td><strong>Diatoms</strong></td>
<td>Laminarin (β-1,3-glucan)</td>
<td>Vacuole</td>
</tr>
<tr>
<td><strong>Brown algae</strong></td>
<td>Mycolaminarin (β-(1,3;1,6)-glucan)</td>
<td>Vacuole</td>
</tr>
<tr>
<td><strong>Oomycetes</strong></td>
<td>Chrysolaminarin (β-(1,3;1,6)-glucan)</td>
<td>Vacuole</td>
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<tr>
<td><strong>Haptophytes</strong></td>
<td>Chrysolaminarin (β-(1,3;1,6)-glucan)</td>
<td>Vacuole</td>
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<td><strong>Cryptomonads</strong></td>
<td>Starch (α-1,4-glucan)</td>
<td>Periplastidial space</td>
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<td><strong>Apicomplexans</strong></td>
<td>Starch (α-1,4-glucan)</td>
<td>Cytosol</td>
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<td><strong>Dinoflagellates</strong></td>
<td>Starch (α-1,4-glucan)</td>
<td>Cytosol</td>
</tr>
<tr>
<td><strong>Ciliates</strong></td>
<td>Glycogen</td>
<td>Cytosol</td>
</tr>
</tbody>
</table>

In the first column, blue indicates primary endosymbiosis; green indicates secondary endosymbiosis originated from green algae; Red indicates secondary endosymbiosis originated from red algae. As for the storage polysaccharide column, green indicates β-1,4-glucans including starch and glycogen; yellow indicates β-1,3-glucans including paramylon and laminarin. For the localization column, grey indicates cytosol or vacuole or periplastidial space; green indicates chloroplast. Modified from (Suzuki and Suzuki 2013).
1.4. The functions of β-1,3-glucan in prokaryotes and eukaryotes

In addition to serving as a storage polysaccharide, β-1,3-glucans play a variety of roles in different organisms (Fig. 1.2). They are essential components of the cell wall in fungi, oomycetes, and plants (Levy and Epel 2009; Myklestad and Granum 2009). Besides cell wall formation, a form of β-1,3-glucan termed callose in land plants, is also involved in a variety of processes including cell division, plasmodesmata dynamics and the development of pollen and embryo sacs, (Levy and Epel 2009; Newbigin et al. 2009). In prokaryotes, such as *Agrobacterium* spp. and *Cellulomonas* spp., β-1,3-glucans are reserved as extracellular capsules, assisting in bacterial survival, colonization and cell to cell interactions (Stanisich and Stone 2009).

It has been demonstrated by Waterkein and Bienfait that, in some diatom species, β-1,3-glucans also appear as callose, an insoluble fiber, associated with three parts of the cell wall: i) between the two valves, ii) as two strips called the “nebenlinien” associated with the cingulum and, iii) transiently associated with the newly formed valves during division (Waterkeyn and Bienfait 1987). However, in 2013, Tesson and Hildebrand observed different localization of β-1,3-glucans in different species (Tesson and Hildebrand 2013). They also proposed β-1,3-glucans to be involved in the shaping of the valves at the microscale (Tesson and Hildebrand 2013).
β-1,3-glucans are essential components of the cell wall in fungi, oomycetes and plants (Levy and Epel 2009; Myklestad and Granum 2009). In land plants, β-1,3-glucan termed callose, is involved in a variety of processes including cell division, plasmodesmata dynamics and the development of pollen and embryo sacs, (Levy and Epel 2009; Newbigin et al. 2009). In prokaryotes, β-1,3-glucans are stored as extracellular capsules, assisting in bacterial survival, colonization and cell to cell interactions (Stanisich and Stone 2009).

**Figure 1.2.** Presentation of β-1,3-glucan functions in organisms
1.5. Chrysolaminarin in diatoms and β-1,3-glucans stramenopiles

Diatoms are unicellular photoautotrophic eukaryotes, contributing around 20 and 40% of primary production to global and oceanic biogeochemical cycles, respectively (Falkowski et al. 1998). Chrysolaminarin is the principal energy storage polysaccharide which is stored in intracellular vacuoles in a non-crystalline form (Beattie et al. 1961a; Chiovitti et al. 2004; Ford and Percival 1965a). The extraction approaches of β-1,3-glucan are well established. It can be purified by ethanol precipitation after dilute acid or hot water extraction (Allan G et al. 1972; Chiovitti et al. 2004; Granum et al. 2002). In *Phaeodactylum tricornutum*, chrysolaminarin is the principle β-1,3-glucan, with an average degree of polymerization (DP) of 20, occasionally branched with glucose units at C-6 (Myklestad and Granum 2009)(Fig. 1.3). Whereas, in diatom *Skeletonema costatum*, chrysolaminarin has branch sites at C-2 and C-6 (Paulsen and Myklestad 1978). In the sea ice diatom *Stauroneis amphioxi*, β-1,3-glucan is branched at C-2, C-4 and C-6. The degrees of polymerization are variable depending on different species, for instance, in *S. amphioxi*, the DP is about 24, whereas the DP of the marine diatom *Thalassiosira weissflogii* is 5-13.

During different growth phases β-1,3-glucans can constitute between 10 and 80% of organic dry weight in diatoms (Myklestad 1974). They are both a short-term day-and-night rhythm reserve and a long-term storage polysaccharide. They are also known to devote the carbon skeletons of the saccharide units to the synthesis of amino acids and it also serves as respiratory substrate (Granum et al. 2002; Granum and Myklestad 1999, 2001; Vårum and Myklestad 1984). Besides diatoms, vacuolar β-1,3-glucans branched with β-1,6-glucan have also been characterized in brown algae and oomycetes, which are known as laminarin and mycolaminarin, respectively (Read et al. 1996; Wang and Bartnicki-Garcia 1974). The components of laminarin is somewhat different from chrysolaminarin or mycolaminarin, which terminates with either a reducing β-1,3-linked glucose residue (G-chain), or with a non-reducing β-1,1-linked mannitol residue (M-chain) (Read et al. 1996). The presence of both G and M chains in laminarin were confirmed in several brown algae species (Chizhov et al. 1998).
Chrysolaminarin comprises a linear β-1,3-glucan backbone branched with glucose units at the C-6 position.

1.6. The synthesis of chrysolaminarin (β-1,3-glucans)

β-1,3-glucans, which are distributed widely in living organisms, serve as storage polysaccharides in haptophytes, stramenopiles, euglenids and chlorarachniophytes (Chang 1984; Chiovitti et al. 2004; McFadden et al. 1997; Michel et al. 2010b; Parke et al. 1971). However, the biochemical synthesis pathway of chrysolaminarin or β-1,3-glucans in organisms, are essentially unknown.

1.6.1. UDP-glucose pyrophosphorylase (UGP)

In plants, carbohydrates are produced via the carbon assimilatory pathway. Some of them are converted by ADP-glucose pyrophosphorylase (AGP) into ADP-glucose, which is the activated substrates for starch synthesis (Geigenberger et al. 2005). However, it has been demonstrated the specific genes, that are involved in the glycogen/starch metabolism such as starch/glycogen synthase, as well as AGP, are completely absent in brown algae, diatoms and oomycetes genomes (Michel et al. 2010b). Instead of ADP-glucose, it was proposed UDP-glucose, catalyzed by UDP-glucose pyrophosphorylase (UGP), severed as activated sugar for the synthesis of chrysolaminarin in diatoms Cyclotella cryptica (Roessler 1987). UGP has been identified in the genomes of brown algae Ectocarpus siliculosus and oomycetes (Michel et al. 2010b).
1.6.2. β-1,3-glucan synthase (GS)

β-1,3-glucan synthase (GS) is essential for the synthesis of β-1,3-glucans, since it can add the activated glucose donors, UDP-glucose, to the β-1,3-glucan polymer (Fig. 1.4) (Roessler 1987). The evolution of GSs in Eukaryotes is consistent with the phylogeny of Eukaryotes indicating β-1,3-glucans are an important metabolite (Michel et al. 2010b). GSs are separated into two glycosyltransferases (GTs) families: GT2, a polyspecific family including bacterial β-1,3-glucan synthases, and GT48, a family only comprising eukaryotic β-1,3-glucan synthases (GSs) (Michel et al. 2010b). *E. siliculosus* possesses 11 GT2 family proteins, homologous to cellulose synthases, and are not significantly related to bacterial β-1,3-glucan synthases (Michel et al. 2010b). However three proteins from GT48 family in *E. siliculosus* share remarkable similarity to the β-1,3-glucan synthase in plants (Michel et al. 2010b). The GSs are likely to function as a multi-subunit membrane-associated complex. For example, in yeast, the characterized GSs complexes (FKS1 and FKS2) consist of a regulated subunit (Rho1p, a Rho-type small GTPase) and a β-1,3-glucan synthase catalytic subunit (Mazur and Baginsky 1996; Okada et al. 2010). In plants, a callose synthase 1 (CalS1) subunit is demonstrated to bind to a UDP-glucose transferase (UGT1) which attaches to Rop1, a homologue of Rho (Hong et al. 2001; Verma and Hong 2001).

1.6.3. Kre6-like protein

Yeast proteins Kre6 and Skn1 belong to a family-16 glycosyl hydrolases which include proteins involved in the 1,6-β-branching of cell wall β-1,3-glucans (Kurita et al. 2011; Nakamata et al. 2007; Roemer and Bussey 1991; Roemer et al. 1993). In brown algae *E. siliculosus*, two proteins which are homologues to Kre6 were found. Interestingly, this Kre6-like protein is fused to β-1,3-glucan synthase in oomycetes *Phytophthora infestans* (Michel et al. 2010b). These Kre6-like proteins might represent good candidates for synthesis of β-1,6-linked branches of β-1,3-glucan (Fig. 1.4).
Figure 1.4. Putative synthesis pathway of chrysolaminarin β-1,3-glucans.

The primary CO₂ fixation product of the Calvin cycle is 3-PGA which provides the original substrate for synthesis of β-1,3-glucans. Glucose-6-phosphate is converted into activated sugar UDP-glucose by the catalysis of UGP. Subsequently, UDP-glucose is incorporated into the linear β-1,3-glucan polymer by β-1,3-glucan synthase. TGS adds glucose unit to the C-6 site of β-1,3-glucan, forming into branched β-1,3-glucan (chrysolaminarin). PGM: phosphoglucomutases, UGP: UDP-glucose pyrophosphorylase. GS: β-1,3-glucan synthase, TGS: β-1,6-transglycosylases. 3-PG: 3-Phosphoglycerate. 3-PGA: glyceraldehyde-3-phosphate.
1.7. Questions and Aims

The biosynthetic pathways of chrysolaminarin is essentially unknown. Here, in order to elucidation the chrysolaminarin synthesis pathway, we need to identify the proteins which are potentially involved in the synthesis pathway of chrysolaminarin by screening the genome of *P. tricornutum*, which is the model organism of diatoms. After the identification of the proteins involved in synthesis pathway of chrysoaminarin, we are aiming to reveal the net transport of carbohydrates from the plastid (where photosynthesis takes place) to the vacuole (where the chrysolaminarin is stored), as well as whether these proteins are involved in synthesizing cell wall, by localizing and characterizing these essential proteins in *P. tricornutum*. Since the storage polysaccharide is the carbon sink of the phototrophic organisms and the photosynthetic system supplies carbon sources to organisms, we are interested in investigating the coordination between storage polysaccharide biosynthetic pathway and the photosynthetic system.
Chapter 2

2. Diatom vacuolar 1,6-β-transglycosylases can functionally complement the respective yeast mutants

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Author contributions

H.W. designed the project, carried out the experiments and wrote the manuscript. C.RB. carried out the phylogenetic tree. P.K. conceived the project and finalized the manuscript.

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Citation

2.1. Abstract

Diatoms are unicellular photoautotrophic algae, which can be found in any aquatic habitat. The main storage carbohydrate of diatoms is chrysolaminarin, a non-linear β-glucan, consisting of a linear 1,3-β-chain with 1,6-β-branches, which is stored in cytoplasmic vacuoles. The metabolic pathways of chrysolaminarin synthesis in diatoms are poorly investigated, therefore we studied two potential 1,6-β-transglycosylases (TGS) of the diatom *Phaeodactylum tricornutum* which are similar to yeast Kre6 proteins and which potentially are involved in the branching of 1,3-β-glucan chains by adding D-glucose as 1,6-side chains. We genetically fused the full-length diatom TGS proteins to GFP and expressed these constructs in *P. tricornutum*, demonstrating that the enzymes are apparently located in the vacuoles, which indicates that branching of chrysolaminarin may occur in these organelles. Furthermore, we demonstrated the functionality of the diatom enzymes by expressing TGS1 and 2 proteins in yeast which resulted in a partial complementation of growth deficiencies of a transglycosylase-deficient Δkre6 yeast strain.

2.2. Key words

Chrysolaminarin; 1,6-β-transglycosylases; functional complementation; vacuolar sorting motif.

2.3. Introduction

Diatoms are unicellular photoautotrophic eukaryotes which contribute considerably to global net primary production (Falkowski et al. 1998). In addition to their ecological importance, diatoms are also very interesting regarding their cell biology for a number of reasons. They have evolved via secondary endosymbiosis, which is the uptake of a eukaryotic photosynthetic endosymbiont into a eukaryotic host cell. This process resulted in plastids with four surrounding membranes and a nuclear genome comprising genes of the former cyanobacterial endosymbiont, as well as those of the primary and the secondary host cells (Archibald 2009). Chrysolaminarin is a unique polysaccharide and, besides lipids, constitutes the principal energy storage compound of diatoms and related algae within the Heterokontophytes (Beattie et al. 1961b; Ford and Percival 1965b). The water soluble 1,3-β-glucan of diatoms may constitute 10-30% of the organic dry weight in the exponential growth phase of the algae, but it may accumulate up to 80% in the stationary phase (Myklestad 1974). Due to the relatively high contribution of chrysolaminarin to marine
particulate matter, diatoms do play an essential role in the global and oceanic biogeochemical cycles (Falkowski et al. 1998; Hama and Handa 1992; Handa 1969; Kroth et al. 2008; Van Oijen et al. 2004). Chrysolaminarin serves both as a short-term diurnal carbohydrate reserve compound and as a long-term storage polysaccharide. The degradation of the polysaccharides may deliver the carbon skeletons required for the synthesis of amino acids as well as the substrates for respiration (Granum et al. 2002; Granum and Myklestad 1999, 2001; Vårum and Myklestad 1984). Chrysolaminarin represents a branched 1,3-β-glucan with glucose units added at the C-6 position at a ratio of eleven 1,3-β-glucan units to one 1,6-β-glucan unit (Beattie et al. 1961b; Ford and Percival 1965b). In diatoms, chrysolaminarin is supposed to be stored in intracellular vacuoles in a non-crystalline form (Chiovitti et al. 2004).

The biosynthetic pathway of chrysolaminarin in diatoms as well as the respective enzyme are poorly investigated, therefore we have screened the JGI *Phaeodactylum tricornutum* genome database (Bowler et al. 2008) for genes encoding proteins that are potentially involved in chrysolaminarin synthesis or modification. We identified three gene models encoding putative 1,6-β-transglycosylases (TGS) in the diatom *P. tricornutum*, which we consider to be involved in the synthesis of 1,6-β-linked branches of chrysolaminarin. These three proteins share moderate similarity to conserved domains of Kre6 and Skn1 in *Saccharomyces cerevisiae* (Kroth et al. 2008). These two yeast proteins belong to family-16 glycosyl hydrolases that include proteins involved in the 1,6-β-branching of cell wall 1,3-β-glucans in yeast (Kurita et al. 2011; Nakamata et al. 2007; Roemer and Bussey 1991; Roemer et al. 1993). The mode of formation of 1,6-β-linkages in side-chain branched 1,3-β-glucans from diatoms and related algae is mostly unknown. However, the modification of 1,3-β-glucan chains by transferring the D-glucose units to the C-6 position by transglycosylases as proposed for fungi is very likely (Bulone 2009).

As there had been reports that in diatoms polysaccharides may be involved in silica cell wall formation by forming an organic layer (Tesson and Hildebrand 2013), it is unclear yet whether the identified *P. tricornutum* TGSs may be involved in either chrysolaminarin modification or the synthesis of the cell wall or both. Therefore, the localization and the functional characterization of the TGSs are essential for the elucidation of the biosynthetic pathway of chrysolaminarin in diatoms. Moreover, the localization of TGS proteins may be indicative for the site of biosynthesis of mature chrysolaminarin within the cell.
We have chosen to study \textit{P. tricornutum}, which is one of the model systems for diatoms because of the availability of both a genome sequence (Bowler et al. 2008) as well as tools like genetic transformation (Karas et al. 2015; Zaslavskaia et al. 2000), gene silencing (De Riso et al. 2009; Lavaud et al. 2012). Recently the transcription activator-like effector nuclease (TALEN) technique for the targeted knocking out of genes has also been successfully implemented in \textit{P. tricornutum} (Daboussi et al. 2014; Weyman et al. 2015). However, such knock down/out approaches may reveal a certain phenotype, but they may not directly demonstrate the nature of the enzymatic activity of the enzyme of interest. We therefore here demonstrate the functionality of the TGS enzymes by achieving functional complementation of \textit{S. cerevisiae} deletion mutants by a homologous gene of interest from a diatom. This kind of complementation assays have been frequently used for genes from plants and humans (Hsu et al. 1993; Schild et al. 1990), but to our knowledge this is the first demonstration that diatom proteins can complement proteins in yeast.

The expression of GFP fusion proteins has been demonstrated to be a useful tool to study the intracellular location of proteins in a variety of organisms including diatoms (Gruber et al. 2007). In this report, we show that both TGS1 and 2 contain signal peptides as well as typical signal sorting motifs for endosomes in potential cytoplasmic tails (CTs). When expressed in \textit{P. tricornutum}, GFP fusion proteins of TGS1::GFP and TGS2::GFP were located in or in vicinity of vacuolar membranes indicating that TGS1 and 2 are tonoplast-associated proteins.
2.4. Materials and methods

2.4.1. Strains, plasmids and media

The wild type and Δkre6 yeast strains BY4741 (MATa; his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and Y05574 (MATa; his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; kre6Δ::kanMX4) were used for the complementation experiments. They were obtained from EUROSCARF (EUROpean Saccharomyces Cerevisiae ARchive for Functional Analysis Frankfurt, Germany). BY4741 is the parent strain of Y05574 possessing different selectable markers (Brachmann et al. 1998). Kre6 had been deleted by integrating kanMX4 in Y05574. The yeast cDNA expression vector pAG300 (www.addgene.org) was used for complementation with the diatom genes (Horbach et al. 2009). This expression system is comprised of 2 µ origin of replication (2 µ ori), ura3 selectable marker, and alcohol dehydrogenase 1 (ADH1) promoter and terminator (Vernet et al. 1987).

Yeast cells were grown in YPD (1% Bacto yeast extract (Becton Dickinson and Company, Le Pont de Claix, France), 2% Bacto-peptone (Becton Dickinson and Company, Le Pont de Claix, France), 2% glucose) or in synthetic complete medium (SC) (0.67% yeast nitrogen base without amino acids (BD Biosciences), 0.01% amino acids without uracil (adenine, arginine, cysteine, leucine, lysine, threonine), 0.05% amino acids (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine), 2% glucose, 2% Difco-agar (Difco laboratories, Detroit Michigan, USA).

2.4.2. Synthesis of TGS1 and 2 cDNA

Cells of *P. tricornutum* were harvested by centrifugation at 5,000 g and the pellet was frozen in liquid nitrogen. Total RNA extraction and synthesis of cDNA was done as described previously (Vugrinec 2011).

2.4.3. Plasmid construction and transformation

*P. tricornutum* Bohlin (University of Texas Culture Collection, strain 646) was cultivated and transformed as described previously (Kroth 2007). The amplified tgs1 and 2 genes have been cloned into the pPha-T1-Hpal-GFP and the pPha-T1-StuI-GFP vectors respectively as described in (Vugrinec 2011). These two vectors are derived from pPha-T1 vectors (GenBank accession AF219942.1) (Zaslavskaia et al. 2000). To clone the genes into the yeast vector, we used primers
overlapping with pAG300 and amplified $tgs1$ and 2 $egfp$ from the respective $P. tricornutum$ vectors. The Gibson Assembly® Master Mix kit (New England Biolabs GmbH, Frankfurt, Germany) has been used to assemble genes into the vector pAG300 (Barnes 1994; Gibson et al. 2010; Gibson et al. 2009). Oligos overlapping 15-16 nucleotide pairs with the vector pAG300 have been used for amplifying $tgs1$ and 2.

$tgs1$, $tgs2$, as well as $tgs1::egfp$ or $tgs2::egfp$ gene fusions were assembled together with the linear vector pAG300 that had been digested with Xbal and Xhol using Gibson Assembly® Master Mix kit generating the vectors $V_{pt}TGS1$, $V_{pt}TGS1::eGFP$, $V_{pt}TGS2$ and $V_{pt}TGS2::eGFP$. Sequences of the fragments were confirmed by DNA sequencing. The plasmids were transformed into $S. cerevisiae \Delta kre6$ strain BY05574 using a protocol that was modified from the “Quick and Dirty” transformation protocol (Amberg et al. 2006) to yield the complemented yeast strains: $T_{pt}TGS1$, $T_{pt}TGS1::eGFP$, $T_{pt}TGS2$ and $T_{pt}TGS2::eGFP$. As a control, BY4741 was transformed with the empty vector pAG300. After transformation, the yeast cells were grown on YSCM agar lacking uracil.

2.4.4. Fluorescence microscopy and the staining of vacuoles in $P. tricornutum$

The transformed cell lines have been examined with an epifluorescence microscope, Olympus BX51 (Olympus Europe, Hamburg, Germany), being equipped with a Zeiss AxioCam MRm digital camera system (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). To view transmitted light images (100 x UplanFL objective, Olympus), Normarski’s differential interference contrast (Dickson and Kirst) illumination was used.

For visualization of the structure of vacuoles in $P. tricornutum$, a green fluorescent vacuole membrane marker MDY-64 was applied (Molecular Probes, Leiden, Netherlands). 1 µl of 1 mM MDY-64 was diluted into 499 µl liquid samples (containing about $10^6$ cells) of $P. tricornutum$ in modified f/2 medium with artificial half-concentrated sea salts (16.6 g l$^{-1}$) and 0.09 µM MnCl$_2$ (Guillard 1975; Guillard and Ryther 1962). The final working concentration of MDY-64 was 2 µM, the cells were incubated at room temperature for 2 minutes. Green fluorescence was analyzed with the epifluorescence microscope.
2.4.5. **In silico protein analyses**

For signal peptides and transmembrane helices predictions, the software HMMER and SignalP were used (http://hmmer.janelia.org/, http://www.cbs.dtu.dk/services/SignalP-3.0/) (Bendtsen et al. 2004; Finn et al. 2011). For protein targeting prediction TargetP was utilized (www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al. 2000).

2.4.6. **Phylogenetic analyses**

Data Sampling was performed by blasting each *P. tricornutum* TGS protein sequence at the NCBI server (http://www.ncbi.nlm.nih.gov/) for non-redundant protein sequence (nr). Sequences from the following organisms were retrieved: metazoa, fungi, red algae, viridiplantae, alveolata, haptophyta, cryptophyta, stramenopiles, rhizaria, archaea and prokarya. To obtain further stramenopile sequences we screened JGI genome portal (www. http://genome.jgi.doe.gov/). The data sets were reduced by discarding redundant sequences, too short sequences and sequences that were too divergent and not able to align to the *P. tricornutum* sequences. This way a data set of 66 fungal and stramenopile sequences was obtained. After aligning the amino acid sequences using ClustalW with the default settings for slow/accurate alignment and the output format Phylip (http://www.genome.jp/tools/clustalw/), the alignment was manually curated, gaps and ambiguously aligned regions were discarded, yielding 530 amino acid positions (see Supplement document 1). Phylogenetic tree was constructed using maximum likelihood with the online program PhyML (Guindon et al. 2010; Guindon and Gascuel 2003). We used the PhyML version with automatic model selection by smart model selection and the Akaike Information Criterion (http://www.atgc-montpellier.fr/phylml-sms/). The substitution model WAG +G6 +I was selected by the program (Whelan and Goldman 2001). Bootstrap analysis with 100 replicates was performed. The tree was displayed by the program Tree v 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).
2.5. Results and discussion

2.5.1. Sequence analyses

In order to identify potential transglycosylases, we screened the *P. tricornutum* genome database (JGI, version 2.0) by blasting Kre6 (Saccharomyces genome database (SGD) ID: S000006363) and Skn1 (SGD ID: S000003375) sequences from *S. cerevisiae* against *P. tricornutum* sequences. This way we identified three genes encoding putative 1,6-β-transglycosylases (gene models: 50238 (TGS1), 56509 (TGS2), 48300 (TGS3)). To confirm the similarity of the identified gene models to other transglycosylases, we performed alignments using the conserved TGS domains of known TGS protein sequences from yeast, showing that the *P. tricornutum* sequences cluster with other TGS sequences (Fig. S1). The deduced full protein sequences of the three putative *P. tricornutum* TGSs show a similar structure (Fig. 2.1A). They all contain an N-terminal signal peptide and a single transmembrane helix at the C-termini followed by a short domain that may represent a cytoplasmic tail (CT). This domain structure is clearly different to that of the respective yeast proteins which have a C-terminal transmembrane helix flanked by a long N-terminal cytoplasmic tail domain without any signal peptide (Fig. 2.1A). Despite these structural domain differences between the *P. tricornutum* and *S. cerevisiae* TGS proteins, the conserved parts of TGS1, 2 and 3 still share a strong similarity of 37.5%, 35.5% and 33.1% amino acid identity, respectively, to the Kre6 protein of *S. cerevisiae* (Fig. S1). Our phylogenetic analyses support these findings (Fig. 2.2). A deep blast search and a stringent removal of ambiguous sequences, resulted in a TGS tree with only stramenopile and fungal sequences. Interestingly, all TGS sequences in the obtained tree apparently have a common ancestor. The fungal sequences belong to the family 16 glycoside hydrolases (Barbeyron et al. 1998). The obtained tree shows Ascomycota and Basidomycota as sister groups (100 Bootstrap support), while both fungal clades are also close to the Oomycete sequences, which are non-photosynthetic stramenopiles. The photosynthetic stramenopiles form a single branch, clearly separating TGS3 sequences from TGS1 and 2 sequences.

According to TargetP and SignalP presequence predictions, all three proteins (TGS1, 2 and 3) have a functional signal peptide, indicating that they are targeted to the secretory/ER system. In eukaryotic cells, the trafficking of proteins within the secretory pathways may rely on various additional sorting signals (Bonifacino and Traub 2003; Trowbridge et al. 1993). Most of the signals
of proteins for the ER-Golgi-endosome network can be found in so-called cytoplasmic tails (CTs). Membrane proteins are initially synthesized in the ER, after correct folding and assembly they are delivered to the Golgi apparatus via the coat protein complex II (COPII) transport vesicles (Barlowe et al. 1994; Bi et al. 2002). Specific sorting signals are required for exporting the protein from the ER to COPII. In yeast, mammalian, and plant cells, typical export signals from the ER to COPII of membrane cargo proteins have been identified, which include the di-acidic motif (DXE), the di-hydrophobic motif (LL), the di-aromatic motif (FF, YY), the di-basic motif (RR, RK), and the Tyr-based motif YXXØ (X is any amino acid, and Ø is a bulky hydrophobic group) (Barlowe 2003; Giraudo and Maccioni 2003; Hanton et al. 2005; Sato and Nakano 2002). Furthermore, for targeting to the trans-Golgi network (TGN), one more sorting motif is required at the cytoplasmic tail, which can be recognized by adaptor protein (AP) complexes leading to transport vesicles. The major type of transport vesicles mediating post-Golgi traffic is clathrin-coated vesicles (CCV). The resulting transport vesicles are carrying proteins to the final destinations such as vacuolar membranes and plasma membranes (Hwang and Robinson 2009; Robinson and Pimpl 2014). Two major classes of signals recognized by AP complexes have been identified and characterized in plants, yeast and in animals. These are Tyr-based sorting signals (YXXØ, with X being any amino acid and Ø an amino acid with a bulky hydrophobic residue) and di-Leu–type sorting signals [D/E]XXXL[L/I]. There are also different adaptors which can recognize other Tyr-based signals (NPXY) and other di-Leu–type signals (DXXLL) (Bonifacino and Traub 2003; Pandey 2010; Robinson 2004; Wolfenstetter et al. 2012).

We have analyzed the cytoplasmic tails of the identified TGSs from diatoms (*P. tricornutum, Thalassiosira pseudonana*) and a brown alga (*Ectocarpus siliculosus*). The amino acid sequences of the potential CTs of *P. tricornutum* TGS1 and *T. pseudonana* TGS (protein ID: 3105) are very similar, they both contain the YXXØ as well as the [D/E]XXXL[L/I]-type motifs (Fig. 2.1B). We could not find indications that this combination of signal motifs has been identified in other organisms before. YXXØ motifs can, besides binding to APs, also be recognized by COPII (Sato and Nakano 2002). Therefore we propose that the YC[V/M]I motif of TGS1 is required for exporting proteins from the ER, while the DGRL[V/L] motif is required for targeting to the trans-Golgi network. The potential CTs of *P. tricornutum* TGS2 and *E. siliculosus* TGS have similar motifs; they both possess the di-basic motif RR next to the transmembrane helices (Fig. 2.1B). Di-basic motifs
in the cytoplasmic tail of animal glycosyltransferases, have been demonstrated to be important for exporting protein from the ER (Giraudo and Maccioni 2003). Furthermore, at the C-terminus of both sequences, a Tyr-based motif YXXØ has been identified (Fig. 2.1B). In conclusion, both, TGS1 and 2, contain ER-exiting-like and endosomes-like sorting motifs, indicating that TGS1 and 2 may be sorted to the vacuolar or the plasma membranes of *P. tricornutum*. As the sorting motifs of both TGS1 and 2 are different, they might be recognized by different adaptor proteins. Within the respective domain of *P. tricornutum* TGS3, we could not detect any typical of such sorting signals. As furthermore the phylogenetic analyses indicate that TGS3 sequences form a clade separate from the TGS1/2 sequences, and screening of the *P. tricornutum* EST database (http://www.diatomics.biologie.ens.fr/EST3/index.php) (Maheswari et al. 2009; Maheswari et al. 2005) imply that TGS3 is only very poorly expressed, we decided to focus only on TGS1 and 2 in the following experiments.
Figure 2.1. Predicted domain structures of TGS proteins from different organisms.

A. Putative structures of TGSs from the yeast *Saccharomyces cerevisiae* (Kre6 SGD ID: S000006363; Skn1 SGD ID: S000003375), the diatoms *Phaeodactylum tricornutum* (JGI ID: TGS1 50238, TGS2 56509, TGS3 48300), *Thalassiosira pseudonana* (TGS1, XP_002288656) and the brown alga *Ectocarpus siliculosus* (NCBI: CBJ31299). CD: conserved TGS domain; CT: potential cytoplasmic tail; SP: signal peptide; TM: transmembrane helix. B. Domains of the potential cytoplasmic tails of TGS1 and 2: Blue: di-basic RR motif; purple: Tyr-based motif; yellow: di-Leu-type motif.
Figure 2.2. Maximum likelihood tree using PhyML.

This tree is based on 66 TGS sequences and 530 amino acid positions. *P. tricornutum* TGS sequences are boldfaced. Numbers at nodes correspond to bootstrap values. The accession numbers are listed behind the strain names, and correspond to GenBank entries, unless otherwise noted. The used alignment for this tree is listed in supplement document 1. The tree was midpoint rooted. The Oomycete, Basidomycota and Ascomycota subtrees are drawn in collapsed form.
2.5.2. Intracellular localization of TGS1 and 2 in *P. tricornutum*

In order to be able to address vacuolar structures in the diatom *P. tricornutum*, we established a staining procedure for diatom vacuoles, using the fluorescent vacuole membrane marker MDY-64. We found that incubation of the cells for 2 min at a concentration of 2 µM MDY-64 yielded the best results. Two different fluorescent staining patterns of vacuoles were observed. While most of the cells of *P. tricornutum* contain 2-3 big vacuoles associated with a few vesicles (Fig. 2.3B), some of the cells revealed a high number of vesicles associated with the vacuoles forming a continuous membraneous net (Fig. SA). In a next step, we had genetically fused the *egfp* gene to the 3′ end of both the genes *PtTgs1* and *PtTgs2* (Fig. 2.3A) and expressed the fusion proteins in *P. tricornutum*. We decided to fuse the GFP protein to the C-termini of the TGS proteins because: i) a fusion to the N-terminus would clearly have blocked the signal peptide, and ii) there are reports from *Arabidopsis* and tobacco BY2 cells, that GFP, even when fused to the C-termini of the respective tonoplast proteins, does not affect the tonoplast localization/targeting (*Czempinski et al. 2002; Wolfenstetter et al. 2012*). Fluorescence microscopy images yielded similar fluorescent green morphology patterns as previously obtained with the vacuole dye, indicating that TGS1 and 2 may represent tonoplast associated proteins (Fig. 2.3C, D; Fig. 2.4B,C). This finding supports the prediction that the identified DGRLV and YRLF motifs of TGS1 and 2 indeed may serve as tonoplast sorting motifs in *P. tricornutum*. However, besides a location at the vacuole-like membranes, green fluorescent vesicle membranes associated to vacuoles were also observed in TGS2::GFP expressed cell lines. These vesicles may participate in transporting the proteins to vacuoles. Nevertheless, they were not obtained in TGS1::GFP expressed cell lines. These different fluorescent vacuolar membranes patterns may be explained by the different vacuolar sorting motifs of TGS1 and 2 (see above).

The Kre6 protein of yeast, which is involved in the synthesis of the cell wall 1,6-β-glucans, has been localized in yeast cell membranes of the endoplasmic reticulum (ER), Golgi apparatus and the plasma membrane (PM) (*Kurita et al. 2011; Roemer et al. 1994*). So far, there is no evidence that Kre6 may be associated with vacuolar membranes of yeast. These different localizations, together with the different domain structure may indicate that TGS1 and 2 in contrast to Kre6, may not be involved in the synthesis of the organic cell wall of *P. tricornutum*. 
Figure 2.3. Analysis of the cellular localization of TGS1 and 2 in *P. tricornutum*.

**A.** Constructs of TGS1 and 2::GFP. TGS1 or 2 was C-terminally fused to GFP. **B.** Staining of vacuoles with MDY-64. Upper left: bright filed image, upper right: chlorophyll fluorescence of the plastids; lower left: MDY-64 fluorescence; lower right, merged image. **C. and D.** Targeting of full length TGS1::GFP and TGS2::GFP fusion proteins. From left to right: bright field image, chlorophyll fluorescence, GFP fluorescence, merged image. Bars indicate length in 5 µm.
2.5.3. **Expression of GFP fusion proteins in Δkre6 yeast strain BY05574**

In order to demonstrate whether TGS1 and 2 from *P. tricornutum* can complement Kre6 deficient yeast cells, we expressed these genes in the respective yeast mutant using a yeast vector. The expression of heterologous genes can be hampered, if the codon usage of the recipient strain is not suitable. Therefore, to demonstrate successful expression, we attempted to visualize gene expression of TGS1 and 2 in the yeast cells by genetically fusing GFP as a reporter protein to the C-termini of TGS1 and 2 from *P. tricornutum*, respectively. We inserted the fusion constructs into the vector pAG300, and expressed these constructs in yeast cells. The obtained mutant cell lines showed green fluorescence (Fig. 2.4), indicating that indeed the diatom proteins can be expressed in required amounts in yeast. Interestingly, the GFP fluorescence of the fusion proteins was found to be present throughout the yeast cells, while not being evenly distributed. This could indicate that some further transport signals for protein sorting in yeast cells may be missing or misinterpreted, resulting in an ambiguous targeting of the diatom proteins. It also has been reported that Kre6 in yeast cells may be transported from the endoplasmic reticulum (ER) to the plasma membrane, but also to budding growth sites (Kurita et al. 2011). As figure 2.4 shows, the green fluorescence of overexpressed TGS1 and 2::GFP proteins in Δkre6 cells has been observed in the cytosol, the ER, possibly the tonoplast and some secondary pathway organelles, but never at the sites of developing budding daughter cells.
Figure 2.4. Images of TGS1 and 2::GFP fusion proteins expressed in the yeast strain BY05574.

A. Expression of TGS1::GFP. B. Expression of TGS2::GFP. From left to right: bright field image, GFP fluorescence, merged images. Bars indicate length in 5µm.
2.5.4. Determination of the function of TGS1 and 2: complementation of Δkre6 yeast strain with TGS1 and 2

In a next step, we tested the functionality of the diatom TGS proteins by expressing TGS1 and 2 (with and without attached GFP) in the kre6-deficient S. cerevisiae strain Δkre6 (Kurita et al. 2011; Roemer et al. 1993; Roemer et al. 1994) and by screening for complementation. Deletion of kre6 in yeast shows a clear phenotype due to distinct cell wall defects, resulting in a very retarded growth combined with a hypersensitivity to the stain Calcofluor White (CFW). The kre6 mutant cells fail to grow in the presence of CFW, which is not lethal to wild-type cells (Kurita et al. 2011; Ram et al. 1994). The goal of this approach was the demonstration that the diatom TGS enzyme may rescue Δkre6 cells in the presence of CFW.

We performed complementation experiments by characterizing wild-type yeast cells, the Δkre6 strain and Δkre6 strains overexpressing TGS1 or 2 of P. tricornutum. When growing on agar plates, the wild type cells (transformed with an empty vector) were not sensitive to a concentration of 30μg CFW, while the Δkre6 cells showed a clear growth inhibition. Although the TGS1 or 2 expressing yeast mutants both showed some growth inhibition, they clearly were less affected by CFW than the Δkre6 strain alone, indicating that TGS1 and 2 can partially complement the missing Kre6 protein (Fig. 2.5A, B). Similar results were obtained for the TGS1::GFP and TGS2::GFP fusion proteins, indicating that the GFP protein, when fused to the C-termini possibly does not affect enzymatic activities. We furthermore studied cellular growth of wild type and mutant yeast cells in liquid culture. The cells were grown in SC medium including 15 μg/ml of CFW and growth was measured by following OD600 in a spectrophotometer. Figures 2.6A and B show the growth curves of the different yeast strains, demonstrating that Δkre6 cells transformed with empty vectors show a strong growth inhibition, while all strains complemented with TGS1, 2, TGS1::GFP or TGS2::GFP were able to grow. Interestingly, TGS1 is apparently complementing Δkre6 better than TGS2, an effect that was also, though to a lesser extent, visible in the plate assays (Fig. 2.5A). The results indicate that cell wall defects of the Δkre6 strain can be partially rescued by overexpression of TGS1 or 2 in the mutant cell lines, confirming that both proteins are the active 1,6-β-transglycosylases. TGS1 and 2 only partially rescued the phenotypes
of the \( \Delta kre6 \) strain, which is consistent with the complementation by Kre6 itself in \( S. \text{ cerevisiae} \) (Kurita et al. 2011; Nakamata et al. 2007). The complementation result is somewhat surprising, considering that Kre6 is lacking the signal peptide which is found in TGS1 and 2, indicating that they may reside in different compartments. However, this could either be explained by potentially different targeting mechanisms to the tonoplast in yeast cells and in diatoms (which possess a chloroplast ER), or by the rather ambiguous targeting of TGS1 and 2 in the yeast cells described above.

Recently, it has been reported that the amount of chrysolaminarin in diatoms was significantly reduced by knocking down UDP-glucose pyrophosphorylase (UGP) (Zhu et al. 2015). UDP-glucose has been demonstrated to supply activated glucose units for chrysolaminarin synthesis (Kroth et al. 2008; Roessler 1987). Two putative UDP-glucose pyrophosphorylase gene models (UGP1 (50444), UGP2 (23639)) have been described earlier (Kroth et al. 2008). Interestingly, UGP1 is a fusion protein that contains both UDP-glucose pyrophosphorylase and phosphoglucomutase (PGM) domains (Kroth et al. 2008). According to TargetP and SignalP predictions, UGP1 and 2 are targeted to the cytosol and the plastid, respectively. To elucidate the compartment in which chrysolaminarin is initially synthesized, it is essential to determine which UGP is involved in chrysolaminarin synthesis. Among the two UGPs, only the cytosolic UGP1 was studied so far (Daboussi et al. 2014; Zhu et al. 2015). After knocking down UGP1 in \( P. \text{ tricornutum} \), the cells apparently reallocated carbon flux from carbohydrate to lipid storage (Zhu et al. 2015). These results indicate that UGP1 may take part in the process of chrysolaminarin synthesis and that the synthesis of chrysolaminarin may be initiated in the cytosol, involving a potential glucan synthase located in the vacuolar membrane that may translocate the glucan backbone into the vacuole where it can be modified by the transglycosylases TGS1 and 2.
Figure 2.5. Complementation of a Δkre6 yeast strain by overexpression of TGS1 or 2.

A. Sensitivity of strains overexpressing TGS1 to Calcofluor White (CFW). B. CFW sensitivity of strains overexpressing TGS2. (A, B) Cells were grown in SC medium at 30°C overnight until OD\textsubscript{600nm}=1, and then serially diluted 4 times by ten-fold. Eight µl of these samples were spotted on an SC plate with or without 30 µg/ml CFW, and incubated at 25 °C overnight and then at 22 °C for 2d. One T\_ptTGS1 strain and two independent isolates of T\_ptTGS1::GFP (T\_ptTGS1::eGFP\_a and T\_ptTGS1::eGFP\_b) have been chosen. One T\_ptTGS2 strain and one T\_ptTGS2::GFP strain have been selected. wt + empty V: BY4741 transformed with the empty pAG300 vector; Δkre6+empty V: Y05574 transformed with the vector.
Figure 2.6. Growth of strains overexpressing TGS1 or 2 in liquid medium with CFW.

A. Growth of Δkre6, wild-type and strains expressing TGS1 in SC with CFW. B. Growth of Δkre6, wild-type and strains expressing TGS2. Cells were grown in SC medium with 15µg/ml CFW at 28°C. Two independent cell lines of Δkre6 transformed with the empty vector (Δkre6 + empty V_a, Δkre6+emptyV_b) were used.
2.6. Conclusion

Diatoms, Oomycetes and brown algae possess 1,3-β-glucans, termed as chrysolaminarin, mycolaminarin and laminarin, respectively. All these polysaccharides have similar structures based on linear 1,3-β-glucan chains branched infrequently with 1,6-β-linked residues. Laminarins furthermore contain mannitol residues which are not found in mycolaminarin and chrysolaminarin (Percival and Ross 1951; Read et al. 1996; Wang and Bartnicki-Garcia 1974). Therefore, the common occurrences of Kre6-like transglycosylases in diatoms, oomycetes and brown algae as well as the functional complementation of Δkre6 by TGS1 and 2 support our hypothesis that TGS1 and 2 might be involved in the synthesis of 1,6-β-linked residues of chrysolaminarin in diatoms. The tonoplast localization of TGS1 and 2 in *P. tricornutum* is a further indication for this role, as within the tonoplast the enzymes may enzymatically modify chrysolaminarin. For the future investigation of chryolaminarin synthesis, it will be important to study the functionality of the TGS enzymes as well as the intracellular location of the key enzyme of this process, the glucan synthase.

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Chapter 3

3. Silencing of a vacuolar β-1,3-glucan synthase in the diatom Phaeodactylum tricornutum increased the soluble sugars and compromised the photosynthetic activity

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3.1. Abstract

Diatoms use β-1,3-glucan, chrysolaminarin, for carbohydrates storage. Chrysolaminarin is comprised of β-1,3-glucan chain with branches of glucose units at C-6 positions. However, the mechanism of chrysolaminarin synthesis in diatoms is barely known. The enzyme β-1,3-glucan synthase (GS) apparently catalyses the synthesis of the β-1,3-glucans by transferring UDP-glucose to form the β-1,3-glucan bond. In this paper, we characterized the only GS (protein ID: 56808, PtGS) in the diatom Phaeodactylum tricornutum (PtGS) by expression of GFP fusion proteins and by creating and investigating silencing mutants of PtGS. We demonstrate that PtGS is located in the vacuolar membrane. The knock down strains gs_a, gs_b and gs_c show significantly lower growth rates, but increases in soluble sugar contents. They also exhibit lower photosynthetic capacities as well as higher photoprotective abilities compared to the wild type cells. The correspondingly increased amounts of xanthophyll cycle pigments in the gs mutants were located within the fucoxanthin chlorophyll protein complexes. The most strongly silenced gs_c mutant furthermore shows aberrations of the usually very regular thylakoid membrane patterns compared to the wild type, including an increase in the thylakoid thickness, a reduction in the numbers of thylakoids per plastid and an increase in the numbers of lamellae (around 5 or more instead of 3) per thylakoid stack. We propose that a reduction of chrysolaminarin synthesis by knocking down PtGS results in a backlog of carbohydrates, accordingly affects photosynthesis.

3.2. Introduction

Diatoms are unicellular photoautotrophic eukaryotes, contributing around 20 and 40% of primary production to global and oceanic biogeochemical cycles, respectively (Falkowski et al. 1998). The principal energy storage polysaccharide of diatoms is chrysolaminarin, a linear non-crystalline β-1,3-glucan with branching β-1,3-glucan units at the C-6 position(Beattie et al. 1961a; Ford and Percival 1965a). In diatoms, chrysolaminarin is stored in intracellular vacuoles (Chiovitti et al. 2004). Vacuolar β-1,3-glucans are also found within other stramenopiles like oomycetes and brown algae (Percival and Ross 1951; Read et al. 1996; Wang and Bartnicki-Garcia 1974). β-1,3-glucans are typical metabolites in stramenopiles (Michel et al. 2010), which widely exist in living organisms. They play a variety of roles in organisms, such as participating in cell
division in plants, and in forming cell wall in fungi, oomycetes, plants, and brown algae, assisting in colonization and cell to cell interactions in prokaryotes organisms (Levy and Epel 2009; Myklestad and Granum 2009; Stanisich and Stone 2009). Besides β-1,3-glucans, in diatoms, the other abundant polysaccharide is chitin which has been found within *Thalassiosira* and *Cyclotella* (Gügi et al. 2015). Chitin consisting of β-1-4-linked N-acetylg glucosamine, an insoluble polysaccharide, is involved in the cell wall formation of the most diatoms (Gügi et al. 2015; Morozov and Likhoshway 2016). However, chitin fibres have not been identified so far, in the cell wall of *P. tricornutum*. The cell wall polysaccharide of *P. tricornutum* is mostly composed of mannose (Gügi et al. 2015). Nevertheless, the genes involved in the synthesis of chitin were identified within the genome of *P. tricornutum* (Kroth et al. 2008).

During different growth phases, the β-1,3-glucans can constitute between 10 and 80% of organic dry weight in diatoms (Myklestad 1974). Due to this high cellular content of the β-1,3-glucans, a better understanding of the molecular synthesis of β-1,3-glucans is essential for elucidating the carbon allocation in diatoms. However, important issues like β-1,3-glucans biosynthetic pathways in diatoms as well as in other stramenopiles are still mysterious, including the net transport of carbohydrates from the plastid (where photosynthesis takes place) to the vacuole (where the chrysolaminarin is stored), the coordination between the storage polysaccharide biosynthetic pathway (the sink of carbon flux) and the photosynthetic system (the source of carbon flux), but also the mobilization in the dark.

β-1,3-glucan synthase (GS) is essential for the synthesis of β-1,3-glucans, it can polymerize the activated glucose donor, UDP-glucose, to a β-1,3-glucan polymer (Roessler 1987). GSs are separated into two glycosyltransferase (GT) families: GT2, a polyspecific family including bacterial β-1,3-glucan synthases, and GT48, comprising only eukaryotic β-1,3-glucan synthases (Michel et al. 2010b). The GSs are likely to function as a multi-subunit membrane-associated complex. For example, in yeast, the characterized GS complexes (FKS1 and FKS2) consist of a regulated subunit (Rho1p, a Rho-type small GTPase) and a β-1,3-glucan synthase catalytic subunit (Mazur and Baginsky 1996; Okada et al. 2010). In plants, a callose synthase 1 (CalS1) subunit binds a UDP-glucose transferase (UGT1), which attaches to Rop1, a homologue of Rho (Hong et al. 2001; Verma and Hong 2001). So far, the GSs in diatoms and in other algae have not been characterized.
Here, we describe the identification, characterization and knock-down mutagenesis of the unique GS (protein ID: 56808) in *Phaeodactylum tricornutum* (PtGS). We reveal that PtGS is a tonoplast-located protein. The knock-down strains of PtGS show an interesting phenotype, which includes the rearrangements of the thylakoid membranes, a dramatically reduced number of thylakoids and the increased lamellae in thylakoid stack. Moreover, all of the silenced gs strains studied here, exhibit decreased photosynthetic capacities and increased xanthophyll cycle pigments as well as an increased NPQ. Furthermore, the analysis of the pigment-protein complexes of gs_c thylakoids show the increased diadinoxanthin (Dd) and diatoxanthin (Dt) are located within fucoxanthin chlorophyll protein complexes (FCP).

### 3.3. Materials and Methods

#### 3.3.1. Cell Culturing and Light Treatments

*P. tricornutum* (strain UTEX646, UTEX Culture Collection of Algae, University of Texas, http://www.bio.utexas.edu/research/utex/) was grown at 20°C, in modified f/2 medium with artificial half-concentrated sea salts (16.6 g l⁻¹) and 0.09 μM MnCl₂ (Guillard 1975; Guillard and Ryther 1962). The cells were grown with continuous white light at 70 μmol photons m⁻² s⁻¹ and harvested at the exponential growth phase for the following experiments.

#### 3.3.2. Phylogenetic analyses

Homologous proteins were searched against UniProt Reference Proteomes using HMMER (http://hmmer.janelia.org/), NCBI server (http://www.ncbi.nlm.nih.gov/) and JGI genome portal (www. http://genome.jgi.doe.gov/), the significant hits were further classified based on their domain architectures into “G”: proteins only containing Glucan_synthase domain; “GG”: proteins containing two Glucan_synthase domains; “GF”: proteins containing Glucan_synthase and FKS1_dom1 domains; “GFV”: proteins containing Glucan_synthase, FKS1_dom1, and Vta1domains; “GS”: proteins containing Glucan_synthase, and SKN1 domains; “GFS”: proteins containing Glucan_synthase, FKS1_dom1, and SKN1 domains; “GFR”: proteins containing Glucan_synthase, FKS1_dom1, and RVT_1 domains; “GFD”: proteins containing Glucan_synthase, FKS1_dom1, and DUF4203 domains; “GH”: proteins containing Glucan_synthase and Glyco_hydro domains and “GFSD”: proteins containing Glucan_synthase, FKS1_dom1, SKN1, and...
DUF4203 domains. Redundant and too short sequences were removed. Totally, 173 proteins were selected for the further phylogenetic analysis. The selected homologous proteins were first aligned using T-Coffee v11 (Notredame et al. 2000) (in accurate mode) and MAFFT E-INS-I with default parameters (Katoh et al. 2002), then the two alignments were further processed using TrimAl v1.3 (Capella-Gutierrez et al. 2009) to keep columns with a consistency score more than 0.5 (option ct=0.5), and with gaps less than 50% (optiongt=0.5). The TrimAl processed alignment was further subjected to ProtTest v3.4 to find the best model among all distributions, with BEST tree search operation (-s option), empirical frequency estimation (-F option), and other default parameters. The estimated best model is LG+I+G+F according to maximized log likelihoods and bayesian information criterion. With the estimated best model, RAxML v8.1.20 (Stamatakis 2014) was used to infer a best-scoring maximum likelihood tree from a parsimony random seed 12345 with 20 maximum likelihood searches. Then 100 slow bootstrap searches were performed to infer bootstrap replicate trees using the same model, finally the bootstrap values were projected to the best maximum likelihood tree.

3.3.3. Generation of vectors for gene silencing and eGFP fusion genes

The 110 bp gs antisense fragment flanked by restriction enzymes was generated by Eurofins MWG Operon (Ebersberg, Germany) (Support information, SI Materials and Methods). The first intron of PtNTT1 (JGI_ID_49533) (128bp) was used as the linker between sense and antisense strands. The sense strand, PtNTT1 linker and antisense strand were sequentially assembled with the vector pPha-NR (GenBank accession number JN180663.1; (Stork et al. 2012)) via the standard cloning procedures and Gibson Assembly® Master Mix kit (New England Biolabs GmbH, Frankfurt, Germany) (Barnes 1994; Gibson et al. 2010; Gibson et al. 2009; Sambrook et al. 1989; Zaslavskaia et al. 2000). Hence, vector pPTV_GSsi was generated. The sequence of the 110 bp gs antisense fragment synthesized by Eurofins MWG Operon is:

\[
\text{GTTAAC(HpaI)ACAGTTGCGGGACCAGAAGTACTCGTTAAAGTCATCGTAGTTCCGTTTATCGGGATGA}
\text{TCCGCATCAGACCTCATGTCTTGCGCCACGATGTCATAAATTGGTGTAATCAAGCTT(HindIII)GAATT}
\text{C(EcoRI)}.
\]

The isolation of \textit{P. tricornutum} genomic DNA and cDNA respectively was performed as described previously (Ewe 2015; Vugrinec 2011). Gene gs was assembled with the modified pPha-T1 vector (GenBank accession AF219942.1) equipped with the \textit{egfp} gene, by Gibson Assembly® Master Mix
kit and the standard cloning procedures. (Barnes 1994; Gibson et al. 2010; Gibson et al. 2009; Sambrook et al. 1989; Zaslavskaya et al. 2000). Thereby, vector pPTV\_GS::eGFP was constructed. Vectors were transformed into wild type *P. tricornutum* cells using the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA, USA) (Zaslavskaya et al. 2000).

### 3.3.4. Protein expression in wild type, gs and GS::GFP cell lines

Proteins were extracted using the lysis buffer (Urea 8M; Thiourea 3M; SDS 1%; 1x lysis buffer containing protease inhibitor “complete EDTA-free” (Roche, Basel, Switzerland)). Further proteins were separated using the SDS-PAGE in 8% SDS gel (Laemmli 1970). In order to obtain a better shift between GS the protein and the fusion protein GS::GFP, SDS-PAGE was performed in a 4 °C constant-temperature room for 3 hours. Next, proteins were transferred onto a nitrocellulose membrane (Whatman Protran BA 79, Whatman Inc., Maidstone, Kent, UK) using Trans-Blot Turbo (Bio-Rad, Hercules, California, USA). The expression level of protein GS in wild type and gs cell lines was detected using the antisera against GS (Agrisera, Vännäs, Sweden). A specific antibody against green fluorescent protein (GFP) (Invitrogen, Carlsbad, California, USA) and the antisera against GS were used for the detection of the fusion protein GS::GFP in wild type and GS::GFP expressed cell lines.

### 3.3.5. Determination of soluble sugar levels

Soluble sugars were quantified in the soluble extract. For quantification the identical number of cells (volume approximately 40 ml) were harvested by vacummfiltration. Cells (including filters) were disrupted by grinding in liquid N2. Soluble sugars were extracted by adding double-distilled water and vigorous mixing. Insoluble material was removed by centrifugation at 10 000 g for 2 min at 4° and the supernatant was collected. Enzymatic reactions were terminated by incubation at 95°C for 5 min and soluble sugars were subsequently quantified via ion chromatography (Compact IC; Metrohm AG, http://www.metrohm.com/). Sugars were separated with a RCX30 anion-exchange column (Hamilton Company, http://www.hamiltoncompany.com: solvent 0.1 M NaOH). Sugars were normalized according to the identical amount of ground material (filters were substracted).
3.3.6. **Fluorescence microscopy, electron microscopy and the staining of vacuoles in *P. tricornutum***

The transformed cell lines expressed with GFP fusion proteins have been examined with LSM 510 META (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). The LSM 510 META was conducted using a Plan-Apochromat 63 ×/1.4 Oil DIC objective.

Electron microscopy has been conducted using a Zeiss TEM 912 Omega (Jena, Germany) (performed at the Electron Microscopy Center of the Universität Konstanz, Germany) as described by Ewe (Ewe 2015) and a Zeiss Libra 120 electron microscope at 80kV at the Nanostructure Laboratory of the Universität Konstanz, Germany.

The pattern of vacuoles in *P. tricornutum* was detected using a green fluorescent vacuolar membrane marker MDY-64 as described by (Huang et al. 2016). The stained cell lines were detected using an epifluorescence microscope, Olympus BX51 (Olympus Europe, Hamburg, Germany).

3.3.7. **Oxygen evolution determination and chlorophyll fluorescence**

Oxygen measurements were performed using a Clark-type electrode (Hansatech Instruments Ltd; Norfolk, United Kingdom). Briefly, the cells were harvested during the exponential growth phase. Cell densities were adjusted to 5 × 10^6 cells ml⁻¹ using f/2 medium. Chlorophyll fluorescence was measured using an Aquapen AP-100 (Photon Systems Instruments; Drasov, Czech Republic). The cell densities were adjusted into 4 × 10^6 cells ml⁻¹. Non-photochemical quenching (NPQ) was based on (Fm – Fm’)/Fm’ (Bilger and Bjorkman 1990). Fm’ stands for the maximal fluorescence value in light-adapted state. The maximum photosynthetic efficiency of photosystem II (PSII) was calculated as (Fm - Fo)/Fm = Fv/Fm. Fm and Fo refer to maximum and minimum fluorescence values in dark-adapted state.

3.3.8. **Pigment analysis and preparation of pigment-protein complexes**

Pigments extraction and HPLC analysis were performed as described previously (Jakob et al. 1999; Lepetit et al. 2013). Thylakoids and pigment protein complexes were isolated as described by (Lepetit et al. 2007). Here, a ratio of β-dodecyl maldoside (DM) per Chl of 30 was used to solubilize thylakoids. Fractions were harvested with a syringe from the bottom to the top in order to
prevent mixture, and absorbance of individual fractions was measured in a GE Healthcare Ultrospec photometer.

3.4. Results

3.4.1. Phylogenetic analysis

Phylogenetic analysis revealed PtGS (protein ID: 56808) is not related to the prokaryotic GS and exhibits strong similarities to the GSs of plants (Fig. 3.1 and S3, and (Michel et al. 2010b)). Eukaryotic GSs are separated into two distinct groups which are composed of i) Fungi and ii) Euglenids, Stramenopiles, Alveolates, Rhizaria, Haptophytes and Viridiplantae (Fig. 3.1 and S3). Very interestingly, PtGS seems to be closely related to the GSs of Rhizaria and Alveolates (Fig. 3.1 and S3), supporting the recent evolutionary theory (Burki et al. 2007) that SAR (Stramenopiles+Alveolates+Rhizaria) is a new super assemblage of eukaryotes. This phylogenetic tree also shows that, during evolution, some phyla evolved variable domains in addition to the β-1,3-glucan synthase catalytic domain (Fig. 3.1 and S3).

![Phylogenetic tree of GS](image)

**Figure 3.1.** Radial phylogenetic tree of GS.

Homologous proteins were searched against UniProt Reference Proteomes using HMMER. Totally, 173 proteins were selected for phylogenetic analysis. The alignment used for this tree is listed in supplement document.
3.4.2. GS is associated with the vacuolar membrane

PtGS (protein ID: 56808) consists of 2150 amino acid residues with a calculated molecular mass of 242 kDa. It consists of two functional domains: the class I domain of yeast β-1,3-glucan synthase FKS1 (FKS1_domain1) and a β-1,3-glucan synthase central catalytic domain (GS central catalytic domain). The polypeptide of PtGS contains 23 transmembrane helices, which are distributed at both termini of FKS1_domain1 and GS central catalytic domain, whereby two functional hydrophilic loops are formed (Fig. 3.2A). This transmembrane and catalytic domain organization is distinct from the GS domain structures demonstrated in yeast and A. thaliana, where the FKS1_domain1 and GS central catalytic domain are not split by the transmembrane helices, but rather forming a single hydrophilic loop (Okada et al. 2010; Verma and Hong 2001). We could not detect any N-terminal signal peptide in PtGS by bioinformatics tools like SignalP.

To examine the cellular localization of PtGS, the Ptgs gene was C-terminally fused to egfp (Fig. 3.2A). The fusion protein PtGS::GFP was expressed in P. tricornutum (Fig. 3.2B_2). Obtained transformants were screened and characterized by fluorescence microscopy, revealing that PtGS is targeted to the vacuolar membranes. The fluorescent pattern in cells expressing protein PtGS::GFP is consistent with the pattern of the vacuoles stained by the vacuolar membrane maker MDY-64 (Fig. 3.2B). In agreement with the vacuolar location of β-1,3-glucan reported previously for the diatoms P. tricornutum, Cylindrotheca fusiformis, and T. pseudonana, (Chiovitti et al. 2004), this indicates that the β-1,3-glucans are synthesized at the vacuolar membrane and simultaneously imported into the vacuoles by PtGS. Because of the large size of the Ptgs gene, there is the possibility that in some transformants, only fragments may have been inserted into the genome, which could result in mistargeting of the fusion protein. Therefore we performed immunoblots, which confirmed that the large fusion protein PtGS::GFP is completely translated in the transformants. A GFP antibody labeled a 280 kDa band, which is the size of the GS::GFP fusion, that is only present in PtGS::GFP expressed cell lines (Fig. 3.2C_1). In addition, we applied an antiserum that we had raised against the PtGS protein. This antiserum specifically labeled a 250kDa GS bands in both the wild type (WT) and PtGS::GFP expressing cell lines (Fig. 3.2C_2), while it also targets a 280 KDa band of the fusion protein in the PtGS::GFP strains.
Figure 3.2. Localization of *PtGS* C-terminally fused to GFP in *P. tricornutum* (Scale bars: 5μm).

(A) Constructs used for cellular localization. Within the brace: the putative PtGS model. Blue cylinder: the class I domain of yeast β-1,3-glucan synthase FKS1. This domain is hypothesized to interact with one or more factors for β-1,3-glucan synthesis (Okada et al. 2010). Red cylinder: β-1,3-glucan synthase central catalytic domain. Black rectangles: transmembrane helices. (B-1) Staining of vacuoles with MDY-64. From left to right: MDY-64 fluorescence, chlorophyll fluorescence, merged image. (B-2) Images of *PtGS::GFP* fusion protein expressed in *P. tricornutum*. From left to right: GFP fluorescence, chlorophyll fluorescence, merged image. (C) Analysis of the expression of fusion protein *PtGS::GFP* in *P. tricornutum*. (1) Immunoblotting using the antibody against GFP. (2) Immunoblotting using the antibody against PtGS. WT: wild type cell line. GS::GFP_a and GS::GFP_b: two independent cell lines of PtGS::GFP.
3.4.3. Silencing PtGS results in impaired growth and increases of soluble sugar levels in gs mutants

In order to knock down the expression level of PtGS in *P. tricornutum*, RNA silencing using constructs containing inverted repeat sequences of PtGS was applied. After transformation of this construct by particle bombardment in *P. tricornutum*, we obtained three GS silencing lines named gs_a, gs_b and gs_c, which all showed reduced GS expression levels compared to the WT cells. By quantifying the western blot bands, the amounts of GSs protein in gs_a, gs_b and gs_c were calculated to be reduced by 90, 68, and 22%, respectively, compared to the WT (Fig. 3.3A_1). The growth of gs_a, b and especially gs_c, is markedly impaired (Fig. 3.3B) indicating that knock-down of PtGS has a severe impact on the cell physiology.

To investigate the effects on the changes of metabolites after knocking down PtGS, we determined soluble sugar levels in WT and gs_c cell lines. As figure 3.3C shown, the gs_c cells show a 4.5 fold increase in the glucose concentration, in contrast to WT cells. Interestingly, an increase in the concentration of another soluble sugar, a disaccharide-like sugar, was also observed in gs_c cells, compared to the WT cells.
Figure 3.3. Effect of knocking down PtGS on growth and sugars.

(A) GS protein levels by western blot detection. A_1: Immunoblotting using the antibody against PtGS in WT and gs mutants. The expression levels of PtGS are shown below the western blot. A_2: loading controls. (B) Growth curves of WT, gs_a, b and c. (C) Determination of soluble sugar levels in WT and gs_c cell lines. Glucose concentration in WT was set to 100% (Standard error is 21%). Glucose concentration in gs_c cells was calculated according to the value of WT. Soluble sugar 2 (possibly a disaccharide) concentration in WT was set to 100% (Standard error is 6.7%). Soluble sugar 2 (possibly a disaccharide) concentration in gs_c cells was calculated according to the value of WT.
3.4.4. **A lower expression of GS causes pigment alterations, a reduced photosynthetic efficiency and a higher photoprotection capacity**

We characterized the physiological properties of the *PtGS* silencing mutants and detected a significant enhancements of the total amounts of diadinoxanthin (Dd) and diatoxanthin (Dt) in all *gs* mutants (Fig. 3.4A and Table. 3.1). These pigments are involved in the so-called xanthophyll cycle (XC) (Stransky and Hager 1970), which contributes to photoprotection (reviewed in (Goss and Lepetit 2015)). The enzymatic de-epoxidation from Dd to Dt in the antenna enables excited chlorophyll molecules to dissipate their absorbed light energy as heat, which releases excitonic pressure on photosystem II, preventing reactive oxygen species from inducing cell damage. Due to the parallel decrease of fluorescence emission, this mechanism is called non-photochemical fluorescence quenching (NPQ) (reviewed in (Goss and Lepetit 2015)). In all silencing lines, there is a significant induction of de-epoxidation already under low light cultivation conditions compared to the WT cells, indicating some kind of stress for the mutants (Fig. 3.4B). Moreover, enhanced NPQ capacities in *gs_a, b* and *c* were observed, with the highest NPQ capacity in *gs_c*, showing the lowest amounts of GS (Fig. 3.4C). The NPQ capacities exhibit a reverse relationship with the maximum PSII quantum yield (Fv/Fm) between the WT and the mutants (Fig. 3.4C), with again the lowest quantum yield in *gs_c*. The varying values of NPQ and Fv/Fm show a positive correlation with the different silencing levels of *PtGS*. However, a 10% reduction of *PtGS* is already sufficient to induce physiological and metabolic changes (Fig. 3.3A and Fig. 3.4).

In diatoms, the Dd+Dt pools are distributed in different areas of the thylakoid membranes. Dd+Dt is localized in: 1) antenna proteins within the the peripheral antenna (FCP) for participating in NPQ, 2) antenna proteins within photosystem I (PSI), possibly for harvesting light, and 3) monogalactosyldiacylglycerol (MGDG) lipid layers where they are assumed for antioxidant function (Lepetit et al. 2010). We investigated the localization of the increased amount of Dd+Dt in the mutants by solubilization of the thylakoids and analysis of the pigment-protein complexes. The additional pigments were exclusively localized in the FCP (fraction 6 and 7) and the free pigment fraction (fraction 8), but not in the photosystems (fraction 1-4) (Fig. 3.5B). Interestingly, the number of antenna per photosystem is increased in the *gs* mutants (Fig. 3.5C). Overall, the knock down strain exhibits increased amounts of Dd+Dt amounts which both are located in the
FCP. Here, the Dd+Dt pool is likely involved in the NPQ mechanism, thus leading to the higher NPQ capacity.

Gs_a, b and c show 1.3, 1.5 and 2.2-fold lower maximum oxygen evolution rates compared to WT cells (Fig. 3.4D). The reduced oxygen evolution rates, the enhanced NPQ capacity, the higher de-epoxidation state (DES) and the decreased PSII quantum yield in gs mutant cells reveal that less absorbed light energy is effectively transferred from the antennae to the photosystems and less biomass is created by the same amount of absorbed light energy compared to the WT.
Figure 3.4. Effects of GS gene silencing on photosynthetic properties.

(A) Xanthophyll cycle pool pigments (diadinoxanthin (Dd) and diatoxanthin (Dt)), normalized to Chlorophyll a (Chla). (B) The deepoxidation states (Briolay et al.) of the xanthophyll cycle pool, calculated as Dt/(Dd+Dt). (C) Non-photochemical quenching (NPQ) and maximum PSII quantum yield. NPQ was calculated as \((F_m - F_m')/F_m\); the PSII quantum yield was calculated as \((F_m - F_o)/F_m = F_v/F_m\). (D) Oxygen evolution rates. Light intensities were: 0, 10, 15, 20, 50, 70, 100, 200, 300, 500, 750, and 1000 μmol photons m\(^{-2}\) s\(^{-1}\). Errors bars represent standard error of mean. The two-sided Student’s t-Test was performed. * denotes significant level less than 0.05, ** denotes significant level less than 0.01.
Table 3.1. Pigment composition of the wild type and gs cell lines (in mmol (mol Chla)$^{-1}$).

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>gs_a</th>
<th>gs_b</th>
<th>gs_c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlc/Chla</td>
<td>129.62 ± 1.61</td>
<td>121.06 ± 2.51</td>
<td>123.70 ± 1.33</td>
<td>166.20 ± 0.80</td>
</tr>
<tr>
<td>Fx/Chla</td>
<td>525.86 ± 9.00</td>
<td>471.63 ± 13.24</td>
<td>514.36 ± 2.93</td>
<td>661.50 ± 13.07</td>
</tr>
<tr>
<td>β–Car/Chla</td>
<td>56.85 ± 1.43</td>
<td>54.12 ± 2.09</td>
<td>54.54 ± 0.70</td>
<td>35.99 ± 0.40</td>
</tr>
<tr>
<td>Dd/Chla</td>
<td>84.63 ± 0.87</td>
<td>106.97 ± 3.23</td>
<td>105.96 ± 1.77</td>
<td>104.75 ± 1.07</td>
</tr>
<tr>
<td>Dt/Chla</td>
<td>0.16 ± 0.09</td>
<td>2.61 ± 0.29</td>
<td>2.89 ± 0.33</td>
<td>3.14 ± 0.21</td>
</tr>
</tbody>
</table>

Cells were grown under constant light at a light intensity of 70μmol photons m$^{-2}$ s$^{-1}$. Cells were harvested at the early exponential phase. chlc, chlorophyll c; chla, chlorophyll a; Fx, fucoxanthin; β-car, β- carotene; Dd, diadinoxanthin; Dt, diatoxanthin. Values are means ± SEM for 5-6 biological replicates. Bold numbers indicate the pigments increased in all of these three gs (gs_a, b, c) mutants.
Figure 3.5. Analysis of pigment-protein complexes.

(A) Separation of the pigment protein complexes of WT and gs_c by Suc density gradient centrifugation with a n-dodecyl β-D-maltoside (DM) to Chl ratio of 30. Eight different fractions were obtained from the bottom to the top of the full gradient which in this order contain the PSI, PSII, FCP, and free pigments fractions. (B) Absorption spectra of the eight fractions. Based on the previous studies on absorption spectra of different pigments (Lepetit et al. 2010; Roy 2011; Szabó et al. 2010), the absorption at the wavelength of 440nm represents the relative amount of the total pigments; the absorption at 490nm represents the relative amount of the total carotenoids (basically Fx, Ddx); the absorption at 680nm represents the relative amount of Chla; and the absorption at 540nm represents the relative amount of Fx. The absorptions of the pigments are normalized to Chla. A two-sided paired Student’s t-Test was performed with the mearments of F6, F7 and F8, based on the means for comparing two series data. (C) The ratio of fucoxanthin chlorophyll protein complexes (FCP) and photosystem (The amount of FCP was caculated by integrating the absorbance of the fractions from 6 to 8 at the wavelength of 680nm; the amount of PS was caculated by integrating the absorbance of the fractions from 1 to 4 at 680nm).
3.4.5. Blocking of the β-1,3-glucan synthesis in diatoms alters the thylakoid membrane organization

Electron microscopical (EM) analyses of the gs cells indicate considerable changes of the thylakoid arrangement patterns compared to WT cells (Fig. 3.6A-F). As shown in Fig. 3G, thickness of thylakoid stacks in WT cells is generally around 50nm. Here, we observed an enlargement of the thickness of thylakoid stacks in the gs cell lines in comparison to the WT. Particularly in the gs_c cells, a width of around 66 nm was measured which is a 1.32 fold increase(Fig. 3.6G). Interestingly, accompanied with the increased thylakoid diameter, a decrease (1.37 fold) in the number of thylakoid stacks is also observed in the gs_c cells (Fig. 3.6H). This striking reverse correlation between the increased thylakoid thickness and the reduced number of thylakoids indicate drastic changes in the thylakoid structure. The mutant gs_b does not exhibit such drastic changes, here only a slight increase of the thylakoid thickness was detected (Fig. 3.6G). While in gs_a cells, no differences compared to the WT cells were observed. Very interestingly, the strict triple lamellar morphology of thylakoid stack is affected, as the number of the lamellae per thylakoid stack are increased in the gs_c cells (Fig. 3.8 and Table. 3.2). In contrast to the WT cells, we often observed five or even more lamellae forming into one thylakoid stack in the gs_c cell lines (Fig. 3.6F, Fig. 3.8C and Table. 3.2).As studied previously (Pyszniak and Gibbs 1992), thylakoid stacks in the plastids of P. tricornutum cells are usually organized as three lamellae stacks, occasionally while rarely two or four lamellae stacks are found (Fig. 3.6B, Fig. 3.8A and Table 3.2).
Figure 3.6. Electron micrographs of the wild-type *P. tricornutum* and the *gs* cell lines. 

(A, C, E) Overview of the WT (A), the *gs_b* (C) and the *gs_c* cells (E). (B, D, F) Thylakoids of the WT (B), the *gs_b* (D) and the *gs_c* cells (F). Bars 500 nm (A, B, C) and 250 nm (E). Whites lines: the lamellae grouped into one thylakoid stack, M: mitochondria, N: nucleus, P: plastid. (G) Thickness of the thylakoid stack in the plastids of the WT, *gs_b* and *gs_c*. (H) Numbers of the thylakoid stacks in the plastids of the WT, *gs_b* and *gs_c*. Red box: mean value of the dot plot. The two-sided Student’s t-Test was performed. ** denotes significant level less than 0.01.
Figure 3.7. Analysis of thylakoids

(A) Analysis of thylakoid lumenal space (TLS), and interthylakoidal space (TS) in the wild type and gs cells. Red box: mean value of the dot plot. (B) Schematic presentation of the thylakoid stack in diatoms. Three lamellae, occasionally two or four lamellae are organized into one thylakoid stack (Pyszniak and Gibbs 1992).
Figure 3.8. Electron micrographs of the wild-type *P. tricornutum* and the *gs* cell lines.

(A) The wild type cells and thylakoids of the WT. From up to down, all of the bars indicate 500 nm. (B) The *gs_b* cells and thylakoids of *gs_b*. From left to right, the bars indicate 500, 500 and 200 nm. (C) The *gs_c* cells and thylakoids of *gs_c*. Bars indicate 250, 500, 500, and 250 nm from up to down. Here, the thylakoid stacks with clear lamellaer structures are labeled with white lines and the number of lamellae in one stack is listed in Table 3.2. G: Golgi apparatus, M: mitochondria, N: nucleus, P: plastid. Bars were shown on the images.
Table 3.2. Analysis of the lamellar numbers in thylakoid stacks of the wild type and gs cell lines

<table>
<thead>
<tr>
<th>lamellar numbers per thylakoid</th>
<th>WT</th>
<th>gs_b</th>
<th>gs_c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line_1</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Line_2</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Line_3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Line_4</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Line_5</td>
<td>3</td>
<td>3</td>
<td>above 5</td>
</tr>
<tr>
<td>Line_6</td>
<td>3</td>
<td>3</td>
<td>above 3</td>
</tr>
<tr>
<td>Line_7</td>
<td>3</td>
<td>3</td>
<td>above 3</td>
</tr>
<tr>
<td>Line_8</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Line_9</td>
<td>3</td>
<td>3</td>
<td>above 5</td>
</tr>
<tr>
<td>Line_10</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

Line 1-10 were shown with white lines on Fig. 3.8.

3.5. Discussion

3.5.1. The molecular synthesis pathway of chrysolaminarin in diatoms

UDP-glucose (UDPG) has been demonstrated to supply activated glucose substrates for chrysolaminarin synthesis (Roessler 1987, Kroth et al. 2008). Two putative UDPG pyrophosphorylase gene models have been described earlier: (UGP1/PGM (50444) (a fusion protein of UGP and phosphoglucomutases (PGM)), UGP2 (23639)) (Kroth et al. 2008). According to TargetP and SignalP predictions, UGP1 and 2 are targeted to the cytosol and into the plastid, respectively. Hence, the experimental determination of the cellular localization of PtGS is essential for elucidating the site of β-1,3-glucans synthesis. Here, we demonstrated that PtGS is targeted into the vacuolar membranes. This result indicates that the PtGS may access UDPG from the cytosol, initiating the synthesis of β-1,3-glucans in the cytosol. Later the polymers of β-1,3-glucans maybe translocated into the vacuole by PtGS (see Fig. 3.9). In addition, there is experimental evidence that UGP1/PGM, which is predicted to reside in the cytosol is likely involved in the synthesis of chrysolaminarin, since after knocking-down UGP1, the amount of...
Chrysolaminarin in diatoms is significantly reduced (Zhu et al. 2015). After the translocation of the β-1,3-glucans into vacuoles, the β-1,3-glucan backbone probably may be modified by the β-1,6-transglycosylases to form the mature chrysolaminarin. We recently identified three putative gene models that encode TGS (gene models: 50238 (TGS1), 56509 (TGS2), 48300 (TGS3)). TGS1 and TGS2 were targeted into the vacuolar membranes in *P. tricornutum* (Huang et al. 2016). Moreover *tgs1* and *tgs2* were confirmed to encode active β-1,6-transglycosylases by complementation of a transglycosylase-deficient yeast strain (Huang et al. 2016). Overall, the polymers of β-1,3-glucans are initiatively synthesized from cytosol by PtGS and then translocated into vacuoles where chrysolaminarin is formed by adding the β-1,6-glucan bond to the β-1,3-glucan backbones.

3.5.2. Knocking down the expression of PtGS induces strong physiological changes of the photosynthetic apparatus via metabolic feedback on photosynthesis

The accumulation of glucose has been shown in Figure. 3.3C. In addition, an increase in the content of a disaccharide-like molecule was also observed. Knocking down the GS enzyme may induce the accumulation of UDP-glucose, glucose-6-phosphate (G6P) and the other intermediates involved in the synthesis of chrysolaminarin (Fig. 3.9). To investigate the potential candidate of this increased disaccharide-like molecule, the genome of *P. tricornutum* was screened, we identified two isoforms of the fusion protein: Trehalose-6-phosphate synthase/Trehalose-6-phosphatase (TPS/TPP) (protein ID: 46137 and 48682), by which UDP-glucose and Glu-6-P are firstly synthesized and then dephosphorylated into trehalose. Therefore, this disaccharide might be trehalose.

Here we propose that silencing of GS results in a backlog mechanism caused by the accumulated soluble sugars, which could regulate the photosynthesis in the mutants. In photosynthesis, electrons are transported across the thylakoid membrane towards NADP, yielding NADPH, while protons are translocated in the lumen from where they return to the stroma via the ATP synthase, which leads to the production of ATP from ADP. Both, NADPH and ATP is then consumed in the Calvin cycle for fixation and reduction of CO₂. The primary CO₂ fixation product of the Calvin cycle is 3-PGA which provides the original substrate for synthesis of β-1,3-glucans in *P. tricornutum* (Fig. 3.9). Once β-1,3-glucan synthesis is inhibited, triose
phosphates may accumulate, which might inhibit the consuming reactions of available ATP and NADPH, as the carbohydrate acceptors for ATP and NADPH are not replenished (Fig. 3.9). Consequently, also the amounts of ADP and NADP will decrease, and electrons and protons of the photosynthetic light reaction will not be consumed which may lead to a partial electron backlog in the thylakoid membrane and an increased thylakoidal proton gradient. Accordingly, the absorbed light energy in the antennae cannot be used to funnel electrons in the electron transport chain and hence the energy has to be emitted by other means. One possibility is the energy transfer on oxygen (either directly in the Mehler reaction or indirectly via triplet chlorophyll), which eventually would lead to cell death by oxidation processes. One of the main mechanisms of diatoms to prevent this photodamage is NPQ. The stronger proton gradient apparently activated the Dd to Dt conversion already at low light intensities in the gs mutants. As in diatom cells, NPQ almost completely relies on the amount of Dt (Lavaud et al. 2002), the increased amount of Dt within FCPs in the gs mutants leads to the higher NPQ and thus increased photoprotection. Moreover, the block of NADPH consumption leads to a higher reduction state of the electron transport chain in the gs mutants. As a reduced plastoquinone pool (PQ-pool) induces total Dd+Dt synthesis (Lepetit et al. 2013), the amount of Dd+Dt is strongly increased in the mutant compared to the WT, adding up to the photoprotective capacity in the gs mutants.

3.5.3. Blocking the synthesis of β-1,3-glucans strikingly alters the morphological architecture of the thylakoids

In diatoms, HL leads to a reduction in the numbers of the thylakoid stacks, but the number of three lamellae per stack remains constant (Janssen et al. 2001). Here, blocking the synthesis of chrysolaminarin leads to a reduction of thylakoid stack numbers, similar to HL effects in diatoms. However, we also observed an increase of the number of lamellae per stack, which, to our knowledge, has not been demonstrated so far in diatoms. The increased thylakoid diameter could result from three parameters: e.g. 1) the thylakoid lumenal space (including the thickness of the thylakoid membranes) (TLS), 2) the interthylakoidal space (distance between the neighboring lamellae) (TS), and 3) the number of the lamellae arranged into one thylakoid stack. Compared to the WT cells, there are no alterations in the TLS and the TS in the mutants (Fig. 3.7A). However, the numbers of the lamellae per thylakoid stack are increased in the gs_c mutants (Fig. 3.8 and Table. 3.2). There are two possibilities that could explain this: more lamellae form a stack, or two
or more thylakoid stacks could converge. Stacking more lamellae may lead to an increased ratio of inner to outer thylakoid membranes in the mutant than in the WT. As (Lepetit et al. 2012) suggested, the inner parts of the stacks might be enriched in the neutral galactolipid monogalactosyldiacylglycerol (MGDG), embedded to a larger extent in PSII (not completely free of PSI) and the peripheral FCP complexes. MGDG supplies a reservoir for the high concentrations of Dd+Dt cycle pigments. The outer lamellae of the stacks consist of the negatively charged lipid Sulfoquinovosyldiacylglycerol (SQDG) and a high number of PSI (not completely free of PSII) complexes with their specific antennae. According to these different components in the inner and outer membranes, the higher ratio of inner to outer thylakoid membranes may enlarge the area of MGDG and thereby leading to an increase of the ratio of FCP to PS, the Dd+Dt cycle pigments and the Dd de-epoxidation. This hypothesis is supported by our HPLC pigments and the pigment-protein complexes analyses, which demonstrate that the antenna per photosystem, the Dd+Dt pool, and the DES are increased in the mutant. In addition, besides increasing the capability of photoprotection, the increased number of lamellae per stack as well as the reduced thylakoids may reduce the total area of the thylakoid. Also, it would increase the self-shading of the pigments by closely located adjacent pigments. Both effects would result in harvesting less photons in gs_c cells, and hence photosynthesis would be less efficient and relieve the plastids from taking up too much light engery. As the other possibility, the thickening of the stacks may also be caused by the union of multiple thylakoids, resulting in thylakoids which have a lamellae number as a multiple of three. However, as shown in Table. 3.2, the lamellar numbers in the gs_c cells, are variable and do not correspond to a multiple of three. Nevertheless, the possibility of increasing the thylakoid diameter via convergent of thylakoids cannot currently be completely ruled out.

3.5.4. Blocking the synthesis of β-1,3-glucans might induce much more complex consequences on photosystem compared to what are found in higher plants

In A. thaliana, ADP-Glc pyrophosphorylase (Bowler et al.) is involved in the synthesis of starch, knocking out of which leads to a significant decrease of starch in the plants (Lin et al. 1988). Mutant adg1-1, under high light, exhibits a significant increase on soluble sugar, most prominently glucose (Schmitz et al. 2012). Furthermore, under low light or normal light, adg1-1 shows hyperstacking of grana thylakoids, and an increased number of plastoglobules compared
to the wild type (Hausler et al. 2009). These phenotypes of \textit{adg1-1}, were also observed in our \textit{gs} cell lines. However, \textit{adg1-1} mutant does not show physiological differences of the photosynthetic apparatus, such as the maximum PSII quantum yield the pigmentation (Schmitz et al. 2012), which we observed when silencing \textit{PtGS} in \textit{P. tricornutum}. The triose phosphate/phosphate translocator (\textit{tpt}) plays essential role on exporting the photoassimilate products out of stroma (Häusler et al. 1998). A double knock out mutant (\textit{adg1-1/tpt-2}), under high light, showed: in addition to a changed chloroplast ultrastructure, also a high chlorophyll fluorescence phenotype (Schmitz et al. 2012). However, in these mutants, the content of soluble sugar is not affected, neither under low light, nor under high light (Schmitz et al. 2012), while the \textit{gs} mutants, under 70 \(\mu\text{mol photons m}^{-2} \text{s}^{-1}\) have shown a clear increase in soluble sugars. Whereas, in \textit{Arabidopsis} the starch synthases knock out strains showed low photosynthetic capacity as well as the accumulation of soluble sugars (Ragel et al. 2013), which are both similar to the phenotypes shown in \textit{gs} mutants. However, the morphological effects on starch synthases knock out mutants were not discussed. Nevertheless, silencing \textit{PtGS} in \textit{P. tricornutum} might result in much severe changes by impairing the starch biosynthesis and inhibiting the exportation of triose phosphate from stroma.

Here, we demonstrated that the photosynthetic capacity is strongly reduced in the \textit{gs} cell lines balancing the increased amounts of free hexoses. According to our results, we suggest that this decreased photosynthetic capacity may be caused by two mechanisms: the reduction of light harvesting, and the dissipation of excessive light energy. In the \textit{gs} silencing cells, the reduced amount of thylakoids and the increased number of lamellae per thylakoid may lead to a reduction of the total surface used for light absorption by self-shading processes. Hence, the total number of harvested photons is reduced. Moreover, the increased xanthophyll cycle pigments result in the increased NPQ, thereby dissipating more excessive excitation light energy. The higher amount of lamellae may be a way to decrease the chlorophyll \(a\) specific absorption, the amount of \textit{Dd+Dt} (located in MGDG in the inner stacks), and NPQ (which relies on the conversion of \textit{Dd} to \textit{Dt}, most efficient in MGDG, Lepetit et al. 2012). However, as so far a phenotype of five lamellae has not been observed in heterokonts, we also have to take into account that chrysolaminarin synthesis may have a direct influence on thylakoid architecture. In summary, the effect of
inhibiting chrysolaminarin synthesis has dramatic consequences on the morphological and physiological performance.

**Figure 3.9.** Schematic presentation of the model of chrysolaminarin synthesis pathway and related reactions in *P. tricornutum*. 3-PGA: glyceraldehyde-3-phosphate; 3-PG: 3-Phosphoglycerate; GAPDH: glyceraldehyde-phosphate dehydrogenase; GPI: Glucose-6-phosphate isomerase; PGM: phosphoglucomutases, PFK: Phosphofructokinase; FBA: fructose-1,6-bisphosphate aldolase; FBP: fructose-1,6-bisphosphatase; RUBISCO: ribulose-1,5-bisphosphate; TPI: triose-phosphate isomerase; UGP: UDP-glucose pyrophosphorylase. Dashed red cross: assumed indirect reactions blocked by knocking down PtGS in the gs mutants. In this model, the *PtGS* is located on the vacuolar membrane leaving two functional hydrophilic loops in the cytosol, where the UDP-glucose are accessed by GS and the synthesis of the backbone of chrysolaminarin is initiated. Metabolites colored with red are assumed to accumulate after silencing *PtGS*. 
Acknowledgements

We thank Dr. Heiko Wagner (University of Leipzig) for help with the experiments. We also thank Lauretta Nejedli, Dr. Michael Laumann and Dr. Jochen Hentschel at the Electron Microscopy Center of the Universität Konstanz for cell preparation and TEM-microscopy. We thank Doris Ballert for help of transformation of *P. tricornutum* cells. This work was supported by a stipend of the CSC (China Scholarship Council) to W.H., by the Graduate School Chemical Biology (KoRS-CB), and by the University of Konstanz.
Chapter 4

4. Characterizations of TGS1 and 2

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Author contributions: W.H. and P.G.K. designed research; W.H., and S.Y. performed research; W.H., and S.Y. analyzed data; and W.H. wrote the paper.

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4.1. Background

Chrysolaminarin, the essential polysaccharide storage compounds in diatoms, is comprised of the β-1,3-glucan main chain branched with β-1,3-glucan units at C-6 (Bulone 2009). The formation of the β-1,6-glucan bond from chrysolaminarin is catalysed by β-1,6-transglycosylases (TGSs). Three gene models of TGSs were indentified in the diatom *Phaeodactylum tricornutum*. They were TGS1 (50238), TGS2 (56509), TGS3 (48300), among which TGS1 and 2 were located in the vacuolar membrane. It was also demonstrated in chapter 2 that TGS1 and 2 can encode active β-1,6-transglycosylases by complementation of a transglycosylase-deficient yeast strain (Huang et al. unpublished). As chrysolaminarin is stored in the vacuoles of diatoms, therefore we conclude TGS1 or 2 might be involved in transferring the Glucosyl (Glc) residues to the C(O)6 position of chrysolaminarin β-1,3-glucan backbone in diatoms.

4.2. Materials and methods

4.2.1. Cell Culturing and Light Treatments

*P. tricornutum* (strain UTEX646, UTEX Culture Collection of Algae, University of Texas, http://www.bio.utexas.edu/research/utex/) was grown at 20°C, in modified f/2 medium with artificial half-concentrated sea salts (16.6 g l\(^{-1}\)) and 0.09 μM MnCl\(_2\) (Guillard 1975; Guillard and Ryther 1962). The cells were grown with continuous white light at 70 μmol photons m\(^{-2}\) s\(^{-1}\). Cells are harvested at the exponential growth phase for extraction of the total RNA. The cells used for BODIPY staining are in stationary phase.

4.2.2. Synthesis of TGS1 and 2 cDNA

Cells of *P. tricornutum* were harvested by centrifugation at 5000 g and the pellet was frozen in liquid nitrogen. Total RNA were isolated with an RNA extraction kit (peqGOLD Total RNA Kit s-line, peqlab, Erlangen, Germany). And cDNA was performed with PrimeScript® RT reagent Kit (PrimeScript® RT reagent Kit with gDNA Eraser, TaKaRa, Shiga, Japan).

4.2.3. Generation of gene silencing vectors

We applied gene silencing using constructs containing inverted repeat sequences of targeted genes. The sense repeat sequences of *tgs1* and *tgs2* were generated from the CDNA of *tgs1* and
2, respectively. The sense sequences of \( tgs1 \) (\( tgs1s \)) and 2 (\( tgs2s \)) are:

\[
\text{CGCGCACACGTCATGGGTGTTGAATATTACTACCGTGGAAAAGGAAAACGTTTATAAAGCATTTACGAGCACACAAAGCAGGTACCGGATAAGAAATATGTGCAGAGCGGGATGCTACAGAGTTGGAACAAAGTTTTGTTTCTGTT}
\]

and

\[
\text{CCGCGTGAACGAGGAGGCGAACTAGTGATTCACACCGAAGGGCGGATACAGATGTCGTTGGC}
\]

\[
\text{TTTGACGACGTAAATCGAAAAAGAACGCACGTCACTAAACACTTTCGATCCGCTATGCTTCAAT}
\]

\[
\text{CGTGGAAATAAGTTCTGCTTTAC.}
\]

The first intron of \( \text{PtNTT1 (JGI_ID_49533)} \) (128bp) was used as the linker between sense and antisense strands. The sense strand and \( \text{PtNTT1 linker} \) were firstly sequentially assembled with the vector \( \text{pPha-T1 (GenBank accession number AF219942.1)} \) via Gibson Assembly\textsuperscript{®} Master Mix kit (New England Biolabs GmbH, Frankfurt, Germany), after this, the antisense strand was cloned into the StuI restriction site of this so-called “sense-NTT1” vector by the standard cloning procedures (Barnes 1994; Gibson et al. 2010; Gibson et al. 2009; Sambrook et al. 1989; Zaslavskaia et al. 2000). Hence, vector \( \text{pPTV\_tgs1 (or 2)} \) was generated. Primers applied for cloning \( \text{pPTV\_tgs1 (or 2)} \) vector are listed in Table. 4.1. Vectors were transformed into wild type \( \text{P. tricornutum} \) cells using the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA, USA) (Zaslavskaia et al. 2000). Transformants were confirmed to contain corresponding plasmids via PCR.

### 4.2.4. Detections of the transcriptional abundances in wild type and knock-down strains

To detect the transcriptional levels of \( tgs1 \) or 2 from different strains, quantitative real-time polymerase chain reaction (qRT-PCR) was used. GoTaq\textsuperscript{®} qPCR Master Mix kit (Promega, USA) was applied for qRT-PCR. Primers used for qRT-PCR are: \( \text{TGS1si fw: GCGAACCGGGAGATTTACCA, TGS1si rev: GTACCAGTGACCCTCGTGTG, TGS2si fw: GGATTGATGCACATGCGGAC and TGS2si rev: TGAGAACCACCAAAGCCTC.} \)
4.2.5. Intracellular lipids detection with BODIPY 493/503

To determine the lipid content in wild type (WT) and knock-down strains, flow cytometry was applied with the Cell Lab Quanta SC MPL (Beckman Coulter, Germany), which is equipped with a 488 nm laser and variable emission filter sets. BODIPY® 493/503 (Molecular Probes, Inc. Leiden, Netherlands), a lipophilic bright green fluorescent dye (Govender et al. 2012), was used to monitor the relative triacylglycerol (TAG) accumulation in different cell lines. To optimize cell staining time, fluorescence intensity was measured at 1, 10, 20, and 30 min of incubation in the dark at room temperature. 40 μl of 1 μg/ml BODIPY 493/503 was diluted into 1960 μl liquid samples, hence the final working concentration of BODIPY is 20 ng/ml.

4.3. Results

4.3.1. Knocking down of tgs1 or tgs2 reduces the growth of the mutants

According to our qRT-PCR results, one tgs1 knock down strain and three tgs1 and 2 double knock down strains were obtained. The tgs1 transcriptional abundance in the tgs1 strain was 3.4-fold lower compared to WT (Fig. 4.1A). The tgs1 and 2 double knock-down strains: tgs(1+2)_5, tgs(1+2)_12 and tgs(1+2)_14, exhibit 3.5, 3 and 2-fold reductions, respectively, in the tgs1 transcriptional level (Fig. 4.1A). However, the mRNA transcripts of tgs2 in these three strains are not reduced to the same extent as the tgs1 transcriptional level reductions (Fig. 4.1B). The tgs2 mRNA in double knock-down strains, tgs(1+2)_5, 12 and 14, were decreased by 70%, 24% and 41% of the wild-type mRNA respectively (Fig. 4.1B). A tgs2 silencing strain was also obtained, where the tgs2 mRNA was, however not significantly, reduced by around 18% of the wild-type mRNA. As Figure. 4.1C shown, the growth of knock-down strains, tgs1 and tgs(1+2)_5, 12, 14, is dramatically impeded compared to WT. The doubling times of tgs1 and tgs(1+2)_5, 12, 14 strains are 16.3, 16.8, 14.5, and 13.9 respectively. In combination with the tgs1 or 2 mRNA abundance, a positive correlation between the growth and the tgs1 mRNA level maybe implied.
4.3.2. Intracellular lipids in \textit{tgs}(1+2) double knock-down strains are increased

To determine the lipid content in wild type (WT) and knock-down strains, flow cytometry was applied, using BODIPY® 493/503, a lipophilic bright green fluorescent dye (Govender et al. 2012), to monitor the relative triacylglycerol (TAG) accumulation in different cell lines. To optimize cell staining time, fluorescence intensity was measured at 1, 10, 20, and 30 min of incubation in the dark at room temperature. As Fig. 4.2A shown, fluorescence intensities stay constant between 10 to 20 min. Hence, BODIPY 493/503 with a final working concentration of 20 ng/ml is incubated with \textit{P. tricornutum} cells for 10min under darkness before the fluorescence intensity is determined. As Figure. 4.2B shown, in contrast to WT, the increased TAG which is the predominant lipid reserve in diatoms (Hu et al. 2008), were observed in all the \textit{tgs}(1+2) double knock-down strains, with the highest content in \textit{tgs}(1+2)_14.

4.4. Discussion

4.4.1. Less expression of TGS1 or 2 might alter the vacuolar osmotic pressure

Here, we proposed a possible mechanism which might induce the impaired growth of \textit{Pttgs}1 or 2 cells. TGS1 or 2 might be involved in adding the glucose (Glc) residues to the C(O)6 of chrysolaminarin β-1,3-glucan dominant chain in diatoms. Knocking down of TGS1 or 2, could reduce the adding of Glc residues to chrysolaminarin, which may result in more free Glc molecules accumulated in the vacuoles. According to the osmotic pressure formula form Jacobus van ‘t Hoff “\( \Pi=[C_{\text{solute}}]RT \)” (where \( \Pi \) is osmotic pressure, \( C_{\text{solute}} \) is molar concentration, \( R \) is gas constant, \( T \) is the temperature in kelvins), osmotic pressure is simply based on the number of the molecules in solution. Hence, the accumulated Glc molecules may induce an increase of the vacuolar osmotic pressure in TGS1 or 2 or the double knock down strains. It has been reported, the osmotic pressure can activate the channels in plant vacuole (Alexandre and Lassalles 1991). In \textit{P. tricornutum} the osmotic pressure is balanced with the inorganic cations \( \text{Na}^+ \) and \( \text{K}^+ \), with their accompanying anions, and the organic solutes (Dickson and Kirst 1987). Hence, in response to the change of the vacuolar osmotic pressure, the intracellular salinity or organic solutes might be changed which may lead to the effect on the growth. Unfortunately, no experiment has been performed to confirm this speculation.
4.4.2. Knocking down of TGS1 or 2 might increase the lipid contents

*Kre6* encodes β-1,6-transglycosylases in yeast, deletion of which results in a severe reduction of β-1,6-glucans (Roemer and Bussey 1991). Moreover, the *kre6* deficient strain shows a clear phenotype due to distinct cell wall defects, resulting in a very retarded growth combined with a hypersensitivity to the stain Calcofluor White (CFW) which can interact with β-glucans and accordingly disrupt the protein matrix of the cell wall (Albani 2001; Nakamata et al. 2007). Here, silencing PtTGS1 or 2 resulted in an increase of the lipid content in the mutants (Fig. 4.2). This might result from that the potentially accumulating glucose molecules in vacuoles of TGS1 or 2 silencing strains might be transported into cytosol. The increased glucose residues in the cytosol may be used for the synthesis of fatty acids. However, to confirm the effect on lipid content caused by less TGS1 or 2; the determination of the relative TAG amount in *tgs1* and 2 knock-down strains and the further analysis for fatty acid profiling are required.
Figure 4.1. Transcriptional level and growth.

A. Relative $tgs1$ transcriptional level in WT, $tgs1$ and $tgs$ (1+2) strains. B. Relative $tgs2$ transcriptional level in WT, $tgs2$ and $tgs$ (1+2) strains. C. Doubling time of WT, $tgs1$, $tgs2$ and $tgs$ (1+2) strains.
Figure 4.2. Analysis of lipid accumulation.

A. Analysis of the fluorescence intensity at different staining time: 1, 10, 20, and 30 min. 1960 μl liquid sample was incubated with 40 μl of 1 μg/ml BODIPY 493/503 under darkness. BODIPY fluorescence per cell was the average of the population for three replicates of WT in stationary phase.

B. TAG contents in WT and tgs (1+2) strains monitored by BODIPY fluorescence. 1960 μl liquid sample in stationary phase was incubated with 40 μl of 1 μg/ml BODIPY 493/503 under darkness, for 10 min. The fluorescent intensities of 5000 cells per sample were collected. Data was analyzed with R program. The results of three biological replicates for each strain are shown. Horizontal line in box plot indicates the mean value.
Table 4.1. Primers used for generating TGS1 or 2 silencing vectors

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGS1_pPhaT Fwd</td>
<td>TCTAGAGTCGACCTGCAAG</td>
</tr>
<tr>
<td>TGS1_pPhaT Rev</td>
<td>GAATTCTCGAACAAGGCAAG</td>
</tr>
<tr>
<td>TGS1_sense_50238 Fwd</td>
<td>gcgggttgagaatcCGGGCACACCGTCCAATGG</td>
</tr>
<tr>
<td>TGS1_sense_50238 Rev</td>
<td>acggtaaccACGAACAAACTTGTCCAACCTTG</td>
</tr>
<tr>
<td>TGS1_NTT Fwd</td>
<td>tgttttcgtGTTACGGTACCGTTACGTATC</td>
</tr>
<tr>
<td>NTT Rev overlap V</td>
<td>gcaggtcgacatcagaTCTAGAAAGGCTCTTGTAAGAG</td>
</tr>
<tr>
<td>TGS1Anti-sense Fwd2</td>
<td>ACGAAACAAACTGTCCTCAACTC</td>
</tr>
<tr>
<td>TGS1Anti-sense Rev2</td>
<td>CGCGACACGTCCAATGG</td>
</tr>
<tr>
<td>TGS2_pPhaT Fwd</td>
<td>TCTAGAGTCGACCTGCAAG</td>
</tr>
<tr>
<td>TGS2_pPhaT Rev</td>
<td>GAATTCTCGAACAAGGCAAG</td>
</tr>
<tr>
<td>TGS2_sense_56509Fwd</td>
<td>gcgggttgagaatcCGCGCTGAAACGAGAGG</td>
</tr>
<tr>
<td>TGS2_sense_56509 Rev</td>
<td>acggtaaccGTAAGACGAGACTTATCCACGATTG</td>
</tr>
<tr>
<td>TGS2_NTT Fwd</td>
<td>cgtcttacGTTACGGTACCGTTACGTATC</td>
</tr>
<tr>
<td>TGS2Anti-sense Fwd2</td>
<td>GAAACGCCAAGACTCTCCGCGATTG</td>
</tr>
<tr>
<td>TGS2Anti-sense Rev2</td>
<td>CGCGTGAACGAAGAGGC</td>
</tr>
</tbody>
</table>

TGS1_pPhaT Fwd and Rev are used for amplifying the pPhaT vector which is the backbone of the gibson assembly system. The pair primers of TGS1_sense_50238 Fwd and TGS1_sense_50238 Rev, which are used for amplifying tgs1s, are overlapped with one end of the pPhaT backbone and PtNTT1, respectively. TGS1_NTT Fwd and NTT Rev overlap V, overlapped with tgs1s and the other end of the pPhaT backbone respectively, are used for amplifying the TGS1_PtNTT1. TGS1Anti-sense Fwd2 and Rev2 are used for amplifying the antisense repeat of the tgs1 silencing construct. The corresponding TGS2 primers represent the same application as the TGS1 primers.

Acknowledgements

We thank Doris Ballert for help of transformation of *P. tricornutum* cells. We also thank Dr. Stefanie Bürger for assisting of applying flow cytometry. This work was supported by a stipend of the CSC (China Scholarship Council) to W.H., by the Graduate School Chemical Biology (KoRS-CB), and by the University of Konstanz.
Chapter 5

General Discussion
5. General discussion

5.1. The putative β-1,3-glucans synthesis models in diatoms and oomycetes

Diatoms are unicellular photoautotrophic eukaryotes, contributing around 20 and 40% of primary production to global and oceanic biogeochemical cycles, respectively (Falkowski et al. 1998). Chrysolaminarin is the principal energy storage polysaccharide which is stored in intracellular vacuoles in a non-crystalline form (Beattie et al. 1961a; Chiovitti et al. 2004; Ford and Percival 1965a). We screened the genome of P. tricornutum and identified the proteins which are potentially involved in the synthesis pathway of chrysolaminarin (Table 5.1). Two isoforms of UGP were found, one of which is a UGP/PGM (phosphoglucomutase) fusion protein. This fusion protein was also found in the diatom Thalassiosira pseudonana, brown algae and oomycetes (Kroth et al. 2008; Michel et al. 2010b). Interestingly, these two UGP isoforms are predicted to target to different compartments in P. tricornutum (Table 1.2). UGP1/PGM (50444) is predicted to localize to the cytosol, whereas, UGP2 (23639) is predicted to be plastidially localised. Due to these two different locality predictions of UGP, the question of where GS accesses UGP-glucose is raised.

A unique β-1,3-glucan synthase in the genome of P. tricornutum which contains 23 transmembrane helices was identified. All known β-1,3-glucan synthases (GSs) are membrane associated protein. In oomycetes, the cell-wall-synthesizing β-1,3-glucan synthases have been localized on the plasma membrane via particulate membrane fractions (Girard and Fevre 1984). Besides oomycetes, the plasma membrane associated β-1,3-glucan synthases, FKS1 involved in the cell wall synthesis of yeast and callose synthase5 from Arabidopsis have also been demonstrated (Okada et al. 2010; Shi et al. 2014). It is very likely that the β-1,3-glucan synthases participating in cell-wall synthesis are located on the plasma membrane. However, signal peptide was not found in PtGS. In addition to serving as storage polysaccharides (chrysolaminarin), β-1,3-glucans are also the components of the cell wall in some diatom species (Waterkeyn and Bienfait 1987). Given that there is only one β-1,3-glucan synthase in the genome of P. tricornutum, the localization and characterization of PtGS is essential for elucidating the synthesis mechanisms of chrysolaminarin and the cell wall. Three isoforms of Kre6-like protein were found in P. tricornutum, termed β-1,6-transglycosylases (TGS1, 2 and 3), which are the candidates for adding
glucose units at the C-6 position of β-1,3-glucan to form the mature chrysolaminarin. Three isoforms of TGSs may imply they play different roles in *P. tricornutum*. As mentioned, in yeast, Kre6 is involved in the 1,6-β-branching of cell wall β-1,3-glucans (Kurita et al. 2011; Nakamata et al. 2007; Roemer and Bussey 1991; Roemer et al. 1993). Hence, in addition to the synthesis of chrysolaminarin, TGS1, 2 and 3 may also be candidates for the synthesis of cell wall.

**Table 5.1.** Putative proteins involved in the synthesis pathway of chrysolaminarin in *P. tricornutum*.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein ID</th>
<th>Predicted location</th>
<th>Experimental localization</th>
<th>Transmembrane domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGP1/PGM</td>
<td>50444</td>
<td>Cytosol</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>UGP2</td>
<td>23639</td>
<td>Plastid</td>
<td>Plastid</td>
<td>0</td>
</tr>
<tr>
<td>GS</td>
<td>56808</td>
<td>None</td>
<td>VM</td>
<td>23</td>
</tr>
<tr>
<td>TGS1</td>
<td>50238</td>
<td>SP</td>
<td>VM</td>
<td>1</td>
</tr>
<tr>
<td>TGS2</td>
<td>56509</td>
<td>SP</td>
<td>VM</td>
<td>1</td>
</tr>
<tr>
<td>TGS3</td>
<td>48300</td>
<td>SP</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

UGP/PGM was predicted to localize in cytosol. UGP2 was predicted to reside in plastid, which was confirmed to locate in plastid by expressing fusion protein UGP2::GFP in *P. tricornutum*. No signal peptide was found in GS, whereas, by expressing fusion protein GS::GFP in *P. tricornutum*, GS was located in the vacuolar membrane. All of the three isoforms of TGS are predicted in secretory pathway. After expression of TGS1 or 2::GFP fusion protein, TGS1 or 2 was targeted to the vacular membrane. PGM: phosphoglucomutases, UGP: UDP-glucose pyrophosphorylase. GS: β-1,3-glucan synthase, TGS: β-1,6-transglycosylases. SP: secretory pathway (ER; Golgi; Vacuole; plasma membrane, extracellular part). VM: vacuolar membrane.
5.2. The putative β-1,3-glucans synthesis model in diatoms

Here we observed that β-1,3-glucan synthase in *Phaeodactylum tricomutum* (PtGS) targeted to the vacuolar membrane. In combination with the vacuolar localization of chrysolaminarin, apparently, PtGS is involved in the synthesis of chrysolaminarin. This might indicate that β-1,3-glucan synthases (GSs), which are involved in the synthesis of vacuolar β-1,3-glucans in oomycetes, brown algae, haptophytes, are also vacuolar associated proteins.

*PtGS* has been located in the vacuolar membrane, hence, for the biosynthesis of vacuolar chrysolaminarin, transport of the activated substrate (UDP-glucose) from cytosol to vacuoles might be required. However, in *Arabidopsis* it has been proposed callose (β-1,3-glucans) synthases (CaLSs), which are used for extracellular matrices β-1,3-glucans synthesis, are very likely to have dual functions: both transporter and transferase activities (Davis 2012) (Fig. 5.1). In this dual function model, the central catalytic domain is in cytosol. This catalytic domain incorporates active sugars from cytosolic substrate pools to an elongating polymer that extends across the membrane through the pore (Fig. 5.1) (Davis 2012). Based on this model, we proposed that, in *P. tricomutum* UDP-glucose might be approached by vacuolar *PtGS* from cytosol, to elongate the β-1,3-glucan polymers and simultaneously transported in to vacuoles (Fig. 5.2A). Therefore, UGP1/PGM predicted to localize to the cytosol might be used for supplying UDP-glucose to *PtGS*, instead of UGP1 which is located in the plastid (Fig. S6 and Table. 5.1). However, this dual function model has not been confirmed, due to multiple TMHs and many embeded in the catalytic domain (Fig. 3.2).

In *Pinnularia* diatoms, besides being deposited as vacuolar chrysolaminarin, β-1,3-glucans are associated with the cell wall as callose (Tesson and Hildebrand 2013; Waterkeyn and Bienfait 1987). The β-1,3-glucan synthases associated with cell wall formation in oomycetes yeast and *Arabidopsis* are located in the plasma membrane (Girard and Fevre 1984; Okada et al. 2010; Shi et al. 2014). Hence, in analogy to β-1,3-glucan synthases these organisms, the GSs involved in the cell wall synthesis in diatoms are expected to reside in the plasma membrane. Here, we showed that PtGS is in the vacuolar membrane. Moreover, only one β-1,3-glucan synthase was indentified by us in each of the annotated genomes of the diatoms, *Phaeodactylum tricomutum, Thalassiosira oceanica, Thalassiosira pseudonana, Pseudonitzschia multiseries* (Fig. S4). This
implies that, as shown in Fig. 5.2A, the cell-wall associated β-1,3-glucan might be transported via vesicles across plasma membrane from vacuoles in diatoms. As the genome of *Pinnularia* has not been sequenced so far, we are not able to rule out that these species may contain two or more β-1,3-glucan synthases. One of these could be located in vacuolar membrane for synthesizing chrysolaminarin, while another GS could be located in the plasma membrane.

Besides β-1,3-glucans, the other abundant polysaccharide in diatoms is chitin consisting of β-1-4-linked N-acetylglucosamine, which has been found within *Thalassiosira* and *Cyclotella* (Gügi et al. 2015). Chitin, an insoluble polysaccharide, is involved in the cell wall formation of most diatoms (Gügi et al. 2015). However, chitin fibers have not been identified so far in the cell wall of *P. tricornutum*. Nevertheless, the genes involved in the synthesis of chitin were identified within the genome of *P. tricornutum* (Table 5.2) (Kroth et al. 2008). Proteins involved in cellulose synthesis are completely absent from diatoms (Table 5.2) (Michel et al. 2010a). However, it has been proposed, in *P. tricornutum* the cell wall polysaccharide is considered to consist of branched and sulfated glucuronomannans, being mostly composed of mannose (Gügi et al. 2015). In 2010, Gurvan Michel showed *P. tricornutum* diatoms contain almost all of the enzymes involved in the synthesis of sulfated fucans which consists of mannose (Michel et al. 2010a).
**Figure 5.1.** A model for dual-function GS transport mechanism.

The transmembrane helices (TMHs) of the enzyme are depicted as cylinders connected by curved lines representing the extra-membrane regions of the protein. A polymer is shown being transported through a pore formed by the TMHs, from the cytosolic active site where sugars are added, across the membrane to the extracellular space or lumen of the secretory pathway. This image and interpretation are from (Davis 2012).
Table 5.2. List of polysaccharides synthases in some organisms.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>β-1,3-glucan synthase</th>
<th>Cellulose synthase</th>
<th>Chitin synthases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diatoms</td>
<td>1</td>
<td>0</td>
<td>2-6</td>
</tr>
<tr>
<td>Oomycetes</td>
<td>2 or 3</td>
<td>4</td>
<td>1-4</td>
</tr>
<tr>
<td>Brown algae</td>
<td>3</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>12 (callose synthase)</td>
<td>13?</td>
<td>0</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>3? (FKS1, FKS2, Fks3p)</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

Diatoms contain only one β-1,3-glucan synthase, 2 (in *P. tricornutum*) or 6 (in *Thalassiosira pseudonana*) chitin synthases (Morozov and Likhoshway 2016). Cellulose synthase is completely absent from diatoms. Oomycetes contain all these three synthases (Michel et al. 2010a; Morozov and Likhoshway 2016). Chitin synthase is absent from brown algae as well as *Arabidopsis*. Yeast does not contain cellulose synthase.
5.3. The putative β-1,3-glucans synthesis model in oomycetes

According to our phylogenetic analyses, in contrast to diatoms, most of the organisms from oomycetes possess two β-1,3-glucan synthases, whereas *Aphanomyces astaci* and *Saprolegnia diclina* contain three GSs (Table. 5.2 and Fig. S4). These GSs belong to two different groups (Fig. S4). Group I clusters with plants, whereas, Group II is specific for diatoms and brown algae. Moreover, these two groups consist of different domain architectures (Fig. S5). The GSs from GroupI harbor the same domain structure as *PtGS*. They comprise a “GF” like domain structure, where G represents a Glucan_synthase catalytic domain and F represents a FKS1_dom1 domain (Fig. S5). In contrast, GSs from GroupII basically contain a “GFS” domain architecture. Besides the “G” and “F” domain, they still have an Skn1 domain which is homologous to Kre6 in yeast, representing β-1,6-transglycosylase.

The occurrences of two groups of β-1,3-glucan synthases are coincident with two distinct β-1,3-glucan forms in oomycetes, one of which is mycolaminarin, stored in vacuoles, resembling chrysolaminarin (Wang and Bartnicki-Garcia 1974). Moreover, β-1,3-glucan is also the major component of cell wall which is vital for oomycetes infection pathology (Blaschek et al. 1992). This indicates that the two different GSs in oomycetes might be responsible the synthesis of mycolamianrin and cell wall, respectively. In brown algae, β-1,3-glucan is only used as a storage polysaccharide (laminarin). Furthermore, brown algal cell wall consists of cellulose consisting of β-1,4-glucan, sulfated fucans and some unique polysaccharides (alginates), but it does not contain β-1,3-glucan (Michel et al. 2010a). Hence, the brown algal GSs might be exclusively used for synthesizing laminarin which indicates one of the two GSs from one oomycetes organism, which is clustered with brown algae and diatoms, termed *MycoGS*, may be responsible for the formation of mycolaminarin, rather than for the synthesis of cell wall. Since mycolaminarin is also stored in vacuoles, the synthesis pathway might be similar to chrysolaminarin in diatoms. The other GS grouped with plants is assumed to specifically form the cell-wall β-1,3-glucan (Fig. 5.2B).
**Figure 5.2.** Representation of the putative synthesis pathways of storage β-1,3-glucan and cell wall β-1,3-glucan in diatoms (A) and in oomycetes (B).

(A). Diatoms only harbor one β-1,3-glucan synthase which is located in vacuolar membrane. Glucose-6-phosphate is catalyzed into activated sugar UDP-glucose by the catalysis of UGP2/PGM. Subsequently, UDP-glucose is taken up and incorporated into the β-1,3-glucan polymer, and meanwhile β-1,3-glucan is translocated into vacuoles by the critical enzyme: β-1,3-glucan synthase in vacuolar membrane. Since only one β-1,3-glucan synthase has been found in organism of diatoms to date, the diatom cell-wall β-1,3-glucan might be transported by vesicles from vacuoles to extracellular compartments. (B). Two or more β-1,3-glucan synthases have been identified in the organism of oomycetes. The β-1,3-glucan synthase (MycoGS) clustered with brown algae and diatoms, is assumed to be responsible for the formation of mycolaminarin. The other GS grouped with plants might be specifically for the cell-wall β-1,3-glucan formation.

5.4. The gs mutants as a tool for investigating the metabolite signaling in diatoms

It had been reported for decades that accumulation of glucose, glucose analogs, fructose and metabolic intermediates may repress photosynthesis in higher plants (Krapp et al. 1993; Rolland et al. 2002). Recently, it was reported that in diatoms, after the shift from low energy red light to high energy blue light, the absolute amount of the soluble sugars involved in glycolysis were increased. Accompanied with the increased sugar contents, NPQ as well as Dd+Dt were enhanced, the redox state of plastoquinone pool (PQ) was more oxidized (Jungandreas et al. 2014). This might imply that the alterations of photosynthesis are affected by the increased sugar concentrations (Jungandreas et al. 2014). Similarly, as mentioned in chapter 3, blocking chrysolaminarin synthesis results in the increases of glucose and a disaccharide-like sugar, in gs cell lines, and causes striking physiological and morphological changes of the photosynthetic apparatus. Hence, the gs mutants might represent an important tool for investigating metabolite signaling in diatoms.

5.5. HXK-dependent sugar sensing and signaling

For any living organism, metabolites often may function as signaling molecules, modulating the metabolism and physiology, in response to the changes of variable environments. One of the important signaling metabolites is glucose, which is essential for regulating physiology, metabolism and development, in bacteria, yeasts, mammals and plants (Cho et al. 2007; Santangelo 2006; Stülke and Hillen 1999). In yeast, mammals, and plants, hexokinases or hexokinase-like proteins (HXKs) are the evolutionarily most conserved glucose sensors which coordinate nutrients and hormone signaling networks (Bolouri-Moghaddam et al. 2010; Cho et al. 2006; Entian 1980; Moore et al. 2003; Wilson 2003). HXKs are predominantly associated with mitochondria, but were also observed in the nuclei of mammalian and plant cells (Bolouri-Moghaddam et al. 2010; Cho et al. 2007). It was already demonstrated that, in Arabidopsis, the nuclear HXK interacts with another two partners: the vacuolar H⁺-ATPase B (VHA-B), and the 19S regulatory unit of proteasome subunit (RPT5B), forming into a regulatory complex (Cho et al. 2007). It was proposed that, glucose may diffuse freely into the nucleus, where it binds to nuclear HXK. The binding initiates a conformational change of HXK, leading to HXK acting together with VHA-B and RPT5B as a transcriptional repressor. The whole HXK complex interacts with the
transcription factor (TF), thereby expression of target gene is suppressed (Bolouri-Moghaddam et al. 2010; Cho et al. 2007). Besides HXK-dependent sugar sensing and signaling pathway, numerous HXK-independent pathways have been demonstrated, for instance, the FRUCTOSE INSENSITIVE1 protein serves as a regulatory factor in Fructose signaling (Hausler et al. 2014).

5.6. Trehalose

Trehalose is a disaccharide and widespread in organisms, which has also been identified as a major regulatory factor (Iturriaga et al. 2009). In yeast, Trehalose-6-phosphate is an efficient inhibitor of yeast HXK, and the yeast Trehalose-6-phosphate synthase (TPS1) may play a role in regulation of glycolytic flux (Bonini et al. 2000; Van Vaeck et al. 2001). In Arabidopsis, inhibition of trehalase resulted in the accumulation of trehalose content and strong reduction in sucrose and starch contents, which indicates trehalose may play a role in regulating the carbohydrate mobilization in plants (Müller et al. 2001). Trehalose may also participate in regulating photosynthesis in response to high glucose content (Avonce et al. 2004).

Figure 5.3. Representation of sugar signaling in P. tricornutum after knocking down GS

UGP/PGM: UDP-glucose pyrophosphorylase/phosphoglucomutases; GS: β-1,3-glucan synthase; TPS/TPP: Trehalose-6-phosphate synthase/Trehalose-6-phosphatase; GLK: Glucokinase; HXK4: Hexokinase; VHA-B: B subunit of the peripheral V1 complex in vacuolar-type H-ATPase (V-ATPase) and is responsible for noncatalytic ATP binding (Cho et al. 2006). RPT5B is an isoform of one of the six AAA-ATPase subunits in the base of the 19S regulatory particle, which binds either end of the 20S proteasome to provide ATP dependence and the specificity for ubiquitinated proteins (Cho et al. 2006).
5.7. Assumed sugar signaling in *P. tricornutum*

We screened the genome of *P. tricornutum*, and identified HXK, VHA-B and RPT5B homologs. One PtVHA-B (protein ID: 17847) and 3 isoforms of PtRPT5B (protein ID: 17169, 28219 and 45122) were found in *P. tricornutum*. Protein 17169 has the highest similarity to RPT5B from *A. thaliana*. However, HXKs are absent from *P. tricornutum*, whereas a glucokinase (GLK) (protein ID: 48774), which belongs to HKX4 family, is present. GLKs have been discovered as a sugar sensor in mammalian insulin and liver cells (Danial et al. 2003; Dentin et al. 2004). Therefore, GLK is considered as the candidate of sugar sensor in *P. tricornutum*, which might imply *P. tricornutum* possess, instead of a HXK signaling pathway, a GLK signaling pathway. Here, we propose, in the nucleus of *P. tricornutum* the accumulated glucoses binds to PtGLK, whereby the conformation of PtGLK is altered. Subsequently, PtVHA-B and PtRPT5B are able to interact with PtGLK, forming into a GLK complex regulating transcriptional factors (Fig. 5.3).

In *P. tricornutum*, we identified two isoforms of the fusion protein: Trehalose-6-phosphate synthase/Trehalose-6-phosphatase (TPS/TPP) (protein ID: 46137 and 48682). Through the catalysis of TPS/TPP, UDP-glucose and Glu-6-P are firstly synthesized and then dephosphorylated into trehalose. According to this catalytic reaction, it might indicate the synthesis of trehalose is related to the biosynthesis of chrysolaminarin. Trehalose might compete UDP-glucose with chrysolaminarin. Therefore, blocking chrysolaminarin synthesis may result in the increase of trehalose content (Fig. 5.3). Here, we propose that the increased disaccharide-like sugar which was observed in *gs_c* cells might be trehalose (chapter 3). However, the experimental analyses are still required to determine the disaccharide-like sugar.

Trehalose could be hydrolyzed into free glucose. Unfortunately, we could not identify proteins used for hydrolyzing trehalose into glucose in *P. tricornutum*. The accumulation of the glucose might initiate the GLK sugar signaling in *P. tricornutum*, repressing or increasing the expressions of target proteins. However, as Figure. 5.3 shows, in addition to glucose, the increased trehalose could also serve as a regulator, inducing a signaling pathway in *gs* cell lines.
5.8. Outlook

Gs mutants may represent a valuable tool for investigating the regulation of photosynthesis by soluble sugars. To reveal this regulatory process, future experiments should include the transcriptional abundance of the genes involved in photosynthesis, and the determination of the redox state of the plastoquinone (PQ) pool. To study sugar sensing, the localizations and characterizations of the proteins which are assumed in this pathway, such as GLK or TPS/TPP, should be studied. Since the transcription activator-like effector nuclease (TALEN) technique for the targeted knocking out of genes has also been successfully implemented in P. tricornutum (Daboussi et al. 2014; Weyman et al. 2015), therefore TALEN can be applied to knock out our interested proteins. As silencing constructs often do not show a stable phenotype over a long term, obtaining the PtGS knockout mutants is necessary for maintaining phenotypes and facilitate future investigation.
6. Supplements

Su Figure. 1. Conserved domain alignment of TGS1, TGS2, TGS3, Kre6 and Skn1.

Yellow colour: Conserved active amino acids and green colour: Conserved catalytic amino acids, both according to the NCBI conserved domains database (accession number: cd02180).
**Su Figure. 2.** Analysis of the cellular localization of TGS1 and 2 in *P. tricornutum*.

A. Staining of vacuoles with MDY-64. From left to right: bright filed image, chlorophyll fluorescence of the plastids, MDY-64 fluorescence, merged image. B. and C. Targeting of full length TGS1::GFP and TGS2::GFP fusion proteins. From left to right: bright field image, chlorophyll fluorescence, GFP fluorescence, merged image. Bars indicate length in 5µm.
**Su Figure. 3.** Phylogenetic tree of GS.

The used alignment for this tree is listed in supplement document. “GF”: Glucan synthase and FKS1_dom1 domains; “GFV”: Glucan synthase, FKS1_dom1, and Vta1domains; “GS”: Glucan synthase, and SKN1 domains; “GFS”: Glucan synthase, FKS1_dom1, and SKN1 domains; “GFR”: Glucan synthase, FKS1_dom1, and RVT_1 domains; “GFD”: Glucan synthase, FKS1_dom1, and DUF4203 domains; “GH”: Glucan synthase and Glyco_hydro domains and “GFSD”: Glucan synthase, FKS1_dom1, SKN1, and DUF4203 domains.
**Su Figure. 4.** Phylogenetic tree of GS where the information of GSs in oomycetes are shown. GS from the same oomycetes species are marked with the same-colored triangles. GSs from oomycetes are classified into two groups. GroupI is related to plants, GroupII is clustered with diatoms and brown algae. The used alignment for this tree is listed in supplement document. “GF”: Glucan_synthase and FKS1_dom1 domains; “GFV”: Glucan_synthase, FKS1_dom1, and Vta1domains; “GS”: Glucan_synthase, and SKN1 domains; “GFS”: Glucan_synthase, FKS1_dom1, and SKN1 domains; “GFR”: Glucan_synthase, FKS1_dom1, and RVT_1 domains; “GFD”: Glucan_synthase, FKS1_dom1, and DUF4203 domains; “GH”: Glucan_synthase and Glyco_hydro domains and “GFSD”: Glucan_synthase, FKS1_dom1, SKN1, and DUF4203 domains.
Su Figure. 5. Presentation of GSs domain architectures.

1: “GF”, Glucan_synthase and FKS1_dom1 domains. PtGS and GSs from oomycetes Group I contain “GF“-like domain structure.

2: “GFV”, Glucan_synthase, FKS1_dom1, and Vta1 domains.


4: “GFS”, Glucan_synthase, FKS1_dom1, and SKN1 domains. Some GSs from oomycetes Group II contain “GFS”-like domain structure.

5: “GFR”, Glucan_synthase, FKS1_dom1, and RVT_1 domains.

6: “GFD”, Glucan_synthase, FKS1_dom1, and DUF4203 domains.

7: “GFSD”: Glucan_synthase, FKS1_dom1, SKN1, and DUF4203 domains. Some GSs from oomycetes Group II contain “GFSD”-like domain structure.
**Su Figure. 6.** Analysis of the cellular localization UGP2 in *P. tricornutum*.

(A) Expression of fusion protein, UGP2 presequence (80 amino acids) fused to GFP, in *P. tricornutum*. From left to right: bright field, GFP fluorescence, chlorophyll fluorescence, merged image. (B) Expression of fusion protein, UGP2 full-length fused to GFP, in *P. tricornutum*. From left to right: merged image, GFP fluorescence, chlorophyll fluorescence. The bottom one is three dimensional image.
**Supplements**

**Document 1.** Manually curated ClustalW alignment used for the calculation of the TGS tree.

**Abbreviations:**
1 TGS1 Phaeodactylum tricornutum TGS1; 3 Thalassiosira oceanica EJK73120; 4 Thalassiosira pseudonana XP_002288656; 5 Aureococcus anophagefferens XP_009034007; 6 Ectocarpus siliculosus CBJ31299; 7 Ectocarpus siliculosus CBN78134; 9 Thalassiosira oceanica EJK63543; 12 Thalassiosira pseudonana XP_002289582; 14 Thalassiosira oceanica EJK77715; 25 Phytophthora sojae XP_009532889; 32 Phytophthora infestans XP_002997803; 36 Pseudozyma hubeiensis XP_012193160; 54 Pseudozyma Antarctica XP_014653806; 60 Albugo candida CCI44651; 73 Sporisorium reilianum CBQ73463; 78 Dichomitus squalens XP_007370618; 85 Saccharomyces cerevisiae NP_015485.1, SGD ID: S000006363, KRE6; 92 Albugo laibachii CCA25842; 93 Auricularia subglabra XP_007344048; 95 Candida maltosa EMG46054; 136 Candida tropicalis XP_002547983; 137 Scheffersomyces stipitis XP_001382588; 139 Candida orthopsilosis XP_003868696; 140 Candida maltose EMG50487; 169 Candida tropicalis XP_002547982; 196 Candida dubliniensis XP_002419480; 227 Albugo candida CCI44158; 235 Phytophthora sojae XP_009516495; 249 Phytophthora parasitica ETP42409; 254 Phytophthora infestans XP_002901590; 259 Phytophthora parasitica ETL91042.1; 262 Phytophthora sojae XP_009522629; 265 Albugo laibachii CCA16055; 266 Phytophthora infestans XP_002895499; 271 Phytophthora sojae XP_009516489; 274 Phytophthora sojae XP_009516500; 275 Phytophthora parasitica XP_008906752; 279 Phytophthora infestans XP_002901572; 280 Phytophthora parasitica ETL44522; 281 Phytophthora infestans XP_002895304; 283 Phytophthora infestans XP_002895302; 284 TGS2 Phaeodactylum tricornutum TGS2; 483 Pyrenophora tritici-repentis XP_001935327; 488 Stemphylium lycopersici KNG49989; 512 Yarrowia lipolytica XP_501820; 523 Kluyveromyces marxianus BAP70535; 532 Rhizoctonia solani CEL51940; 533 Bipolaris maydis XP_014072634; 534 Wickerhamomyces ciferrii XP_011271191; 549 Malassezia symposialis CCU98853; 595 Phaeodactylum tricornutum TGS3; 636 Punctularia strigosozonata XP_007382047; 640 Tulasnella calospora KIO25209; 657 Dichomitus squalens XP_007366602; 680 Phytophthora parasitica ETP42371; 681 Phytophthora sojae XP_009516476; 682 Phytophthora sojae XP_009516477; 685 Phytophthora sojae XP_009535227; 691 Phytophthora parasitica ETI44496; 695 Plasmopara halstedii CEG42791; 696 Albugo laibachii CCA26187; 794 Saccharomyces cerevisiae SGD ID: S000003375 SKN1; Fragi1_jgi Fragilariopsis cylindrus jgiID:188850; Psemu1_jgi Pseudo-nitzschia multiseries jgiID:292157; Psemu2_jgi Pseudo-nitzschia multiseries jgiID:63024; Psemu3_jgi Pseudo-nitzschia multiseries jgiID:260509
Author Contributions

Chapter 2

Diatom vacuolar 1,6-β-transglycosylases can functionally complement the respective yeast mutants
Weichao Huang*, Carolina Río Bártulos, Peter G. Kroth

H.W. designed the project, carried out the experiments and wrote the manuscript. C.RB. carried out the phylogenetic tree. P. G. K. conceived the project and finalized the manuscript.

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Chapter 3

Silencing a vacuolar β-1,3-glucan synthase in the diatom Phaeodactylum tricornutum increased the soluble sugars and compromised the photosynthetic activity
Weichao Huang*, Ilka Haferkamp, Carolina Río Bártulos, Bernard Lepetit, Shengwei Hou, and Peter G. Kroth

Author contributions: W.H. and P.G.K. designed research; W.H., I.H., B.L., C.RB., and S.H. performed research; W.H., I.H., B.L., C.RB., and P.G.K. analyzed data; and W.H. wrote the manuscript.

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Chapter 4

Characterations of TGS1 and 2
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W.H. and P.G.K. designed research; W.H., and S.Y. performed research; W.H., and S.Y. analyzed data; and W.H. wrote the paper.

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