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**Title:** Structurally reduced monosaccharide transporters in an evolutionary conserved red alga

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## SYNOPSIS

The unicellular red alga *Galdieria sulphuraria* is a facultative heterotrophic member of the Cyanidiaceae, a group of evolutionary highly conserved extremophilic red algae. Uptake of various sugars and polyols is accomplished by a large number of distinct plasma membrane transporters. We have cloned three transporters (*GsSPT1*, *GsSPT2*, *GsSPT4*), followed their transcriptional regulation and assayed their transport capacities in the heterologous yeast system. SPT1 is a conserved type of sugar / H<sup>+</sup> symporter with twelve predicted transmembrane spanning domains, while SPT2 and SPT4 represent monosaccharide transporters, characterized by only nine hydrophobic domains. Surprisingly, all three proteins are functional plasma membrane transporters, as demonstrated by genetic complementation of a sugar uptake-deficient yeast mutant. Substrate specificities were broad and largely redundant, except for glucose which was only taken up by SPT1. Comparison of SPT1 and truncated SPT1( $\Delta$ 1-3) indicated that the N-terminus of the protein is not required for sugar transport or substrate recognition. However, its deletion affected substrate affinity as well as maximal transport velocity and released the pH dependency of sugar uptake. In line with these results, uptake by SPT2 and SPT4 was active but not pH-dependent, making a H<sup>+</sup> symport mechanism unlikely for the truncated proteins. We postulate SPT2 and SPT4 as functional plasma membrane transporters in *G. sulphuraria*. Most likely, they originated from genes encoding active monosaccharide / H<sup>+</sup> symporters with twelve transmembrane spanning domains.

**Key words:** Cyanidiaceae, *Galdieria*, polyol transport, Rhodophyta, sugar transport

## INTRODUCTION

Unicellular algae are ideal candidates to study transport processes across the plasma membrane. The hexose uptake machinery (HUP1, HUP2, HUP3) of the green alga *Chlorella kessleri* was the first to be characterized within the green plant lineage and it still serves as a model for conserved hexose transport in higher plants [1 – 6]. However, sugar transport within the branch of non-green plants is not well characterized. Cyanidiaceae, a group of evolutionary conserved, unicellular acidophilic red algae, have been described as the oldest extant members of the red lineage and are most likely located at the basis of secondary endosymbiosis [7, 8], an event that was dated back some 1260 +/- 30 million years and gave rise to the highly diverse group of chromalveolates [9]. Following current taxonomy, three genera are classified as Cyanidiaceae: *Cyanidium*, *Cyanidioschyzon*, and *Galdieria* [10, 11]. These algae have a cosmopolitan distribution but are restricted to habitats with pH values between 0.05 and 3 and temperatures not above 56°C. Due to a high sensitivity towards desiccation, cells often grow endolithically and, as a result, are subjected to severe light limitation, hampering autotrophic growth [12, 13]. *G. sulphuraria* can survive periods of light limitation by metabolizing carbohydrates (disaccharides, hexoses, pentoses, deoxysugars), polyols (hexitols, pentiols, tetriols and triols), amino acids and organic acids [14 – 16]. Many sugars are taken up and metabolized in the D- as well as in the L-configuration [16, 17]. This large spectrum of substrates for heterotrophic growth is unique among eukaryotes and, therefore, *G. sulphuraria* is well suited as a model for sugar transport in the non-green plant lineage, comprising such diverse groups as the cryptomonads, haptophytes, heterokonts, dinoflagellates and apicomplexa [18].

*In vivo* studies with intact cells indicated at least 14 different sugar and polyol transporters with a pronounced pH dependency and a partly overlapping spectrum of substrates [17]. However, up to now, a detailed characterization of the uptake machinery for sugars and polyols in *G. sulphuraria* has been hampered by a lack of genomic sequence information and thus access to individual transporters. The recent initiation of genome projects for *Galdieria sulphuraria* [19] (<http://genomics.msu.edu/galdieria/index.html>) and the obligate autotrophic *Cyanidioschyzon merolae* [20] (<http://merolae.biol.s.u-tokyo.ac.jp/>) has now made it possible to identify putative sugar transporters encoded by these red algal genomes. Comparative genomics of the physiologically very different Cyanidiaceae revealed surprisingly few differences. One of them is a large number of putative sugar transporters, likely up to 28, encoded only by *G. sulphuraria* [21].

In this paper, we report the cloning of three monosaccharide and polyol transporters of *G. sulphuraria*. We follow their transcriptional regulation, and characterize the functions of these proteins in the heterologous yeast system.

## EXPERIMENTAL

### Cultivation of algae

*Galdieria sulphuraria* strain 074G (Mt. Lawu, Java, Indonesia [16]) was cultivated at ambient temperature in inorganic salt medium at pH 2 [22]. Autotrophic cultures were illuminated with white incandescent light ( $120 \mu\text{E m}^{-2} \text{s}^{-1}$ ) and supplied with sterile air enriched with 2%  $\text{CO}_2$  from the bottom of the flask. Heterotrophic cultures were kept in the dark on a rotary shaker (120 rpm) and supplied with 25 mM organic substrate. Cell growth was monitored by following the optical density of the culture at 800 nm. For quantification, cells were diluted to give a reading between 0.05 and 0.15.

### Extraction of RNA

Cells were harvested by centrifugation at 3,000 g and washed twice with 20 mM Tris-HCl, pH 7.5. The cell pellet was either stored at  $-20^\circ\text{C}$  or immediately used for extraction. Cells (1 g fw) were disrupted in 4 ml of lysis buffer (100 mM Tris-HCl, pH 8.6, 2% (w/v) Sarkosyl, 25 mM EDTA, 25 mM EGTA, 100 mM  $\beta$ -mercaptoethanol) using a bead beater (Biospec. Products, Bartlesville, USA) operated for one minute at  $4^\circ\text{C}$ . Cell debris and membranes were removed at 35,000 g (15 min). RNA was isolated according to Sheen and Bogorad [23]. It was resuspended in a suitable volume DEPC- $\text{H}_2\text{O}$ , precipitated over night at  $4^\circ\text{C}$  with 0.5 volume 6 M LiCl and collected at 14,000 g (20 min,  $4^\circ\text{C}$ ), followed by a second precipitation with 2.5 volumes 96% ethanol (1 h,  $-20^\circ\text{C}$ ) and centrifugation at 14,000 g. The pellet was washed twice in 70% ethanol, dried and resuspended in DEPC- $\text{H}_2\text{O}$ .

### Northern blot analysis

30  $\mu\text{g}$  of RNA were separated on 1.4% (w/v) agarose-formaldehyde gels and transferred to Hybond-N+ nylon membrane by capillary blotting. Hybridization was carried out in 0.12 M  $\text{Na}_2\text{HPO}_4$  (pH 7.2), 0.25 M NaCl, 7% (w/v) SDS and 50% formamide at  $42^\circ\text{C}$ . Filters were washed twice for 15 min in 2x SSC, 0.1% SDS at  $65^\circ\text{C}$ , then twice for 20 min in 0.1x SSC, 0.1% SDS at  $65^\circ\text{C}$ .

### Construction of cDNA libraries

cDNA libraries of *G. sulphuraria* (strain 074G) were constructed as  $\lambda$  ZAP cDNA libraries following the instructions of the provider (Stratagene, La Jolla, USA). Autotrophic and heterotrophic (grown in the dark with glucose as carbon source) cells were harvested during exponential growth.

### Heterologous expression of transporters in yeast

For heterologous expression in *Saccharomyces cerevisiae*, the coding sequences of the three sugar and polyol transporters *GsSPT1*, *GsSPT2* and *GsSPT4* were cloned into the yeast expression vector pAUR123, under the control of the alcohol dehydrogenase promoter (TAKARA Bio Inc, Japan). Resistance against aureobasidine was used as selective marker for transformands. Transporter *GsSPT1* was also cloned as a 5' deletion construct (omitting the first 444 nucleic acids), termed *GsSPT1*( $\Delta$ 1-3). As expression system, we used the sugar uptake-deficient yeast strain EBY.VW4000 (*MAT a  $\Delta$ hxt1-17  $\Delta$ gal2  $\Delta$ stl1  $\Delta$ agt1  $\Delta$ mph2  $\Delta$ mph3 leu2-3,112 ura3-52 trp1-289 his3- $\Delta$ 1 MAL2-8<sup>c</sup> SUC2*), kindly provided by Prof. E. Boles (Johann Wolfgang Goethe University, Frankfurt/Main, Germany) [24]. Cells were routinely cultivated in yeast minimal medium (YNB), supplemented with leucine, uracil, tryptophan and histidine, containing 2% maltose as carbon source unless indicated otherwise. Transformation of yeast cells was done as described [25]. After transformation, cells were plated onto minimal selective medium containing 2% maltose and aureobasidine A (3  $\mu$ g / ml) and incubated for 3-4 days at 30°C. Transformation of colonies was verified by PCR with transporter- and vector-specific primers. Transformed cells were used for complementation studies of yeast growth on minimal selective medium containing 2% sugars and polyols, respectively, and also for uptake of radiolabeled substrates (see below).

### Uptake studies with radiolabeled substrates

Yeast cells were harvested during exponential growth at an optical density between 0.5 – 0.8 (600 nm), washed twice and resuspended in distilled water to give a final concentration of 100 mg fw ml<sup>-1</sup>. 20  $\mu$ l of the cell suspension (2 mg fw) were mixed with one volume of 100 mM Tris/citrate buffer, pH 5.0. After 2 min preincubation at 30°C, uptake of radiolabeled substrate was initiated by rapid addition to give a final volume of 50  $\mu$ l. As labelled substrates we used L-[C-6-<sup>3</sup>H]fucose (3152.4 GBq mmol<sup>-1</sup>), D-[C-1-<sup>3</sup>H]ribose (740 GBq mmol<sup>-1</sup>), L-[C-1-<sup>3</sup>H]arabinose (111 GBq mmol<sup>-1</sup>), L-[C-1-<sup>3</sup>H]rhamnose (185 GBq mmol<sup>-1</sup>), D-[U-

$^{14}\text{C}$ ]glucose ( $10.7 \text{ GBq mmol}^{-1}$ ), D-[U- $^{14}\text{C}$ ]fructose ( $11.2 \text{ GBq mmol}^{-1}$ ), D-[U- $^{14}\text{C}$ ]sorbitol ( $9.5 \text{ GBq mmol}^{-1}$ ), D-[U- $^{14}\text{C}$ ]mannitol ( $1.9 \text{ GBq mmol}^{-1}$ ) (Moravek Biochemicals and Radiochemicals, Brea, CA, USA). The final sugar concentration was  $0.1 \text{ mM}$  with a specific activity of  $18.5 \text{ MBq mmol}^{-1}$  for fucose, rhamnose, ribose, arabinose and glucose and  $9.25 \text{ MBq mmol}^{-1}$  for fructose, mannose and sorbitol. Incubation was done for  $20 \text{ s}$  at  $30^\circ\text{C}$  unless indicated otherwise. Uptake was stopped by the addition of  $1 \text{ ml}$  ice-cold distilled water. Cells were harvested on a glass-fiber filter (GE Healthcare, Little Chalfont, England) and washed 4 times with  $1 \text{ ml}$  cold distilled water. Radioactivity was monitored using  $3 \text{ ml}$  scintillation cocktail and a Beckman liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA). Assays were performed in duplicates and repeated at least twice in independent experiments. Untransformed cells were used as a blank. Their intake as well as binding of radioactive material to the surface was subtracted from the samples in all experiments.

## RESULTS

### Cloning of a novel type of sugar transporter

We have cloned three sugar (monosaccharide) and polyol transporters from the acidophilic red alga *Galdieria sulphuraria* (strain 074G). They were termed *GsSPT1*, *GsSPT2*, and *GsSPT4* and their coding sequences were deposited into the NCBI GenBank as EF166070, EF166071, and EF166072, respectively. *SPT1* was isolated from cDNA libraries of autotrophic as well as of glucose-grown heterotrophic cells. *SPT2* could only be isolated from cDNA of autotrophic cells, while *SPT4* was specific for cDNA of glucose-grown cells. Attempts to amplify PCR fragments of *SPT2* and *SPT4* from the respective other type of cDNA yielded no results, suggesting a sugar-dependent expression of transporters. The cDNA of *SPT1* comprises a coding sequence of  $1671 \text{ bp}$ . cDNA sequences of *SPT2* and *SPT4* were found to contain stop codons upstream of the first ATG. *SPT2* contains three stop codons at  $-120$ ,  $-147$ , and  $-204 \text{ bp}$ , leaving a  $1206 \text{ bp}$  coding sequence of the transporter. *SPT4* contains only one stop codon at  $-381 \text{ bp}$ , resulting in a  $1236 \text{ bp}$  coding sequence. These stop codons were confirmed by multiple and independent RT-PCR. The gDNA sequences of *SPT1*, *SPT2*, and *SPT4* were identical to the corresponding cDNA sequences and did not contain introns. *SPT1* translates into a mature protein of  $557$  amino acids. *SPT2* translates into  $402$ , and *SPT4* into  $412$  amino acids. A protein alignment indicates an overall identity of  $25\%$  for all three transporters. Closest homologs to the algal proteins are myo-inositol transporters from yeast.

Secondary structures of the deduced protein sequences were predicted using the public DAS – transmembrane prediction server for putative membrane proteins. Apparently, SPT1 follows the highly conserved structure of sugar transporters, exhibiting twelve transmembrane spanning domains with a large cytosolic loop in the middle of the protein (predicted 6+6 structure). SPT2 and SPT4 were predicted to consist of only nine transmembrane spanning domains: three at the N-terminus, six at the C-terminus. They are separated by a cytosolic middle loop, resulting in a 3+6 structure of the transporters. Going back into the putatively non-translated 5' region of the cDNA sequences of *SPT2* and *SPT4*, we found the three 'missing' N-terminal domains present in both sequences. However, as stop codons separate the corresponding nucleotides from the putative start of translation, the proteins have to be regarded as N-terminally deleted. The new N-termini of both 3+6 transporters were predicted on the outer side of the plasma membrane, with 8 - 10 (*SPT2*) and 10 (*SPT4*) amino acids reaching into the periplasmatic space.

### **Transcriptional regulation of transporters**

The fact that *SPT2* could only be isolated from cDNA of autotrophic and *SPT4* only from glucose-grown heterotrophic algae, indicated a sugar-dependent transcriptional regulation of protein expression. We used Northern blot analysis to investigate this regulation in more detail (Fig. 1). Algae were grown autotrophically as well as heterotrophically on hexoses (glucose, fructose), pentoses (xylose, L-arabinose), polyols (mannitol, sorbitol, dulcitol, xylitol, arabitol), and deoxysugars (L-fucose). They were harvested and extracted during early exponential growth. Transporter-specific probes were used for RNA-hybridization. *SPT1*- and *SPT2*-transcripts were detected in autotrophic as well as in heterotrophic cells. Transcript levels, however, were much higher in heterotrophic cells. The induction of *SPT1* was independent of the type of substrate provided for heterotrophic growth, while induction of *SPT2* was highly specific for dulcitol. Transcript levels of *SPT4* were generally very low. In autotrophic cells, no signal was detected. Under heterotrophic conditions, only glucose- and arabitol-grown cells exhibited a faint signal. We conclude that the regulation of monosaccharide and polyol transporters takes place – at least in part – on the transcriptional level in *G. sulphuraria*. Transcription of all three transporters was upregulated under heterotrophic conditions. For *SPT2* and *SPT4*, this induction was substrate-specific, while *SPT1*-transcription was unspecifically upregulated in the presence of monosaccharides or polyols.

## Functional characterization of transporters in the heterologous system

### *Complementation of yeast cell growth*

To determine the functionality and substrate specificity of both, the predicted 6+6 and 3+6 type of transporters, we expressed SPT1, SPT2 and SPT4 in a sugar uptake-deficient yeast strain [24]. We assessed transformants for cell growth on various substrates and determined their uptake capacities for radiolabeled substrates. To evaluate the importance of the first three domains for transport, we deleted the corresponding 148 amino acids from SPT1 to obtain the artificial 3+6 transporter SPT1( $\Delta$ 1-3).

For complementation of growth, yeast cells were pregrown on maltose before spotting them onto minimal selective plates, supplemented with different carbon sources. We assessed cell growth of transformants on hexoses (glucose, galactose, mannose, fructose), polyols (mannitol, dulcitol, arabitol), pentoses (arabinose, xylose) and deoxysugars (fucose, rhamnose). All carbon sources were used in the D-configuration as yeast is not known to metabolize L-sugars. Heterologous expression of the algal transporters in uptake-deficient yeast, did not complement growth on any of the substrates tested (data not shown).

### *Uptake of radiolabeled substrates*

To determine the substrate specificities of transporters independent of cell growth, we followed uptake of radiolabeled substrates in the heterologous expression system. Cells were pregrown on maltose, and subsequently assessed for their uptake capacities. Results are summarized in figure 2. In contrast to the above results obtained from complementation studies, all three algal transporters, SPT1, SPT2 and SPT4, mediated uptake of hexoses, polyols, pentoses, and deoxysugars. Glucose was specifically taken up by SPT1, while fructose transport was also mediated by SPT2 and SPT4. Obviously, the growth defect of yeast transformants on hexoses was not due to a general lack of hexose uptake. We therefore assessed hexose phosphorylation capacities of our yeast strain. We confirmed the presence of glucokinase I, hexokinase I and hexokinase II in the sugar-uptake deficient host by PCR and by following enzyme activities in crude cell extracts (data not shown). Complementation of yeast growth on glucose and fructose should thus be feasible and the negative results can only be explained if uptake as mediated by the algal transporters was not sufficient to sustain yeast

cell growth. For comparison, glucose uptake of wild type yeast was about 15 times higher than in transformants expressing GsSPT1 (Fig. 2).

In contrast to yeast, *Galdieria sulphuraria* can take up and metabolize a number of substrates in the L-configuration [16, 17]. In the heterologous yeast system, uptake of these substrates by SPT1, SPT2, and SPT4 could only be assessed by using radiolabelled compounds. As demonstrated in figure 2, all three algal transporters mediated high uptake of L-fucose, while another deoxysugar, L-rhamnose, was not taken up. Transport of L-arabinose was very poor and only mediated by SPT4. In general, substrate specificities of SPT1 and its deletion construct SPT1( $\Delta$ 1-3) were identical, while uptake capacities differed significantly. This effect was most pronounced for L-fucose and D-ribose. Their uptake by SPT1( $\Delta$ 1-3) was twice that mediated by SPT1. For mannitol, the effect was inverse. Uptake of glucose and fructose was only slightly affected by the deletion (Fig. 2). We conclude, that the N-terminus of SPT1 does not determine the transporter's substrate specificity, i.e. take part in substrate recognition, while, on the other hand, it does seem to be involved in determining transport capacities.

### ***Uptake kinetics***

Uptake kinetics were determined for L-fucose, as uptake of this deoxysugar was highest for all transporters (Fig. 3). Substrate affinity was highest for SPT1( $\Delta$ 1-3) ( $K_m = 17 \mu\text{M}$ ) and lowest for SPT1 ( $K_m = 36 \mu\text{M}$ ), indicating a significant influence of the 148 N-terminal amino acids of SPT1 on substrate binding. SPT2 and SPT4 exhibited similar  $K_m$  values of 27 and 29  $\mu\text{M}$ , respectively. Maximal uptake velocity was highest for SPT1( $\Delta$ 1-3) ( $417 \text{ nmol} \times \text{g}^{-1} \text{ fw} \times 20 \text{ s}^{-1}$ ). Apparently, the N-terminal truncation of SPT1 resulted in a reduced sterical hindrance of L-fucose uptake.  $V_{\text{max}}$  values for SPT1 and the predicted 3+6 transporter SPT4 were identical ( $357 \text{ nmol} \times \text{g}^{-1} \text{ fw} \times 20 \text{ s}^{-1}$ ), that for SPT2 was lowest ( $303 \text{ nmol} \times \text{g}^{-1} \text{ fw} \times 20 \text{ s}^{-1}$ ) (Fig. 3).

In order to distinguish between active and passive transport, we incubated yeast cells expressing the algal transporters with saturating concentrations of L-fucose (0.1 mM) until a steady state was reached (Fig. 4). Intracellular L-fucose concentrations were calculated, estimating an average yeast cell volume of 20 fl and  $10 \times 10^9$  cells  $\times \text{g}^{-1} \text{ fw}$  [26, 27]. In SPT1 as well as in SPT1( $\Delta$ 1-3) expressing cells, uptake was saturated after 2 min at a level of  $750 \text{ nmol} \times \text{g}^{-1} \text{ fw}$ . SPT4 expressing cells reached the same steady state after 4 min, while SPT2 mediated uptake of only  $600 \text{ nmol} \times \text{g}^{-1} \text{ fw}$ . Intake of  $600 - 750 \text{ nmol L-fucose} \times \text{g}^{-1} \text{ fw}$  gives an intracellular concentration of 3.0 – 3.75 mM, roughly corresponding to a 30 – 40-fold

enrichment as compared to the medium. This intracellular accumulation of sugars has to be based on active transport, and we therefore classify SPT1, SPT2 and SPT4 as active transporters.

As there are different types of active transporters (e.g. H<sup>+</sup> or Na<sup>+</sup> cotransporters, ABC-transportes), we followed the pH-dependency of sugar uptake in yeast (Fig. 5). Only SPT1 exhibited a pronounced pH-dependency of L-fucose transport with maximal uptake at pH 4.5. The predicted 6+6 transporter is therefore likely to function as an active sugar / H<sup>+</sup> symporter. Deletion of the three N-terminal transmembrane spanning domains resulted in a significant reduction of this pH-dependency in SPT1( $\Delta$ 1-3) expressing cells, while uptake mediated by SPT2 and SPT4 was completely independent of external pH in the range of pH 4.5 – 8.0. The 3+6 transporters are therefore unlikely to function as H<sup>+</sup> symporters and have to rely on alternative energy sources for active transport.

## DISCUSSION

Heterotrophy of *Galdieria sulphuraria* is mediated by transporters in the plasma membrane which are specifically encoded in the *Galdieria*-genome but absent from that of the closely related but obligate autotrophic red alga *Cyanidioschyzon merolae* [21]. We have identified two types of transporters in *G. sulphuraria* – one that follows the conserved structure of twelve transmembrane spanning domains with a large cytosolic central loop (predicted 6+6 structure), the other one consisting of nine transmembrane spanning domains with a cytosolic loop between the third and the fourth hydrophobic domain (predicted 3+6 structure). GsSPT2 and GsSPT4 are two examples of transporters with a putative 3+6 configuration. Their coding regions are significantly shorter than that of GsSPT1, a conserved 6+6 type of transporter, while the cDNA sequences as well as the algal genome still encode the missing N-terminal domains within the putatively non-translated 5' regions. We suggest that the 3+6 transporters were generated by mutations within the conserved 6+6 type of transporters. The introduction of stop codon(s) has left the resulting proteins with nine instead of twelve transmembrane spanning domains. Based on molecular clock estimates, Cyanidiaceae are probably more than 1.5 billion years old and thus likely some of the oldest extant eukaryotic organisms [7, 8]. This phylogenetic age increases the chance for an accumulation of mutations – as long as they do not impair the cell's viability and evolutionary fitness. From a comparison of SPT1 with its deletion construct SPT1( $\Delta$ 1-3) – both expressed in the heterologous yeast system – we

conclude that loss of the three N-terminal transmembrane spanning domains does not impair the basic function of active sugar uptake. It has to be kept in mind, though, that uptake specificity as well as kinetics could be affected by heterologous expression. In yeast, both proteins mediated an intracellular accumulation of monosaccharides and polyols and had the same spectrum of substrates. On the other hand, transport kinetics ( $K_m/V_{max}$ ) and especially the pH-dependency of uptake were significantly altered in SPT1( $\Delta$ 1-3) expressing cells.  $H^+$  symport has previously been suggested as the mechanism for sugar and polyol uptake in *Galdieria* [17]. And while this still holds true for SPT1, N-terminal truncation released the pH dependency of sugar uptake and therefore appears to significantly impair  $H^+$  symport. At the same time, intracellular accumulation of sugars was still possible in SPT1( $\Delta$ 1-3) expressing yeast cells, and transport therefore had to be active. In line with these results, the predicted 3+6 transporters SPT2 and SPT4 were functional and active transporters when expressed in yeast. Their spectrum of substrates did not differ significantly from that of SPT1, yet uptake was pH-independent and therefore unlikely to be mediated by  $H^+$  symport. Our attempts to complement the growth defect of an hexose uptake-deficient yeast strain by expressing the algal transporters, however, were not successful. This is quite surprising as we could clearly demonstrate uptake of radiolabeled hexoses by the corresponding yeast transformands. Apparently, hexose uptake rates as mediated by the algal transporters were not sufficient to support yeast cell growth. A second explanation is based on concurrent activity of a sugar exporter in the uptake-deficient yeast strain. EBY.VW4000, deleted in at least 20 transporters [24], still encodes a putative hexose exporter (E. Boles, pers. com.). Low hexose uptake by the algal transporters would thus be nullified by this endogenous exporter. We conclude that the inability of the *Galdieria* transporters, GsSPT1, GsSPT2, and GsSPT4, to complement cell growth of the uptake-deficient yeast strain on hexoses, is most likely due to endogenous features of the yeast strain in combination with low uptake rates of the algal transporters in the heterologous system.

With as many as 28 distinct sugar and polyol transporters present in the plasma membrane of *Galdieria* [21], a highly coordinated regulation of their activity has to be applied. Based on *in vivo*  $^{14}C/^3H$ -uptake studies with native cells, we have previously reported a sugar-dependent regulation of transporter activity in the alga [28]. We now corroborate these previous results by Northern blot analyses and conclude that regulation is, at least in part, achieved on the transcriptional level. Transcripts of *SPT1* and *SPT2* are detectable in autotrophic algae but massively up-regulated under heterotrophic conditions. *SPT1* was non-specifically induced by all substrates tested in our study, *SPT2* on the other hand was

specifically induced by dulcitol. *SPT4* transcript was hardly detectable under any of the conditions tested, implying either very high turnover rates or very low transcription of the transporter. For several sugar transporters from higher plants, a stress-related induction has been reported [29, 30]. It may well be that *SPT4* serves a similar purpose in *G. sulphuraria* and we did not apply the appropriate conditions for maximal gene induction. Protein phosphorylation as a way of regulating transporter activity [31] was not investigated in this study, but it should not be excluded as a regulatory level in *G. sulphuraria*.

The presence of low transcript levels of *SPT1* and *SPT2* in autotrophic cells is in agreement with previous data indicating marginal sugar uptake already in autotrophic cells [17, 28]. Several explanations for this early gene activation come to mind: one is a direct role for *SPT1* and *SPT2* in sugar sensing, i.e. signaling the presence of extracellular substrates to the cell. *Rgt2* and *Snf3* are two signaling transporter-homologues known from the yeast system [32, 33]. They are no longer active in the uptake of substrates but involved in the regulation of the high- and low-affinity hexose transporters (*Hxt1-7*) of yeast. *Rgt2* and *Snf3* exhibit extended cytosolic C-termini which are needed for interaction with the signal transducing proteins *Std1* and *Mth1* [34, 35]. Neither *SPT1* nor *SPT2* have such putative signaling domains (data not shown). In addition, they proved to be functional transporters when expressed in yeast. A primary role in sugar sensing is therefore unlikely. However, an indirect role of *SPT1* and *SPT2* in sugar sensing by mediating early uptake of sugars and polyols prior to full activation of the cell's heterotrophic machinery appears possible. In a third model, the presence of *SPT1* and *SPT2* transcript in autotrophic cells reflects a 'ready-to-go' status of the alga. This could speed up the generation of mature transporters upon the arrival of organic carbon.

Until recently, sugar uptake in *G. sulphuraria* could only be studied in intact cells with an unknown set of transporters obscuring the results. The cloning of *SPT1*, *SPT2* and *SPT4* made it possible to assess transporters separately for the first time, to distinguish between active transport and facilitated diffusion, and to determine substrate specificities and uptake kinetics. In the heterologous yeast system, substrate specificities of *GsSPT1*, *GsSPT2* and *GsSPT4* were largely redundant, except for glucose which was only transported by *SPT1*. These results corroborate previous *in vivo* studies on intact *Galdieria* cells, indicating transporters with a broad and partly overlapping spectrum of substrates [17]. However, in contrast to previous models, transporters were not specific for a certain class of substrates, e.g. polyols or hexoses. Furthermore, they mediated transport of substrates in the D-configuration (e.g. fructose) as well as in the L-configuration (e.g. L-fucose). This is in

accordance with the spectrum of substrates used for heterotrophic growth of *G. sulphuraria* [16]. Fucose uptake rates were very high for all three *Galdieria* transporters, suggesting an important role for this deoxysugar as an *in vivo* substrate for the alga.  $K_m$  values ranged between 27 and 36  $\mu\text{M}$ , corresponding to about 5 mg fucose  $\text{l}^{-1}$ . In general, the level of organic compounds is very low at the natural habitat [12, 13]. Living in endolithic cell mats, it has been suggested that *G. sulphuraria* draws substrates from dead cell material of neighboring Cyanidiaceae and other species. Dissolved organic carbon is highly concentrated by the dense packing of mats and directly available to heterotrophic cells. If fucosylated cell walls should be abundant in these communities, the deoxysugar could be a major source of organic material for *Galdieria*. N-terminal truncation of conserved 6+6 transporters apparently leads to increased uptake velocity and substrate affinity (as shown for SPT1 and SPT1( $\Delta$ 1-3)). Expressing these truncated transporters, cells would thus be able to rapidly deplete organic substrates from the extracellular space.

Comparing the monosaccharide and polyol transporters of *G. sulphuraria* to that of the green plant line, substrate specificities appear broader in the red alga. The *Chlorella kessleri* hexose transporters HUP1 and HUP3 are specific for hexoses (glucose, fructose and mannose), while HUP2 takes up glucose, galactose and xylose [2, 6]. In higher plants, sugar transporters for glucose, fructose, xylose, arabinose, mannose and galactose [36 – 40] as well as some polyol-specific transporters [41 – 45] have been described. To our knowledge, only one transporter from higher plants (*Arabidopsis thaliana*) exhibits a spectrum of substrates that is comparable to that of *G. sulphuraria* transporters: AtPLT5 takes up a large number of polyols in addition to hexoses and pentoses [46]. None of the transporters mentioned has been described as active also after truncation of the three N-terminal transmembrane spans. In this respect, the red algal transporters are unique in the plant kingdom.

While higher green plants and red algae are only distantly related, our studies on the monosaccharide and polyol transporters of *G. sulphuraria* will widen the understanding of sugar transport and heterotrophic capacities within the red line of evolution.

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## FIGURE LEGENDS

### **Figure 1. Northern Blot analysis for *GsSPT1*, *GsSPT2*, and *GsSPT4*.**

*Galdieria sulphuraria* was grown autotrophically or heterotrophically on different substrates, as indicated. RNA was isolated during early exponential growth. Ribosomal RNA was used as loading control for total RNA.

### **Figure 2. Uptake of radiolabeled substrates by yeast cells expressing transporters from**

*Galdieria sulphuraria*. *GsSPT1* (black), *GsSPT2* (dark grey), *GsSPT4* (white), and the deletion construct *GsSPT1*( $\Delta$ 1-3) (light grey) were expressed in the sugar uptake-deficient yeast strain EBY.VW4000. Transformands were assessed for their uptake capacities for radiolabeled substrates. Uptake of cells expressing the empty vector (below resolution, last column position) as well as of wild type cells (striped) are given as reference. Assays were done in triplicate as described.

### **Figure 3. Michaelis-Menten (A) and Lineweaver-Burk (B) plots of uptake kinetics for L-**

**fucose.** The sugar uptake-deficient yeast strain EBY.VW4000, expressing *GsSPT1* (closed circles), *GsSPT2* (crosses), *GsSPT4* (closed squares), and the deletion construct *GsSPT1*( $\Delta$ 1-3) (open circles), respectively, was incubated with L-fucose concentrations ranging between 0 and 0.2 mM. Assays were done in triplicate as described.  $K_m$  and  $V_{max}$  values are given in the text.

### **Figure 4. Time-dependent uptake of L-fucose.**

The sugar uptake-deficient yeast strain EBY.VW4000, expressing *GsSPT1* (closed circles), *GsSPT2* (crosses), *GsSPT4* (closed squares), and the deletion construct *GsSPT1*( $\Delta$ 1-3) (open circles), respectively, was incubated with saturating L-fucose concentrations (0.1 mM) for up to 6 min. Assays were done in triplicate as described.

### **Figure 5. pH-dependet uptake of L-fucose.**

The sugar uptake-deficient yeast strain EBY.VW4000, expressing *GsSPT1* (closed circles), *GsSPT2* (crosses), *GsSPT4* (closed squares), and the deletion construct *GsSPT1*( $\Delta$ 1-3) (open circles), respectively, was incubated

with saturating L-fucose concentrations (0.1 mM) at external pH values ranging from pH 4.5 to 8.0. Assays were done in duplicate as described.









