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Microbial rhodopsins on leaf surfaces of terrestrial plants

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Summary

The above-ground surfaces of terrestrial plants, the phyllosphere, comprise the main interface between the terrestrial biosphere and solar radiation. It is estimated to host up to 10^{26} microbial cells that may intercept part of the photon flux impinging on the leaves. Based on 454-pyrosequencing-generated metagenome data, we report on the existence of diverse microbial rhodopsins in five distinct phyllospheres from tamarisk (*Tamarix nilotica*), soybean (*Glycine max*), *Arabidopsis* (*Arabidopsis thaliana*), clover (*Trifolium repens*) and rice (*Oryza sativa*). Our findings, for the first time describing microbial rhodopsins from non-aquatic habitats, point towards the potential coexistence of microbial rhodopsin-based phototrophy and plant chlorophyll-based photosynthesis, with the different pigments absorbing non-overlapping fractions of the light spectrum.

Received 27 February, 2011; accepted 20 June, 2011. For correspondence. *E-mail beja@tx.technion.ac.il; Tel. 972-4-8293961; Fax 972-4-8225153; **E-mail shimshon@vms.huji.ac.il; Tel. 972-2-6584192; Fax 972-2-6585559. †Present address: Department of Earth and Planetary Science, University of California, Berkeley, CA 94720, USA. ‡N.A.-I. and O.M.F. contributed equally to this work.

Introduction

Solar radiation is the main source of energy for both marine and terrestrial organisms, with terrestrial plants and aquatic phytoplankton performing an equivalent ecological function as chlorophyll-based photosynthetic primary producers (Field *et al.*, 1998). Marine surface waters are now known to harbour an additional type of phototrophy; several lineages of bacteria and archaea utilize rhodopsins (Béjà *et al.*, 2000; 2001; de la Torre *et al.*, 2003; Balashov *et al.*, 2005; Giovannoni *et al.*, 2005; Sabehi *et al.*, 2005; Frigaard *et al.*, 2006; Gómez-Consarnau *et al.*, 2007; 2010; Oh *et al.*, 2010), retinal-containing *trans*membrane proteins, as light-driven proton pumps. The first microbial rhodopsin was reported four decades ago in the archaeon *Halobacterium salinarum* from hypersaline environments (Oesterhelt and Stoeckenius, 1971). Further studies revealed the existence of microbial rhodopsins in diverse habitats including freshwater, sea ice, hypersaline and brackish environments (Rusch *et al.*, 2007; Atamna-Ismaeel *et al.*, 2008; Sharma *et al.*, 2008; 2009; Koh *et al.*, 2010). To date, microbial rhodopsins have been reported exclusively for aquatic habitats.

As light is an abundant resource on land, we tested the hypothesis that microbial rhodopsins also exist and play an important role in terrestrial niches. The leaf surface of terrestrial plants covers a surface area of an estimated 6.4×10^8 km^2 and comprises the main interface between terrestrial biomass and solar photon flux. This habitat harbours an immensely diverse microbial community of up to 10^6–10^7 cells per cm^2 leaf surface (Lindow and Brandl, 2003). A mode of phototrophy that is compatible with the plant's photosynthesis would offer a significant ecological advantage to microbes inhabiting this environment.

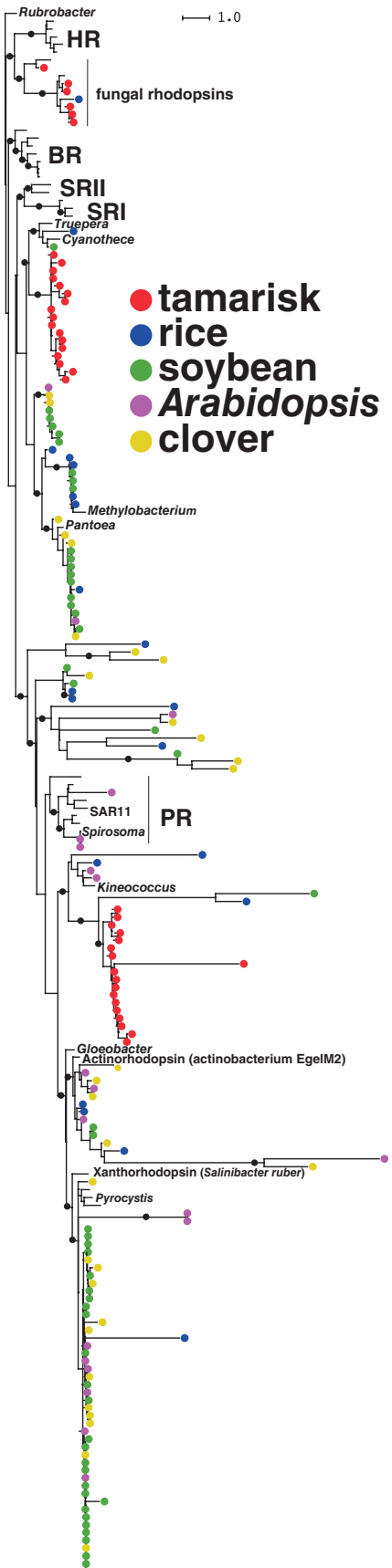

Fig. 1. A phylogenetic tree of rhodopsin amino acid sequences (deduced from the metagenomic data) from the phyllospheres of tamarisk, rice, soybean, *Arabidopsis* and clover. Following alignment computation (see *Experimental procedures*), a FastTree version 2.1.1 was used for the calculation of the approximately maximum-likelihood phylogenetic tree using settings for high accuracy. Bootstraps above 60% are shown as black circles at the junctions. PR, proteorhodopsins; HR, halorhodopsins; BR, bacteriorhodopsins; SRI, sensory rhodopsins-I; SRII, sensory rhodopsins-II.

Results and discussion

We have identified 156 microbial rhodopsin sequences in five phyllosphere metagenomes (Supporting material S1, S2, S3, S4 and S5 in *Supporting information*), from different terrestrial plants: soybean (*Glycine max*) (Delmotte *et al*., 2009), tamarisk (*Tamarix nilotica*), clover (*Trifolium repens*), rice (*Oryza sativa*), and from a wild population of the model plant *Arabidopsis thaliana*. The size of the different metagenomes obtained was 261 Mb, 448 Mb, 234 Mb, 831 Mb and 250 Mb for soybean, tamarisk, clover, rice and *Arabidopsis* with an average read length of 235, 328, 235, 357 and 233 bp, respectively.

Phylogenetic analysis revealed that some phyllosphere microbial rhodopsins have branched away from known rhodopsin families within the bacterial and eukaryal domains (Fig. 1). Some of these sequences clustered with fungal rhodopsins, while another group clustered with xanthorhodopsins (Balashov *et al*., 2005; Lanyi and Balashov, 2008) and actinorhodopsins (Sharma *et al*., 2008; 2009). However, most phyllosphere rhodopsins appear on novel branches, with no representatives from either culture-based or environmental data sets, thus rendering them with an as yet uncertain phylogenetic affiliation. In most cases, the leaf surface rhodopsins from tamarisk clustered separately from other phyllosphere rhodopsins (Fig. 1) with a statistically significant phylogenetic signal [calculated using Mesquite (Maddison and Maddison, 2010)], indicating that they reside in distinct microbial taxa, probably adapted to the unique hypersaline environment of the tamarisk phyllosphere (Qvit-Raz *et al*., 2008).

In contrast with soil metagenomes, which do not contain any rhodopsin reads, the five phyllosphere data sets were found to contain microbial rhodopsins, but at frequencies lower than those found in marine and freshwater metagenomes (Fig. 2). While some of the phyllosphere rhodopsins lack the retinylidene Schiff base proton donor carboxylate and are thus likely sensory rhodopsins, others contain both proton acceptor and donor carboxylates at helix C (bacteriorhodopsin positions 85 and 96 respectively; see Supporting material S1, S2, S3, S4 and S5) and may be considered as potential proton pumps. Compared with the marine environment, where they make up only 3% of all microbial rhodopsins (Spudich, 2006),

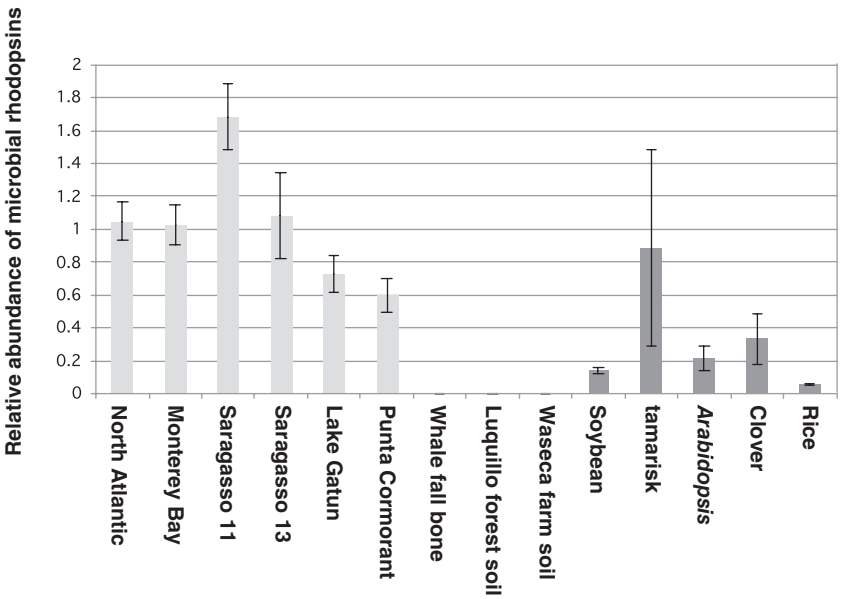

Fig. 2. Relative abundance of microbial rhodopsins in different metagenomes. MG-RAST (Meyer *et al*., 2008) accession numbers of the different data sets can be found in *Experimental procedures*. Abundance was normalized relative to the numbers of *rplA*, *rplC*, *rplD*, *rpoA*, *rpoB* and *rspJ* genes (Frank and Sorensen, 2011) in each environment.

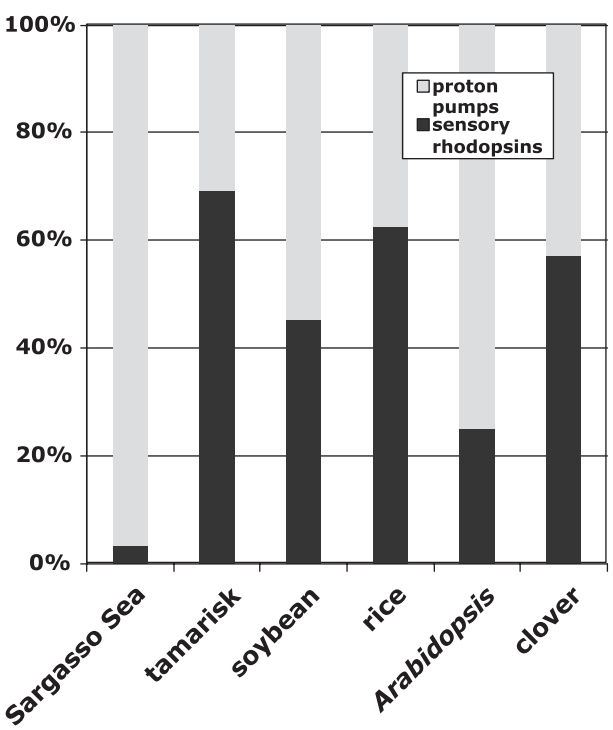

Fig. 3. Sensory rhodopsins and proton pumps in different environments. Proportions of sensory rhodopsins and rhodopsin proton pumps were calculated only from reads containing the region surrounding the proton acceptor and donor carboxylates at helix C (bacteriorhodopsin positions 85 and 96, respectively); Sargasso Sea (Spudich, 2006) ($n = 732$), tamarisk ($n = 13$), soybean ($n = 31$), rice ($n = 8$), *Arabidopsis* ($n = 4$) and clover ($n = 7$).

the contribution of sensory rhodopsins to phyllospheres is much higher (25–70%; Fig. 3). This suggests that microorganisms in the phyllosphere are intensively engaged in light sensing, to accommodate the effects of fluctuations in light quality, intensity and UV radiation at the leaf surface (Ballaré *et al*., 1990; Beattie and Lindow, 1999).

Interestingly, all detected phyllosphere rhodopsins carry a leucine residue at position 105 (Fig. 4; based on sequence reads that contain this region; not all reads cover the entire gene), which renders them as putative green light absorbing pigments (Man *et al*., 2003), thus avoiding an overlap with the absorption spectrum of the plant's leaf and possibly even masking out the negative role of green light on plant growth (Folta and Maruhnich, 2007). This is opposed to blue light absorbing rhodopsins (Béjà *et al*., 2001; Sabehi *et al*., 2005), which contain a glutamine instead of leucine at position 105, and are abundant in marine habitats (Béjà *et al*., 2001; Rusch *et al*., 2007; Sabehi *et al*., 2007).

Another indication that this may indeed be the case in the tamarisk phyllosphere is presented by the absorption spectra in Fig. 5; it is demonstrated that the microbes washed off the leaves have an absorption maximum around 545 nm, a region of the spectrum where there is no light absorption by the tamarisk leaves and where the absorption of microbial rhodopsins is maximal. This absorption peak, however, could also be the result of the presence of carotenoids-containing pink-pigmented *Methylobacterium* spp. (Kutschera, 2007) in the leaf wash.

This is the first report on the existence of microbial rhodopsins in terrestrial habitats; whether it portrays commensalism or mutualism should be a matter of further investigations. We show that rhodopsin sequences have

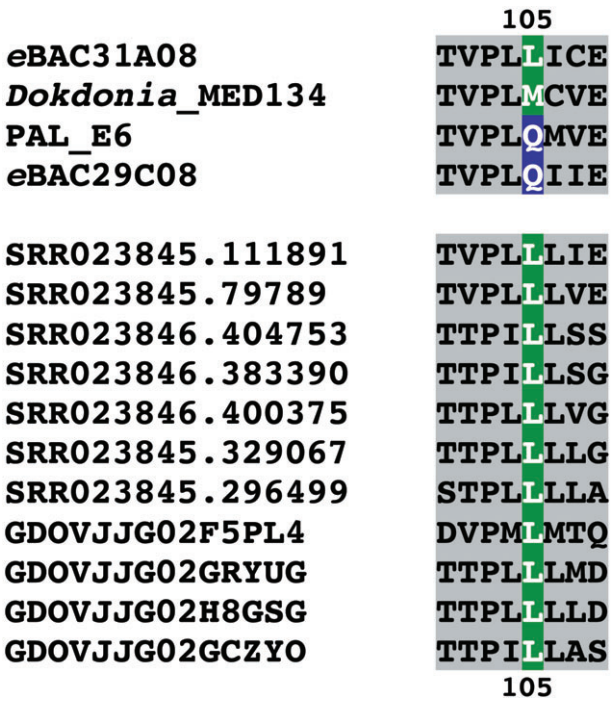

Fig. 4. Protein alignment of phyllosphere rhodopsins. Amino acid position 105 is marked with green or blue backgrounds according to the predicted absorption spectra of the rhodopsin pigments. Only the vicinity of amino acid 105 is shown. Examples from confirmed green absorbing proteorhodopsins *e*BAC31A08 (Béjà *et al*., 2000), *Dokdonia* MED134 (Gómez-Consarnau *et al*., 2007) and confirmed blue absorbing proteorhodopsins PAL-E6 (Béjà *et al*., 2001), *e*BAC49C08 (Sabehi *et al*., 2005) are shown for reference at the top. Names of rhodopsins from the soybean phyllosphere start with SRR and from the tamarisk start with GDOVJJ. Only a subset of the phyllosphere rhodopsins is shown. See Supporting material S1, S2, S3, S4 and S5 for more variations.

been found to be abundant both in the harsh environment of the tamarisk phyllosphere (Qvit-Raz *et al*., 2008) and on the leaves of cultivated plants; furthermore, they are common to diverse leaf shapes and plant growth characteristics, but are absent from both agricultural and forest soils. This indicates that microbial rhodopsins may be selected for in the phyllosphere environment, thus conferring an important adaptive trait onto this microbial niche. We propose that rhodopsin light interception by phyllosphere bacteria needs to be taken into account in global energy balance and biomass production by the terrestrial biosphere.

Experimental procedures

Phyllosphere sampling

Leaf samples were collected from a *T. nilotica* tree in an oasis by the Dead Sea (31°42′41.06″N 35°27′19.32″E), and processed within 1 h of sampling (Qvit-Raz *et al*., 2008). Briefly, 50 g of leaves were placed inside a 250 ml sterile glass Erlenmeyer flask, immediately immersed in sterile phosphate-buffered saline (1 g leaf per 5 ml PBS, pH 7.4), and cavitated in a sonication bath [Transistor/ultrasonic T7 (L&R Manufacturing Company)] for 2 min at medium intensity. The preparations were then vortexed 6 × 10 s at 5 min intervals, and the leaf wash was separated from the leaf debris by decanting and kept for analysis. *Arabidopsis*, clover and rice phyllospheres were prepared according to the previously reported soybean phyllosphere preparation (Delmotte *et al*., 2009).

DNA extraction and pyrosequencing

The leaf wash was filtered on a 0.22 μm membrane filter (Millipore), which was subjected to total community DNA extraction, using a Power Soil Microbial DNA extraction kit (MoBio). Sequencing was performed on the Genome Sequencer FLX system using 3 μg of DNA at a concentration of 17 ng μl^{-1} (as determined by a NanoDrop spectrophotometer). The resulting reads were annotated using the MG-RAST rapid annotation platform (Meyer *et al*., 2008). Using this platform, rhodopsin-containing reads were located within each of the compared metagenomes using an *e*-value cutoff of 10^{-5}. For the phylogenetic analysis, hits with higher *e*-values were included as well. The number of reads was normalized against the average number of selected single-copy genes found in the data sets using an *e*-value cut-off of 10^{-20}.

All non-phyllosphere datasets used are publicly available on the MG-RAST website. The soybean phyllosphere metagenome can be found in the GenBank SRA database. The rhodopsin-containing reads from the phyllosphere metagenomes are provided in Supporting material S1, S2, S3, S4 and S5 in *Supporting information*.

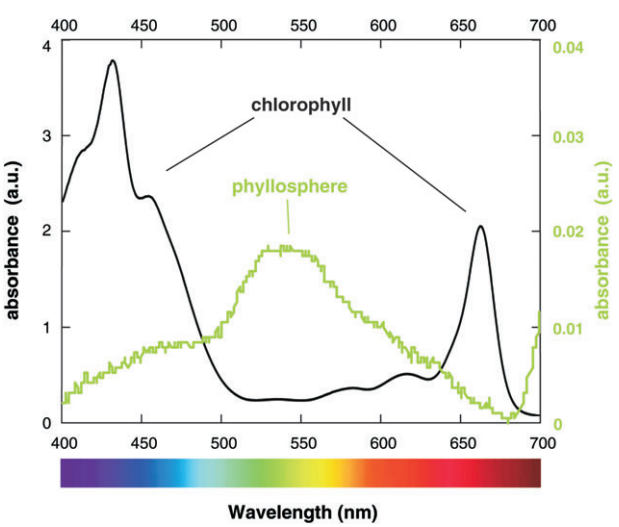

Fig. 5. Absorbance spectra of tamarisk leaves and phyllosphere wash. Absorbance of tamarisk chlorophylls (acetone extract) and of phyllosphere leaf buffer-wash are shown; note the different scales used.

Phylogenetic tree analysis

In this work, we tried several methods for multiple sequence alignment calculation [MUSCLE, ProbCons, MAFFT and PROMALS, see references within Kemena and Notredame (2009)]. In an effort to automatically identify the most reliable multiple sequence alignment for a given protein family, we used the AQUA protocol for automated quality improvement of multiple sequence alignments (Muller *et al*., 2010). We performed several alignments using MUSCLE, MAFFT, ProbCons, along with one refinement program (RASCAL) and one assessment program (NORMD). According to this protocol the MAFFT alignment refined by RASCAL produced the most reliable alignment (highest NORMD value) and was used to produce the phylogenetic tree. Following the alignment computation, we used FastTree version 2.1.1 SSE3 (Price *et al*., 2009) for the calculation of the phylogenetic tree using settings for high accuracy [-spr 4 (to increase the number of rounds of minimum-evolution SPR moves) and -mlacc 2 -slownni (to make the search for the most likely alternative topology more exhaustive)]. These parameters can produce slight increases in accuracy. To estimate the reliability of each branching point, FastTree uses a Shimodaire-Hasegawa test on the three alternative topologies (NNIs) around that split (Guindon *et al*., 2009). Phyllogenetic protein trees were visualized and edited using Dendroscope software version 2.7.3 (Huson *et al*., 2007).

To test if the phylogenetic signal we observe is statistically significant we used the Mesquite program (Maddison and Maddison, 2010). This was done using a randomization test (to see if the observed number of changes on the tree is less than 95% of the null values). The 10 000 reshufflings of the characters (five different plants and other environments) allowed constructing a character chart of parsimonious changes between the six characters assigned.

Relative abundance of microbial rhodopsins in different metagenomes

Frequency of rhodopsin blast hits with an *e*-value $\leq 10^{-5}$ was determined for 14 metagenomes from phyllosphere (5), marine (5), freshwater (1), hypersaline (1) and soil (2) environments. Rhodopsin abundance was normalized with the abundance of *rplA*, *rplC*, *rplD*, *rpoA*, *rpoB* and *rspJ* genes (Frank and Sorensen, 2011) (blast hits with an *e*-value $\leq$ 1e-20) according to Yutin and colleagues (2007) and Howard and colleagues (2008).

Metagenomic data sets used for comparison (Fig. 2)

Freshwater: GS020, Lake Gatun, Panama (MG_RAST accession: 4441590.3)
Hypersaline: GS033, Punta Cormorant hypersaline lagoon, Galapagos (MG-RAST accession: 4441599.3)
Open Sea: GS000a, Sargasso Station 11 (MG-RAST accession: 4441570.3) and GS000b, Sargasso Station 13 (MG-RAST accession: 4441573.3)
Estuary: Monterey Bay (MG-RAST accession: 4443712.3)
Whale Fall: Whale fall Bone (MG-RAST accession: 4441619.3)
Forest Soil: Luquillo experimental forest soil, Puerto Rico (MG-RAST accession: 4446153.3) and Waseca farm soil (MG-RAST accession: 4441091.3)
Soybean: SRA accession: SRX008324 (http://www.ncbi.nlm.nih.gov/sra/SRX008324?report=full)

Absorbance spectra of tamarisk leaves and phyllosphere wash (Fig. 5)

Phyllosphere absorbance was calculated as the difference between two measurements of reflectance spectra (intact tamarisk leaves and phosphate-buffered saline washed, sonicated leaves), obtained at room temperature with a Labsphere DRA-CA-30I diffuse reflectance accessory. Leaves were densely arranged on a slide and covered with another slide. Two empty slides were used as a blank. Measurements were performed on four different leaf samples from different dates. For chlorophyll absorbance, tamarisk leaves were ground with 90% acetone and filtered through GFF filters. The extract was measured using a Cary 100 spectrophotometer.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Supporting material S1. *Tamarix* phyllosphere microbial rhodopsin sequences.
Supporting material S2. Soybean phyllosphere microbial rhodopsin sequences.
Supporting material S3. Rice phyllosphere microbial rhodopsin sequences.
Supporting material S4. *Arabidopsis* phyllosphere microbial rhodopsin sequences.
Supporting material S5. Clover phyllosphere microbial rhodopsin sequences.

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