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SUMMARY

Chlorella viruses are a source of interesting membrane transport proteins. Here we examine a putative K$^+$ transporter encoded by virus FR483 and related chlorella viruses. The protein shares sequence and structural features with HAK/KUP/KT-like K$^+$ transporters from plants, bacteria and fungi. Yeast complementation assays and Rb$^+$ uptake experiments show that the viral protein, termed HAKCV (high-affinity K$^+$ transporter of chlorella virus), is functional, with transport characteristics that are similar to those of known K$^+$ transporters. Expression studies revealed that the protein is expressed as an early gene during viral replication, and proteomics data indicate that it is not packaged in the virion. The function of HAKCV is unclear, but the data refute the hypothesis that the transporter acts as a substitute for viral-encoded K$^+$ channels during virus infection.

Keywords: virus K$^+$ transporter, chlorella virus membrane proteins, HAK/KUP/KT-like potassium transporter, HAK5, viral K$^+$ channel Kcv.

INTRODUCTION

Chlorella viruses are members of a large, rapidly expanding genus (*Chlorovirus*, Family *Phycodnaviridae*) of plaque-forming dsDNA viruses that infect certain unicellular, exsymbiotic, chlorella-like green algae (Wilson *et al.*, 2009). The viruses have large genomes (290–370 bp), are structurally similar, have an internal membrane surrounded by a glycoprotein coat, and exhibit considerable diversity with regard to the proteins they encode. Chlorella viruses are present in fresh water all over the world, and titres as high as 100 000 infectious particles per millilitre of indigenous water have been reported (Van Etten, 2003; Yamada *et al.*, 2006). The chlorella viruses have three known hosts: viruses that infect *Chlorella* NC64A (recently renamed *Chlorella variabilis*; these viruses are called NC64A viruses), viruses that infect *Chlorella* SAG 3.83 (recently renamed *Chlorella heliozoae*; these viruses are called SAG viruses), and viruses that infect *Chlorella* Pbi (recently renamed *Micractinium conductrix*; these viruses are called Pbi viruses).

Collectively, the chlorella viruses encode many unexpected proteins, including several channel/transporter pro-

teins such as an aquaglyceroporin (Gazzarrini *et al.*, 2006), a calcium-transporting ATPase (Bonza *et al.*, 2010) and a small K$^+$ channel protein named Kcv. This Kcv channel is the subject of intensive investigation (e.g. Plugge *et al.*, 2000; Gazzarrini *et al.*, 2003; Kang *et al.*, 2004; Hertel *et al.*, 2010). Unexpectedly, one Pbi virus, FR483, does not encode a Kcv ortholog. Instead, FR483 encodes a putative K$^+$ transporter (Fitzgerald *et al.*, 2007a). The SAG virus *Acanthocystis turfacea* chlorella virus-1 (ATCV-1) encodes both a putative K$^+$ transporter protein and a Kcv protein (Fitzgerald *et al.*, 2007b).

The two putative K$^+$ transporters from viruses FR483 and ATCV-1 are orthologues of the HAK/KUP/KT-like K$^+$ transporters from plants, bacteria and fungi (Grabov, 2007; Gierth and Maser, 2007). No crystal structures have been reported for HAK/KUP/KT-like K$^+$ transporters, and so only limited information is available on their structure. Hydrophobicity plots of HAK/KUP/KT-like K$^+$ transporters suggest that they have 10–14 transmembrane (TM) domains, with a large hydrophylic loop between the second and third TM domains. The second TM domain contains the sequence

VFGD/IYGD, which is conserved among all HAK/KUP/KT-like K$^+$ transporters and is thought to act as the filter sequence (Rigas *et al.*, 2001). The number of subunits required to form a functional HAK/KUP/KT-like K$^+$ transporter is unknown.

HAK/KUP/KT-like K$^+$ transporters cannot discriminate between K$^+$ and Rb$^+$. Consequently, Rb$^+$ is an important tool for characterizing the transport properties of these proteins (Kim *et al.*, 1998; Rigas *et al.*, 2001). The general picture that emerges from these studies is that HAK/KUP/KT-like K$^+$ transporters preferentially transport K$^+$. Like K$^+$ channels, they also often transport Cs$^+$ (Zhu and Smolders, 2000), but do not transport NH$_4^+$ and are even inhibited by NH$_4^+$ (Rodríguez-Navarro and Rubio, 2006). Na$^+$ is also transported by HAK/KUP/KT-like K$^+$ transporters, albeit with low affinity (Rodríguez-Navarro and Rubio, 2006). HAK/KUP/KT-like K$^+$ transporters facilitate K$^+$ transport by symport with H$^+$ (Szczerba *et al.*, 2009).

Unlike K$^+$ channels, HAK/KUP/KT-like K$^+$ transporters are not encoded by all organisms. They are present in plants (e.g. tiny root hair-1 K$^+$ transporter TRH1 in *Arabidopsis thaliana*; Rigas *et al.*, 2001), fungi (e.g. high-affinity K$^+$ transporter 1 HAK1 in yeast; Banuelos *et al.*, 1995; Ramos *et al.*, 2011) and bacteria (e.g. K$^+$ uptake protein 1 KUP1; Schleyer and Bakker, 1993). To date, no HAK/KUP/KT-like K$^+$ transporters have been reported in animals. This paper describes the first functional characterization of a virus-encoded K$^+$ transporter protein.

RESULTS

Sequence and predicted structure of the putative viral K$^+$ transporter

Annotation of the Pbi virus FR483 genome (Fitzgerald *et al.*, 2007a) revealed a 660 amino acid protein-encoding sequence N110R, which has 37% amino acid identity to HAK/KUP/KT-like K$^+$ transporters TRH1 and HAK5 from *A. thaliana* (Rigas *et al.*, 2001) (Figure 1, the alignment was performed using ClustalW, http://www.ebi.ac.uk/Tools/msa/clustalw2/. The TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM/) predicts that N110R has at least 12 TM domains. The HAK5 transporter, which is 125 amino acids longer than N110R, also has 12 predicted TM domains. Both proteins are predicted to contain a large loop between the second and third TM domains and a long cytoplasmic C-terminus. The main difference between the two proteins is the length of the C-terminus: that of HAK5 is approximately 100 amino acids longer.

Recent sequencing of 38 additional chlorella viruses revealed six more virus-encoded HAK/KUP/KT-like K$^+$ transporter orthologues (J. L. Van Etten *et al.*, unpublished results): one in a Pbi virus and five in SAG viruses (Table 1). The occurrence of K$^+$ transporters (in eight viruses) and K$^+$ channel proteins (in 37 viruses) among the three groups of chlorella viruses can be summarized as

follows: (i) NC64A viruses only encode K$^+$ channels, no K$^+$ transporter encoding genes were detected in the 16 NC64A viruses sequenced to date, (ii) all SAG viruses encode a K$^+$ channel and six of the 13 SAG viruses also encode a K$^+$ transporter, and (iii) two Pbi viruses encode a K$^+$ transporter (FR483 and NW665.2), and all encode a K$^+$ channel protein except for FR483.

Phylogenetic analysis of the viral K$^+$ transporter

To examine the origin and evolution of the putative viral transporter N110R, we constructed a phylogenetic tree of HAK/KUP/KT-like K$^+$ transporters from plants, fungi, eubacteria, archaea and the green alga *Chlamydomonas reinhardtii*. The phylogenetic tree was obtained by the neighbour-joining method (Figure 2), and shows a clear separation between a prokaryal group and a eukaryal group. The prokaryal transporters further separate into eubacterial and archaeal branches. The eukaryal branch separates into plant and fungal branches. The viral transporters are clearly separated from the other organisms. The same results were obtained by Bayesian estimate of phylogenies (data not shown). The results of these phylogenetic analyses suggest that it is unlikely that the viral transporter genes were recently obtained from their *Chlorella* hosts. However, this interpretation is speculative as genomic data are not available for the viral hosts *Chlorella* Pbi and *Chlorella* SAG 3.83. A BLAST search against the genome of the related and fully sequenced *Chlorella* NC64A (Blanc *et al.*, 2010) revealed a small 282 amino acid protein (fgenesh3_pg.C_scaffold_30000010) with slight resemblance to N110R (Figure 3). However, the *Chlorella* NC64A protein is only predicted to have five TM domains (data not shown). Therefore, we believe that it is unlikely that this protein is a HAK/KUP/KT-like K$^+$ transporter. These results support the conclusion, based on the phylogenetic tree, that the *n110r* gene was probably not acquired from its host.

To examine the distribution of the transporter gene among the chlorella viruses, we prepared a phylogenetic tree based on the DNA polymerase genes from all the chlorella viruses, using other members of the Phycodnaviridae, namely *Ostreococcus tauri* virus 5 (OsV5), *Emiliana huxleyi* virus 86 (EhV86) and *Ectocarpus siliculosus* virus 1 (EsV-1) as outgroups (Figure 4). The DNA polymerase gene is a useful genetic marker in studies on phycodnaviruses because all members contain this gene (Chen and Suttle, 1995; Chen *et al.*, 1996; Hanson *et al.*, 2006). The three chlorella virus groups are clearly separated. The K$^+$ transporter protein occurs in several viruses. However, the transporter is not virus species-specific or related to a particular host.

Functional characterization of N110R in heterologous expression systems

To test the putative K$^+$ transporter N110R for functionality, we used the $\Delta trk1 \, \Delta trk2$ mutant of *Saccharomyces cerevisiae*

```
HAK5    MDGEEHQIDGDEVNNHENKLNEKKKSWGKLYRPDSFIIEAGQTPTNTGRRSLMSWRTTMS 60
N110R   ---------------------------MSETGVVTIEQEEKILELGRKNIRGWS-LVI 30
                                   : .... . ** :.  :  **:.:  .*   :

HAK5    LAFQSLGVVYGDIGTSPLYVYASTFTDGINDKDD--VVGVLSLIIYTITLVALLKYVFIV 118
N110R   LSLASLGVVFGDIGTSPLYVLPAIFGELRHQPTENFILGVFSTIFWTITLMVLVKYVWFT 90
        *:: *****:********** .: * :  :: :  ::**:* *::****:.*:***::.

HAK5    LQANDNGEGGTFALYSLICRYAKMGLIPNQEPEDVELSNYTLELPTTQLRRAHMIKEKLE 178
N110R   LAIDDHGEGGVFALYSIIRRAITS-----KPSDFGVDTQEEKIPS-------KTKDFLE 137
        *  :*:****.*****:* *  .       :*.*. :..  ::*:         *: **

HAK5    NSKFAKIILFLVTIMGTSMVIGDGILTPSISVLSAVSGIKSLG---QNTVVGVSVAILIV 235
N110R   NNKWARKVIMGIVITCASLTMADGILTPSISVISATEGIQFHTGISHDTVIFITIGILVG 197
        *.*:*: ::: :.*  :*:.:.**********:**.:.**:    ::**: :::.**:

HAK5    LFAFQRFGTDKVGFSFAPIILVWFTFLIGIGLFNLFKHDITVLKALNPLYIIYYFRRTG- 294
N110R   LFSIQFLGTGKVGVIFGPTMLVWFVNLSVGVYNVTKMPG-VFRAFSPHYMYYFWEEFGS 256
        **:: *  :**.***. *.*  ***** *. .:*::*: *    *:*::.* *: *:::. *

HAK5    RQGWISLGGVFLCITGTEAMFADLGHFSVRAVQISFSCVAYPALVTIYCGQAAYLTKHTY 354
N110R   WEAFKLLGEVFLAITGVEALYADMGHLNAMSIRISFSAIVYPSLVMNYLGQTAVVLLDYN 316
         :.:   ** ***.***.**::**:**:..  :::****.:.**:**   * **:* :  .

HAK5    NVSNTFYDSIPDPLYWPTFVVAVAASIIASQAMISGAFSVISQSLRMGCFPRVKVVHTSA 414
N110R   TSSSLYWSSIPAKLAWPSLAIAASAAVIASQALITGTFTIVQQAMHANVFPRVAIFQTNK 376
        . *. ::.***   * **::..*.:.*:*:*****.*:*:*:::.*::: . ****  ..*.

HAK5    KYEGQVYIPEINYLLMLACIAVTLAFRTTEKIGHAYGIAVVTVMVITTLMVTLIMLVIWK 474
N110R   KHAGQIYIPVVNFALLVGSISVVLIFQSSSKIVSAYGFAVSIVVVLTHIFFCIVLHIQGR 436
        *: **:***  :*: *::..*:*.* *:::.**  ***:** *.*:* *:.:. *

HAK5    TNIVWIAIFLVVFGSIEMLYLSSVMYKFTSGGYLPLTITVVLMAMMAIWQYVHVLKYRYE 534
N110R   -NKLFSFVFSSFFGVISIAFAASLTIKIPKGAWFSAAIGSALIFVSLVWHRGHRMKVRYI 495
         * ::  :*  .** *.: : :*:  *:...*.::. :* .:: :  :*:  * :* **

HAK5    LREKISRENAIQMATSPDVNRVPGIGLFYTELVNGITPLFSHYISNLSSVHSVFVLISIK 594
N110R   KINRLSARQVFSKPSNNSKN-----IVFYNELTDGIVPAYNQLENLITISGTNNIVLSVR 550
         :::*  .:.:. .:.  .  *       :**.**.:**.* :.:  : :   : :::*::

HAK5    TLPVNRVTSSERFFFRYVGPKDSGMFRCVVRYGYKEDIEEPDEFERHFVYYLKEFIHHEH 654
N110R   KMTIPRVREDQRFLITGYD----GVYHVVARYGYAEIIDHGNCFARKLCQAVN------- 599
        .:.: **  ..:**:.. *:     *::: *.**** * *:.: * * *::  :

HAK5    FMSGGGGEVDETDKEEEPNAETTVVPSSNYVPSSGRIGSAHSSSSDKIRSGRVVQVQSVE 714
N110R   ----------------------------------------------------------

HAK5    DQTELVEKAREKGMVYLMGETEITAEKESSLFKKFIVNHAYNFLKKNCREGDKALAIPRS 774
N110R   --------AESSDVVFVMGRTKLLTTNTS--FYNKAVIAMYSLLVKLSSWTTDTFNTPTS 649
                *....:*::**.*:: : * *  *: *   *.:* * .   .:: * *

HAK5    KLLKVGMTYEL 785
N110R   KLIIFEASYEI 660
        **: . :**:
```

Figure 1. Alignment of the putative K⁺ transporter HAKCV encoded by coding sequence N110R of chlorella virus FR483 with that of high-affinity K⁺ transporter 5 (HAK5) from *A. thaliana*.
Transmembrane domains of the two proteins predicted using the TMHMM algorithm are highlighted in grey.

Table 1 Predicted HAK/KUP/KT-like K⁺ transporter proteins encoded by chlorella viruses

Virus	Host	Length (amino acids)	ORF
FR483	*Chlorella* Pbi	660	N110R
NW665.2	*Chlorella* Pbi	660	R1M11_755R
TN603	*Chlorella* SAG 3.83	644	R1M1_2359R
Br0604L	*Chlorella* SAG 3.83	644	R2M1_1283L
MN0810.1	*Chlorella* SAG 3.83	644	R2M5_1262R
GM0701.1	*Chlorella* SAG 3.83	676	R2M4_1084R
OR0704.3	*Chlorella* SAG 3.83	644	R2M10_35R
ATCV-1	*Chlorella* SAG 3.83	644	Z696R

as an expression system. This yeast strain lacks endogenous K⁺ uptake systems (Minor *et al.*, 1999) and is unable to grow on medium containing low K⁺ (Ko and Gaber, 1991). We

transformed yeasts with either the functional K⁺ channel Kcv from chlorella virus *Paramecium bursaria* chlorella virus-1 (PBCV-1) (Chatelain *et al.*, 2009; Gebhardt *et al.*, 2011) as a positive control, empty vector pYES2 as a negative control, or *n110r*. The yeast cells were grown in liquid transformation medium, washed twice with water, and diluted to an optical density of 600 nm (OD_{600}) of 1. Cells were spotted on a non-selective plate containing 100 mM K⁺ to test whether the protein is toxic for the yeasts. Cells were also spotted on selective plates containing 1 or 0.5 mM K⁺ to test for functionality. Subsequent dilutions were performed to avoid artifactual growth. All yeasts grew on the 100 mM K⁺ control plate at all dilutions (Figure 5a). Hence none of the expressed proteins is deleterious to the cells. Cells expressing *n110r* and the viral K⁺ channel Kcv$_{PBCV-1}$ grew at all dilutions on the selective plates, but yeast transformed with the
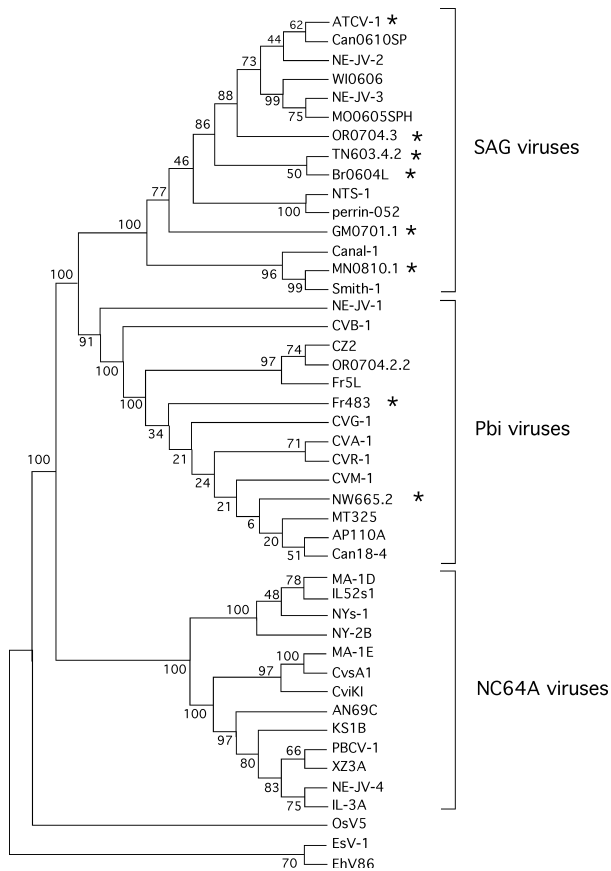
Figure 2. Phylogenetic tree of HAK/KUP/KT-like K$^+$ transporters.
Phylogenetic tree of HAK/KUP/KT-like K$^+$ transporters from plants, fungi, algae (*Chlamydomonas reinhardtii*), Archaea, bacteria and viruses. The tree was generated by the neighbour-joining method using MEGA4 (Tamura *et al.*, 2007).

```
HAKCV-1    ------MSETGVVTIEQEEKILELGRKNIRG--WSLVILSLASLGVVFGDIGTSPLYVLP 52
CNP        MAGQLTVSSLWAHDADLQKQVEEAHRKRMGVGTWSLLAMAFSTLGIVYGDIGTSPLYVFA 60
             :*.  .   :  : ::::  *  **.:    ***: :::::**:*:**********:.

HAKCV-1    AIFGELRHQPTENFILGVFSTIFWTITLMVLVKYVWFTLAIDDHGEGGVFALYSIIRRAI 112
CNP        SIFPDG--PPSAEVTLGAASTIFWSITGIVVVKYIVFTLQADDNGEGGIFALYALLCRAV 118
            :** :     *: :. **. *****:** :*:***: ***  **:****:****::: **:

HAKCV-1    TSK----------------------------PSDFGVDTQ---------EEKIPSKT 132
CNP        SIRSGSLLHEADLSLSQYQAPDPPAQARASPSPPHTSGAGTCGGPGAAYTRWRQSVVARA 178
            : :                           *   *..*         .:.: :::

HAKCV-1    KDFLENNKWARKVIMGIVITCASLTMADGILTPSISVISATEGIQFHTGISHDTVIFITI 192
CNP        RASLEGSRAAQGILLAVVLLAANMILSDGVLTPAISVVSAVEGIEYQTGISRGTVVGIAV 238
            :  **..: *: :::.:*: .*.: ::**:***:***:**.***::::****:.**: *::

HAKCV-1    GILVGLFSIQFLGTGKVGVIFGPTMLVWFVFNLSVGVYNVTKMPGVFRAFSPHYMYYFWE 252
CNP        GILVCLFAVQPWGTQRVAVMFSPLVFLWFAS---------------------------- 269
            **** **::*   ** :*.*:*.* :::**.

HAKCV-1    EFGSWEAFKLLGEVFLAITGVEALYADMGHLNAMSIRISFSAIVYPSLVMNYLGQTAV// 660
CNP        ----------------------------LSGIGEALGLAA----------------- 281
                                        *..:.  :.::*
```

Figure 3. Alignment of N110R encoded by virus FR483 with an uncharacterized protein coded by *Chlorella* NC64A (CNP).
The alignment was performed using ClustalW. Asterisks and colons indicate identical and conserved residues, respectively.

empty vector did not. The results of these experiments suggest that N110R is functional and complements the K$^+$ uptake defect of the yeast mutants.

For a more quantitative analysis, the experiment was repeated in liquid medium containing 0.5 mM K$^+$. Cell growth was monitored at OD_{600} over 72 h. The OD_{600} value was normalized to the value measured at $t = 0$; the

data are reported as the relative increase in OD_{600} over the starting value as a function of time. Cells expressing *n110r* and the positive control Kcv_{PBCV-1} grew similarly, but yeast transformed with the empty vector (negative control) did not (Figure 5b). These results indicate that *n110r* complements the deficiency in K$^+$ uptake in the mutant yeast, and the efficiency of complementation is
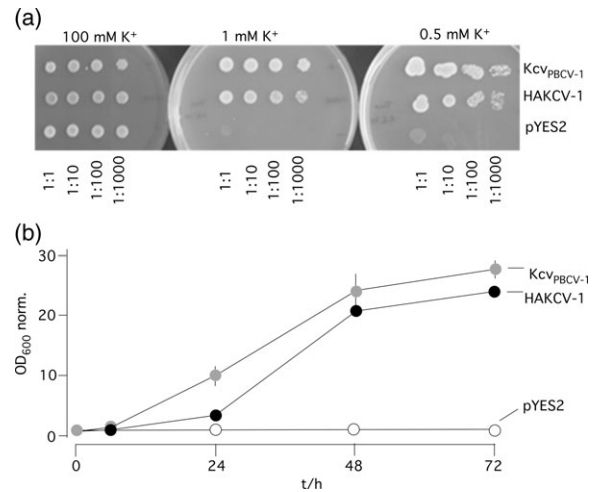
Figure 5. The putative K+ transporter N110R from virus FR483 rescues a Δ*trk1* Δ*trk2* mutant of *S. cerevisiae*.
(a) Complementation of a Δ*trk1* Δ*trk2* mutant of *S. cerevisiae* using Kcv$_{PBCV-1}$ (positive control), N110R and empty vector (negative control). The transformed yeasts were grown under non-selective conditions (100 mM K+) or selective conditions (1 and 0.5 mM K+). Subsequent dilutions (1:1–1:1000) were used to avoid artifactual growth.
(b) Growth of a Δ*trk1* Δ*trk2* mutant of *S. cerevisiae* carrying the *n110r* gene, Kcv$_{PBCV-1}$ or the empty vector pYES2. Cells were grown in liquid 0.5 mM K+ medium lacking uracil and methionine for 3 days (30°C, 220 rpm). OD$_{600}$ was determined between 0 and 72 h after inoculation.

Figure 4. HAK/KUP/KT-like K+ transporting proteins in chlorella viruses. The tree was generated using the DNA polymerase genes of all fully sequenced chlorella viruses plus the polymerase genes from phycodnaviruses *Ostreococcus tauri* virus 5 (OsV5), *Emiliana huxleyi* virus 86 (EhV86) and *Ectocarpus siliculosus* virus 1 (EsV-1) as outgroups. The viruses indicated by asterisks encode HAK/KUP/KT-like K+ transporting proteins. The tree was generated by the neighbour-joining method using MEGA4 (Tamura *et al.*, 2007).

similar to that of the viral K+ channel. N110R was given the name HAKCV$_{FR483}$ (high-affinity K+ transporter from chlorella virus FR483).

HAKCV$_{FR483}$ transports Rb+

HAK/KUP/KT-like K+ transporters cannot distinguish between K+ and Rb+; Rb+ flux experiments can therefore be used to monitor the function of these proteins (Kim *et al.*, 1998; Rigas *et al.*, 2001; Rodriguez-Navarro and Ramos, 1984; Rodríguez-Navarro and Rubio, 2006). We performed uptake experiments with Rb+ to examine the kinetics and mechanisms of HAKCV$_{FR483}$ transport. Yeast cells were transformed with either *hakcv*$_{FR483}$ or empty vector pYES2 as a negative control, and grown in synthetic medium containing 50 mM K+. At time zero, 50 mM RbCl was added to the medium and Rb+ uptake into the cells was monitored. The same experiment was repeated with cells grown in 50 mM K+ synthetic medium and then K+-starved for 2 h in

K+-free synthetic medium, in order to show that the control strain did hardly transport Rb+ at low millimolar concentrations, and that enhanced Rb+ uptake is due to the expressed protein rather than stress-induced up-regulation of endogenous transport proteins (Ramos *et al.*, 1985). After 2 h, 0.5 mM RbCl was added to the medium and Rb+ uptake was monitored. In both experiments, the amount of Rb+ inside the cells increased, showing that the cells took up Rb+ (Figure 6a,b). The cells expressing HAKCV$_{FR483}$ took up approximately three or four times more Rb+ than the empty vector control. These results confirm that HAKCV$_{FR483}$ is a functional K+ transporter.

To examine the transport mechanisms of HAKCV$_{FR483}$ in more detail, the *hakcv*$_{FR483}$ gene was expressed in *Xenopus laevis* oocytes. No specific current was detected by changing the K+ concentration in the external buffer. Based on the assumption that the protein is functionally expressed in the plasma membrane of the oocyte, these experimental results suggest that the transporter is electroneutral (data not shown).

Expression of hakcv during virus replication

Northern hybridization experiments were performed to determine when FR483 and SAG virus ATCV-1 express *hakcv* genes. Probes for the *hakcv* genes *n110r* (FR483) and *z696r* (ATCV-1) were constructed and hybridized to RNA extracted at various times from virus-infected *Chlorella* Pbi and
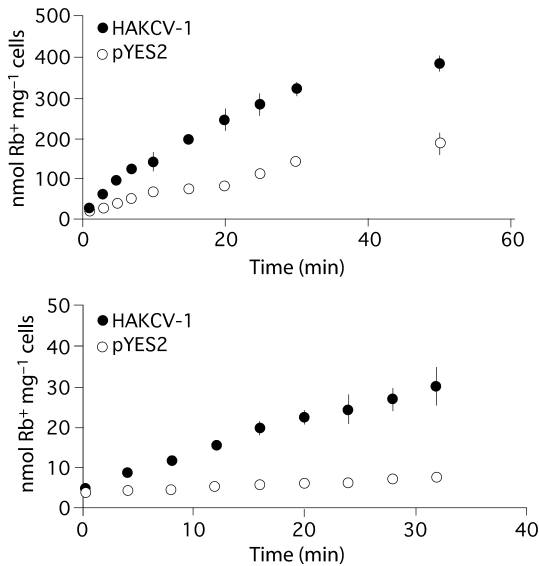
Figure 6. The putative K$^+$ transporter N110R from virus FR483 enhances Rb$^+$ uptake.
(a) *S. cerevisiae* cells transfected with either the empty vector pYES2 or *n110r* were grown in 50 mM K$^+$ synthetic medium. At time zero, 50 mM Rb$^+$ was added to the medium.
(b) Yeast cells transfected with either the empty vector pYES2 or *n110r* were grown in 50 mM K$^+$ synthetic medium and then starved in K$^+$-free synthetic medium for 2 h. Following to this preincubation, 0.5 mM Rb$^+$ was added to the medium at time zero.

Chlorella SAG 3.83, respectively. Northern blots (Figure 7) indicate that a strong transient transcript of approximately 2000 bp appears for both viral-infected algae; the transcripts are the expected size for the 660 and 645 amino acid K$^+$ transporter proteins from the FR483 and ATCV-1 viruses, respectively. For FR483, the signal appears at 20 min post-infection, with the strongest signal at 45 min post-infection, followed by a decrease (Figure 7a). Expression of *hakcv* in virus ATCV-1 is similar, appearing at 20 min, peaking at 30 min and disappearing after 90 min (Figure 7b). Studies with the prototype chlorella virus PBCV-1 have established that virus DNA replication begins 60–90 min post-infection (Van Etten *et al.*, 1984). Genes expressed before virus DNA replication are classified as early genes. Assuming that viruses FR483 and ATCV-1 have similar replication cycles to PBCV-1, the *hakcv* genes are classified as early genes.

DISCUSSION

Collectively, the results presented here show that the 660 amino acid protein N110R coded by Pbi virus FR483 is a functional K$^+$ transporter, named HAKCV$_{FR483}$. Functionality was established by both yeast complementation assays and Rb$^+$ flux experiments. HAKCV$_{FR483}$ is an ortholog of the HAK/KUP/KT-like K$^+$ transporters found in all organisms except for animals (Corratge-Faillie *et al.*, 2010). HAKCV$_{FR483}$ is predicted to have 12 TM domains, with a large insert between the second and the third TM domains. Thus, in

addition to sequence similarity, the viral protein has the same predicted structural architecture as HAK/KUP/KT-like K$^+$ transporters. Also like the other HAK/KUP/KT-like K$^+$ transporters, the viral protein contains a conserved putative filter sequence, VFGD, in the second TM domain (Rigas *et al.*, 2001). This VFGD sequence resembles the K$^+$ channel filter sequence TxxTxGYGD, and is a good candidate for future mutational studies on functionality and selectivity of the protein.

The results from the flux experiments indicate that cells transformed with HAKCV$_{FR483}$ have three to four times higher Rb$^+$ transport rates than negative controls. The experiment with K$^+$-starved cells shows that the uptake of Rb$^+$ is due to the expressed protein and is not the result of an up-regulated endogenous transport protein. Comparable Rb$^+$ uptake was also reported for yeast cells expressing other HAK/KUP/KT-like K$^+$ transporters, e.g. HAK1 from barley (Figure 4 in Santa-Maria *et al.*, 1997) and HAK1 from the yeast *Debaryomyces hansenii* (Figure 5 in Martínez *et al.*, 2011). These results indicate that the viral transporter has a transport capacity that is comparable to that of related transporters.

It is interesting that the chlorella viruses encode proteins that are associated with K$^+$ transport or K$^+$ channel activity, including a small K$^+$ channel protein named Kcv that is encoded by most chlorella viruses (e.g. Plugge *et al.*, 2000; Gazzarrini *et al.*, 2003, 2009; Kang *et al.*, 2004). The fact that all the chlorella viruses encode either a channel protein or a transporter protein and some encode both suggests that potassium regulation must be important in replication of the viruses. Our current hypothesis on the biological role of Kcv is that Kcv is packaged in the internal membrane of the virus particle. During infection, the virus membrane fuses with the host plasma membrane and the large conductance of the Kcv channel results in depolarization of the host plasma membrane (Frohns *et al.*, 2006) and loss of K$^+$ from the host (Neupärtl *et al.*, 2008). This process reduces the turgor pressure of the host and eases ejection of the virus genome into the alga cell (Thiel *et al.*, 2010). Several experimental results are consistent with this model.

FR483 is the only chlorella virus that lacks a gene encoding a Kcv channel (Fitzgerald *et al.*, 2007a). Thus, it is reasonable to consider the possibility that HAKCV$_{FR483}$ substitutes for Kcv's role in virus replication. However, this explanation is unlikely for several reasons. First, proteomic analyses of FR483 failed to detect HAKCV$_{FR483}$ in the virion (Dunigan, D.D., Cerny, R., and Van Etten, J.L., unpublished results). However, it is possible that the protein is not detected due to low abundance. Second, the *hakcv* gene is expressed early during replication of both FR483 and ATCV-1 viruses, whereas *kcv* genes are expressed late. Typically, genes that are expressed early in viral replication cycles do not encode proteins that are packaged in virions. Third, six SAG viruses encode both K$^+$ transporters and channels, suggesting that they serve different functions.
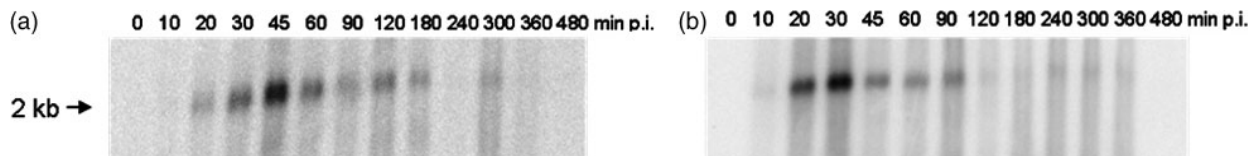
Figure 7. The viral-encoded K+ transporter genes are expressed as early genes during virus replication.
Northern hybridization of potassium transporter genes encoded by Pbi virus FR483 (a) and SAG virus ATCV-1 (b).

Collectively, therefore, the data suggest that HAKCV transporters do not substitute for K+ channels. The fact that only eight of the 43 chlorella viruses that have been sequenced have a *hakcv* gene suggests that the protein may not be essential for virus replication. Currently, we can only speculate about the function of the K+ transporter. As *hakcv* is expressed early, we assume that it is used for manipulating the host, e.g. by changing the ionic environment within the cell. Transporters generally have a higher affinity for K+ than channels, and transport this cation using the energy provided by another ion against its gradient. These properties support a hypothesis that accumulation of K+ is beneficial at some stage of viral replication. As K+ transport by HAK/KUP/KT transporters is coupled to H+ transport (Grabov, 2007), modulation of the host cell pH may also be relevant.

Phylogenetic comparison of HAKCV with HAK/KUP/KT-like K+ transporter proteins from other organisms indicates that viral transporters make up their own clade that is clearly distinct from the fungal and plant clades (Figure 2). The results indicate that the various clades came from a common ancestor but that this probably occurred a very long time ago. It is unlikely that the viral HAKCV genes were captured from their host algae because the HAK/KUP/KT-like K+ transporter protein encoded by the green alga *C. reinhardtii* clearly falls into the plant lineage. However, this interpretation is speculative as genomic data are not available for the viral hosts *Chlorella* Pbi and *Chlorella* SAG 3.83. A BLAST search using HAKCV$_{FR483}$ against the related and fully sequenced *Chlorella* NC64A alga (Blanc *et al.*, 2010) revealed a small 282 amino acid protein with slight resemblance to N110R (Figure 3). However, the *Chlorella* NC64A protein is only predicted to have five TMs. Therefore, it is unlikely that this protein is a HAK/KUP/KT-like K+ transporter. These data support the interpretation of the phylogenetic tree in that the *n110r* gene was probably not acquired from its host, at least not recently.

Only two of the three chlorella virus groups, namely Pbi viruses and SAG viruses, encode a HAKCV ortholog. The phylogenetic tree based on the viral DNA polymerase gene in Figure 4 suggests that the common ancestor of Pbi viruses and SAG viruses possessed the *hakcv* gene, and that repeated loss occurred mostly within the Pbi viruses. The repeated loss of a gene is more likely than repeated acquisition of the gene, in accordance with Dollo's law of irreversibility (Dollo, 1893; Collin and Miglietta, 2008).

In summary, unexpected genes encoded by chlorella viruses continue to be discovered; often the genes are only present in a few of the viruses, even those that infect the same host. The unexpected gene products are usually functional, suggesting that they play an unknown role in viral replication cycles. HAKCV, which is the first-described K+ transporter protein encoded by a virus, is the latest such example. There is increasing evidence that viruses modify the ion homeostasis of their hosts via virus-coded channels (e.g. Hyser *et al.*, 2010). The discovery of new viral transport proteins such as HAKCV provides new molecular tools to understand the complex strategies that viruses use to efficiently infect and replicate in their host cells.

EXPERIMENTAL PROCEDURES

Isolation and cloning of the *n110r* gene

PCR using Phusion DNA polymerase (New England Biolabs, http://www.neb.com) was performed to amplify the *n110r* gene from chlorella virus FR483, using primers containing specific restriction sites. A diluted virus suspension was directly added to the PCR mixture as template, and the reaction produced a single transcript of approximately 2000 bp. For expression in yeast, the *n110r* gene was cloned into a modified version of the expression vector pYES2/CT (Minor *et al.*, 1999) at *Bam*HI and *Xho*I sites using primers *n110r*-BamHI-for (5′-TATGGATCCATGTCTGAAACAGGAGTT-3′) and *n110r*-Xhol-rev (5′-TATCTCGAGTCAAATTTCATAGGATGC-3′).

Yeast complementation assays

Selection experiments were performed as described previously (Minor *et al.*, 1999; Balss *et al.*, 2008). The *n110r* construct was transformed into *S. cerevisiae* strain SGY1528 (MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 trk1::HIS3 trk2::TRP1) (Tang *et al.*, 1995), which lacks endogenous K+ uptake systems. Yeasts from the same stock were grown in parallel under non-selective conditions on plates containing 100 mM KCl, and under selective conditions on agar containing 1 or 0.5 mM KCl. Growth experiments were performed at 30°C for 2–3 days. The experiment was repeated in liquid culture. Cells were grown in liquid 0.5 mM K+ medium lacking uracil and methione for 3 days (30°C, 220 rpm). OD$_{600}$ was determined between 0 and 72 h after inoculation.

Rb+ uptake experiments

Two experiments were performed. In the first experiment, yeasts transformed with either the *n110r* gene or empty vector pYES2 were grown in 50 mM K+ synthetic medium (1.75 g/L Yeast Nitrogen Base (YNB)) without amino acids, ForMedium™ UK, http://www.formedium.com; the pH was adjusted to 5.8 with ammonium hydroxide

Table 2 Similarity of viral K$^+$ transporters to corresponding proteins from other organisms

Organism	Domain	Accession no.	% similarity to N110R
ATCV-1	Virus	YP_001427177	55
TN603	Virus	ORF Y68_050R	57
Arabidopsis lyrata subsp. *lyrata*	Plant	XP_002863130	36
Arabidopsis thaliana (HAK5)	Plant	NP_567404	36
Populus trichocarpa	Plant	XP_002303014	34
Arabidopsis thaliana (TRH1)	Plant	NP_194095.2	35
Zea mays	Plant	NP_001148930	37
Ricinus communis	Plant	XP_002521896	32
Thellungiella halophila	Plant	ABO76902	35
Phytolacca acinosa	Plant	AAX13997	36
Vitis vinifera	Plant	XP_002264560	34
Oryza sativa Japonica Group	Plant	NP_001045298	34
Phragmites australis	Plant	BAE93348	35
Mesembryanthemum crystallinum	Plant	AAK53758	35
Physcomitrella patens subsp. *patens*	Plant	XP_001773728	35
Chlamydomonas reinhardtii	Green alga	XP_001700451	35
Botryotinia fuckeliana B05.10	Fungus	XP_001554184	32
Talaromyces stipitatus ATCC 10500	Fungus	XP_002482094	32
Magnaporthe oryzae 70-15	Fungus	XP_365422	32
Arthroderma otae CBS 113480	Fungus	XP_002847150	30
Aspergillus oryzae RIB40	Fungus	XP_001821738	29
Aspergillus clavatus NRRL 1	Fungus	XP_001273808	29
Aspergillus niger	Fungus	XP_001399699	30
Aspergillus flavus NRRL3357	Fungus	XP_002379685	30
Neurospora crassa OR74A	Fungus	XP_964946	33
Methanosarcina acetivorans C2A	Archaea	NP_618072	30
Methanosarcina barkeri str. Fusaro	Archaea	YP_306942	31
Candidatus *Methanoregula boonei* 6A8	Archaea	YP_001404526	34
Candidatus *Methanosphaerula palustris* E1-9c	Archaea	YP_002466744	31
Methanospirillum hungatei JF-1	Archaea	YP_502350	30
Chlorobium phaeobacteroides DSM 266	Bacteria	YP_912218	34
Thiobacillus denitrificans ATCC 25259	Bacteria	YP_315823	32
Chlorobium limicola DSM 245	Bacteria	YP_001943875	33
Aromatoleum aromaticum EbN1	Bacteria	YP_159072	32
Burkholderia thailandensis E264	Bacteria	ZP_05587240	33
Chlorobaculum parvum NCIB 8327	Bacteria	YP_001999537	33
Bdellovibrio bacteriovorus HD100	Bacteria	NP_968854	32
Laribacter hongkongensis HLHK9	Bacteria	YP_002794126	32
Desulfovibrio magneticus RS-1	Bacteria	YP_002952960	33

solution before autoclaving), to an OD_{600} of approximately 0.3 at 28°C; 50 mM RbCl was then added to the medium (time zero) and Rb$^+$ uptake was monitored. In the second experiment, yeasts transformed with either the *n110r* gene or empty vector pYES2 were grown in 50 mM K$^+$ synthetic medium and then starved for 2 h in K$^+$-free starvation medium (1.75 g/L YNB without amino acids, ForMedium™ UK, CYN7505; the pH was adjusted to 5.8 with ammonium hydroxide solution before autoclaving) at 28°C. After 2 h, 0.5 mM RbCl was added to the medium and Rb$^+$ uptake was monitored. Rb$^+$ was quantified by atomic emission spectrophotometry (Rodriguez-Navarro and Ramos, 1984). Both experiments were performed three times. Values are means ± SD.

Northern blot hybridization

Chlorella Pbi cells (2×10^9) or *Chlorella* SAG 3.83 cells (3×10^9) were collected at intervals from 0 to 480 min post-infection with FR483 (multiplicity of infection = 3) or ATCV-1 (multiplicity of infection = 5), respectively. Cells were frozen in liquid nitrogen

and stored at −80°C. RNA was extracted using TRIzol reagent (Invitrogen, http://www.invitrogen.com/), denatured using formaldehyde, separated on a 1.5% denaturing formaldehyde/agarose gel, and then transferred to nylon membrane. Full-length probes for the *hakcv* genes *n110r* (FR483) and *z696r* (ATCV-1) were amplified by PCR. Probes were labelled with ^{32}P-dATP using an Invitrogen random primers DNA labelling kit. The membrane was pre-hybridized in 10 ml Church buffer (1% BSA, 1 mM EDTA, 0.5 M NaPO$_4$, 7% SDS, pH 7.2) for 2 h at 65°C, and hybridized in Church buffer and the probe was denatured for 16 h at 65°C. After hybridization, the membrane was washed four times for 3 min at 50°C with $0.1 \times$ SSC, 0.1% SDS. Signal detection was performed using a Storm Phosphorimager and ImageQuant software (Molecular Dynamics Inc., http://www.gelifesciences.com).

Chlorella cell culture and virus purification

Chlorella Pbi was grown in FES medium (Reisser *et al.*, 1986). Virus FR483 was produced and purified as described for NC64A virus

PBCV-1 (Van Etten *et al.*, 1983). *Chlorella* SAG 3.83 was grown in MBBM medium (Bubeck and Pfitzner, 2005). Virus ATCV-1 was purified as described previously (Bubeck and Pfitzner, 2005).

Phylogeny

A BLASTP search using the N110R amino acid sequence (accession number YP_001425742) was performed using the NCBI non-redundant protein sequences database (http://blast.ncbi.nlm.nih.gov/) with default settings. The sequence was BLAST searched against genomes of plants, eubacteria, archaea, fungi, animals and viruses. Proteins with significant E-values ($<e^{-50}$) were used for the phylogenetic analyses and are listed in Table 2.

Phylogenetic trees were constructed by the neighbour-joining method using MEGA4 (Kumar *et al.* 2008) and by Bayesian inference run for 10^7 generations using MRBAYES version 3.1.2 (Huelsenbeck and Ronquist, 2001 Ronquist and Huelsenbeck, 2003). Trees were sampled every 100 generations. The first 25% of the trees were excluded from the analysis (burn-in). The 'sump' command of MRBAYES was used to compute clade posterior probabilities. Trees were displayed using FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

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