Preference of *Proteomonas sulcata* anion channelrhodopsin for NO$_3^-$ revealed using a pH electrode method

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Ion channel proteins are physiologically important molecules in living organisms. Their molecular functions have been investigated using electrophysiological methods, which enable quantitative, precise and advanced measurements and thus require complex instruments and experienced operators. For simpler and easier measurements, we measured the anion transport activity of light-gated anion channelrhodopsins (ACRs) using a pH electrode method, which has already been established for ion pump rhodopsins. Using that method, we successfully measured the anion transport activity and its dependence on the wavelength of light, i.e. its action spectra, and on the anion species, i.e. its selectivity or preference, of several ACRs expressed in yeast cells. In addition, we identified the strong anion transport activity and the preference for NO$_3^-$ of an ACR from a marine cryptophyte algae *Proteomonas sulcata*, named *PsuACR*$_{353}$. Such a preference was discovered for the first time in microbial pump- or channel-type rhodopsins. Nitrate is one of the most stable forms of nitrogen and is used as a nitrogen source by most organisms including plants. Therefore, *PsuACR*$_{353}$ may play a role in NO$_3^-$ transport and might take part in NO$_3^-$-related cellular functions in nature. Measurements of a mutant protein revealed that a Thr residue in the 3rd transmembrane helix, which corresponds to Cys102 in *GtACR1*, contributed to the preference for NO$_3^-$ of *PsuACR*$_{353}$. These findings will be helpful to understand the mechanisms of anion transport, selectivity and preference of *PsuACR*$_{353}$.

In living cells, there are several kinds of proteins that transport a variety of ions and small molecules across cell membranes. The membrane transport of those substrates is directly or indirectly connected to a variety of life phenomena, such as the maintenance of cell homeostasis, cell signaling, the generation of action potential in neurons, and so on. Therefore, membrane transport proteins are physiologically important and their molecular properties, including their structures and functions, have been intensively investigated by many research groups. In this study, we focused on one type of ion transporter, ion channels.

Ion channels passively transport ions according to the electrochemical potential gradients of ions across cell membranes. No external energy is needed for that type of transport, however some factors, such as membrane potential change, binding of ligands as well as physical or mechanical stimuli, are necessary to open and close those channels. In 2002, the first light-gated ion channel-type microbial rhodopsin for cations (cation channelrhodopsin, abbreviated as CCR) was reported, which originated from a green algae *Chlamydomonas reinhardtii* and was named channelrhodopsin-1 (abbreviated as ChR1)$^1$. As the name suggested, channelrhodopsins belong to a family of microbial rhodopsins, each composed of a seven transmembrane α-helical protein opsin and a chromophore all-trans-retinal$^1$. A year later, another CCR that originated from the same algae, named channelrhodopsin-2 (abbreviated as ChR2), was reported$^2$. Both ChR1 and ChR2 transport not only H$^+$ but also other monovalent cations, such as Li$^+$, Na$^+$ and K$^{+}$.$^{1,3,4}$ In 2015, natural light-gated anion channelrhodopsins (abbreviated as ACRs) that originated from a cryptophyte algae *Guillardia theta*, named *GtACR1* and *GtACR2*, were reported$^5$. Both of those ACRs are capable of transporting monovalent anions, such as F$^-$, Cl$^-$, Br$^-$, I$^-$ and...
NO$_3^-$, since then, many more CCRs and ACRs have been identified, characterized and engineered\textsuperscript{6–26}. Especially for ACRs, they are novel proteins that have just been discovered and therefore their molecular characteristics and their biological roles remain to be clarified.

The molecular functions of ion channels have been investigated using several experimental techniques. Especially, electrophysiological methods, such as the patch-clamp method, are highly quantitative and accurately characterize ion channel functions. In cases of not only ACRs but also CCRs, a series of electrophysiological analyses, such as current–voltage relationship and ion selectivity or preference, have been precisely performed. In addition, the introduction of a short-pulsed laser as an actinic light source into the electrophysiological measurement system enables the measurement of transient current changes during a single sequential photochemical reaction of microbial rhodopsins, called the photocycle\textsuperscript{1,2,6,16,17,20,27,28}. This technique is beneficial for identifying which photo-intermediates facilitate ion conducting and non-conducting states in the photocycle. Therefore, electrophysiological analysis is powerful and necessary to deeply understand the ion channel mechanisms of ACRs and CCRs. However, in exchange for such accuracy, complex instrumental setups and experienced operators are required. This is a bottleneck not only for non-experts in electrophysiology but also for scientists who want to measure ion transport activity more simply and easily.

We conceived the idea to measure the anion transport activity of ACRs using the pH electrode method, which had already been established for ion pump-type microbial rhodopsins expressed as recombinant proteins in \textit{Escherichia coli} cells. Simple instrumental setups and usability of the pH electrode method are a great advantage although the method is less quantitative compared to the electrophysiological method. In addition, the pH electrode method is expected to easily measure the dependence of the activities of ACRs on the wavelength of excitation light, i.e. action spectra, and on the anion species, i.e. selectivity or preference, even if precise measurements for reversal potentials, current density, kinetics, and so on, are impossible to perform. However, \textit{E. coli} cells cannot be used as host cells to express a variety of recombinant ACRs except for \textit{GtACR2}\textsuperscript{29–31}. In most cases so far, insect, mammalian and yeast cells have been used as host cells for the expression of recombinant ACRs. Among them, yeast cells are as easy to culture as \textit{E. coli} cells and to exchange buffers for the measurement of anion transport activity. Therefore, the pH electrode method is expected to be applicable for ACRs expressed in yeast cells to measure anion transport activity and anion dependence in a relatively simple way.

In this research study, we first developed and demonstrated the feasibility of the pH electrode method to measure the anion transport activity of ACRs expressed in yeast cells. We used \textit{GtACR1}, which is a well-studied ACR at present, to test the usefulness of the system. The action spectrum and anion dependence of the activity of \textit{GtACR1} measured using the pH electrode method were almost the same as previously measured using the electrophysiological method except for F$^-$ and SO$_4^{2-}$ probably due to the difference in the experimental condition\textsuperscript{5}. To apply this method to other ACRs, we measured the anion transport activities of 7 ACRs that were successfully expressed in yeast cells. As a result, we found that an ACR that originated from a marine cryptophyte algae \textit{P. sulcata}, previously named as \textit{PsaACR}_353\textsuperscript{19}, showed the strongest activity among the ACRs that we tested and preferably transported nitrate (NO$_3^-$) to other monovalent anions, such as Cl$^-$ and Br$^-$. NO$_3^-$ is one of the most stable forms of nitrogen on earth and is used as a nitrogen source by most organisms including plants\textsuperscript{3}. Therefore, the light-gated \textit{PsaACR}_353 may play a role in NO$_3^-$ transport and might possibly take part in nitrate sensing and signaling, and furthermore, the nitrogen assimilation by \textit{P. sulcata} in nature. Measurement of a mutant protein revealed that the Thr108 residue in the 3rd transmembrane helix, which corresponds to Cys102 in \textit{GtACR1}, contributed to the NO$_3^-$ preference in \textit{PsaACR}_353. These findings will be helpful to understand the mechanisms of anion transport, selectivity and preference of \textit{PsaACR}_353.

### Results

#### Concept of the pH electrode method.

Figure 1 illustrates the concept of the pH electrode method for measuring the anion transport activity of ACRs expressed in yeast cells. The fundamental concept was almost the same as that for the system using \textit{E. coli} cells. The yeast cell suspension was poured into the glass vial container and the pH electrode was placed into it. The cell suspension was stirring during the measurement to keep the suspension homogeneous. Continuous LED light (10 mW/cm$^2$ on average) was illuminated for 2 min from the side of the vial container. To reduce undesirable artifacts, we were careful not to let the light directly hit the light-sensitive part of the pH electrode.

Yeast cells have a cell wall outside and a cell membrane inside and ACRs are localized in the cell membrane. Once the light activated ACRs, anions were passively transported into the cells due to the electrochemical potential gradient of anions being higher outside the cells (300 mM) than inside (nearly 0 mM). This anion influx resulted in making the membrane potential more negative. To compensate for that, protons (H$^+$) in the bulk solution penetrate into the cells by passing through the cell wall and cell membrane. As a result, we can indirectly observe anion transport using the pH electrode as a pH change of the suspension. In this study, we observed pH increases that reflect the anion transport into yeast cells.

#### Demonstration of the pH electrode method to measure the activity of \textit{GtACR1}.

To ensure that the system works, we used \textit{GtACR1} as a test sample. First, we successfully expressed \textit{GtACR1} in yeast \textit{P. pastoris} cells according to previous studies\textsuperscript{28,33}. We then measured the Cl$^-$ transport activity of \textit{GtACR1} using the pH electrode method, and as shown in Fig. 2a, the activity of \textit{GtACR1} was successfully measured. The signal for the pH increase was clearly larger than that in the absence of CCCP. In most cases for anion and sodium-ion pump rhodopsins, the measurements were carried out in the presence of a proton-selective ionophore, carbonyl cyanide m-chlorophenyl hydrzone (abbreviated as CCCP), which facilitates the proton influx into cells. As a result, the signal of the pH change becomes larger than that in the absence of CCCP.
not protons. However, in the case of GtACR1, the signal intensity of the pH change was decreased in the presence of CCCP (Fig. 2a) although protons are not transport substrates of GtACR1. As shown in Fig. 2b, the color of the cell suspension was changed from pink to yellow, indicating that something undesirable happened in the presence of CCCP. Therefore, all subsequent measurements were carried out in the absence of CCCP. At present, however, we have no idea that explains why the signal has been decreased in the presence of CCCP. As the absorption spectra of purified GtACR1 shows (Supplementary Figure S3), the CCCP little affects the visible absorption property of active form GtACR1 (at around 515 nm), meaning that the CCCP or its solvent ethanol, whose concentrations are very low (10 μM and 0.001%(v/v), respectively), do not significantly induce the denaturation of GtACR1 and the deprotonation of the retinal protonated Schiff base.

We then measured the dependence of the excitation light wavelength on the Cl⁻ transport activity, i.e. the action spectrum (Fig. 2c). Stronger signals were obtained when the sample was illuminated using 505 nm and 530 nm light than by using 590 nm light. The amplitudes of the initial slope obtained from each condition were averaged and were statistically compared (Fig. 2d). As a result, the activities at 505 nm and 530 nm were not significantly different but were stronger than that at 590 nm. This result correlated well with the absorption spectrum of purified GtACR1 (Fig. 2e) and with the action spectrum previously measured using the electrophysiological method. Therefore, the pH electrode method can be used to investigate the action spectra of ACRs.

The anion-dependent transport activity of GtACR1 was also measured using 530 nm LED light for activation. Six monovalent anions (F⁻, Cl⁻, Br⁻, I⁻, NO₃⁻ and aspartate (abbreviated as Asp⁻)) and one divalent anion (SO₄²⁻) at a concentration of 300 mM were tested (Fig. 2f,g). As a result, significant transport activities were obtained for the anions except for F⁻ and Asp⁻ compared to the negative control cells. As suggested by a previous electrophysiological study, GtACR1 transports wide variety of anions and thus has less anion selectivity. Therefore, the pH electrode method in this study closely reproduced such an anion dependence. Therefore, we concluded that the pH electrode method can potentially be applied to measure anion transport activity, the action spectrum and the anion dependence of ACRs expressed in yeast cells.

Closely looking at the anion-dependent transport activity of GtACR1 measured using the pH electrode method, our results showed that GtACR1 transported SO₄²⁻ but not F⁻ (Fig. 2f,g), which was opposite of the previous electrophysiological study. The exact reason was unclear, however there were some differences in the experimental conditions, for example pH condition, between the two methods. In our case, the initial pH was adjusted to around 5 on average. On the other hand, in the previous electrophysiological study, the pH was estimated to be from neutral to weakly alkaline due to the use of HEPES buffer. We checked the effect of pH on the Cl⁻ transport activity of GtACR1 (Supplementary Fig. 4). As a result, the maximum activity was obtained
Figure 2. Anion transport activity of GtACR1 measured using the pH electrode method. (a) Comparisons of Cl⁻ transport activities in the absence (black line) or presence of 10 μM CCCP (red line); the negative control (grey line) was yeast cells without integration of the GtACR1 gene. 530 nm LED light (10 mW/cm²) was illuminated for 2 min as shown on a white background. (b) The color of pigmented yeast cell suspension expressing GtACR1 changed from pink to yellow by adding 10 μM CCCP. (c) Comparisons of Cl⁻ transport activities at various excitation light wavelengths (10 mW/cm²). LED lights were illuminated for 2 min as shown on a white background. (d) Statistical comparisons of Cl⁻ transport activities at various excitation light wavelengths. Data are reported as means and S.E.M.; the numbers in parentheses indicate the number of independent experiments. One-way ANOVA followed by Tukey’s test was performed (p value; *** < 0.0006). (e) Visible absorption spectrum of purified GtACR1 suspended in 10 mM MOPS (pH 7) buffer containing 1 M NaCl and 0.05% n-Dodecyl-β-D-maltoside (DDM). (f) Comparisons of anion-dependent transport activities. 530 nm LED light (10 mW/cm²) was illuminated for 2 min as shown on a white background. (g) Statistical comparisons of anion-dependent transport activities. Data are reported as means and S.E.M.; the numbers in parentheses indicate the number of independent experiments. One-way ANOVA followed by Dunnett’s test was performed (p values; **** < 0.0001, *** < 0.001, ** < 0.01). The negative control was yeast cells without integration of the GtACR1 gene resuspended in 300 mM NaCl.
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light was kept at an average of 10 mW/cm². As a result, the signals of pH change were obtained in most ACRs but nearly 0.15 unit, which is ca. 3-times larger than even for the Cl Gc ACRs using the pH electrode method. For activation, 505 nm LED light was used for ZipACR, the intensities were very weak (Fig. 3c). However, only indicating its significant Cl Gt ACR15. Corresponding to Cys102 in Gt ACR15.

Application of the pH electrode method to other ACRs. The pH electrode method was then used to test other ACRs. At present, several ACR genes have been identified by transcriptome analysis. Among them, we tested 8 ACRs (3 from Proteomonas sulcata and 5 from Geminigera cryophila; see Table 1) for functional expression in yeast cells. Five of the 8 ACRs tested in this study belong to the Cys-type (see below) as does GtACR1, 2 belong to the Thr-type as does RapACR reported recently19, and 1 belongs to the Val-type as does Gt161302 reported previously5. Note that G. cryophila has 5 ACRs that belong to all three types.

ACRs can be roughly classified into three types, the Cys-type, the Thr-type and the Val-type (Fig. 3a). One of the factors contributing to that classification is the amino acid conserved at position 102 in Gt (Fig. 2g), however reference is not well understood. No anion transport activity was reported for the effect of such diversity in the amino acid conservation on anion transport activity, anion selectivity or preference in yeast cells. Five of the 8 ACRs tested in this study belong to the Cys-type as does RapACR reported recently19, and 1 belongs to the Val-type as does Gt161302 reported previously5. Note that G. cryophila has 5 ACRs that belong to all three types.

As shown in Fig. 3b, 7 of the 8 ACRs, except Gc_ACR_439, were pigmented in the presence of all-trans-retinal, indicating their successful expression in yeast cells. We then measured the Cl⁻ transport activities for these ACRs using the pH electrode method. For activation, 505 nm LED light was used for ZipACR, Gc_ACR_145 and Gc_ACR_457, while 530 nm LED light was used for PsuACR1, PsuACR_353, Gc_ACR_197 and Gc_ACR_201, by taking into account the colors of the cells and the action spectral maxima reported previously16–19. The intensity of light was kept at an average of 10 mW/cm². As a result, the signals of pH change were obtained in most ACRs but the intensities were very weak (Fig. 3c). However, only PsuACR_353 generated a strong signal of a pH increase, indicating its significant Cl⁻ transport activity compared to the other ACRs.

Characterization of the anion transport activity of PsuACR_353. To characterize the anion transport activity of PsuACR_353, we measured the action spectrum and anion dependence of its transport activity using the pH electrode method. Figure 4a shows that the Cl⁻ transport activities of PsuACR_353 depend on the excitation light wavelength. Statistical comparisons of the initial slope amplitudes revealed that the activity at 530 nm was significantly larger than that at 590 nm (Fig. 4b). This result correlated well with a previous electrophysiological study19. We then measured the anion dependence of the transport activity of PsuACR_353 using 530 nm light. Strong signals were observed not only for Cl⁻ but also for Br⁻, I⁻, NO₃⁻ and even SO₄²⁻ despite the low level of expression (Figs. 3b, 4c and 4b explained below). Especially for NO₃⁻, the pH was increased by nearly 0.15 unit, which is ca. 3-times larger than even for the Cl⁻ transport of GtACR1 (Fig. 2f). In addition, statistical analysis clearly showed the strongest activity for NO₃⁻, which was more than 2-times larger than that for the other anions (Fig. 4d). These results indicated that PsuACR_353 had less anion selectivity as was GtACR1 (Fig. 2g), however PsuACR_353 preferably transported NO₃⁻.

To quantitatively compare the anion transport activities of GtACR1 and PsuACR_353, their expression levels were estimated by Western blotting (Fig. 5a,b). The results showed that the relative level of expression of PsuACR_353 was ca. 0.46-times smaller than that of GtACR1. Figure 5c and Supplementary Fig. S2 show statistical comparisons of the anion transport activities after correction to account for the expression levels of GtACR1 and PsuACR_353. These results clearly showed that the transport activities of PsuACR_353 for Cl⁻ and NO₃⁻ were ca. 2- and 5-times larger than that for GtACR1, respectively, and therefore the preference for NO₃⁻ of PsuACR_353 was far greater than GtACR1 and other anion species.
Figure 3. ACRs tested using the pH electrode method. (a) Phylogenetic relationship of 45 ACRs analyzed by MEGA 7 software. According to amino acids conserved at position 102 in GtACR1, ACRs are classified into three types, Cys-type (blue), Thr-type (green) and Val-type (orange). (b) Pictures of pigmented yeast cells due to the functional expression of each ACR in the presence of all-trans-retinal. The negative control was yeast cells without integration of any ACR gene. (c) Comparisons of Cl⁻ transport activities of ACRs. LED lights were illuminated for 2 or 3 min as shown on a white background.
Anion transport activities of *Gt*ACR1-C102T and *Psu*ACR_353-T108C mutants. Finally, the anion transport activities of mutant ACRs were investigated using the pH electrode method. As described in the previous section, one difference in the amino acid sequence between *Gt*ACR1 and *Psu*ACR_353 is the amino acid conserved in the 3rd transmembrane helix, i.e. *Gt*ACR1 has Cys102 whereas *Psu*ACR_353 has Thr108 at...
Figure 5. Quantitative comparisons of the anion transport activities of GtACR1, PsuACR_353 and their mutants. (a) Western blotting using anti-His-tag mAb after SDS-PAGE; each ACR has monomer and dimer bands as indicated by filled and open triangles, respectively. The sum of band intensities was used to estimate protein expression levels. Original image of full-length membrane was shown in Supplementary Figure S5. (b) Statistical comparisons of relative expression levels. Data are reported as means and S.E.M.; the numbers in parentheses indicate the number of independent experiments. One-way ANOVA followed by Dunnett’s test was performed (p values; **** < 0.0001, * 0.0124). (c) Statistical comparisons of the transport activities of Cl⁻ (left) and NO₃⁻ (right). Data were corrected by the relative expression level and are reported as means and S.E.M.; the numbers in parentheses indicate the number of independent experiments. One-way ANOVA followed by Tukey’s test was performed (p values; **** < 0.0001, ** < 0.0049, * < 0.0492).
that position (Table 1, Figs. 3a, 6a, and Supplementary Fig. S1). We investigated the effects of that difference on anion transport activity, anion selectivity and preference of GtACR1-C102T and PsuACR_353-T108C mutants using the pH electrode method.

For the GtACR1-C102T mutant (Fig. 6b,c), the intensities of the pH change and initial slope increased compared to wild-type GtACR1 (Fig. 2f,g). However, the anion dependence was almost the same as the wild-type (Fig. 6b,c). The expression level of the GtACR1-C102T mutant was also not significantly different from the wild-type (Fig. 5a,b). Taking this into account, a statistical comparison showed that the transport activities of anions other than F− were almost the same as those of the wild-type (Fig. 5c and Supplementary Fig. S2). On the other hand, when compared with the transport activities of wild-type PsuACR_353, the transport activities of the GtACR1-C102T mutant were significantly increased and the preference for NO3− was only slightly enhanced (Fig. 5c and Supplementary Fig. S2). Therefore, the Cys-to-Thr mutation at position 102 in GtACR1 resulted in almost no effect either on the anion transport activity or the selectivity or preference.

However, for the PsuACR_353-T108C mutant (Fig. 6d,e), decreases in transport activities were observed for all anions by compared with wild-type PsuACR_353 (Fig. 4c,d). In addition, the intensities of pH changes and initial slopes resulted in decreases to almost the same level (Fig. 6d,e), indicating that the anion dependence was completely lost due to that mutation. Taking into account the expression level of the PsuACR_353-T108C mutant, which was about 7-times larger than wild-type GtACR1 (Fig. 5a,b), we concluded that the Thr-to-Cys mutation at position 108 in PsuACR_353 caused a significant loss of function (Fig. 5c and Supplementary Fig. S2). These results indicate that the effects of the corresponding amino acids at positions 102 in GtACR1 and 108 in PsuACR_353 on anion transport activity were completely different from each other, which would reflect the phylogenetic difference (Fig. 3a).

Discussion
In this study, we used the pH electrode method to measure the anion transport activity of ACRs expressed as recombinant proteins in yeast cells. That method has already been established and used for ion pump-type microbial rhodopsins expressed in E. coli cells. Therefore, this is the first study that measured the activity of ion channel-type rhodopsins expressed in yeast cells. The advantages of this method include the simple instrumental setup and the use of a glass electrode pH meter, and the ease of measurement even for non-experts in electrophysiology. Indeed, electrophysiological techniques are powerful and are required for quantitative and precise measurements of ion channel functions. However, as we demonstrate here, the action spectrum and the substrate dependence of anion transport activity can be easily measured using the pH electrode method. We assume that this method will also be useful as a screening technique to explore and engineer ACRs with unique absorptions, anion selectivities, and so on.

We newly characterize the strong anion transport activity and NO3− preference of PsuACR_353 in this study. Govorunova et al. already performed electrophysiological measurements for various ACRs including PsuACR_35319. However, no results were reported for such preferences probably because they did not perform the electrophysiological measurements using various anions other than Cl−. One reason for that we suppose is that the light-induced current of PsuACR_353 caused by Cl− transport was significantly smaller than not only the other ACRs tested but also for GtACR119. That may be due to the much lower expression levels of PsuACR_353 in their mammalian cell expression system because its expression level was also significantly small even in our yeast expression system (Fig. 5a,b). We noticed that the magnitude relationship of the Cl− transport activities of PsuACR_353 and GtACR1 reported by Govorunova et al.19 were completely different from our results obtained using the pH electrode method (Fig. 5c). This may also reflect differences in the expression levels of GtACR1 and PsuACR_353 in each protein expression system.

On the other hand, the anion-dependent transport activity determined by each method can be considered equivalent. In the first report of GtACR1, its anion dependence was revealed by an electrophysiological method4. The result was that GtACR1 transported Cl−, Br−, I− and NO3− almost equally but transported F− more weakly. In this study, we were successfully reproduced such a dependence using the pH electrode method (Fig. 2g). GtACR1 has less anion selectivity, which is similar to the anion pump rhodopsins but different from the potassium channel. It is speculated that a structure or a mechanism working as an anion selective filter is not necessarily sophisticated in GtACR1. Conversely, such a less anion selectivity may be connected to its biological roles in the alga G. theta, which awaits clarification in further studies. In contrast, PsuACR_353 was revealed to transport NO3− preferentially to the other anions tested in this study (Figs. 4c, 5c and Supplementary Fig. S2). In the sense that PsuACR_353 transported Cl−, Br− and I− almost equally, which was the same as GtACR1, and even SO42−, PsuACR_353 also showed less anion selectivity. However, the preference for NO3− was far greater than the other anions. To our knowledge, such a preference has not been discovered in any microbial pump- or channel-type rhodopsins reported so far.

Nitrate (NO3−) is one of the most stable forms of nitrogen on earth and is used as a nitrogen source by most organisms including plants32. For NO3− transport, both pump- and channel-type transporters exist in nature. Among them, a slowly activating anion channel 1 named SLAC1 and its homologous proteins named SLAH2 and SLAH3 expressed in plants are known to transport NO3− preferably and take part in nitrate acquisition in roots, in nitrate translocation from roots to shoots, and in guard cell closure32,33. Therefore, PsuACR_353 may play a role in NO3− transport and might possibly take part in nitrate sensing and signaling, and furthermore, in the nitrogen assimilation in P. sulcata in nature. Indeed, it is known that P. sulcata senses the concentration of nitrate outside the cell and accumulates nitrogen as a form of protein-pigment, phycoerythrin, which contributes to the light-harvesting function for photosynthesis35,36. It should be noted that we tried to purify PsuACR_353 to more deeply understand the mechanism and role of NO3− transport using spectroscopic techniques, but those efforts failed because the protein was unstable in the presence of a detergent.
Figure 6. Anion transport activities of GtACR1-C102T and the PsuACR_353-T108C mutants. (a) Location of the Cys102 residue in the GtACR1 structure (PDB ID 6CSM) drawn by PyMOL software. Cys102 and the all-trans-retinylidene chromophore are shown in stick representation by PyMOL. (b, d) Comparisons of anion-dependent transport activities of (b) GtACR1-C102T and (d) PsuACR_353-T108C mutants. 530 nm LED light (10 mW/cm²) was illuminated for 2 min as shown on a white background. (c, e) Statistical comparisons of anion-dependent transport activities of (c) GtACR1-C102T and (e) PsuACR_353-T108C mutants. Data are reported as means and S.E.M.; the numbers in parentheses indicate the number of independent experiments. One-way ANOVA followed by Dunnett’s test was performed (p values; for GtACR1-C102T **** < 0.0001, * 0.0197; for PsuACR_353-T108C **** < 0.0001, *** < 0.0007, * 0.0338). Negative controls are yeast cells without integration of any ACR gene resuspended in 300 mM NaCl.
We wonder why *PsuACR_353* is able to preferably transport NO$_3^-$-... One possible explanation is that the ionic radii or hydrated radii of anions contribute to the preference$^{37}$. However, no sequential correlation is seen because the ionic and hydrated radii of NO$_3^-$ are 0.177 nm and 0.316 nm (Table 2), respectively, which is almost the same as those of Cl$^-$ (0.180 nm and 0.319 nm, respectively). The crystallographic structure of *GtACR1* in the dark state revealed that there is a continuous tunnel spanning through the protein, which is constructed by the 1st–3rd, and 7th transmembrane helices (Supplementary Fig. S1), and the radius of that tunnel is the same or smaller than the ionic radius of Cl$^-$ (0.1–0.2 nm)$^{38,39}$. Of course, a transient change of the tunnel radius accompanied by a structural change of the protein should also be considered because the structures of *GtACR1* represent the state before light activation$^{38,39}$, and the less anion selectivity of *GtACR1* can be explained by the size of the tunnel radius and the ionic radius of anions. Another possible explanation is that when *PsuACR_353* transports NO$_3^-$ upon light activation, some spaces or sites are transiently formed, where NO$_3^-$ is preferably trapped or passes through, during the photocycle. Among the anions we tested in this study, only NO$_3^-$ is not spherically symmetrical. Thus, NO$_3^-$ has two hydrated radii, one for the axial radius and another for the equatorial radius (Table 2)$^{37}$. Therefore, it is speculated that a possible mechanism for the NO$_3^-$ preference is that *PsuACR_353* transiently recognizes the asymmetrical structure of NO$_3^-$ and therefore transports NO$_3^-$ being hydrated.

We suppose that Thr108 in *PsuACR_353* is one of the residues contributing to the NO$_3^-$ preference, which is based on comparisons of *PsuACR_353*-T108C and *GtACR1*-C102T mutants (Figs. 5c, 6, and Supplementary Fig. S2). We investigated the effects of differences in amino acid residues at positions 102 and 108 in *GtACR1* and in *PsuACR_353*, respectively, on their anion transport activities, anion selectivity or preference for the first time in this study. Because the phylogenetic relationship between these ACRs is distant from each other (Fig. 3a), it is conceivable that the anion conducting paths and mechanisms in each ACR are different. The crystal structure of *GtACR1* suggested that Cys102 is not involved in the anion conducting path$^{38,39}$. On the other hand, in *PsuACR_353*, Thr108 may be a part of a conducting path dedicated for NO$_3^-$.

**Conclusion**

In this study, we successfully demonstrated the usefulness of the pH electrode method to measure the anion transport activity of ACRs. This method allows us to measure the action spectra and the anion dependence of transport activity simply and easily, with results comparable to previous results obtained using electrophysiological methods. In addition, we identified the strong anion transport activity and the preference for NO$_3^-$ of *PsuACR_353* for the first time. Nitrate is one of the most stable forms of nitrogen and is used as a nitrogen source by most organisms including plants. Therefore, *PsuACR_353* may play a role in NO$_3^-$ transport and might take part in NO$_3^-$-related cellular functions in nature. Furthermore, we successfully demonstrated that Thr108, which corresponds to Cys102 in *GtACR1*, contributes to the NO$_3^-$ preference of *PsuACR_353*. Such a preference has been discovered for the first time in microbial pump- or channel-type rhodopsins. This finding will be helpful to understand the anion transport mechanism of *PsuACR_353*. We did not address the potential use of NO$_3^-$-preferred *PsuACR_353* for optogenetics because NO$_3^-$ is rare in mammalian cells. Possibly, *PsuACR_353* may be useful for optogenetics in plants.

**Methods**

**DNA constructs of ACRs.** Gene information about ACRs used in this study was obtained from GenBank and is summarized in Table 1. An 8 histidine tag was attached to the C-terminus of each ACR. The genes encoding ACRs with codon optimization for expression in *Pichia pastoris* were purchased from GENERWIZ (South Plainfield, NJ, U.S.A.). The procedures for constructing the pPICZ B vector (Thermo Fisher Scientific, Waltham, MA, U.S.A.) for *P. pastoris* were the same as our previous report$^{33}$. The genes for *GtACR1*-C102T and *PsuACR_353*-T108C mutants were prepared using PrimeSTAR Max DNA Polymerase (Takara Bio Inc., Shiga, Japan). The correctness of all nucleotide sequences was verified by dyeodexy sequencing.

**Protein expression.** The methylotrophic yeast *Pichia pastoris* SMD1168H strain (Thermo Fisher Scientific) was used as the protein expression host. The procedures for transformation of yeast and for the protein expression were almost the same as our previous report$^{33}$. Briefly, transformed *P. pastoris* SMD1168H cells were pre-cultured in BMGY medium containing 100 μg/mL Zeocin (Thermo Fisher Scientific) for 1 day at 30°C. The medium was exchanged to BMMY containing 100 μg/mL Zeocin, 2% (v/v) methanol (Fuji Film Wako Chemi-

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| Anion   | Ionic radius/nm | Hydrated radius/nm |
|---------|------------------|--------------------|
| F$^-$   | 0.124            | 0.263              |
| Cl$^-$  | 0.180            | 0.200              |
| Br$^-$  | 0.198            | 0.237              |
| I$^-$   | 0.225            | 0.265              |
| NO$_3^-$ mean | 0.177        | 0.236              |
| NO$_3^-$ axial | –                | 0.265              |
| NO$_3^-$ equatorial | –          | 0.345              |
| SO$_4^{2-}$ | 0.242           | 0.382              |

Table 2. Summary of ionic and hydrated radii of anions tested in this study. Data taken from$^{37}$. 

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Anion transport activity measurement and data analysis. To measure anion transport activity, *P. pastoris* SMD1168H cells expressing ACRs were washed with 300 mM salt solution (NaCl, NaF, NaBr, NaI, NaNO₃, Na₂SO₄, sodium aspartate; all from Fuji Film Wako Chemical Industries) 4 times by centrifugation (3,000 rpm, 5 min, 4°C; himac CF16RN equipped with a T9A31 rotor). The cells were finally suspended in the same salt solution used for washing. As a negative control, *P. pastoris* SMD1168H cells without integration of any ACR gene were prepared. The optical density at 660 nm of each cell suspension was measured using a UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan) and adjusted to 1.9 on average. Anion transport activity was measured at temperatures below room temperature (16°C on average) by monitoring pH changes using a LAQUA F-72 pH meter equipped with a standard ToupH pH electrode (HORIBA, Ltd., Kyoto, Japan). The initial pH was 5 on average. To measure the dependence of transport activity on the wavelength of light, blue-green (peak wavelength is 505 nm), green (peak wavelength is 530 nm) and orange (peak wavelength is 590 nm) LED light was illuminated. The light intensity was adjusted to 10 mW/cm² on average, which was measured using a power meter (ORION, Ophir Optronics Solutions Ltd., Jerusalem, Israel). Anion transport activity was estimated by the initial slope of the first 10 s after LED light illumination for the time-dependent pH changes. More than three independent measurements were averaged. For statistical analysis, one-way ANOVA followed by Dunnett’s test and Tukey’s test were performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, U.S.A.).

SDS-PAGE and Western blotting. SDS-PAGE with 12% (v/v) polyacrylamide gels and Western blotting using an anti-His tag antibody conjugated with horseradish peroxidase (anti-His-tag mAb-HRP-Direct, MBL Co., Ltd., Nagoya, Japan) were performed using standard protocols. *P. pastoris* cell samples were prepared as previously published40. Briefly, 1 mL of each cell suspension at an OD₆₆₀ of ca. 1.9 was centrifuged to collect cell pellets. The wet weight of the cell pellets was 10 mg on average. The cell pellets were then resuspended in 1 mL 0.1 M NaOH and incubated for 5 min at room temperature. After centrifugation to remove the supernatant, the cell pellets were suspended in 250 μL SDS-PAGE sample buffer (60 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) glycerol, 4% (v/v) β-mercaptoethanol and 0.0025% (w/v) bromophenol blue) and then boiled at 95°C for 5 min. Finally, after centrifugation again, 6 μL of each supernatant diluted by one-sixth was loaded on the gel. To estimate the total amount of each ACR expressed in *P. pastoris* cells, the band intensities were analyzed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, U.S.A.) and 5 independent measurements were averaged. For statistical analysis, one-way ANOVA followed by Dunnett’s test was performed using GraphPad Prism 9 software (GraphPad Software).

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Author contributions
T.T. designed the research. C.K., H.K., T.W. and T.T. performed experiments and analyzed data. M.D. and T.K. provided technical assistance. C.K. and T.T. wrote the paper. All authors discussed and confirmed the results of the paper.

Competing interests
The authors declare no competing interests.

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