Measurements of plasma membrane potential changes in *Saccharomyces cerevisiae* cells reveal the importance of the Tok1 channel in membrane potential maintenance

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Abstract

K⁺ is one of the cations (besides protons) whose transport across the plasma membrane is believed to contribute to the maintenance of membrane potential. To ensure K⁺ transport, *Saccharomyces cerevisiae* cells possess several types of active and passive transporters mediating the K⁺ influx and efflux, respectively. A diS-C₃(3) assay was used to compare the contributions of various potassium transporters to the membrane potential changes of *S. cerevisiae* cells in the exponential growth phase. Altogether, the contributions of six K⁺ transporters to the maintenance of a stable membrane potential were tested. As confirmed by the observed hyperpolarization of *trk1 trk2* deletion strains, the diS-C₃(3) assay is a suitable method for comparative studies of the membrane potential of yeast strains differing in the presence/absence of one or more cation transporters. We have shown that the presence of the Tok1 channel strongly influences membrane potential: deletion of the *TOK1* gene results in significant plasma membrane depolarization, whereas strains overexpressing the *TOK1* gene are hyperpolarized. We have also proved that plasma membrane potential is not the only parameter determining the hygromycin B sensitivity of yeast cells, and that the role of intracellular transporters in protecting against its toxic effects must also be considered.

Introduction

The maintenance of membrane potential (∆Ψ) is a highly regulated process in all living cells. In *Saccharomyces cerevisiae*, the potential (negative inside) is controlled mainly by the regulation of cation fluxes (H⁺, K⁺) (Gaber, 1992). Protons are actively extruded from cells at the expense of ATP hydrolysis by the H⁻-ATPase Pma1 (Serrano et al., 1986), which maintains the electrochemical pH gradient necessary for ion and nutrient transport. It has been shown that certain mutations in Pma1 ATPase cause the depolarization of *S. cerevisiae* cells (Perlin et al., 1988, 1989), proving the importance of this transport system in membrane potential generation.

Besides protons, another cation involved in membrane potential regulation is K⁺. Potassium is a vital component of living cells, and all cells depend on K⁺ uptake to grow and to keep themselves alive. Under nonstressed conditions, potassium cations are accumulated in yeast cells against large concentration gradients via the proteins Trk1 and Trk2 (Ko & Gaber, 1991; Ramos et al., 1994), and the membrane potential might be the energy source for this process (Rodriguez-Navarro, 2000). It has been shown that strains with both the *trk1* and *trk2* deletions are hyperpolarized (Madrid et al., 1998), proving the important role of K⁺ transporters in membrane potential regulation.

Several other K⁺ transporters have been identified in the plasma membrane of *S. cerevisiae*, contributing to the regulation of intracellular K⁺ concentration. In specific conditions (for example KCl stress), potassium can be actively extruded from cells by Ena/Pmr2 ATPases (encoded by a tandem array of several nearly identical genes, the precise number of which depends on the particular yeast strain (Haro et al., 1991; Wieland et al., 1995) or by the Nha1 antiporter (Bañuelos et al., 1998). Both these K⁺ efflux systems are able to transport other alkali-metal cations as well (e.g. Na⁺, Li⁺; Haro et al., 1991; Bañuelos et al., 1998). The expression of the *ENA1* or the *NHA1* gene in an
en1-4Δnha1Δ strain showed the complementary functions of both systems in the maintenance of optimal intracellular K⁺ (and Na⁺) concentration. Ena/Pmr2 ATPases are responsible for cell growth at high concentrations of NaCl and KCl at high external pH values, while the Nha1 antiporter is necessary at acidic pH levels (Banuelos et al., 1998). Nha1p is a typical secondary active transport system, which uses the proton gradient created by Pma1 ATPase as the energy source for K⁺(Na⁺) export.

Another potassium-specific plasma membrane transport system has been named Tok1p (Ketchum et al., 1995). The biophysical properties of this K⁺ channel are well described (Bertl et al., 1993), but the physiological role of Tok1p has not yet been fully characterized. Tok1 is a voltage-gated K⁺ channel with two pore domains in tandem (Ketchum et al., 1993), but the physiological role of Tok1p has been later questioned (Breinig et al., 1999; Sesti et al., 2001). It was also shown that Tok1p is phosphorylated by the Hog1 kinase, indicating a role of the channel in the response to osmotic shock (Proft & Struhl, 2004).

The Kha1 antiporter was previously believed also to extrude potassium from cells (Ramirez et al., 1998), but we have recently discovered that this protein does not carry out that function (Maresova & Sychrova, 2005), and, as shown in this work, the presence of Kha1p does not affect the membrane potential of cells.

In this study, we used a series of isogenic mutant yeast strains and measured the fluorescence potentiometric probe distribution in cell suspensions [diS-C₃(3) assay; Plasek et al., 1994; Denksteinova et al., 1997] to study the contribution of various potassium transporters to the regulation of membrane potential in S. cerevisiae cells.

### Materials and methods

#### Strains

All S. cerevisiae strains used in this study are derivatives of the W303-1A strain (ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mali10) (Wallis et al., 1989). Additional mutations are listed in Table 1.

The tok1 deletion strains were prepared by homologous recombination according to the protocol described by Guldener et al. (1996). PCR was run on the pUG6 plasmid as a template using the primers TokKAN-sense (5’-atgaacaggtcatgaacagctttgccaaacaaacgctgggatatgttcgtacgctgcaggtcgggc-3’) and TokKAN-antisense (5’-tcacaaggtctttctatgctcaccaaaaatttttttgctgcatggccacttgg-3’). The resulting deletion cassette was used for the transformation of the W303 (wt), CW25 (nha1Δ), GW19 (en1-4Δ) and BW31 (en1-4Δnha1Δ) strains. Cells were transformed by electroporation (Blok et al., 1992) and selected on YPD medium containing 800 µg.mL⁻¹ G418 (MP Biomedicals). The resulting strains were named TOW (tok1Δ), TOC (nha1Δtok1Δ), TOG (en1-4Δtok1Δ) and TOB (en1-4Δnha1Δtok1Δ), respectively (cf. Table 1).

The correct insertion of the deletion cassette was verified by PCR, using primers from the TOK1 ORF, its surroundings, and from the kanMX deletion cassette: Upstream-TOK1 (5’-tcgtagatgctcgatga-3’) with KanX-R1 (5’-ctctggcgcatcgggc-3’), Downstream-TOK1 (5’-atgaacaggtcatgaacagctttgccaaacaaacgctgggatatgttcgtacgctgcaggtcgggc-3’) with KanX-F1 (5’-tcacaaggtctttctatgctcaccaaaaatttttttgctgcatggccacttgg-3’), and Inside-TOK1 (5’-attacaaaaagatgatgagcagc-3’) with Downstream-TOK1.

#### Plasmids

The pYEX plasmid used in this study is a multicopy vector containing the URA3 selection marker, an inducible CUP1 promoter and the sequence encoding GFP (green fluorescent protein) for N-terminal protein fusion. Both the empty vector and the construct containing the TOK1 gene were obtained from J. Ludwig, Universität Tübingen, Germany.

### Table 1. Saccharomyces cerevisiae derivatives of W303

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
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<tr>
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<td>MATa</td>
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<td>TOG</td>
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<tr>
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<td>LMB 01</td>
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<td>LMB 02</td>
<td>MATa</td>
<td>Maresova &amp; Sychrova (2005)</td>
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<tr>
<td>MAB 2d</td>
<td>MATa</td>
<td>Maresova &amp; Sychrova (2005)</td>
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Media and growth assays

Cells were grown in YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose, adenine 15 μg mL⁻¹; 2% agar for solid media) sometimes supplemented with KCl as indicated in the text. For hygromycin B sensitivity tests and for the cultivation of strains transformed with a plasmid, YNB medium was used (0.17% yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 2% glucose; 2% agar for solid media) with auxotrophic supplements added after autoclaving.

The hygromycin B gradient plate was prepared by the successive pouring of two layers of media differing in composition. The first layer (YNB containing 500 μg mL⁻¹ hygromycin B) was poured into a moderately inclined square Petri dish. After solidification of the sloping layer, the plate was placed in a horizontal position and the second layer (YNB without hygromycin B) was poured on top.

For the assessment of growth phenotypes, fresh cells of each tested strain were resuspended in water, adjusted to the initial OD of 1.0, and thirteen 3-μL aliquots of each suspension were spotted in a line on the YNB agar plate along the hygromycin B gradient. The plate was incubated at 30°C for 2 days and a digital greyscale image was obtained using a Nicon Coolpix 4500 digital camera. The more tolerant to hygromycin B a particular strain was, the more drops in the line were able to grow.

Fluorescence measurement of membrane potential [diS-C₃(3) assay]

The fluorescence assay of membrane potential was performed according to Denksteinova et al. (1997). Yeast cells were cultured in liquid YPD media. A preculture was cultivated for 15–20 h at 30°C. The main culture was grown in 10 mL of the same medium at 30°C with an initial OD of 1.0, and thirteen 3-μL aliquots of the suspension to a final probe concentration of 0.2 μM were grown until the OD₅₇₈ nm reached 0.2–0.4 (exponential suspension to a final probe concentration of 0.2 μM). The stock solution in ethanol) was added to 3 mL of yeast cell layer (YNB without hygromycin B) was poured on top. The fluorescence emission spectra were measured on a FluoroMax 2 spectrofluorimeter equipped with a xenon lamp. The excitation wavelength was 531 nm, the emission range 560–590 nm, and duration of one spectral scan 20 s. Scattered light was eliminated by an orange glass filter with a cut-off wavelength at 540 nm.

The staining curves (i.e., the dependence of the wavelength of maximum emission λₘₐₓ(t) on the duration of staining t (Gaskova et al., 1998) were fitted as described in Malac et al. (2005), and the position of the emission maximum at equilibrium (λₑₐₗ) was estimated.

The spectral properties of diS-C₃(3) λₘₐₓ(t) change when the probe is bound to intracellular components, and the position of the emission spectrum therefore reflects the actual intracellular probe concentration (Plasek et al., 1994). The probe is known to enter yeast cells according to their membrane potential and simultaneously to be actively expelled by the MDR-pumps Pdr5 and Snq2 (Gaskova et al., 2002). It is important to note that, in this study, the diS-C₃(3) assay is only used for comparative purposes in cells that are entirely isogenic except for the genes considered in each case, so the activity of the MDR-pumps was the same and the differences in λₑₐₗ reflected purely the differences in membrane potential.

Results

The diS-C₃(3) assay of membrane potential changes

To make sure that the diS-C₃(3) assay was a suitable method for monitoring membrane potential changes in yeast cells, we first compared the fluorescence spectra of the BW31 and MAB 2d strains. Both these strains carry the ena1-4 and nha1 deletions, but MAB 2d also carries the trk1 and trk2 deletions. As was reported earlier, the deletion of the TRK1 and TRK2 genes results in cell membrane hyperpolarization caused by the inability to actively import potassium cations (Madrid et al., 1998).

The MAB 2d strain does not grow in media without K⁺ addition (Maresova & Sychrova, 2005). For the standard cultivation of trk1Δtrk2Δ strains, media with 100 mM KCl were used. The MAB 2d strain grew just as well as the BW31 strain under these conditions, and their membrane potentials were nearly the same. However, when transferred to standard YPD without the addition of KCl for 1 h before potential measurement, the MAB 2d strain was hyperpolarized compared with BW31, shown by a higher λₑₐₗ for the MAB 2d strain than for the BW31 strain (data not shown). This corresponds well with the observation of Madrid et al. (1998), that K⁺ starvation causes the hyperpolarization of yeast cells (BW31 does not experience K⁺ starvation, because the K⁺ included in standard YPD is sufficient for a strain with functional Trk1 and Trk2 proteins). A very similar situation occurs when the strains are cultivated in...
YPD supplemented with 10 mM KCl from the beginning of the experiment – the MAB 2d strain, which is not able actively to import K⁺, grows slightly more slowly under these conditions, and is hyperpolarized compared with the control BW31 strain. These results confirm that the diS-C₃(3) assay is a suitable method for comparative studies of the membrane potential of yeast strains differing in the presence/absence of one or more cation transporters.

**Membrane potential changes caused by various K⁺ transporters**

In our previous work, we found that the deletion of the *KHA1* gene resulted in sensitivity to hygromycin B (Maresova & Sychrova, 2005), an aminoglycosidic antibiotic known to enter cells in amounts proportional to their membrane potential (Mulet et al., 1999). It was previously shown that the depolarized *pmal* mutants were more tolerant to hygromycin B than the wild type (Perlin et al., 1988, 1989), while the hyperpolarized *trk1Δtrk2Δ* strain was hypersensitive to hygromycin B (Madrid et al., 1998).

Therefore we wanted to know whether the membrane potential of *S. cerevisiae* cells was dependent on the presence of the Kha1 protein.

As the location of the KHA1 gene on the left arm of chromosome X is just next to the TOK1 gene (encoding another K⁺ transporter in *S. cerevisiae*) and we had prepared both *kha1Δ* and *tok1-kha1Δ* mutants (Maresova & Sychrova, 2005), we tested both of them together. In contrast to the case for KHA1, the absence of the TOK1 gene did not affect cell tolerance to the toxic cation hygromycin B (Fig. 1). This observation could mislead to the erroneous conclusion that deletion of the KHA1 gene hyperpolarized the cells, while deletion of the TOK1 gene did not affect the membrane potential.

The diS-C₃(3) fluorescence measurements have, however, shown that this is not the case. Our results strongly indicate that membrane potential is not the only feature determining cell sensitivity to hygromycin B. The BW31 (*ena1-4Δnha1Δ*), LMB 01 (*ena1-4Δnha1Δkha1Δ*) and LMB 02 (*ena1-4Δnha1Δtok1-kha1Δ*) strains were subjected to diS-C₃(3) assay for membrane potential comparison (data not shown). The *kha1* deletion did not influence membrane potential (the LMB 01 strain was stained to the same level as the BW31 strain), while the LMB 02 staining curve (and equilibrium emission maximum) was significantly lower. Now further questions arose – was it the *tok1* deletion itself that caused the depolarization of the LMB 02 strain, or was it the fact that it was the deletion of the last K⁺ exporting system in a strain without the *ENA1-4* and *NHA1* genes? Would the *tok1* mutant be depolarized even if the cells had a functional K⁺ exporter, Ena1p or Nha1p?

We therefore decided to prepare *tok1Δ* mutants with various combinations of deletions of genes encoding the other plasma membrane K⁺ exporters (*Ena1-4* and *Nha1*) and to compare changes in their membrane potential by diS-C₃(3) assay.

As shown in Fig. 2, each deletion of a K⁺ exporting system caused a slight decrease of λₑq max. The most depolarized strain was the triple deletion mutant (*enal-4Δnha1Δtok1Δ*), showing that not only potassium importers Trk1 and Trk2, but also potassium exporters play an important role in membrane potential regulation. It was interesting to compare the double deletion mutants (TOG, TOC and BW31). If one of the deleted genes was *TOK1* (strains TOG and TOC), λₑq was much lower than for the combination *ena1-4Δnha1Δ* (BW31). In other words, if we consider the K⁺ exporters left in the double mutants, the Tok1 channel alone (in the BW31 strain) is able to keep the membrane potential at a level comparable to that of the wild type, while we cannot say that about the Nha1 antiporter (in the TOG strain) nor about the Ena1-4 ATPases (in the TOC strain). A possible explanation is that Ena1-4 and Nha1 transporters perform separate complementary functions in membrane potential maintenance, both of which can be fully substituted by Tok1p.

**Complementation of the *tok1Δ* depolarization effect**

To make sure that the main depolarization phenotype was really connected to the *tok1* deletion, we transformed four of the tested strains (W303, TOW, BW31 and TOB) with a plasmid designed for *TOK1* overexpression. All of them were

![Fig. 1. Hygromycin B sensitivity of yeast strains carrying various combinations of mutations on YNB medium. Drop lines of equal initial OD were spotted on YNB medium with hygromycin B concentration increasing gradually from left to right and incubated for 2 days at 30 °C. The drop lines 1–3 correspond to the BW31 (*ena1-4Δnha1ΔTOK1 KHA1*), LMB 01 (*ena1-4Δnha1ΔTOK1 kha1Δ*) and LMB 02 (*ena1-4Δnha1Δtok1-kha1Δ*) strains, respectively.](http://femsyr.oxfordjournals.org/)

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transformed with the GFP-labelled TOK1 gene in the pYEX vector under the regulation of the inducible CUP1 promoter, and with the same vector without the TOK1 gene for control. All the transformed strains were inspected by fluorescence microscopy for the correct localization of the Tok1 protein. Figure 3 shows an example of the GFP-labelled Tok1 protein localization in the plasma membrane, while GFP expressed from the empty pYEX vector is dissolved in the cytosol. It is important to note that the fluorescence parameters of GFP (excitation 450–480 nm, emission 490–515 nm) are considerably different from those of the diS-C3(3) probe (excitation 531 nm, emission 560–590 nm), so the presence of GFP did not interfere with diS-C3(3) fluorescence measurements.

For the transformed strains, the incubation medium had to be changed – YNB supplemented with adenine, histidine, leucine and tryptophan was used instead of rich YPD medium, and 20 μM CuSO₄ was added for CUP1 promoter induction.

Overexpression of the TOK1 gene from the plasmid in the TOB strain (ena1-4Δnha1Δtok1Δ) resulted not only in regeneration of the parental-strain level of membrane potential, but even in hyperpolarization of the cells (Fig. 4). The BW31 cells (ena1-4Δnha1Δ) were also slightly hyperpolarized when overexpressing the TOK1 gene from the plasmid (Fig. 4). Similar results, namely a slight hyperpolarization, were also obtained for the W303 and TOW strains overexpressing the TOK1 gene (not shown). These results

Fig. 2. Relative membrane potential of yeast cells grown on YPD media, as reflected by the equilibrium position of the emission spectrum maximum \( \lambda_{\text{eq, max}} \) (nm). At least four samples from at least two independent experiments were measured; the average values are shown and error bars correspond to the SD. Strain genotypes: W303 (wild type), TOW (tok1Δ), GW19 (ena1-4Δ), TOG (ena1-4Δtok1Δ), CW25 (nha1Δ), TOC (nha1Δtok1Δ), BW31 (ena1-4Δnha1Δ) and TOB (ena1-4Δnha1Δtok1Δ), respectively.

Fig. 3. The fluorescence of W303 cells expressing N-terminally green-fluorescent-protein-tagged Tok1p (a) or green fluorescent protein alone (b). Differential interference contrast photos were obtained using Nomarski optics.

Fig. 4. Relative membrane potential of transformed yeast cells grown on YNB media, as reflected by the equilibrium position of the emission spectrum maximum \( \lambda_{\text{eq, max}} \) (nm). At least four samples from at least two independent experiments were measured; the average values are shown and error bars correspond to the SD. Strain genotypes: BW31 (ena1-4Δnha1Δ) transformed with empty pYEX or with the TOK1 gene in pYEX, TOB (ena1-4Δnha1Δtok1Δ) transformed with empty pYEX or with the TOK1 gene in pYEX.
confirm the important function of the Tok1 protein in membrane potential maintenance.

**Discussion**

We compared the influence of potassium transporters with various transport mechanisms on the maintenance of plasma membrane potential in *S. cerevisiae* cells using a diS-C$_3$(3) fluorescence assay. The method proved to be applicable to such a comparative study, as verified by the observed hyperpolarization of a trk1 trk2 deletion strain (published previously by Madrid et al., 1998).

The proteins studied in this work comprise the electrogenic Ena1-4 ATPases, the Nha1 antiporter, previously believed to be electroneutral (transporting 1 sodium cation against 1 proton) but recently shown to transport more H$^+$ per Na$^+$ cation (Ohgaki et al., 2005), a voltage-gated potassium channel Tok1, and an intracellular antiporter Kha1.

The deletion of the KHA1 gene did not cause any change in the membrane potential of the cells. This means that the hygromycin B sensitivity of the kha1 deletion strains (Fig 2; Maresova & Sychrova, 2005) was probably caused by a feature other than an increased influx of the toxic cation drawn by the negative charge inside the cells. Similar results were shown previously for another intracellular cation antipporter, Nhx1p: the nhx1A mutants were hypersensitive to hygromycin B (Gaxiola et al., 1999; Kinclova-Zimmermannova et al., 2006), but their membrane potential was not changed (Kinclova-Zimmermannova et al., 2006). Nhx1p is believed to influence hygromycin B tolerance by intracellular pH regulation and subsequently compartmentation of the toxic compound (Brett et al., 2005); the role of Kha1p could be similar. The kha1A mutants are sensitive to increased pH, showing the possible role of Kha1p in intracellular pH regulation as well (Maresova & Sychrova, 2005). Further evidence that intracellular transport systems play an important role in hygromycin B sensitivity was shown for the Gef1 chloride channel localized in the membrane of the Golgi apparatus (Gaxiola et al., 1999), but the influence of Gef1p on membrane potential has not been studied yet.

The deletion of the ENA1-4 and NHA1 genes in a strain expressing the TOK1 gene did not change the membrane potential more than the tok1 deletion, although both these transport systems are electrogenic and able to export K$^+$ actively. This could be a result of the fact that, under the standard cultivation conditions used in this study (low pH of the growth media, no salt stress), both of these systems are relatively weakly expressed (Banuelos et al., 1998). It was shown recently that the overexpression of Nha1p hyperpolarized the cells (Kinclova-Zimmermannova et al., 2006), but the overexpression of Ena1p has not yet been explored.

The presence of Ena1-4p and Nha1p was more important for the membrane potential regulation of cells lacking the TOK1 gene: a triple mutant (ena1-4Δnha1Δtok1Δ) was obviously more depolarized than a single tok1Δ mutant, indicating that these transporters could partially compensate for the function of Tok1p in membrane potential regeneration. However, in order to keep the potential without the contribution of the Tok1 channel at a value comparable to that of the wild type, both systems (Nha1 and Ena1-4) must be present, while the Tok1 channel is able to carry out this function on its own.

The significant influence of Tok1p on membrane potential regulation (shown both by the depolarization of tok1 mutants and by the hyperpolarization of cells overexpressing the TOK1 gene) was quite surprising, as tok1 deletion does not influence the sensitivity of the cells to high or extremely low K$^+$ concentrations (Bertl et al., 2003; Maresova & Sychrova, 2005), nor to the toxic cation hygromycin B. Moreover, under standard cultivation conditions, there was no measurable K$^+$ efflux from BW31 cells (ena1-4Δnha1Δ) at pH 5.5 or 8.0 (Kinclova et al., 2001); although these cells possessed the TOK1 gene, the intracellular K$^+$ concentration was about 300 mM and cells were incubated in a K$^+$-free buffer. Most probably the membrane potential was keeping the Tok1 channel closed and K$^+$ cations inside the cells in spite of a strong concentration gradient.

An alternative explanation of the Tok1 contribution to $\lambda_{\text{max}}^{\text{ep}}$ changes would be possible if the Tok1 channel were a conduit of cyanine dyes, as was shown, for example, for DDA$^+$ (dibenzyl(dimethylammonium) and thiamine uptake systems (Barts et al., 1980). However, it does not seem very likely that a channel highly specific for K$^+$ would transport organic cations [diS-C$_3$(3) and other used fluorescence probes] into the cells.

Although channels are not connected to energy sources and cannot transport ions actively, there is good evidence that Tok1p displays strong outward rectification and its gating is precisely regulated by membrane voltage, by intracellular and extracellular K$^+$, and by extracellular Ca$^{2+}$ (Bertl et al., 1992, 1993). Our results correspond well with electrophysiological measurements showing that depolarization of the plasma membrane activates the Tok1 channel (Bertl et al., 1993). The depolarization of the plasma membrane is probably not a direct consequence of tok1 deletion. However, when the potential decreases (for example via K$^+$ uptake, which is a natural physiological action), cells lacking the TOK1 gene cannot react by activating Tok1p and therefore they stay depolarized, as shown by the lower diS-C$_3$(3) staining level of tok1Δ mutants. These results support the hypothesis that one of the physiological roles of the Tok1 channel might be the regeneration of membrane potential when the cells become depolarized.
Acknowledgements

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Authors contribution

L.M. and E.U. contributed equally to this work.

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