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Review

Secrets of the fungus-specific potassium channel TOK family

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Several families of potassium (K^+) channels are found in membranes of all eukaryotes, underlining the importance of K^+ uptake and redistribution within and between cells and organs. Among them, TOK (tandem-pore outward-rectifying K^+) channels consist of eight transmembrane domains and two pore domains per subunit organized in dimers. These channels were originally studied in yeast, but recent identifications and characterizations in filamentous fungi shed new light on this fungus-specific K^+ channel family. Although their actual function *in vivo* is often puzzling, recent works indicate a role in cellular K^+ homeostasis and even suggest a role in plant–fungus symbioses. This review aims at synthesizing the current knowledge on fungal TOK channels and discussing their potential role in yeasts and filamentous fungi.

Highlights

The tandem-pore outward-rectifying potassium (K^+) channel family (TOK) is found only in fungi. Therefore, a specific role in fungus physiology can be assumed.

ScTOK1 was the first TOK channel characterized in *Saccharomyces cerevisiae* that displayed a unique structure: two pore domains and eight transmembrane domains per subunit. ScTOK1 induces mainly outward K^+ currents upon membrane depolarization, and is highly regulated.

The gating model of TOK channels has evolved over the years and involves external and internal K^+ concentration, potential binding sites, and membrane potential.

TOK channels have also been found recently in filamentous fungi, and light has been shed on their potential role in beneficial and pathogenic interactions with host organisms.

Potassium channel families in fungi

In yeast and **filamentous fungi** (see Glossary), multiple families of potassium (K^+) transport systems have been identified and characterized [1–3]. Among the known K^+ **ion channels** in living organisms [4], two main groups can be described: channels with two-**pore domains**, TOK-types (tandem-pore outward-rectifying K^+, Figure 1A) and K2P-types (two-pore domain K^+, Figure 1B); and those with a single pore domain, SKC-types (*Shaker*-like K^+ channels, Figure 1C) and Kir-types (inward rectifier K^+, Figure 1D). SKC-type channels have been found in animals [5,6], plants [7,8], and fungi [9]; **K2P channels** in plants and animals [10]; and **Kir channels** in animals [11], plants [12], and bacteria [13]. Strikingly, **TOK channels**, initially described in yeast [14,15], seem to be fungus-specific. A phylogenetic analysis of putative TOK sequences identified in selected genomes representative of different fungal phyla, and of different lifestyles, revealed that two subfamilies (TOK1 and TOK2) exist in Ascomycetes and Basidiomycetes [16]. Interestingly, many **ectomycorrhizal** (**ECM**) or **endophytic** fungal species have at least one TOK-type sequence [2,16,17] (see also for further ongoing genome data: https://mycocosm.jgi.doe.gov/mycocosm/home [18,19]). Intriguingly, none have been identified in the genomes of **arbuscular mycorrhizal fungi** available so far [20–28]; however, these fungi have other types of K^+ channels and transporters [2]. Because of this fungus-specific feature, and the efforts recently made to understand the role of these channels in fungal biology, here we decided to synthesize the historical development of research on TOK-type channels and the current knowledge of their function in yeast and filamentous fungi.

*Sc*TOK1 from *Saccharomyces cerevisiae*, the first TOK channel identified

In the 1990s, advanced patch-clamp techniques on *S. cerevisiae* spheroplasts and protoplasts allowed the functional characterization of new ion channels *in vivo* before the corresponding genes and proteins had been identified [29,30]. Later, the first member of a new family of K^+ channels was described in *S. cerevisiae* by four laboratories almost simultaneously and was named TOK1, YKC1, DUK1, and YORK [14,15,31,32]. A consensus on the name '*Sc*TOK1' was

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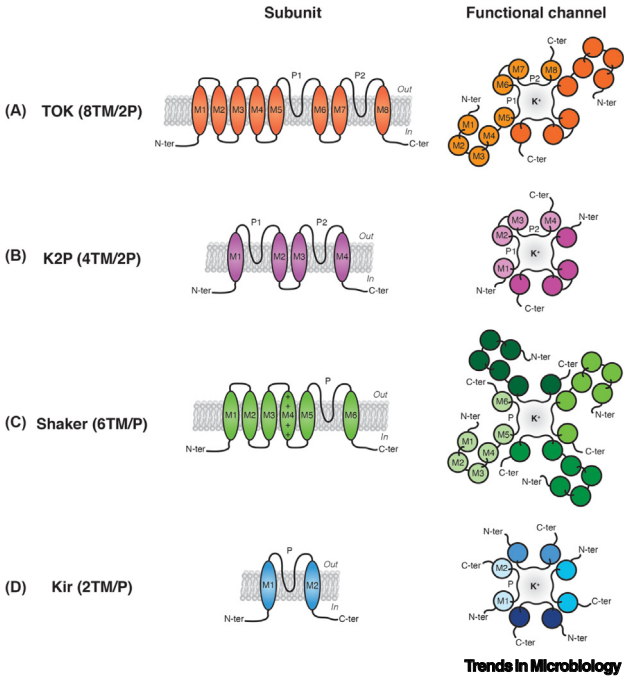

Figure 1. Structure of the four main types of potassium channels. For each type, one subunit and the functional potassium (K^+) channel are described. (A) The tandem-pore outward-rectifying K^+ (TOK) channel subunit consists of eight transmembrane domains (TMs) and two pore (P) domains (8TM/2P) between TM 5 and 6 and between TM 7 and 8. Functional TOK channels are dimers and were identified in fungi only. (B) The two-pore domain (K2P) channel subunit consists of four TMs and two P domains (4TM/2P) between TM 1 and 2 and between TM 3 and 4. Functional K2P channels are dimers and were identified in plants and animals, but not in fungi. (C) The *Shaker* channel subunit consists of six TM and one P domain (6TM/P) between TM 5 and 6. The fourth transmembrane domain (TM4) contains positively charged amino acids with a voltage-sensing function. Functional *Shaker* channels are tetramers and were identified in animals, plants, and fungi. (D) The inward rectifier K^+ (Kir) channel subunit consists of two TMs and one P domain (2TM/P). Functional Kir channels are tetramers and were identified mainly in animals, rarely in plants. The bacterial KcsA channel is thought to be the basic structure of the K^+ selective channels.

chosen. This channel harbors two pore domains in tandem with a conserved eight-amino-acid sequence, determining the selectivity, and eight **transmembrane domains** (**TMs**), assembling putatively as dimers (Figure 1A) [33–35]. This first description of channels with two pore domains in the same polypeptide marked the discovery of a brand-new type of K^+ channels. Excitingly, the discovery of *Sc*TOK1 resulted in the later identification of other types of two-pore K^+ channels, having only four TMs, named K2P (Figure 1B) in animals [15,32,36] and plants [37–39].

It was originally thought that the structure of *Sc*TOK1 resulted from the duplication of the pore domains [32]. However, a more recent strategy of splitting *Sc*TOK1 into two cationic channels, named TOK1A and TOK1B, revealed that both were functional, indicating that TOK channels could have evolved from the fusion of a ***Shaker*-type channel** (six TMs) and an inwardly rectifying subunit (two TMs) [40]. Additionally, the origin of fungal TOK channels seems to be distinct from animal or plant two-pore channels, according to differences in the conservation of the GYGD pore motif that determines K^+ selectivity [16,40]. In fact, in animal two-pore channels, the first pore is rather conserved (GYGx) but the second pore varies between species (GL/FG). In contrast, *Sc*TOK1 harbors GLGD in the first pore, but GYGD in the second one. A remarkable trait of *Sc*TOK1 is the lack of the voltage-sensing TM described for voltage-gated channels. More recently, TOK channels have also been found and described in other yeasts [41], including non-conventional yeasts *Kluyveromyces marxianus* and *Rhodotorula toruloides* [42], *Ca*TOK (*Candida albicans*) [33,43], *Cn*TOK (*Cryptococcus neoformans* var. *neoformans*), and H99TOK (*C. neoformans* var. *grubii*) [33,41,44]. Interestingly, compared to the other TOKs, the *Cn*TOK and H99TOK of *C. neoformans* display flipped pore domains, GYGD in pore #2 and GFGD as pore #1, like the animal 4-TM two-pore channels K2P. This raises questions regarding the evolutionary and functional properties of these channels (see Outstanding questions).

Glossary

Arbuscular mycorrhizal fungi: fungi forming symbiotic associations with the roots of most land plants; they belong to the phylum Mucoromycota, subphylum Glomeromycotina.

Depolarization: a shift in the distribution of charges across a biological membrane, where the cytosol is less negative than at hyperpolarized resting potentials.

Ectomycorrhizal (ECM) fungi: fungi forming symbiotic associations with roots from trees and shrubs; they belong to the phyla Ascomycota and Basidiomycota.

Endophytic fungi: fungi that internally colonize terrestrial plant tissues. Some of them can be beneficial and others can have a neutral effect on plants.

Equilibrium potential: an electrochemical gradient for which no net currents/fluxes will flow across the cellular membrane; it is determined by the membrane potential and the external and internal ion concentrations.

Filamentous fungi: a generic name for fungi (non-taxonomic group) that describes the way they grow: a network of mycelium made of hyphae that look like filaments.

Gating: conformational change of an ion channel by membrane potential in which concentrations or ligands allow the opening or closure of the permeation pathway.

Ion channel: membrane proteins, belonging to transport systems, mediating ion currents across cellular membranes, mostly regulated by membrane potential or ligands.

K2P channel: two-pore domain potassium (K^+) channel, identified in animals and plants, formed by two subunits organized in dimers. Each subunit consists of four transmembrane domains and two pore domains.

Kir channel: inwardly rectifying potassium channel formed by four subunits organized in tetramers, mostly found in animals (but rarely also in plants). Each subunit consists of two transmembrane domains and one pore domain, a minimal structure corresponding to the bacterial KcsA member.

Membrane potential: the difference in electrical potential between the cytosol and the extracellular medium, caused by concentration gradients of charged ions and molecules.

Functional properties and regulation of *Sc*TOK1

Concerning its function, *Sc*TOK1 elicited mainly **outwardly rectifying** K^+ **currents** upon membrane **depolarization** in yeast and when expressed in *Xenopus laevis* oocytes, and was found strongly selective for K^+ over sodium (Na^+) [14,15,29–32,45]. However, small inward currents were later detected in some TOKs in relation to K^+ concentrations when the **membrane potential** was below the **equilibrium potential** for K^+ (E_K) [34,46,47], explaining the uptake of K^+ observed previously in yeast growth complementation assays [48]. Concerning K^+ dependence of the outward currents, it has been shown that the activity of *Sc*TOK1 is affected by extracellular K^+ since changes in the concentration caused shifts in the activation threshold of the channel, displacing the current curve on the voltage axis [49,50]. In *X. laevis* oocytes and in yeast cells, the activation potential of *Sc*TOK1 decreased with lower K^+ concentration towards more negative potentials, indicating potential current activation at more physiological membrane potentials and an overall stronger activity [14,49,50]. Surprisingly, it has additionally been proposed that *Sc*TOK1 activity was also controlled by the internal K^+ concentrations [50], indicating that high cytosolic K^+ would favor shift of the activation potential towards more negative potentials and thus allow opening of the channel leading to K^+ efflux. The sensing of K^+ concentrations at each side of the membrane would drive its activity, rather than the gradient between them, contrasting with previous data obtained in yeast [31].

Inhibition and pH regulation were studied by the external use of several cations or other chemicals known to block other K^+ channels (Figure 2). For example, *Sc*TOK1 was inhibited by the external application of the classical blocker tetraethylammonium (TEA) [14,15,31] (Figure 2), but not by cesium (Cs^+) [15,48] or external protons (H^+) [15,50]. The physiological reason for external pH independence might be that *S. cerevisiae* can live in a broad range of pH and would need its ion channels to be insensitive to fluctuating proton concentrations in order to regulate the homeostasis of electrogenic ions. However, *Sc*TOK1 was inhibited by internal acidic pH [15], probably through the protonation of histidine residues in the intracellular segments (Figure 2). Divalent barium cations (Ba^{2+}) are another inhibitor of the channel, affecting its response to positive voltages [14,15,29,49] (Figure 2). In contrast, Zhou *et al.* [31] and Lesage *et al.* [15] confirmed that the **gating** of *Sc*TOK1 was not affected by the external presence of divalent cations, such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), as was initially assumed [14].

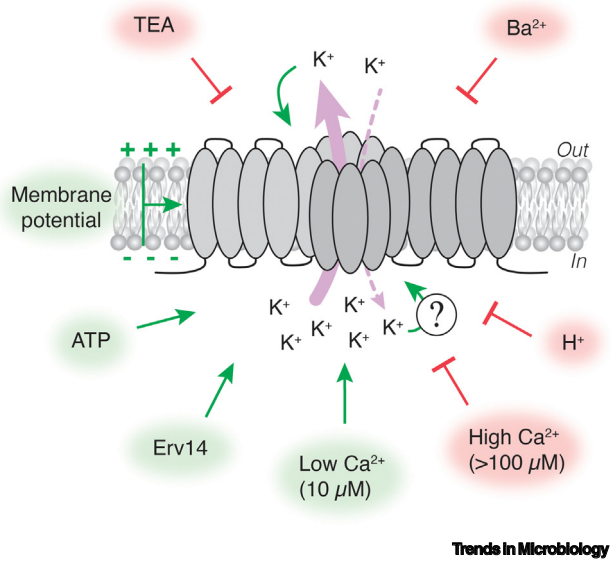

Figure 2. Regulation of *Sc*TOK1, the tandem-pore outward-rectifying K^+ (TOK) channel in *Saccharomyces cerevisiae,* by extracellular and intracellular factors. This figure describes regulations by various factors that can impact the activation or inhibition of the function of *Sc*TOK1 from *S. cerevisiae*. Inhibition is mediated by external application of tetraethylammonium (TEA) and barium (Ba^{2+}), as well as by cytosolic acidification (H^+) and high calcium (Ca^{2+}) concentrations (>100 μM). Activation of *Sc*TOK1 is mediated by external potassium concentrations (K^+), as well as intracellular factors such as cytosolic ATP, low Ca^{2+} concentrations (10 μM), membrane depolarization, and the direct interaction of the channel with the endoplasmic reticulum protein ERV14. Changes in intracellular K^+ concentrations might also regulate *Sc*TOK1.

Outwardly rectifying currents: ion currents mediated by an ion channel that opens only upon depolarization, thus allowing efflux of the given ion.
Pathogenic fungi: fungi that cause diseases in other species such as animals or plants. They can lead to severe symptoms and even death of their host. These fungi are mostly intracellular pathogens and take advantage of their 'relationship' with their host to maintain their own life cycle.
Pore domain: part of a transport system that allows ions or molecules to cross (selectively) the membrane.
***Shaker*-type channel:** voltage-dependent potassium-selective channel (also called Kv) formed by four subunits organized in tetramers. Each subunit consists of six transmembrane domains and one pore domain. These channels have been identified in animals, plants, and fungi and can mediate inwardly or outwardly directed K^+ currents.
TOK channel: fungus-specific tandem-pore outward-rectifying K^+ channel formed by two subunits organized in dimers. Each subunit consists of eight transmembrane domains and two pore domains.
Transmembrane domain (TM): part of a protein that is integrated in the lipid bilayer of the cellular membrane.

However, in addition to pH, internal Ca^{2+} concentrations seemed to play an important role in the regulation of *Sc*TOK1 (Figure 2). Free cytosolic Ca^{2+} concentrations in yeast cells are around 350 nM [51]. A moderate release of Ca^{2+} in the cytosol (around 10 μM) induced the opening of *Sc*TOK1, whereas higher Ca^{2+} concentrations (>100 μM) blocked K^+ transport through the channel [30,31]. The activity of *Sc*TOK1 also depends on cytosolic ATP [50] (Figure 2). This ATP-related regulation could be mediated in two ways: either by fixation at a putative ATP-binding site between the TM 4 and 5, or through several putative phosphorylation domains for protein kinases PKA and PKC. While the former still remains to be demonstrated, the latter was confirmed by Lesage *et al.* [15]. The most promising predicted phosphorylation sites are grouped in the long intracellular segment between the two pore domains. More recently, the study by Zimmermannová *et al.* [52] demonstrated the interaction of *Sc*TOK1 with *Sc*Erv14 (Figure 2). *Sc*Erv14 is a transmembrane protein required for the selective trafficking of proteins in COPII vesicles, from the endoplasmic reticulum to the Golgi body. The absence of the *ERV14* gene impaired the localization of *Sc*TOK1 to the plasma membrane and thus its activity in yeast complementation assays.

Additional regulations could arise from the protein itself, namely from conformational changes in response to cytosolic conditions. The so-called 'N-type inactivation', a rapid block of the open channel pore by a configuration change of the N terminus, has been discussed [50], and there is evidence that the C-terminal tail regulates the gating of the inner pore [53,54] (see gating models in the following section).

Regarding the activity of TOK channels in other yeast species (*C. albicans* and *C. neoformans*), H99TOK channels are strictly selective for K^+ while *Cn*TOK and *Ca*TOK transport K^+ and Na^+ [44]. H99TOK showed small inward currents at potentials below E_K. Pharmacological studies have been done for *Ca*TOK, using known inhibitors from animal two-pore channels [55].

Gating of *Sc*TOK1, an evolving model

Models for the gating and regulation of *Sc*TOK1 have evolved over time (Figure 3), starting with electrophysiological studies *in vivo* to heterologously expressed channels. Originally, Bertl and

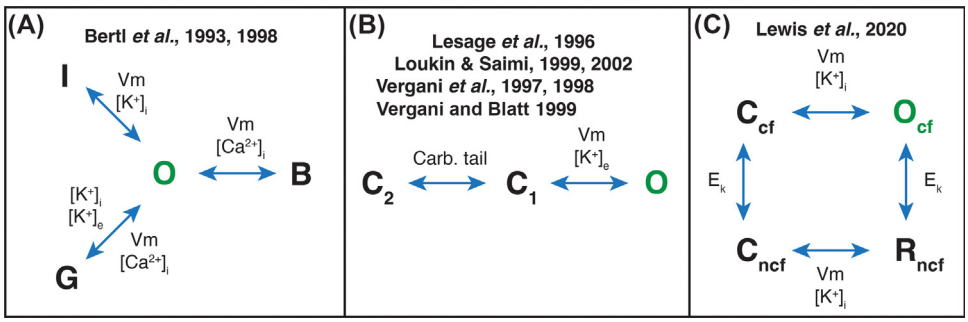

Figure 3. Evolution of the gating model describing the tandem-pore outward-rectifying K^+ (TOK) channel opening and closing. (A) The gating model proposed by Bertl *et al.* [30,50] described the functioning of *Sc*TOK1 with one open state (O) that can switch to three possible closed states named interrupt (I), gap (G), and block (B). The transitions are regulated by the membrane potential (V_m), the internal and external K^+ concentrations ($[K^+]_i$, $[K^+]_e$), and the internal calcium concentration ($[Ca^{2+}]_i$). (B) The gating model proposed by Lesage *et al.* [15], Loukin *et al.* [45], Loukin and Saimi [53,58], Vergani *et al.* [49,56], and Vergani and Blatt [57] is based on an open (O) and (at least) two closed states (shallow state C_1, deep state C_2). Upon depolarization, C_1 would switch to O instantaneously whereas C_2 would change to C_1 and then O more slowly. The transition from C_1 to O would be regulated by V_m and $[K^+]_e$. The switch from C_1 to C_2 would require structural conformation changes of the protein's carboxyl tail (Carb. Tail). (C) The gating model proposed recently by Lewis *et al.* [44] suggests that TOK channels require at least two independent gating mechanisms to open (or close) (such as C_{cf} <–> O_{cf} and R_{ncf} <–> O_{cf}). It is based on the ion-binding sites at the pore entry, within the pore and at an inner cavity. It relies on the dependence on K^+ concentrations at both sides of the membrane and V_m. E_K represents the equilibrium potential for potassium ions, cf means conductive filter, and ncf means nonconductive filter.

colleagues [30,50] mathematically simulated the behavior of this channel in a 4-state model, considering its response to membrane potential and K^+ and Ca^{2+} concentrations (Figure 3A). Their model proposed an open state (O) that can switch to three possible closed states depending on the duration: I (interrupt, less than 1 ms), B (block, 2–3 ms), and G (gap, hundreds of milliseconds), with different transition mechanisms (and dynamics/kinetics). The G➔O transition is sensitive to external and internal K^+ concentrations, I➔O to internal K^+ concentration and B➔O to none of them. In this model, cytoplasmic Ca^{2+} plays a role in the activation of the channel by inducing the G➔O state at increased concentrations. However, at very positive voltages, Ca^{2+} causes a block of the channel (Figure 3A). The authors of this model speculated that *Sc*TOK1 could execute conformational changes that affect the gating process.

Similarly, Lesage *et al.* [15] tried to elucidate the gating mechanism of *Sc*TOK1 (Figure 3B) by using two-electrode voltage- and patch-clamp approaches in *X. laevis*. This second model suggested that the protein would have three different conformations: a deep blocked state (C2), a shallow blocked state (C1), and an open state (O) (Figure 3B). A peptide region of the protein would block the pore by binding to two sites that correspond to the C2 and C1 conformations. In a normal situation at polarized (negative) membrane potentials, the channels would remain closed at C1 or C2. Upon depolarizing membrane potentials, the proteins at C1 would change to O (open channels, instantaneous component of the observed currents), while those at C2 would change first to C1 and subsequently to O (delayed, time-dependent current component). This model was further elaborated by detailed functional analyses of *Sc*TOK1 wild-type and mutant channels. Vergani *et al.* [49,56] and Vergani and Blatt [57] demonstrated that not only voltage played a role in the transitions between C1, C2, and O, but the external K^+ concentrations also affected the process between C1 and C2. Similarly, Loukin and Saimi [58] underlined the importance of voltage and external K^+, as well as temperature, for the gating. Moreover, the role of the pore domain in the channel gating was introduced, in addition to the classical concept of selectivity [45,56,57]. Their model involved K^+ binding sites in the pore region responsible for conformation changes mediating gating. The authors supported this hypothesis as the one that best suited the results obtained in electrophysiology. Later, this model was further expanded by introducing the carboxyl-tail domain interacting with an inner gate [53].

In addition, regulation of TOK channels by an N-type inactivation was discussed, as there is some homology with other ion channels with such a mechanism [50]. Furthermore, a possible gating by protein phosphorylation was mentioned [45].

To summarize, the regulation of the gating of *Sc*TOK1 is a controversial issue in the literature. As shown before, *Sc*TOK1 is regulated by the K^+ concentrations at both sides of the membrane (Figure 2). Bertl *et al.* [50] supported the hypothesis of two binding sites for K^+, one at each side of the membrane. If this were the case, K^+ permeation would indeed depend on both K^+ concentrations, rather than the gradient between them. In this context, a relatively simple gating model was more recently developed based on the assumption of ion binding sites in the pore domain, at the pore entry, within the pore, and at an inner cavity, as well as on the interaction of two independent gating mechanisms [44]. K^+ concentrations would determine the ion occupancy of the binding sites, thus playing a role in the K^+-dependent gating, and the membrane potential would determine the internal gate. Altogether, outward currents are only mediated in the case of an open gate and a conductive filter (Figure 3C). In all three closed situations, outward currents were not enabled. This model nicely explained the dependence on K^+ concentrations at both sides of the membrane and was validated by experimental data with different TOKs from **pathogenic fungi** and yeasts and with channel mutants [44]. Similarly, gating via the selective pore (C-type gating) was recently described in a structural study of a representative

member of the animal two-pore channels [59]. Even if all these detailed electrophysiological studies and modeling appear to be a playground of sophistic and theoretic analyses, such studies might help to understand the physiological role of these channels, explaining their ongoing interest.

Role of *Sc*TOK1 in yeast physiology

Despite the numerous (mostly biophysical) studies on *Sc*TOK1, the exact role of TOK channels in yeast is currently unknown. Indeed, many researches have failed to demonstrate or explain a specific situation in which their absence or mutation is deleterious. The natural abundance of *Sc*TOK1 in yeast cells was estimated at approximately 40 proteins per cell (5–6 μm in diameter) [30]. *Sc*TOK1 is likely involved in K^+ homeostasis, membrane potential maintenance, and osmoregulation [60], and it could have implications in the early response to osmotic stress [61]. Additionally, there was suspicion of a more complicated function of *Sc*TOK1 in *S. cerevisiae* [50]. In natural physiological conditions and in laboratory cultures, the membrane potential of yeast cells is very negative, ranging between –100 and –200 mV, which is far from the threshold of *Sc*TOK1 activation. In this situation, the channel could mediate influx of K^+. With plasma membrane depolarization, the channel would be activated and stabilize the membrane potential near the equilibrium potential for K^+. This would reduce the gradient between its internal and external concentrations and substitute the K^+-driven transport by H^+-coupled nutrient uptake. However, this putative role still remains to be demonstrated.

Another function has been described for these K^+ efflux channels in the pathogenic yeast *C. albicans*. Several authors have proposed that their activation might be the first step in programmed cell death induced by the antimicrobial proteins lactoferrin [62] and histatin 5 [33], by the silymarin extract from milk thistle [63], or by chlorogenic acid [64]. In these cases, the release of K^+ from the cytosol would result in cell shrinkage in apoptotic processes. Similarly, the viral-coded polypeptide toxin K1 caused cell death in *S. cerevisiae* by the activation of the plasma membrane channel TOK1p, demonstrated by an increase of the open-state probability at the level of single channel activities [65]. Interestingly, the toxin effect on yeast cell survival and K^+ fluxes was clearly linked to the presence of the TOK channel since its genetic deletion conferred resistance to the toxin, whereas its overexpression increased toxin-induced K^+ efflux.

TOK channels in filamentous fungi

TOK channels have also been identified in filamentous fungi, and some have been functionally characterized (Table 1) – first in *Neurospora crassa* with *Nc*TOKA [34]. To determine the biophysical properties of this channel, heterologous expression of *Nc*TOKA in yeast was used in combination with the patch-clamp technique. Whole-cell outward currents were recorded, indicating an efflux activity. Additionally, deficient yeasts for K^+ uptake (*trk1&2* mutants) were complemented with *Nc*TOKA and were able to grow on low K^+ media, indicating that *Nc*TOKA could also be involved in K^+ influx. As seen in *Sc*TOK1, the gating regulation of *Nc*TOKA also involved variation in extracellular K^+ concentrations, TEA, and quinine, but also extracellular Ca^{2+}.

More recently, three members of the TOK channel family have been identified in the genome of the pine-associating ECM fungus *Hebeloma cylindrosporum*, and their role in symbiotic plant nutrition was investigated [16,66]. The three TOK systems have been named *Hc*TOK1, *Hc*TOK2.1, and *Hc*TOK2.2 according to their different structure and separation into two subfamilies. The two-electrode voltage-clamp approach was used to determine their functional characterization in *X. laevis* oocytes (Table 1). *Hc*TOK1 and *Hc*TOK2.1 clearly showed outwardly rectifying

Table 1. Fungal species in which TOK channels have been identified and characterized

	Phylum	Species	TOK channel name	Refs
Yeasts				
	Ascomycota	*Saccharomyces cerevisiae*	*Sc*TOK1[a,b]	Ketchum *et al.* [14] Lesage *et al.* [15] Reid *et al.* [32] Zhou *et al.*[31]
		Candida albicans	*Ca*TOK[a,c]	Baev *et al.* [33] Lewis *et al.* [44]
	Basidiomycota	*Cryptococcus neoformans* var. *grubii*	H99TOK[a]	Lewis *et al.* [44]
		Cryptococcus neoformans var. *neoformans*	*Cn*TOK[a]	Lewis *et al.* [44]
Filamentous fungi				
	Ascomycota	*Aspergillus fumigatus*	*Af*TOK[a]	Lewis *et al.* [44]
		Neurospora crassa	*Nc*TOKA[d]	Roberts [34]
		Fusarium graminearum	*Fg*TOK1[a]	Manville *et al.* [68]
		Mycosphaerella graminicola	*Mg*TOK1[a]	Manville *et al.* [68]
	Basidiomycota	*Hebeloma cylindrosporum*	*Hc*TOK1[a,e] *Hc*TOK2.1[a] *Hc*TOK2.2[e]	Guerrero-Galán *et al.* [16,66]

Methods of characterization: [a]characterized by electrophysiological assays with heterologous expression in *X. laevis* oocytes; [b]characterized by growth complementation and electrophysiological assay in *S. cerevisiae* strain αW303zJJO911 (αW303, *ykclΔ*::URA3); [c]characterized by growth complementation and electrophysiological assays with *C. albicans* strain CAI4 and subsequently generated deletion strains DBT-1, -2, -3, -4; [d]characterized by growth complementation and electrophysiological assays in *S. cerevisiae* mutant strain *WΔ3TOK1Δ* (*MATa ura3 his3 trp1 ade2 trk1Δ::LEU2 trk2Δ::HIS3 tok1Δ::TRP1*); [e]characterized by growth complementation assays in *S. cerevisiae* mutant strain PLY246 (*trk1Δ trk2Δ tok1Δ*).

currents, suggesting that they are capable of K^+ efflux, while no result was obtained in oocytes expressing *Hc*TOK2.2. Contrary to what is described in yeast (Figure 2), currents from *Hc*TOK2.1 seem to be activated at low pH, which can be a potential advantage in soils having pH ranges like the pine forest soils where *H. cylindrosporum* is living. *Hc*TOK1 and *Hc*TOK2.2 were also able to complement K^+-transport deficient yeast strains, suggesting their role in K^+ influx. To investigate their role in symbiotic association with pine roots, *in situ* hybridization and overexpression approaches were performed for the first time with any TOK channel. Although all *HcTOK* transcripts were localized in the area where nutrients are exchanged between pine roots and the fungus, only *Hc*TOK2.1 and *Hc*TOK2.2 were able to provide more K^+ to the plants when overexpressed [16]. Moreover, *HcTOK2.2* expression was induced in the presence of the host root and in mycorrhizal association compared to free-living hyphae, indicating a role of this channel for K^+ delivery towards host trees. Consequently, since members of the TOK2 subfamily have not been identified in yeast, and not in all filamentous fungi, it is worth hypothesizing that these channels could be specifically involved in symbiotic relationships with host organisms. Investigation of TOK2-type genes and proteins in other fungi is therefore needed to assess their physiological function in axenic and symbiotic conditions.

Concerning *Hc*TOK1, no symbiotic role was suggested, and it seems to be involved in the overall K^+ homeostasis of *H. cylindrosporum* [66]. Altogether, these studies revealed the first description of TOK channels in the context of a symbiotic interaction with plants, where they might play a critical role in plant adaptation to K^+-limited soils. Additional research concerning this specific role in plant nutrition mediated by beneficial symbiosis is needed since TOK channels were identified in

many ECM fungi [2,16]. Moreover, TOK channels have also been found and described in endophytic fungi [17], among them the well-studied endosymbiotic model fungus *Serendipita indica* (former *Piriformospora indica*) [67]. Interestingly, one TOK member, named *SiTOK1*, showed a slight induction in contact with the host [67], reminiscent of the mentioned finding with *HcTOK2.2*. Phylogenetically, this TOK member seems to be more related to the TOK2-type channels. However, the physiological function of the K^+ efflux channel TOK for this kind of fungal lifestyle has not yet been revealed.

Several other TOKs from pathogenic fungi were identified and characterized (Table 1), first from phytopathogenic fungi [68], *Mg*TOK1 from *Mycosphaerella graminicola* (current name: *Zymoseptoria tritici*; wheat leaf blotch), *Fg*TOK1 from *Fusarium graminearum* (wheat head blight), and *Af*TOK1 from a filamentous human pathogen *Aspergillus fumigatus* [44]. All of them operate as K^+ outward rectifiers, obviously mediating K^+ efflux. Like other characterized TOK channels, *Af*TOK1 produced strong outwardly rectifying K^+ currents, strictly selective for K^+, in two-electrode voltage-clamp experiments in *X. laevis* oocytes. Contrary to other fungi described above, no inward currents were observed. Regarding its gating, *Af*TOK1 can be accommodated by a similar three-state model as described by Loukin *et al.* [45], but a new model was proposed (Figure 3C; see previous text). Moreover, *Mg*TOK1 was tested pharmacologically for known inhibitors from animal channels [55] and was found to be regulated by protein kinases PKC and PKA [69].

As for the three members of *Hc*TOK in *H. cylindrosporum*, the studies of TOKs from pathogenic fungi, and also yeasts (see previous text), indicate that members of the TOK channel family display specific rather than redundant functions regarding their respective properties. However, more structure–function studies would be needed to enable prediction of functional properties. So far, the subfamily TOK2, having a specific structure, namely a longer linker region between TM 6 and 7, was found only in Basidiomycota [16], and may fulfill specific physiological functions in these fungal species. Expansion of fungal gene families was recently discussed in the context of adaptation to different lifestyles and ecological conditions and was called adaptasome [70]. The TOK channel family might be part of this fungus-specific plasticity and adaptation linked to host interactions.

Concluding remarks and future perspectives

Altogether, recent identification of TOK channel members in mycorrhizal, endophytic, and pathogenic fungi open a new field of studies to advance the understanding of their physiological roles in fungi (or/and in interaction with their hosts), as well as our understanding of their structure–function relationships. Biophysical, transcriptional, and functional studies with these new members, distributed in two different subfamilies, will certainly clarify their functioning, regulation, and gating. Characterization and localization of three TOK members within the ECM fungus *H. cylindrosporum* [16,66] represent a first step in this direction but have yet to be completed. Symbiosis-induced expression of one of these members highlighted a potential specific role of the TOK2-type channel subfamily in mycorrhizal association. Identification and characterization of new members, among them four TOK members from four different human pathogenic fungi, also contribute to the knowledge of this ion channel family [44]. The presence of several members of this channel family in a single fungal species raises the question about their specific activities and biological roles. Moreover, this finding also allows speculation about a possible formation of heteromers [71] that would further increase physiological diversity and regulation. Finally, from the evolutionary point of view, it is fascinating that fungi have developed this specific TOK-type ion channel family. Probably, this ion channel family might be part of a fungus-specific plasticity and adaptation linked to host interactions. We believe that the TOK channel family has evolved in tight relation with the fungal lifestyle(s) in interaction with their hosts, and that we are rather at the beginning of understanding the origin and functional roles of these channels (see Outstanding questions).

Outstanding questions

What is the evolutionary origin of the unique structure of TOK-type channels?

Why are TOK-type channels not present in the genomes of arbuscular mycorrhizal fungi, despite their presence in ectomycorrhizal, endophytic, pathogenic fungi and in yeasts?

What is the specific function of TOK-type channels in yeasts and fungi?

What are the physiological roles of TOK-type channels during beneficial interactions with plants? Would the TOK2-type subfamily play a specific role in symbiosis?

What is the meaning of several TOK channels in one fungus, redundancy or specific roles?

What are the physiological roles of TOK-type channels during pathogenic interactions with their hosts?

Can the TOK channels be specifically targeted to fight against fungal pathogens?

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Declaration of interests

No interests are declared.

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