A Functional EXXEK Motif is Essential for Proton Coupling and Active Glucosinolate Transport by NPF2.11

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The proton-dependent oligopeptide transporter (POT/PTR) family shares a highly conserved E1X1X2E2RFXYY (E1X1X2E2R) motif across all kingdoms of life. This motif is suggested to have a role in proton coupling and active transport in bacterial homologs. For the plant POT/PTR family, also known as the NRT1/Ptr family (NPF), little is known about the role of the E1X1X2E2R motif. Moreover, nothing is known about the role of the X1 and X2 residues within the E1X1X2E2R motif. We used NPF2.11—a proton-coupled glucosinolate (GLS) symporter from Arabidopsis thaliana—to investigate the role of the E1X1X2E2R motif variant in a plant NPF transporter. Using liquid chromatography–mass spectrometry (LC-MS)–based uptake assays and two-electrode voltage clamp (TEVC) electrophysiology, we demonstrate an essential role for the E1X1X2E2R motif for accumulation of substrate by NPF2.11. Our data suggest that the highly conserved E1, E2, and K residues are involved in translocation of protons, as has been proposed for the E1X1X2E2R motif in bacteria. Furthermore, we show that the two residues X1 and X2 in the E1X1X2E2R[K/R] motif are conserved as uncharged amino acids in POT/PTRs from bacteria to mammals and that introducing a positive or negative charge in either position hampers the ability to overaccumulate substrate relative to the assay medium. We hypothesize that introducing a charge at X1 and X2 interferes with the function of the conserved glutamate and lysine residues of the E1X1X2E2K motif and affects the mechanism behind proton coupling.

Keywords: Defense compound transporters • EXXEK motif • NPF2.11 • POTs • Proton-coupling • TEVC electrophysiology.

Abbreviations: GLS, glucosinolates; LC-MS, liquid chromatography–mass spectrometry; I3M, indol-3-ylmethyl glucosinolate; 1MO-13M, 1-methoxyindol-3-ylmethyl glucosinolate; 6MO-13M, 6-methoxyindol-3-ylmethyl glucosinolate; 8MSO, 8-methylsulfanyloctylglucosinolate; 4MTB, 4-methylsulfanybutyl glucosinolate; 4M, 4-methylsulfinylbutyl glucosinolate; NPF, NRT1/Ptr family; POT/PTR, proton-dependent oligopeptide transporter; TEVC, two-electrode voltage clamp; YFP, yellow fluorescent protein.

Introduction

The proton-dependent oligopeptide transporter (POT/PTR) family of transporters are H+substrate symporters found across all kingdoms of life (Daniel et al. 2006, Leran et al. 2014). The POT/PTR family is hypothesized to utilize a common alternating-access transport mechanism, in which two six-transmembrane domain bundles flip around a central substrate-binding cavity. This leads to alternating access of the substrate-binding cavity to either the exterior or interior of the cell, with an intermediate step where the substrate cavity is occluded (reviewed by Law et al. 2008, Forrest et al. 2011, Newstead 2015). POT/PTR transporters share a highly conserved E1X1X2E2RFXYY motif (E1X1X2E2R) (Daniel et al. 2006, Newstead 2015). In the bacterial PepT family, this motif was suggested to have an important role in proton coupling (Solcan et al. 2012). In a recent mutational study of the plant nitrate transporter NRT1.1 (NPF6.3), removal of charged residues in the E1X1X2E2R motif abolished nitrate ion transport (Sun et al. 2014). Apart from this study, the role of the E1X1X2E2R motif in the plant NRT1/Ptr family (NPF) remains poorly characterized and, moreover, the importance of the two amino acid positions denoted by X remains unknown.

The Arabidopsis thaliana genome encodes 53 members of the NPF of transporters characterized by highly diverse substrate specificities spanning from specialized metabolites such as glucosinolates (GLS) (Nour-Eldin et al. 2012) and plant phytohormones (auxin and ABA) (Krouk et al. 2010, Kanno et al. 2012), to primary metabolites, e.g. di- and tripeptides (Dietrich et al. 2004, Komarova et al. 2008, Weichert et al. 2012) and the nitrate ion (Tsy et al. 1993). Among the A. thaliana NPF transporters, two versions of the E1X1X2E2R motif occur, with either a terminal lysine or a terminal arginine. To date, the E1X1X2E2K motif has not been functionally characterized.

In this study, we investigated the role of the E1X1X2E2K motif variant E57T58F59E60K61 found in NPF2.11, a plasma membrane-localized, high-affinity H+/GLS symporter (Nour-Eldin et al. 2012). By mutating key residues of the E1X1X2E2K motif and characterizing mutant transporters by liquid chromatography–mass spectrometry (LC-MS) and two-electrode voltage clamp...
(TEVC) electrophysiology, we showed that the E<sub>1</sub>X<sub>1</sub>X<sub>2</sub>K motif was essential for overaccumulation of GLS substrate relative to the assay medium. Furthermore, we demonstrated that positions X<sub>1</sub> and X<sub>2</sub> influenced the transport properties of NPF2.11 and appeared constrained as neutral residues. How X<sub>1</sub> and X<sub>2</sub> may influence the transport mechanism is discussed.

Results

Charged residues in the E<sub>57</sub>T<sub>58</sub>F<sub>59</sub>E<sub>60</sub>K<sub>61</sub> motif are required for overaccumulation relative to the assay medium

NPF2.11 is a H<sup>+</sup>/GLS symporter that utilizes the inwardly directed proton electrochemical gradient (Δ<sub>Δ</sub>H<sub>U</sub>) between the plant apoplast (pH 5.5) and cytoplasm (pH 7.4) to drive the import of GLS (Nour-Eldin et al. 2012). Here we tested the ability of NPF2.11 to accumulate 4-methylsulfinylbutyl glucosinolate (4MTB) relative to the assay medium. LC-MS analysis of extracts from Xenopus oocytes expressing NPF2.11 showed overaccumulation of 4MTB to levels several fold higher than the concentration in the assay medium (Fig. 1A). The absolute overaccumulation depended on assay time and varied between oocyte batches. This is exemplified with a 3-fold overaccumulation after 1 h uptake (Fig. 1A) and a 10-fold overaccumulation after 1 h (Fig. 6B). We proceeded by substituting the three charged residues in the NPF2.11-E<sub>57</sub>T<sub>58</sub>F<sub>59</sub>E<sub>60</sub>K<sub>61</sub> motif to alanine. The mutant versions were expressed in X. laevis oocytes and effects on transport activity were characterized using LC-MS-based uptake assays and TEVC electrophysiology (see Fig. 1C for a model of NPF2.11 with Glu57 and Glu60 highlighted in green, Thr58 and Phe59 highlighted in magenta and Lys61 highlighted in red).
relative to that of external medium after 1 h of incubation was reduced to 0.1–0.2 (Fig. 1A). Furthermore, currents elicited from oocytes expressing E57A, E60A and K61A were indistinguishable from background currents of non-injected oocytes (Fig. 1B).

**Sequence analysis of the Arabidopsis NPF family E1X1X2E2[K/R] motif**

The roles of the second and third residue (X1 and X2) in the E1X1X2E2[K/R] motif are unknown, and no mutants in these residues, in any POT/PTR gene, have been characterized to date. The denotation by X indicates that these residues could be occupied by any amino acid. We aligned the E1X1X2E2[K/R] motifs in the 53 members of the A. thaliana NPF of transporters and homologs from bacterial, fungal, worm and human POT/ PTR transporters. A subset of the NPF does not encode the canonical E1X1X2E2[K/R] motif (Fig. 2), as also previously reported (Sun et al. 2014). These belong to the NAXT and NRT1.5 subclades (At1g32450, At4g21680 and At5g19640), which appear to encode nitrate transporters (Segonzac et al. 2007, Lin et al. 2008, Li et al. 2010). Among the NPF transporters and POT/PTR homologs encoding the canonical E1X1X2E2[K/R] motif, none contained a charged amino acid in either of the X positions (Fig. 2), suggesting an evolutionary constraint enforced on these residues. In consequence, we hypothesized that the X1 and X2 residues have an important role as uncharged residues to avoid interfering with the E1X1X2E2[K/R] motif and thus the proton-coupling mechanism.

**Mutations of Thr58 and Phe59 abolish the ability to overaccumulate substrate**

Introduction of charged residues instead of T58 or F59 in the NPF2.11-E57T58F59E60K61 motif was performed to test the hypothesis that position X1 and X2 are evolutionarily constrained as uncharged residues to avoid disruption of the proton-coupling mechanism. Mutation of F59 to either a positive residue (arginine) or a negative residue (aspartate) and of T58 to a positive (arginine) or negative residue (aspartate) of the original T58 and F59 residue completely abolished 4MTB accumulation in oocytes (Fig. 3A) and resulted in 4MTB-induced currents indistinguishable from background currents in non-injected oocytes (Fig. 3B). In comparison, the NPF2.11-T58D (T58D) mutant transported 4MTB into oocytes, but accumulation after 1 h was only 10% of NPF2.11 uptake (Fig. 3A). Like the other mutants, T58D did not elicit 4MTB-induced currents (Fig. 3B).

The reduced and apparent non-electrogenic transport activity of the T58D mutant prompted further characterization to reveal the role of the uncharged X1 and X2 residues in the NPF2.11-E57T58F59E60K61 motif with regards to pH dependency, and the ability to overaccumulate substrate.

**Thr58 is not essential for transporter function**

T58 was mutated to a less bulky alanine residue, and the transport activity and kinetics of the NPF2.11-T58A mutant were measured by LC-MS-based uptake assays and TEVC electrophysiology. T58A elicited quantifiable 4MTB-induced currents that enabled determination of its affinity constant towards 4MTB. Plotting currents at –60 mV as a function of increasing 4MTB concentrations yielded a saturation curve, which was fitted by a Michaelis–Menten equation with an apparent affinity constant (Km) of 20.7 ± 1.0 μM and similar 4MTB uptake per oocyte as NPF2.11 (Fig. 4). The Km value is identical to those elicited by wild-type NPF2.11 protein when expressed in oocytes (Nour-Eldin et al. 2012). In contrast, mutation of F59 to leucine caused a decrease in 4MTB transport to 25% of NPF2.11 uptake, indicating that a phenylalanine at position X2 is integral for a functional NPF2.11 (Fig. 4A).

**Does T58D have a reduced expression level?**

An alternative cause for reduced transport activity of T58D could be a reduced expression or changed localization (e.g. to oocyte endomembranes). In one experiment, single oocytes expressing T58A or T58D and non-injected oocytes were analyzed for 4MTB-induced currents and protein expression by Western blot (Fig. 5). T58D-expressing oocytes did not elicit any 4MTB-induced currents, whereas T58A-expressing oocytes had wild-type-like induced currents (Fig. 5A). No significant difference was observed when expression levels of T58A and T58D were quantified by densitometric analysis (Fig. 5C). In a subsequent experiment, localization of T58A and T58D was determined using confocal laser scanning microscopy. Both proteins associated with the periphery of the X. laevis oocytes, indicating an identical localization to the plasma membrane (Fig. 5D–F).

**Does the charge mutant T58D have altered pH dependency?**

We investigated the relative pH dependency of NPF2.11 and T58D transport by conventional LC-MS-based uptake assays because 4MTB-induced currents of T58D-expressing oocytes were below the detection limit. The uptake activity of T58D-expressing oocytes was very low relative to that of NPF2.11-expressing oocytes at all pH values. Therefore, to compare pH dependency between NPF2.11 and T58D, accumulation at pH 5, pH 6 and pH 7 was normalized to the uptake at pH 5 for each transporter. For both NPF2.11 and T58D, uptake at pH 7 was below the detection limit (Fig. 6A). Interestingly, at pH 6, T58D-expressing oocytes had a significantly lower relative uptake of 4MTB compared with oocytes expressing NPF2.11. This indicates that insertion of an aspartate residue at this position could influence the pKa of E57 (the closest amino acid of the NPF2.11-E57T58F59E60K61 motif).

**Is T58D a passive facilitator?**

We speculated that the T58D mutation could have transformed the NPF2.11 transporter into a passive facilitator of 4MTB; therefore, we investigated the concentration of imported 4MTB over time. After incubation in 100 μM 4MTB for 1 h, oocytes expressing NPF2.11 contained an 11-fold higher concentration of 4MTB compared with the external assay medium. In comparison, oocytes expressing T58D only contained 0.7 times the concentration of the external medium.
**Fig. 2** Alignment of the E$_1$X$_1$X$_2$E$_2$[K/R] motifs of the 53 Arabidopsis NPF transporters and characterized bacterial, fungal, worm and mammalian POT/PTR transporters. Highlighted in black and gray are, respectively, conserved glutamine residues and the less conserved arginine and lysine residues. (A) Aligned E$_1$X$_1$X$_2$E$_2$[K/R] motifs from 53 Arabidopsis transporters that are grouped based on experimental characterization. Abbreviations: ARABIDOPSIS THALIANA GLUCOSINOLATE TRANSPORTER (GTR) (Nour-Eldin et al. 2012), NITRATE EXCRETION TRANSPORTERS (NAXT) (Segonzac et al. 2007), ABA-IMPORTING TRANSPORTER (AIT) (Kanno et al. 2012) and NITRATE TRANSPORTERS (NRT) (Tsay et al. 1993, Chiu et al. 2004, Almagro et al. 2008, Fan et al. 2009, Li et al. 2010, Wang and Tsay 2011, Hsu and Tsay 2013). (B) Alignment of the E$_1$X$_1$X$_2$E$_2$R motifs from characterized bacterial, fungal, worm and mammalian POT/PTR transporters. Transporters are identified by gene identifier code (GI) and grouped based on species. Transporters have previously been described (Daniel et al. 2006a, Newstead et al. 2011, Solcan et al. 2012, Doki et al. 2013, Guettou et al. 2013).
Non-injected oocytes did not accumulate GLS above the detection level at any time point during the 4 h uptake assay (data not shown). The internal concentration of 4MTB in oocytes expressing NPF2.11 relative to the concentration of the external assay medium increased to approximately 10-fold after 1 h incubation and continued to increase to 19-fold after 4 h incubation. For oocytes expressing T58D, the internal concentration of 4MTB after 1 h incubation was 0.7-fold relative to that in the external assay medium. After 4 h, the internal concentration of 4MTB was only approximately 1.6-fold relative to concentration in the external medium (Fig. 6C). This indicates that the T58D mutant is still able to overaccumulate 4MTB relative to the concentration in the external assay medium but at a severely reduced rate.

Finally, we tested whether the T58D mutation rendered the transport protein capable of transporting 4MTB out of the cell against a proton gradient. 4MTB was injected into oocytes to an estimated internal concentration of 1 mM in T58A-expressing, T58D-expressing and non-expressing oocytes. The 4MTB-injected oocytes were incubated for 1 h in 4-MTB-free Kulori pH 5 buffer, and subsequently the internal 4MTB content was measured. During the 1 h assay, both T58A- and T58D-expressing oocytes lost approximately 20% of the injected 4MTB relative to T0 (control oocytes injected with 4MTB that were analyzed for 4MTB content immediately after injection). Loss of 4MTB in non-expressing oocytes probably occurs because the injection wound does not close immediately. Consequently, a small proportion of the injected 4MTB is expected to be lost from the oocyte during the assay. However, it
is possible that the apparent efflux of injected 4MTB may be caused by a carrier endogenous to X. laevis oocytes that facilitates export of GLS. We did not measure a significant difference in 4MTB content of the T58A-expressing, T58D-expressing and non-expressing oocytes (Fig. 6D, which indicates that neither the T58D nor the T58A mutant facilitates additional 4MTB efflux.

Is substrate specificity affected in the charge mutant T58D?

To investigate if the ability of T58D mutants to transport different classes of GLS was affected to the same extent, we measured the transport activity of NPF2.11-expressing, T58D-expressing and non-injected oocytes exposed to GLS isolated from an A. thaliana Ler accession that primarily contain long-chained, aliphatic 8-methylsulfinyloctylglucosinolate (8MSO) and indolyl GLS [sum of indol-3-ylmethyl glucosinolate (I3M); 4-methoxy-3-indolylmethylglucosinolate (4MO-I3M) and 1-methoxy-3-indolylmethylglucosinolate (1MO-I3M)]. Uptake of 8MSO and indole GLS was as drastically reduced in oocytes expressing T58D (Fig. 7) compared with oocytes expressing NPF2.11, as was observed for 4MTB (Fig. 3A). This showed that the reduced transport activity observed for T58D was not substrate specific and was independent of side chain structure of the GLS.
In this study, we investigated the E1X1X2E2K variant of the E1X1X2E2R motif in the GLS transporter NPF2.11 with special focus on the uncharacterized X1 and X2 residues that are conserved as uncharged residues within the Arabidopsis NPF.

The E57T58F59E60K61 motif is essential for active transport

NPF2.11 was previously characterized as a H+/GLS symporter that utilizes the ΔpH between the plant apoplast (pH 5.5) and cytoplasm (pH 7.4) to drive the import of GLS (Nour-Eldin et al. 2012). When we tested the ability of NPF2.11 to overaccumulate 4MTB in Xenopus oocytes (i.e. to a concentration above the concentration in the assay medium), a several fold overaccumulation of 4MTB relative to the assay medium was observed. For NPF2.11, the arginine residue in the canonical E1X1X2E2R motif is changed to a lysine, i.e. E57T58F59E60K61. Approximately 30% of Arabidopsis NPFs contain the E1X1X2E2K motif, whereas the remaining members contain the canonical E1X1X2E2R motif. The arginine to lysine substitution is considered a conservative substitution but may influence the transport properties as lysine only has one amino group whereas arginine has a guanidium group capable of forming a greater number of hydrogen bonds (Barnes 2007). Our finding—that NPF2.11 overaccumulates GLS against its concentration gradient—is the first experimental evidence that a transporter encoding the atypical E1X1X2E2K motif can overaccumulate substrate relative to the concentration in the assay medium.

Mutational studies of the E1X1X2E2R motif in the bacterial PepT5s suggested an important role for the E1X1X2E2R motif in proton coupling (Solcan et al. 2012). In addition, the E1X1X2E2R motif has been suggested to translocate a single proton per transport cycle, with both E1 and E2 as potential proton acceptors (Doki et al. 2013). The negative currents elicited by NPF2.11 when exposed to the negatively charged organic GLS...
and运输活动。在实验介质中（仅残留吸收），E57A、E60A和K61A突变体相对于浓度
进一步支持了E57T58F59E60K61氨基酸侧链的保守性。整体而言，不带电荷的残基在E1X1X2E2[K/R] motif中起作用。同样，正电荷的 arginine residue引入
于盐桥形成（在和之后），这在E1 residue与E2和E3 residue的motif之间形成盐桥，在靠近X1 residues的
其形成和断裂对E1X1X2E2R motif的形成和破断有影响。当mutating PepTSt-E2和-R residues to glutamine
(mimicking a protonated glutamate)，突变体丢失了的能力积累
盐桥的存在。这表明，E1 residues的位置有助于
图7 长链脂肪酸8MSO和吲哚基GLS. NPF2.11和T58D被分别在15个Xenopus卵中，以及
运输活动是在介质中（仅残留吸收）的。在Xenopus卵中，E57A、E60A和K61A

图8 GMS累加到NPF2.11

中

6.6±0.5 for AE57E and 4.6±0.3 for E57A. ND = below
detection limit.

在

1.5

GMS accumulation relative to NPF2.11

0.8

0.6

0.4

0.2

0.0

A

A

A

B

B

C

ND ND

NPF2.11

T58A

T58D

Non-injected

2

4

6

8

10

8

6

4

2

0

2347

indole GLS were quantified by LC-MS in

Fig. 7 Uptake of long-chain aliphatic 8MSO and indolyl GLS. NPF2.11 and T58D were expressed separately in 15 Xenopus oocytes, and transport activity was measured in the presence of 134 μM 8MSO, 31 μM 13M, 10 μM 4MO-13M and 37 μM 1MO-13M for 1 h at pH 5. Accumulated 8MSO and indolyl GLS were quantified by LC-MS in 3 x 5 oocytes for each gene. Groups are determined by one-way ANOVA (P < 0.001) vs. NPF2.11. Error bars: SD, n = 3. ND = below
the detection limit.

图7 长链脂肪酸8MSO和吲哚基GLS. NPF2.11和T58D被分别在15个Xenopus卵中，以及
运输活动是在介质中（仅残留吸收）的。在Xenopus卵中，E57A、E60A和K61A

图8 GMS累加到NPF2.11

中

6.6±0.5 for AE57E and 4.6±0.3 for E57A. ND = below
detection limit.

研究到目前为止，对保守的X位置在E1X1X2E2[K/R] motif中起作用的
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中

6.6±0.5 for AE57E and 4.6±0.3 for E57A. ND = below
detection limit.
Oocyte preparation and cRNA injection

Xenopus oocytes (stages V–VI) were prepared as previously described (Carpaneto et al. 2005) or purchased as defolliculated Xenopus oocytes (stages V–VI) from Ecoocyte Biosciences. Injection of 50 nl of cRNA (500 ng l−1) into Xenopus oocytes was done using a Drummond NANOJECT II (Drummond Scientific Company). Oocytes were incubated for 3 d at 17°C in Kulori medium (90 mM NaCl, 1 mM KCl, 1 mM MgCl2, 10 mM MES) pH 7.4.

Glucosinolate uptake/export assays

4MTB was obtained from C2 Bioengineering (Denmark). GLS uptake assays were carried out by injecting 50 nl of a 20 mM 4MTB solution per oocyte followed by transfer of injected oocytes to a standard Kulori-based solution (90 mM Na-gluconate, 1 mM K-gluconate, 1 mM Ca-gluconate, 1 mM Mg-gluconate, 1 mM LaCl3 and 10 mM MES pH 5). The oocyte was impaled by current and potential electrodes at a 45° angle and allowed to heal and equilibrate until the membrane potential was stable. The amplifier was switched to voltage clamp at −60 mV and the oocyte was allowed to establish a stable baseline, and currents in the absence of GLS substrate were measured in the voltage range between −30 and −170 mV in 10 mV decrements. (iv) When the baseline was stable, a standard Kulori-based solution with GLS substrate was perfused over the oocyte and currents were recorded in the voltage range between −30 and −170 mV in 10 mV decrements. TEVC data were extracted from pCLAMP10 software as a Microsoft Excel-compatible worksheet and analyzed in Excel. GLS-induced currents were calculated by subtracting currents before addition of GLS substrate from currents after addition of GLS substrate. SigmaPlot version 12.3 (Systat software) was used for statistical analysis and data plotting. Michaelis–Menten equation (Equation 1) to calculate the apparent Km value was done using SigmaPlot version 12.3 (Systat software).

\[ I = \frac{I_{\text{max}} \times [4\text{MTB}]}{[4\text{MTB}]+K_{\text{m}}} \]  

(iii) The amplifier was switched to voltage clamp at –60 mV and the oocyte was allowed to establish a stable baseline, and currents in the absence of GLS substrate were measured in the voltage range between −30 and −170 mV in 10 mV decrements. When the baseline was stable, a standard Kulori-based solution with GLS substrate was perfused over the oocyte and currents were recorded in the voltage range between −30 and −170 mV in 10 mV decrements. TEVC data were extracted from pCLAMP10 software as a Microsoft Excel-compatible worksheet and analyzed in Excel. GLS-induced currents were calculated by subtracting currents before addition of GLS substrate from currents after addition of GLS substrate. SigmaPlot version 12.3 (Systat software) was used for statistical analysis and data plotting. Michaelis–Menten equation (Equation 1) to calculate the apparent Km value was done using SigmaPlot version 12.3 (Systat software).

\[ I = \frac{I_{\text{max}} \times [4\text{MTB}]}{[4\text{MTB}]+K_{\text{m}}} \]  

Calculation of oocyte volume

The oocyte concentration of 4MTB was calculated based on an estimated oocyte water content of approximately 70% (de Laat et al. 1974) and a maximal oocyte diameter of 1.5 mm. Based on this, the oocyte cytosolic volume was calculated to be 1 μl.

Glucosinolate analysis of Xenopus oocytes by LC-MS

ESI-LC-MS analysis of GLS from Xenopus uptake assays was performed as described previously (Nour-Eldin et al. 2012).

Electrophysiological measurements and data analysis

All measurements were performed with a TEVC system composed of an NPI TEC-03X amplifier (NPI electronic GmbH) connected to a PC with pCLAMP10 software (Molecular Devices) via an Axon Digidata 1440a digitizer (Molecular Devices).

Western blot

Samples were separated by SDS–PAGE and transferred to PVDF membranes. For detection of YFP-tagged proteins, we used a polyclonal rabbit-anti-green

### Table 1 USER primers for epitope tagging of wild-type and mutant transporters

| Primer | Name | Sequence |
|--------|------|----------|
| NPF2.11 HA-tagged mutants | | |
| 1 | NPF2.11 FW1 | GGGTTAAAUATGGGACAGAAAGCCTCTTGAAAC |
| 2.1 | NPF2.11 TS8 FW (T→R) | ATTATTGGAATUGAGACGTGGAGCTGGATCT |
| 2.2 | NPF2.11 FS9 FW (F→D) | ATTATTGGAATUGAGACGTGGAGCTGGATCT |
| 2.3 | NPF2.11 FS9 FW (F→R) | ATTATTGGAATUGAGACGTGGAGCTGGATCT |
| 2.4 | NPF2.11 E57 FW (E→A) | ATTATTGGAATUGAGACGTGGAGCTGGATCT |
| 2.5 | NPF2.11 E60 FW (E→A) | ATTATTGGAATUGAGACGTGGAGCTGGATCT |
| 2.6 | NPF2.11 K61 FW (K→A) | ATTATTGGAATUGAGACGTGGAGCTGGATCT |
| 2.7 | NPF2.11 K61 FW (K→D) | ATTATTGGAATUGAGACGTGGAGCTGGATCT |
| 3 | TS8 RV_1 (X→R/D/A) | ATTATTGGAAAUATGGGACAGAAAGCCTCTTGAAAC |
| 4 | NPF2.11 RV- HA | GGTTCATAUTCAACTGGAACATCGTATGGGTAGGCAACGTTCTTGTCTTG |

NPF2.11 YFP-tagged mutant | | |
| 1 | NPF2.11 FW1 | GGGTTAAAUATGGGACAGAAAGCCTCTTGAAAC |
| 2 | NPF2.11 TS8 FW (T→D) | ATTATTGGAATUGAGACGTGGAGCTGGATCT |
| 3 | TS8 RV_1 (T→D) | ATTATTGGAAAUATGGGACAGAAAGCCTCTTGAAAC |
| 4 | NPF2.11 no stop RV2 | AGGCCTGGUTATTACGCCGCAACCTGCTTTGCTTTG |

TEVC recordings were performed as follows. (i) An oocyte was placed in the recording chamber and perfused with a standard Kulori-based solution (90 mM Na-glucanate, 1 mM K-glucanate, 1 mM Ca-glucanate, 1 mM Mg-glucanate, 1 mM LaCl3 and 10 mM MES pH 5). (ii) The oocyte was impaled by current and potential electrodes at a 45° angle and allowed to heal and equilibrate until the membrane potential was stable. (iii) The amplifier was switched to voltage clamp at −60 mV and the oocyte was allowed to establish a stable baseline, and currents in the absence of GLS substrate were measured in the voltage range between −30 and −170 mV in 10 mV decrements. When the baseline was stable, a standard Kulori-based solution with GLS substrate was perfused over the oocyte and currents were recorded in the voltage range between −30 and −170 mV in 10 mV decrements. TEVC data were extracted from pCLAMP10 software as a Microsoft Excel-compatible worksheet and analyzed in Excel. GLS-induced currents were calculated by subtracting currents before addition of GLS substrate from currents after addition of GLS substrate. SigmaPlot version 12.3 (Systat software) was used for statistical analysis and data plotting. Michaelis–Menten equation (Equation 1) to calculate the apparent Km value was done using SigmaPlot version 12.3 (Systat software).

\[ I = \frac{I_{\text{max}} \times [4\text{MTB}]}{[4\text{MTB}]+K_{\text{m}}} \]  

Michaelis–Menten equation: I is the current, and I_{\text{max}} is the maximal current achieved by the transporter at saturating concentrations of 4MTB.

### Xenopus oocyte protein extraction

Membrane and soluble proteins were isolated from Xenopus oocytes expressing wild-type or mutated NPF2.11 by the following protocol: one oocyte was homogenized in 100 μl of homogenization medium [20 mM Tris–HCl, pH 7.6, 0.1 M NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), buffer described in Galili et al. (1995)] and left on ice for 20 min. The homogenate was then cleared by centrifugation for 2 min at 10,000 g. The supernatant was transferred to a new tube and 25 μl of SDS sample buffer was added (60 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromphenol blue).

### Western blot

Samples were separated by SDS–PAGE and transferred to PVDF membranes. For detection of YFP-tagged proteins, we used a polyclonal rabbit-anti-green...
fluorescent protein (GFP) antibody (Invitrogen A11122). For endogenous actin detection, we used polyclonal rabbit-anti-β-actin antibody (abcam-ab8227).

Western blot development
SuperSignal West Pico was used as chemiluminescent substrate and chemiluminescence was measured using a UVF autochem system (UVF Bioimaging Systems).

Relative quantification of Western blot bands
Western blots were developed and analyzed by the standard ‘gel analysis’ tool in ImageJ (http://rsb.info.nih.gov/ij). To achieve a relative quantification, we compared the intensity of endogenous actin bands with the intensity of heterologously expressed transporter.

Oocyte fixation and bioimaging
Oocytes expressing NPF2.11::YFP and T58D:YFP were fixed in the manner described by Sayers et al. (1997). Briefly, oocytes were fixed in 4% paraformaldehyde for 1 h and washed in phosphate-buffered saline (PBS). Oocytes were then sliced in two and mounted on a glass slide for bio imaging by confocal scanning microscopy using a SPX5-X Point-scanning Confocal from Leica Microsystems.

Sequence alignment
Aligned bacterial, fungal, worm, animal and plant POT/PTR transporters were previously described (Daniel et al. 2006, Newstead et al. 2011, Solcan et al. 2012, Doki et al. 2013, Guettou et al. 2013). All sequences were retrieved from NCBI or TAIR and aligned using CLC genomic workbench and ClustalW. The EXXE[K/R] motif was identified and all sequences were trimmed to show only the five amino acids of the EXXE[K/R] motif.

Homology modeling
NPF2.11 sequences were submitted to the SWISS-MODELS server and the highest scoring templates was used to generate homology models (swissmodel.expasy.org/) (Biasini et al. 2014). NPF2.11 was modeled on the NRT1.1 structure (PDB: 4OH3) (Sun et al. 2014). PDBsum was used to calculate Ramachandran Plot statistics where NPF2.11 had 1% of residues in disallowed regions (de Beer et al. 2014). Pymol was used for visualization of homology models and preparation of illustration.

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Disclosures
The authors have no conflicts of interest to declare.

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