Functional identification of the glycerol permease activity of *Arabidopsis thaliana* NLM1 and NLM2 proteins by heterologous expression in *Saccharomyces cerevisiae*

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Abstract NLM proteins (NOD26-like major intrinsic proteins) from plants contain amino acid sequence signatures which can be found in aquaporins including plant plasma membrane intrinsic proteins and tonoplast intrinsic proteins and glycerol permeases such as the *Escherichia coli* GlpF and the yeast FPS1 proteins. Heterologous expression of two members of the NLM subgroup from *Arabidopsis thaliana* (AtNLM1 and AtNLM2) in baker's yeast demonstrated the glycerol permease activity in addition to the previously described aquaporin activity of AtNLM1. The transport was non-saturable up to 100 mM extracellular glycerol concentration. Longer-chain sugar alcohols did not compete with the transport of radiolabelled glycerol and hexoses were also not transported through the pore.

Key words: Major intrinsic protein; Aquaporin; Aquaglyceroporin; Water transport; Glycerol transport; Heterologous expression

1. Introduction

The major intrinsic protein (MIP) gene family [1] of plants consists of at least four subgroups based on sequence comparisons: the tonoplast intrinsic proteins (TIPs), the plasma membrane intrinsic proteins (PIPs), the NOD26-like MIPs (NLMs) [2] and a small subgroup named small and basic intrinsic proteins (presented by Urban Johanson at the MIP2000 meeting, July 1–5, 2000, Göteborg, Sweden). This MIP family has recently been subdivided into two major functional clusters, aquaporins and aquaglyceroporins (including glycerol permeases) [3,4]. Moreover, specific amino acid residues along the peptide sequence can be used to distinguish between aquaporins and aquaglyceroporins [5]. While most plant PIPs and TIPs belong to the aquaporin group of MIPs based on the amino acid signature, the NLMs exhibit signatures of both subgroups, the aquaporins and the aquaglyceroporins.

The aquaporin activity of *Arabidopsis thaliana* AtNLM1 and *Glycine max* (soybean) GmNOD26 has been shown in *Xenopus* oocytes, while glycerol transport activity was only known for GmNOD26 [2,6,7]. In order to find out whether the NLM proteins in *Arabidopsis* also form mixed-functional solute permeases (aquaglyceroporins), we used *Saccharomyces cerevisiae* as a powerful and versatile heterologous expression system for transport tests with heterologously expressed NLM proteins. Because the peribacteroid membrane of soybean nodules, where GmNOD26 is inserted, is absent in the non-nodulating plant *A. thaliana*, we assume that the physiological role of NLM proteins in *Arabidopsis* is not the same as in soybean. Therefore establishing a versatile expression system for detailed investigation of the substrate specificity and transport kinetics of AtNLM proteins should help us to elucidate the physiological functions of this class of transport proteins in non-nodulating plants.

2. Materials and methods

2.1. Cloning of AtNLM2

Specific oligonucleotide primers (ATNLM2F1B: 5′-TGG ATC CAG TCG TTT AAA GTC TGA TCC-3′; AtNLM2R1B: 5′-TGG ATC CAC AAC TTA ACC TCC GAT GAC-3′) with *Bam*HI sites at the 5′ ends were designed corresponding to the putative 5′ and 3′ untranslated regions of the *AtNLM2* gene. Reverse transcription-PCR experiments were carried out with cDNA from leaves, flowers, siliques, bolts, cauline leaves and roots of adult *A. thaliana* (ecotype Columbia) plants. A DNA fragment of the expected size (genomic sequence without putative introns) was subcloned into a *Sma*I-digested pBluescript II KS+ giving plasmid pN2-2 and sequenced.

2.2. Yeast strains and expression plasmids

Transport tests were performed in the following *S. cerevisiae* strains: YSH6.114.-2A [8], an *fps1* mutant, and EBY.VW4000 [9], a multiple hexose carrier deletion mutant which can be grown on maltose containing media. For heterologous expression, *Bam*HI fragments of pNCA4 (*AtNLM1*, [2]) and pN2-2 (*AtNLM2*, this study) were subcloned into the *Bam*HI site of the yeast expression vector pDR195 [10] adjacent to the yeast plasma membrane ATPase promoter PMA1 yielding the plasmids pN1DR1 (*AtNLM1*) and pN2DR1 (*AtNLM2*), respectively. The sense orientation of the cDNA inserts towards the promoter was confirmed by restriction analysis. Yeast cells were transformed with pN1DR1, pN2DR1 and the empty vector as described in [11] and maintained on selective minimal media MMA (2% glucose, 0.67% yeast nitrogen base w/o amino acids); glucose was replaced by other sugars in sugar transport experiments.

2.3. Transport experiments and glycerol measurements

Yeast cells were grown at 30°C in selective medium to an optical density of 0.7–1.3 OD_{600nm}. Cells were harvested at 4°C by centrifugation, washed twice with phosphate buffer (50 mM sodium phosphate, pH 5.5) and resuspended in the same buffer to a density of about 10 OD_{600nm} (for uptake experiments under hyper-osmotic conditions, which ran over several hours, the phosphate buffer was supplemented with glycerol and/or sorbitol and 1% glucose as an energy source for yeast cells). Uptake experiments were performed in glass vials in a water bath shaker at 30°C. The specific radioactivity of glycerol in the different uptake experiments (0.1–500 mM) varied, but a 100 μl sample of the uptake experiment contained about

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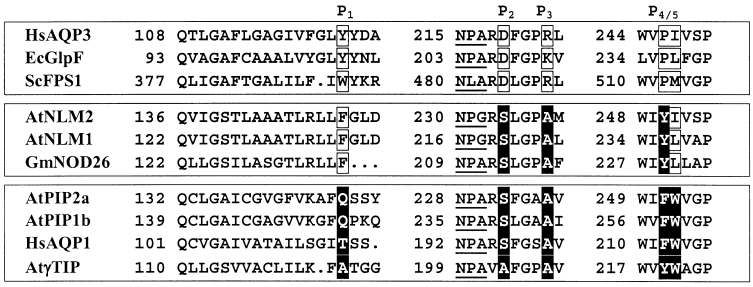

Fig. 1. Amino acid signature of aquaporins and aquaglyceroporins (after [5]). The upper box contains aquaglyceroporins from human (Hs), *Escherichia coli* (Ec) and yeast (Sc), the lower box contains aquaporins from *Arabidopsis* (At) and human. The middle box shows NLMs from *Arabidopsis* and soybean (Gm). The five specific positions are indicated at the top. Aquaglyceroporin-type amino acid residues are marked with white boxes, aquaporin-type amino acid residues with black boxes, the C-terminal 'NPA' box is underlined. The respective amino acid position of each sequence is indicated at the left.

40 000 disintegrations per minute (dpm) of [^{14}C]glycerol (Moravek Biochemicals, Brea, CA, USA) or 350 000 dpm of [^{3}H]glycerol (NEN, Cologne, Germany), respectively. For competition experiments, longer-chain sugar alcohols were added in 10-fold excess (1 mM final concentration) to the cell suspension together with the radioactive glycerol (0.1 mM final concentration). Prior to the addition of radiolabelled substrates, the yeast cells were pre-incubated for about 2 min in the water bath for temperature equilibration. Cell samples (100 μl) were withdrawn at given time points and washed on glass filters (AE-91, Schleicher and Schuell, Dassel, Germany) with ice-cold phosphate buffer. The glass filters containing the yeast cells were transferred to scintillation vials, overlaid with scintillation fluid and the radioactivity was determined in a scintillation counter. The packed cell volume in each uptake experiment was determined using centrifuge vials with μl markings ('leucocyte counters'). Generally, 30% intracellular volume was subtracted from the packed cell volume to obtain the cell volume. The glycerol contents of yeast cells and incubation media were determined using a NAD-coupled enzymatic test (R-Biopharm, Darmstadt, Germany). Yeast cells were washed on glass filters as described above, transferred into 5 ml 80% ethanol, incubated at 65°C for 30 min and cleared by centrifugation at 4100×*g*/4°C for 10 min. The clear extract was used for the enzymatic test (final ethanol concentration below 0.22%) as instructed by the manufacturer. The cell volume of each culture was determined in each experiment. The media were cleared by centrifugation and the supernatants were directly used for the enzymatic test.

2.4. Phenotype analysis

Yeast cells were grown in MMA medium (supplemented with 1 M glycerol, as indicated), adjusted to the same OD_{600nm} and 5 μl of 10-fold serial dilution was spotted onto agar plates. The cultures were incubated at 30°C for 1–2 days; for anaerobe conditions, the plates were placed into an Anaerocult® container (Merck, Darmstadt, Germany) at 30°C.

3. Results

3.1. Identification and cloning of AtNLM2

When we searched the *Arabidopsis* genome database for homologous genes of *AtNLM1*, seven additional *NLM* genes with significant similarity could be identified [12]. The sequence from the genomic clone F13C5 (accession number AL021711) contained both, the entire *AtNLM1* gene and a second gene, *AtNLM2*, in about 53 kb distance. Comparison of the *AtNLM1* gene sequence with the cloned *AtNLM1* cDNA sequence [2] revealed four introns within the coding region. Computer programs predicted a similar gene structure for *AtNLM2*. Using primers complementary to the putative 5′ and 3′ untranslated region of *AtNLM2*, we could amplify a full-length cDNA fragment out of root cDNA. Sequence analysis of cDNA clone pN2-2 (for details, see EMBL accession number AJ250668) confirmed the intron–exon structure as predicted by computer programs and was identical to the genomic sequence. The deduced amino acid sequence is 81.3% and 66.4% identical to AtNLM1 and GmNOD26, while the identity to the *Arabidopsis* γTIP and PIP1b aquaporins is only 30.8% and 26.3%, respectively. As described earlier for AtNLM1 [2], one of the two MIP specific NPA amino acid boxes reads NPG in the C-terminal half of AtNLM2 (Fig. 1). The phosphorylation site of GmNOD26 close to the C-terminus is conserved in AtNLM1 and AtNLM2.

3.2. Aquaporin signature of Arabidopsis NLM proteins

A recent survey of more than 150 MIPs of different organisms pointed to five amino acid positions, which are significant either for aquaporins or glycerol permeases [5]. Exchange of amino acid residues at two of the five positions (P4 and P5) was later shown to convert an insect aquaporin into a glycerol permease and therefore most likely play a role in transport selectivity [13]. With respect to the five amino acid positions described above, AtNLM1 and AtNLM2 stand in between the aquaporins and the glycerol permeases/aquaglyceroporins (Fig. 1). At three positions (P2, P3 and P4) in AtNLM1 and AtNLM2, the amino acid residues are similar to comparable residues in aquaporins such as AtγTIP, AtPIP1b/2a or the human aquaporin HsAQP1. The other two positions (P1 and P5) contain amino acid residues as they can be found in

Table 1
Glycerol content of yeast cells and growth media

Yeast strain transformed with	MMA medium		MMA medium+1 M sorbitol	
	cells[a]	medium[b]	cells[a]	medium[b]
Vector	74.1	1.0 (0.163)	284	11.9 (1.49)
AtNLM1	14.6	6.9 (1.21)	131	31.8 (5.43)
AtNLM2	3.77	5.9 (1.25)	160	16.0 (16.0)

[a]Glycerol content (mmol/(l cells)).
[b]Glycerol concentration (mM) and glycerol production (mmol/(ml cells)/day) in brackets.

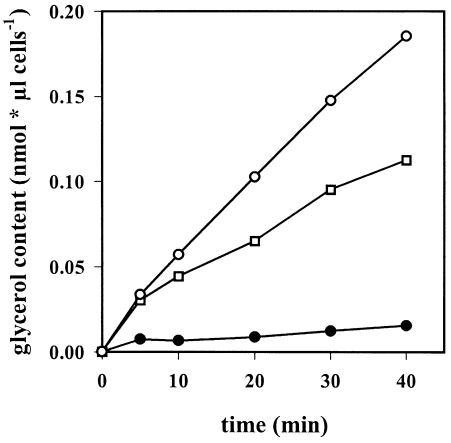

Fig. 2. Glycerol uptake into *AtNLM1*- (□), *AtNLM2*- (○) and vector-transformed (●) *fps1* yeast mutants.

bacterial or yeast glycerol permeases or the human aquaglyceroporin HsAQP3.

3.3. Glycerol content of transformed fps1 cells

Yeast cells grown on selective medium already contained considerable amounts of intracellular glycerol (Table 1). While *fps1* cells were able to retain most of their glycerol inside the cells, *AtNLM1*- and *AtNLM2*-transformed yeast cells lost glycerol close to the concentration equilibrium (since we do not know the subcellular compartmentation of glycerol, we calculated the cellular values in Table 1 in 'mmol/l cells' rather than in 'mM'). Yeast cells obviously tried to compensate this glycerol loss through the AtNLM proteins by a 7–8-fold higher production rate. Similar results were observed when yeast cells were grown under hyper-osmotic stress (1 M sorbitol): the intracellular glycerol content increased in control cells to about 284 mmol/l cells, but also *AtNLM1*- and *AtNLM2*-transformed yeast cells contained 91.6 and 112 mmol/l cells, respectively (Table 1). However, *AtNLM1*- and *AtNLM2*-transformed cells grew slower than the control strain and the glycerol released to the medium calculated per cell volume was much higher in these two strains (Table 1, brackets).

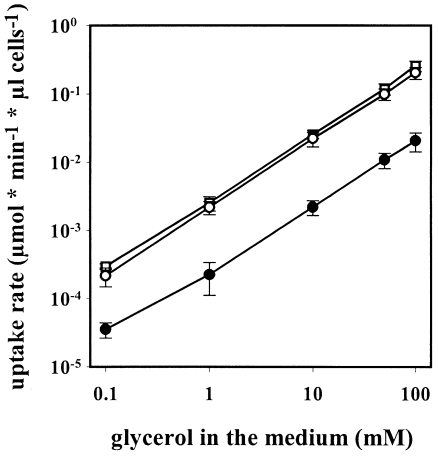

Fig. 3. Initial uptake rates at different extracellular glycerol concentrations into *AtNLM1*- (□), *AtNLM2*- (○) and vector-transformed (●) *fps1* yeast cells grown in minimal medium.

3.4. Glycerol uptake under normal osmotic conditions

We used *S. cerevisiae* rather than *Xenopus* oocytes as a heterologous expression system to directly analyse glycerol transport activities of AtNLM1 and AtNLM2 for the following reasons: first, according to the German 'Tierschutzgesetz' (animal protection law), we are not allowed to perform surgery on frogs for oocytes preparation. Second, stable expression of foreign genes via expression plasmids in yeast cells is more versatile compared to individually injected oocytes before each experiment. Third, the yeast expression system is routinely used in our lab for the expression of plant membrane proteins. The *S. cerevisiae* genome contains four MIP homologs [14]; for two of them, *FPS1* and *AQY1*, functional expression has been demonstrated: *FPS1* encodes a glycerol permease [15] while *AQY1* is an aquaporin [14]. A deletion mutant of *FPS1* shows greatly reduced glycerol uptake activity and was used to characterise the glycerol transport activity of heterologously expressed AtNLM1 and AtNLM2 proteins.

We performed glycerol uptake experiments at a low glycerol concentration (0.1 mM) in the medium to minimise water efflux from the yeast cells. Since AtNLM1 is also an aquaporin, addition of glycerol at higher concentrations could cause an efflux of water out of the cells possibly via AtNLM1 (and the putative aquaporin AtNLM2, too). Because we did not know whether water and glycerol use the same molecular path through the proteins, we tried to keep an experimentally induced water flow in the opposite direction of a glycerol flow through AtNLMs as low as possible.

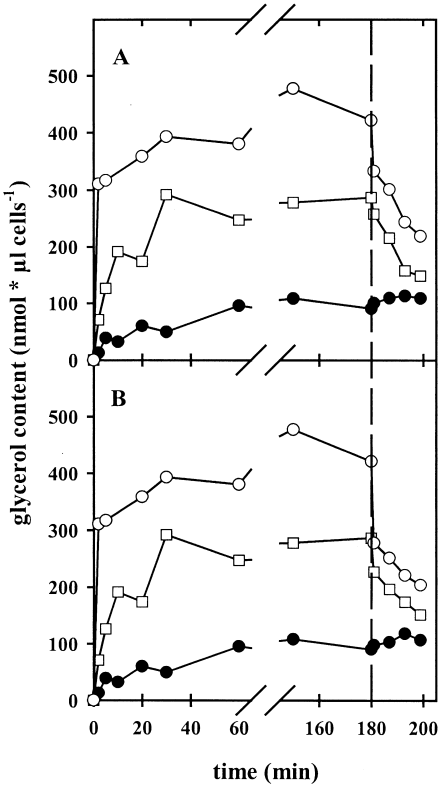

Fig. 4. Glycerol uptake into and efflux out of *AtNLM1*- (□), *AtNLM2*- (○) and vector-transformed (●) *fps1* yeast cells under hyper-osmotic conditions (0.5 M glycerol, 0.5 M sorbitol). After 3 h (dashed line), the medium glycerol concentration was lowered 10 times without changing the overall medium osmolarity (A), or the medium osmolarity was lowered by 40% without changing the medium glycerol concentration (B).

AtNLM1- and *AtNLM2*-transformed yeast cells took up glycerol at higher rates than vector-transformed cells (Fig. 2). Although the washing steps of the cultures replaced much of the medium, glycerol release (see above) did certainly continue, e.g. in *AtNLM1*- and *AtNLM2*-transformed yeast cells during preparation for the uptake experiment. However, with a linear relationship between glycerol uptake and extracellular glycerol concentration (Fig. 3), the uptake of comparable amounts radioactive glycerol is independent on the total glycerol concentration. Our results presented in Fig. 2 were calculated on the assumption that at least 0.1 mM glycerol was present in the incubation medium.

3.5. Influx and efflux of glycerol under hyper- and hypo-osmotic conditions

Since water or solute transport through aquaporins or glycerol permeases is thought to be non-active (e.g. no proton cotransport), we tested whether intracellular glycerol would also exit the cells through AtNLM1 or AtNLM2. In order to pre-load cells with radioactive glycerol, *AtNLM1*- and *AtNLM2*-transformed yeast cells were grown in the presence of 0.5 M glycerol and 0.5 M sorbitol in the medium and [^{3}H]glycerol uptake experiments were performed under these hyper-osmotic conditions. *AtNLM1*- and *AtNLM2*-transformed yeast cells grew faster under these conditions compared to 1 M sorbitol, because a loss of intracellular glycerol was prohibited by the high extracellular glycerol concentration. Since the yeast cells were prepared for the uptake experiment using a phosphate buffer containing 0.5 M glycerol and 0.5 M sorbitol, the glycerol gradient across the plasma membrane was probably kept close to equilibrium. Under these conditions, equilibration of radioactive glycerol could be observed, which was considerable faster in *AtNLM1*- and *AtNLM2*-transformed yeast cells than in control cells (Fig. 4). After about 3 h, when radioactive glycerol had equilibrated between the medium and the cells at least in *AtNLM1*- and *AtNLM2*-transformed cells, we lowered the extracellular glycerol concentration by diluting the cell suspension with nine volumes of 1 M sorbitol (in phosphate/glucose buffer) without lowering the overall medium osmolarity. Efflux of radioactivity as the result of the imposed outward-directed glycerol concentration gradient was clearly observed in *AtNLM1*- and *AtNLM2*-transformed yeast cells and the kinetics showed that the influx and efflux rates were quite similar (Fig. 4A). In parallel experiments, the extracellular osmolarity was lowered from about 1.105 osmol/l to about 0.655 osmol/l by adding nine volumes of 0.5 M glycerol (in phosphate/glucose buffer) without changing the extracellular glycerol concentration. Again, rapid efflux of radioactive glycerol under a situation close to the concentration equilibrium indicated a fast diffusive glycerol permeation through AtNLM1 and AtNLM2 (Fig. 4B).

3.6. Complementation of the fps1 phenotype

fps1 specific growth phenotypes have been observed under anaerobiosis and under hypo-osmotic shock [15]; in these experiments, *fps1* cells cannot release excessive intracellular glycerol which results in increased cell turgor and finally in reduced cell growth rates. Moreover, yeast cells expressing a mutant form (*fps1*-Δ*1*) of the yeast glycerol permease, lacking the N-terminal regulatory domain, showed severe growth defects under hyper-osmotic conditions [15]. While under aerobe conditions *AtNLM1*-, *AtNLM2*- and vector-transformed *fps1* cells grew indistinguishable from each other, the *NLM*-expressing strains grew faster under anaerobic conditions (Fig. 5A,B). Under hyper-osmotic conditions, the three yeast strains grew at comparable rates only when the net efflux of glycerol was prohibited by supplying the compatible osmolyte via the medium (Fig. 5C). On a comparable hyper-osmotic medium without glycerol, *AtNLM1*- and *AtNLM2*-transformed yeast cells showed dramatically reduced growth rates (Fig. 5D). However, when the yeast strains were subjected to a steep hypo-osmotic shock, *AtNLM1*- and *AtNLM2*-transformed *fps1* cells grew better than the vector-transformed *fps1* strain because they could release excessive amounts of intracellular glycerol, preventing increase in cell turgor which reduced the growth rate in the *fps1* yeast (Fig. 5E).

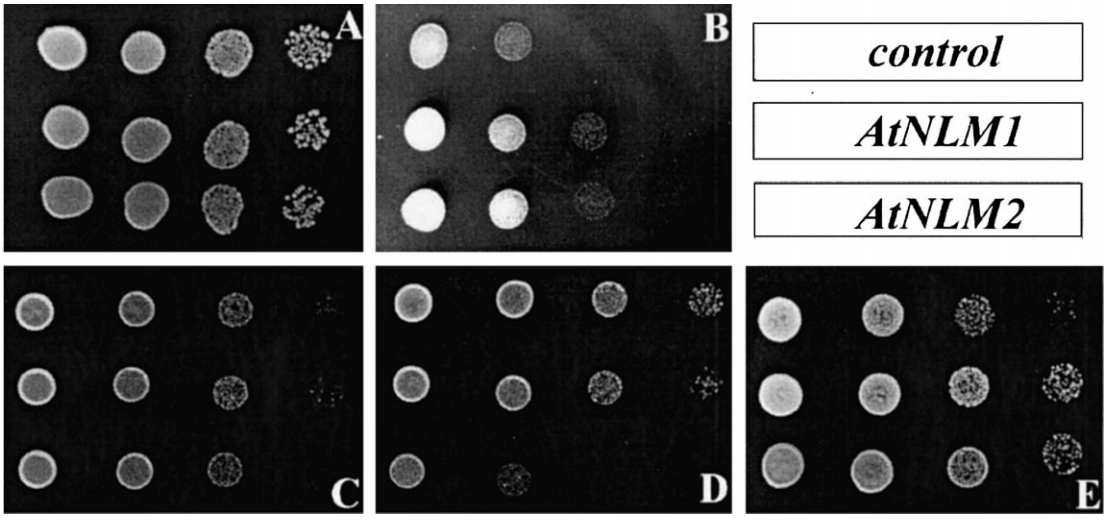

Fig. 5. Growth of *AtNLM1*-, *AtNLM2*- and vector-transformed *fps1* cells under various aerobic (A, B) and osmotic (C–E) conditions: yeast cells were spotted in serial dilutions onto selective agar medium and grown under aerobic (A) or anaerobic (B) conditions. For osmotic treatments, yeast cells were pre-grown in selective liquid medium containing 1 M glycerol and then transferred onto selective agar medium containing 1 M glycerol (C), 1 M sorbitol (D) or medium without any additional osmotica (E).

3.7. Hexose and sugar alcohol transport experiments

For hexose transport experiments, the *AtNLM1* and *ATNLM2* cDNAs were expressed in the yeast strain EBY.VW4000 [9]. This yeast mutant is deleted in most of the hexose transporters and two glucose sensors and will not grow on many hexose media. Growth experiments on different carbon sources showed that only EBY.VW4000 cells transformed with the *RcHEX3* cDNA (a castor bean glucose carrier [16]) were able to grow on glucose, fructose or galactose (data not shown). *AtNLM1*-, *AtNLM2*- and vector-transformed yeast cells did not grow at detectable rates on any of these hexose containing media. In control experiments, growth on maltose plates was comparable for all four transformants since EBY.VW4000 yeast still contains the MAL31 maltose transporter. Uptake experiments using radiolabelled hexoses confirmed these findings: *AtNLM1*- or *AtNLM2*-transformed yeast cells took up neither glucose nor fructose or galactose. Vice versa, radioactive glycerol uptake could be detected only in *AtNLM1* and *AtNLM2* but not in *RcHEX3* transformants (data not shown).

The addition of unlabelled sugar alcohols (erythritol, adonitol, mannitol, sorbitol) with a chain length from C-4 to C-6 to *AtNLM1*- and *AtNLM2*-transformed *fps1* cells did not reduce the initial uptake rates of ^{3}H-labelled glycerol (data not shown). Therefore, a specific interaction of the substrate with the transport protein – as it has been shown for sugar or amino acid carriers – is unlikely for AtNLM1 and AtNLM2.

4. Discussion

The *Arabidopsis* genome contains at least eight *NLM* genes; five of them (*AtNLM1, -2, -4, -5* and *-7*) are expressed since cDNA clones could be isolated [2,12]. Specific amino acid residues [5] indicate that NLMs form an 'intermediate' group between the classical 'aquaporins' (plant PIPs and TIPs, animal AQP1 etc.) and glycerol facilitators/aquaglyceroporins (GlpF, FPS1, AQP3 etc.) (Fig. 1). We should, however, keep in mind that glycerol transport activities have also been described for two plant 'aquaporins' (NtAQP1 and NtTIPa; [17,18]), and not all 'aquaglyceroporins' (e.g. GlpF and FPS1) as described in [4,14] exhibit water transport activity. Therefore, aquaglyceroporins can be found in many subgroups of eukaryotic MIPs, but the plant NLM cluster and the animal AQP3 cluster seem to contain many aquaglyceroporins.

AtNLM1 and AtNLM2 form functional glycerol permeases in *S. cerevisiae* as shown by influx and efflux experiments with radioactive glycerol (Figs. 2 and 4). Transport of glycerol was linear over several minutes, which was also described for the FPS1 glycerol permease from yeast [15]. Glycerol efflux out of *AtNLM1*- and *AtNLM2*-transformed *fps1* cells was slow enough to maintain an at least 4.1-fold and 10-fold glycerol gradient, respectively, in batch cultures under hyper-osmotic conditions (Table 1, right side). Influx into and efflux out of *AtNLM1*- and *AtNLM2*-transformed yeast cells showed similar kinetics; this behaviour is different from FPS1 wild-type cells which release glycerol faster than they take up glycerol [15].

In batch cultures, the significantly increased glycerol content of the medium pointed already to functional glycerol permeases in *AtNLM1*- and *AtNLM2*-transformed yeast cells, but at least *AtNLM1*-transformed yeast cells showed an outward-facing steady state glycerol gradient which is maintained by balancing glycerol production and glycerol efflux. However, we did not determine whether this gradient still existed at the beginning of the uptake experiment. Uptake of radioactive glycerol was observed under the experimental conditions, possibly against a concentration gradient. This is a thermodynamic necessity, irrespective of whether a concentration equilibrium or a steady state concentration gradient exists and irrespective of the net flow of glycerol. First, concentration equilibrium over a membrane simply means that the rate of import is equal to the rate of export. Therefore, after the addition of radioactive glycerol on one side, uptake has to occur until a new equilibrium for the radioactive compound is reached. Second, under a steady state concentration gradient across a membrane, the transport rate in one direction is larger than the transport rate in the opposite direction, but both transport directions still co-exist. Again, uptake of radioactive glycerol even against a net flow of unlabelled glycerol in the opposite direction has to occur until a new steady state concentration gradient for the radioactive compound is reached.

Complementation of the *fps1* phenotype on different osmotic media and under anaerobiosis showed that *AtNLM1*- and *AtNLM2*-transformed cells behave like yeast cells expressing a continuously open glycerol permease, such as *fps1-Δ1*, a truncated FPS1 glycerol permease lacking the regulatory N-terminal domain [15]. Radioactive transport experiments performed in at least three other yeast strains, W303-1A [19], SEY2102 [20] and CLY1 (a glycerol kinase mutant; M. Kielland-Brand, Carlsberg Laboratory, unpublished), showed similar uptake kinetics as in the *fps1* background. Since *AtNLM1* and *AtNLM2* mRNA of the expected size could be detected on RNA gel blots (data not shown), we assume that the AtNLM proteins were present in the plasma membrane of transformed yeast cells. Antibodies will be raised against specific domains to finally proof the presence of the respective proteins in yeast cell membranes.

Since the initial uptake rate at different extracellular glycerol concentrations is not saturable and unlabelled sugar alcohols do not compete with radiolabelled glycerol transport, the model of a size specific pore rather than a substrate specific carrier is further supported, although it is still unclear why GlpF or FPS1 transport glycerol but not water. Therefore, there must be some specificity besides size selectivity in MIPs to explain the different activities of aquaporins, aquaglyceroporins and glycerol permeases. Whether AtNLM1 or AtNLM2 transport longer-chain sugar alcohols has to be investigated by independent experiments. Recently, the oligomerisation state of GlpF (monomer) and AQPcic (tetramer) has been correlated with either glycerol or water transport, respectively [21]. Whether AtNLM proteins exist in different oligomeric forms remains to be determined.

To our knowledge, this is the first time that plant NLM proteins were functionally expressed in *S. cerevisiae*. The possibility of stable expression of plant transport proteins in an organism, which is easy to handle and susceptible to genetic manipulations, enabled us to identify transported solutes via NLM proteins and to characterise basic transport parameters. These techniques will allow us to screen for other transport activities than glycerol and water, too.

To date, NLM proteins are published from six plant species (without EST entries): GmNOD26 from *G. max* [22], LiMP2

from *Lotus japonicus* [23], OsrMIP from *Oryza sativa* [24], NaPIP28 from *Nicotiana alata* (GenBank accession number P49173), PsNLM1 from *Pisum sativum* (GenBank accession number AJ243308) and the AtNLMs from *A. thaliana* (described in this paper). Detailed information is only known for GmNOD26, where this gene is expressed in nodules upon infection with rhizobia. Recently, a orthologous protein (LiMP2) was described from another legume, *L. japonicus*, which is also expressed exclusively in nodules [23]. In contrast, the *N. alata* protein seems to be pollen specific, the *P. sativum* protein was found in seed coats (see description of the respective database entries), and the rice protein is expressed in shoots [24]. However, the so far investigated *Arabidopsis* NLM gene transcripts could be found either exclusively (AtNLM1 and AtNLM5) or predominantly (AtNLM2 and AtNLM4) in roots [2,12]. Since *Arabidopsis* is not known to live in symbiosis with rhizobia, the physiological function of the AtNLM proteins will most likely be different from that in legumes even when the water and solute transport activities are similar.

Further experiments are underway on the (sub)cellular localisation of the NLM proteins, on the role of glycerol in plants (which unfortunately is widely unknown) and on additional transport activities of AtNLM proteins in order to get closer to the physiological role of this subclass of MIPs in plants.

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