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Structural features of the aromatic/arginine constriction in the aquaglyceroporin GintAQPF2 are responsible for glycerol impermeability in arbuscular mycorrhizal symbiosis

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ABSTRACT

Carbon transport in arbuscular mycorrhizal (AM) symbiosis is of fundamental importance. However, the role of glycerol transport in AM symbiosis has not yet been resolved. Glycerol transport across the cell membrane is mediated by aquaglyceroporins (AQGPs), whereas our previous study revealed that it was disfavoured by GintAQPF2, an AQGP from AM fungi (AMF). Here, we analysed the function of two amino acid residues in the aromatic/arginine (ar/R) constriction known as the major selectivity filter in AQGPs. Replacement of phenylalanine-94 (Phe-94) by alanine (Ala) enlarged the diameter of the ar/R constriction and resulted in an increased intracellular glycerol accumulation and thus survival rate of yeast cells at high glycerol levels, while individual or joint replacement of Phe-94 and Ala-234 by tryptophan and glycine induced a closed state of GintAQPF2, suggesting that the potential double gates (Phe94-Phe243 and arginine-249) of the ar/R constriction also likely determined solute permeability. To figure out whether GintAQPF2 functions were relevant to the establishment of AM symbiosis, genomic analyses of four representative fungi with different lifestyles were performed. We found that glycerol facilitators existed in the facultative fungi (the ectomycorrhizal fungus *Laccaria bicolor* and hemibiotrophic pathogen *Magnaporthe oryzae*), but not in the obligatory fungi (the AMF *Rhizophagus irregularis* and necrotrophic pathogen *Fusarium verticillioides*), revealing a conserved pattern of glycerol transport in symbionts and pathogens. Our results suggested that glycerol blocks due to the special structural features of the ar/R constriction in the only AMF AQGP could potentially play a role in the establishment of AM symbiosis.

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Introduction

Arbuscular mycorrhizal (AM) symbiosis, established between AM fungi (AMF) and roots of higher plants, can be found in most terrestrial ecosystems (Remy *et al.* 1994). AMF form highly branched structures called 'arbuscules' in root cortical cells, mediating bidirectional nutrient exchange between the symbiotic partners (Parniske 2008). The symbiosis is a mutualistic association, in which plants provide carbohydrates for fungal growth and obtain enhanced uptake of minerals, principally phosphate, in return (Bonfante & Genre 2010). Nuclear magnetic resonance and radiorespirometric studies have shown that AMF can absorb and utilize hexose from plants, most likely in the form of glucose (Shachar-Hill *et al.* 1995; Solaiman & Saito 1997; Pfeffer *et al.* 1999). Molecular evidence demonstrated that the AMF high-affinity monosaccharide transporter 2, functioning at the plant–fungal interface, was capable of transporting not only glucose but also other monosaccharides, such as xylose, mannose and fructose (Helber *et al.* 2011). In fact, AMF did not derive all their carbon from host plants (Jennings 1995). Isotope labelling analysis with [^{13}C]-glycerol supplied to the extraradical mycelium (ERM) revealed that AMF could uptake a small portion of glycerol by diffusion (Bago *et al.* 2003).

Glycerolipids, including phospholipids and triacylglyceride (TAG), act as important cellular signalling molecules, major energy reserves and crucial regulators of fungal life cycle (Jabaji-Hare 1998; Henry *et al.* 2012; Pannkuk *et al.* 2014). Glycerolipid are formed from the incorporation of fatty acids to the glycerol backbone (Bates & Browse 2011). Thus, glycerol supply is essential for fungal survival. Glycerol can either diffuse directly through the lipid bilayer of biological membranes or be facilitated by aquaglyceroporins (AQGPs), belonging to the superfamily of integral membrane proteins called major intrinsic proteins (MIPs) (Nehls & Dietz 2014). However, glycerol conduction mediated by AQGPs occurs at much higher rates compared with membrane permeability (Hansen *et al.* 2002; Nehls & Dietz 2014). Two constriction regions within AQGPs, known as the asparagine-proline-alanine (NPA) motifs located at the center of the pore and the aromatic/arginine (ar/R) constriction at the extracellular pore mouth, determine transport selectivity (de Groot & Grubmüller 2005). The ar/R constriction is formed by four amino acids and is narrower than the NPA region, whose diameter and polarity determine whether glycerol could pass through MIPs (Beitz *et al.* 2006; Hub & de Groot 2008). Therefore, the wider and less polar ar/R constriction renders AQGPs permeable for glycerol and co-instantaneously impairs water permeability (Sui *et al.* 2001; Tajkhorshid *et al.* 2002).

Our previous investigations indicated that both GintAQPF1 and GintAQPF2 from the AMF *Rhizophagus irregularis* AH01 cannot mediate transmembrane transport of glycerol (Li *et al.* 2013). Surprisingly, phylogenetic analysis identified GintAQPF2 as an 'AQGP', which commonly conducts glycerol to alleviate osmotic stress (Li *et al.* 2013). Considering the critical role of the ar/R region as the selective filter, we sought to determine whether solute permeability of GintAQPF2 is defined solely by the diameter and polarity, or other parameters of the ar/R constriction. In contrast to carbon transport in AM symbiosis where significant progress has been made (Bago *et al.* 2000), the mechanism underlying glycerol transport remains largely unknown.

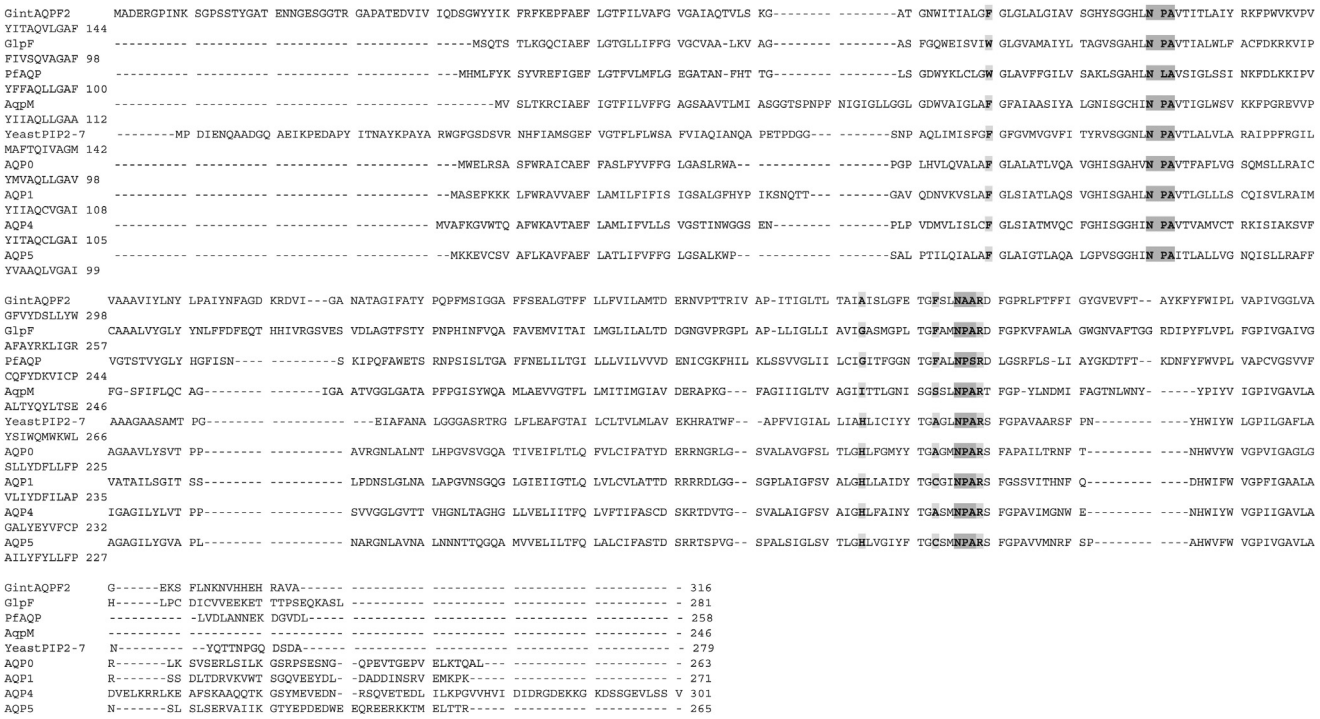

Fig 1 – Protein sequence alignment of GintAQPF2 and other major intrinsic proteins (MIPs) with X-ray structures. Shading in grey shows the sites of the aromatic/arginine (ar/R) constriction, dark grey indicates positions of asparagine-proline-alanine motifs.

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Table 1 – Mutagenic primers.

Primers		5′→3′
GintAQPF2-F94A	Forward primer	GGTGCTGGTTTAGGTCTCGC
	Reverse primer	TAAAGCTATAGTGATCCAATTTC
GintAQPF2-F94W	Forward primer	CTTTAGGTTGGGGTTTAGGTC
	Reverse primer	CTATAGTGATCCAATTTCC
GintAQPF2-A234G	Forward primer	CCATTGGTATTTCACTTGGG
	Reverse primer	CCGTTAAAGTTAGACCAATG

To unravel the relationship between the structural features of the ar/R constriction in GintAQPF2 and glycerol transport ability, we carried out a series of point mutations at ar/R site and analysed the consequences of these alterations in yeast. In addition, comparative genomic analysis could identify the presence or absence of genes in a genome across other genomes, and establish phylogenetic profiles for the genes and subsequently differentiate metabolic traits of corresponding organisms (Załuga *et al.* 2014). Therefore, in terms of the technical limitations in studying AMF genetics (Helber & Requena 2008), genomic analyses of four fungal species with different lifestyles were performed to set up a relationship of glycerol transport with AM symbiosis, which has not been identified to date.

Materials and methods

Comparative modelling of a bacterial glycerol facilitator (GlpF) and GintAQPF2

Protein sequences of MIPs with published three dimensional structures were retrieved from the National Center for Biological Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The amino acid sequence alignment was performed with ClustalW (a program in *MEGA* version 4) to identify the conserved ar/R region in GintAQPF2 (Fig 1). BLAST was used to search for the best homology modelling template from the MIP protein sequences. The 2.2 Å structure of GlpF from *Escherichia coli* (i.e. sharing the largest identity with target) was used as a template to produce a structure model of the ar/R constriction of GintAQPF2 by using the Swiss-model server (http://swissmodel.expasy.org). Figures were prepared using PyMOL software (DeLano Scientific, San Francisco, USA).

Site-directed mutagenesis of GintAQPF2

Point mutations were introduced into *GintAQPF2* by PCRs using a MutanBEST Kit (TAKARA, Dalian, China). Primers with respective nucleotide changes were listed in Table 1. All mutations were confirmed by DNA sequencing.

Heterologous expression in yeast and growth assays under osmotic stress

For functional analysis of the *GintAQPF2* mutations, heterologous expression and growth assays were performed, as described by Li *et al.* (2013). Mutated versions of *GintAQPF2* were PCR-amplified and cloned into pGEM-T Easy vector (Promega Corp., Wisconsin, USA) between the *EcoR* I and *Xho* I sites. Then they were cut out from pGEM-T and inserted into the corresponding sites of the *Pichia* overexpression vector pGAPZ B (Invitrogen, Carlsbad, USA), which has been demonstrated to have high efficiency in the expression of recombinant proteins in *Pichia pastoris* (Waterham *et al.* 1997). The recombinant plasmids were linearized with *Avr*II (New England Biolabs, Hitchin, UK) and transformed into *P. pastoris* GS115 cells by electroporation. Positive transformants were selected on the medium containing Zeocin™. For growth assays, *P. pastoris* GS115 cells were pre-grown in yeast extract peptone dextrose (YPD) medium to an optical density at 600 nm (OD_{600}) of 0.2. Fifty microlitres of each were taken and added to 10 ml YPD containing a final concentration of 25 % polyethylene glycol (PEG) or 1 M glycerol or no exogenous osmolytes. Samples were incubated at 30 °C and collected at 9, 12, 15, 18, 21 and 30 h. Changes in cell density were monitored

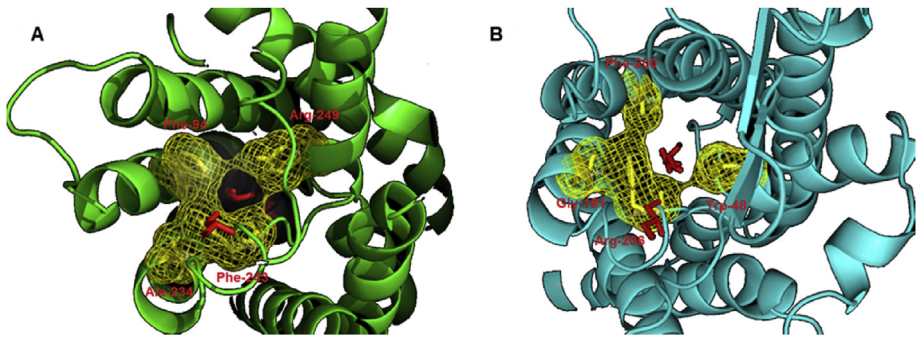

Fig 2 – Modelling and electron density ($2F_o$–F_c) (yellow lines) of the ar/R constriction in (A) GintAQPF2 and (B) *Escherichia coli* glycerol facilitator GlpF. Numbering of the four key residues and simulation of the shape and electron density of the ar/R constriction of GintAQPF2 were based on the structure of GlpF. Trp, tryptophan; Phe, phenylalanine; Gly, glycine; Ala, alanine; Arg, arginine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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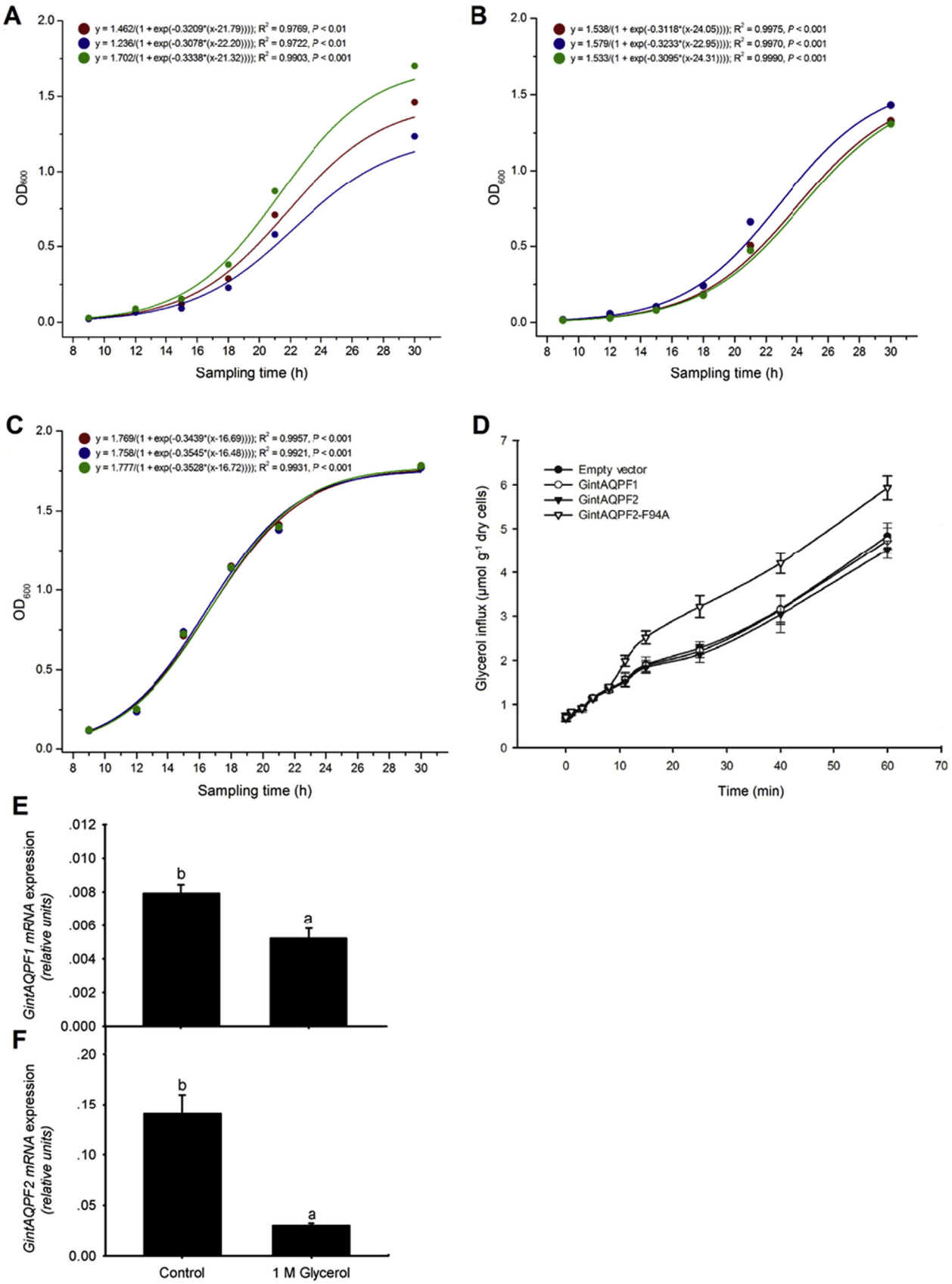

Fig 3 – Evidence for GintAQPF2 disfavouring glycerol transport. Growth responses to osmotic stress of transformed yeast overexpressing *GintAQPF2* and *GintAQPF2-F94A* (A, B, and C). GintAQPF2-F94A, a GintAQPF2's mutant is generated by replacing residue phenylalanine (F)-94 with residue alanine (A). Each data point represents the mean of the values from three

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by OD_{600} using Microplate Reader Spectra (SPECTRA max190, Molecular Devices, San Francisco, USA). Each assay was replicated independently three times.

Measurement of glycerol permeability

Intracellular glycerol content was determined according to Tamás *et al.* (1999), with minor modifications. After pregrowth in YPD medium to an OD_{600} of 2.0, *Pichia pastoris* GS115 cells were harvested, washed and suspended in ice-cold MES buffer (10 mM MES, pH 6.0) to a density of 25 mg cells/ml. Glycerol uptake was measured with an external concentration of 100 mM ice-cold glycerol. After exposing these cells for 0, 1, 3, 5, 8, 11, 15, 25, 40, and 60 min, the reaction was stopped by diluting with 5 ml ice-cold water. Aliquots of 50 μl were collected by filtration and then immediately washed three times with ice-cold water. Glycerol concentrations were measured using a glycerokinase/lactate dehydrogenase-coupled assay (Roche, Mannheim, Germany). Filters with cells were dried at 105 °C for 48 h to a constant weight. Data were expressed as μmol per gram of dry cells and representative of one experiment repeated three times independently with similar results.

Gene expression analysis

The monoxenic culture system for AMF was adopted to confirm the responses of both *GintAQPF1* and *GintAQPF2* in ERM of *Rhizophagus irregularis* AH01 to high concentrations of glycerol. Spores were surface sterilized according to Bécard & Fortin (1988) and placed near the apex of the Ri-T DNA transformed carrot (*Daucus carota* L.) root piece in two-compartment Petri dishes (St-Arnaud *et al.* 1996). The root compartment contained solid minimal medium (M medium) to initiate the monoxenic culture, and the other compartment for ERM development was filled with liquid M medium without sucrose. After cultivation for three months, ERM was incubated in liquid M medium containing a final concentration of 1 M glycerol for 4 d. Total RNA from ERM was extracted by using the RNeasy Plant Mini Kit (Qiagen, Santa Clarita, USA). Following cDNA synthesis with the PrimeScript® RT Reagent Kit (TAKARA), quantitative real-time PCR (qRT-PCR) was performed in a Bio-Rad iQ5 Optical system (Bio-Rad, Hercules, USA), monitored by SYBR Green I fluorescence (TAKARA), as described by Li *et al.* (2013). *Gint18S rRNA* was used as reference. qRT-PCR was conducted in triplicate using at least three independent cDNA samples.

MIP searching in fungal genomes

Based on the two highly conserved NPA motifs located in MIPs, we searched the fungal genome databases in NCBI for candidate MIP genes. The sequences of selected genes were translated to protein sequences by using the Translate tool on the ExPASy Server (http://web.expasy.org/translate), followed by the prediction of hypothetical transmembrane topology using the HMMTOP transmembrane topology prediction server (Gabor & Istvan 2001) to ensure all candidates conform to MIP family-specific sequence signature (six transmembrane domains), as reported by Pettersson *et al.* (2005). Fungal genome databases include *Rhizophagus irregularis* DAOM 197198 (taxid: 1432141), *Laccaria bicolor* (taxid: 29883), *Magnaporthe oryzae* (taxid: 242507) and *Fusarium verticillioides* (taxid: 117187).

Statistical analysis

Data were expressed as the means ± standard deviations (SDs) of observations from independent experiments. For curve fitting of yeast cell growth, nonlinear regression and curve comparison with a sum-of-squares F test were performed by using Origin 8.6.0 (32-bit) Sr2b98 (OriginLab Corporation, Massachusetts, USA). To analyse glycerol uptake and *GintAQPF1* and *GintAQPF2* expression, data were subjected to t testing in Microsoft® Excel 2007.

Results

Structural prediction of the ar/R constriction

We proposed the structure of GintAQPF2 by homology modelling using the 2.2 Å crystal structure of GlpF as our template. As shown in Fig 2, the ar/R constriction of GintAQPF2 consisted of phenylalanine-94 (Phe-94), alanine-234 (Ala-234), Phe-243 and arginine-249 (Arg-249). Phe-243 in loop E_1 (LE_1) and Arg-249 in LE_2 of GintAQPF2 were well conserved with GlpF Phe-200 and Arg-206, whereas variations of the other

independent experiments. Curve fitting by the Slogistic1 model, $y = a/\{1 + \exp[-k(x-x_c)]\}$, in Origin 8.6.0 Sr2b98. Red, blue, and green curves represent the growth curves of *Pichia pastoris* overexpressing Empty Vector, *GintAQPF2* and *GintAQPF2-F94A*, respectively. (A) *P. pastoris* GS115 in 1 M glycerol. There were significant differences ($P < 0.05$) in parameter '*a*' among the curves, but no significant differences among the curves for x_c and *k*. (B) *P. pastoris* GS115 in 25 % polyethylene glycol (PEG). Parameter '*a*' was significantly different between Empty Vector/*GintAQPF2-F94A* and *GintAQPF2* ($P < 0.05$), but with no significant difference between Empty Vector and *GintAQPF2-F94A*. No significant difference among curves for x_c and *k*. (C) *P. pastoris* GS115 under normal conditions. No significant differences among the curves for *a*, x_c and *k*. (D) Uptake profile of glycerol by transformed yeast overexpressing *GintAQPF1*, *GintAQPF2*, and *GintAQPF2-F94A*. Glycerol uptake was measured as the accumulation of intracellular glycerol as a function of time after addition of glycerol to a final concentration of 100 mM. Empty vector, close circles; *GintAQPF1*, open circles; *GintAQPF2*, close triangles; *GintAQPF2-F94A*, open triangles. The values given are means ± SD of three independent experiments. (E, F) *GintAQPF1* and *GintAQPF2* expression in extraradical mycelium of *Rhizophagus irregularis* in expose to 1 M glycerol. Different letters above the columns indicate significant differences (t-test, $P < 0.05$) between treatments. The error bars represent SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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two residues from helices 2 (H2) and 5 (H5) decrease the diameter of the ar/R constriction of GintAQPF2.

Glycerol permeability

To examine the effects of variable residues on GintAQPF2 function, we generated four mutated versions of GintAQPF2 (GintAQPF2-F94A, GintAQPF2-F94W, GintAQPF2-A234G, and GintAQPF2-F94W/A234G) and assessed their selectivity against glycerol.

Individual or joint replacement of Phe (F)-94 and Ala (A)-234 by tryptophan [Trp (W)] and glycine (G) (refer to the last three mutants listed above), same as in GlpF, did not affect glycerol permeability (data not shown). GintAQPF2-F94A with substitution of Phe-94 by Ala led to a significant increase in the OD_{600} of transformed cells in response to 1 M glycerol compared with the cells transformed with empty pGAPZ B vector or *GintAQPF2* (Fig 3A). However, in the presence of 25 % PEG, the mutant gene was not functional, and no significant differences in the alleviation of PEG stress compared with the control were observed (Fig 3B). Moreover, we did not note growth differences among the three types of transformants in the absence of exogenous osmolytes, demonstrating that growth alterations upon glycerol and PEG treatments were caused by the mutations (Fig 3C).

Consistent with the results from the yeast growth assays at high glycerol levels, glycerol uptake assays indicated that *GintAQPF2-F94A* overexpression resulted in a significant increase in intracellular accumulation of glycerol after 11 min of exposure compared with that of cells expressing either empty vector, GintAQPF2 or water-specific GintAQPF1 (Fig 3D). No significant differences in glycerol uptake were observed between the control, GintAQPF2 and GintAQPF1, confirming that glycerol was only permeated through GintAQPF2-F94A.

Gene expression in ERM

The qRT-PCR data indicated that high glycerol concentrations significantly decreased *GintAQPF1* and *GintAQPF2* expression in ERM (Fig 3E, F).

AQGP distribution among the selected fungi

In this study, four representative fungi were selected due to their different strategies for accessing nutrients from hosts and were classified into two branches: obligatory fungi and facultative fungi. The biotrophic AMF *Rhizophagus irregularis* and necrotrophic pathogen *Fusarium verticillioides* belong to the former group, exhibiting only one type of lifestyle (Güimil *et al.* 2005; Geurts & Vleeshouwers 2012). The latter group, consisting of the ectomycorrhizal (ECM) fungus *Laccaria bicolor* and hemibiotrophic pathogen *Magnaporthe oryzae*, has a dual saprotrophic (*L. bicolor*)/necrotrophic (*M. oryzae*) and biotrophic lifestyle (Mendgen & Hahn 2002; Martin *et al.* 2008). We revealed that only obligatory fungi lacked the capacity to transport glycerol. For the facultative fungi, *M. oryzae* possessed six MIPs, two out of which were classified into AQGPs (GenBank accession numbers XM_003711660 and XM_003717454) and responsible for efficient glycerol transport (Birch 2013). Similarly, one AQGP (JGI identification number 391485) in *L. bicolor* exhibited a strong glycerol conductance (Dietz *et al.* 2011). However, for the obligatory fungi, no MIPs existed in *F. verticillioides*. Although *R. irregularis* had three MIPs, they showed no glycerol permeability based on our results and those of Aroca *et al.* (2009), who carried out GintAQP1 transport activity assays in *Xenopus laevis* oocytes.

Discussion

AMF are obligate biotrophs due to their nutritional deficiencies resulting from lack of some metabolic pathways. Previous study demonstrated that carbon metabolism and transport contribute significantly to AM symbiosis (Bago *et al.* 2000). Whether glycerol fluxes are correlated with AM symbiotic establishment, however, remains an open question. In this study, we confirmed that GintAQPF2 was impermeable to glycerol because of the special structural features of its ar/R constriction. The function of GintAQPF2 is likely associated with AMF survival strategies.

According to Fu *et al.* (2000), three ar/R residues located at H2, LE_1, and LE_2 were key determinants conferring AQGP transport behaviours. In rat aquaporin 1, Phe at H2 and cysteine at LE_1 were strong indicators of its water specificity, whereas the presence of Trp at H2 and Phe at LE_1 led to a larger and more hydrophobic ar/R filter of GlpF, which greatly favours glycerol transport (Fu *et al.* 2000; Sui *et al.* 2001). These findings suggest that specificity switch (water to glycerol transport) can be achieved by changing the polarity and diameter of ar/R constriction. Interestingly, we found that replacing GintAQPF2 with GlpF H2/H5 residues (GintAQPF2-F94W and GintAQPF2-F94W/A234G) had no effect on glycerol permeability (data not shown). It has been reported that side-chain conformations of the conserved Arg residue at LE_2 modulate the formation of ring stacking between the methylindole ring of Trp and the toluene ring of Phe, which could close the channel and thus block glycerol transport (Xin *et al.* 2012). Consistently, the Ala substitution of Phe at H2 (GintAQPF2-F94A) resulted in loss of ring stacking and elevated glycerol permeation (Fig 3A, D) by creating a much wider pore aperture than the Trp substitution (GintAQPF2-F94W). Theoretically, replacing the phenyl ring of Phe should enhance polarity and water permeability (Beitz *et al.* 2006), while our data showed no effect on water transport (Fig 3B), implying that water transport through GintAQPF2 was dependent on the ar/R diameter rather than polarity.

Fu *et al.* (2000) reported that the LE_1-Phe located at the narrowest position of the GlpF channel. Its phenyl ring provided a hydrophobic corner favourable for Van der Waals interaction with the alkyl chain of glycerol and glycerol flux. Surprisingly, GintAQPF2 was still an efficient water facilitator (Li *et al.* 2013), even with Phe at LE_1. This finding reconfirmed that the ar/R diameter determined solute permeability. Moreover, new double-gate mechanisms likely existed. As mentioned above, the ar/R residue at H5 was dispensable for AQGP transport behaviours (Fu *et al.* 2000). However, our data showed that GintAQPF2-A234G (alteration at H5) was in a closed state and disfavoured water or glycerol transport (data not shown). Hence, we speculated that Arg249 as the primary steric gate could arrange the conformation of toluene rings of both Phe-94 and Phe-243, which located around the narrowest region

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inside GintAQPF2, consequently making Phe94-Phe243 an additional gate for water flow. In addition, Arg-249 likely donated three hydrogen bonds to GintAQPF2, as in most other water-specific channels, to allow water passage (Newby *et al.* 2008). Further studies should focus on how the dual gates cooperate and function.

The previous studies indicated that intracellular structures formed during the interaction with AMF were similar to those with biotrophic pathogenic fungi, which resulted in the prediction that plants might use the same genetic programs for responding to pathogens (Parniske 2000). Similarly, some evolutionarily conserved proteins (e.g. mitogen-activated protein kinases) of the fungi could function to contribute to an interconnected molecular dialogue between the plant and the fungus, but relying on some unknown mechanisms, the fungi could regulate some modules to realize different strategies (Hamel *et al.* 2012). On account of this, in view of the difficulties of studying AMF genetics, we have endeavoured to provide evidence for a role of glycerol transport in AM symbiosis by analysing AQGP distribution among four types of fungi with different lifestyles. Interestingly, conservation of glycerol transport pattern was observed either in obligatory or in facultative symbionts and pathogens. Nonetheless a set of genes encoding the main enzymes involved in *de novo* synthesis of TAG were conserved in all the fungi (unpublished data). These led to the prediction that glycerol facilitator might have evolved to support a dual lifestyle of facultative fungi. Glycerol transport was critical for metabolic interactions between plants and fungal pathogens with a biotrophic lifestyle (Wei *et al.* 2004), whereas ECM formation modified gene expression profiles of glycerol facilitator, consequently making ECM fungi disfavour substantial glycerol permeability (Dietz *et al.* 2011). In spite of the difference, with glycerol supply, they both could potentially synthesize TAG, complete their life cycle and thus exhibit a saprotrophic (ECM fungi)/necrotrophic (pathogenic fungi) phase. Conversely, glycerol blocks might be important for interactions between plants and obligatory fungi. The results from the heterologous system showed that the only one AMF AQGP (GintAQPF2) disfavoured glycerol transport due to the special structural features of its ar/R constriction (Fig 3A, D). In addition, AMF reduced water loss by down-regulating expression of only two functional *MIP* genes, *GintAQPF1* and *GintAQPF2*, in ERM at the high glycerol level (Fig 3E, F) to maintain its water potential rather than taking up glycerol. Furthermore, supplying labelled permeant glycerol to ERM resulted in labelling of a small portion of fungal storage carbohydrates including trehalose (with only 11 % ± 2 % labelling) and glycogen (too low to quantify reliably), which were synthesized in the ERM from TAG (Bago *et al.* 2003). These actions excluded the possibility that AMF had efficient glycerol channels. Consequently, this could ensure that the glycerol backbone originated almost entirely from conversion of hexose taken up from host plants (Pfeffer *et al.* 1999) and likely make AMF an obligate symbiont.

Conclusions

We found that the ar/R diameter and the potential dual gates (Arg-249 and Phe94-Phe243) determined water permeability and glycerol impermeability of GintAQPF2. Future studies will investigate the gating mechanisms based on the crystal structure of GintAQPF2.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2016.09.006.

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