**Mesophyll conductance is unaffected by expression of Arabidopsis PIP1 aquaporins in the plasmalemma of Nicotiana**

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**Abstract**

In plants with C₃ photosynthesis, increasing the diffusion conductance for CO₂ from the substomatal cavity to chloroplast stroma (mesophyll conductance) can improve the efficiencies of both CO₂ assimilation and photosynthetic water use. In the diffusion pathway from substomatal cavity to chloroplast stroma, the plasmalemma and chloroplast envelope membranes impose a considerable barrier to CO₂ diffusion, limiting photosynthetic efficiency. In an attempt to improve membrane permeability to CO₂ and increase photosynthesis in tobacco, we generated transgenic lines in *Nicotiana tabacum* L. cv Petite Havana carrying either the Arabidopsis PIP1;2 (*AtPIP1;2*) or PIP1;4 (*AtPIP1;4*) gene driven by the constitutive dual 2x35S CMV promoter. From a collection of independent T₀ transgenics, two T₂ lines from each gene were characterized, with western blots confirming increased total aquaporin protein abundance in the *AtPIP1;2* tobacco lines. Transient expression of *AtPIP1;2-mGFP6* and *AtPIP1;4-mGFP6* fusions in *Nicotiana benthamiana* identified that both *AtPIP1;2* and *AtPIP1;4* localize to the plasmalemma. Despite achieving ectopic production and correct localization, gas exchange measurements combined with carbon isotope discrimination measurements detected no increase in mesophyll conductance or CO₂ assimilation rate in the tobacco lines expressing *AtPIP*. We discuss the complexities associated with trying to enhance $g_m$ through modified aquaporin activity.

**Keywords:** Carbon isotope discrimination, mesophyll conductance, *Nicotiana tabacum*, photosynthesis, PIP aquaporin genes, transgenic.

**Introduction**

Enhancing photosynthetic processes has increasingly been a research target due to the need to improve crop yields to feed a growing global population in the face of changing climates and diminishing resources (Ray et al., 2013; Bailey-Serres et al., 2019). A key first step in C₃ photosynthesis is the diffusion of atmospheric CO₂ into leaves where it is fixed by Rubisco within chloroplasts. Improving the conductance to CO₂ diffusion within leaves is predicted to increase photosynthetic
capacity and ultimately crop yields, while also improving water use efficiency (Lundgren and Fleming, 2020). Several points of resistance for CO2 diffusion occur on the path from atmosphere into chloroplasts (Clarke et al., 2021). Initially, CO2 diffuses through the leaf boundary layer and stomatal pores, whose aperture limits the ease with which CO2 passes into the substomatal cavity and regulates water loss from the leaf. CO2 diffusing between substomatal cavity airspaces and mesophyll tissue encounters resistance from the cell wall, plasma membrane, cytosol, chloroplast envelope, and stroma (the aqueous chloroplast phase). The sum of all these resistances is termed mesophyll resistance, and its inverse, mesophyll conductance ($g_m$), captures how efficiently CO2 can move through mesophyll tissue to the chloroplast stroma where it is fixed by Rubisco.

We know little about the exact resistance to CO2 associated with the cell membranes. Experiments using artificial membranes found that CO2 diffuses rapidly through simple lipid bilayers, arguing that there is no need for facilitated transmembrane transport of CO2. However, subsequent work highlighted that unlike simple lipid bilayers, biological membranes have high protein and sterol content that substantially reduces their permeability to CO2, suggesting the need for embedded membrane channels or transporters (Endeward et al., 2014, 2017). However, others argue that the solubility–diffusion model (also known as the Meyer–Overtorn rule) alone still accounts for gaseous CO2 transfer across biological membranes (Misser and Pohl, 2009). Modelling of the diffusion resistances in plants, suggests that plant cell membranes represent a significant component of $g_m$ in leaves (Evans et al., 1994, 1999; Tholen and Zhu, 2011; von Caemmerer and Evans, 2015; Evans, 2021), and that factors that increase membrane CO2 permeability should increase $g_m$ and consequently CO2 assimilation rate.

Manipulation of genes to alter the transmembrane protein composition of biological membranes to facilitate CO2 diffusion has been an active area of research over the past 25 years. Nakhoul et al. (1998) first reported that heterologous expression of human AQUAPORIN 1 (hAQP1) in Xenopus oocytes increased plasma membrane permeability to CO2. Aquaporins (AQP)s, such as hAQP1, are pore-forming membrane-spanning proteins belonging to the larger Major Intrinsic Protein (MIP) family. Originally named after their ability to passively move water across membranes, AQP$s$ have since been reported to facilitate the transfer of many different substrates across biological membranes, including gases (Maurel et al., 2015). Several members of the Plasma membrane Intrinsic Protein (PIP) subfamily, which are homologous to hAQP1, have evidence of facilitating CO2 transport across cell membranes (Uehlein et al., 2017). Consistent with a role in regulating $g_m$, PIP proteins generally localize to the plasma membrane, with some isoforms also detected in chloroplast envelopes by western blot and proteomic analysis (Uehlein et al., 2008; Beebo et al., 2013).

Using Xenopus oocytes and yeast heterologous systems, NtAQP1, also known as NtPIP1;5s (De Rosa et al., 2020; Groszmann et al., 2021), was the first plant AQP identified to be permeable to CO2 (Uehlein et al., 2003; Otto et al., 2010). Since then, a number of other CO2–permeable PIPs have been identified using heterologous systems, including AtPIP1;2 (Heckwolf et al., 2011), AtPIP2;1 (Wang et al., 2016), Hordeum vulgare HvPIP2;1, HvPIP2;2, HvPIP2;3 and HvPIP2;5 (Mori et al., 2014), Zea mays ZmPIP1;5 and ZmPIP1;6 (Heinen et al., 2014), and Setaria italica SiPIP2;7 (Ermakova et al., 2021).

In planta studies revealed that decreasing NtAQP1/NtPIP1;5s transcript abundance by RNA interference (RNAi), resulted in tobacco plants with reduced photosynthetic rate and mesophyll conductance (Flexas et al., 2006; Uehlein et al., 2008). Similarly, T-DNA knock-out mutants of an Arabidopsis homolog, AtPIP1;2, and co-suppression of rice Oryza sativa OsPIP genes also resulted in a reduced $g_m$ (Hanba et al., 2004; Heckwolf et al., 2011). On the other hand, Kromdijk et al. (2019) failed to observe any difference in $g_m$ between wild-type (WT) and single knockout mutants of AtPIP1;2, AtPIP1;3 or AtPIP2;6 in Arabidopsis.

Conversely, there are reported examples where overexpression of PIP AQPs has improved photosynthesis. Overexpression of NtAQP1/NtPIP1;5s in its native host tobacco led to an increase in $g_m$ by 20% compared with controls, with a corresponding increase in CO2 assimilation rate (Flexas et al., 2006). Similarly, overexpression of SiPIP2;7 increased $g_m$ and CO2 assimilation in the C4 photosynthetic species S. italica (Ermakova et al., 2021), while overexpression of OsPIP1;2 increased $g_m$ and photosynthesis, and improved productivity in rice (Xu et al., 2019). Cross–species expression of some AQP isoforms have also been shown to increase mesophyll conductance including, tobacco NtAQP1/NtPIP1;5s in Arabidopsis (Sade et al., 2014) and tomato (Kelly et al., 2014), barley HvPIP2;1 in rice (Hanba et al., 2004), and Mesembryanthemum crystallinum, MmMIPB (PIP1-subtype), in tobacco (Kawase et al., 2013). Thus, the use of foreign AQPs represents another potential avenue to engineer improvements in $g_m$ and CO2 assimilation in species of interest.

Tobacco is a popular model species that is closely related to crops of economic interest such as tomatoes, potatoes, eggplants, and peppers, and itself has renewed commercial applications in the biofuel and plant-based pharmaceutical sectors. Tobacco is capable of scaling from the laboratory to the field and as such is a key model for trialing transgenic manipulations to improve photosynthesis before translation into food crops. In this study, we investigated the effects on membrane permeability to CO2 in tobacco plants expressing the Arabidopsis PIP1 AQPs AtPIP1;2 and AtPIP1;4. We confirm protein expression and subcellular localization to the plasma membrane of tobacco mesophyll cells for these AtPIPs, but could not detect an increase in mesophyll conductance. We discuss the complexities associated with trying to enhance $g_m$ through modified aquaporin activity.

**Materials and methods**

*Assembly of constructs*

AtPIP1;2 and AtPIP1;4, and β-glucuronidase (GUS) (non-AQP expression control) protein-coding sequences (CDS) were commercially synthesized.
(Genscript) as gateway-enabled entry vectors (i.e. included flanking attL sites) incorporating dicot optimal Kozak translation start site sequences (AGAACCAGTGGGA). A second set of AtPIP1;2 and AtPIP1;4 genes without the stop codon were also made, for use in green fluorescent protein (GFP) C-terminal fusion constructs. The AtPIP1;2, AtPIP1;4, and GUS CDSs were cloned into expression vectors of the pMDC Gateway-compatible Agrobacterium sp. binary vector system (Curtsius and Grossniklaus, 2003) using Gateway LR Clonase II enzyme mix (Thermo Fisher Scientific). Full length CDSs (i.e. including stop codon) were inserted into pMDc32 (MG0100 in this study) resulting in a final cassette of RB-2x35S:AtPIP1;2:nosT-Hyg'-LB, RB-2x35S:AtPIP1;4:nosT-Hyg'-LB, and RB-2x35S:GUS:nosT-Hyg'-LB ArPIp CDSs without the stop codons were cloned into pMDc83 (MG0100 in this study) to create C-terminal GFP fusions with the final cassette being RB-2x35S:AtPIP1;2-GFP6his:nosT-Hyg'-LB and RB-2x35S:AtPIP1;4-GFP6his:nosT-Hyg'-LB. All E. coli cloning steps used One Shot OmniMAX 2 T1R Chemically Competent E. coli cells (Thermo Fisher Scientific). All final plasmids were Sanger sequenced to confirm accuracy of the clones using Wizard Plus SV Miniprep DNA Purification Systems (Promega), BigDye sequencing chemistry (Thermo Fisher Scientific), and ZR DNA Sequencing Clean-Up Kit (Zymo Research). Final expression vectors were transformed via electroporation into Agrobacterium tumefaciens strain GV3101 and PCR genotyped with primers flanking the AtPIP or GUS CDSs (2x3SS forward (fwd): 5'-TCTAGAGACATGACTATCCAGCAAG and NOS-term: 5'-GCAAAGACGGCGAAGAAGATT). Stable transgenic plants

Surface-sterilized seeds of WT Nicotiana tabacum, L. cv Petite Havana were germinated in sterile Magenta boxes containing half-strength Murashige and Skoog (MS) medium (pH 5.7), 3% (w/v) sucrose, and 0.3% (v/v) agarose and grown for 4 weeks at 28 °C at 350 ± 100 μmol photons m⁻² s⁻¹ illumination and 16 h daylength. Four-week-old tobacco plants were used for transformation via the leaf disc method on co-cultivation medium (Horsch et al., 1985). Leaf discs were then transferred to regeneration medium consisting of MS-based medium containing agar (0.6 g l⁻¹), hygromycin (50 mg l⁻¹), timentin (50 mg l⁻¹), 6-benzylaminopurine (BAP; 1 mg l⁻¹), 1-naphthaleneacetic acid (NAA; at 1 mg l⁻¹), myo-inositol (100 mg l⁻¹), and thiamine (1 mg l⁻¹) for 2–3 weeks to stimulate callus formation. Thereafter, the calli were transferred to fresh regeneration medium (agar (0.6 g l⁻¹), hygromycin (50 mg l⁻¹), timentin (50 mg l⁻¹), BAP (1 mg l⁻¹), NAA (1 mg l⁻¹), myo-inositol (100 mg l⁻¹) and thiamine (1 mg l⁻¹)) to stimulate shoot growth. Prominent shoots were excised and placed onto rooting plates consisting of MS agar medium with hygromycin (50 mg l⁻¹) and timentin (50 mg l⁻¹). Ten hygromycin-resistant primary transformants for each construct (T₁ generation) with established root systems were transferred to soil and allowed to self-fertilize. The presence of the transgene was confirmed via PCR genotyping on gDNA using the above 2x3SS fwd and NOS-term reverse primer set.

qPCR to quantify transgene expression

T₁ generation seedlings were selected on hygromycin (50 mg l⁻¹) MS-based agar medium for 18 d post-germination at 16 h light, 22 °C. and light intensities between 100 and 120 μmol m⁻² s⁻¹. Three biological replicates per independent transgenic line, each consisting of aerial tissue from five T₁ seedlings, were harvested and snap frozen in liquid nitrogen. Tissue was ground using a Qiagen TissueLyser II and RNA extracted using the ISOLATE II RNA Plant Kit (Meridian Bioscience). RNA was quality checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and diluted to 200 ng μl⁻¹. One microgram of RNA was aliquoted and DNaseI treated as per the manufacturer’s instructions (cat. no. 18068015; Thermo Fisher Scientific). cDNA was generated using the sensiFAST cDNA Synthesis Kit (Meridian Bioscience) as per the manufacturer’s instructions and diluted 1:10. Real-time reactions were set up using SensiFAST SYBR Lo-ROX Kit (Meridian Bioscience) chemistry as per the manufacturer’s instructions using 1 μl of cDNA (~5 ng). qPCR reactions were performed in a 384-well plate format on a ViiA 7 Real-Time system (Applied Biosystems/Thermo Fisher Scientific) using the cycle format: 95 °C, 2 min (×1); 95 °C, 5 s; 60 °C, 10 s; 72 °C, 10 s (×40); and finished with a melt curve between 95 °C and 60 °C. QuantStudio Real-Time PCR Software (Thermo Fisher Scientific) was used to capture and analyse the data. Primers specific to the 3’ end of the AtPIP CDS and within the 3’ transcribed region of the NOS-terminator were used to detect transgene abundance; AtPIP1;2 (fwd: 5’-TTCGTCGTCTCTTACCACTGGT and reverse (rev): 5’-GGAATTCCGAGCTCCACCCG) and AtPIP1;4 (fwd: 5’-TCTAGACGACTATATCCAGCAAG and rev: 5’-GGAATTCCGAGCTCCACCCG). Data were analysed using the ΔCt method, with NUBG2 expression used for normalization (Schmidt and Delaney, 2010) (fwd: 5’-AGCTGCATATCGACTTGATCCA and rev: 5’-TCTCACTGAACATGGTGCT). Transient expression in Nicotiana benthamiana

Wild-type Nicotiana benthamiana plant were grown for 4 weeks in a CONVIRON (Winnipeg, Canada) growth chamber under a 16 h/8 h day/night cycle at 28°C/22°C with 60% humidity and with approximately 100 μmol photons m⁻² s⁻¹ light intensity. Agroinfiltration was performed as described in Rolland (2018). Briefly, Agrobacterium tumefaciens GV3101(pMP90) (Koncz and Schell, 1986) was transformed with plasmids containing C-terminal GFP fusions with AtPIP1;2 or AtPIP1;4 and grown on LB medium containing rifampicin (50 μg ml⁻¹) and kanamycin (25 μg ml⁻¹). Cultures were grown at 28°C with shaking at 220 rpm. Nicotiana benthamiana leaves were co-infiltrated onto their abaxial surface with either AtPIP1;2-GFP or AtPIP1;4-GFP vectors and a vector containing the P19 protein to inhibit post-translational gene silencing and allow the PIP–GFP fusion constructs to be expressed (Roth et al., 2004).

Protoplast preparation and confocal microscopy

Leaf sections of approximately 4 cm x 4 cm were harvested 3 d post-infiltration from N. benthamiana leaves transformed with either AtPIP1;2-GFP + P19 or AtPIP1;4-GFP + P19. Protoplasts were isolated as detailed in Rolland et al. (2016). In two independent experiments, around 100 protoplasts (per independent experiment) expressing GFP-tagged constructs were observed, and a selection were imaged using an upright Zeiss LSM780 confocal laser-scanning microscope (Carl Zeiss), a ×40 water immersion objective (NA 1.1) and the Zen Blue software package (Carl Zeiss). GFP and chlorophyll were excited at 488 nm and emission recorded at 499–535 nm and 630–735 nm, respectively. Higher resolution images (with the same excitation and emission spectra) were obtained on an upright Zeiss LSM800 with Airyscan (Carl Zeiss) fitted with a ×63 oil immersion objective (NA 1.4), and Zen Blue software package (Carl Zeiss).

Further imaging on selected protoplasts was conducted using the Stellaris 8 Falcon (Leica) utilizing FAST FLIM to separate chlorophyll autofluorescence and GFP signals, under the following conditions: ×40 water lens (NA 1.1), excitation 473 nm, emission 493–550 nm and 10 line accumulations. Additional confocal imaging on the Stellaris excited GFP at 482 nm (emissions at 525–542 nm), and chlorophyll at 650 nm (emission 667–755 nm), using a ×40 water lens (NA 1.1).

Plant growth

Tobacco (Nicotiana tabacum, L. cv Petite Havana) was grown in a naturally lit glasshouse with day/night temperatures set at 28/18 °C in 5-litre pots filled with Debo Green Wizard commercial potting mix supplemented
with slow release fertilizer at 7 g L⁻¹ (Osmocote Exact, Scotts, NSW, Australia). Plants were grown between October and November 2019 in Canberra (Australia), and watered daily. Average light intensity at midday during the growing period was 1400 µmol m⁻² s⁻¹.

**Gas exchange measurements**

CO₂ response curves of CO₂ assimilation rate and chlorophyll fluorescence were measured together with a LI-6800 portable photosynthesis system (LI-COR Biosciences, USA) at a leaf temperature of 25 °C, irradiance of 1500 µmol quanta m⁻² s⁻¹, relative humidity of 55%, 21% O₂ and varying reference CO₂ concentrations (0, 50, 75, 100, 200, 300, 400, 600, 800, 1000, 1200 µmol mol⁻¹). All gas exchange measurements were made on the youngest expanded leaf of 4-week-old plants. Curves were analysed to derive estimates of maximum Rubisco activity, Vc₅₀, and the rate of electron transport, J (Sharkey et al., 2007). Vc₅₀ was estimated from measurements below C₅₀=400 µbar and J was estimated from measurements between C₅₀=400 and 800 µbar. Triose phosphate utilization (TPU) was not assigned as a limitation in the calculations of J. Direct measurements of gₛ (0.5 mol m⁻² s⁻¹ bar⁻¹) from carbon isotope discrimination measurements on the same plants (see below) were used when fitting the model.

**Concurrent measurements of gas exchange and carbon isotope discrimination to quantify mesophyll conductance**

Gas exchange and carbon isotope discrimination measurements were made as described by Tazoe et al. (2011) using a 6 cm² chamber of the LI-6400 with a red blue light emitting diode (LED) light source (LI-COR). Two LI-6400 chambers and the plants were placed in a temperature-controlled cabinet with fluorescent lights (TRIL1175, Thermoline Scientific Equipment, Smithfield, NSW, Australia). The CO₂ in the leaf chamber was set at 380 µmol mol⁻¹, flow rate at 200 µmol s⁻¹ and irradiance at 1500 µmol quanta m⁻² s⁻¹. Leaf temperature was controlled at 25 °C, N₂ and O₂ were mixed by mass flow controllers (Omega Engineering Inc., Stamford, CT, USA) to generate 2% O₂, which was supplied to the LI-6400s after humidification of the air by adjusting the temperature of water circulating around a Nafion tube (Perma Pure LLC, Toms River, NJ, USA, MH-110-12P-4). Gas exchange was coupled to a tunable diode laser (TDL; TGA100a, Campbell Scientific, Inc., Logan, UT, USA) for concurrent measurements of carbon isotope composition. Measurements were made at 4-min intervals for 20 s, with 10–12 measurements per leaf and the last five measurements were averaged. The δ¹³C of CO₂ gas cylinders (δ¹³Cmed) used in the LI-6400 CO₂ injector system was −10.5 ± 0.5‰. Gas exchange was calculated using the equations presented by Evans et al. (1986). The average value of δ was 6.9 with a standard deviation of 2.32, where δ = Cᵥref / (Cᵥref - Cₓmed) and Cᵥref and Cₓmed are the CO₂ concentrations of dry air entering and exiting the leaf chamber, respectively, measured by the TDL. Measurements were taken on four 6-week-old plants on the youngest expanded leaf. Mesophyll conductance, gₛ, was calculated as described by Evans and von Caemmerer (2013).

**Western blots**

To isolate protein from leaves, leaf discs of 0.71 cm² corresponding to area where gas exchange was measured were collected and frozen immediately in liquid N₂. One disc was ground in ice-cold glass homogenizer in 0.5 ml of protein extraction buffer: 100 mM Tris–HCl, pH 7.8, supplemented with 25 mM NaCl, 20 mM EDTA, 2% SDS (w/v), 10 mM dithiothreitol, and 2% (v/v) protease inhibitor cocktail (Sigma, St Louis, MO, USA). Protein extracts were incubated at 65 °C for 10 min and then centrifuged at 13 000g for 1 min at 4 °C to obtain clear supernatant. Protein extracts were diluted into 4× SDS Sample buffer containing 0.25M Tris–HCl pH 6.8, 40% (v/v) glycerol, 8% SDS, 4% bromophenol blue, 0.5% β-mercaptoethanol, and incubated at 95 °C for 5 min. Samples were loaded on a leaf area basis and separated by polyacrylamide gel electrophoresis (Nu-PAGE 4–12% Bis-Tris gel, Thermo Fisher Scientific) in running buffer (pH 7.5) containing 50 mM MES, 50 mM Tris, 1.0% SDS (w/v), 20 mM EDTA. Proteins were transferred to a nitrocellulose membrane and probed with antibodies against PIP proteins (Agrisera cat. no. A09487, Växhus, Sweden) at 1:1000 dilution. Quantification of western blots was performed with Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA).

**Statistical analysis**

All statistical analyses were performed using two-way analysis of variance. Comparison of means was made using a 0.05 significance level using Tukey’s post hoc test (OriginPro 2020, OriginLab Corp.).

**Results**

Ten independent T₀ transgenic tobacco (cv Petite Havana) lines were generated for both the 2x35S::AtPIP1;2 and 2x35S::AtPIP1;4 transgenes. PCR genotyping using primers specific to the AtPIP transgenes confirmed their presence in the T₀ lines. Positive T₀ tobacco lines were allowed to self-pollinate and produce T₁ seeds. The T₁ lines were sown on hygromycin medium to select for progeny carrying the transgene. Segregation counts were used to indicate transgene locus number, and RNA extracted from five pooled 18-day-old seedlings was used to profile AtPIP transgene expression (see Supplementary Fig. S1). We selected AtPIP1;2 T₁ lines 6 (single insertion) and 10 (double insertion), and AtPIP1;4 T₁ lines 5 (double insertion) and 10 (single insertion) for preliminary physiological analysis. AtPIP1;2 T₁ line 6 and AtPIP1;4 T₁ line 5 showed increased photosynthetic and mesophyll conductance and T₂ lines derived from AtPIP1;2 line 6 (single insertion) and AtPIP1;4 line 5 (double insertion) were then analysed further.

AtPIP protein levels in T₂ lines were assayed in leaf tissue collected from 6-week-old plants by western blot with a α-PIP antibody, which is reported (Agrisera, Sweden) to react to all five AtPIP1 proteins (AtPIP1;1, AtPIP1;2, AtPIP1;3, AtPIP1;4, and AtPIP1;5) and potentially, but less likely, AtPIP2 proteins as the epitope is located within the C loop that is somewhat conserved between PIP1 and PIP2. In non-AtPIP expressing control plants (a stable 2x35S::GUS line, used as a surrogate WT transgenic control), a western signal was detected at 28 kDa, consistent with the expected size of the PIP1 monomer. This cross-reactivity with NtPIPS is unsurprising given the close structural conservation of PIP1 proteins across dicot angiosperms (De Rosa et al., 2020). A faint band was also detected at approximately 50 kDa, which likely corresponds to undenatured PIP dimers (not shown). Lines expressing AtPIP1;2 had significantly increased PIP protein signal over
control plants, while lines expressing AtPIP1;4 were not significantly different from controls (Fig. 1).

Subcellular localization patterns of the AtPIP1 proteins in tobacco were determined using C-terminal GFP fusions driven by the 2x35S promoter and transiently expressed in the close relative of tobacco, Nicotiana benthamiana (Schiavinato et al., 2020). When imaged under confocal microscopy at 488 nm excitation, the GFP-tagged AtPIP proteins were observed in the green channel (499–535 nm), while the autofluorescence from chlorophyll was captured in the magenta channel (630–735 nm). AtPIP1;2–GFP and AtPIP1;4–GFP each localized to the extreme periphery of the cell, consistent with plasma membrane localization (Figs 2, 3). Signal was also detected in the GFP channel associated with the chloroplast and chloroplast envelope of isolated tobacco mesophyll protoplasts (Fig. 2). To determine if the chloroplast envelope signal was PIP–GFP or chlorophyll bleed-through into the GFP channel, isolated protoplasts were imaged with Fluorescence Lifetime Imaging (FAST FLIM, Leica Stellaris Falcon). GFP signal was observed only on the plasma membrane for both AtPIP1;2–GFP and AtPIP1;4–GFP (see Supplementary Fig. S2). Further, the optimal excitation and emission wavelengths for PIP–GFP were determined using a lambda lambda scan (Stellaris Falcon, Leica), and imaging of AtPIP1;4–GFP under these conditions eliminated chlorophyll bleed-through into the GFP channel (Fig. 3).

Two T2 progeny of single insertion line #6 of AtPIP1;2 (lines 6.1 and 6.2) and two from double insertion line #5 of AtPIP1;4 (lines 5.3 and 5.4) were grown for physiological analysis. The AtPIP1;2 and AtPIP1;4 lines showed similar leaf mass per area to the GUS control lines (Table 1). Steady state CO2 assimilation rates (measured at 300 ppm CO2) were comparable to GUS controls (Fig. 4A). Mesophyll conductance and the draw-down of CO2 into the chloroplasts (C5-C4) was also unchanged in the AtPIP1;2 and AtPIP1;4 transgens (Fig. 4B, C). The CO2 response of assimilation was measured, and the expression of the AtPIP1;2 or AtPIP1;4 transgene did not significantly increase assimilation rates (Fig. 5A). Vc,max values derived from gas exchange data and our sequential measurements of mesophyll conductance were not significantly different from controls (Table 1). Electron transport rate (f) calculated from gas exchange data was not significantly different from controls for any of the AtPIP1 transgenic lines (Table 1).

Discussion

Crop plants like tobacco are characterized by relatively thin cell walls, and analysis suggests that membranes may represent up to 50% of the overall mesophyll CO2 diffusion resistance in these leaves (Evans et al., 1994; von Caemmerer and Evans, 2015; Clarke et al., 2021; Evans, 2021). Increasing membrane permeability to CO2 is one way to increase gmax and subsequently photosynthetic rates. Improving gmax would also improve drought tolerance in plants and be complementary to other improvements of photosynthesis, such as increasing the efficiency of Rubisco and RuBP regeneration (Long et al., 2015). A subset of PIP AQPs isoforms have been identified as capable of permeating CO2, which has led to research investigating the role AQPs play in facilitating CO2 diffusion across the mesophyll plasma membrane and chloroplast envelope and their potential for engineering improvements in photosynthesis (for review see Groszmann et al., 2017).

There are several examples where overexpression of PIP aquaporins has resulted in increases in gmax (see Introduction). Overexpression of NtAQPI in tobacco led to an increase in gmax by 20% compared with controls, with a corresponding increase in CO2 assimilation rate (Flexas et al., 2006). However, the increase in CO2 assimilation rate unexpectedly occurred at higher Ci and no changes in initial slope of the CO2 response curves were observed (Flexas et al., 2006). Expression of the PIP proteins HvPIP2;1 or OsPIP1;2 in rice also increased gmax (Hanba et al., 2004; Xu et al., 2019). AtPIP1;2, which is a close homolog of the CO2 transporting NtAQPI/NtPIP1;5s, shows evidence of influencing gmax in its native Arabidopsis. This includes; Atpip1;2 T-DNA knock-out mutants having reduced gmax (Heckwolf et al., 2011) and a 50% reduction in the

![Fig. 1. Analysis of AtPIP overexpression lines. (A) Western blot dilution series at 80, 65, 50, and 35% loading for GUS transgenic control and T2 ectopic expression lines of AtPIP1;2.6.1, AtPIP1;2.6.2, AtPIP1;4.5.3, and AtPIP1;4.5.4 with an anti-PIP1 antibody (Agisera) which reacts to both native tobacco PIP1s and Arabidopsis PIP1s. (B) Only the AtPIP1;2.6.2 expressing line had significantly more overall PIP protein than the control (n=3, two way analysis of variance with post hoc Tukey test, P<0.05).]
CO₂ permeability of chloroplasts (Tolleter et al., 2017), and overexpression of AtPIP1;2 in the Atpip1;2 mutant background restoring \( g_m \) back to WT levels (Heckwolf et al., 2011). More recently, however, Kromdijk et al. (2019) failed to replicate the mutant observations, with no differences observed in \( g_m \) between WT and the single lines for AtPIP1;2 (or AtPIP1;3 and AtPIP2;6) in Arabidopsis. Our results are somewhat analogous in that we did not observe any differences in mesophyll conductance when ectopically expressing Arabidopsis AtPIP1;2 in tobacco, but knock-down and ectopic expression studies are not directly comparable and are subject to other influencing factors.

We worked on transgenic lines with the greatest transgene expression. Ectopic expression of AtPIP1 in tobacco was driven by the 2x35S promoter, which has previously successfully driven ectopic expression of tobacco AQP1 in tomato to phenotype (Kelly et al., 2014). qRT-PCR data showed that our transgenes were expressed (Supplementary Fig. S1), and our confocal data confirmed protein production and incorporation of AtPIP1;2–GFP and AtPIP1;4–GFP into the plasma membrane (Figs 2, 3). The localization of AQPs to the chloroplast envelope through confocal microscopy is complicated by the difficulty in distinguishing GFP fluorescence from chlorophyll autofluorescence at the chloroplast, as observed here in Fig. 2. This can be resolved through spectral unmixing or fluorescence lifetime imaging techniques. Here, lifetime imaging clearly showed no AtPIP1;2–GFP or AtPIP1;4–GFP signal was present at the chloroplast envelope (see Supplementary Fig. S2). With optimized excitation and emission wavelengths, bleed-through of chlorophyll autofluorescence into the GFP channel can also be eliminated (Fig. 3). A plasma membrane localization of AtPIP1;2 and AtPIP1;4 is consistent with the localization pattern of the \( g_m \)-enhancing OsPIP1;2, with OsPIP1;2-GFP localizing to the plasma membrane in rice protoplasts derived from culms (stems) of dark grown plants (Xu et al., 2019). In Xu et al. (2019) confocal images of rice culm cells expressing OsPIP1;2, fluorescence is evident around an internal structure that was not specified, but is likely an etioplast (differentiating chloroplast), but without further analysis it is not clear if this is a true GFP signal. PIP proteins have previously been detected in the chloroplast envelope by proteomics (Kleffmann et al., 2004; Ferro et al., 2010; Simm et al., 2013), but contamination from plasma and vacuolar membranes cannot be excluded (Beebo et al., 2013). Uehlein et al. (2008), using immuno-gold labelling in tobacco, reported plasma membrane localization for NtAQPI/NtPIP1;5s and gold particles were also observed on the chloroplast envelope indicating NtAQPI at least is present on the chloroplast envelope in tobacco.

Quantifying AQP protein content was complicated by the close homology of PIPs across species and the inevitable cross-reactivity of \( \alpha \)-PIP antibodies to both the transgenic
AtPIP1 expression in Nicotiana

We chose not to attach an epitope tag to our AtPIP1;2 and AtPIP1;4 transgenes for fear they may obscure the channel passage, given that both the N- and C-terminal tails reside adjacent to the cytosolic channel opening in the tertiary structure, and are important steric regulators of PIP channel activity (reviewed in Groszmann et al., 2017; Tyerman et al., 2021). Western blots indicated a modest increase of up to 40% in leaf PIP AQP protein content above controls in the AtPIP1;2 overexpressing lines. Total PIP protein content was similar between control and AtPIP1;4 lines, despite active transgene expression. As we cannot distinguish between tobacco and Arabidopsis PIPs, it is possible that the native NtPIP isoforms could have been down-regulated in response to ectopic AtPIP1;4 production. Interaction between ectopically expressed PIP proteins with native tobacco PIP proteins is an important consideration as PIP2 proteins are involved in the recruitment of PIP1 proteins to the plasma membrane (see Groszmann et al., 2017). As AtPIP1;2 was detected on the plasma membrane, we can assume it must be interacting with the native PIP2 proteins and forming heterodimers. It is possible, however, that these cross-species heterodimers have altered functionality and may not transfer CO2 across the membrane.

Fig. 3. Subcellular localization of the AtPIP1;4-mGFP fusion protein in Nicotiana benthamiana mesophyll cells, with GFP excitation at 482 nm. AtPIP1;4-mGFP localized to the plasma membrane. (A–D) Whole protoplast imaged on a Leica Stelar-Falcon confocal microscope (scale bars, 20 µm). (A) Brightfield; (B) GFP signal (green; excitation at 482 nm, emission 525–542 nm); (C) chlorophyll autofluorescence (magenta; excitation at 650 nm, emission 667–755 nm); (D) a composite image of GFP and chlorophyll autofluorescence channels. Under these excitation and emission conditions, no chlorophyll bleed-through at the chloroplast was observed in the GFP channel.
We used combined measurements of gas exchange and carbon isotope discrimination, which is one of the most robust techniques for quantifying $g_m$ (Pons et al., 2009). The plants were well-watered with values of $g_m$ for our control tobacco lines (0.5 mol m$^{-2}$ s$^{-1}$ bar$^{-1}$) similar to our previous measurements of WT tobacco based on carbon isotope discrimination (Evans et al., 1994; Yamori et al., 2010; von Caemmerer and Evans, 2015; Clarke et al., 2021). Despite all of the above, we did not detect an improved $g_m$ phenotype. There are several factors that may have influenced a change in phenotype and we discuss these below.

Modelling of various gas-exchange parameters (Fig. 6), suggested that at our high basal $g_m$ values for controls (0.5 mol m$^{-2}$ s$^{-1}$ bar$^{-1}$), it would have been more difficult to statistically detect differences in the CO$_2$ response curves (Fig. 6A) or increased CO$_2$ assimilation rates (Fig. 6B) because of the diminishing increases in these traits per unit improvement in $g_m$. However, differences in $g_m$ and $C_i-C_c$ would have likely been apparent (Fig. 6C). Our $g_m$ values are greater than $g_m$ values reported by Flexas et al. (2006) (0.32 mol m$^{-2}$ s$^{-1}$ bar$^{-1}$) and the exceptionally low values reported by Kawase et al. (2013) (0.108 mol m$^{-2}$ s$^{-1}$ bar$^{-1}$). However, these differences are not surprising as it is well known that photosynthetic rate and $g_m$ vary with growth conditions (Evans and von Caemmerer, 1996; Yamori et al., 2010). Our higher basal $g_m$ values, which are expected in agricultural systems, would therefore make detection of improvements more difficult than those observed in systems with lower basal $g_m$ values (Fig. 6).

Tobacco is a recently emerged allotetraploid and its highly duplicated genome encodes 84 AQP genes, of which 30 belong to the PIP subfamily (Groszmann et al., 2021). For comparison, the Arabidopsis genome encodes for only 35 AQPs in total, with 13 being PIPs (Quigley et al., 2001).

Fig. 4. Expression of AtPIP1;2 or AtPIP1;4 did not affect CO$_2$ assimilation rate (A), mesophyll conductance (B), or the draw-down of CO$_2$ into the chloroplasts ($C_i-C_c$) (C) compared with control plants (black). Measurements taken at 25 °C, 380 ppm CO$_2$ and 2% O$_2$, n=4. No significant differences were observed between any lines (two-way analysis of variance, $P>0.05$).

Table 1. Summary of physiological and biochemical parameters measured and modelled for AtPIP1 overexpression lines in tobacco

| Parameter                              | GUS control | PIP1;2.6.1 | PIP1;2.6.2 | PIP1;4.5.3 | PIP1;4.5.4 |
|----------------------------------------|-------------|------------|------------|------------|------------|
| Hygromycin copy number                 | 0           | 2          | 2          | 2–4        | 2–4        |
| PIP protein levels (relative signal)   | 1.00        | 1.25 ± 0.19| 1.41 ± 0.07*| 0.78 ± 0.06| 1.02 ± 0.23|
| Leaf mass per area (g m$^{-2}$)        | 25.9 ± 2.0  | 25.8 ± 1.5 | 23.3 ± 1.5 | 21.0 ± 1.9 | 22.7 ± 1.4 |
| Steady state assimilation rate (μmol m$^{-2}$ s$^{-1}$) | 21.6 ± 1.2 | 23.1 ± 0.9 | 21.5 ± 1.6 | 21.6 ± 1.9 | 21.3 ± 1.6 |
| Steady state $C_i$ (380 ppm CO$_2$) (μbar) | 211.0 ± 3.2 | 204.6 ± 3.0 | 208.8 ± 6.5 | 200.5 ± 5.7 | 204.9 ± 7.2 |
| Stomatal conductance (CO$_2$) (μmol m$^{-2}$ s$^{-1}$) | 0.30 ± 0.03 | 0.29 ± 0.01 | 0.29 ± 0.02 | 0.27 ± 0.05 | 0.27 ± 0.01 |
| Mesophyll conductance (μmol m$^{-2}$ s$^{-1}$ bar$^{-1}$) | 0.50 ± 0.04 | 0.52 ± 0.02 | 0.53 ± 0.04 | 0.50 ± 0.05 | 0.53 ± 0.04 |
| $V_{cmax}$ (gas exchange) (μmol m$^{-2}$ s$^{-1}$) | 85.1 ± 5.2 | 90.5 ± 3.6 | 81.5 ± 8.0 | 90.0 ± 3.5 | 84.3 ± 9.1 |
| $J$ (gas exchange) (μmol m$^{-2}$ s$^{-1}$) | 111.5 ± 4.9 | 124.5 ± 3.4 | 118.3 ± 11.7 | 114.8 ± 3.3 | 113.5 ± 13.7 |

Steady state assimilation, steady state $C_i$, stomatal conductance, and mesophyll conductance measurements were made at 380 ppm CO$_2$ and 2% O$_2$. $V_{cmax}$ and $J$ (gas exchange) were calculated with the model of Sharkey et al. (2007). *Significant difference from control (n=3, $P<0.05$)

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Our understanding of the effects of individual resistance components on CO$_2$ transfer capacity is limited by our inability to accurately isolate and measure the impact of each component. However, recent studies have helped to refine our
estimations of these resistance components, through anatomical measurements and mutant analyses (Tosens et al., 2012; Clarke et al., 2021; Evans, 2021). The plasma membrane and chloroplast envelope appear to account for around half of the mesophyll CO₂ diffusion resistance in crop species with thin mesophyll cell walls, such as tobacco (Evans et al., 1994; Yamori et al., 2010; von Caemmerer and Evans, 2015; Clarke et al., 2021; Evans, 2021). However, our modelling shows that significant increases in membrane-associated CO₂ conductance improve overall mesophyll conductance by a much smaller fraction (see Supplementary Fig. S3). For example, a 50% increase in CO₂ conductance across the membranes would yield only a 20% improvement in mesophyll conductance (Supplementary Fig. S3). This modelling is supported by experimental data, such as Flexas et al. (2006), where doubling PIP protein levels increased mesophyll conductance by only 40%.

We observed an increase of up to 40% in PIP proteins in the membranes of the mesophyll cells (AtPIP1;2.6.2, Fig. 1), and assuming this was all active and functional, our modelling suggests this would improve $g_m$ by ~16% which translates into an increase from our basal $g_m$ of 0.50 to 0.58 mol m$^{-2}$ s$^{-1}$ bar$^{-1}$ (see Supplementary Fig. S3). This is only slightly greater than the error rate (biological and technical replication errors) of $g_m$ in this study of around 10% (Supplementary Fig. S3; Table 1). We might therefore be achieving an increase in $g_m$, but it is indistinguishable from the background variation of our measurements.

The normal basal $g_m$ value may be an important factor influencing the ease with which $g_m$ might be improved through transgenic engineering. Environmental factors during the growth of plants (e.g. photoperiod, light intensity, day/night temperature, nutrient supply, watering, and humidity) can impact anatomical and biochemical traits that determine $g_m$. Lipid and protein composition of membranes can be strongly remodelled in response to environmental cues (Uemura et al., 1995). Under growth conditions that lead to a higher basal
grow, increasing \( g_m \) further through engineering becomes more challenging. For instance, Kelly et al. (2014) failed to observe an improvement in \( g_m \) when overexpressing NtAQPI in tomato until basal \( g_m \) values were tempered by overexpressing hexokinase (AtHXT1). Conditional effects also seem to plague the understanding of PIPs in their native roles in \( g_m \) and \( \text{CO}_2 \) assimilation through mutant analysis. Although several studies show that a loss of \( \text{CO}_2 \)-permeable PIPs reduces \( g_m \) (Hanba et al., 2004; Flexas et al., 2006; Uechlein et al., 2008; Heckwolf et al., 2011), other studies have failed to corroborate these findings, with the contrasting results interpreted as differences in growth conditions between studies (Kromdijk et al., 2020). Recently such conditional responses (growth conditions and growth stage) between PIP loss-of-function mutants and declines in photosynthetic rates and \( g_m \) have been observed in rice (Huang et al., 2021), and in tomato a SLPPIP1;2 knockout mutant only reduced \( g_m \) when mutants were grown under \( \text{CO}_2 \) enrichment (Zhang et al., 2021).

It appears that the conditional relevance of PIPs and their involvement in regulating \( g_m \) and photosynthetic rates requires further study in order to more intricately assess and improve the consistency of engineering efforts. To detect an increase in \( g_m \), we may need to investigate different growth conditions. Higher transgene expression appears a strong necessity but may be an overly simplistic view. We have a limited understanding of the capacity of cellular membranes to support additional integral membrane proteins, along with the composition and regulation of the AQP tetramers. The assembly of AQPs into functional tetramers is complex and the specific monomers and their ratios can influence substrate specificity (Jozefkowicz et al., 2017). For instance, increasing ratios of NtAQPI over NtPIP2;1 in a tetrameric complex gradually switches specificity from water to \( \text{CO}_2 \) with mingled transport specificities in between (Otto et al., 2010). PIP AQPs can also move in and out of membranes in response to environmental cues, and their channel activity and substrate specificity is regulated by phosphorylation gating mechanisms (Groszmann et al., 2017; Qiu et al., 2020). Phosphomimetic versions may help ensure transgenic PIPs remain in their desired transport state and embedded within membranes (Qiu et al., 2020).

Conclusion

Ectopic expression of Arabidopsis aquaporins AtPIP1;2 and AtPIP1;4 in the plasma membrane of tobacco mesophyll cells did not increase mesophyll conductance to \( \text{CO}_2 \), or other photosynthetic parameters. While it has been shown that some PIP AQP isoforms are capable of transporting \( \text{CO}_2 \) in heterologous systems, translation of this capability to plants to improve \( g_m \) and \( \text{CO}_2 \) assimilation has had varied results. Plant growth and environmental conditions may play a significant role in the ability for AQPs to alter \( g_m \). Further studies are needed to better understand aquaporin function. These could include conditional transgene expression, co-expression of PIPs to induce desirable heterodimers/tetramers or alterations to phosphorylation states to enhance channel activation and membrane integration.

Supplementary data

The following supplementary data are available at JXB online. Fig. S1. Transgene expression in T1 lines. Fig. S2. Lifetime imaging of GFP and chlorophyll fluorescence signals in mesophyll cells expressing AtPIP1;4-GFP localization construct. Fig. S3. Modelled effect of increased membrane conductance of \( \text{CO}_2 \) on total mesophyll conductance.

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Authors contributions

VC designed and conducted physiology, microscopy and biochemistry experiments, analysed data and wrote the manuscript; ADR designed and created the 2x35S-GUS lines; BM conducted biochemistry experiments; AG made the PIP transformants and genotyped them; JE contributed resources and facilities and edited the manuscript; SvC planned the study, contributed resources and facilities and wrote the manuscript; and MG planned the study, designed the constructs, analysed data and wrote the manuscript.

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Data availability

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

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