

Plasma membrane intrinsic proteins PIP1;1 and PIP1;3 contribute to the tolerance to nitrogen deficiency in potato

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Abstract

Nitrogen (N) deficiency is one of the major constraints for potato (*Solanum tuberosum* L.) production, due to its shallow root system and poor capabilities of N mobilization from the deeper layers of the soil. The aim of this study was to elucidate plasma membrane (PM) proteins potentially involved in the tolerance towards N deficiency. We compared the PM proteome of the two potato cultivars ‘Lambada’ and ‘Topas’, contrasting in their N deficiency response under in vitro conditions. Using mass spectrometry we identified 65 proteins, which were differentially abundant in the two cultivars when submitted to N deficiency. Amongst those, candidate proteins were selected based on their potential to be involved in N transport under deficient conditions. Two members of the aquaporin family, StPIP1;1 and StPIP1;3, were studied in more detail. By means of a yeast growth assay we showed that these proteins do not facilitate transport of ammonia. The *Arabidopsis* (*Arabidopsis thaliana*) knockout mutants AtPIP1;1 and AtPIP1;3 showed a reduced N content and accumulation of anthocyanins under N deficiency. We conclude that PIP1;1 and PIP1;3 are indirectly involved in the N uptake under N deficient conditions and contribute to an increased N deficiency tolerance.

Summary statement

Root plasma membrane proteome profiling of two potato cultivars reveals the participation of the two members of the aquaporin family PIP1;1 and PIP1;3 in the cultivar specific response to nitrogen deficiency.

Introduction

The productivity of most field crops is sensitive to the availability of N in the soil. Globally, around 85-90 metric tons of nitrogenous fertilizer is added to arable soils each year, an amount predicted to be more than doubled by 2050 (Tilman, 1999). The production of nitrogenous fertilizer is energy and cost demanding in modern crop production systems. Due to the effects of nitrate leaching, denitrification and ammonia volatilization, which have been proved environmentally damaging (Masclaux-Daubresse et al., 2010; Shaviv & Mikkelsen, 1993), it has been estimated that only 30-50% of fertilizer-supplied N is taken up by crop plants, a range which reflects the combined influence of soil type, environment and plant species (Good, Shrawat, & Muench, 2004). Potato is an important source of food and industrial starch. N utilization efficiency is especially poor in potato, on account of its shallow root system (Iwama, 2008) exacerbated by the fact that the crop is frequently grown on highly leachable sandy soils (Cambouris, Zebarth, Nolin, & Laverdiere, 2008). The aim to improve N use efficiency has become important in crop plant breeding research (Chao & Lin, 2015; Garnett, Plett, Heuer, & Okamoto, 2015; Guo, Wang, Fan, Chen, & Cui, 2016). In this respect,

progress has been made in sugarcane, maize and rice (Hajari, Snyman, & Watt, 2015; Ju et al., 2015; P. C. Li et al., 2015), but not in potato.

In plants, the interaction with various biotic and abiotic stress agents induces alterations in the protein composition of the PM (Komatsu, 2008). A better understanding of the PM proteome response to various environmental stimuli – and in particular to N deficiency – is seen as providing a route to the development of stress tolerant plants (Cordwell & Thingholm, 2010; Komatsu et al., 2009). The PM acts both, as a selective barrier between the cell and the external environment and as a sensor for the prevailing environmental conditions (Barkla & Pantoja, 2010). It controls a multiplicity of cellular processes such as transport of metabolites and ions, cell differentiation and proliferation, endocytosis, and supports energy generation and signal transduction (Komatsu, 2008; Ray, Kassan, Busija, Rangamani, & Patel, 2016). The transport across membranes, required for acquisition and release of nutrients and signaling molecules, is achieved primarily through integral membrane proteins (Frommer et al., 1994). Members of four transporter families participate in root nitrate uptake, distribution and storage, including proteins from the nitrate transporter 1/peptide transporter family (Leran et al., 2014), the nitrate transporter 2 family (NRT2) (Orsel, Krapp, & Daniel-Vedele, 2002), the chloride channel family (Barbier-Brygoo et al., 2011), and slow anion associated channel homologs (Negi et al., 2008). Ammonium uptake is facilitated by members of the ammonium transporter (AMT) family (Loque & von Wiren, 2004).

Since the PM constitutes a complex platform allowing the translation of external signals into finely tuned appropriate acclimation responses, the ability of plants to exploit several sources of N displays an intricate network in which signaling and regulatory proteins play a key role (Gronnier, Gerbeau-Pissot, Germain, Mongrand, & Simon-Plas, 2018). Getting insights into this complex network through proteomic approaches is hampered, because the PM proteins are underrepresented when proteome analysis is performed. Thus, membrane-specific approaches are necessary to study the roles played by PM proteins. However, the number of studies on root PM proteomes remain scarce (Cheng et al., 2009; Hashimoto, Toorchi, Matsushita, Iwasaki, & Komatsu, 2009; Voothuluru, Anderson, Sharp, & Peck, 2016; Witzel et al., 2018) and are lacking for potato. To unveil key contributors involved in the low N tolerance, we made use of the contrasting response to N deficiency of the two potato cultivars ‘Lambada’ (sensitive) and ‘Topas’ (tolerant). Both cultivars were already characterized and described to differ at the physiological (Schum & Jansen, 2012, 2013, 2014; Schum, Meise, Jansen, Seddig, & Ordon, 2017) and at the whole proteome (Jozefowicz, Hartmann, Matros, Schum, & Mock, 2017; Meise et al., 2017) level. Here, we report on the effect of N deficiency on the root PM proteome composition of the two potato contrasting cultivars and present candidate proteins likely playing a role in the acclimation of the tolerant cultivar to N deficiency.

Materials and Methods

Plant material - potato

Plant cultivation and stress treatments of two genotypes contrasting in their N deficiency response, namely ‘Lambada’ (sensitive) and ‘Topas’ (tolerant), were performed exactly as described (Jozefowicz et al., 2017). At the time of root appearance, after one week of cultivation, the nutrient solution was exchanged. Control plants were moved to fresh medium containing the original 20.6 mM NH_4NO_3 and 18.8 mM KNO_3) and the plants for deficiency treatment were transferred to a medium with 1/16 of the N level. The loss of K (as a result of withholding KNO_3) was compensated by the addition of KCl. After seven days, roots were harvested, snap-frozen in liquid N_2 and stored at -80°C . The experiment was performed in triplicate with six vessels per condition and cultivar.

Plant material - Arabidopsis thaliana

Experiments on Arabidopsis were performed using the wild type (WT) ecotype Col-0 and the two T-DNA insertion lines GABI_437B11 (*Atpip1;1*) (Rosso et al., 2003) and SALK_051107 (*Atpip1;3*) (Alonso et al., 2003) obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, United Kingdom). Homozygous mutants were selected by PCR with specific primers listed in Supplementary Table S1. Homozygous lines were selected based on the absence of a wild type amplicon and the presence of the T-DNA insertion

(Supplementary Fig. S1). For the cultivation of plants on soil, ‘Fruhstorfer Nullerde’ (white-peat volcanic clay mixture) was used. The soil substrate was prepared as described previously (Pommerrenig et al., 2018) with some modifications. The pH of a white-peat volcanic clay mixture was first adjusted to around pH 5.5 with CaO (3 g kg⁻¹) and CaCO₃ (2 g kg⁻¹). After 1 week, the substrate was fertilized with 250 ml kg⁻¹ of a modified Hoagland solution containing: 60 mM NH₄NO₃, 23.5 mM KH₂PO₄, 2.8 mM K₂SO₄, 13.3 mM MgSO₄, 420 μM CuSO₄, 360 μM ZnSO₄, 2.5 mM MnCl₂, 3.3 μM NaMoO₄, 152 μM NaFeEDTA and 226 μM H₃BO₃. N deficiency was achieved by reducing the NH₄NO₃ concentration to 30 mM NH₄NO₃. Leaves (8 plant replicates per condition, three independent repetitions of the experiment) were harvested after 5 weeks of growth, snap-frozen in liquid N₂ and stored at -80 °C. The N and C contents of the leaves were measured using a Euro EA elemental analyser (HEKAtech, Wegberg, Germany). Statistical analysis was performed using SigmaPlot v13 (Systat Software, Erkrath, Germany) utilizing a two way analysis of variance (ANOVA) and the Holm-Sidak post hoc test. Additionally for the qRT-PCR analysis plants were grown using *in vitro* based conditions as described previously (Schlesier, Breton, & Mock, 2003). To impose N deficiency, the medium’s N content was reduced to 2 mM.

Enrichment of plasma membrane

For the PM enrichment of potato roots, all six vessels obtained from each independent biological experiment were pooled. The two phase PM enrichment protocol described by Hynek, Svensson, Jensen, Barkholt, and Finnie (2006) was modified to treat potato roots as described recently (Jozefowicz, Matros, Witzel, & Mock, 2018). Briefly, the microsomal fraction of potato roots was obtained by differential centrifugation (15 min 10,000x *g*, 50 min 50,000 x *g*, 4 °C). Subsequently, 3 g of membrane suspension were loaded onto 9 g two-phase mixture composed of 6.2% Dextran T500, 6.2% PEG 3350, 8 mM KCl pH 7.8, 300 mM sucrose, 5 mM potassium phosphate buffer. Loosely bound and soluble proteins trapped in vesicles were removed by washing the PM enriched upper phase pellet in Brij-58 buffer (0.33 M sucrose, 0.2 M KCl, 5 mM phosphate buffer (pH 7.8), 0.2% (w/v) Brij-58) (Alexandersson, Saalbach, Larsson, & Kjellbom, 2004). Protein quantification was performed according to Bradford (1976). Proteins (5 μg per fraction) were separated by electrophoresis through 11.25% sodium dodecyl sulphate polyacrylamide gels (Laemmli, 1970) and subsequent Western blot analyses performed as described elsewhere (Amme et al., 2005). The following antisera were used: anti-H⁺-ATPase (diluted 1:3000), anti-V-ATPase (1:3000), anti-VDAC (voltage dependent anion channel, 1:2000), anti-BiP (luminal binding protein, 1:8000) (Agrisera, Vannas, Sweden) and self-raised anti-MDAR (monodehydroascorbate reductase, 1:1000). The universal secondary antibody used was the goat polyclonal anti-rabbit conjugated with IRDye 800CW (1:15000), which enabled infrared fluorescence detection via a LI-COR Biosciences scanner (Bad Homburg, Germany). Relative quantification of the fluorescence signal was performed using Odyssey 3.0 software and mean values were compared using the Student’s *t*-test.

Protein extraction and digestion

Protein extraction and tryptic digestion of the PM enriched upper phase were performed according to the protocol described previously (Jozefowicz et al., 2018). Prior to the processing by LC-MS, the resulting peptide mixtures were resuspended in 1% (v/v) acetonitrile with 0.1% (v/v) formic acid.

Label-free quantification of proteins and data analysis

The LC-MS analysis of peptides was performed using a Q-TOF Premier MS device coupled to a NanoAcquity system device (Waters Corporation, Eschborn, Germany) as described elsewhere (Jozefowicz et al., 2017). Two technical repetitions were run for the three independent experiments. Protein identification was performed using the ProteinLynx Global SERVER v2.5.3 software (Waters Corp.). The following parameters were used: automatic mass tolerance and fragment mass tolerance, one missed cleavage and variable modification: oxidation (Met), carbamidomethyl (Cys). MS data were searched against a potato database, based on the sequences from *Solanum tuberosum* group Phureja DM1-3 (PGSC_DM.v3.4.pep.-representative, 39,031 entries) (X. Xu et al., 2011) annotated by means of the Blast2GO software (09.2014) (Conesa & Gotz, 2008) and supplemented with sequences of human keratin and trypsin. Additional annotation using the UniProt database (<http://www.uniprot.org/>) was performed for the proteins of inter-

est. Protein quantification was carried out using Progenesis QI for proteomics software (Nonlinear Dynamics, Newcastle, UK). Only proteins with more than one unique peptide were included in the analysis and those with ANOVA p-values < 0.05 and a fold change > 1.5 were considered as showing a significant degree of variation. Transmembrane regions were predicted by reference to the TMHMM Server v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) (Krogh, Larsson, von Heijne, & Sonnhammer, 2001). Principal component analysis (PCA), Z-score normalization and hierarchical clustering based on the Euclidean distance method were carried out using the Perseus Framework (Tyanova et al., 2016). A full listing of the differentially expressed proteins has been placed, together with the raw data, in the IPK Gatersleben system e!DAL (Arend et al., 2014), available at: <https://doi.ipk-gatersleben.de/DOI/15c49da1-7f23-4c8f-9227-5695c1218b6a/2a5bce80-0e3b-484f-8878-0a4f3afe62b9/2/1847940088>.

Quantitative real time (RT)-PCR

The RNA extraction as well as qRT-PCR was performed as described previously (Jozefowicz et al., 2017) The reference sequences for potato were L2 and Aprt (primers as suggested by Nicot, Hausman, Hoffmann, and Evers (2005), while the primers targeting aquaporin genes were taken from Venkatesh, Yu, and Park (2013). The primers for the *A. thaliana* aquaporin genes were those described by Alexandersson et al. (2010) and the reference genes were *tip41* and *expr* (Czechowski, Stitt, Altmann, Udvardi, & Scheible, 2005).

Extraction and UPLC-PDA profiling of phenylpropanoids

Phenylpropanoid extraction and detection was performed as described (D. R. Xu et al., 2019). PDA-detection of phenylpropanoids was performed in a range between 210 and 800 nm, at a resolution of 1.2 nm and a sampling rate of 20 points s⁻¹. Spectra for phenylpropanoids and anthocyanins were recorded at 280 and 520 nm, respectively. Total phenylpropanoid and anthocyanin contents were quantified as the total peak area of the compounds detected at 280 and 520 nm, respectively, eluting between 2 and 7 min. Data analysis was performed using the software packages Compass Data Analysis V4.4 and QuantAnalysis V4.4 (Bruker Daltonik GmbH, Bremen, Germany). Statistical analysis was performed using SigmaPlot v13 with a two way ANOVA and Holm-Sidak post-hoc test.

Yeast strains and growth assay

StPIP1;1 and *StPIP1;3* were cloned from ‘Topas’ root cDNA using primers listed in Supplementary Table S2. PCR products were cloned in the USER-yeast expression vector pYeDP60u (Hamann & Moller, 2007) and pRS426-pTPIu using an uracil excision-based improved high-throughput USER cloning technique (Nour-Eldin, Hansen, Norholm, Jensen, & Halkier, 2006). A *S. cerevisiae* deletion mutant strain ($\Delta mep1-3(-ura/-leu)$ (Anna Maria Marini’s lab collection)) was co-transformed with either the two empty vectors (EV) pRS426-pTPIu and pYeDP60u (negative control transformant), or *hAQP8* (in pYeDP60u) and an EV (pRS425-pTPIu) (positive control transformant). The two PIP isoforms, *StPIP1;1* or *StPIP1;3* in pYeDP60u, were co-transformed with maize *ZmPIP2;5* in pRS425-pTPIu to localize the individual PIPs to the PM for transport assays. Co-transformation of *StPIP1;1* or *StPIP1;3* in pRS426-pTPI:N-terminal-green fluorescent protein (GFP) with *ZmPIP2;5* in pRS425-pTPIu was performed for transport assays and for confocal microscopy (G. P. Bienert, Heinen, Berny, & Chaumont, 2014). Transformed yeast was selected on synthetic medium containing 2% glucose, 50 mM succinic acid/Tris base, pH 6.5, 0.7% yeast nitrogen base without amino acids (Difco) and 0.2% proline. For the ammonium transport assay, yeast cells were diluted to different OD₆₀₀ values (1, 0.01, and 0.0001) and spotted on synthetic medium containing 2% galactose, 50 mM succinic acid/Tris base, pH 6.5, 0.7% yeast nitrogen base without amino acids (Difco) and different concentrations of ammonium (2, 4, 5, 10, 20 mM ammonium, supplied as ammonium sulfate) or 0.2% proline as positive control. Growth was documented after 8-10 days at 30 °C.

Microscopy

Yeast cells expressing *StPIP1;1* or *StPIP1;3* GFP fusion proteins together with either the EV or *ZmPIP2;5* were grown in liquid YPG medium. At an optical density at 600 nm of approx. 1, the fluorescence signal was observed. The localization was detected with a Zeiss LSM 780 confocal laser scanning microscope (Carl

Zeiss, Jena, Germany) using a 488 nm laser line for excitation. GFP signals were detected at 491-536 nm.

Results

The effect of N deficiency on the growth of potato in vitro

The potato cultivars ‘Lambada’ (sensitive) and ‘Topas’ (tolerant) were differentiated from a set of potato cultivars according to their Membership Function Value of stress tolerance calculated from previously published data (Jozefowicz et al., 2017; Schum et al., 2017). Here, the root PM proteome was analyzed after seven days of N deficiency. Under these conditions, the sensitive cultivar displayed a decrease in root fresh weight, whereas the growth of the tolerant cultivar remained unaffected. N deficiency at this time point did not lead to differences in the root N content or in the shoot fresh weights (Supplementary Fig. S2). This allowed us to analyze mechanisms leading to the early plant acclimation towards low N, excluding the secondary effects of long-term N deficiency, such as senescence. However, when the culture duration was prolonged, the differences between cultivars development and root architecture were more pronounced (Schum & Jansen, 2012, 2013, 2014).

Enrichment of PM from potato roots

PM enrichment was critical for the subsequent membrane proteome analysis. The effectiveness of the two-phase partitioning PM enrichment method was evaluated immunologically using compartment-specific antibody markers (Fig. 1). The abundance of the PM marker H⁺-ATPase was 13.3-fold higher in the PM fraction compared to the original crude extract (CE), and three-fold higher than in the microsomal (M) fraction (Supplementary Fig. S3). In contrast, both the cytosolic marker MDAR (highly abundant in the soluble phase (S)) and the mitochondrial marker VDAC (highly abundant in the microsomal and the lower phase Dextran T500 phase (M and LP)), were barely detectable in the upper PM-enriched phase (PMe). BiP was represented in the PM fraction, but to an intensity three-fold lower than in the M fraction. V-type ATPase was present at a comparable levels in the M, LP and PMe fractions. These results indicate that the PM proteins were enriched in the upper phase, which was used in our comparative proteome analysis.

Identification of N deficiency responsive root PM proteins

A total of 1512 unique peptides corresponding to 215 proteins were identified after subjecting the root PMe fraction to label-free LC-MS. The PCA of the LC-MS data shows that the first principal component was responsible for 75.3% of the explained variance and the second for the 14.4% (Supplementary Fig. S4). The clustering of the biological replicates in the scores plot demonstrates that the major variance in the dataset was due to the differences displayed between the contrasting cultivars under N deficiency, whereas their protein profiles were similar under N-sufficient conditions. About 33% of the overall identified proteins were predicted to harbor a transmembrane helix (Supplementary Fig. S5), of which 39 proteins harbored at least two transmembrane domains. N deficiency significantly induced changes in the abundance for 65 of the 215 identified proteins (Table 1).

K-means clustering assembled the differentially changed proteins into seven groups differing in their abundance patterns (Fig. 2). A single protein, namely defensin-like protein was assigned to cluster I, showing the lowest abundance in the sensitive cultivar ‘Lambada’ under N sufficiency. Cluster II comprised two proteins decreasing in abundance in ‘Lambada’ under N deficiency. Cluster III grouped two proteins displaying a decreased abundance under N deficiency in the tolerant cultivar ‘Topas’ and unchanged, low abundance in the sensitive cultivar ‘Lambada’. Cluster IV comprised 22 proteins, which showed an increased abundance under N deficiency in ‘Topas’, but not in ‘Lambada’. The overrepresented functional assignments in this cluster were ‘transporters’ (12 proteins) and ‘protein destination and storage’ (three proteins). Two proteins grouped in cluster V were more abundant in ‘Lambada’ under both N regimes. In cluster VI, eight proteins participating in various biological processes, showed a decreased abundance in the tolerant cultivar. Finally, cluster VII included 28 proteins which decreased in both cultivars under N deficiency. These included proteins assigned to the categories ‘transporters’ (five proteins), ‘signal transduction’ (three proteins), ‘cell structure’ (three proteins) and ‘energy’ (three proteins). The proteins that were exclusively increased in the

tolerant genotype ‘Topas’ under N deficiency (Cluster IV), showing the highest contribution to the variance explained by the first principal component on the PCA (Supplementary Fig. S4) and harboring at least one transmembrane helix, were considered as integral root PM proteins likely involved in potato N deficiency tolerance.

Candidate proteins involved in the adaptation of potato to N deficiency

A protein identified as NRT2.1 (accession number- 400031304) increased 8.2-fold in ‘Topas’ and decreased in ‘Lambada’. Three isoforms of the PM H⁺-ATPase (400083041, 400065168, 400010497) increased between 1.9- and 4.1-fold in ‘Topas’, while in ‘Lambada’ their abundance remained unchanged. Four other proteins also displayed this behavior, including a sugar carrier protein C (400057709) and two ABC transporters (400018820- ABCB family member, 400046727- ABCA family member). In addition, two proteins belonging to the PM intrinsic protein (PIP) aquaporin subfamily increased in ‘Topas’ compared to ‘Lambada’ under N deficiency. The aquaporin isoforms PIP1;1 (400012780) and PIP1;3 (400032134) were, respectively, four and 2.5 times higher in N-deficient ‘Topas’, but remained unchanged in ‘Lambada’ (Fig. 2, marked with asterisks). Additionally, both isoforms were more abundant in ‘Topas’ (1.6 and 1.8-fold in PIP1;1 and PIP1;3, respectively) than in ‘Lambada’ under N-sufficient conditions. As aquaporins have not only proved to play an important role in water conductance, but also to facilitate the transport of other small uncharged solutes such as urea or ammonia (Jahn et al., 2004; Loque, Ludewig, Yuan, & von Wiren, 2005), we further investigated the transcript levels of the *PIP1;1* and *PIP1;3* genes during N deficiency and their potential role in N uptake.

The expression of PIP1;1 decreased in the tolerant cultivar under low N

In the sensitive cultivar, there was no significant difference in the *PIP1;1* transcript abundance under both N-sufficient and N-deficient conditions (Fig. 3). In ‘Topas’, contrary to the protein abundance, N deficiency caused a two-fold reduction in the *PIP1;1* transcript level. The transcription of *PIP1;3* was unaffected by N deficiency in both cultivars.

Potato aquaporins StPIP1;1 and StPIP1;3 do not facilitate the transport of ammonia across yeast membranes

In order to test the permeability of aquaporins to solutes such as urea or ammonia, the heterologous expression system *S. cerevisiae* is commonly used. Here we investigated whether StPIP1;1 and StPIP1;3 can channel ammonia across biological membranes thereby potentially directly contributing to N uptake under N limiting growth conditions (Fig. 4). We expressed the two *StPIP*s in a *S. cerevisiae* deletion mutant strain ($\Delta mep1-3$ (-ura/-leu)). This mutant has a deletion in all three ammonium transporter (*mep*) genes and is therefore unable to grow on medium containing lower than 5 mM ammonium (Marini, Soussi-Boudekou, Vissers, & Andre, 1997). An additional source of N (*e.g.* in form of proline) is essential to ensure its growth. As PIP1 aquaporins were repetitively shown to traffic to the PM as PIP1/PIP2 heterodimers and cannot reach the PM membrane when expressed alone (G. P. Bienert et al., 2014; M. D. Bienert, Diehn, Richet, Chaumont, & Bienert, 2018; Fetter, Van Wilder, Moshelion, & Chaumont, 2004), *StPIP*s were either expressed individually or in combination with *ZmPIP2;5* in the heterologous expression system. To investigate protein localization in *S. cerevisiae* we used GFP:PIP-fusion proteins of the respective aquaporin expressed in yeast cells. Yeast transformed with *GFP:StPIP1;1* or *GFP:StPIP1;3* alone showed a GFP signal in the internal structures, whereas co-expressed with *ZmPIP2;5* GFP was clearly fluorescent in the PM (Supplementary Fig. S6). Neither yeast cells expressing *StPIP1;1* or *StPIP1;3* alone (Supplementary Fig. S7), nor in combination with *ZmPIP2;5* were able to grow on ammonia concentrations below 5 mM (Fig. 5). The expression of the positive control *hAQP8* (Saparov, Liu, Agre, & Pohl, 2007) complemented the mutant and allowed growth on the medium with low ammonium concentration. This led to the conclusion that StPIP1;1 and StPIP1;3 are not able to channel ammonia.

The absence of PIP1;1 and PIP1;3 leads to a lower N content in the leaves of Arabidopsis

In Arabidopsis, PIP aquaporins were recently shown to impact on nitrate acquisition by increasing the root hydraulic conductivity (Li et al., 2016). At the amino acid level, the Arabidopsis AtPIP1;3 and AtPIP1;1 share 87.2% and 85.9% of sequence identity with the potato StPIP1;3 and StPIP1;1, respectively. When

Arabidopsis WT was grown under *in-vitro* based N-deficient conditions, we observed a decrease in the expression of both *AtPIP* genes (Fig. 5). Subsequently, the Arabidopsis T-DNA insertion mutants *Atpip1;1* and *Atpip1;3* were used to elucidate the functional properties and potential significance of these two aquaporins in the N deficiency tolerance. The WT and the T-DNA insertion lines were cultivated on the soil substrate fertilized as described earlier (Pommerrenig et al., 2018) with the exception that N was supplemented either in limiting (30 mM) or sufficient amounts (60 mM). Under N-sufficient conditions, the shoot fresh weights of the *Atpip1;1* and *Atpip1;3* mutants were comparable to those of the WT. When the WT and the mutants were grown under N deficiency, a reduction in the leaf biomass was observed in all plants (Fig. 6) to a similar extent. The N content of the leaves, which is a representation of N assimilated by the plant, comprised around 5 to 6 % N of the leaf dry weight in all examined plants when N was supplied in sufficient amounts (Fig. 7A). When the level of N in the soil was reduced, the WT plants contained 3.4 % of N in the leaf dry weight. Interestingly, the accumulation of N was significantly lower in both *pip* mutants, where the N content was as low as 2.4% in *Atpip1;1* and 2.5% in *Atpip1;3* plants. In contrast, the C content increased under N deficiency similarly in both, the leaves of WT and the *pip* mutants. In line with these results, the *pip* mutants displayed a purple coloration of the leaves when the level of N in the soil was reduced (Fig. 6). Since the purple coloration of Arabidopsis leaves was attributed to anthocyanin accumulation (Kovinich, Kayanja, Chanoca, Otegui, & Grotewold, 2015), we analyzed the soluble phenylpropanoids levels. The total soluble phenylpropanoid levels of the leaves were similar in all plants when N was sufficient. As shown in Fig. 7B, under N deficiency, the content of total phenylpropanoids increased in all plants, but this change was higher in the *pip* mutants compared to the WT. In agreement with the observed coloration, the same trend was observed in the anthocyanin content, with an average 2.5, 2.1 and 1.7-fold increase in the *Atpip1;1*, *Atpip1;3* and WT plants under N deficiency, respectively. These results show that the lack of either *AtPIP1;1* or *AtPIP1;3* in Arabidopsis leads to an increased susceptibility to N deficiency, since low N typically results in increased C/N ratios and the accumulation of secondary metabolites, including phenylpropanoids and flavonoids (Fritz, Palacios-Rojas, Feil, & Stitt, 2006).

Discussion

N deficiency induced variation in the PM proteome of potato cultivars

Plants as sessile organisms must constantly adapt their PM composition to properly sense and respond to the variations in their environment. N deficiency is known to cause changes in the expression of genes participating in the nitrate and ammonia uptake (as reviewed by Kiba and Krapp (2016)), signaling, as well as hormone translocation (O'Brien et al., 2016). Information about the PM protein composition under stress conditions and in particular, in response to N deficiency, remains however absent for potato. Two potato cultivars that show contrasting N deficiency tolerance (Jozefowicz et al., 2017; Meise et al., 2017; Schum & Jansen, 2014; Schum et al., 2017) ('Lambada'-sensitive, and 'Topas'-tolerant) formed the basis of the present study to identify PM proteins associated with N deficiency tolerance. Our study revealed that changes in the root PM composition occur in potato upon N deficiency. The alterations in the root PM proteome are cultivar specific, as we observed that many proteins respond in a cultivar dependent manner (Clusters I, III, IV, V, Fig. 2). Those proteins which were higher abundant only in the tolerant cultivar under N deficiency, were considered as potential candidates for improving tolerance to N shortages in potato.

PM proteins involved in the tolerance of potato to N deficiency

Proteins functioning in the transport of ions across the membrane were overrepresented in the group of responsive proteins. The abundance of the majority was largely unaffected by N deficiency in 'Lambada', while increasing in 'Topas'. During N deficiency, four *NRT2* genes (Krapp et al., 2011; Lezhneva et al., 2014) and six *AMT* genes were described to be upregulated in Arabidopsis (Yuan et al., 2007). Probably due to the low abundance of the N transporters or to the lack of accessible trypsin cleavage sites, only one protein, namely NRT2.1, was identified in our study. NRT2.1 is a major contributor to the high-affinity transport system operating under low levels of external nitrate (Lezhneva et al., 2014; Orsel, Filleur, Fraiser, & Daniel-Vedele, 2002). The increase in StNRT2.1 protein abundance confirmed that the growth settings used in this study induced the N deficiency response. In Arabidopsis, N deficiency induced expression of *NRT2.1* in the

cortex cells of the older part of primary and lateral roots (Wirth et al., 2007). Additionally, it was observed that the expression of *AtNRT2.1* increased transiently during short time starvation and decreased to the level slightly higher than before the treatment during prolonged N starvation (Lezhneva et al., 2014). In potato, *StNRT2.1* was shown to be higher expressed in the cultivars accumulating more nitrate in the tubers, suggesting that also in potato NRT2.1 is a key factor for nitrate uptake (M'hamdi, Abid, Chikh-Rouhou, Razgallah, & Hassen, 2016). An increase in the StNRT2.1 abundance under N deficiency may likely be linked to a decrease in the tissue nitrate concentration in 'Topas', inducing the transcriptional upregulation of this high affinity transport system. In 'Lambada' the slight decrease in NRT2.1 abundance might be the consequence of severe N deficiency, as it was previously described that 'Lambada' absorbs all the N available in culturing medium faster than 'Topas' (Schum & Jansen, 2012). The higher abundance of NRT2.1 is probably one of the key factors determining the more tolerant behavior of the cultivar 'Topas' under selected conditions. As the role of NRT2.1 in the nitrate uptake is well characterized (Filleur et al., 2001; Okamoto, Vidmar, & Glass, 2003; Orsel, Filleur, et al., 2002; Wirth et al., 2007), we thus decided to elucidate proteins which have not yet been associated with contrasting N deficiency response.

A few other membrane transporters have been identified to be differentially abundant in 'Topas' compared to 'Lambada'. Amongst these are three PM H⁺-ATPases (Table 1). These proteins are involved in generating an electrochemical proton gradient across the PM which is the basis for the active transport of various ions and metabolites (Palmgren, 2001). Also, nitrate transport depends on the activity of PM H⁺-ATPases (Forde, 2000; Meharg & Blatt, 1995). All the H⁺-ATPases identified in this proteome analysis correlate positively in their abundance with that of NRT2.1 (Pearson R > 0.9, Supplemental Fig. S8), supporting the hypothesis that the sensitive cultivar 'Lambada' responds differentially to N deficiency in respect to its transport properties at the PM.

Two putative ABC transporters (one of the A subfamily and one of the B subfamily, ABCB) were present in significantly higher amounts in 'Topas' plants exposed to N deficiency (Table 1). In Arabidopsis, the ABCB transporters are known to play a complex role in auxin transport (Blakeslee et al., 2007; Geisler et al., 2005; Hwang et al., 2016) and therefore also in the regulation of root traits (Overvoorde, Fukaki, & Beeckman, 2010). For instance, *AtABCB4* participates in the regulation of root hair elongation (Santelia, Fukao, Martinoia, & Geisler, 2006), whereas *AtABCB1* impacts on lateral root formation (Geisler et al., 2005). In previous experiments, N deficiency increased the lateral root length in 'Topas', but not in 'Lambada' (Schum & Jansen, 2013), but so far there is no evidence for the role of ABCB transporters in the auxin transport and in the modification of root architecture in potato.

PIP1;1 and PIP1;3 are indirectly involved in the N uptake

Interestingly, two aquaporins, StPIP1;1 and StPIP1;3 were induced under N deficiency in the tolerant cultivar 'Topas'. PIPs are channel proteins located in the endoplasmic reticulum and the PM, involved in promoting the transport of water and small neutral solutes such as hydrogen peroxide, carbon dioxide or arsenite across the PM (Dynowski, Schaaf, Loque, Moran, & Ludewig, 2008; Fetter et al., 2004; Mosa et al., 2012; Uehlein, Lovisolo, Siefritz, & Kaldenhoff, 2003). Previously, it was shown that certain aquaporin isoforms of the Tonoplast Intrinsic Protein (TIP) family facilitate the transport of ammonia across biological membranes (Jahn et al., 2004; Kirscht et al., 2016; Loque et al., 2005). Here, we tested whether StPIP1;1 and StPIP1;3 are directly involved in N uptake by sharing the selectivity properties with TIPs. The expression of StPIP1;1 and StPIP1;3 did not increase the growth of yeast mutants under low N supply (Fig. 4), strongly suggesting that these isoforms do not facilitate the transport of ammonia and do not contribute directly to N uptake in plants.

PIPs were described to be efficient water channels (Maurel et al., 2015) and high PIP expression correlates with high root hydraulic conductivity (L_{pr}) (Gambetta et al., 2013; Perrone et al., 2012). Remarkably, it was shown that the local and/or overall nitrate availability influences the L_{pr} and thereby, N uptake by impacting the diffusion of nitrate towards the root surface (Gorska, Ye, Holbrook, & Zwieniecki, 2008; Ishikawa-Sakurai, Hayashi, & Murai-Hatano, 2014; G. Li, Tillard, Gojon, & Maurel, 2016). Generally, it is observed that N limitation decreases whereas N resupply stimulates, L_{pr} and PIP expression. In contrast

to these observations, StPIP1;1 and StPIP1;3 protein abundance increased along with StNRT2.1 in the N deficiency tolerant cultivar ‘Topas’, whereas the transcript levels decreased for *StPIP1;1* and remained unchanged for *StPIP1;3* (Fig. 3). Discrepancies between aquaporin transcript and protein levels are not uncommon and might be reasoned by more dynamic mRNA turnover, protein stability or by regulating protein activity by post-translational modifications (Hachez et al., 2012; Velez-Bermudez & Schmidt, 2014).

In order to further elucidate the possible function of these PIP isoforms in respect to N deficiency tolerance, we used T-DNA knock-out mutants of the homologous genes of *StPIP1;1* and *StPIP1;3* in Arabidopsis (*AtPIP1;1* and *AtPIP1;3*) and investigated these plants in more detail. In WT plants, *AtPIP1;1* and *AtPIP1;3* transcript levels were downregulated in roots under N-deficient conditions (Fig. 5), which is in line with observations in other plant species in which the transcript levels of *PIPs* were studied under N limitation (Ishikawa-Sakurai et al., 2014).

Mass flow of N towards the root surface, which is stimulated by the close interrelation between transpiration, convection and L_{pr} , is one of the factors contributing to efficient N uptake (Tyerman, Wignes, & Kaiser, 2017). Working with hydroponic- or *in vitro*-based plant cultivation systems, limits the impact of these parameters on the mass flow towards the roots and might mask the contribution of PIPs to these processes. We established a soil substrate-based cultivation system to overcome this limitation. Interestingly, both insertion lines displayed a higher sensitivity to N deficiency reflected in their lower N content in the leaves, as well as in their larger total anthocyanins content (Fig. 6). This indicates that despite their transcriptional downregulation under N limiting conditions in Arabidopsis, the complete lack of these proteins leads to an increase in N deficiency sensitivity.

Recently, it was proposed that in Arabidopsis NRT2.1 in its action as a nitrate sensor/transducer, impacts on PIP expression and thereby, on the L_{pr} (G. Li et al., 2016). It was shown that *PIP1* and *PIP2* expression and protein abundance were largely unaffected under both high and low N treatments in the WT, suggesting regulatory events on the post-translational level. PIP expression in the *Atnrt2;1* mutant was lower than in the WT, which co-occurred with a decrease in L_{pr} (G. Li et al., 2016). In respect to N availability, it was shown that despite of a decrease in PIP expression under N deficiency, L_{pr} was rapidly recovered under N resupply (Gloser, Zwieniecki, Orians, & Holbrook, 2007; Gorska, Ye, et al., 2008; Gorska, Zwieniecka, Holbrook, & Zwieniecki, 2008). This is probably due to a high degree of post-translational modifications, such as phosphorylation, which enables a rapid response towards changing N availabilities (di Pietro et al., 2013). It was already argued that aquaporins activity is locally regulated by the N availability of roots (Gorska, Ye, et al., 2008; Ishikawa-Sakurai et al., 2014). Therefore, it is conceivable, that irrespectively of an overall transcriptional downregulation of *AtPIP1;1* and *AtPIP1;3* under N limitation in Arabidopsis, the protein is of importance to sustain local or overall L_{pr} at places with higher N availability or under prolonged N deficiency, respectively. Furthermore, a higher level of certain PIP isoforms might be of advantage under N-deficient conditions to enable rapid changes in protein activity.

We observed a concurrently increased abundance of StNRT2.1 with StPIP1;1 and StPIP1;3 in the tolerant potato cultivar. This indicates that in the cultivar ‘Topas’, these two PIP aquaporins might be in their inactive state, awaiting their local and rapid activation, to ensure efficient N delivery to the root surface. Another explanation might be, that StPIP1;1 and StPIP1;3 are important isoforms which ensure a steady state mass flow of water towards the root under N limiting conditions, which points towards a function of these PIPs in contributing to the N deficiency tolerance in cultivar ‘Topas’.

In conclusion, N limitation has been shown to modify the potato root PM proteome, and clear differences have been identified between the two varieties contrasting in their response to N deficiency. It has been possible to identify several candidate proteins likely involved in the mechanisms underlying N deficiency tolerance. Our data suggest that the increased abundance of StPIP1;1 and StPIP1;3 together with StNRT2.1 is one of the factors contributing to N deficiency tolerance in potato. In addition, evidence is presented that *AtPIP1;1* and *AtPIP1;3* interplay with efficient N uptake under N limiting conditions in Arabidopsis, which is in accordance with the working model proposed previously (G. Li et al., 2016; Tyerman et al., 2017). To the best of our knowledge, it has not been reported previously, that the lack of individual PIP aquaporins results

in a decrease in N deficiency tolerance.

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Tables

Table 1. Label-free quantification of the set of 65 proteins showing significantly altered abundance in the potato root PM induced by nitrogen (N) deficiency treatment. The proteins were clustered according to

their genotype- and treatment-specific abundance into seven groups (see Fig. 2). Their accession number, description, functional classification (where known) and their predicted content of transmembrane helices as well as the abundance ratios are given. Accession numbers are given without the PGSC003DMT prefix. Abbreviations: LamC- Lambada N sufficient, LamS- Lambada N deficient, TopC- Topas N sufficient, TopS- Topas N deficient, TMH -Transmembrane helices. Full details of the protein identification are stored together with raw data.

Accession	Description	Function	TMH
Cluster 1	Cluster 1	Cluster 1	Cluster 1
400039130	Defensin-like protein	disease/defence	1
Cluster 2	Cluster 2	Cluster 2	Cluster 2
400079443	GLOBIN domain-containing protein	unclassified	0
400083124	GSDH domain-containing protein	unclassified	1
Cluster 3	Cluster 3	Cluster 3	Cluster 3
400005571	Glutathione S-transferase-like protein	disease/defence	0
400040518	Putative cinnamyl alcohol dehydrogenase	secondary metabolism	0
Cluster 4	Cluster 4	Cluster 4	Cluster 4
400035262	Sucrose synthase	metabolism	0
400047710	Luminal-binding protein 5	protein destination and storage	1
400035380	ADP,ATP carrier protein	transporters	3
400018820	Putative ABC transporter B family member	transporters	9
400025670	RING-type E3 ubiquitin transferase	protein destination and storage	0
400083041	Plasma membrane ATPase	transporters	8
400019462	Uncharacterized protein	unclassified	0
400036847	Pyrophosphate- energized vacuolar membrane proton pump 1	transporters	13
400031304	High-affinity nitrate transporter 2.1	transporters	11
400005236	Hypersensitive induced response protein 2 like	disease/defence	0
400001904	Ras-related protein RABG3f like	intracellular traffic	0
400057709	Sugar carrier protein C	transporters	12
400029027	Tetraspanin-8	disease/defence	4
400027156	Ubiquitin	protein destination and storage	0
400046727	Putative ABC transporter A family member	transporters	6
400046278	Calmodulin-2/4	signal transduction	0
400032134	Aquaporin PIP1;3	transporters	6
400065168	Plasma membrane ATPase	transporters	8
400036643	Transmembrane protein	unclassified	6
400012780	Aquaporin PIP1;1	transporters	6
400010497	Plasma membrane ATPase	transporters	8
400005889	CSC1-like protein	transporters	10
Cluster 5	Cluster 5	Cluster 5	Cluster 5
400004106	1-phosphatidylinositol phosphodiesterase	metabolism	0
400051789	AvrRpt-cleavage domain-containing protein	unclassified	0
Cluster 6	Cluster 6	Cluster 6	Cluster 6
400073713	70-kD heat shock protein	disease/defence	0
400076528	GTP-binding nuclear protein	intracellular traffic	0
400016422	14-3-3 protein	unclassified	0
400075291	40S ribosomal protein S6	protein synthesis	0
400014945	Putative vacuolar proton ATPase subunit E	transporters	0
400068704	Vacuolar proton pump subunit B	transporters	0
400004125	Peptidyl-prolyl cis-trans isomerase	protein destination and storage	0
400033202	Fasciclin-like arabinogalactan protein	cell structure	0

Accession	Description	Function	TMH
Cluster 7	Cluster 7	Cluster 7	Cluster 7
400047481	Actin	cell structure	0
400058342	CASP-like protein	unclassified	4
400024318	FAS1 domain-containing protein	unclassified	0
400062832	GSDH domain-containing protein	unclassified	0
400022574	Alpha-tubulin	cell structure	0
400070407	ATP synthase D chain	transporters	0
400035497	Putative plant intracellular Ras-group-related LRR protein 4-like	unclassified	0
400078507	ATP synthase subunit beta	transporters	0
400023732	Protein kinase domain-containing protein	signal transduction	2
400010174	Actin 1	cell structure	0
400058377	Pollen-specific lysine-rich protein SBgLR	unclassified	0
400039213	Protein kinase domain-containing protein	signal transduction	2
400073467	Phospholipase D	metabolism	0
400045607	Protein kinase domain-containing protein	signal transduction	1
400041634	14-3-3 protein	unclassified	0
400007182	Dihydrolipoamide Dehydrogenase	energy	0
400079310	60S ribosomal protein L13	protein synthesis	0
400012216	Cupredoxin superfamily protein isoform 1	unclassified	1
400057418	Glycerophosphodiesterase-like protein	metabolism	1
400008687	60S ribosomal protein L12	protein synthesis	0
400061841	Xyloglucan endotransglucosylase/hydrolase	cell growth/division	0
400036347	PLAT domain-containing protein	disease/defence	1
400006894	Fructose-bisphosphate aldolase	energy	0
400010366	V-type proton ATPase subunit E2	transporters	0
400013297	Putative patellin-2-like	cell growth/division	0
400008800	V-type proton ATPase subunit B2	transporters	0
400044217	Malate dehydrogenase	energy	0
400074197	Vacuolar H ⁺ -ATPase A1 subunit isoform	transporters	0

Figure legends

Figure 1. Enrichment for plasma membrane (PM) proteins. A-Western blot analysis of the sub-cellular fractions obtained from the roots of ‘Topas’ plants grown under nitrogen (N)-sufficient conditions. The compartment-specific marker proteins were: H⁺-ATPase (PM marker), VDAC (mitochondrial), BiP (ER), MDAR (cytosolic), V-ATPase (vacuolar). Abbreviations are used as follows, C- crude extract, S- soluble fraction, M- microsomal fraction, LP- lower phase, PMe- plasma membrane fraction.

Figure 2. Heat map representation of proteins with differential abundance (p-value < 0.05, fold change > 1.5) in the two potato cultivars contrasting in their nitrogen (N) deficiency response, grown under either sufficient or deficient N conditions. According to the K-means algorithm, the proteins formed seven clusters. Relative abundance has been color-coded following Z-score normalization. Each column represents one treatment. Abbreviation used: HN- N sufficient treatment, LN- N deficient treatment. The aquaporin proteins PIP1;1 and PIP1;3 are marked with an asterisk each.

Figure 3. Effect on nitrogen (N) deficiency on the *StPIP1;1* and *StPIP1;3* transcript abundances in roots of the potato cultivars ‘Lambada’ and ‘Topas’. The expression of genes was quantified using qRT-PCR. The expression of genes was measured using qRT-PCR. Bars (black: N-sufficient, white: N-deficient conditions) labeled by a different letter indicate statistically significant differences (p < 0.05), error bars represent ±SD.

Figure 4. The ammonium transport ability of PIP1;1 and PIP1;3 studied in yeast by a growth comple-

mentation assay. The $\Delta mep1-3$ mutant was co-transformed with the combination of empty vector (EV) and indicated *PIP* cDNA. Cultures spotted on the medium containing indicated amount of ammonium or 0.2% proline (positive control) as nitrogen sources. The growth of the transformants was recorded after 8-10 days of incubation at 30 °C. Displayed image represents a group of images assembled from different growth conditions.

Figure 5. Effect on nitrogen (N) deficiency on the *AtPIP1;1* and *AtPIP1;3* transcript abundances in the roots of *in-vitro* grown Arabidopsis WT plants. The expression of genes was quantified using qRT-PCR. Bars represent N-sufficient (black) and N deficient (white) treatment. The statistical significance in transcript abundance induced by N deficiency is indicated by asterisks (Student's t-test ,***: $p < 0.001$), error bars represent \pm SD.

Figure 6 . Effect of nitrogen (N) deficiency on growth of *Arabidopsis thaliana pip* mutants. Plants were grown in the presence of either 60 mM (sufficient) or 30 mM (deficient) NH_4NO_3 . Leaves fresh weight was determined after 5 weeks of cultivation. Bars represent N-sufficient (black) and N deficient (white) treatment. The data present the mean of 8 plants per treatment for 3 independent growth experiments. Letters above the histogram columns indicate significant differences in mean values, as predicted by a two way ANOVA (p -value < 0.05), error bars represent \pm SD.

Figure 7 . The response of *Arabidopsis* WT and *pip* mutants to nitrogen deficiency. A- Nitrogen (N) and carbon (C) content of the leaves. B- Leaf phenylpropanoid and anthocyanin contents. Bars represent N-sufficient (black) and N deficient (white) treatment. The data present the mean of 8 plants per treatment for 3 independent growth experiments. Letters above the histogram columns indicate significant differences in mean values, as predicted by a two way ANOVA (p -value < 0.05), error bars represent \pm SD.

Supplementary Data

Supplementary Table S1 . Primers used for identification of mutation homozygotes among the progeny of Arabidopsis T-DNA insertion *pip* mutant lines.

Supplementary Table S2 . Primers used for cloning of potato aquaporins.

Supplementary Fig. S1 . Identification of mutation homozygotes among the progeny of Arabidopsis T-DNA insertion *pip* mutant lines.

Supplementary Fig. S2 . Effect of nitrogen deficiency on growth of potato genotypes. **Supplementary Fig. S3 .** Relative quantification of Western blot signal for determination of PM enrichment.

Supplementary Fig S4 . LC-MS based plasma membrane protein profiling of the two cultivars under N deficiency and control conditions analyzed by principal component analysis. **Supplementary Fig. S5 .** Number of transmembrane helices (TMH) predicted for the identified proteins.

Supplementary Fig. S6 . Localization of GFP:StPIPs in yeast cells.

Supplementary Fig. S7 . The ammonium transport ability of PIP1;1 and PIP1;3 studied in yeast by a growth complementation assay (without ZmPIP2;5 co-expression).

Supplementary Fig. S8 . Correlation between the abundance of PM ATPase and NRT2.1.

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