Overexpression of MfPIP2-7 from *Medicago falcata* promotes cold tolerance and growth under NO$_3^-$ deficiency in transgenic tobacco plants

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**Abstract**

**Background:** Plasma membrane intrinsic proteins (PIPs), which belong to aquaporins (AQPs) superfamily, are subdivided into two groups, PIP1 and PIP2, based on sequence similarity. Several PIP2s function as water channels, while PIP1s have low or no water channel activity, but have a role in water permeability through interacting with PIP2. A cold responsive PIP2 named as MfPIP2-7 was isolated from *Medicago falcata* (hereafter *falcata*), a forage legume with great cold tolerance, and transgenic tobacco plants overexpressing MfPIP2-7 were analyzed in tolerance to multiple stresses including freezing, chilling, and nitrate reduction in this study.

**Results:** MfPIP2-7 transcript was induced by 4 to 12 h of cold treatment and 2 h of abscisic acid (ABA) treatment. Pretreatment with inhibitor of ABA synthesis blocked the cold induced MfPIP2-7 transcript, indicating that ABA was involved in cold induced transcription of MfPIP2-7 in *falcata*. Overexpression of MfPIP2-7 resulted in enhanced tolerance to freezing, chilling and NO$_3^-$ deficiency in transgenic tobacco (*Nicotiana tabacum* L.) plants as compared with the wild type. Moreover, MfPIP2-7 was demonstrated to facilitate H$_2$O$_2$ diffusion in yeast. Higher transcript levels of several stress responsive genes, such as *NtERD10B*, *NtERD10C*, *NtDREB1*, and 2, and nitrate reductase (NR) encoding genes (*NtNIA1*, and *NtNIA2*) were observed in transgenic plants as compared with the wild type with dependence upon H$_2$O$_2$. In addition, NR activity was increased in transgenic plants, which led to alterations in free amino acid components and concentrations.

**Conclusions:** The results suggest that MfPIP2-7 plays an important role in plant tolerance to freezing, chilling, and NO$_3^-$ deficiency by promoted H$_2$O$_2$ diffusion that in turn up-regulates expression of NIAs and multiple stress responsive genes.

**Keywords:** Cold, Hydrogen peroxide, *Medicago falcata*, MfPIP2-7, Nitrate reductase, NO$_3^-$ deficiency, Tolerance

**Background**

Aquaporins (AQPs) form a superfamily of intrinsic channel proteins and function as diffusion facilitators for water and small molecules such as CO$_2$, glycerol, ammonium, and urea cross plasma and intracellular membranes in plant cells [1–4]. Plant AQPs are divided into five subgroups consisting of the plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins, nodulin 26-like intrinsic proteins, small basic intrinsic proteins, and X intrinsic proteins [5]. The PIPs can be further subdivided into PIP1 and PIP2, based on sequence similarity. Several PIP2s function as water channels, while PIP1s have low or no water channel activity, but are associated with water permeability through interacting with PIP2 [6–8].

Responses of PIP expression to abiotic stresses are variable, with up-, down- or no regulation, depending on species or tissues [9–14]. Most of AtPIPs are less affected...
by salinity, except for AtPIP1-5 and AtPIP2-6 which are down-regulated in roots and shoots respectively [9]. Transcripts of AtPIPs are generally down-regulated in leaves upon gradual drought stress, but AtPIP1-4 and AtPIP2-5 transcript levels are up-regulated [12]. Osmotic water permeability of protoplasts is decreased by down-regulation of certain PIP, which leads to a higher susceptibility to drought and osmotic stress [15–17], while overexpression of PIP genes generally increases root osmotic hydraulic conductivity and transpiration in transgenic plants [10, 18, 19]. The transgenic tobacco and Arabidopsis plants overexpressing AtPIP1-4 or AtPIP2-5 display enhanced water loss under dehydration stress [20]. The responses of PIPs to water stress and ABA are different between upland rice and lowland rice [10, 11]. For example, OsPIP1-3 is up-regulated by osmotic stress in highland rice, while OsPIP1-3 transcript is unaltered in lowland rice, indicating that OsPIP1-3 is associated with the differential avoidance to drought in the two varieties [10]. Salt and drought tolerance are enhanced in transgenic plants overexpressing either OsPIP1-1 or OsPIP2-2 [13]. GhPIP2-7 expression is up-regulated in leaves after drought treatments, and overexpression of GhPIP2-7 in Arabidopsis leads to an enhanced drought tolerance in transgenic plants [21]. TaAQP8, a wheat PIP1 gene, is induced by NaCl, which involves ethylene and H2O2 signaling. Overexpression of TaAQP8 in tobacco increases root elongation under salinity, with increased K+/Na+ ratio and Ca2+ content and reduced oxidative damages [22].

Most of PIP subfamily members in Arabidopsis thaliana are down-regulated by cold treatment, but AtPIP2-5 is up-regulated [9]. Overexpression of AtPIP2-5 alleviates the inhibition of low temperature on plant growth in transgenic Arabidopsis [23] and facilitates seed germination under cold stress [20]. Chilling results in decreased expression of some PIPs in rice seedlings, but higher transcript levels of OsPIP1-1, OsPIP2-1, OsPIP2-7 in shoots and OsPIP1-1, OsPIP2-1 in roots were observed in a chilling-tolerant variety than a chilling-sensitive one during the recovery at room temperature, indicating an important role of PIPs in re-establishing water balance after chilling conditions [24]. OsPIP1-3 plays an important role in chilling tolerance through interacting with members of OsPIP2 subfamily and improving water balance [8].

Medicago falcata is closely related to alfalfa (Medicago sativa), the most important perennial forage legume, with better cold tolerance [25–27]. Higher levels of sucrose, myo-inositol, galactitol, and raffinose family oligosaccharides (RFOs) are accumulated in falcata than in alfalfa during cold acclimation [27]. Transcript levels of myo-inositol phosphate synthase (MIPS), galactitol synthase (GalS), and myo-inositol transporter-like (INT-like) genes are accordingly induced in falcata [27–29]. In addition, expression of S-adenosylmethionine synthetase (SAMS) and a temperature induced lipocalin (TIL) are also induced by low temperature, and these genes are associated with cold tolerance in falcata plants [30, 31].

In our previous investigation a fragment encoding a PIP was harvested in a cDNA library of falcata responsive to cold [32], and no other PIP genes was found in the library, implying a potential role of the PIP in cold tolerance of falcata. We isolated the cold responsive PIP from falcata, which was highly homologous to MtPIP2-7. However, there is no report on the role of plant PIP2-7 in regulation of cold tolerance. The objective of this study was to investigate the role of the PIP2-7 gene (MfPIP2-7) in cold tolerance of falcata. MfPIP2-7 transcript in response to low temperature was analyzed, and transgenic tobacco plants overexpressing MfPIP2-7 were generated for examining tolerance to abiotic stresses such as cold and nitrate reduction.

Results

Characterization of MfPIP2-7

A cDNA sequence of MfPIP2-7 (910-bp) was cloned from falcata leaves. It contains an open reading frame (ORF) of 864 bp (GenBank accession number F607305) and encodes a deduced polypeptide of 30.9 kDa (GenBank accession number ACM50914). Sequence blast showed that MfPIP2-7 was most homologous (97.2 %) in AA sequence to a PIP2-7 (MTR_2g094270) in M. truncatula. A phylogenetic tree of MfPIP2-7 and all PIPs from Arabidopsis showed that MfPIP2-7 is most similar to AtPIP2-7 (Additional file 1: Figure S1). A multiple alignments of three PIPs indicated that six amphipathic channels/transmembrane helices and two signature motifs, which characterize major intrinsic protein, were found in MfPIP2-7 protein (Additional file 1: Figure S2). MfPIP2-7 was predicted to be localized in plasma membrane using PSORT Prediction (http://psort.hgc.jp/form.html) and cross checking with CELLO v.2.5 prediction software (http://cello.life.nctu.edu.tw/).

MfPIP2-7 transcript in response to abiotic stress

No tissue-specific expression of MfPIP2-7 was observed in falcata plants, although roots had 76 % higher level of MfPIP2-7 transcript than leaves or stems (Fig. 1a). MfPIP2-7 transcript in leaves was initially induced at 4 h and reached to the peak at 8 h after cold treatment, followed by a decline after 12 h (Fig. 1b). MfPIP2-7 transcript was also induced by 2 h of ABA treatment (Fig. 1c). ABA is signaling in plant adaptation to abiotic stress as well as in cold acclimation of falcata [30]. Involvement of ABA in cold-induced MfPIP2-7 transcript was examined. The expression of MfPIP2-7 induced by cold was blocked by pretreatment with naproxen (NAP) (Fig. 1d), inhibitor of ABA synthesis [30, 33], indicating that ABA were involved in MfPIP2-7 expression induced by cold.
Tissue-specific expression of $MfPIP2-7$ and influence of cold, and abscisic acid (ABA) on $MfPIP2-7$ transcripts. Mature leaflets, stem, and lateral roots were detached from 2-month-old seedlings (a). Plants were exposed to 5 °C in a growth chamber for cold treatment (b). Detached leaves placed in 0.1 mM ABA solution or H$_2$O as control for 12 h (c), or pretreated in 1 mM naproxen solution for 2 h, followed by 8 h of cold treatment at 5 °C, while those continuously placed in H$_2$O under room temperature were used as non-stressed control (d). Relative expression levels were determined by qRT-PCR and normalized to $actin$ expression. The same letter above a column indicates no significant difference by Duncan’s test at $P < 0.05$.

Analysis of transgenic tobacco plants
DNA blot hybridization showed that transgenic tobacco plants overexpressing $MfPIP2-7$ had hybridization signals, whereas no cross-hybridization was observed in the wild type, indicating that the transgene was integrated into the genomes of the transgenic tobacco lines (Fig. 2a). qRT-PCR data showed that $MfPIP2-7$ was expressed in transgenic plants (Fig. 2b).

Freezing tolerance was evaluated using survival rate and LT$_{50}$. Most of the wild type plants could not survive after freezing treatment, while 52 to 69 % transgenic plants could survive (Fig. 2c, d). Compared to a $-1.3$ °C of LT$_{50}$ in the wild type, lower levels of LT$_{50}$ were observed in transgenic lines than in the wild type (Fig. 2d). Moreover, the difference in LT$_{50}$ was blocked by pretreatment with dimethylthiourea (DMTU), a scavenger of H$_2$O$_2$, and DMTU treatment resulted in increased LT$_{50}$ in all plants (Fig. 2e), indicating that the differential LT$_{50}$ between transgenic plants and the wild type was associated with H$_2$O$_2$.

Chilling tolerance was assessed by measuring ion leakage and photosynthesis. Both the wild type and transgenic plant had similar levels of ion leakage, maximal photochemical efficiency of photosystem II ($F_v/F_m$), and net photosynthetic rate ($A$) under control conditions. Chilling led to an enhanced ion leakage and decreased $F_v/F_m$ and $A$ in all plants, and transgenic plants maintained lower levels of ion leakage and higher levels of $F_v/F_m$ and $A$ than the wild type (Fig. 3a, b, c).
Transgenic plants and the wild type showed similar growth on ½ Murashige and Skoog (MS) medium, which contained 10 mM NO$_3^-$ and was used as a control condition in the study (Fig. 4a, b). Plant growth declined under conditions of low level of NO$_3^-$ (0.2 mM) or without NO$_3^-$ (0 mM), but transgenic plants had higher levels of plant fresh weight and relative growth than the wild type (Fig. 4a, b, c). For example, relative growth of the wild type on the medium without NO$_3^-$ or containing 0.2 mM NO$_3^-$ was 31 % and 24 %, respectively, while that of transgenic plants was 41 to 44 % and 31 to 34 %, respectively, after growing for 8 weeks (Fig. 4c).

Sensitivity of yeast cells expressing MfPIP2-7 to externally supplied H$_2$O$_2$
Sensitivity of yeast cells transformed with AQP or with an empty vector (control) to externally supplied H$_2$O$_2$ was used to evaluate the permeability of AQP to H$_2$O$_2$ [3, 34]. Yeast cells transformed with MfPIP2-7 or with an empty showed no difference in growth on the medium containing 0.5 mM H$_2$O$_2$ or without H$_2$O$_2$. However, expression of MfPIP2-7 markedly reduced growth and cell survival on the medium containing 1 or 2 mM H$_2$O$_2$ (Fig. 5). The reduced growth of yeast expressing MfPIP2-7 was due to increased oxidative stress as the result of increased uptake of H$_2$O$_2$ from the external medium [34]. The results suggest that expression of MfPIP2-7 facilitated H$_2$O$_2$ diffusion in yeast cells.

Abiotic stress responsive genes were induced in transgenic plants
Transcripts of abiotic stress responsive genes, such as early response to drought 10 (ERD10B, ERD10C), nitrate...
reductase1 (NIA1), NIA2, and dehydration responsive element binding protein (DREB), were analyzed using transgenic tobacco plants in comparison to the wild type. Higher levels of NtERD10B, NtERD10C, NtNIA1, NtNIA2, NtDREB1, and NtDREB2 transcripts were observed in transgenic plants than in the wild type (Fig. 6a to f), while there was no difference in NtDREB3 and 4 transcripts between the two type plants (data not shown). Pretreatment with DMTU blocked the difference in transcripts of above genes between the two types of plants (Fig. 6a to f), indicating that the higher transcript levels in transgenic plants were associated with H$_2$O$_2$.

NR activity was higher in leaves than in roots in all plants, while 61 to 71 % or 55 to 70 % higher activities were observed in leaves or roots of transgenic plants than that in the wild type, respectively (Fig. 7a, b). The results were consistent with that transgenic plants had higher transcript levels of NIA1 and NIA2 than the wild type.

**Fig. 5** Yeast growth and survival test on medium containing H$_2$O$_2$. After a series of dilution of the yeast cells transformed with either an empty pYES2 as control or derivate of pYES2 carrying MfPIP2-7 (pYES2-PIP2-7) at an A$_{600nm}$ of 0.6, 10 μl was spotted on medium containing various concentrations of H$_2$O$_2$ as indicated. Growth was recorded after 4 days at 30 °C.

**Fig. 6** Analysis of transcript levels of ERD10C (a), ERD10B (b), NIA1 (c), and NIA2 (d), DREB1 (e), and DREB2 (f) in transgenic tobacco plants in comparison to the wild type. The expression levels were normalized to that of actin using qRT-PCR. Means of three repeats and standard errors are presented; the same letter above the column indicates no significant difference at $P < 0.05$. 
Amino acid levels were altered in transgenic plants

Significantly higher (29 %) level of total free amino acids was observed in roots but not in leaves of the transgenic line 3-1 than in the wild type (Table 1). Levels of most of the free amino acids in leaves or/and roots showed significant difference between the transgenic line and the wild type, but there was no difference in glutamic acid and phenylalanine levels (Table 1). Compared to the wild type, significantly higher levels of asparagine, threonine, leucine, tyrosine, tryptophan, lysine, histidine, and ornithine levels were observed in both leaves and roots of transgenic line. In addition, higher levels of proline, arginine, and γ-amino butyric acid, and α-aminoacidic acid and lower levels of serine, glycine, alanine, aspartic acid, and citrulline were observed in leaves, and lower levels of glutamate, isoleucine, phospho-serine were observed in roots of the transgenic line as compared with the wild type (Table 1).

Discussion

An ORF encoding MfPIP2-7 was cloned from falcata. MfPIP2-7 has the highest AA similarity with MtPIP2-7 or AtPIP2-7 among PIP proteins in M. truncatula or Arabidopsis. MfPIP2-7 transcript was induced by cold and ABA treatment. ABA is signaling in regulation of downstream stress responses, including expression of multiple down-stream genes with relevance to abiotic stress tolerance [35–37]. ABA is also involved in cold acclimation of falcata [30]. In this study, ABA was demonstrated to be involved in MfPIP2-7 expression induced by cold, suggesting that ABA-regulated MfPIP2-7 plays an important role in cold tolerance in falcata.

The role of MfPIP2-7 in cold tolerance was documented using transgenic plants. Overexpression of MfPIP2-7 resulted in enhanced tolerance to freezing and chilling stresses in transgenic tobacco plants, suggesting that MfPIP2-7 expression is associated with elevated cold tolerance. Similarly, expression of GhPIP2-7 leads to an improved growth under osmotic stress in transgenic Arabidopsis [21], while transgenic Arabidopsis overexpressing OsPIP1-1 or OsPIP2-2 showed improved root growth under osmotic or salt stress [13]. The altered osmotic or drought stress in transgenic plants up- or down-regulating PIP genes expression is associated with the increase or decrease in hydraulic conductivity and transpiration [10, 15–17]. In addition, plant PIPs function to facilitate H₂O₂ diffusion across plasma membrane apart from as water channels [3, 34]. In this study MfPIP2-7 was found to facilitate H₂O₂ diffusion through expressing in yeast cells. Transgenic plants had higher levels of NtERD10B, NtERD10C, NtNIA1, NtNIA2, NtDREB1, and NtDREB2 transcripts, which were blocked by scavenger of H₂O₂, suggesting that the high transcript levels in transgenic plants were associated with elevated H₂O₂. Intercellular H₂O₂, mainly produced by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, is an important signal in

Table 1 Analysis of free amino acid levels (nmol g⁻¹ FW) in transgenic plant line (3-1) in comparison with the wild type (WT)

| Amino Acids          | Leaves | Roots |
|----------------------|--------|-------|
| Glycine              | 2256   | 1664**| 28.2 | 44.3**|
| Glutamine            | 2143   | 1986  | 131  | 258**|
| Glutamic acid        | 1801   | 1848  | 171  | 172  |
| Serine               | 1260   | 860** | 79.2 | 107**|
| Aspartic acid        | 1094   | 946*  | 79.9 | 86.2 |
| Asparagine           | 924    | 1844**| 70.1 | 140**|
| Threonine            | 754    | 1173**| 53   | 101**|
| Proline              | 682    | 1174**| 208  | 211  |
| Alanine              | 622    | 489*  | 56.9 | 76.5*|
| Phenylalanine        | 368    | 352   | 29.8 | 34.8 |
| Arginine             | 313    | 595** | 20.6 | 26.1 |
| Leucine              | 239    | 332** | 74.5 | 102* |
| Phospho-serine       | 221    | 241   | 78.7 | 92.5**|
| Histidine            | 177    | 308** | 26.1 | 38.3**|
| Tyrosine             | 176    | 294** | 13.1 | 22.3**|
| γ-Amino butyric acid | 143    | 197** | 130  | 163  |
| Tryptophan           | 142    | 227** | 19.5 | 42.3**|
| Lysine               | 130    | 297** | 34.6 | 49.4**|
| Isoleucine           | 114    | 141   | 10.7 | 22.6**|
| 1-Methyl-histidine   | 58.3   | 80.5**| 117  | 88.4*|
| Citrulline           | 52.8   | 32.9**| 5.85 | 7.55 |
| α-Aminoadipic acid   | 24.5   | 43.0**| ND   | ND   |
| Ornithine            | 18.4   | 27**  | 26.8 | 33.2**|
| Ethanolamine         | ND     | ND    | 131  | 110  |
| Carnosine            | ND     | ND    | 173  | 232**|

Means of three independent samples and standard errors are presented; an asterisk * or ** indicates significant difference between WT and transgenic plant at P < 0.05 or P < 0.01, respectively.
regulating expression of multiple genes associated with abiotic stress tolerance [38, 39]. H₂O₂ is also involved in cold and/or drought induced gene expression, such as MfMIPS and MfSAMs, in *falcata* [27, 30] and NIA1 in tobacco plants [40]. NtDREB1, 2, 3, and 4 belong to DREB1/CBFs (C-repeat binding factors) transcription factors, which are induced in response to cold in tobacco plants [31], while CBFs regulate cold acclimation and expression of cold responsive genes. ERD10B and ERD10C belong to the dehydrin (DHN) family [41]. They are induced by drought and cold [42], and protect plant cells against stress induced damages by potent chaperone activity and membrane-binding capacity for increased stabilization of diverse proteins and membrane systems [43]. Nevertheless, the higher transcript levels of *NtERD10B*, *NtERD10C*, *NtDREB1*, and *NtDREB2* in MfPIP2-7 transgenic plants are associated with the elevated cold tolerance.

It is interesting that *NtNIA1* and *NtNIA2* transcript levels were up-regulated in transgenic plants with dependence upon H₂O₂, which led to enhanced NR activity in both leaves and roots. NR is a key enzyme in nitrate reduction and nitrogen metabolism [44]. The elevated NR activity resulted in alterations in free amino acid components and concentrations in transgenic plants, indicating that expression of MfPIP2-7 influences N metabolism. An elevated concentration of total free amino acids in roots may provide transgenic plants with more nitrogen under NO₃⁻ deficiency and thus promote NO₃⁻ deficiency tolerance in transgenic tobacco plants. In addition, NR-dependent NO production is involved in cold acclimation and freezing tolerance by modulating proline accumulation in *Arabidopsis* [45]. Apart from proline, many free amino acid concentrations, such as argine, ornithine, and γ-amino butyric acid, were higher in transgenic tobacco than in the wild type. Many free amino acids can modulate membrane permeability and ion uptake and function as osmolyte in plants [46]. γ-Aminobutyric acid is involved in cold acclimation and freezing tolerance in barley and wheat [47]. Ornithine and argine are the precursor of polyamine biosynthesis, while polyamines are involved in cold tolerance [48]. Thus the alterations in free amino acid are proposed to be associated with the elevated cold tolerance in transgenic plants.

**Conclusions**

MfPIP2-7 was characterized in this study. MfPIP2-7 transcript level is induced by cold and ABA, while ABA is involved in the cold induced expression of MfPIP2-7. MfPIP2-7 showed facilitation of H₂O₂ diffusion in yeast cells. Overexpression of MfPIP2-7 led to enhanced cold tolerance in transgenic tobacco plants, which was associated with the induced expression of stress responsive genes, such as *NtERD10B*, *NtERD10C*, and CBF transcription factors. Moreover, the higher levels of *NtNIA1* and *NtNIA2* transcripts and NR activity led to alterations of free amino acid in components and concentrations which are associated with the elevated tolerance to NO₃⁻ deficiency and cold.

**Methods**

**Isolation of MfPIP2-7 cDNA from falcata**

*Medicago sativa* subsp. *falcata* (L.) Arcang. cv. Hulunbeir seeds were provided by Institute of Animal Science, Chinese Academy of Agricultural Sciences. Total RNA was isolated from leaves of cold-treated *falcata* plants (0.1 g) [31], cDNA was synthesized from two micrograms of total RNA in the presence of 160 U of M-MLV reverse transcriptase (Promega, Madison, WI, USA) and oligo (dT)₁₈ in a 20 μl reaction mixture [28]. Primers RT59 (5'-GAACACAAACATGGGCAAAGA-3') and RT60 (5'-CAAATCATATAATAATTGAAACCA-3') were designed for amplification of MfPIP2-7, based on assembly of EST sequences from the GenBank using SeqMan (DNASTAR Inc, Madison, WI, USA). PCR reaction mixture contain the first-strand cDNA as the template, primers RT59 and RT60, and Ex Taq DNA polymerase (Takara Bio Inc., Dalian, China). After sequencing of the PCR product, the deduced amino acid sequence was analyzed using DNAMAN software.

**Transgenic tobacco generation**

An expression plasmid pBI-MfPIP2-7 was constructed by inserting the ORF of MfPIP2-7 into the pBI121 binary vector and used for generation of transgenic tobacco plants as described previously [27]. Seeds of the wild type (*Nicotiana tabacum* L. cv. Zhongyan 90) were initially provided by Crops Research Institute, Guangdong Academy of Agricultural Sciences and harvested in our laboratory. Seeds of homozygous transgenic tobacco plants (T₃) and the wild type used for investigation in this study were harvested at the same time.

**Plant growth and treatments**

Homozygous lines of transgenic tobacco and the wild type plants of tobacco and *falcata* were grown in a greenhouse for 2 months as described previously [27]. *Falcata* plants were placed in a growth chamber at 5 °C for 4 days for cold treatment [27]. In addition, the detached leaves were placed in distilled water for 1 h to eliminate the potential wound stress influence, followed by moving into new beakers: (1) containing 100 μM ABA for 12 h for detecting effect of ABA on MfPIP2-7 expression; (2) containing H₂O or 1 mM NAP for 2 h, followed by transferring to a growth chamber at 5 °C for 8 h as cold treatment for detecting involvement of ABA in cold-induced expression of MfPIP2-7 as described previously [30], while those placed in beakers containing H₂O under room temperature 8 h were used as a non-stressed control. In addition, tobacco leaf discs were
placed in beakers containing H₂O (control) or 5 mM DMTU for 2 h before determinations of freezing tolerance, gene expression, or NR activity. The experiments were repeated for three times.

**DNA blot hybridization**

Genomic DNA was extracted from tobacco leaves using hexadecyltrimethylammonium bromide (CTAB) as previously described [27]. DNA samples (15 μg) were separated by electrophoresis on 0.8 % agarose gel after digestion overnight with HindIII, followed by transfer to Hybond XL nylon membrane (Amersham, GE Healthcare Limited, Buckinghamshire, UK). Hybridization was conducted using [α-³²P] dCTP labeled fragment (407 bp) of MfPIP2-7 as probe. The hybridization signals were detected using Typhoon Trio (General Electric Company, Fairfield, CT).

**Real time quantitative reverse transcription PCR (qRT-PCR)**

One μg of total RNA was used for synthesis of first-strand cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara). After dilution the cDNAs were used as template in 10-μl PCR reactions containing 200 nM forward and reverse primers and 5 μl SYBR Premix Ex Taq (Takara), and qRT-PCR was conducted in Mini-Option Real-Time PCR System (Bio-Rad, Hercules, CA) [28]. Parallel reactions to amplify actin were used to normalize the amount of template. We use actin as reference gene because it had been demonstrated to be reliable in M. falcata and M. truncatula [25]. The primers and their sequences used in this study are listed in Additional file 1: Table S1. Three technical and two biological replicates were conducted in each experiment.

**Abiotic stress tolerance assessment**

Survival rate and the temperature (LT₅₀) that resulted in 50 % lethal were measured to evaluate freezing tolerance as previously described [30]. For measurement of survival rate, 6-week-old tobacco plants were placed in a growth chamber under light of 700 μmol photon m⁻² s⁻¹, with decreasing temperature from 25 to -3 °C within 6 h and maintained for 3 h [31]. The experiments contained five replicates and 20 plants each line per replicate. Plant survival rate was calculated 3 d after plants were moved to room temperature for recovery. LT₅₀ was calculated using a fitted model plot based on ion leakage data after leaf discs detached from 6-week-old tobacco plants were treated with freezing [26, 30]. For assessment of chilling tolerance, 10-week-old pot plants were chilled at 3 °C for 4 d under light of 200 μmol photon m⁻² s⁻¹ in a growth chamber with a 12-h photoperiod. Ion leakage, F₄/Fₐ₀, and A were measured as previously described [28, 30]. For nitrogen-deprivation treatment, tobacco seeds were sterilized and germinated on half strength of MS medium, followed by transferring to a half strength of MS medium containing 0 or 0.2 mM NO₃⁻ and growing in a growth room with a 12-h photoperiod under light of 200 μmol photon m⁻² s⁻¹ at 25 °C, while those growing on ½ MS medium were used as a control. Compared to ½ MS medium that contained 10 mM NO₃⁻, the nitrogen deprivation medium was made by replacing NH₄NO₃ with (NH₄)₂SO₄ so that KNO₃ was the sole nitrate source at 0 or 0.2 mM. The K⁺ concentration was adjusted to 10 mM by the addition of K₂SO₄ in all media [49]. Plant fresh weight was weighed at the eighth week after transplanting.

**Yeast growth assay**

Yeast growth assay was conducted according to the method described by Bienert et al. [34] with modifications. The Saccharomyces cerevisiae strain INVSc1 was transformed with either an empty pYES2 (Invitrogen) as control or derivate of pYES2 carrying MfPIP2-7 coding sequence. Yeast cells were grown on SD/-Ura synthetic medium containing 2 % glucose until an A₆₀₀nm of 0.6 to 0.8, followed by two times washing with liquid SG/-Ura synthetic medium containing 2 % galactose to an A₆₀₀nm of 0.6. After a series of dilution, 10 μl were spotted on solid SG/-Ura medium containing various concentrations of H₂O₂ as indicated. Differences in growth and survival were recorded after 4 days of incubation at 30 °C.

**Measurement of NR activity**

Tobacco leaves (0.5 g) were ground in a mortar with pestle in 5 ml of 50 mM phosphate buffer (pH 7.8) containing 2 % (w/v) polyvinylpyrrolidone (PVP), 2 mM EDTA and 5 mM dithiothreitol (DTT) at 4 °C. The homogenate was centrifuged at 12,000 x g for 15 min for recovery of the supernatant. Nitrate reductase activity and protein content were measured as described previously [40]. The enzyme reaction mixture (2 ml) contained 50 mM K-phosphate buffer (pH 7.5), 60 mM KNO₃ and 0.25 mM NADH. The reaction was started by addition with 400 μl of the supernatant and incubated at 25 °C for 30 min, followed by addition of 1 ml of 1 % sulphanilamide in 1.5 M HCl and 1 ml of 0.01 % 1-naphthylamine. After incubated for 15 min, the mixture was centrifuged for 5 min at 10,000 x g and absorbance at 540 nm of the supernatant was measured to determine nitrite production. One unit of NR was defined as the amount of enzyme required for catalyzing the production of one μmol NO₂⁻ within one hour. Protein content in the enzyme extracts was determined using Coomassie Brilliant Blue G-250.

**Analysis of free amino acids**

Free amino acids were extracted from leaves (0.4 g) by grinding in 1 ml of 6 % (w/v) 5-sulfosalicylic acid at 4 °C. The extract was centrifuged for 15 min at 12,000 rpm. The supernatant was subjected to derivatization by phenyl
isothiocyanate, followed by filtration (0.45 μm). 20 μl of the filtrate was injected into a Hitachi model L-8800 amino acid analyzer (Hitachi Co. Ltd., Tokyo, Japan), supplied with Hitachi chromatographic column 855–350, for measurement of amino acids.

Additional files

Additional file 1: Table S1. Primers for quantitative real-time PCR.
Figure S1. Phylogenetic analysis of MfPIP2-7 in falcata with PIPs in Arabidopsis. Figure S2. Alignment of the deduced amino acid sequences of MfPIP2-7, MfPIP2-7, and AtPIP2-7. (DOC 108 kb)

Abbreviations
A, net photosynthetic rate; ABA, abscisic acid; AQPs, aquaporins; CBFs, C-repeat binding factors; CTAB, hexadecyltrimethylammonium bromide; DHA, dehydro; DMTU, dimethylthiourea; DREB, dehydration responsive element binding protein; DTT, dithiothreitol; ERD, early response to drought; GolS, galactinol synthase; INT-like, myo-inositol transporter-like; LEAs, late embryogenesis abundant proteins; MIPS, myo-inositol phosphate synthase; MS, Murashige and Skoog; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NAP, naproxen; NIA, nitrate reductase; NIPs, nodulin 26-like intrinsic proteins; NR, nitrate reductase; ORF, open reading frame; PCR, Polymerase chain reaction; pl, isoelectric point; PIPs, Plasma membrane intrinsic proteins; PVP, polyvinylpyrrolidone; RFOs, raffinose family oligosaccharides; SAMS, S-adenosylmethionine synthetase; SiPs, small basic intrinsic protein; SSH, suppression subtractive hybridization; TIL, temperature induced lipocalin; TIPS, tonoplast intrinsic proteins; XIPs, X intrinsic proteins

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Availability of data and materials
All datasets supporting the results of this study are included in the article and the additional files.

Authors’ contributions
CZ analyzed gene expression in falcata and performed all experiments using transgenic tobacco plants; TW generated transgenic tobacco and conducted DNA blot analysis; ZG designed the study, analyzed the data, and wrote the paper; SL designed the study with ZG. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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