Short Communication
Expression of plasma membrane H$^+$-ATPase in cluster roots of white lupin under phosphorus deficiency

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Abstract

It was previously shown that plasma membrane (PM) H$^+$-ATPases of active cluster roots are acclimated to phosphorus (P) deficiency. In the present study, we followed the question whether the qualitative acclimation of PM H$^+$-ATPase is based on isoform-specific expression. Therefore, three different PM H$^+$-ATPase isoforms (*LHA1–LHA3*) were investigated at the transcriptional level. Our data indicate that LHA1 may play a key role in the qualitative acclimation of PM H$^+$-ATPase in cluster roots to P deficiency.

Key words: ATPase isoforms / *Lupinus albus* / phosphorus deficiency / transcription / translation

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1 Introduction

An insufficient supply of phosphorus (P) often leads to limitation of agricultural production. Some crops use strategies for a better acquisition of P from the soil. This also includes white lupin (*Lupinus albus* L.) which secretes root exudates from cluster roots. Cluster roots are second-order laterals with determinate growth (Fig. 1). These cluster roots which are induced by P deficiency enable the plant to acidify intensively micro compartments in the rhizosphere (*Yan* et al., 2002; *Zhu* et al., 2005). Protons are released by the PM H$^+$-ATPase (*Palmgren*, 2001), which helps to dissolve Ca phosphates (*Dinkelaker* et al., 1989) and to provide low pH conditions necessary for activity of acid phosphatase (*Gilbert* et al., 1999).

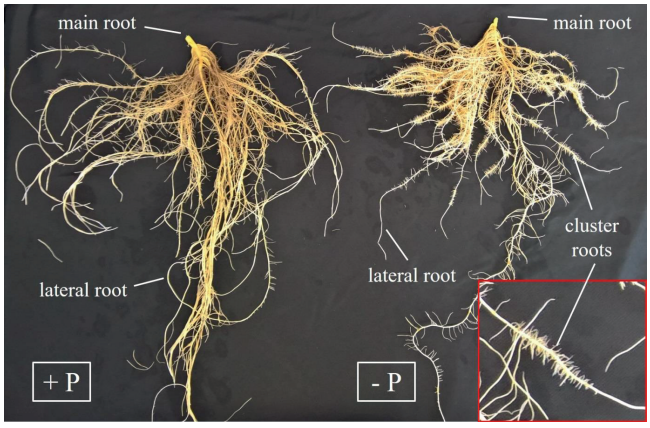

Figure 1: Roots of plants grown under sufficient P supply (+P; left) and P deficiency (–P; right). Insert shows magnification of cluster roots.

Yan et al. (2002) determined the activity of PM H$^+$-ATPase in lateral and cluster roots of P-sufficient and P-deficient white lupin. They showed that PM H$^+$-ATPases of active cluster roots are quantitatively and qualitatively acclimated to P deficiency. The quantitative acclimation of PM H$^+$-ATPase (higher hydrolytic activity, higher H$^+$-pumping activity) was ascribed to a higher steady-state enzyme concentration in cluster roots from P-deficient plants in comparison to roots of P-sufficient plants. As qualitative acclimation the authors reported significant changes of pH optimum, K_m value, and vanadate sensitivity of the PM H$^+$-ATPase of active cluster roots under P deficiency. In the present study, we followed the question whether the qualitative acclimation of PM H$^+$-ATPase to P deficiency can be explained in terms of isoform-specific gene expression.

For this purpose, gene expression was investigated at the transcriptional level *via* qRT-PCR and at the translational level *via* Western blot. With isoform-specific primers, three different PM H$^+$-ATPase isoforms (*LHA1–LHA3*) were investigated at the transcriptional level. It was hypothesized that the transcriptional pattern of the three isoforms changed under P deficiency. Based on the results of *Yan* et al. (2002), it was hypothesized that P deficiency leads to an increased amount of PM H$^+$-ATPase protein in cluster roots of white lupin.

2 Material and methods

Seeds of white lupin (*Lupinus albus* L. cv. Amiga) were grown in hydroponics in a climate chamber under controlled conditions. The day/night cycle was 16 h/8 h at 22°C/15°C at a relative humidity of 50%. The full-strength nutrient solution according to *Yan* et al. (2002) with a slight modification (additional supply of 0.1 μM $NiSO_4$) for P-deficient plants had the

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following composition: 0.5 mM $Ca(NO_3)_2$, 1.75 mM K_2SO_4, 0.25 mM KCl, 1.25 mM $MgSO_4$, 25 μM H_3BO_3, 1.5 μM $MnSO_4$, 1.5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.025 μM $(NH_4)_6Mo_7O_{24}$, 20 μM Fe(III)-EDTA. For the control plants, 0.25 mM KH_2PO_4 were added to the nutrient solution. The plants were harvested at the age of 25 d. Fresh and dry masses of roots and shoots were determined. The phosphate concentration of roots and shoots was determined following the vanado-molybdate colorimetric method (*Gerike* and *Kurmies*, 1952).

For the determination of the transcription and translation of PM H^+-ATPase in lateral and cluster roots (Fig. 1), the main roots were removed. Lateral and cluster roots were frozen in liquid nitrogen and ground with mortar and pestle. Ground root material was kept at –80°C until analyses. Total RNA was extracted from lateral and cluster roots. RNA extractions were performed using the RNeasy® Plant Mini Kit (Qiagen) following the manufacturer's instructions. Extracted RNA was transcribed to cDNA by means of "QuantiTect® Reverse Transcription Kit" (Qiagen). The relative mRNA abundance of PM H^+-ATPase isoforms *LHA1–LHA3* and of reference gene *polyubiquitin* was determined *via* Rotor Gene Q 2plex (Qiagen). As reference gene *polyubiquitin* was used, whose transcription in white lupin roots under sufficient and deficient P supply was shown by *Liu* et al. (2005). Primer sequences were selected as described by *Tomasi* et al. (2009): for *LHA1* (AY989893), CCA TTC ATT TCT CTT TTG GGA TA (forward) and GAA GAC AAA GCT CAA TAA CCA GAA (reverse); for *LHA2* (AY989895), GGA GAC TGG CCG AAG ACT T (forward) and CGG GAA TTG AGG CAA TAC TC (reverse); for *LHA3* (AY989894), CAG GGC AAT TTT CCA AAG AA (forward) and ACC TCC AGA GCA AGG CAA TA (reverse); for *polyubiquitin* (DQ118117), GCA CCC TAG CCG ACT ACA AC (forward) and CCG GTA AGG GTC TTG ACA AA (reverse). For amplification of sequences of interest SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich) and primers (forward and reverse, 0.3 μM) were used. The cycle program (40 cycles) consisted of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 30 s. Analysis of transcription data was performed using the Relative Expression Software Tool (REST©) of *Pfaffl* et al. (2002). Water instead of template served as negative control. Four biological replicates (with two technical replicates of each sample) for each treatment and root segment were performed. Specificity of amplification was confirmed by melt curve analysis.

Proteins were extracted from lateral and cluster roots with the protein extraction buffer (PEB, Agrisera). The total protein concentration in the extracts was determined by means of the DC Protein Assay Kit (Bio-Rad). The procedure followed the protocol specified by the manufacturer. For the separation of the proteins, 15% SDS polyacrylamide gel loaded with 50 μg of total protein were used. There were three biological replicates for each treatment and root segment. Separated proteins were transferred to a polyvinylidene difluoride membrane. After blotting, the "Plasma membrane H^+-ATPase (rabbit antibody)" (Agrisera) with a dilution of 1 : 5000 was used as first antibody for the PM H^+-ATPase. "Goat anti-rabbit IgG" (Agrisera) was chosen as second antibody with a dilution of 1 : 8000. PM H^+-ATPase proteins were detected by alkaline phosphatase staining. Band intensity was evaluated densitometrically *via* the image processing program "ImageJ". Western blotting was repeated in two independent experiments.

3 Results and discussion

After 25 d, the fresh and dry masses of P-deficient plants were significantly reduced in both shoots and roots (data not shown). Growth is determined by extension growth and cell division. Neither the ratio between dry and fresh mass nor the water concentration in relation to the fresh mass was significantly reduced in the P-deficient plants (data not shown). These two parameters indicate that extension growth was not affected due to P deficiency. Therefore, it is likely that cell division was inhibited under P deficiency leading to reduced growth. These results are consistent with those obtained in similar studies (*Yan* et al., 2002; *Funayama-Noguchi* et al., 2015; *Müller* et al., 2015).

We followed the question whether the qualitative acclimation of PM H^+-ATPase in active cluster roots, which was observed by *Yan* et al. (2002), is caused by a differential expression of PM H^+-ATPase isoforms. The transcription pattern of PM H^+-ATPase isoforms, measured *via* qRT-PCR, in the cluster roots of P-deficient plants was different from the pattern in the lateral roots of control plants: There was a significant increase in the relative mRNA abundance of the isoform *LHA1* (Fig. 2). The hypothesis that the transcriptional pattern of the three isoforms changes in cluster roots under P deficiency is therefore supported.

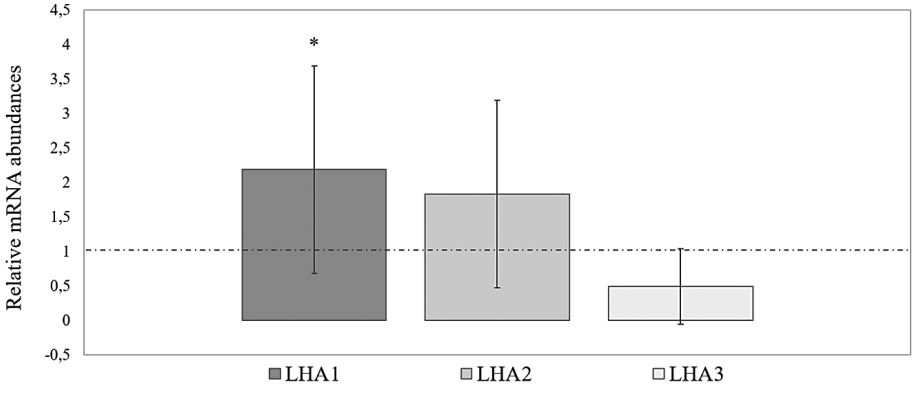

Figure 2: Effect of P deficiency in white lupin on the relative mRNA abundance of the PM H^+-ATPase isoforms *LHA1, LHA2*, and *LHA3* in cluster roots of P-deficient plants (columns) with reference to the lateral roots of control plants (dotted line). Presented are the mean values of four biological replicates and the associated standard errors. Significant differences between the isoforms with $^*P < 5\%$.

The changes in the transcriptional pattern due to P deficiency may explain the observed qualitative changes in enzyme kinetics observed by *Yan* et al. (2002). These authors reported that pH optimum, K_m value, and vanadate sensitivity of the PM H+-ATPase of active cluster roots were significantly changed due to P deficiency. It was shown by *Palmgren* and *Christensen* (1994) that heterologously expressed PM H+-ATPase isoforms of *Arabidopsis thaliana* differ strongly in their affinity to ATP and sensitivity to vanadate. Therefore, it is likely that isoform *LHA1* plays a key role in the change of enzyme characteristics of PM H+-ATPase in cluster roots. Analysis of the protein sequences of the three isoforms [by multiple sequence alignment, Clustal O (1.2.4)] showed that the protein lengths of the three tested isoforms do not differ much (LHA1: 956 amino acids; LHA2: 953 amino acids; LHA 3: 951 amino acids), but LHA1 amino acid sequence differs from LHA2 and LHA3 in 3% and 12% of amino acids, respectively. We speculate that a five amino acid-long sequence present in the actuator domain of LHA1, which is responsible for the dephosphorylation of the phosphoenzyme (*Farley*, 2012), absent in LHA2 and LHA3 could be responsible for the changes in enzyme characteristics. Unfortunately, the genome of white lupin is not yet fully sequenced. Hence, the picture of contribution of specific isoforms to PM H+-ATPase activity in cluster roots is still incomplete. However, the results of *Tomasi* et al. (2009) underline the importance of isoform LHA1. They determined mRNA abundance of *LHA1–LHA3* in cluster roots during a light period of 11 h. They observed a strong transient upregulation of *LHA1* (about 20-fold) 3 h after start of illumination. This upregulation was associated with a strong increase in PM H+-ATPase protein.

As a quantitative acclimation of gene expression at the translational level, an increased amount of PM H+-ATPase protein was detected in the cluster roots of white lupin under P deficiency compared to the lateral roots of control plants (Fig. 3). Based on this result, the second hypothesis concerning the increase in the amount of PM H+-ATPase in the cluster roots is also supported.

This is in line with the results obtained by *Yan* et al. (2002). They also observed an increased amount of PM H+-ATPase protein in the cluster roots, which may explain the increase in hydrolytic ATPase activity and H+-pumping activity reported by *Yan* et al. (2002). Due to the fact that the antibody was only protein family-specific, no statement can be made about an acclimation of specific PM H+-ATPase isoforms at the translational level. However, the significantly increased mRNA abundance of the isoform *LHA1* (Fig. 2) suggests that protein abundance of this isoform might make a contribution to the increase of PM H+-ATPase protein quantity observed due to P deficiency (Fig. 3). The Western Blot showed two protein bands. The predicted protein size of the known isoforms LHA1, LHA2, and LHA3 is very similar (LHA1: 956 amino acids, LHA2: 953 amino acids, LHA3: 951 amino acids). Therefore, the presence of two protein bands indicates the existence of one or more further isoform(s) which differ in molecular weight of known isoforms. The availability of

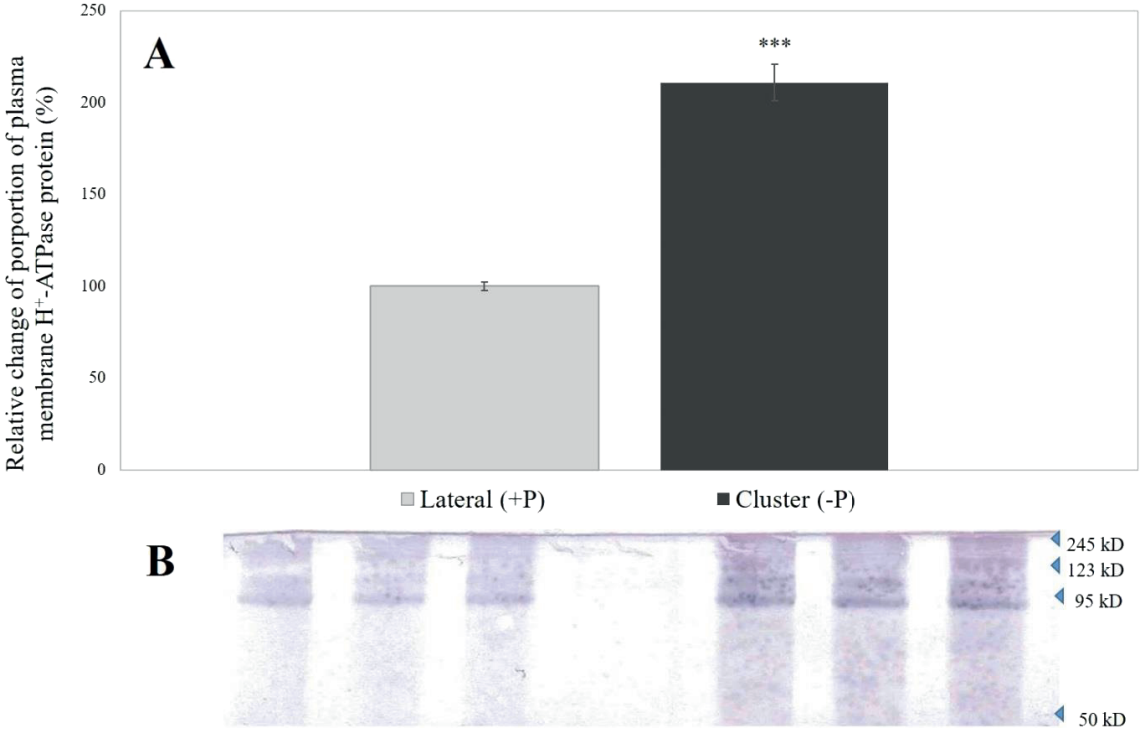

Figure 3: Effect of P deficiency in white lupin on the relative proportion of PM H+-ATPase protein to the total protein in the cluster roots of P-deficient plants in relation to the lateral roots of control plants. (A) Presented are the mean values of three biological replicates and the associated standard errors. Significant differences between the roots with ***P < 0.1%. (B) Corresponding Western Blot of proteins from lateral roots (+P) and cluster roots (–P). Double bands of H+-ATPase are visible in the 95 kDa region.

full-genome sequence and further gene expression analyses of white lupin will help to clarify.

4 Conclusion

During P deficiency of white lupin, alterations in the expression of PM H^+-ATPase in cluster roots occur at both transcriptional and translational levels. The changes in the transcriptional pattern due to P deficiency may explain the observed qualitative changes in enzyme kinetics. At translational level, the quantitative acclimation of the enzyme is realized by an increased amount of PM H^+-ATPase protein.

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References

Dinkelaker, B., *Römheld, V.*, *Marschner, H.* (1989): Citric acid excretion and precipitation of calcium citrate in the rhizosphere of white lupin (*Lupinus albus* L.). *Plant Cell Environ.* 12, 285–292.

Farley, R. A. (2012): Active Ion Transport by ATP-Driven Ion Pumps, in Sperelakis, N. (ed.): Cell Physiology Sourcebook, 4th Ed., Academic Press, New York, NY, USA, pp. 167–177.

Funayama-Noguchi, S., *Noguchi, K. O.*, *Terashima, I.* (2015): Comparison of the response to phosphorus deficiency in two lupin species, *Lupinus albus* and *L. angustifolius*, with contrasting root morphology. *Plant Cell Environ.* 38, 399–410.

Gerike, S., *Kurmies, B.* (1952): Die colorimetrische Phosphorsäurebestimmung mit Ammonium-Vanadat-Molbydat und ihre Anwendung in der Pflanzenanalyse. *Z. Düng. Pflanz. Bodenkunde* 104, 235–247.

Gilbert, G. A., *Knight, J. D.*, *Vance, C. P.*, *Allan, D. L.* (1999): Acid phosphatase activity in phosphorus-deficient white lupin roots. *Plant Cell Environ.* 22, 801–810.

Liu, J., *Samac, D. A.*, *Bucciarelli, B.*, *Allan, D. L.*, *Vance, C. P.* (2005): Signaling of phosphorus deficiency-induced gene expression in white lupin requires sugar and phloem transport. *Plant J.* 41, 257–268.

Müller, J., *Gödde, V.*, *Niehaus, K.*, *Zörb, C.* (2015): Metabolic adaptations of white lupin roots and shoots under phosphorus deficiency. *Front. Plant Sci.* 6. DOI: https://doi.org/10.3389/fpls.2015.01014.

Palmgren, M. G. (2001): Plant plasma membrane H^+-ATPases: Powerhouses for nutrient uptake. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 817–845.

Palmgren, M. G., *Christensen, G.* (1994): Functional comparisons between plant plasma membrane H^+-ATPase isoforms expressed in yeast. *J. Biol. Chem.*. 269, 3027–3033.

Pfaffl, M. W., *Horgan, G. W.*, *Dempfle, L.* (2002): Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 30. DOI: https://doi.org/10.1093/nar/30.9.e36.

Tomasi, N., *Kretzschmar, T.*, *Espen, L.*, *Weisskopf, L.*, *Fuglsang, A. T.*, *Palmgren, M.*, *Neumann, G.*, *Varanini, Z.*, *Pinton, R.*, *Martinoia, E.*, *Cesco, S.* (2009): Plasma membrane H^+-ATPase-dependent citrate exudation from cluster roots of phosphate-deficient white lupin. *Plant Cell Environ.* 32, 465–475.

Yan, F., *Zhu, Y.*, *Müller, C.*, *Zörb, C.*, *Schubert, S.* (2002): Adaptation of H^+-pumping and plasma membrane H^+-ATPase activity in proteoid roots of white lupin under phosphate deficiency. *Plant Physiol.* 129, 50–63.

Zhu, Y., *Yan, F.*, *Zörb, C.*, *Schubert, S.* (2005): A link between citrate and proton release by proteoid roots of white lupin (*Lupinus albus* L.) grown under phosphorus-deficient conditions? *Plant Cell Physiol.* 46, 892–901.

