PLASMA-MEMBRANE H^+-ATPase-DEPENDENT CITRATE EXUDATION FROM CLUSTER ROOTS OF PHOSPHATE-DEFICIENT WHITE LUPIN

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ABSTRACT

White lupin (*Lupinus albus* L.) is able to grow on soils with sparingly available phosphate (P) by producing specialized structures called cluster roots. To mobilize sparingly soluble P forms in soils, cluster roots release substantial amounts of carboxylates and concomitantly acidify the rhizosphere. The relationship between acidification and carboxylate exudation is still largely unknown. In the present work, we studied the linkage between organic acids (malate and citrate) and proton exudation in cluster roots of P-deficient white lupin. After the illumination started, citrate exudation increased transiently and reached a maximum after 5 hours. This effect was accompanied by a strong acidification of the external medium and alkalinization of the cytosol, as evidenced by *in vivo* NMR analysis. Fusicoccin, an activator of the plasma-membrane H^+-ATPase, stimulated citrate exudation, whereas vanadate, an inhibitor of the H^+-ATPase, reduced citrate exudation. The burst of citrate exudation was associated with an increase in expression of

the *LHA1* plasma-membrane H^+-ATPase gene, an increased amount of H^+-ATPase protein, a shift in pH optimum of the enzyme and post-translational modification of an H^+-ATPase protein involving binding of activating 14-3-3 protein. Taken together, our results indicate a close link in cluster roots of P-deficient white lupin between the burst of citrate exudation and plasma-membrane H^+-ATPase catalyzed proton efflux.

KEYWORDS:
Lupinus albus, organic acids, root exudates, malate, rhizosphere, proton pump, phosphorus, carboxylate release, pH

57 INTRODUCTION

58

59 The availability of phosphate (P) is a wide-spread limiting factor for plant growth (Handreck
60 1997; Marschner 1995). To deal with this situation, plants have developed several strategies to
61 improve P acquisition. The most common one is the association with mycorrhizal fungi. If plants
62 do not engage in symbiosis, they adapt to phosphate-poor soils by increasing the quantity and
63 length of roots and root hairs, and in many cases by increasing the synthesis and the release of P-
64 mobilizing root exudates, such as organic acids, phenolics, and acid phosphatases (Dinkelaker,
65 Römheld & Marschner 1989; Neumann et al. 2000; Tomasi et al. 2008).
66 Some plants have developed special root structures in response to phosphate starvation, such as
67 the dauciform roots found in the Cyperaceae family (Davies, Briarty & Rieley 1973; Lamont
68 1974; Shane, Dixon & Lambers 2005) and the cluster or proteoid roots of many Proteaceae
69 (Purnell 1960). Cluster roots or proteoid roots are generally bottlebrush-like root structures.
70 Many studies have demonstrated the capacity of these roots to increase the availability of soil
71 phosphate (Dinkelaker et al. 1989; Neumann et al. 1999; Shane et al. 2003). Phosphate
72 mobilization is suggested to occur predominantly via the release of large amounts of organic acid
73 anions by the cluster roots (Dinkelaker et al. 1989; Gardner, Parbery & Barber 1982; Jones &
74 Darrah 1994; Lambers et al. 2002).
75 White lupin (*Lupinus albus* L.) has often been used as a model plant for studying P-deficiency
76 response and cluster-root formation (Neumann & Martinoia 2002). In cluster roots of white
77 lupin, carboxylate release follows a spatial and temporal pattern. In the first growing phase,
78 cluster roots release low amounts of organic acids, mainly malate. In mature proteoid roots, a
79 burst of citrate exudation can be observed which is accompanied by acidification of the

rhizosphere (Neumann et al., 2000). Citrate is likely to be exuded via an anion channel, since exudation can be inhibited by anthracene-9-carboxylic acid (Neumann et al. 1999). In a more recent study, a citrate-specific channel from lupin root protoplasts was described (Zhang, Ryan & Tyerman 2004) which could be responsible for massive citrate exudation; however, regulatory mechanisms of carboxylate exudation still need to be elucidated. A correlation between citrate and proton extrusion has been suggested by several authors (Ligaba et al. 2004; Ohno, Koyama & Hara 2003; Sas, Rengel & Tang 2001; Shen et al. 2005; Zhu et al. 2005); however, in these studies different or even contrasting results were obtained, possibly due to the use of different experimental approaches and culture conditions.

The involvement of the plasma-membrane (PM) H^+-ATPase in organic anion release via proton extrusion in white lupin was suggested by Kania et al. (2001) and Yan et al. (2002). Using isolated plasma-membrane vesicles from whole proteoid roots and lateral roots grown in the presence or absence of phosphate, these authors showed that proteoid roots exhibited an increased PM H^+-ATPase activity that was correlated to changes in the amount of the corresponding protein. Ligaba et al. (2004) also observed an enhanced release of citrate, but not of malate, associated with the increased activity of the plasma-membrane proton pump in cluster roots of P-deficient *Lupinus pilosus*. Recently Shen et al. (2005), who investigated citrate exudation in aluminum-stressed soybean, demonstrated that citrate exudation was coupled to changes in the activity of the PM H^+-ATPase, involving transcriptional and post-translational modification of the enzyme. Furthermore it has been reported that release of carboxylates from cluster roots of P-deficient white lupin may show diurnal variations (Watt & Evans 1999).

The goal of this work was to study the linkage between organic acids (malate and citrate) and proton exudation in fully grown cluster roots of P-deficient white lupin, highlighting the involvement of PM H^+-ATPase activity and the regulatory aspects.

MATERIAL AND METHODS

Plant material and growth conditions

White lupin plants (*Lupinus albus* L. cv. Amiga, Südwestdeutsche Saatzucht, Rastatt, Germany) were grown in hydroponic conditions as described by Massonneau et al. (2001), with the exception that 48 plants were grown in 50-liter containers. Seeds were soaked overnight in aerated water, and then kept for 3 days in the dark followed by one day in the light, on filter paper soaked in 0.2 mM $CaCl_2$ to allow them to germinate. Seedlings were transferred to a hydroponic culture medium (0.05 mM Fe(III)-EDTA, 2.5 mM $Ca(NO_3)_2$, 0.9 mM K_2SO_4, 0.8 mM $MgSO_4$, 38 µM H_3BO_3, 12.5 µM $MnSO_4$, 1.25 µM $CuSO_4$, 1.25 µM $ZnSO_4$, 0.33 µM $(NH_4)_6Mo_7O_{24}$, 62.5 µM KCl and with 0.25 mM KH_2PO_4 in case of P-sufficient condition). Plants were grown at 22°C and 65% relative humidity with a light period of 16 h at 200 µmol $m^{-2}s^{-1}$ for 5 weeks. For most of the experiments, the root samples were collected from P-deficient white lupins at different times after the start of the light (HALS). Only the fully grown parts of cluster root were collected (see also Fig. 1), corresponding to the immature and mature parts described by Massonneau et al. (2001). For the measurement of the cytosolic and vacuolar pH under –P and +P condition, we harvested the apical part (the last centimeter) of lateral roots.

Quantification of citrate and malate released by cluster roots

Collection of root exudates was performed according to Massonneau et al. (2001). Fully grown cluster roots were collected, pre-incubated in 10 mM $CaSO_4$ and incubated in 10 mM $CaSO_4$ in 2-(N-morpholino) ethanesulfonic acid and 1,3-bis[tris(hydroxymethyl)-methyloamino] (MES-BTP) 15 mM pH 6 in the absence or presence of 10 µM fusicoccin or 500 µM vanadate for one hour at room temperature with agitation. Root weight was determined and roots were used for the detection of root-induced change of pH (see below). Sample solutions were frozen at –80°C until processing. Citrate or malate content in the exudates was determined using the kit for citric acid or malic acid test (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. Each dataset represents the mean of 3 independent experiments run in triplicates, processed and analyzed statistically (t-test, N=3, $P<0.05$) using Sigma Plot 8 (Systat software, Point Richmond, USA).

Detection of root-induced change of rhizospheric pH

The acidification capability of cluster roots was determined as described by Massonneau et al. (2001). After collection of the exudates, roots were placed on a glass plate covered by a 2-mm layer of 1% agar containing 0.04% (w/v) bromocresol purple at pH 6.0. Roots, which strongly acidify the medium, induce a color shift of the pH indicator from purple to yellow, corresponding to a pH below 5.5. Images were captured after 30 minutes of incubation.

NMR measurements and P concentration

Roots were collected and equilibrated for 10 min in the perfusion medium (0.5 mM $CaSO_4$, 1 mM MES-BTP, pH 6.1). ^{31}P-NMR spectra were recorded on a standard broad-band 10-mm

147 probe on a Bruker AMX 600 spectrometer (Bruker Analytische Messtechnik GmbH,
148 Rheinstetten-Forchheim, Germany) equipped with Indy computer running XWIN-NMR version
149 2.6. *In vivo* experiments were carried out by packing excised root segments in a 10 mm diameter
150 NMR tube equipped with a perfusion system connected to a peristaltic pump in which the
151 aerated, thermo-regulated (26°C) medium [0.5 mM $CaSO_4$, 1 mM MES-BTP (pH 6.1)] flowed at
152 10 ml min^{-1}.
153 ^{31}P-NMR spectra were recorded at 242.9 MHz without lock, with a waltz-based broad-band
154 proton decoupling and a spectral window of 16 kHz. Chemical shifts were measured relative to
155 the signal from a glass capillary containing 33 mM MDP, which is at 18.5 ppm relative to the
156 signal from 85% H_3PO_4. The spectra were determined using a 90° pulse angle and fast
157 acquisition conditions (1^{-s} recycle time). Resonance assignments were performed according to
158 Roberts et al. (1980) and Kime et al. (1982). Intracellular pH values were calculated from the
159 chemical shift of the cytosolic and vacuolar Pi resonance after construction of a standard titration
160 curve (Roberts et al. 1981).
161
162 *Isolation of plasma membranes*
163 Plasma-membrane vesicles were isolated from fully grown cluster roots as following Santi et al.
164 (1995) and Fisher-Schliebs et al. (1994). Briefly, 5 g of roots were homogenized with a mortar
165 and pestle in a freshly prepared ice-cold extraction medium (250 mM sucrose, 2 mM $MgSO_4$, 2
166 mM ATP, 10% (v/v) glycerol, 10 mM glycerol-1-phosphate, 0.16% (w/v) BSA, 2 mM EGTA, 2
167 mM DTT, 5.7% (w/v) choline-iodide, 1 mM PMSF, 20 µg ml^{-1} chymostatin, 10 nM okadeic acid
168 and 25 mM MES-BTP pH 7.6). Four milliliters of medium per g fresh weight of root tissue were
169 used.

The homogenates were filtered through four layers of cheesecloth and the suspensions were subjected to differential centrifugation steps at 2°C: 1,500 *g* for 5 min (pellets discarded); 9,800 *g* for 20 min (pellets discarded); 83,400 *g* for 30 min (pellets recovered); and 83,400 *g* for a further 30 min. Microsomes, gently resuspended in 1.2 mL of homogenization medium, were loaded onto a discontinuous sucrose gradient made by layering 2 mL of sucrose solution (1.13 g cm^{-3}) onto a 3 mL sucrose (1.17 g cm^{-3}) cushion, and centrifuged at 107600g for 2 h. The sucrose solutions were prepared in 5 mM MES-BTP, pH 7.4, and contained all of the protectants present in the homogenization medium. Vesicles migrating to the 1.13/1.17 g cm^{-3} interface were collected, diluted with homogenization medium, and centrifuged at 122,400 *g* for 30 min. The pellets were resuspended in a medium containing 250 mM sucrose, 10 % (v/v) glycerol, 1 mM DTT, 50 µg mL^{-1} chymostatin, 10 nM okadeic acid and 2 mM MES-BTP pH 7.0, were immediately frozen in liquid nitrogen, and stored at -80°C until use.

Measurement of PM H^+-ATPase activity and membrane protein content

Plasma-membrane H^+-ATPase activity was measured at 38°C in a 0.6 mL reaction (50 mM MES-BTP pH 6.5 or pH 6.2 to 8.0 for the pH dependency assay, 5 mM $MgSO_4$, 100 mM KNO_3, 600 µM Na_2MoO_4, 1.5 mM NaN_3, 5 mM ATP-BTP pH 6.5, 0.01 % (w/v) Brij 58 (polyoxyethylene 20 cetyl ether), plus or minus 100 µM V_2O_5; the vanadate-dependent activity was 85±3%). The reaction was started by addition of membrane vesicles containing 0.5 µg of total protein; after 30 min, the reaction was stopped and color developed as previously described by Santi et al. (1995). Inorganic phosphate was quantified spectrophotometrically at 705 nm as described by Forbush (1983).

192 Protein content was determined as described by Bradford (1976), using BSA as standard, after
193 solubilizing membrane vesicles with 0.5 M NaOH (Gogstad & Krutnes 1982).
194
195 *Western blots*
196 Equal amounts of protein isolated at the different time points were loaded, electrophoresed in an
197 8% w/v SDS-PAGE gel and transferred to a Protran BA 83 nylon membrane (0.2 µm, Biorad,
198 Hercules, USA) with a semi-dry transfer system (Trans-blot SD, Biorad, Hercules, USA). For the
199 PM H^+-ATPase blot, a polyclonal antibody against the C-terminal part of the *Arabidopsis*
200 *thaliana* AHA3 plasma-membrane H^+-ATPase. The 14-3-3-protein blot was performed using an
201 antiserum against barley 14-3-3, which was kindly provided by David B. Collinge (Royal
202 Veterinary and Agricultural University, Frederiksberg C, Denmark). Secondary antibodies (Goat
203 anti-rabbit IgG alkaline phosphatase conjugate, Biorad, Hercules, USA) were used and the
204 immunodetection was performed using the standard BCIP/NBT protocol (Promega, Madison,
205 USA). Relative-intensity band quantifications were determined using ImageJ (1.40g;
206 http://rsb.info.nih.gov/ij/).
207
208 Gene expression analysis
209 At the harvesting times (0, 1, 3, 5, 7, 9 and 11 hours after the initiation of the light period), fully
210 grown cluster roots were collected, immediately frozen in liquid nitrogen and conserved until
211 further processing at -80°C. RNA extractions were performed using TRIzol® reagent (Invitrogen,
212 Carlsbad, USA) following manufacturer's instructions and contaminant genomic DNA was
213 removed with 10 U of DNase I (GE Healthcare, Munich, Germany). Total-RNA samples were
214 cleaned up using the phenol:chloroform protocol (Maniatis, Sambrook & Fritsch 1989). 1 µg of

total RNA (checked for quality and quantity using a spectrophotometer, followed by a migration in an agarose gel) of each sample was retrotranscribed using 1 pmol of Oligo $d(T)_{23}VN$ (New England Biolabs, Beverly, USA), 15 U Prime RNase Inhibitor (Eppendorf, Hamburg, Germany) and 10 U M-MulV RNase H^- for 1 hour at 42°C (Finnzymes, Helsinki, Finland) following the manufacturer's instruction. After RNA digestion with 1U RNase A (USB, Cleveland, USA) for 1 hour at 37°C, gene expression analyzes were performed by adding 0.1 µL of the cDNA to FluoCycleTM sybr green (20 µl final volume; Euroclone, Pero, Italy) in a DNA Engine Opticon Real-Time PCR Detection (Biorad, Hercules, USA). Primers used (Tm= 58°C) were the following: for *LHA1* gene (AY989893), CCATTCATTTCTCTTTTGGGATA and GAAGACAAAGCTCAATAACCAGAA, for *LHA2* gene (AY989895), GGAGACTGGCCGAAGACTT and CGGGAATTGAGGCAATACTC, for *LHA3* gene (AY989894), cagggcaattttccaaagaa and acctccagagcaaggcaata, and as housekeeping gene (polyubiquitin; DQ118117), GCACCCTAGCCGACTACAAC and CCGGTAAGGGTCTTGACAAA). Triplicates were performed on three independent experiments; analyses of real-time result were performed using Opticon Monitor 2 software (Biorad, Hercules, USA) and R (version 2.7.0; http://www.r-project.org/) with the qPCR package (version 1.1-4; http://www.dr-spiess.de/qpcR.html). Efficiencies of amplification were calculated following the authors' indications (Ritz & Spiess 2008): PCR efficiencies were 92.3 and 81.4% for *LHA1* and *LHA2* genes, respectively. Computation of the graphical representation and statistical validation (t-test) were performed using SigmaPlot 8.0 (Systat software, Point Richmond, USA), considering the differences in the PCR efficiency and setting up that *LHA1* gene expression at time 0 equal 1.

238

239 RESULTS

240

241 *Citrate, malate and proton exudation by cluster roots*

242 In order to get a better settlement of the diurnal variation of citrate and malate release, we
243 collected exudates released from fully grown white lupin cluster roots (Fig. 1), corresponding to
244 the immature and mature stages according to the nomenclature of Massonneau et al. (2001), over
245 a period of 11 hours after starting the light period (HALS). Figure 2A shows that citrate
246 exudation progressively increased from 1 HALS to reach a maximum at 5 HALS, where it had
247 approximately doubled. Thereafter, citrate exudation decreased to levels lower than those
248 recorded at the beginning of the light period. Exudation rates for malate (Fig 2B) were always
249 lower than those recorded for citrate and did not change significantly. These results indicate that
250 there is a differential regulation between citrate and malate exudations.

251 To investigate whether the enhanced citrate exudation was accompanied by changes in root
252 external medium acidification, fully grown cluster roots were placed on an agar gel containing
253 the pH indicator bromocresol purple that turns yellow in response to acidification. The time
254 dependence of agar acidification by cluster roots (Fig. 3) corresponded well with that of citrate
255 exudation (Fig. 2A). The basal citrate and malate release observed at the beginning or the end of
256 the light period (Fig. 2, A and B) was not accompanied by detectable acidification of the agar
257 medium (Fig. 3).

258 In order to verify whether the PM H^+-ATPase was involved in the development of a higher
259 citrate exudation rate, cluster roots were treated with fusicoccin, which permanently activates the
260 plasma-membrane proton pump (Johansson, Sommarin & Larsson 1993; Palmgren 1998).

Fusicoccin stimulated proton release from cluster roots (Fig. 3) and was accompanied by an increase in exudation of citrate, but not of malate (Fig. 2, A and B). The potency of fusicoccin was strongest in the beginning of the light period and negligible as citrate exudation peaked at 5 HALS (Fig. 2A and 4). These results indicate that activation of the proton pump induces citrate release, however at 5 HALS, citrate exudation cannot be stimulated further suggesting that the proton pump does not respond to fusicoccin, *i.e.* it is already operating at maximal activity.

Vanadate is a well known inhibitor of P-type ATPases, a family of ion pumps to which the PM H^+-ATPase belongs (Cocucci, Ballarin-Denti & Marrè 1980). Application of vanadate to white lupin cluster roots strongly limited medium acidification (Fig. 3). However, citrate release was only partially affected by the inhibitor (Fig. 4), the degree of inhibition being dependent on the time at which cluster roots were treated. At 1 HALS, citrate exudation was almost insensitive to vanadate (Fig. 4) whereas vanadate was able to completely inhibit the enhanced element of citrate release, reverting the exudation rate to the level measured at 1 HALS (Fig. 4). This strongly suggests an involvement of PM H^+-ATPase in the increased level of citrate release in cluster roots of P-deficient white lupin.

Time-course of cytosolic pH fluctuations

In order to verify whether the burst of citrate and proton release in fully grown cluster roots were reflected by changes in the cytosolic pH, an *in vivo* NMR spectroscopy approach was used. For this purpose, the chemical shift (δ) of phosphate (^{31}P) resonance was followed. Cytosolic and vacuolar pH values were calculated using a standard titration curve designed for a “classical” cellular composition (Roberts, Wadejardetzky & Jardetzky 1981; Spickett, Smirnoff & Ratcliffe

283 1992). Figure 5 illustrates typical NMR profiles measured on roots of P-deficient and P-
284 sufficient white lupin.
285 Table 1 shows that the cytosolic pH changed paralleling the release of protons and citrate (Fig
286 2A; Fig. 3). At the beginning of the light period, the cytosolic pH was 7.8 and subsequently
287 increased to reach a maximum of pH 8.2 after 5 hours where citrate exudation also peaked (Fig.
288 2A). This alkalinization could be a consequence of proton extrusion from the cytosol to the
289 external solution (Fig. 3). Following the maximum alkalization at 5 HALS, the cytosolic pH
290 decreased. Addition of fusicoccin similarly resulted in an alkalinization of the cytosol (Table 1)
291 as has also been observed previously (Espen et al. 2000).
292
293 *Activity, amount and regulation of the PM H^+-ATPase*
294 To confirm that the PM H^+-ATPase was activated in concomitancy with organic acid release,
295 plasma-membrane vesicles were isolated from fully grown cluster roots at different time points
296 and characterized their plasma-membrane H^+-ATPase activities (Fig. 6). Notably, the activity of
297 PM H^+-ATPase in vesicles changed during the light period and followed a pattern similar to that
298 observed for citrate and proton exudation (see Fig. 2A and 3). Thus, it progressively increased up
299 to 5 HALS and subsequently decreased down to a basal value at 11 HALS. These results clearly
300 indicate the concomitance of the citrate-exudation burst (Fig. 2A), the acidification of the root
301 external medium (Fig. 3) and the increase in the ATP hydrolytic activity of the PM H^+-ATPase
302 (Fig. 6).
303 In order to determine whether the enhancement of PM H^+-ATPase activity occurred at the
304 transcriptional and/or the post-transcriptional level we determined: i) the expression levels of
305 three PM H^+-ATPase genes isolated from white lupin roots; ii) the amount of PM H^+-ATPase

306 protein; iii) the amount of 14-3-3 bound to the plasma membranes; and iv) the pH-dependency of
307 the PM H^+-ATPase activity, which is a measure of its regulatory state (Palmgren 1998).
308 Transcriptional activity of PM H^+-ATPase genes was investigated by real-time RT-PCR analyses
309 using primers designed from gene sequences of three different H^+-ATPase isoforms known to be
310 present in white lupin: *LHA1* (AY989893), *LHA2* (AY989895) and *LHA3* (AY989894). PM H^+-
311 ATPase genes showed different expression patterns during the light period (Fig. 7). A distinct
312 peak in transcript abundance was observed for *LHA1* at 3 HALS; *LHA2* was expressed at a lower
313 level as compared to *LHA1*, although showing maximal transcript abundance at 7 HALS.
314 Expression of *LHA3* was not detectable over the whole experimental period (data not shown).
315 In order to investigate whether changes in activity of PM H^+-ATPase could be explained by
316 changes in the amount of the corresponding protein, we performed Western blot analyses using
317 polyclonal antibodies targeted against the C-terminal domain of the enzyme (Fig. 8). Following
318 the onset of the light period, an increase in the amount of H^+-ATPase protein was indeed
319 observed, with a peak at 5 HALS; thereafter, the protein level progressively decreased. The
320 changes in PM H^+-ATPase protein levels followed the pattern observed for the ATP hydrolytic
321 activity of the pump (see Fig. 6).
322 14-3-3 protein is involved in the activation of plasma-membrane proton pump by binding to its
323 C-terminal domain (Johansson et al. 1993; Palmgren 1998). The possible occurrence of a post-
324 translational regulation of the plasma-membrane proton pump was analyzed by observing
325 changes in the amount of activating 14-3-3 protein bound to plasma membranes and by
326 analyzing the pH-dependency of the enzyme, which moves to a slightly more alkaline maximum
327 in response to post-translational activation (Palmgren 1998). Indeed, there was a significant
328 increase in the amount of 14-3-3 protein bound to plasma membranes during the first hours (Fig.

8). The pH optimum of PM H^+-ATPase activity was 6.5 at the beginning of the light period (Fig. 9), which is consistent with the optimum of the down-regulated enzyme (Regenberg et al. 1995). During the light period, concomitantly with the burst of citrate exudation, the pH optimum shifted to more alkaline values (6.8 and 7.0 at 3 and 5 HALS, respectively). Taken together, these results suggest that post-transcriptional regulation of the plasma-membrane proton pump had occurred at the time of the enhanced release of citrate.

DISCUSSION

Adaptation of white lupin to low-P soils is generally attributed to its capacity to form cluster roots, from which massive release of carboxylates (citrate and, to a lesser extent, malate) takes place (Gardner, Parbery & Barber 1981; Keerthisinghe et al. 1998). It has been shown that carboxylate release occurs according to a developmentally defined program (Massonneau et al. 2001) and may show diurnal variations, with higher rates being recorded during the light period than in the dark (Watt & Evans 1999).

In the present study we have defined in detail the time-course, during the illumination period, of citrate and malate release rates from immature and mature cluster roots of P-deficient white lupin (Fig. 1). The results show that the citrate exudation progressively increased from 1 to 5 hours after the start of the light period (HALS), then decreased to a minimum at 11 HALS (Fig. 2A). A similar pattern was reported by Hocking and Jeffery (2004) for *Lupinus luteus*. Our data also show that white lupin cluster roots are able to release malate, but interestingly at lower rates and

without showing any significant change during the considered period demonstrating a differential exudation pattern between citrate and malate (Fig. 2, A and B).

A strong acidification of the rhizosphere has been reported to occur concomitantly with the release of carboxylates (Neumann et al. 2000). Several studies have addressed the question whether a link exists between carboxylate and proton exudation. Due to the form (anionic) of carboxylic acids at cytosolic pH values (Ma et al. 2001) and the presence of citrate transport systems at the plasma membrane of white lupin (Zhang et al. 2004), protons are likely to be needed for energization and charge compensation during release of carboxylates. Increased proton release has also been related to the increased cation/anion influx ratio of P-deficient plants (Shen et al. 2005) and to the presence of the rhizobium-legume symbiosis (Sas et al. 2001). Activation of the PM H^+-ATPase has been suggested to be responsible for proton exudation associated with citrate export in white lupin (Kania et al. 2001; Yan et al. 2002). Recently Zhu et al. (2005), studying the effect of the PM H^+-ATPase effectors fusicoccin and vanadate on release of carboxylates (malate and citrate) and several ions (cations and anions) from cluster roots of P-deficient white lupin, concluded that proton extrusion may serve as charge balance for malate release, while sodium or potassium might act as counterions for citrate. Results here presented suggest that the counterions might change in function of how much citrate is released; *i.e.* it seems there are two different mechanisms of citrate release: one might be an anion channel as shown by Zhang et al. (2004) and another might be a multidrug and toxic compound extrusion system (MATE), which transports citrate but not malate, as hypothesized by Furukawa et al. (2007).

In the present study we show that rhizosphere acidification by immature and mature proteoid roots is concomitant with the burst of citrate exudation (Fig. 3). As expected, proton release was

374 enhanced by fusicoccin treatment. Citrate extrusion could also be enhanced by fusicoccin, but
375 not when the rate of release was maximal (at 5 HALS, Fig. 2A and 4). This result indicates that
376 either the proton pump was already fully activated at 5 HALS or that a maximal citrate synthesis
377 or exudation activity has been reached at that time which could not be further increased by
378 activation of the PM H^+-ATPase in response to fusicoccin. After 11 hours of illumination,
379 fusicoccin had only a negligible effect on citrate release while it was still able to stimulate proton
380 efflux, suggesting that at this time the internal supply of citrate might have become limiting. On
381 the other hand, malate release was not affected by the fusicoccin treatment (Fig. 2B).Vanadate
382 virtually abolished rhizosphere acidification (Fig. 3); on the other hand the effect of the inhibitor
383 on citrate release was dependent on the time of application, with a decrease of about 50% being
384 observed at 5 HALS and only of 17% at 1 HALS (Fig. 4).
385 The marked variations in citrate and proton release from the cluster roots during the light period
386 could conceivably influence the cytosolic pH, therefore we performed time-course measurements
387 with NMR instrumentation (Fig. 5) and observed that during the burst of citrate and proton
388 exudation an alkalinization of the cytosol took place (Tab. 1), indicating that the cellular pH-stat
389 could not fully compensate for the release of protons, even if it is known that the capacity to
390 regulate pH should be increased in white lupin cluster roots; in fact, PEP carboxylase, an
391 enzyme, which is postulated to play an important role in pH regulation, was shown to be up-
392 regulated in these tissues (Johnson, Vance & Allan 1996). The lowest pH values were measured
393 concomitantly with a decrease in proton and citrate efflux (from 7 to 11 HALS). Fusicoccin,
394 applied at 1 HALS, also led to an increase in cytosolic pH (Tab. 1), as a consequence of PM H^+-
395 ATPase activation (Espen et al. 2000).

PM H^+-ATPase activity also changed during the light period (Fig. 6) closely paralleling citrate and proton extrusion; these modifications were consistent with variations in cytosolic pH. Data on the cytosolic pH and PM H^+-ATPase activity further highlight the close link between the enhanced exudation of proton and citrate in cluster roots of P-deficient white lupin. Modulation of PM H^+-ATPase activity have been reported to be due either to altered gene expression or to post-translational modifications (Michelet et al. 1994; Palmgren 1998; Shen et al. 2005). The latter type of regulation is achieved by phosphorylation of the C-terminal auto-inhibitory domain of the PM H^+-ATPase which enables binding of activating 14-3-3 protein (Fuglsang et al. 1999; Olsson et al. 1998). In our work changes in PM H^+-ATPase activity (Fig. 6) strictly concomitant with the burst of citrate exudation (Fig. 2A) were paralleled by similar changes in protein amount (Fig. 8); furthermore increased transcript levels for at least one of the genes encoding the three known enzyme isoforms could be recorded (Fig. 7). These data are consistent with a transcriptional regulation of the PM H^+-ATPase. On the other hand, the shift in pH optimum (Fig. 9) and the pattern of changes in 14-3-3 protein amount (Fig. 8) suggest the occurrence also of a post-translational regulation of the enzyme. Up-regulation of PM H^+-ATPase due to transcriptional and post-translational modifications has been reported for aluminum-stressed soybean root tips (Shen et al. 2005); furthermore, a transcriptional regulation of PM H^+-ATPase involving a specific isoform has also been demonstrated in cucumber plants exposed to Fe-deficiency (Santi et al., 2005).

The present study demonstrates for the first time the involvement of a two-component system in organic acid exudation in cluster roots of P-deficient white lupin: one, that sustains a basal rate of malate and citrate release and a second that sustains the burst of citrate exudation which is closely linked to active proton extrusion from the cytosol. Further, the results indicate that two

different mechanisms of organic acid exudation are operating. The first component mediates a basal rate of malate and citrate release corresponding to about 2 μmol of citrate and 1 μmol of malate per hour and gram of fresh weight (Fig. 2, A and B, Fig. 4), and is not directly linked to the PM H^+-ATPase activity. The second one corresponds to the burst of citrate release (which is about two fold higher than the basal citrate release) and is strictly PM H^+-ATPase activity dependent.

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REFERENCES

Bradford M.M. (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Cocucci M., Ballarin-Denti A. & Marrè M.T. (1980) Effects of orthovanadate on H^+ secretion, K^+ uptake, electric potential difference and membrane ATPase activities of higher plant tissues. *Plant Science Letters* **17**, 391-400.

Davies J., Briarty L.G. & Rieley J.O. (1973) Observations on swollen lateral roots of Cyperaceae. *New Phytologist* **72**, 167-174.

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 and Environment* **12**, 285-292.

Espen L., Dell'Orto M., De Nisi P. & Zocchi G. (2000) Metabolic responses in cucumber (*Cucumis sativus* L.) roots under Fe-deficiency: a P-31-nuclear magnetic resonance in-vivo study. *Planta* **210**, 985-992.

Fischer-Schliebs E., Varanini Z. & Lüttge U. (1994) Isolation of H^+-transport-competent plasma-membrane vesicles from corn roots by discontinuous sucrose gradient centrifugation - Effect of membrane protectant agents. *Journal of Plant Physiology* **144**, 505-512.

Forbush B. (1983) Assay of Na,K-Atpase in plasma-membrane preparations - Increasing the permeability of membrane-vesicles using sodium dodecyl-sulfate buffered with bovine serum-albumin. *Analytical Biochemistry* **128**, 159-163.

453 Fuglsang A.T., Visconti S., Drumm K., Jahn T., Stensballe A., Mattei B., Jensen O.N., Aducci P.
454 & Palmgren M.G. (1999) Binding of 14-3-3 protein to the plasma membrane H^+-ATPase AHA2
455 involves the three C-terminal residues Tyr(946)-Thr-Val and requires phosphorylation of
456 Thr(947). *Journal of Biological Chemistry* **274**, 36774-36780.

457 Furukawa J., Yamaji N., Wang H., Mitani N., Murata Y., Sato K., Katsuhara M., Takeda K. &
458 Ma J.F. (2007) An aluminum-activated citrate transporter in barley. *Plant and Cell Physiology*
459 **48**, 1081-1091.

460 Gardner W.K., Parbery D.G. & Barber D.A. (1981) Proteoid root morphology and function in
461 *Lupinus albus*. *Plant and Soil* **60**, 143-147.

462 Gardner W.K., Parbery D.G. & Barber D.A. (1982) The acquisition of phosphorus by *Lupinus*
463 *albus* L. I. Some characteristics of soil/root interface. *Plant and Soil* **68**, 19-32.

464 Gogstad G.O. & Krutnes M.B. (1982) measurement of protein in cell-suspensions using the
465 coomassie brilliant blue dye-binding assay. *Analytical Biochemistry* **126**, 355-359.

466 Handreck K.A. (1997) Phosphorus requirements of Australian native plants. *Australian Journal*
467 *of Soil Research* **35**, 241-289.

468 Hocking P.J. & Jeffery S. (2004) Cluster-root production and organic anion exudation in a group
469 of old-world lupins and a new-world lupin. *Plant and Soil* **258**, 135-150.

470 Johansson F., Sommarin M. & Larsson C. (1993) Fusicoccin activates the plasma membrane H^+-
471 ATPase by a mechanism involving the c-terminal inhibitory domain. *The Plant Cell* **5**, 321-327.

472 Johnson J.F., Vance C.P. & Allan D.L. (1996) Phosphorus deficiency in Lupinus albus. Altered
473 lateral root development and enhanced expression of phosphoenolpyruvate carboxylase. *Plant*
474 *Physiology* **112**, 31-41.

475 Jones D.L. & Darrah P. (1994) Role of root derived organic acid in the mobilization of nutrients
476 from the rhizosphere. *Plant and Soil* **166**, 247-257.

477 Kania,A.; Neumann,G.; Cesco,S.; Pinton,Roberto; Römheld,V. (2001) Use of plasma membrane
478 vesicles for examination of phosphorus deficiency-induced root excretion of citrate in cluster
479 roots of white lupin (*Lupinus albus* L.). In: Plant Nutrition: Food security and sustainability of
480 agro-ecosystems through basic and applied research. XIV International Plant Nutrition
481 Colloquium pp. 546-547. Kluwer Academic Publishers, Dordrecht.

482 Keerthisinghe G., Hocking P.J., Ryan P.R. & Delhaize E. (1998) Effect of phosphorus supply on
483 the formation and function of proteoid roots of white lupin *(Lupinus albus* L.). *Plant Cell and*
484 *Environment* **21**, 467-478.

485 Kime M.J., Ratcliffe R.G., Williams R.J.P. & Loughman B.C. (1982) The application of ^{31}P
486 nuclear magnetic-resonance to higher plant tissue. *Journal of Experimental Botany* **33**, 656-669.

487 Lambers H., Juniper D., Cawthray G.R., Veneklaas E.J. & Martinez-Ferri E. (2002) The pattern
488 of carboxylate exudation in *Banksia grandis* (Proteaceae) is affected by the form of phosphate
489 added to the soil. *Plant and Soil* **238**, 111-122.

490 Lamont B. (1974) Biology of dauciform roots in sedge *Cyathochaete-Avenacea*. *New Phytologist*
491 **73**, 985-996.

492 Ligaba A., Yamaguchi M., Shen H., Sasaki T., Yamamoto Y. & Matsumoto H. (2004)
493 Phosphorus deficiency enhances plasma membrane H^+-ATPase activity and citrate exudation in
494 greater purple lupin (*Lupinus pilosus*). *Functional Plant Biology* **31**, 1075-1083.

495 Ma Y., Jakowitsch J., Maier T.L., Bayer M.G., Muller N.E., Schenk H.E.A. & Loffelhardt W.
496 (2001) ATP citrate lyase in the glaucocystophyte alga *Cyanophora paradoxa* is a cytosolic
497 enzyme: characterisation of the gene for the large subunit at the cDNA and genomic levels.
498 *Molecular Genetics and Genomics* **266**, 231-238.

499 Maniatis T., Sambrook,J. & Fritsch,E.F. (1989) Molecular Cloning: A laboratory manual, Cold
500 Spring Harbor Laboratory Press, New York.

501 Marschner H. (1995) Mineral nutrition of higher plants, Academic Press, London.

502 Massonneau A., Langlade N., Leon S., Smutny J., Vogt E., Neumann G. & Martinoia E. (2001)
503 Metabolic changes associated with cluster root development in white lupin (*Lupinus albus* L.):
504 relationship between organic acid excretion, sucrose metabolism and energy status. *Planta* **213**,
505 534-542.

506 Michelet B., Lukaszewicz M., Dupriez V. & Boutry M. (1994) A plant plasma-membrane
507 proton-atpase gene is regulated by development and environment and shows signs of a
508 translational regulation. *Plant Cell* **6**, 1375-1389.

509 Neumann G. & Martinoia E. (2002) Cluster roots - an underground adaptation for survival in
510 extreme environments. *Trends in Plant Science* **7**, 162-167.

Neumann G., Massonneau A., Langlade N., Dinkelaker B., Hengeler C., Römheld V. & Martinoia E. (2000) Physiological aspect of cluster root function and development in phosphorus-deficient White Lupin (*Lupinus albus* L.). *Annals of Botany* **85**, 909-919.

Neumann G., Massonneau A., Martinoia E. & Römheld V. (1999) Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta* **208**, 373-382.

Ohno T., Koyama H. & Hara T. (2003) Characterization of citrate transport through the plasma membrane in a carrot mutant cell line with enhanced citrate excretion. *Plant and Cell Physiology* **44**, 156-162.

Olsson A., Svennelid F., Ek B., Sommarin M. & Larsson C. (1998) A phosphothreonine residue at the c-terminal end of the plasma membrane H^+-ATPase is protected by fusicoccin-induced 14-3-3 binding. *Plant Physiology* **118**, 551-555.

Palmgren M.G. (1998) Proton gradients and plant growth: Role of the plasma membrane H^+-ATPase. *Advances in Botanical Research, Vol 28* **28**, 1-70.

Purnell H.M. (1960) Studies of the family Proteaceae. Anatomy and morphology of the roots of some Victorian species. *Australian Journal of Botany* **8**, 38-50.

Regenberg B., Villalba J.M., Lanfermeijer F.C. & Palmgren M.G. (1995) C-terminal deletion analysis of plant plasma membrane H^+-ATPase: Yeast as a model system for solute transport across the plant plasma membrane. *The Plant Cell* **7**, 1655-1666.

Ritz C. & Spiess A.N. (2008) qpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis. *Bioinformatics* **24**, 1549-1551.

Roberts J.K.M., Ray P.M., Wadejardetzky N. & Jardetzky O. (1980) Estimation of cytoplasmic and vacuolar pH in higher-plant cells by P-31 NMR. *Nature* **283**, 870-872.

Roberts J.K.M., Wadejardetzky N. & Jardetzky O. (1981) Intracellular pH measurements by P-31 nuclear magnetic-resonance influence of factors other than pH on P-31 chemical-shifts. *Biochemistry* **20**, 5389-5394.

Santi S., Locci G., Pinton R., Cesco S. & Varanini Z. (1995) Plasma membrane H^+-ATPase in maize roots induced for $NO3^-$ Uptake. *Plant Physiology* **109**, 1277-1283.

Sas L., Rengel Z. & Tang C. (2001) Excess cation uptake, and extrusion of protons and organic acid anions by *Lupinus albus* under phosphorus deficiency. *Plant science* **160**, 1191-1198.

Shane M.W., De Vos M., De Roock S. & Lambers H. (2003) Shoot P status regulates cluster-root growth and citrate exudation in *Lupinus albus* grown with a divided root system. *Plant Cell and Environment* **26**, 265-273.

Shane M.W., Dixon K.W. & Lambers H. (2005) The occurrence of dauciform roots amongst Western Australian reeds, rushes and sedges, and the impact of phosphorus supply on dauciform-root development in *Schoenus unispiculatus* (Cyperaceae). *New Phytologist* **165**, 887-898.

Shen H., He L.F., Sasaki T., Yamamoto Y., Zheng S.J., Ligaba A., Yan X.L., Ahn S.J., Yamaguchi M., Sasakawa H. & Matsumoto H. (2005) Citrate secretion coupled with the modulation of soybean root tip under aluminum stress. Up-regulation of transcription, translation, and threonine-oriented phosphorylation of plasma membrane H^+-ATPase. *Plant Physiology* **138**, 287-296.

551 Spickett C.M., Smirnoff N. & Ratcliffe R.G. (1992) Metabolic response of maize roots to
552 hyperosmotic shock : An *in vivo* ^{31}P nuclear magnetic resonance study. *Plant Physiology* **99**,
553 856-863.

554 Tomasi N., Weisskopf L., Renella G., Landi L., Pinton R., Varanini Z., Nannipieri P., Torrent J.,
555 Martinoia E. & Cesco S. (2008) Flavonoids of white lupin roots participate in phosphorus
556 mobilization from soil. *Soil Biology and Biochemistry* **40**, 1971-1974.

557 Watt M. & Evans J.R. (1999) Linking development and determinacy with organic acid efflux
558 from proteoid roots of white lupin grown with low phosphorus and ambient or elevated
559 atmospheric CO_2 concentration. *Plant Physiology* **120**, 705-716.

560 Yan F., Zhu Y., Muller C., Zorb C. & Schubert S. (2002) Adaptation of H^+-pumping and plasma
561 membrane H^+-ATPase activity in proteoid roots of white lupin under phosphate deficiency. *Plant*
562 *Physiology* **129**, 50-63.

563 Zhang W.H., Ryan P.R. & Tyerman S.D. (2004) Citrate-permeable channels in the plasma
564 membrane of cluster roots from white lupin. *Plant Physiology* **136**, 3771-3783.

565 Zhu Y., Yan F., Zorb C. & Schubert S. (2005) A link between citrate and proton release by
566 proteoid roots of white lupin (*Lupinus albus* L.) grown under phosphorus-deficient conditions?
567 *Plant and Cell Physiology* **46**, 892-901.
568

TABLES

Table I. Variations of cytosolic and vacuolar pH of cluster-root cells during the light period. Cluster roots were collected from cluster roots of 5-week-old P-deficient white lupin at different times after the start of the light period. Cytosolic and vacuolar pH values were derived from the chemical shift (δ) of cellular phosphate resonance after construction of a standard titration curve with or without 10 µM fusicoccin (FC), using ^{31}P-NMR in *in vivo* experiments. Values are the means of two independent experiments. Data are presented as differences from the cytosolic pH value determined at 1 HALS which was 7.78. The accuracy is calculated as three times the maximum variation observed in the experiments and is equal to ±0.02 pH units for the cytoplasm and ±0.04 pH units for the vacuole. nd stand for not detectable.

Hours after light start (HALS)	**1**	**1+FC**	**3**	**5**	**7**	**9**	**11**
Δcytosolic pH	-	0.68	0.13	0.42	-0.07	-0.46	-0.36
Δvacuolar pH	-2.51	-2.38	nd	nd	nd	nd	nd

FIGURE LEGENDS

Figure 1. Schematic view of a white lupin plant grown for 5-week in a P-deficient nutrient solution and a detailed cluster root. This figure represent typical cluster root and whole plant of white lupin grown for 5-week in nutrient solution without any external supply of phosphate. Moreover, the different parts of the cluster roots are indicated following the nomenclature of Massonneau et al. (2001). In this work, immature and mature parts were collected together and named fully grown cluster root.

Figure 2. Citrate (A) and malate (B) secretion from cluster roots during the light period. Fully grown cluster roots were harvested from 5-week-old P-deficient white lupin at different times after the start of the light period. Exudates were collected after bathing roots for one hour in 10 mM $CaSO_4$, 15 mM MES-BTP pH 6 with or without 10 µM fusicoccin. Means ± SD of three independent experiments are reported. Capital letters (A, B) refer to statistically significant differences (t-test, N=3, $P<0.05$) in organic acid secretion of control roots (- fusicoccin). Asterisks indicate a statistically significant (t-test, N=3, $P<0.05$) effect of fusicoccin treatment.

Figure 3. Acidification capacity of cluster roots during the light period. Fully grown cluster roots were harvested from 5-week-old P-deficient white lupin at different times after the start of the light period. After incubation for 1 hour in 10 mM $CaSO_4$, 15 mM MES-BTP pH 6 in the absence or presence of 10 µM fusicoccin or 500 µM vanadate, roots were placed for 30 min on an agar gel containing the pH indicator bromocresol purple and adjusted to pH 6. Yellow and purple colors indicate pH values respectively below and above 6.

Figure 4. Effect of vanadate and fusicoccin on citrate secretion. Fully grown cluster roots were harvested from 5-week-old P-deficient white lupin at 1 and 5 hours after the start of the light period (HALS). Exudates were collected after bathing roots for one hour in 10 mM $CaSO_4$, 15 mM MES-BTP pH 6 in the absence or presence of 10 µM fusicoccin or 500 µM vanadate. Means + SD of three independent experiments, are reported; relative values in comparison with the untreated control at 1 HALS are also shown. Capital letters (A, B, C) refer to statistically significant differences (t-test, N=3, $P<0.05$).

Figure 5. *In vivo* ^{31}P-NMRspectra of white lupin cluster roots. Apical segments of lateral roots (the last centimeter) were harvested from plants grown for 5 weeks in the absence (A) or presence (B) of Pi. Each spectrum was recorded using fast acquisition conditions with a recycle time of 1 s and represents the sum of 2,700 scans. The numbered peaks correspond to: 1) cytosolic phosphate; 2) vacuolar phosphate. The region including cytosolic phosphate is shown also on an expanded scale (4x). The chemical shift of the cytosolic phosphate peaks, reflecting the differences in pH values, is highlighted by a vertical dotted line.

Figure 6. Plasma-membrane H^+-ATPase activity in cluster roots during the light period. Plasma-membrane (PM) vesicles were isolated from cluster roots of 5-week-old P-deficient white lupin at different times after the start of the light period. The enzyme activity was measured at pH 6.5. Data are means ± SD of two independent experiments.

Figure 7. Expression analyses of genes coding for different PM H^+-ATPase isoforms of cluster roots during the light period. Total RNA was extracted from cluster roots of 5-week-old P-deficient white lupin at different times after the start of the light period. Relative expression of *LHA1* and *LHA2* genes coding for different PM H^+-ATPase isoforms of white lupin was analyzed by real-time RT-PCR. Data are means ± SD of three independent experiments. Changes in gene expression were calculated on the basis of expression levels of *LHA1* at 1 hour after the start of the light period (HALS); relative expression of *LHA2* gene was also illustrated in the insert with a higher magnification. Asterisks indicate a statistically significant (t-test, N=3, $P<0.05$) difference in expression level of each gene with respect to its expression at 1 HALS.

Figure 8. Western-blot analysis of the PM H^+-ATPase and 14-3-3 proteins in cluster roots during the light period. Purified plasma membranes isolated from cluster roots of 5-week-old P-deficient white lupin at different times after the start of the light period were used for immunodetection of PM H^+-ATPase using antibodies raised against the C-terminal part of the *Arabidopsis* AHA3 PM H^+-ATPase and of 14-3-3-protein using antibodies raised against the barley 14-3-3 protein. Quantification of western-blot signals is expressed as percentage of that recorded at 1 hour after the start of the light period (HALS). Data of a representative experiment are reported.

Figure 9. pH dependency of PM H^+-ATPase activity of cluster roots during the light period. Plasma-membrane (PM) vesicles were isolated from cluster roots of 5-week-old P-deficient white lupin at 1, 3, 5, 9 hours after the start of the light period (HALS). The enzyme activity was measured at different pH values in the range between 6.2 and 8.0. Data are means ± SD of two independent experiments.

Figure 1.

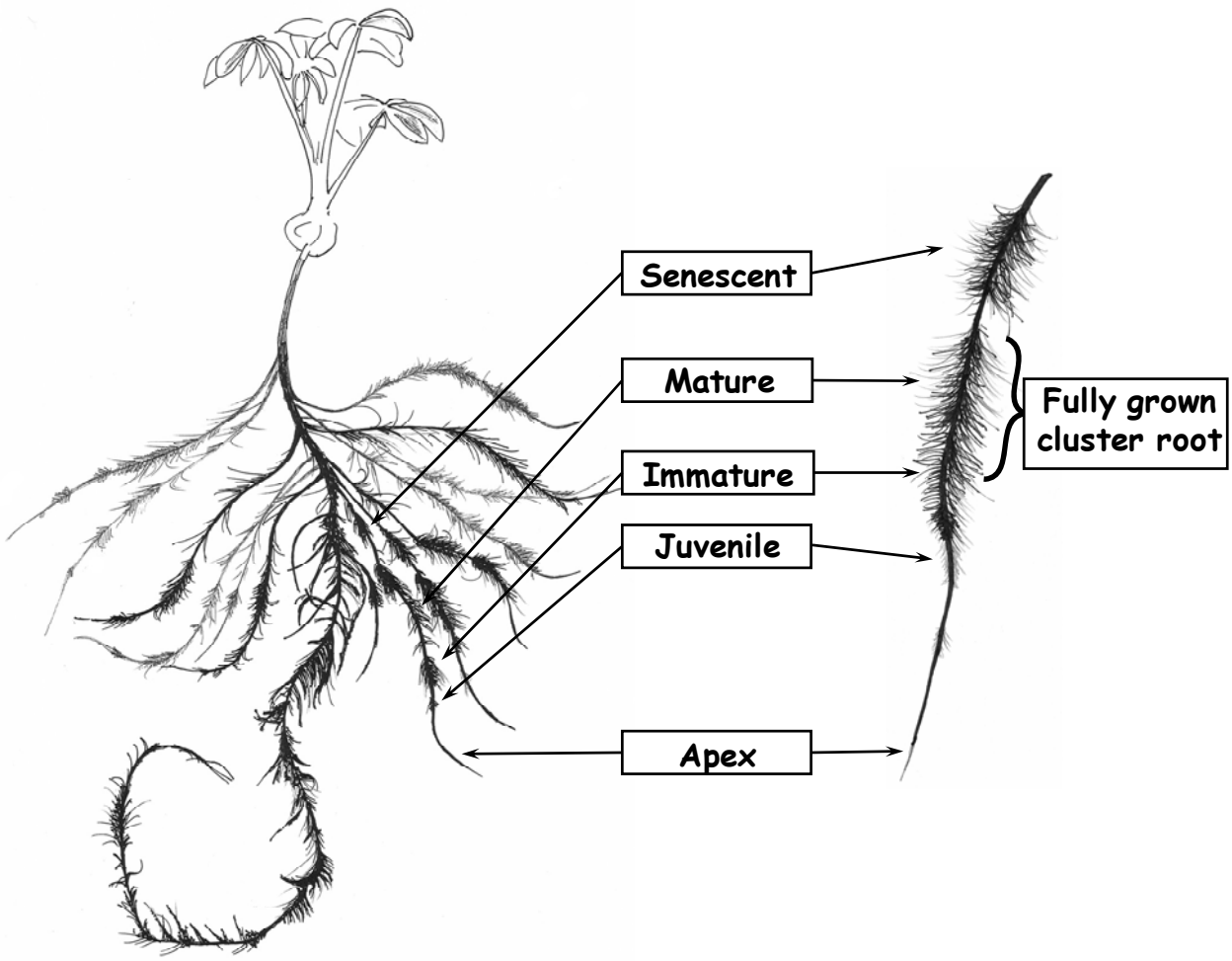

Senescent
Mature
Immature
Juvenile
Apex
Fully grown cluster root

Figure 2.

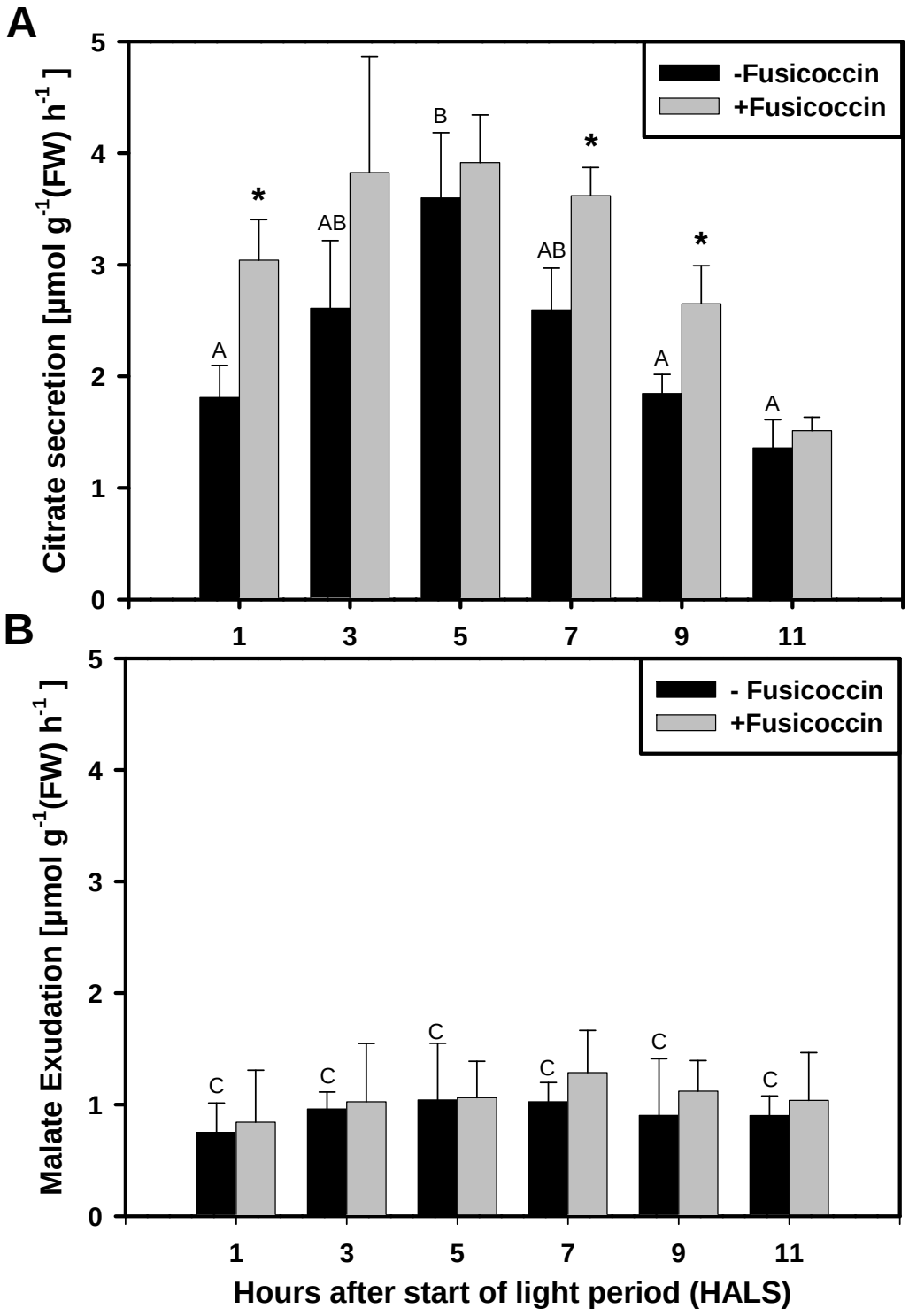

A
Citrate secretion [µmol g-1(FW) h-1]
-Fusicoccin
+Fusicoccin
A
AB
B
AB
A
A
*
*
*
0
1
2
3
4
5
1
3
5
7
9
11
B
Malate Exudation [µmol g-1(FW) h-1]
- Fusicoccin
+Fusicoccin
C
C
C
C
C
C
0
1
2
3
4
5
1
3
5
7
9
11
Hours after start of light period (HALS)

Figure 3.

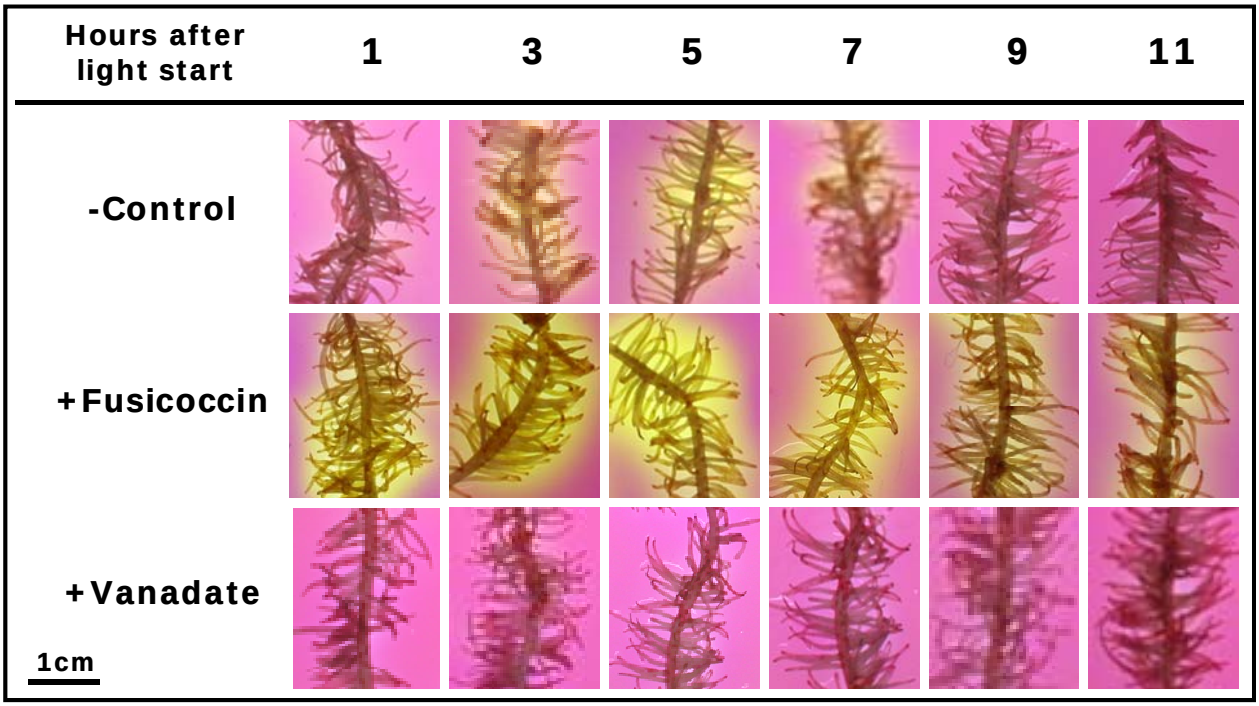

Hours after light start
1
3
5
7
9
11
-Control
+Fusicoccin
+Vanadate
1cm

Figure 4.

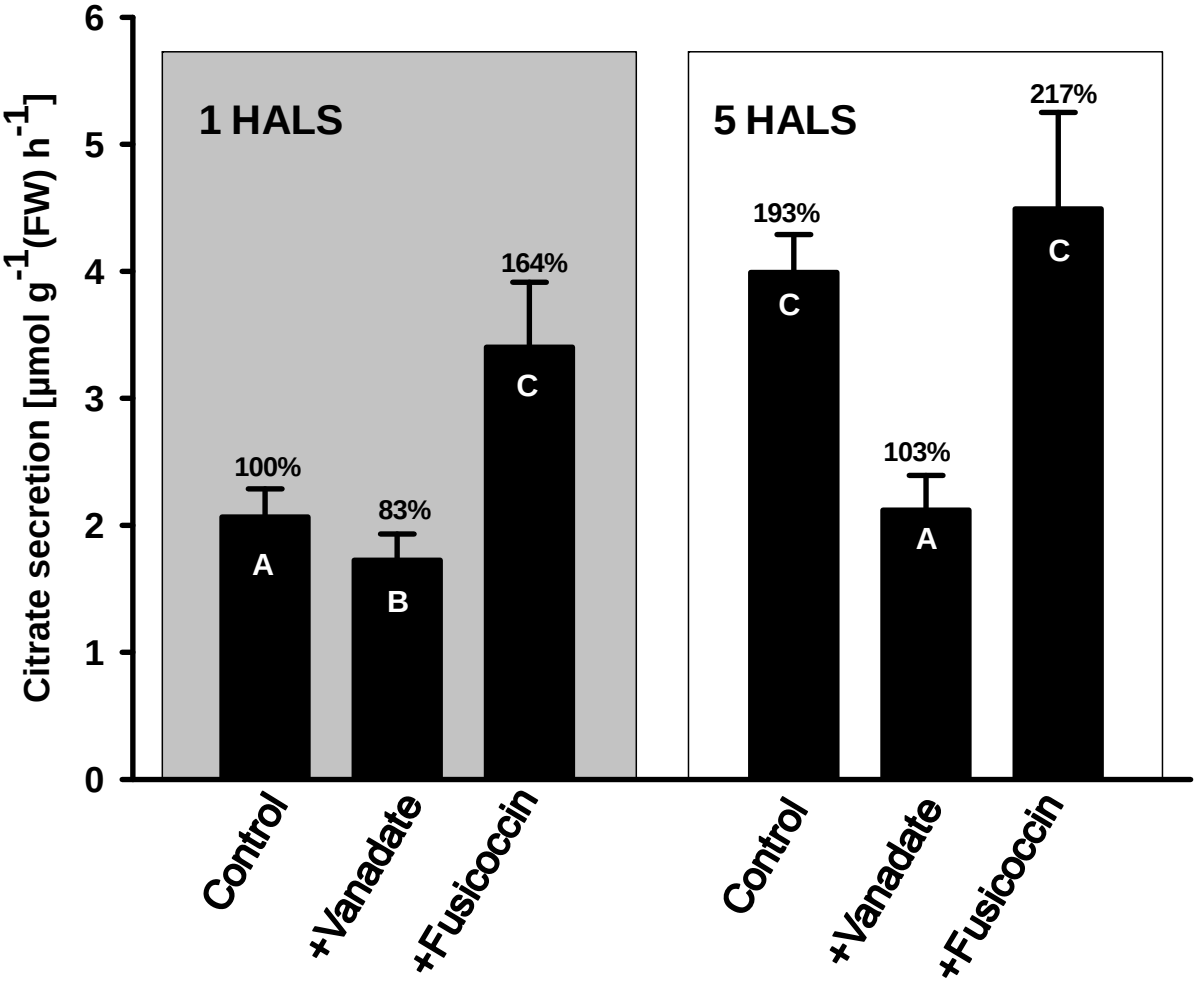

Citrate secretion [µmol g-1(FW) h-1]
0
1
2
3
4
5
6
1 HALS
5 HALS
100%
83%
164%
193%
103%
217%
A
B
C
C
A
C
Control
+Vanadate
+Fusicoccin
Control
+Vanadate
+Fusicoccin

Figure 5.

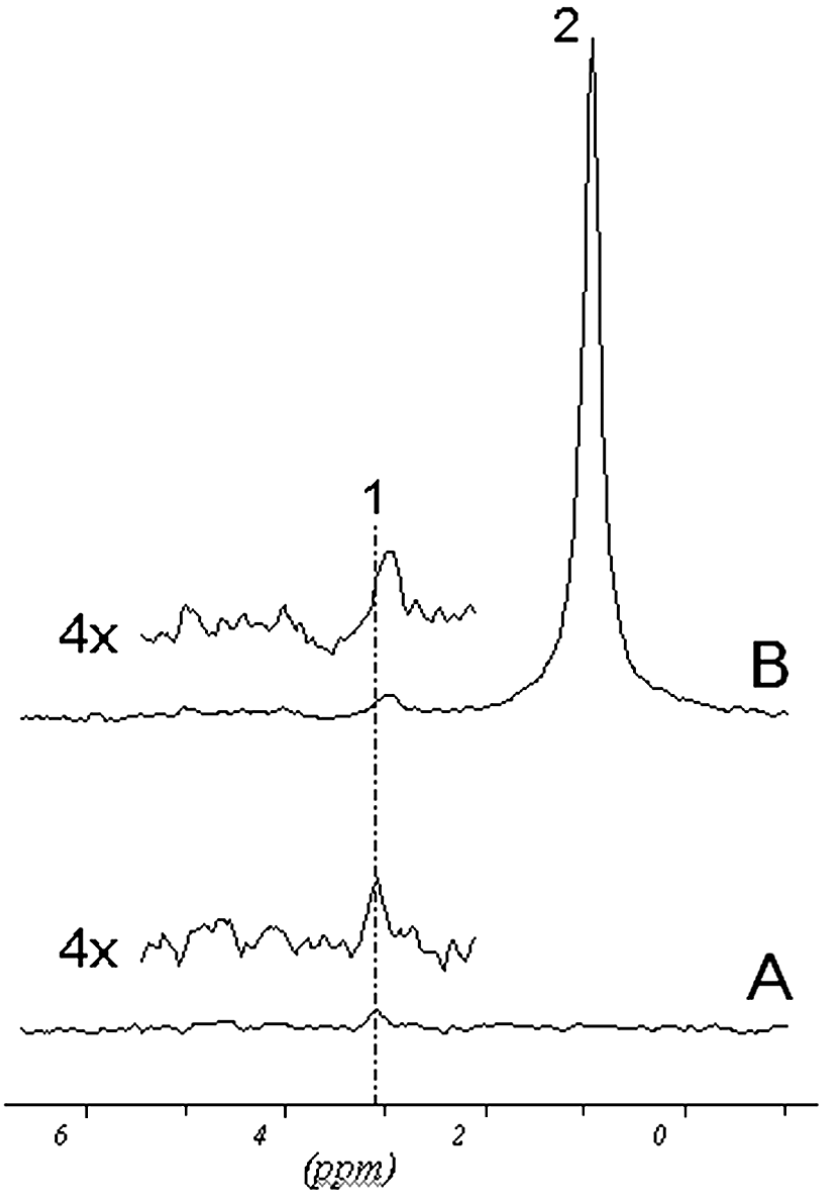


Figure 6.

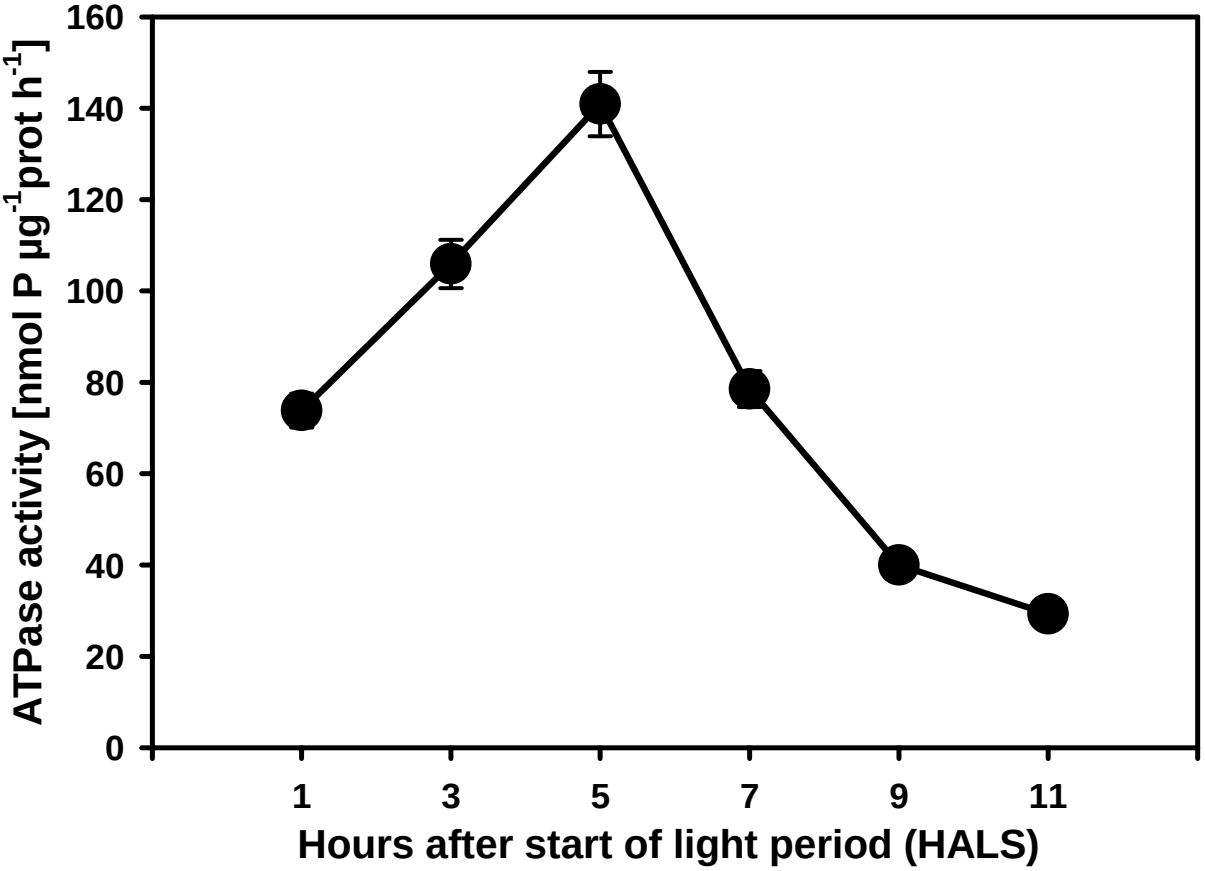

ATPase activity [nmol P µg-1prot h-1]
160
140
120
100
80
60
40
20
0
1
3
5
7
9
11
Hours after start of light period (HALS)

Figure 7.

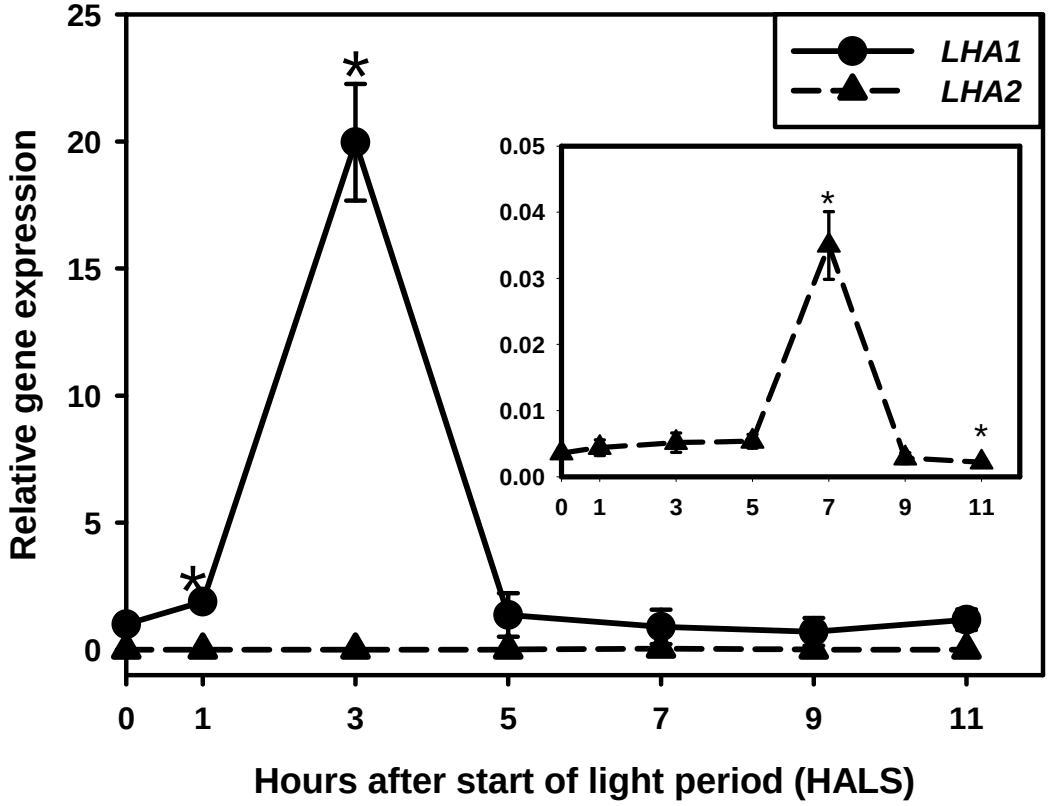

LHA1
LHA2
Relative gene expression
Hours after start of light period (HALS)
25
20
15
10
5
0
0 1 3 5 7 9 11
0.05
0.04
0.03
0.02
0.01
0.00
0 1 3 5 7 9 11
*
*
*
*

Figure 8.

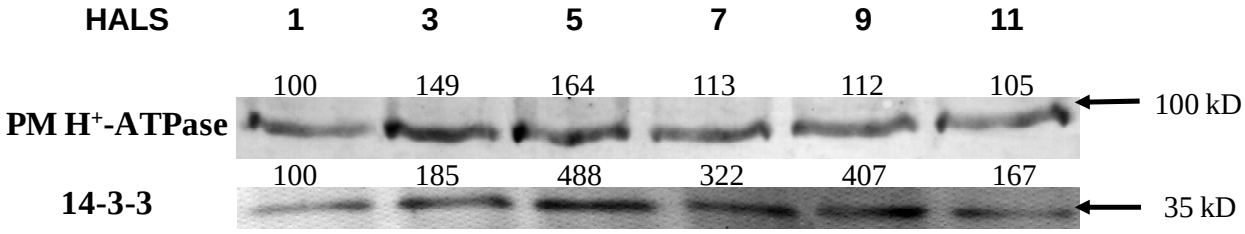
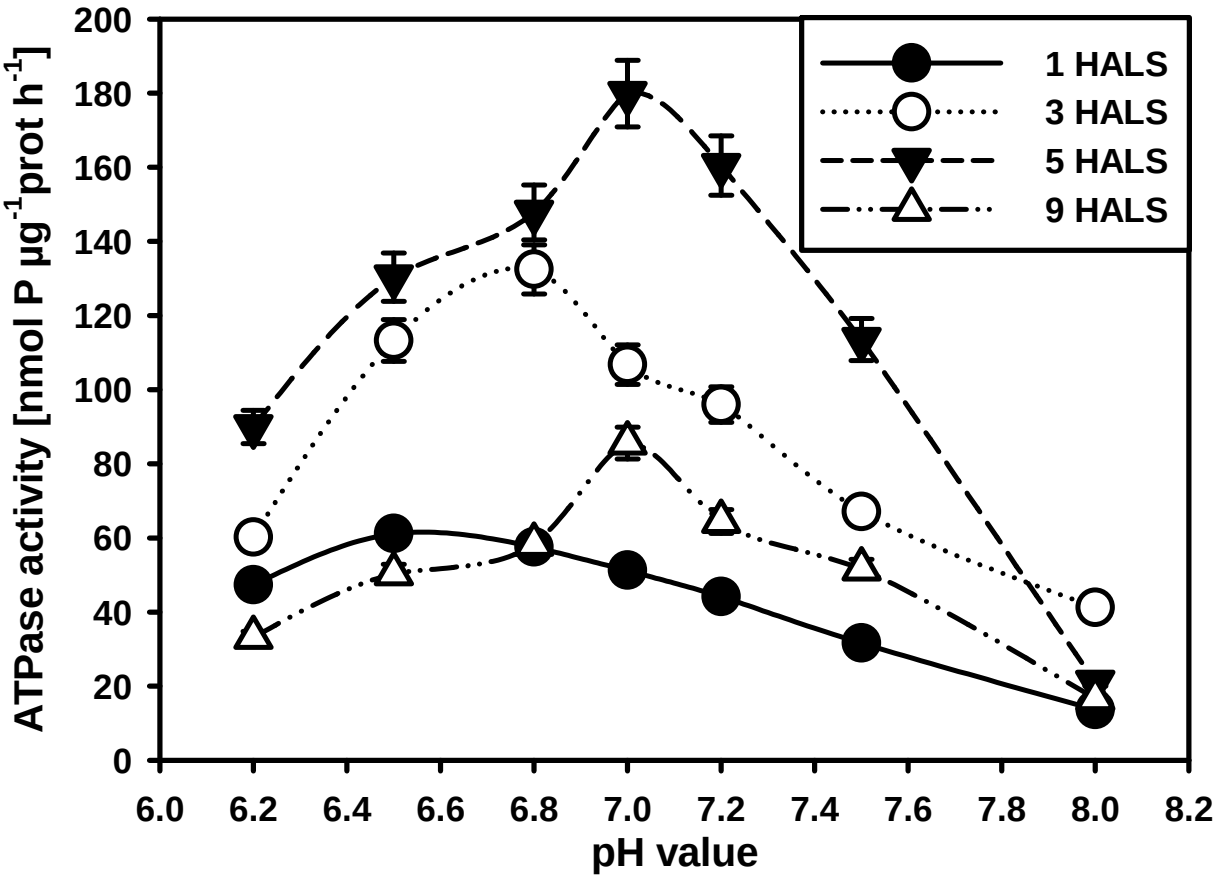

HALS
1
3
5
7
9
11
100
149
164
113
112
105
PM H+-ATPase
100 kD
100
185
488
322
407
167
14-3-3
35 kD

Figure 9.


ATPase activity [nmol P µg-1prot h-1]
pH value
1 HALS
3 HALS
5 HALS
9 HALS
0
20
40
60
80
100
120
140
160
180
200
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2

COVER PAGE

Effects of PM H^+-ATPase modulators on proton extrusion by cluster roots at the time of maximum exudation. Fully grown cluster roots were harvested from 5-week-old P-deficient white lupin at 5 hours after the start of the light period. After incubation for 1 hour in 10 mM $CaSO_4$, 15 mM MES-BTP (pH 6) in the absence or presence of 10 µM fusicoccin or 500 µM vanadate, roots were placed for 30 min on an agar gel containing the pH indicator bromocresol purple and adjusted to pH 6. Yellow color indicates pH values below 6.