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ORIGINAL PAPER

Acid phosphatases and growth of barley (*Hordeum vulgare* L.) cultivars under diverse phosphorus nutrition

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Abstract The morphological and physiological responses of barley to moderate Pi deficiency and the ability of barley to grow on phytate were investigated. Barley cultivars (*Hordeum vulgare* L., Promyk, Skald and Stratus) were grown for 1–3 weeks on different nutrient media with contrasting phosphorus source: KH_2PO_4 (control), phytic acid (PA) and without phosphate (−P). The growth on −P medium strongly decreased Pi concentration in the tissues; culture on PA medium generally had no effect on Pi level. Decreased content of Pi reduced shoot and root mass but root elongation was not affected; Pi deficit had slightly greater impact on growth of barley cv. Promyk than other varieties. Barley varieties cultured on PA medium showed similar growth to control. Extracellular acid phosphatase activities (APases) in −P roots were similar to control, but in PA plants were lower. Histochemical visualization indicated for high APases activity mainly in the vascular tissues of roots and in rhizodermis. Pi deficiency increased internal APase activities mainly in shoot of barley cv. Stratus and roots of cv Promyk; growth on PA medium had no effect or decreased APase activity. Protein extracts from roots and shoots were run on native discontinuous PAGE to determine which isoforms may be affected by Pi deficiency or growth on PA medium; two of four isoforms in roots were strongly induced by conditions of Pi deficit, especially in barley cv. Promyk. In conclusion, barley cultivars grew equally well both on medium with Pi and where the Pi was replaced with phytate and only slightly differed in terms of acclimation to moderate deficiency of phosphate; they generally used similar pools of acid phosphatases to acquire Pi from external or internal sources.

Keywords APase localization · Extracellular acid phosphatase · Intracellular APase · Phytate · Pi deficit · Root exudates

Abbreviations

APase	Acid phosphatase
PA	Phytate
PAGE	Polyacrylamide gel electrophoresis
Pi	Inorganic phosphate
−P	Plants cultured on medium without phosphate
+P	Control plants

Introduction

Availability of phosphorus in the soil strongly determines the plant growth, metabolism, development, reproduction and crop yield (Marschner 1995; Brinch-Pedersen et al. 2002; Hermans et al. 2006). Phosphorus is well known as an essential nutrient important in many processes like photosynthesis, respiration, regulation of enzymes activity and gene expression or signal transduction (Raghothama 1999; Ciereszko and Kleczkowski 2005; Ciereszko et al. 2005; Rychter and Rao 2005; Calderon-Vazquez et al. 2008; Nilsson et al. 2010; Yao et al. 2011). Phosphate deficiency is a common stress condition experienced in many different environments. Generally, only inorganic phosphate (Pi) is freely available to plants and it is absorbed by the roots from the soil solution. Other phosphorus-containing compounds in the soil are mainly insoluble and unavailable for plants, among them aluminum and ferrous phosphates or organic

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forms, like phytate (Marschner 1995; Richardson et al. 2000; Brinch-Pedersen et al. 2002). Plants could adapt to such stress conditions by developing a number of mechanisms increasing Pi uptake from soil and Pi mobilization or recycling from intercellular reservoirs. Acclimation responses of plants to Pi starvation that can increase availability, uptake and transport of orthophosphate are mainly exudation of organic acid to the ground, extracellular enzymes induction or secretion (e.g. acid phosphatases), or enhancement of synthesis of phosphate transporters (Duff et al. 1994; Raghothama 1999; Żebrowska and Ciereszko 2009; Yao et al. 2011). Enhanced organic acid exudation like citrate, malate or oxalate, could improve Pi uptake from the inorganic insoluble soil-P compounds; numerous plant species can release different organic acid anions from roots in response to Pi deficiency (Raghothama 1999; Vance et al. 2003; Gregory 2006; Yao et al. 2011).

Acid phosphatases (orthophosphoric monoester phosphohydrolases, APases, EC 3.1.3.2) hydrolyze different forms of organic phosphorus at low pH, thereby increasing Pi availability for plants. Extracellular APases, usually nonspecific enzymes, are involved in hydrolysis of various organic phosphate monoesters in the soil, whereas intracellular enzymes are important for the remobilization of Pi from rich phosphorus components inside plant cell (Duff et al. 1994; Olczak et al. 2003; Tran et al. 2010). APases expression is mediated by different environmental and developmental factors, including Pi deficit (Duff et al. 1994; Olczak et al. 2003). Under low Pi conditions the activities of extracellular and/or internal APases are often increasing; additionally, some plants might secrete nonspecific APase from roots to the rhizosphere (Duff et al. 1994; Gilbert et al. 1999; Yun and Kaeppler 2001; Wasaki et al. 2008). Specific acid phosphatases that exhibit phytasic activities (phosphohydrolases with the capability of initiating dephosphorylation of myoinositol-hexakisphosphate) are active not only mainly in germinating seeds but also in cells of primary roots (Asmar 1997; Richardson et al. 2000; Gregory 2006). Enhanced activities of various nonspecific APases or phytases under Pi-deficient conditions have been documented for many crop plants (Asmar 1997; Römer and Schenk 1998; Ciereszko et al. 2002; Ciereszko and Balwicka 2005; George et al. 2008). However, recent results by George et al. (2008) indicated that root-associated APases activities are rather a poor indicator of the plant growth and phosphorus nutrition under a natural conditions, when plants grown in soils. Additionally, some earlier studies showed negative relationship between APase activity and Pi uptake under phosphate deficiency conditions (Yan et al. 2001; Yun and Kaeppler 2001). Therefore, the physiological role of acid phosphatases in plant acclimation to low Pi stress is not yet fully understood and needs further investigation.

Plants may acclimate to low Pi nutrition also by adequate modifications in growth and different metabolic processes, most of them are preceded by activation or repression of specific genes (Hermans et al. 2006; Calderon-Vazquez et al. 2008; Nilsson et al. 2010). Especially an increase of the root/shoot mass ratio, as the result of shoot growth reduction and stimulation of root growth, is one of the most characteristic symptoms of Pi starvation. Induction of main root elongation or formation of lateral roots, root hairs, proteoid/cluster roots and remodeling of the root system architecture or aerenchyma formation are common plant reactions to limiting Pi conditions (Raghothama 1999; Gregory 2006; Svistoonoff et al. 2007). Additionally, some plants can activate colonization of roots by mycorrhizal fungi to enhance Pi uptake (Vance et al. 2003). All of these changes allow to increase the total root surface area and facilitate soil exploration and nutrient acquisition under low Pi conditions. Especially first weeks of growth are really important for the development of roots (and tillering) and thus might be strongly affected by Pi starvation (Gregory 2006; Hermans et al. 2006).

Metabolic responses to Pi deficit, alternative pathways or higher intensity of some processes could improve utilization of Pi internal pools. Phosphate deficiency may either decrease photosynthesis or respiration rate or has no effect on these processes, but alterations in carbon partitioning into starch or sucrose and assimilate transport were often observed (Ciereszko et al. 1996, 2005; Ciereszko and Kleczkowski 2005; Rychter and Rao 2005; Hermans et al. 2006). The Pi deficiency-dependent promotions of several reactions in the respiratory carbon metabolism or in photorespiration (and other pathways) were observed (Rychter and Rao 2005); many reactions might require a high activity of specific phosphatases to hydrolyze different phosphorylated substrates (e.g. phosphate esters of sugars).

Phosphorus is one of the most limiting elements for cultivated plants in agriculture, thus application of phosphorus-rich fertilizers is widely recommended for enhancing Pi availability. However, the main source for such fertilizers—inexpensive rock phosphate—probably will be soon depleted (Vance et al. 2003). Phytate, organic compound of phosphorus and known storage form of phosphate in seeds, is considered to be unavailable for plants but sometimes can provide a source of adequate Pi for sustained growth in phytate-rich environments (Asmar 1997; Richardson et al. 2000; Brinch-Pedersen et al. 2002; Gregory 2006). As the Pi sources are nonrenewable but organic phosphorus compounds are rather in excess, further investigations and selection of crop plant genotypes tolerant to depletion of soil minerals or/and able to grow well on e.g. phytate are very important in order to sustain crop productivity (Brinch-Pedersen et al. 2002; Shenoy and Kalagudi 2005; Hammond et al. 2009).

In this study, we have investigated the morphological and physiological responses of barley, a common grain crop, to early and moderate Pi deficiency and the ability of three barley genotypes to grow on phytate as their sole phosphate source. The estimation of variations in secretion, localization and activities of APases—important component of acclimatization mechanism to low Pi conditions—among new barley varieties is the main goal of our study.

Materials and methods

Plant material and culture

Three spring barley (*Hordeum vulgare* L.) commercial cultivars: Stratus (registered in 1999), Skald (registered in 2009) and Promyk (new genotype, will be registered probably in 2011) recommended for farmers were selected in the preliminary experiments among other several barley genotypes and used as a plant material in this study. Seedlings, after 7 days of germination, were grown hydroponically on complete nutrient medium (+P, control), medium with 0.1 mM phytic acid (PA) as the only source of phosphorus or without phosphate (−P) as described by Ciereszko et al. (1996). Barley plants were cultured in plastic containers (15 seedlings per 5 l of nutrient medium). The nutrient media were adjusted to pH 5.7, continuously aerated and replaced every 4–5 days. Plants were cultured in a growth chamber with a light period of 16 h (8 h dark); PAR, 150 µmol m^{-2} s^{-1}; temperature, 23/19°C (day/night) and about 60% relative humidity. Plants were harvested after 1, 2 and 3 weeks of growth on nutrient media (14, 21 and 28 days old plants, respectively); the samples were collected about 3–4 h after the beginning of light period.

Phosphate determination

Inorganic phosphate was determined directly after homogenization and extraction of barley leaves or roots with 10% trichloroacetic acid (at 4°C) using phosphomolybdate colorimetric assay, as described by Ames (1966).

Extracellular acid phosphatase activity measurements

Intact root systems of barley cultivars, from all growth conditions, were washed in distilled water, blot dried, placed into 30–50 ml (depending on root size) of incubation medium with substrate mixture (6 mM *p*-nitrophenyl phosphate—*p*NPP and 1 mM dithiothreitol in 50 mM sodium acetate [Na-acetate] buffer, pH 5.0) and incubated at 25°C, as described before by Ciereszko et al. (2002). 200 µl aliquots of the reaction medium were collected at different intervals for 2 h, added to 200 µl of 4 M NaOH (to stop reaction) and the absorbances were read at 410 nm (Cecil CE 2501). The results after 15 min of incubation are presented; enzyme activity was expressed as µmol *p*NP min^{-1} g^{-1} FW (protein content in incubation media was extremely low).

In vivo APase activity staining and tissue localization of enzymes

Secretion of APases was determined by in vivo activity stain as described by Ciereszko et al. (2011). Intact roots (after 1 week of growth on nutrient media) were rinsed in Na-acetate buffer (0.1 mM, pH 5.0) and embedded in 2% agarose with substrate mixture (0.2% 1-naphtyl phosphate, 0.2% Fast Blue B in 100 mM Na-acetate buffer, pH 5.0). Then the dishes were placed in the refrigerator for 2–24 h. After different periods of incubation, a dark purple color indicated APase activity in the roots and root exudates as compared to heat-killed tissue control.

Root cross sections were rinsed in Na-acetate buffer (0.1 mM, pH 5.0) and put in substrate mixture (0.2% 1-naphtyl phosphate, 0.2% Fast Blue B in 100 mM Na-acetate buffer, pH 5.0). After 20 min of incubation, root fragments were washed in distilled water and photographed under a light microscope (Olympus BX 41). A dark brown/purple color indicated APase activity in different tissues.

Intracellular APase activity measurements

Intracellular acid phosphatase activity was determined in shoots and roots of all studied barley plants after 1–3 weeks of growth on different media. Tissue samples (0.5 g) were ground in liquid nitrogen, extracted in 50 mM Na-acetate buffer, pH 5.0 with 1 mM DTT and centrifuged at 12,000*g* for 10 min at 4°C, similar to Ciereszko et al. (2002). Enzyme extracts were incubated 15 min with 6 mM *p*NPP (in 100 mM Na-acetate buffer, pH 5.0) at 37°C, then the reaction was terminated with NaOH and absorbances were read at 410 nm; APase activities were expressed as µmol *p*NP min^{-1} g^{-1} FW (to compare with extracellular APases).

Analysis of intracellular acid phosphatase isoforms

Proteins from root and shoot tissues of barley cultivars were extracted in buffer (1/4 w/v): 100 mM Na-acetate, pH 5.0; 2 mM EDTA; 5 mM DTT; 20 mM $CaCl_2$, with PVPP; gently mixed at 4°C for 60 min, then centrifuged at 10,000*g*; samples were stored in small aliquots at −80°C until used. 5 µg proteins from roots or 10 µg proteins from shoots, per lane, were loaded into discontinuous native polyacrylamide gel (5% w/v stacking gel and 10% w/v

resolving gel) as described by Tomscha et al. (2004). Electrophoresis was carried out using a mini-gel system (Hoefer SE 260, Amersham). The native gels were run at low temperature (4°C), then washed in 0.1 mM Na-acetate buffer, pH 5.0 (3 times). Approximate masses of APase isoforms were estimated using protein molecular weight marker pre-stained with colors (Full Range Rainbow Molecular Weight Markers; Amersham). Native gels were poured with 300 μg/ml of 4-methylumbelliferyl phosphate in 100 mM Na-acetate, pH 5.0 and 1% agarose (w/v). The fluorescence of methylumbelliferone liberated by phosphatase activity was visualized and documented under UV light (Gel Doc 2000, Quantity One version 4.1, Bio-Rad) after 10 min of incubation.

Protein quantification

Protein concentration in different tissue extracts was determined spectrophotometrically at 595 nm (Cecil CE 2501) according to Bradford method (1976), with the bovine serum albumin as the standard.

Statistical analysis

All experiments were performed in 3–5 independent series, in at least three replicates of assays, and standard deviation (SD) was calculated. The effects of treatments were tested by one-way analysis of variance (ANOVA). Means were compared between the treatments at the 0.05 probability level. The linear regression and correlation coefficients (r) between enzyme activity and Pi content were determined by using the STATISTICA (6.0) software package.

Results

Growth of barley plants and Pi concentration

The growth of barley plants (*Hordeum vulgare* L., cv.: Promyk, Skald and Stratus) for 3 weeks on phosphate-deficient (−P) nutrient medium resulted in lower inorganic phosphate (Pi) in the tissues and significant changes of growth parameters; plants growth on nutrient medium with phytate (PA) was less affected and generally was similar to that on Pi-sufficient medium (+P, control).

The fresh masses of shoots were generally comparable in 14-day-old barley plants, cultured for 1 week on +P, PA and −P nutrient media (Fig. 1). After 2 weeks of growth on −P nutrient medium (21-day-old barley plants) the shoot weight of barley cv. Promyk, Skald and Stratus decreased and was 34, 53 and 64%, respectively, of the control plants (Fig. 1a). Whereas growth on phytate medium (PA) slightly decreased or increased shoot weight—81% for Promyk and 128 and 135% of control for Skald and Stratus, respectively. The reduction of shoot growth was enhanced in 28-day-old barley plants, after 3 weeks of culture on −P medium, when fresh mass of barley cv. Promyk, Skald and Stratus was 15, 24 and 22%, respectively, of control (Fig. 1a). After 3 weeks of culture on PA medium Promyk shoot was similar to control but Skald and Stratus shoots were higher by 33 and 18% than control. The root fresh weight of −P barley plants was similar to +P plants (at the beginning of growth) or lower (after 2–3 weeks of culture). The root fresh mass of barley cv. Promyk, Skald and Stratus, cultured for 2 weeks on −P nutrient media was 58–59%, but after 2 weeks, it was 27, 39 and 43%, respectively, of control plants (Fig. 1b). The changes in dry masses of barley shoots and roots were similar to changes of fresh weight (data not shown). The root fresh mass of barley grown on PA medium was similar (cv. Promyk) or even significantly higher (cv. Skald and Stratus) than in control plants. The ratio root/shoot mass was higher in −P than in +P and PA plants, after 3 weeks of culture; only in Pi-deficient barley cv. Promyk the higher ratio was observed during growth period (Fig. 1). After 2 and 3 weeks of growth on −P medium, the shoot height of barley cv. Promyk was 86 and 77% of the control and Skald was 90 and 73% (Fig. 2a). After 2 weeks of culture on PA medium, the shoot height of Skald and Stratus was 126 and 123%, respectively, of control (Fig. 2a). The root length of barley cv. Skald increased by about 90 and 58% already after first and second week of culture on PA medium; root lengths of barley plants grown under −P conditions were similar (or no significantly lower) to control (Fig. 2b). The ratio of root length to shoot height was generally similar in −P barley plants to +P or PA plants (except Skald, PA medium) (Fig. 2).

The growth on −P nutrient medium strongly decreased the Pi content in the leaves and roots of all plants. Growth on the nutrient medium with phytic acid (PA) as the sole phosphate source, generally had no significant effect on Pi content (or total phosphorus—data not shown). After 1 week of barley growth on −P medium, Pi concentration in the Promyk shoot was only 7% of that found in +P plant, in the Skald shoot was 8% and in Stratus was 11% (Fig. 3a). Similarly, Pi concentration in roots of barley cv. Promyk, Skald and Stratus was 5, 6 and 9%, respectively, of the control (Fig. 3b). The Pi concentration both in shoots and roots of all barley cultivars grown on PA medium was similar to control plants. After 2 weeks of culture on −P medium, shoot Pi content was 2% for Promyk and about 6% for Skald and Stratus; Pi concentration in roots was 6, 8 and 20% of the control for Promyk, Skald and Stratus, respectively (Fig. 3). The Pi concentration in shoots of barley cv. Promyk and Stratus, cultured on PA medium was 64 and 60%, respectively, of the control plants

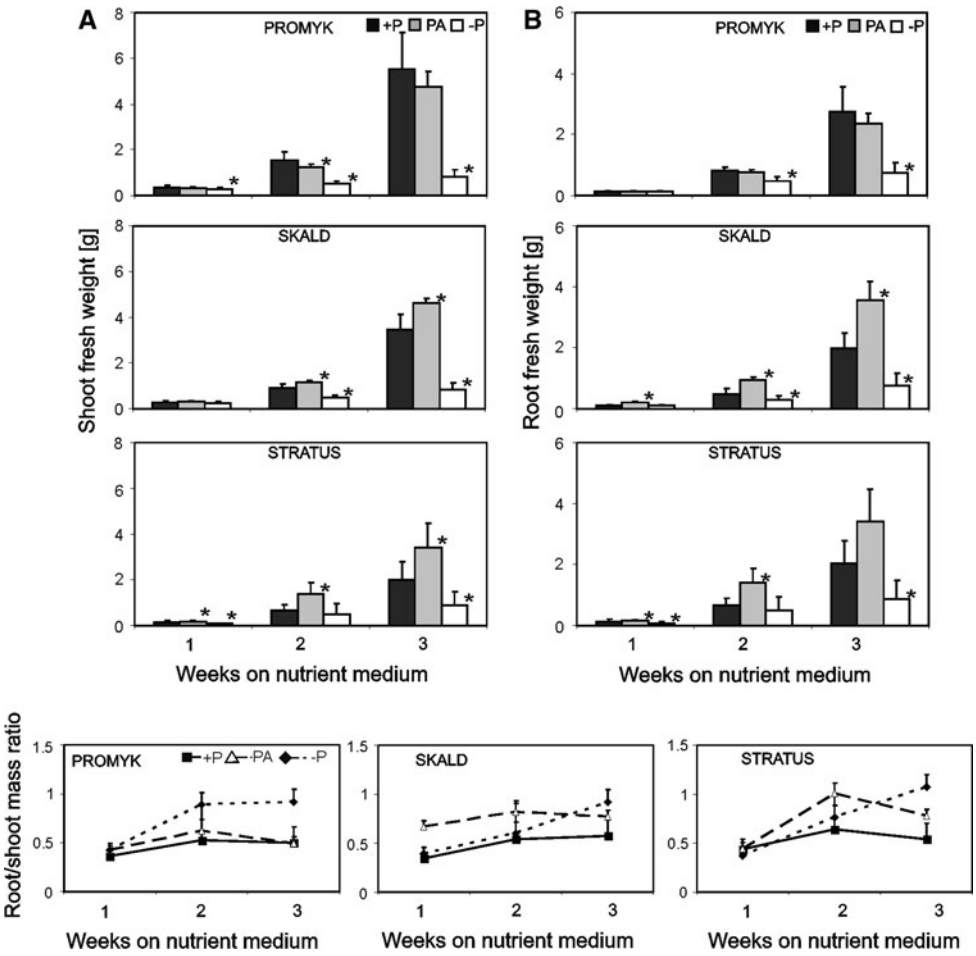

Fig. 1 Fresh mass of shoots (**a**) and roots (**b**) and root to shoot fresh mass ratio (**c**) of three barley varieties (*Hordeum vulgare* L., Promyk, Skald and Stratus) hydroponically cultured for 1–3 weeks on complete nutrient medium (+P), medium with phytic acid (PA) and without phosphate (−P). Mean ± SD values are indicated. *Differences statistically important at 0.05

(Fig. 2a). After 3 weeks of growth on −P medium, shoot Pi level of Promyk, Skald and Stratus was 1.5, 3 and 8%, respectively, of the control and root Pi was 13, 8 and 11% (Fig. 3). The Pi concentration in the roots of barley cv. Skald and Stratus, cultured on PA medium was 73–74% of that found in +P plants (Fig. 3b).

The water content in tissues of all barley cultivars was about 85–90% in shoots and 89–95% in roots and was not significantly affected by Pi deficiency, especially at the beginning of culture (data not shown). However, after 3 weeks of culture Pi deficit decreased slightly the water content in the shoot of barley cv. Promyk and Stratus (not shown).

Extracellular acid phosphatases activity and secretion by barley roots

Secretion of acid phosphatases (APase) from root is rather a common plant reaction to Pi starvation, which can increase Pi availability by breakdown of organic forms of phosphorus. However, the in vivo root staining for secreted and root-associated APases (after 1 week of barley culture on +P and PA or −P nutrient media) showed rather similar enzyme secretion by intact roots of all barley varieties under stress conditions as compared with +P plants (Fig. 4a). APase secretion to the agarose from the roots of the barley cv. Skald grown on Pi-deficient medium was slightly higher than from roots of Promyk or Stratus (Fig. 4a). Histochemical APase activity staining was carried out on cross sections of young and mature parts of roots of barley plants hydroponically grown under Pi-sufficient, Pi-deficient conditions and on medium with phytate (Fig. 4b). The intensity of brown/purple color, indicating APase activity, was slightly stronger in −P roots than in +P roots, or plants grown on PA medium (Fig. 4b). Especially transverse sections of −P roots of the barley cv. Promyk (after 3 weeks of growth—data not shown) and cv. Skald showed strong signal of APase activity. The distribution of APases activity differed between root tissues—the highest activity of APases was found mainly in the vascular tissues of roots but sometimes also near the epidermis (Fig. 4). Similar results were obtained also after 2 and 3 weeks of plants growth on +P, −P and PA media (data not shown).

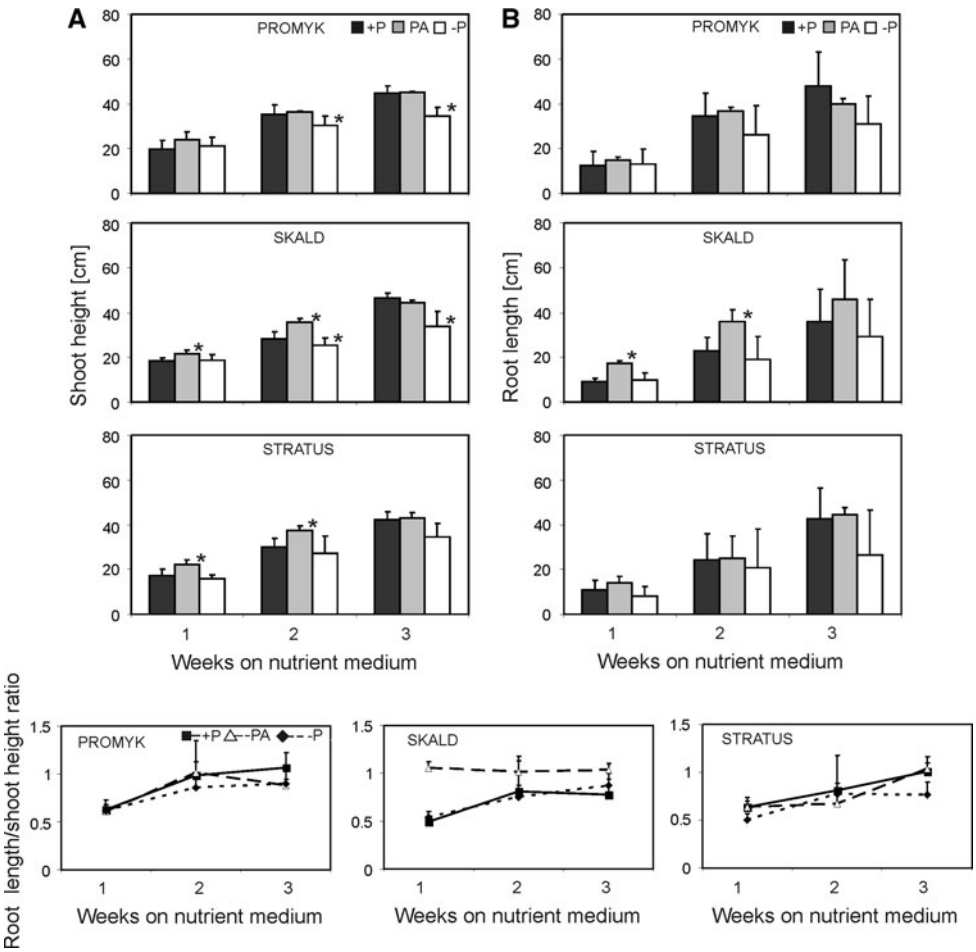

Fig. 2 Shoot height (**a**), root length (**b**) and the ratio of root length to shoot height (**c**) of different barley varieties (*Hordeum vulgare* L., Promyk, Skald and Stratus) cultured for 1–3 weeks on complete nutrient medium (+P), medium with phytic acid (PA) and without phosphate (−P). Mean ± SD values are indicated. *Differences statistically important at 0.05

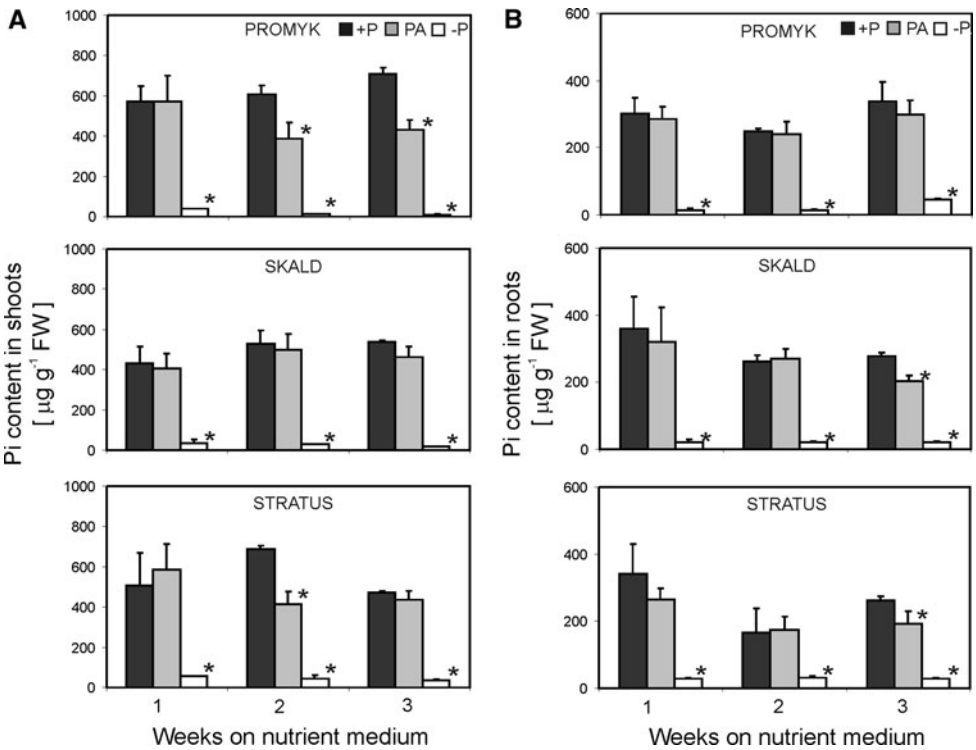

Fig. 3 Inorganic phosphate (Pi) concentration in shoots (**a**) and roots (**b**) of three barley (*Hordeum vulgare* L.) varieties (Promyk, Skald and Stratus) grown for 1, 2 and 3 weeks on complete nutrient medium (+P), medium with phytic acid (PA) and without phosphate (−P). (Mean ± SD). *Differences statistically important at 0.05

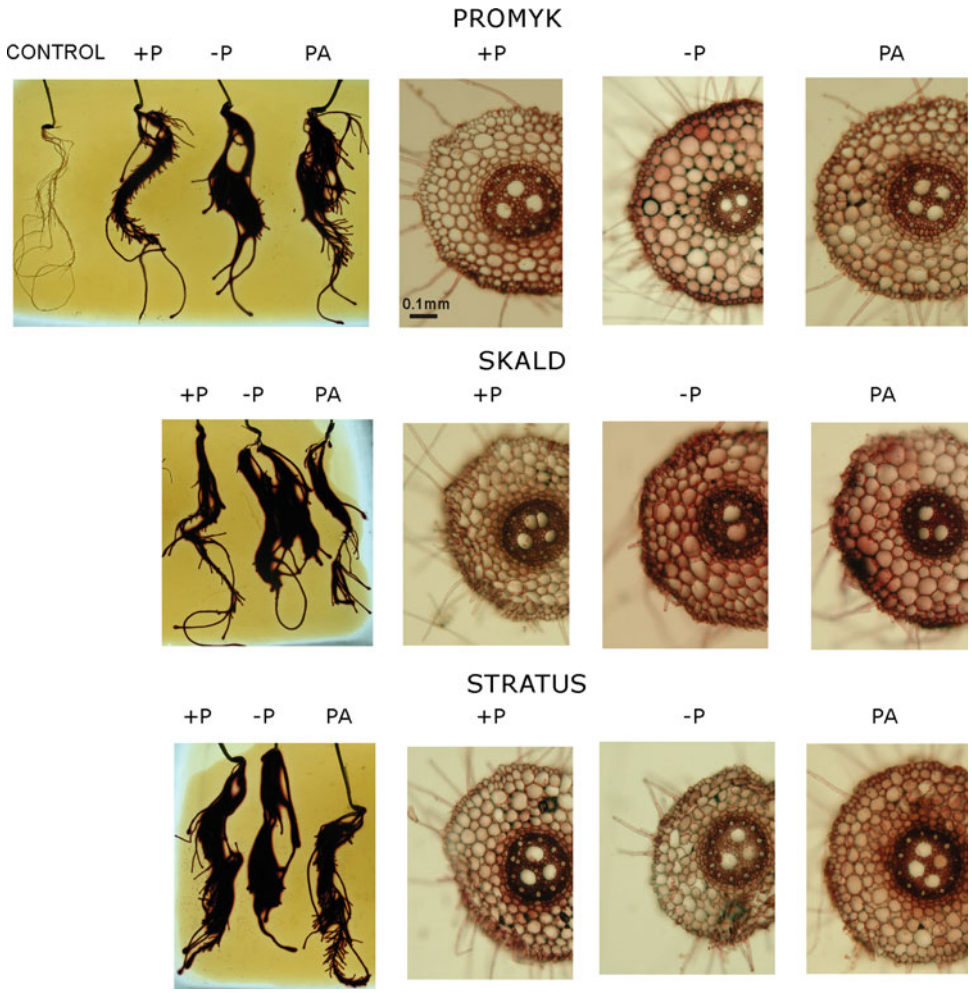

Fig. 4 In vivo staining for acid phosphatase activity in whole roots and root cross sections of three barley (*Hordeum vulgare* L.) varieties (Promyk, Skald and Stratus). The *dark color* indicates acid phosphatase activity in the root tissues and roots exudates as compared to heat-killed tissue control. Plants were cultured for 1 week on complete nutrient medium (+P), medium with phytic acid (PA) and without phosphate (−P)

Extracellular acid phosphatase activity, associated with the roots and secreted to the incubation medium (rich in enzyme substrate—*p*NPP) was altered by growth on PA medium but only slightly by Pi deficiency (Fig. 5). After 1 week of growth on PA nutrient solutions, the extracellular APase activity of barley root cv. Skald and Stratus decreased and was about 60–70% of that found in +P plants; the activity of APases from −P roots was similar to control (Fig. 5). After 2 weeks of culture, the mean extracellular APase activity in the incubation medium with intact roots of the −P barley plants slightly increased (statistically insignificant); on the other hand APase activity after culture on PA medium decreased and was 62 and 31% of control, respectively, for barley root cv. Promyk and Skald (Fig. 5). After 3 weeks of culture, the APase activity in the incubation medium with intact roots of the Pi-deficient barley cv. Promyk and Stratus was about 1.7- and 1.4-fold higher than in control, respectively (Fig. 5). However, after 3 weeks of growth on PA medium the extracellular APase activity of intact roots of three barley cultivars was only about 63–64% of control (Fig. 5).

Intracellular acid phosphatases activity in barley tissues

Intracellular acid phosphatases activity was estimated in extracts from both shoots and roots of all studied barley cultivars (Fig. 6). An increase of intracellular APases activity was observed already at the beginning of the culture, but only in the Pi-deficient barley plants. The growth on medium with phytate (PA) had no effect or decreased APases activity in shoots and roots (Fig. 6). After 1 week of culture on −P solution, the activity of APase increased about 1.5- and 1.9-fold in shoots of barley cv. Skald and Stratus, when compared to control plants (Fig. 6a). After 2 weeks the APase activity increased about 1.5-fold for Promyk, Skald and 1.8-fold for Stratus, and after 3 weeks, it increased by 1.7-, 1.4- and 1.9-fold, as compared to that of the +P plants (Fig. 6a). The plant culture on PA medium did not affect internal APase activity in shoots (except Skald, 1 week). The significant increase of intracellular APase activity in crude extracts from roots of −P barley cv. Promyk and Skald was observed; however in Stratus roots, APase activity slightly decreased, sometimes

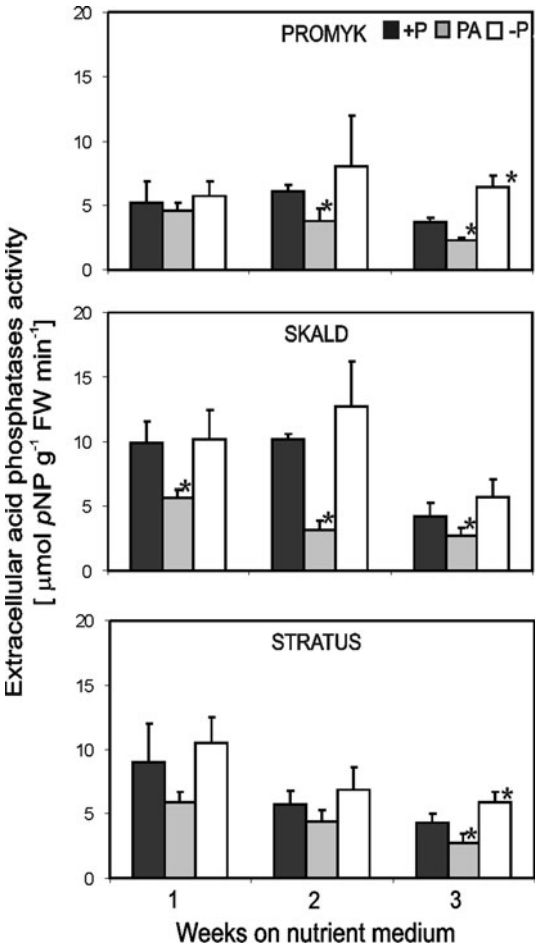

Fig. 5 Extracellular acid phosphatase activity (µmol *p*NP g^{-1} FW min^{-1}) of intact roots of three barley (*Hordeum vulgare* L.) varieties (Promyk, Skald and Stratus) grown for 1, 2 and 3 weeks on complete nutrient medium (+P), medium with phytic acid (PA) and without phosphate (−P). (Mean ± SD). *Differences statistically important at 0.05

increased (by 2.4-fold, 2 weeks) or was not affected, dependent on duration of Pi deficiency (Fig. 6b). The intracellular acid phosphatase activity in the extracts from roots increased after 2–3 weeks of plants growth on −P nutrient solution, when compared with the +P barley plants (Fig. 6b). The APase activity in the roots of −P barley cv. Promyk increased about 2- to 2.4- fold when compared to control and Skald, over 2.4-, 1.5- and 1.9-fold (respectively after 1–3 weeks of culture) as compared to +P plants (Fig. 6b). The barley culture on PA medium did not affect on internal APase activity in root and generally was similar to that of the +P plants, or decreased, even by about 20–50%, especially after 3 weeks of growth (except Stratus root, 1 week) (Fig. 6b). The higher activity was observed in shoots of all barley cultivars than in roots. In barley cv. Promyk and Skald almost all Pi-deficient conditions caused internal APase activity enhancement both in shoot and roots, whereas in shoot of barley cv. Stratus the APases enhancement was well visible, but root enzymes were not affected by low Pi.

Concentration of soluble proteins in enzymatic extracts from the shoots and roots of three barley varieties (Promyk, Skald and Stratus) were not significantly affected by Pi deficiency (Table 1). Culture of barley on PA medium did not affect or change protein concentration, e.g. decrease of protein content was observed after 1–2 weeks of growth in barley shoot cv. Stratus (Table 1).

To examine APase isoforms, the proteins isolated from shoots and roots of three barley cultivars (after 1–3 weeks of culture on +P, PA or −P media) were separated on native PAGE and stained for APase activity (with 4-methylumbelliferyl phosphate). In extracts from shoot tissues, at least three major APase isoforms, with lower and higher mobility on the gel (approximately about 95, 70 and 27 kDa) were detected in the studied barley varieties (Fig. 7). In extracts from barley roots, at least four major APase isoforms (about 95, 80, 70 and 27 kDa) were detected (Fig. 7). Especially two of these isoforms (approximately 80 and 27 kDa) were strongly induced by Pi deficiency in roots, mainly in barley cv. Promyk and Skald roots but also to a lesser extent in Stratus. The growth on medium with phytate (PA) had no significant effect on activity of APase isoforms, it was similar to that of adequate control (Fig. 7). During 3 weeks of hydroponics the isoformic pattern of APase was basically unchanged, although in the barley cultured for 2 weeks a higher activity of some isoforms (approximately 80 and 27 kDa) was observed in the roots of the −P plants than in barley varieties cultured for 1 week or 3 weeks (Fig. 7).

Discussion

Effects of Pi deficiency and organic phosphorus on barley growth

The lack of phosphate in the nutrient medium affected growth of all studied barley varieties (cv. Promyk, Skald and Stratus): shoot and root mass was reduced (e.g. after 3 weeks to 15–24% of control shoot) but root elongation was not significantly affected. It seems that especially the shoot growth reduction is in a good consistency with low Pi concentration in the tissues of barley (Figs. 1, 2, 3). When comparing barley varieties, the highest reduction of Pi concentration was observed in the shoots of Promyk, shoot growth of this cultivar was also affected by Pi deficiency more than in other varieties; the increased ratio of root/shoot mass was also observed mainly in barley cv. Promyk, especially after longer period of culture without Pi (Figs. 1, 2, 3). The increase of root elongation growth and root to

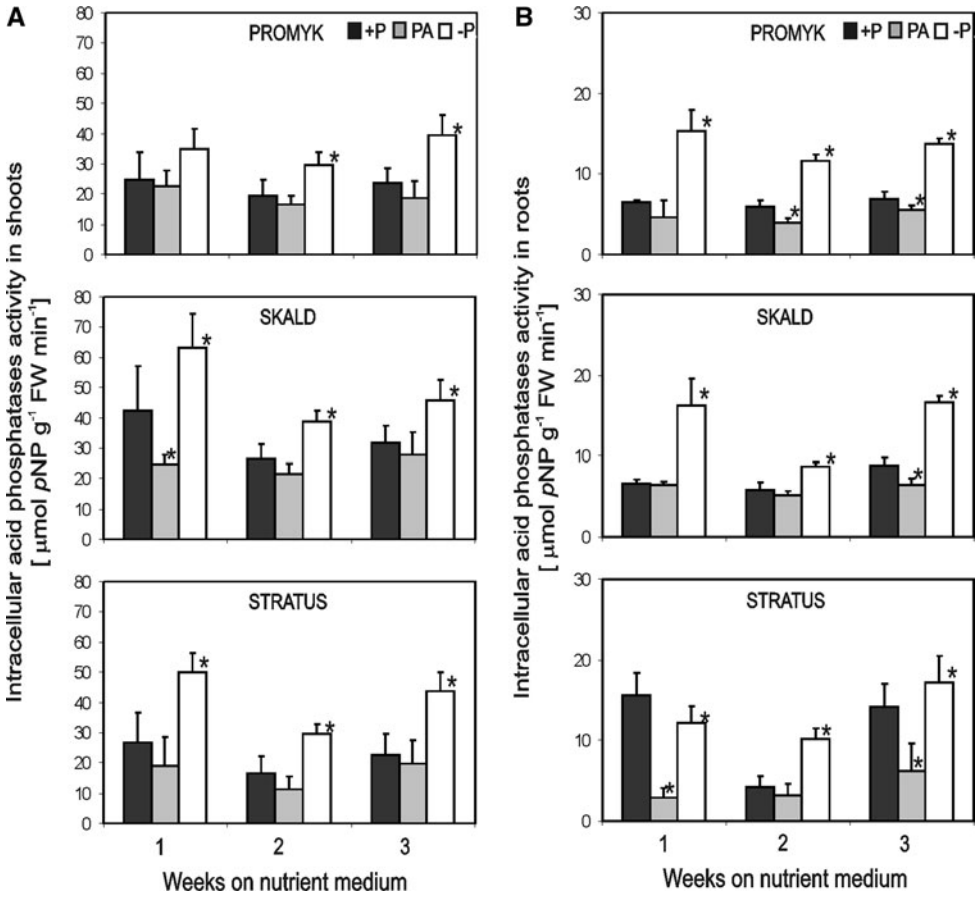

Fig. 6 Intracellular acid phosphatase activity (µmol *p*NP g^{-1} FW min^{-1}) in shoots (**a**) and roots (**b**) of three barley varieties (*Hordeum vulgare* L., Promyk, Skald and Stratus) grown for 1, 2 and 3 weeks on complete nutrient medium (+P), nutrient medium with phytic acid (PA) or without phosphate (−P) (Mean ± SD). *Differences statistically important at 0.05

Table 1 Protein concentration in enzymatic extracts from shoots and roots of three barley (*Hordeum vulgare* L.) varieties (Promyk, Skald and Stratus) grown for 1, 2 and 3 weeks on nutrient medium with inorganic phosphate (+P), with organic phosphate, phytic acid (PA) and without phosphate (−P)

Cultivar	Growth on nutrient medium (weeks)								
	1			2			3		
	+P	PA	−P	+P	PA	−P	+P	PA	−P
Proteins in shoots [mg g^{-1} FW]									
Promyk	4.4 ± 0.9	2.9 ± 0.4	3.5 ± 0.9	5.9 ± 1.0	4.5 ± 0.7	5.0 ± 0.4	8.9 ± 1.3	9.1 ± 1.2	9.9 ± 1.1
Skald	6.3 ± 2.1	3.5 ± 0.9	6.8 ± 2.4	6.9 ± 4.6	4.9 ± 0.9	5.5 ± 0.7	9.9 ± 2.1	8.9 ± 1.7	8.7 ± 1.6
Stratus	4.2 ± 0.7	1.9 ± 0.4	5.1 ± 1.1	6.7 ± 0.8	3.7 ± 0.4	4.8 ± 0.6	10.2 ± 2.3	10.3 ± 2.4	9.5 ± 1.7
Proteins in roots [mg g^{-1} FW]									
Promyk	2.4 ± 0.4	1.1 ± 0.4	2.1 ± 0.3	2.1 ± 0.3	1.7 ± 0.3	1.9 ± 0.4	2.5 ± 0.1	1.9 ± 0.2	2.0 ± 0.1
Skald	0.9 ± 0.1	1.1 ± 0.1	1.3 ± 0.2	1.0 ± 0.3	1.1 ± 0.1	0.9 ± 0.1	1.6 ± 0.3	1.4 ± 0.2	1.7 ± 0.1
Stratus	1.8 ± 0.5	0.8 ± 0.2	2.0 ± 0.2	1.4 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.7 ± 0.2	1.4 ± 0.2	1.5 ± 0.2

Mean ± SD values are indicated

shoot ratio is thought to be a common plant response to the low external level of Pi, important for better soil exploration; even small changes in root diameter and root elongation might have a large effect on Pi uptake from soil (Gregory 2006 and papers cited therein). We have previously investigated the growth of oat (and wheat) plants under Pi-deficient conditions, where reduction of shoot mass was accompanied by significant stimulation of root elongation growth (in contrary to barley root) and a huge increase of the ratio of root/shoot weight was observed (Ciereszko and Balwicka 2005; Ciereszko et al. 2011, and other not published data). The modifications of growth parameters were observed at the early and medium stages of phosphate deficiency (around 2–3 weeks of culture); the

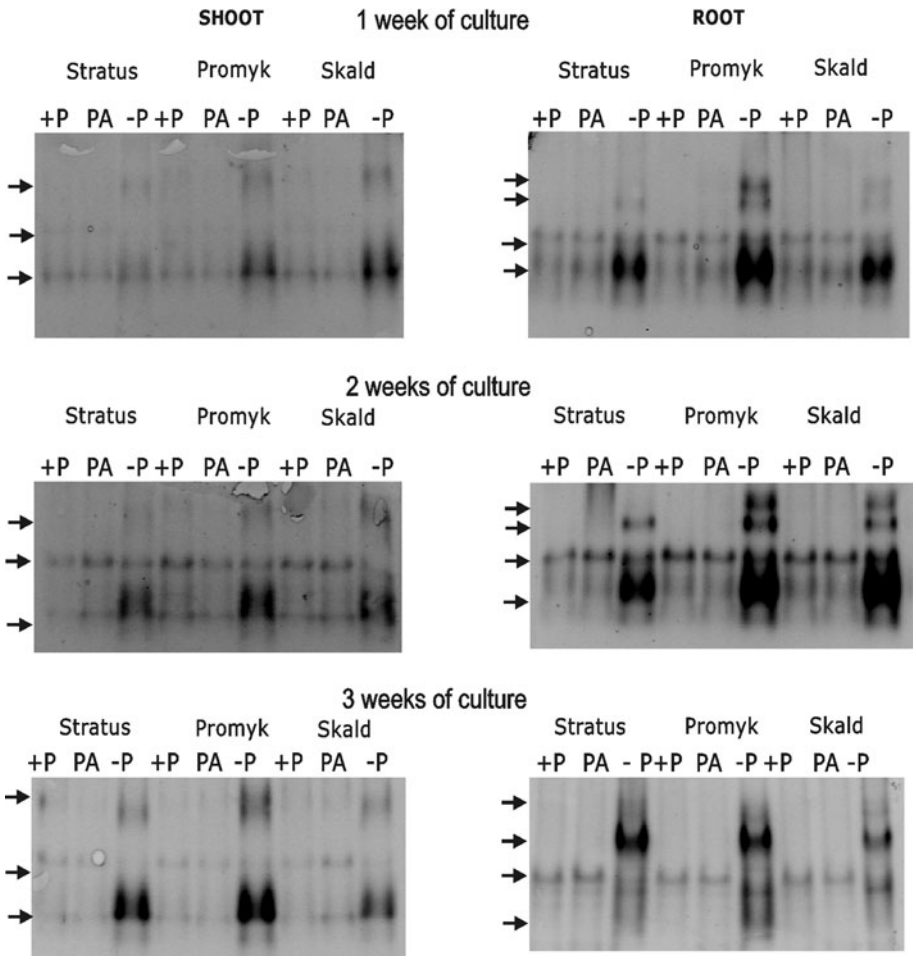

Fig. 7 Profile of APase isoforms in crude protein extracts from shoots and roots of three barley varieties (*Hordeum vulgare* L., Promyk, Skald and Stratus) hydroponically cultured for 1–3 weeks on complete nutrient medium (+P), medium with phytic acid (PA) and without phosphate (−P). Protein extracts from shoots (10 μg protein per lane) and roots (5 μg protein per lane) were run on native discontinous PAGE and stained for APase activity using 4-methylumbelliferyl phosphate and visualized under UV light

effect of more prolongated Pi starvation has not been investigated in these studies.

Phosphorus is one of the most limiting elements for plants and low Pi availability is rather common in many natural and agricultural ecosystems. To improve crop productivity plants has been characterized for better acclimation mechanisms to low Pi conditions. Phosphorus-efficient plant genotypes has usually highly branched root systems with numerous basal roots, while the inefficient plants had smaller, less branched roots (Gregory 2006; Hammond et al. 2009). Several studies have addressed the role played by phytohormones in root architectural changes under Pi deficit. The overexpression of *MYB62* or *ZAT6* also resulted in altered root architecture of plants and Pi uptake (Nilsson et al. 2010; Rouached et al. 2010 and papers cited therein). Additionally, roots with better nutrient acquisition rates possess longer root hairs and their high density, as root hairs are responsible for the most of total Pi uptake from soil. Several barley genotypes with long root hairs are known to sustain high grain yields in low Pi fields (Gahoonia and Nielsen 2004). Quite often such changes in morphology results rather from temporary Pi deficit in the soil (or medium) than from a significant decrease of Pi concentration in plant tissues. It has been shown that physical contact of root tip with low Pi in the ground is sufficient to reduce primary root growth and increase number and length of lateral roots (Svistoonoff et al. 2007). Recent analyses of the transcriptome of hair-less mutants of barley indicated for genes that are involved in the initiation of root hair morphogenesis (Kwaśniewski et al. 2010); perhaps such hair-less mutants are worth to study in the context of acclimation to Pi deficiency or growth on phytate.

However, in spite of the currently available data, it is still unclear whether alteration in root elongation or architecture are the only attribute sufficient to improve the efficiency of Pi acquisition by plants grown in natural conditions. The increased capacity of roots to phosphate uptake is, however, dependent not only on the increased surface of nutrient absorption but also on the metabolic/physiological capacity to enhance secretion of organic acid or enzymes like acid phosphatases to the ground (Gregory 2006 and papers cited therein; Yao et al. 2011). However, in our study the decrease of pH in the nutrient media and rhizosphere was not observed (data not shown), which indicated that barley did not responded to Pi starvation via

increase of protons or organic acids exudation from the roots, at least in our experimental conditions.

We hypothesized that barley would be unable to grow in nutrient medium with phytic acid as the only phosphate source, similarly to results obtained for wheat by Richardson et al. (2000) or George et al. (2008). However, in our studies, within the following several days of culture on phytate medium, the shoot and root growth of all barley varieties was still more similar to that of the +P plants than to Pi-deficient plants, which clearly indicated that they are able to utilize this organic form of phosphorus (Figs. 1, 2). We have investigated also the growth of oat (*Avena sativa* L.) plants on −P and PA media, where Pi deficiency significantly affected growth of all studied varieties, whereas growth parameters of plants supplied with phytate were similar to control (data not published). Barley genotypes with high efficiency of extracellular acid phosphatases activity or secretion from the roots to the soil probably could significantly enhance availability of phosphorus from organic fertilizers.

Acid phosphatases activity and localization—plant response to Pi depletion

Extracellular and intracellular APase activity increase under Pi deficiency is a common phenomenon among various plants. However, the level of APases activity (and secretion from roots) quite often may differ between plant species and between varieties.

Extracellular acid phosphatase activities (APases) in roots of barley grown on −P nutrient medium generally were similar to control, also the activity of secreted APases (detected in vivo) was rather similar in all conditions and barley varieties (Figs. 4, 5). Extracellular acid phosphatase activities (APases) in roots of barley grown on PA medium were lower than in Pi-sufficient and Pi-deficient plants (Fig. 5), which clearly indicates that these enzymes are not involved in the utilization of phytate in the nutrient medium in our experimental conditions. Thus, perhaps root-associated specific APases activity, like phytases, should be checked in future experiments. In one of the previous studies, barley genotypes grown in nutrient solution under Pi stress differed significantly when compared for activity of root-associated and root-released extracellular phytase; in addition a correlation between the activity of root-associated and released extracellular phytase was observed (Asmar 1997). However, other studies suggested that high APases activities and secretion have rather a limited effects in soil rich in insoluble Ca-, Fe-, Al- or Mg-phytates, and these results are sometimes in contrast to that obtained in experimental conditions with artificial substrates (Richardson et al. 2000; George et al. 2008).

Pi deficiency usually has a significant impact on root-associated APase activities (Duff et al. 1994; Olczak et al. 2003; Żebrowska and Ciereszko 2009). The experiments with *pup* mutants of *Arabidopsis* have demonstrated that secreted or cell wall-bound APases are important for phosphate nutrition and homeostasis (Tomscha et al. 2004). However, some studies showed negative relationship between APase activity and efficiency of Pi uptake under Pi starvation (Yan et al. 2001; Yun and Kaeppler 2001). No significant differences in root-associated APase activities among genotypes of white clover, differing in responses to Pi starvation, were found (Hunter and McManus 1999). Experiments on maize varieties with contrasting efficiency of Pi uptake suggested that APase might not be a main part of mechanism for acquiring Pi (Yun and Kaeppler 2001). The recent results suggested that despite differences in surface APases activities of wheat roots, such variability probably has no significant role in the phosphorus nutrition of plants grown in natural soil, and that any benefit derived from the hydrolysis of organic P compounds is common to all studied genotypes of wheat (George et al. 2008). However, the improved phosphorus acquisition of white clover via better utilization of organic phosphorus in response to Pi deficiency were obtained recently by over-expression of plant-derived phytase and acid phosphatase genes (Xiao et al. 2006; Ma et al. 2009). In spite of such conflicting reports, further investigations of new crop cultivars tolerant to low Pi availability in the soil are still important. The identification of differences among acid phosphatase activities/localization/secretion might be also useful for selection of crop plants varieties more tolerant to low Pi stress.

Pi deficiency increased internal APase activities in extracts from barley shoots and roots as compared to Pi-sufficient plants (Fig. 6). The intracellular APase activity in the shoot increased mainly in the Stratus cultivar grown on −P medium, whereas the intracellular APase activity in the roots was highest in the Pi-deficient barley cv. Promyk. The growth on PA medium had no effect or decreased APase activity (Fig. 6). The increase of intracellular APase activity in the roots of plants under Pi deficiency was more intensive than the activity of extracellular APases (Figs. 5, 6). Additionally, a high negative correlation was observed between Pi concentration in shoot/root and activity of intracellular APases in these tissues (data not shown). As our results indicated, the three barley varieties may use similar acid phosphatases to acquire the potentially available Pi from external or internal sources under mild and moderate Pi deficiency (Figs. 6, 7). Intracellular APases are important mainly for remobilization of internal Pi sources, e.g. from the vacuole (Duff et al. 1994; Tomscha et al. 2004; Xiao et al. 2006). In this study, one of the

growth media contained no Pi, thus the measured activities of both APases possibly reflect the potentially maximal plant reaction to the nutrient stress. Additionally, experiments with media containing low amounts of organic sources of phosphorus (for different barley cultivars) indicated that phytate did not affect internal APase activity, which was similar to that of the +P plants, or decreased (after 3 weeks of growth) (Fig. 6).

APase activities were also detected and localized on root transverse sections of all the barley varieties under study. Histochemical visualization on the root cross sections indicated that higher activity of APases was mainly in the vascular tissues and in rhizodermis than in other tissues of barley roots (Fig. 4). No significant differences were observed between barley cultivars. The results obtained in the measurements of APases activity, root-associated and internal, are generally in agreement with visualization of enzyme activity on root transverse sections or on native gels. Our other studies showed the strong activity of APases detected in the root epidermis (and outer cortex cells) of different oat varieties, which indicated that at least a part of these enzymes might be secreted from the roots to the ground (data not published). Histochemical visualization of APases activity in roots of white lupine also showed a strong enzyme activity in the epidermis and root hairs under Pi deficiency (Wasaki et al. 2008); white lupine can produce both cell wall-bound APase and enzymes than can be released into the rhizosphere, LASAP2, a form of secreted APase from white lupin roots (Wasaki et al. 2008). Similarly, in *Arabidopsis* under Pi starvation a strong expression of the promoter region of secreted APases was detected in young lateral roots and in the vascular tissues (Haran et al. 2000). In addition, Pi-deficient nodules of pea roots showed the increase of APase activity in plant cell walls and the infection threads (Sujkowska et al. 2006). It was suggested that such enhancement of APase activity in nodule apoplast might increase the availability of Pi for plant and symbiotic bacteria under low Pi nutrition (Sujkowska et al. 2006).

APase isoforms in plant tissues

Three major APase isoforms were detected in shoot extracts of all the barley varieties, one unique isoform (around 27 kDa) was strongly induced both by mild and severe Pi deficiency (Fig. 7). However, four major APase isoforms were detected in the roots of barley plants; two isoforms (approximately 80 and 27 kDa) was strongly induced by conditions of Pi deficit, especially in roots of barley cv. Promyk. Rather a few differences occurred among barley cultivars, as regards APase isoforms activity. Phytate in the growth medium had no effect on activity of APase isoforms (Fig. 7). It seems that duration of Pi deficiency might have an impact on the activity of APase isoforms as after 3 weeks of culture the activity was lower than after 2 weeks. Generally, in our study a consistency between the activity of intracellular APases (measured spectrophotometrically) and the visualization of isoforms activity was visible, especially for barley cv. Promyk. It seems also that the further studies of the subcellular APase localization and purification of isoforms could be useful in elucidating the role of APases in barley plant tissues similar to investigations of the major cytoplasmic isoenzyme from barley root, which was purified and characterized by Panara et al. (1990).

Under Pi-deficient conditions one unique isoform of two major APase isoforms detected in white lupine root was induced, especially in proteoid roots (Gilbert et al. 1999). On the other hand, three of the four APase isoforms were induced by phosphorus stress in leaves of common bean (Yan et al. 2001). In the tissues of rice, three different APases isoforms were found; two of them were affected by Pi starvation (Lim et al. 2003). The five APase isoforms was secreted from *Arabidopsis* roots and the activity of one increased specifically in response to Pi deficit (Tomscha et al. 2004). On the other hand, six APases isoforms were detected in soybean leaves and surprisingly activity of all detected isoforms increased significantly under low Pi conditions (Tian et al. 2003).

In conclusion, the responses of studied barley cultivars to phosphorus starvation were generally similar to those of other crop plants. Pi deficit reduced shoot growth of all studied plants, the increase of APase activity, mainly intracellular enzymes, was observed. It seems that studied plants acclimation to phosphate deficiency was dependent mainly on duration of stress conditions and less on plant variety. Generally, the barley cultivars only slightly differed in terms of acclimation to early and moderate deficiency of phosphate and used similar pools of acid phosphatases to acquire Pi from external or internal sources. Phytate, known as the storage form of phosphate in seeds, is often a major part of environmental organic form of phosphorus (from fertilizer or agricultural runoff). Barley varieties (Promyk, Skald and Stratus) grew equally well on medium with Pi and on medium in which the Pi was replaced with phytate. These results demonstrate that phytate can provide a source of adequate phosphate for sustained growth of these barley varieties in phytate-rich environments.

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