Volume 56(1):51-55, 2012
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Boiling stable acid phosphatases (BsAPases) in *Triticum aestivum* induced by phosphate (Pi) deficiency

Arun Dev Sharma[1]*, Gurmeen Rakhra[1], Jatinder Singh[2]

[1]Department of Biotechnology, Lyallpur Khalsa College, Jallandhar, Punjab, India, [2]Department of Molecular Biology and Biochemistry, Guru Nanak Dev University, Punjab, India

ABSTRACT Nutrient limitation represents a ubiquitous form of environmental abiotic stress and Pi limitation is especially common due to low availability in most soils. As a result, most natural soils have soluble Pi concentrations lower than cytoplasmic Pi concentrations required for plant growth. Acid phosphatases (APases) are widely found in plants having intracellular and extracellular activities. APases are believed to be important for Pi scavenging and remobilization in plants, but role of boiling stable APases in adaptation to Pi stress at germination level has not been critically evaluated. To address this issue, the effect of low phosphate stress (LPS) on boiling stable acid phosphatases in wheat embryos and endosperm was investigated. With comparison to high phosphate condition (HP), a considerable increase in boiling stable acid phosphatase (BsAPase) activity was observed both in embryos and endosperm under LPS treatment. Post-LPS study revealed that BsAPase activity decreased considerably upon relieving the Pi stress. Further, as compared to HP conditions, SDS-PAGE analysis also detected a differential strong band under LPS conditions, which disappeared upon relieving Pi stress. These findings suggest that changes in the phosphatase enzymes might play important roles in adaptation of germinating seeds under Pi stress conditions. Based upon these results, a possible physiological role of BsAPases in germinating wheat seeds is discussed. **Acta Biol Szeged 56(1):51-55 (2012)**

KEY WORDS

acid phosphatase
boiling stable proteins
wheat

Plants are unable to escape unfavorable conditions due to their sessile nature, and must therefore evolve adaptations to cope with the bevy of biotic and abiotic stresses imposed upon them. The plant responses to water deficit depend upon various factors such as duration and degree of stress, the stage of growth, time of stress exposure and use of various plant organs. This might be reason for the large size of plant genomes and gene families. Against these stresses, plants adapt themselves by different mechanisms including change in morphological and developmental pattern as well as physiological and biochemical responses (Bohnert et al. 1995). Nutrient limitation represents a ubiquitous form of abiotic stress and phosphate (Pi) limitation is especially common due to low availability in most soils, as vast Pi reserves exits in soil as immobilized P-esters. As a result, most natural soils have soluble Pi concentrations lower than cytoplasmic Pi concentrations required for plant growth (Vance et al. 2003). Low availability of phosphate (Pi) to plants in soil is a major constrain which determines plant growth, metabolism, development, reproduction and crop yield. It is estimated that majority of planting areas in Asia are currently under Pi deficiency (Zhang et al. 2009; Ciereszko et al. 2011). Currently, the world phosphate mine reserves, our major source of Pi, are projected to be depleted by the end of this century (Vance et al. 2003). Moreover, Pi deficiency is also known to cause plant oxidative stress and this could be reflected by modulations of an array of stress related proteins (Li et al. 2007; Yao et al. 2010). Adaptation to all these abiotic stresses is associated with metabolic adjustments that lead to the modulation of different proteins (Shinozaki and Shinozaki 1996; Yan et al. 2001). Recent proteomic or microarray studies have documented that a large part of genes or proteins (e.g. PPIases; GST, eIF5A, Maturase, HSP70, chaperonin10) were also up regulated under low phosphorous (LP) stress (Yao et al. 2010). In contrast, Pi stress also caused a broad repression of genes involved in RNA synthesis, processing, protein synthesis (Yao et al. 2010). On the other hand, crop yields are sustained by massive use fertilizers added to soil which pollute water sources and contribute to the process of eutrophication. It is therefore, essential to develop efficient cultivars of crop plants capable of using Pi more efficiently in order to minimize application of fertilizers (Zhang et al. 2009). A central role of modern Biotechnology is characterizing the adaptations of plants to biotic and abiotic stresses which allow growth and reproduction in conditions inclement of crop growth. To this end, it is important to identify the genes and reveal the underlying physiological, biochemical mechanisms for high Pi efficiency in the crop species. Overexpression studies in *Medicago* revealed that ectopic

Accepted Febr 12, 2012
*Corresponding author. E-mail: arundevsharma@hotmail.com, arundevsharma47@rediffmail.com

expression of *AtPAP15* increased the plant ability to acquire Pi during growth on unfertilized soil (Ma et al. 2009).

Plants increase efficiency of Pi use during Pi starvation via up-regulation of phosphate starvation inducible (PSI) hydrolases that are believed to scavenge Pi from non-essential P-esters. Classical PSI hydrolases include phospholipases (PL), ribonucleases (RNases) and acid phosphatases (APases) (Vance et al. 2003; Dick et al. 2011). Acid Phosphatases (APases; EC 3.1.3.2) are one among them which are typified as broad spectrum APases that largely catalyze the hydrolysis of Pi from small molecules, which are believed to be important for many physiological processes, including regulation of soluble phosphorous (Pi) (Yan et al. 2001). Orthophosphate (Pi) plays a central role in virtually all major metabolic processes in plants including: photosynthesis, respiration, energy transfer, metabolic regulation, important structural constituent of biomolecules like phytin bodies in the ungerminated seeds, protein and nucleotide phosphorylation, gene expression and signal transduction (Fincher 1989). APases catalyze the hydrolysis of Pi from Pi monoesters and anhydrides with in the acidic pH range. Induction of intracellular and secreted APases are widespread plant response to nutritional Pi-deficiency. The probable function of intracellular APases is to recycle Pi from expendable intracellular organophosphate pools, whereas as secreted APases likely scavenge Pi from the organically bound Pi that is prevalent in most soil. Although, some abiotic stresses like salt, osmotic and water have been reported to increase phosphatase activities by maintaining a certain level of inorganic phosphate in plant cells (Olmos and Hellin 1997), however, the exact role of phosphatases in the germinated seeds is still not clear, because, metabolism of these compounds can be affected by a number of environmental factors such as stress type, irridance, temperature, and type of ions present (Bohnert et al. 1995). Germination of grains is initiated by water uptake of water and its successful completion is signaled by emergence of the developing root and shoot. Following uptake of water, hormone signals, probably released from the embryos, are believed to result in the synthesis of hydrolytic and other enzymes in endosperm (Fincher 1989). Moreover, like mature plants, germinating seeds and seedlings also can be subjected to environmental stresses. Even when they imbibe water, seeds may be exposed to elements of a hostile environment, which include Pi stress, high temperature of soil, salinity and varying moisture content. Failure to cope with the adversity cause by these extremes results in poor germination, seedling development, and eventually, reduced crop yields. Thus, the variation that occurs in phosphatase activities during germination is poorly understood and information on physiological events involved in this process is scarce. Moreover, it was reported that some abiotic stress-induced proteins (e.g. dehydrins) are highly hydrophilic and remain soluble even after boiling (Close et al. 1989), a characteristic that has been termed "boiling stability" (Jacobsen and Shaw 1989). At present hundreds of genes induced under drought stress have been identified those may allow plants to adapt to water limiting conditions. Because plant responses to environmental stresses are complex and multigenic, the functions of many of the induced genes are still a matter of conjuncture (Bray 2002). These traits are mostly constitutive rather than stress-induced. Even some of the proteins detected in total protein extracts, under drought stress, are lost in the boiling-stable fractions (Pelah et al. 1995). Therefore, to better understand the role of these proteins in abiotic stress tolerance, it is a prerequisite to examine their expression not only under water stress, but also after boiling. Thereafter, the sequencing of the relevant proteins and cloning of the corresponding genes will generate probes for early selection and making abiotic stress resistant genotypes. Therefore, In the continuation of our previous studies (Sharma et al. 2004 Sharma et al. 2005; Sharma and Kaur 2007; Sharma et al. 2007; Sharma and Kaur 2008; Sharma 2010; Sharma and Rakhra 2011), in the present study, we analyzed boiling stable acid phosphatase activity (BsAPase activity) and boiling stable protein (BsP) profiles in the embryos and endosperm of wheat under 5 µm (low phosphate; LP) and 1000 µm (high phosphate condition; HP) KH_2PO_4 solutions.

Materials and Methods

Seed germination and growth conditions

Washed grains of *Triticum aestivum* were surface sterilized with 1% (w/v) mercuric chloride followed by 70% ethanol. Seeds were thoroughly rinsed with deionized water and imbibed for 6 h. After imbibition, seeds were placed in Petri plates containing sterile filter sheets and were incubated at 25 ±1°C in a seed germinator for 12-h. Low phosphate stress (LPS) was imposed by irrigating 12-h germinated seed plates with 5uM KH_2PO_4 solution for 12-h, whereas as control plates were irrigated with 1000 µM KH_2PO_4 solution (HPS). Low phosphate stress (LPS) was relieved again by irrigating LPS plates with 1000 µM KH_2PO_4 solution for 6-h. For biochemical analysis, tissues (embryos and endosperm) from each replicate (40 each/plate) independently of other replicate were combined and used for further studies.

Extraction of boiling stable acid phosphatases

Tissues were homogenized with chilled mortar and pestle in extraction buffer [50 mM sodium acetate buffer (pH 5.0)]. Crude extracts were boiled at 100°C for 10 min, and stored on ice for 10 min followed by centrifugation at 10,000 g for 10 min. Total protein content in the supernatant was determined by the Bradford method (Bradford 1976) using BSA as a standard. The SDS-PAGE analysis of boiling stable proteins (BsP) was carried out as per instructions given in Sambrook et al. (1989).

Estimation of boiling stable acid phosphatase activity (BsAPase activities)

The tissue was ground with mortar and pestle at 0-4°C using 50 mM sodium acetate buffer (pH 5.0) as described above. Phosphatase activities were assayed by measuring the amount of p-nitrophenol produced. Phosphatase activities were measured spectrophotometrically at 410 nm in a final volume of 1 ml. The reaction mixture contained 300 µl of enzyme extract, 0.05 M buffer [Sodium acetate (pH5.0)], 0.1 M NaCl and 0.2 mg/ml BSA, with 5 mM para-nitrophenylphosphate (pNPP) as a substrate. The time of reaction was 10 min. The reaction was stopped by adding 1.5 ml of 0.25 M NaOH. The liberated p-nitrophenol (pNP) was determined at 410 nm and calibration curve of pNP prepared in the same conditions. One unit (U) of BsAPase activity is equivalent to the amount of enzyme liberating 1 µmole of product per min under assay conditions.

Extraction and assay of phosphorous (Pi)

For determination of total soluble Pi, only fresh tissue samples were used, which were homogenized with 5 ml of 10% (v/v) $HClO_4$ at 4ºC. After centrifugation at 5000 g at 4ºC, the supernatant was collected for analysis of Pi. The Pi content of the resultant soluble fraction was measured by the formation of a blue molybdenum complex as described by Tsvetkova and Georgiev (2003). Briefly, appropriate aliquots were mixed with 5 ml 0.1 M acetate buffer pH 4.0, 0.5 ml 1% (w/v) ammonium molybdate in 0.05 N H_2SO_4, 0.5 ml 1% (w/v) Na-ascorbate. To avoid the delay in the conversion of the blue color of molybdate- phosphoric complex, 1 mM $CuSO_4.5H_2O$ was added into the ascorbate solution. The blue color of the complex was obtained after 10 min and the absorption was determined using spectrophotometer at 620 nm.

Statistical analysis

A statview ANOVA program was used for statistical analysis of the data. Values for different treatments with in each tissue were compared using one-way analysis of variance with repeated measures and student's *t*-test for differences between pairs of data if the ANOVA ($LSD_{0.05}$) revealed significance. Means were tested by LSD at P 0.05 level ($LSD_{0.05}$).

Results and Discussion

Low availability of phosphate (Pi) to plants in soil is a major constrain which determines plant growth, metabolism, development, reproduction and crop yield. Pi is well known as an essential nutrient in many plant metabolic and gene regulatory pathways. Pi deficiency is a common stress condition experienced in many different environments. Adaptation to Pi stress is often associated with many morphological and biochemical changes at protein level, however, the role of boiling stable APases is still not well documented. Hence,

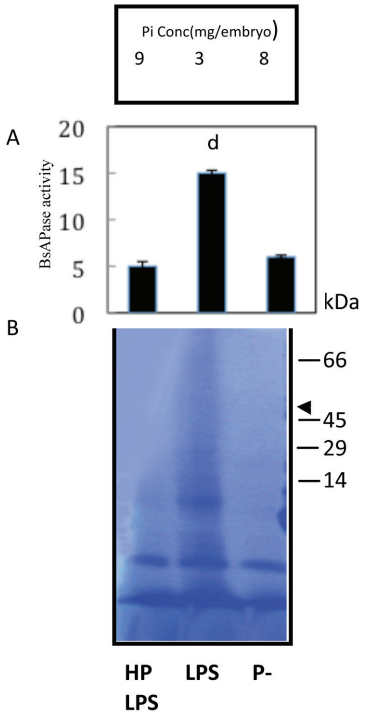

Figure 1. Boiling stable acid phosphatase (BsAPase, µmoles min^{-1}) activity (A), boiling stable protein (BsP) profile (B) of embryos under different LPS, HP and P-LPS conditions. Symbols: LPS: Low phosphorous stress; HP: high phosphorous condition; P-LPS: Post LPS condition. Data shown are average ± SE of three replicates. [d]indicates significant difference vs HP at P≤0.05. Each lane loaded with 60µg of total soluble proteins was resolved on 12% SDS-PAGE.

the present investigation monitored changes caused by low Phosphate stress (LPS) on boiling stable acid phosphatases (BsAPase) in wheat embryos and endosperm. Normally, salt and water stresses affect the physiology and biochemistry of plant cells under *in vitro* and *in vivo* conditions. These stresses have been reported to enhance acid phosphatase activity in pea and wheat (Barrett-Lennard et al. 1982). As shown in Figure 1A, upon imposition of LPS as compared to HP condition, the embryo BsAPase activity increased significantly during Pi nutrient depletion, indicating that BsAPases plays a pivotal role in the Pi metabolism during Pi stress. Internal Pi concentration in the extracted embryos was substantially higher under HPS conditions than LPS. Similar trend was also observed in endosperm (Fig. 2A). This data corroborate previous studies (Yao et al. 2010), where Pi deprivation resulted in 2- to 3-fold increase in APase activity. It may be due to fact that under conditions of Pi stress, growth is restricted and delivery of phosphate is impaired, resulting in the activation of the cellular phosphatases that release soluble phosphate from its insoluble compounds inside or outside of the cells thereby modulate free phosphate uptake mechanism. In embryos, SDS-PAGE analysis of boiling stable proteins

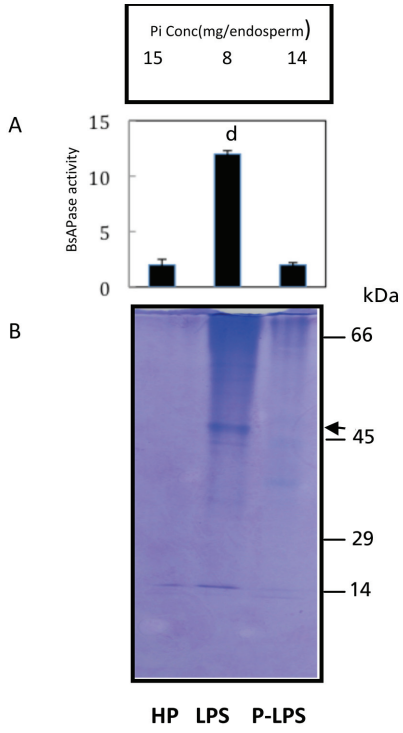

Figure 2. Boiling stable acid phosphatase (BsAPase, µmoles min^{-1}) activity (A), boiling stable protein (BsP) profile (B) of endosperm under different LPS, HP and P-LPS conditions. Symbols: LPS: Low phosphorous stress; HP: high phosphorous condition; P-LPS: Post LPS condition. Data shown are average ± SE of three replicates. [d]indicates significant difference vs HP at P≤0.05. Each lane loaded with 60µg of total soluble proteins was resolved on 12% SDS-PAGE.

(BsP) revealed the presence of few low mt proteins, however, imposition of LPS resulted in the induction of a differential protein band BsP-45 (about 45 kDa), whereas, no BsP-45 was detected under HP conditions. In endosperm, under HP conditions, no substantial protein bands were observed, however, under LPS conditions, a strong differential band (about was observed, again indicating key role of Pi-induced BsPs in low phosphorous adaptation. Olmos and Hellin (1997) observed that acid phosphatases are known to act under salt and water stress by maintaining a certain level of inorganic phosphate which can be co-transported with H^+ along a gradient of proton motive force.

In order to gain further insight into the physiological changes occurred after low Pi stress conditions, we studied post-LPS studies on boiling stable acid phosphatases activities and protein profiles in wheat embryos and endosperm. In earlier research, Gill and Singh (1985) has reported that germination, growth, respiration and other related processes can be affected in seeds that are subjected to environmental stresses. Changes in anyone of these processes can affect other metabolic activities, particularly the enzymes of phosphate metabolism that plays an important role in germination and seed development (Fincher 1989). So as shown in Fig 1A,B, to adapt to low Pi deficiency stress, embryos and endosperm exhibited higher BsAPase activity accompanied by protein band induction. In contrast, Pi re-supply to –Pi plants rapidly represses BsAPase activity accompanied by high internal tissue Pi levels (top panel, Figs. 1 and 2). Similarly in SDS-PAGE, BsP-45 kDa protein band disappeared upon removal of LPS condition (Figs. 1, 2 B), indicating specific role of BsAPases under LPS conditions. Earlier study (Francisca et al. 2008) also demonstrated induction of AtPAP26 during Pi stress and repression of APase genes upon re-supply of Pi. Moreover, Francisca et al. 2008 also showed that ecotopic expression of a soybean mitochondrial PAP (GmPAP23) conferred resistance not only to low Pi but also to oxidative stress in transgenic Arabidopsis plants.

In conclusion, our study indicates that changes observed under LPS condition is associated with adaptation of plants to different environmental conditions that lead to increase or decrease in synthetic activity and associated changes. Further, investigations are needed to enhance our understanding on the effect of LPS stresses and growth hormones during early seed development. Detailed immuobloting studies with more drought tolerant and susceptible cultivars will reveal the potential of this protein as a marker for Pi tolerance.

Acknowledgements

This work was supported by a grant-in-aid for research work from University Grant Commission, New Delhi, India.

References

Barrett-Lennard ED, Robson AD, Greenway H (1982) Effect of phosphorous deficiency and water deficit on phosphatase activities from wheat leaves. J Exp Bot 33:682-693.

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. Plant Cell 7:1099-1111.

Bradford MM (1976) A rapid and sensitive method for quantitation of microorganism quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Bray EA (2002) Abscisic acid regulation of gene expression during water-deficit stress in the era of the Arabidopsis genome. Plant Cell Environ 25:153-161.

Close TJ, Fenton KAA, Chandler PM (1989) A cDNA based comparisons of dehydration-induced proteins (dehydrins) in barley and corn. Plant Mol Biol 13:95-108.

Ciereszko I, Żebrowska E, Ruminowicz M (2011) Acid phosphatases and growth of barley (*Hordeum vulgare* L.) cultivars under diverse phosphorus nutrition. Acta Physiologiae Plantarum 33:2355-2368

Dick CF, Dos-Santos ALA, Meyer-Fernandes JR (2011) Inorganic Phosphate as an Important Regulator of Phosphatases. Enzyme Res 2011: 103980.

Ehsanpour AA, Amini F (2003) Effect of salt and drought stress on acid phosphatase activities in alfalfa (*Medicago sativa* L.) explants under in vitro culture. Afr J Biotechnol 2:133-135.

Fincher GB (1989) Molecular and cellular biology association with endosperm mobilization in germination cereal grains. Annual Rev Plant Physiol Plant Mol Biol 40:305-346.

Francisca Li WY, Shao, G, Lam H (2008) Ectopic expression of GmPAP3 alleviates oxidative damage caused by salinity and osmotic stresses.

New Phytol 178:80-91.
Gill KS, Singh OS (1985) Effect of salinity on carbohydrate metabolism during paddy (*Oryza sativa*) seed germination under salt stress condition. J Exp Biol 23:384-386.
Jacobsen JV, Shaw DC (1989) Heat-stable proteins and Abscisic acid action in barley aleurone cells. Plant Physiol 91:1520-1526.
Li G, Xue WX (2007) *Arabidopsis PLDζ2* regulates vesicle trafficking and is required for auxin response. Plant Cell 19:281-295.
Ma XF, Wright E, Ge Y, Bell J, Xi Y, Bouton J H, Wang ZY (2009) Improving phosphorus acquisition of white clover (*Trifolium repens* L.) by transgenic expression of plant-derived phytase and acid phosphatase genes. Plant Sci 176:479-488.
Olmos E, Hellin E (1997) Cytochemical localization of ATPase plasma membrane and acid phosphatase by cerium -based in a salt-adapted cell line of *Pisum sativum*. J Exp Bot 48:1529-1535.
Pelah D, Shoseyov O, Altman A (1995) Characterization of BspA, a major boiling stable water stress responsive protein in aspen (*Populus tremula*). Tree Physiol 15: 673-678.
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, USA, pp. 18.64-18.75.
Sharma Arun Dev, Thakur M, Rana M, Singh K (2004) Effect of plant growth hormones and abiotic stresses on germination, growth and phosphatase activities in *Sorghum bicolor* (L.) Moench seeds. African J Biotechnol 3:308-312.
Sharma AD, Singh N, Kang JK (2005) Short-term waterlogging-induced changes in phosphatase activities in shoots and roots of sorghum seedlings: role of phosphatases during waterlogging in relationship with phosphorus. General Applied Plant Physiol 31:71-79.
Sharma AD, Kaur R (2007) Drought-induced changes in acid phosphatase activities in wheat in relationship with phosphorus. Emiratus J Agricultural Sciences 19:31-38.
Sharma AD, Kaur P (2008) Drought-stress induced changes in the expression of acid phosphatases in drought tolerant and susceptible cultivars of wheat. World J Agricultural Sciences 4:471-475.
Sharma AD (2010) Expression analysis and Cloning of TaPase phosphatase gene in wheat (*Triticum aestivum*). Emiratus J Agricultural Sciences 22: 483-489
Sharma AD, Rakhra G (2011) Abiotic-stress induced studies on boiling stable acid phosphatases activities (bsA-Pases) in *Triticum aestivum.* Asian J Microbiol Biotechnol Environmental Sciences 13:1-16.
Shinozaki K, Shinozaki-Yamaguchi K (1996) Molecular responses to drought and cold stress. Curr Opinion In Biotechnol. 7:161-167.
Vance CP, Uhde-stone C, Allan DL (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Phytol 157:423-447.
Yan X, Liao H, Melanie CT, Steve EB, Lynch JP (2001) Induction of a major leaf acid phosphatase does not confer adaptation to low phosphorous availability in common bean. Plant Physiol 125:1901-1911.
Yao Y, Sun H, Xu F, Zhang X, Liu S (2010) Comparative proteome analysis of metabolic changes by low phosphorus stress in two *Brassica napus* genotypes. Planta 233:523-537
Zhang H, Huang Y, Ye X, Shi L, Xu F (2009) Genotypic differences in phosphorous acquisition and the rhizosphere properties of *Brassica napus* in response to low phosphorous stress. Plant Soil 320:91-102.