Seed Science Research (2001) **11**, 285–291 DOI: 10.1079/SSR200185

Generation of low phytic acid *Arabidopsis* seeds expressing an *E. coli* phytase during embryo development

Patricia Coello*[1,6], Jeffrey P. Maughan[4], Alex Mendoza[1], Reena Philip[3,5], David W. Bollinger[2], Trygve L. Veum[2], Lila O. Vodkin[3] and Joe C. Polacco[1]

Departments of [1]Biochemistry and [2]Animal Sciences, University of Missouri-Columbia, Columbia, MO 65211; [3]Department of Crop Science, University of Illinois, Urbana, IL 61801; [4]Monsanto Corporation, Ankeny, IA 50021; [5]Gene Logic Inc., 708 Quince Orchard Road, Gaithersburg, MD 20878, USA

Abstract

An *Escherichia coli* phytase gene was introduced into *Arabidopsis* plants using an embryo-specific promoter and a signal peptide for vacuolar targeting. Three independent transgenic lines were analysed. Phytase activity in dry seeds was observed in transgenic lines, whereas no activity was detected in control, untransformed seeds. Transgenic seeds expressing the phytase gene had lower levels of phytic acid than the controls. Concomitant with the decrease in phytic acid was an increase in free phosphate. These results indicated that embryo-expressed phytase can reduce the levels of phytic acid stored during development.

Keywords: *Arabidopsis*, phytase, phytic acid, *Escherichia coli*

Introduction

Phytic acid (PA) is the major form of phosphorus (P) in the seeds of most plants (Maga, 1982). In seed meal, PA (*myo*-inositol [1,2,3,4,5,6] hexa*kis*phosphate) is unavailable to monogastric animals, such as pigs, humans, chickens and fish, and thus becomes a major source of P pollution in large animal operations (Cromwell and Coffey, 1991).

In addition to restricting P availability to the animal, PA complexes divalent cations (Honig and Wolf, 1991). If PA were converted to available P and minerals during the natural maturation of soybean seeds, the savings to swine and poultry producers in dicalcium phosphate supplementation would be approximately US$50 million (Lobo, 1999). This could be achieved, at a one-time cost, by engineering seeds to produce their own phytase during embryo development. By this strategy, newly made PA would be rapidly broken down to phosphate and more digestible low-P esters of inositol.

In this study, an *Escherichia coli* phytase, normally a periplasmic protein, was targeted to the vacuole of *Arabidopsis thaliana*, the reported site of PA accumulation (Lott *et al*., 2000), as a way to obtain low-PA seeds.

Materials and methods

Preparation of a vacuolar-targeted, seed-specific construct of E. coli *phytase gene* appA

The *E. coli appA* gene (Dassa *et al*., 1990) was amplified by polymerase chain reaction (PCR) from *E. coli* genomic DNA, using the following conditions: denaturation at 95°C for 1 min, primer annealing at 55°C for 30 s and polymerization at 72°C for 30 s. The primers used for the amplification were: 5′GTCTAG**GCGGCCGC**CCAGAGTGAGCCGGAGC TG-3′ (N-terminus) and 5′-TTATATG**GCGGCC GC**TTACAAACTGCACGCCGG-3′ (C-terminus). The *Not*I eight-base restriction site is shown in bold; in the N-terminal sequence it precedes the first amino acid (the underlined CAG = glutamine) of the AppA mature periplasmic protein, and in the C-terminal sequence it immediately follows the TAA stop codon (underlined as the reverse complement TTA). The amplified ~1.3 kb fragment (which corresponds to the gene size) was digested with *Not*I endonuclease, cloned into the *Not*I site of pBluescript SK$^+$ and introduced into *E. coli* strain RR1. The sequence of the ends of the fragment was determined to ensure that the open reading frame (ORF) was in frame with the *lacZ* α fragment (27 amino acids upstream of mature AppA), and 5′ to 3′ with respect to the *lac* promoter. After

*Correspondence
Fax: 5622 5329
Email: pcoello@servidor.unam.mx
[6]Present address: Departamento de Bioquímica, Facultad de Química, Universidad Nacional Autónoma de México (UNAM), Mexico, D.F. 04510

induction of the *lac* promoter with 0.3 mM isopropyl β-thiogalactoside (IPTG) by overnight incubation at room temperature in Luria Broth (LB), the cells were osmotically shocked to release endogenous *appA*-encoded periplasmic protein. The cells were then lysed by sonication, and intracellular, recombinant phytase was detected by an activity stain (Elliott *et al*., 1986) of proteins resolved on a 8% native polyacrylamide gel. After electrophoresis the gel was washed in 100 mM sodium acetate, pH 4.8, and incubated in 2 mM α-naphthyl phosphate and 1.5 mM Fast Garnet dye in the same buffer overnight in the dark.

The mature *E. coli appA*-coding sequence was excised from pBluescript SK$^+$ with *Not*I and cloned into the *Not*I site of plasmid pGL-3 (Cho *et al*., 1995), placing the open reading frame in frame with a 32-amino-acid lectin signal sequence shown to be sufficient for vacuolar targeting of β-casein in transgenic soybean seeds (Philip *et al*., 2001). The hybrid coding sequence was under control of the lectin promoter and transcriptional terminator. This entire region was excised with *Xba*I and cloned into the *Xba*I site of pBin19 (Bevan, 1984) to produce the binary plasmid pBinGLPH7–5, which was introduced into disarmed *Agrobacterium* strain GV3101 by freeze-thawing. *Agrobacterium* carrying the GLPH7–5 vector was used to transform *Arabidopsis thaliana* by vacuum infiltration (Bent and Clough, 1998), and putative transformants were selected as kanamycin-resistant seedlings.

Other nucleic acid techniques

In vitro transcription/translation of the lectin phytase cassette (pGLPH-7 plasmid DNA) was performed with a Promega (Promega, Madison, WI, USA) TNT Quick Coupled Transcription/Translation System that uses a rabbit reticulocyte lysate translation mix. Approximately 2 μg of DNA was used per reaction. Out of 50 μl of translated products, 10 μl was mixed with an equal volume of 2× gel loading buffer (2% sodium dodecyl sulphate (SDS), 10% glycerol, 100 mM 1,4-dithiothreitol (DTT), 60 mM Tris–HCl, pH 6.8), and was loaded on to 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). [^{14}C]-labelled protein molecular weight high-range standards from Life Technology (GIBCO BRL, Gaithersburg, MD, USA) were used as markers on the protein gel. The gel was dried and autoradiographed.

Southern blots were performed at high stringency as described by Coello *et al*. (1999). Briefly, genomic DNA was isolated from *Arabidopsis* seedlings according to Sambrook *et al*. (1989). Twenty micrograms of DNA were digested with *Not*I at 37°C overnight and then separated on a 1% agarose gel. The DNA was transferred to nitrocellulose and hybridized at high stringency in 50% formamide. The filter was dried and exposed for 1 d to Kodak X-Omat X-ray film (Fisher Scientific) with intensifying screens.

Assays for phytic acid, phosphate and phytase levels in Arabidopsis *seeds*

Phytic acid (PA) was determined essentially as described by Wade and Morgan (1953), except that extracts (in 0.5 N HCl, pH approx. 0.3) were unfractionated. Wade's reagent is yellow due to a complex of 5-sulphosalicylate with acidic $FeCl_3$. PA competing with 5-sulphosalicylate produces an insoluble white precipitate with $FeCl_3$, reducing absorbance at 500 nm. Since Wade's reagent also reacts weakly with phosphate, values were corrected for phosphate determined by the molybdo-vanadate method (AOAC, 1990). The adjusted PA values were thus reduced by 1–2%. While no correction was made for inositol 5-phosphate and inositol 4-phosphate (intermediate PA breakdown products), confirmation of phytate values in some cases was by AOAC method 986.11 (AOAC, 1990), which gave values about 75% of those reported here.

To determine phytase levels, extracts were prepared by grinding 25 mg of seeds in 1 ml extraction buffer (0.1 M sodium acetate, pH 5.5, 1 mM $CaCl_2$, 0.1 mg ml^{-1} Tween 20). Phytase was assayed as described in Engelen *et al*. (1994). A unit of phytase is defined as the activity that liberates 1 μmole phosphate from PA per min at 37°C (Liu *et al*., 1997).

Characterization of optimal reaction temperatures and pH of E. coli *phytase expressed in* Arabidopsis

These determinations were a modification of the extraction and assay of the *E. coli appA*-encoded phytase (Greiner *et al*., 1993). *Arabidopsis* seeds (25 mg) were ground in 1 ml 0.1 M sodium acetate, pH 5.5, 1 mM $CaCl_2$, 0.1 mg ml^{-1} Tween 20 in a small mortar, followed by centrifugation for 5 min at 12,000 ***g***. To determine the optimal reaction temperature, 10 μl of supernatant was added to 350 μl of 0.1 M sodium acetate, pH 4.5, containing 500 nmol sodium phytate. Reactions were run at various temperatures for 30 min and stopped by adding a mixture of 1.5 ml acetone:5 N H_2SO_4:10 mM ammonium molybdate (2:1:1, v/v) and 100 μl 0.1 M citric acid. To determine the optimal pH, the reaction was run for 30 min in various buffers, all at 0.1 M: glycine/HCl (pH 1–3), sodium acetate/NaOH (pH 4–5), Tris/H-acetate (pH 6–7) and Tris/HCl (pH 7–9). Each pH value was adjusted in the presence of PA. Reaction rates were linear for the 30-min assay period.

https://doi.org/10.1079/SSR200185 Published online by Cambridge University Press

Results

Construction of an E. coli *phytase transgene for embryo-specific expression and vacuolar targeting in* Arabidopsis *seeds*

E. coli phytase (Greiner *et al*., 1993) contains an N-terminus identical in 11 of 12 amino acids to the deduced sequence of a pH 2.5 acid phosphatase encoded by *appA* (Dassa *et al*., 1990). The purified activity qualified as a phytase rather than a relatively non-substrate-specific phosphatase by its much greater catalytic efficiency against phytate (k_{cat}/K_m = 4.8 × 10^7 s^{-1} M^{-1}) versus *p*-nitrophenyl phosphate (k_{cat}/K_m = 6.3 × 10^4 s^{-1} M^{-1}) (Greiner *et al*., 1993). This periplasmic protein is synthesized as a precursor, whose N-terminal signal peptide is cleaved upon movement to the periplasmic space (Dassa *et al*., 1990). We removed the signal peptide in AppA expressed under *lac* promoter control (Materials and methods). Transformation of *E. coli* RR1 with this construct resulted in the cytoplasmic expression of acid phosphatases. This activity is shown in the gel of Fig. 1, in extracts of cells from which the endogenous periplasmic acid phosphatases were released by osmotic shock.

The mature open reading frame of *appA* was cloned into plasmid pGL-3 (Cho *et al*., 1995) by placing it in frame with an N-terminal soybean seed lectin signal sequence, under the control of the seed lectin promoter, to create plasmid pGLPH7–5. The intactness of the construct was tested by sequencing through the fusion and by *in vitro* transcription/translation to examine the size of the protein product produced. Figure 2A shows that a 45 kDa product was obtained by *in vitro* transcription and translation of the pGLPH7–5 DNA. This result agrees with the size expected of the fusion of the *E. coli* phytase to the 3 kDa lectin signal sequence. The empty pGL-3 progenitor expression cassette, containing only the signal sequence, did not produce any labelled polypeptide, while an apparent 30 kDa polypeptide was produced from pGLCH-2, a plasmid that contains the β-casein protein cloned into pGL-3.

The *Xba*I fragment of the lectin–phytase construct was cloned into pBin19 to derive pBinGLPH7–5 (Fig. 2B) and subsequently introduced into *Agrobacterium tumefaciens* for transformation of *A. thaliana* by vacuum infiltration (Clough and Bent, 1998). The presence of the phytase transgene was confirmed by probing genomic DNA of plants transmitting kanamycin resistance. Genomic DNA from two putative transformants (T_3 generation) was digested with *Not*I (flanking the phytase mature sequence); both contained a *Not*I DNA fragment of the predicted size (1.3 kb) which hybridized to the bacterial *appA* (Fig. 3). The differences in band intensity in both lines may be correlated to the copy number of the transgene.

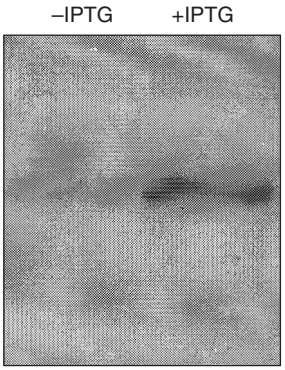


Figure 1. *E. coli* periplasmic phytase (AppA) expressed as an intracellular protein. The mature portion of the *E. coli appA* gene (1.3 kb) was PCR-amplified with *NotI* termini, cloned into pBluescript SK^+ and introduced into *E. coli* strain RR1. After induction of the *lac* promoter with isopropyl β-thiogalactoside (IPTG), cells were osmotically shocked to release endogenous *appA*-encoded periplasmic protein. The cells were then sonicated and intracellular phytase activity was determined by an activity stain (Elliott *et al*., 1986) of proteins (40 µg per lane) resolved on a native gel.

Expression and characterization of phytase activity in transgenic Arabidopsis *seeds*

Three independent transgenic lines were homozygous, as demonstrated by progeny testing; none segregated as white, kanamycin-sensitive seedlings (data not shown). Seeds from these three transgenic lines exhibited significant phytase activity, in contrast with control seeds in which there was virtually no detectable phytase activity (Table 1). Transgenic seed phytase exhibited a pH optimum of pH 4–4.5 (Fig. 4) and a temperature optimum of 50°C (Fig. 5).

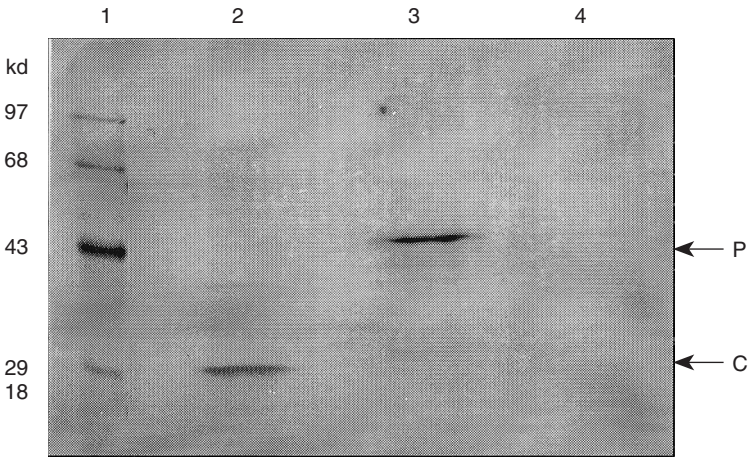
PA and phosphate levels in transgenic Arabidopsis *seeds*

Free phosphate levels were increased three- to fourfold in three independent transgenic lines, while PA content was reduced to approximately one-third the level of the progenitor (Table 1). Thus, at least a portion of the PA produced during embryogenesis was a substrate for the product of the *appA* phytase transgene. Two different procedures for determinations for PA differed by 25% (Materials and methods), suggesting that *myo*-inositol phosphate derivatives of PA, containing fewer than six phosphates, may be differentially detected by the two procedures.

Discussion

PA represents about 75% of total P in grains, but is essentially unavailable to non-ruminants (Lott *et al*.,

https://doi.org/10.1079/SSR200185 Published online by Cambridge University Press



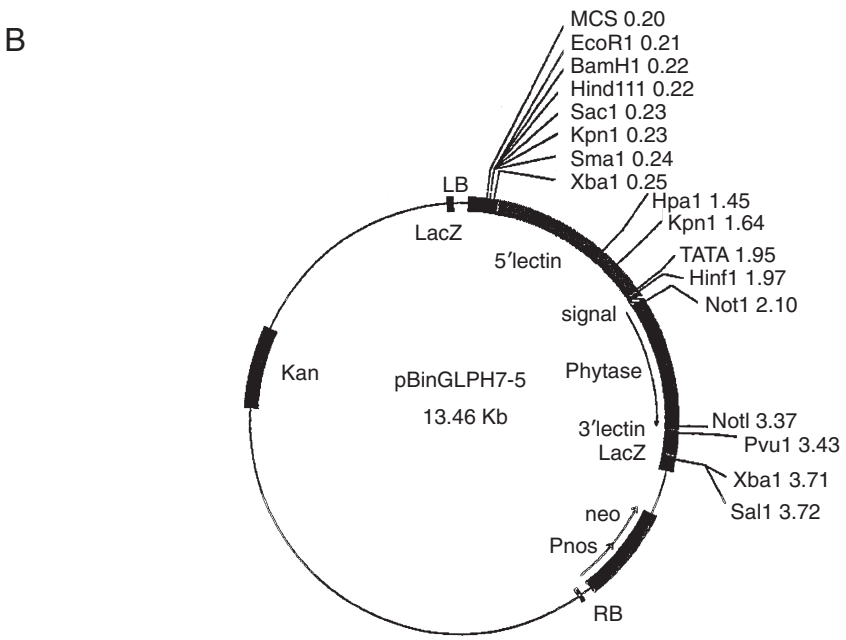


Figure 2. Plasmid pBinGLPH7–5. (A) *In vitro* transcription/translation of the lectin–phytase plasmid. Lane 1: [^{14}C]-labelled protein molecular weight standards. Lane 2: translation products of pGLCH-2 (a casein protein cloned in the lectin cassette). Lane 3: translation products of pGLPH-7, the lectin–phytase construct. Lane 4: translation products using the empty cassette pGL-3. The arrow marked P is the position of the *in vitro* translated 45 kDa phytase, and the C arrow marks the position of the *in vitro* translated 30 kDa casein polypeptides. (B) The *E. coli appA* mature phytase is in frame with an N-terminal soybean seed lectin vacuolar targeting sequence under the control of the seed lectin promoter (Cho *et al.*, 1995).

2000). Mutants are available that have reduced levels of seed PA with or without reducing grain total P (Raboy *et al.*, 2000). Initial breeding efforts to develop low-PA maize hybrids and barley cultivars used selected *lpa1* mutations that have little effect on plant growth and vigour and caused 50–75% reduction in PA (Ertl *et al.*, 1998).

In this study, another strategy was chosen to lower the levels of seed PA, by targeting the *E. coli* phytase to the vacuole or protein body, since this is the reported location of phytin, a PA–protein complex (Gabard and Jones, 1986; Monma *et al.*, 1992) We chose the 32-amino-acid lectin signal peptide since it appears to be sufficient for vacuolar targeting. In transgenic soybean expressing the same cassette used in this study, the lectin signal peptide directed β-casein to seed vacuoles (Philip *et al.*, 2001).

Transgenic *Arabidopsis* seed PA was reduced by 60% compared to untransformed controls. These data are consistent with a vacuolar location for phytase. Recently, we showed that the green fluorescent protein, fused to the lectin signal peptide, was found

https://doi.org/10.1079/SSR200185 Published online by Cambridge University Press

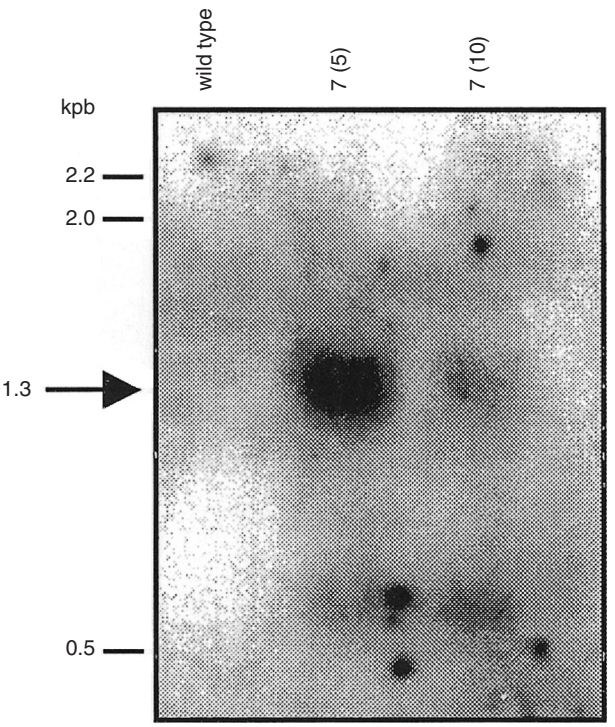


Figure 3. Presence of *appA* in transformed lines of *Arabidopsis*. *Not*I-digested genomic DNA (20 µg) from wild-type and kanamycin-resistant T_3 plants was electrophoresed, blotted and hybridized to the mature periplasmic ORF of *appA*. Southern blot analysis showed a band of approx. 1.3 kb which corresponded to the phytase gene. The wild-type control did not show any hybridization band.

in vacuoles of transgenic *Arabidopsis* seeds (Darnowski and Vodkin, unpublished). The decrease in PA was accompanied by an increase in inorganic phosphate. Differences in phytase activity in different transgenic lines (Table 1) might be related to the copy number of the transgene, suggested by Southern blots of these lines probed with *appA*. However, despite having half of the phytase activity, the GLPH7–5 (10) line had a similar reduction in phytic acid, suggesting that phytase activity in this line is enough to decrease PA levels. Seed PA was probably not hydrolysed by recombinant phytase during extraction since the two extraction methods employed 2.4% (AOAC, 1990) and 0.5 N (Wade and Morgan, 1953) HCl, at pHs equivalent to 0.3 and 0.18, respectively. *E. coli* phytase exhibits no activity at these pHs (Greiner *et al*., 1993). The lack of additivity of P in phosphate and PA, compared to the PA value in Columbia (Table 1) may be due to undetected *myo*-inositol tetra- and pentaphosphate derivatives of PA. If this is indeed the case, production of these more digestible lower phosphate esters of *myo*-inositol will make total P more available.

Lines with lower, or zero, seed PA levels were not recovered in these experiments. Severe reductions in seed PA may diminish seed viability; the transgenic lines of this study had germination frequencies equal to approximately 75% of those of wild-type seeds (data not shown). We are currently quantifying germination, using transformed lines lacking the phytase construct as a control. However, from a practical point of view, it may be feasible to select, or breed, better germinability of high-phytase lines. Another approach for improved germination, which we are currently examining, is to target phytase to the cytosol.

Another advantage of the presence of an active phytase in mature seeds is that this activity can continue to break down residual PA in the meal from the same seed, or in admixed non-transgenic seed meal; soaking increases the availability of PA phosphorus by microbial phytase in a low corn–soybean meal diet for growing pigs (Liu *et al*., 1997).

There was no loss in phytase activity in transgenic seed extracts over a 20-min incubation at 50°C, while 40% of the activity was lost at 55°C and <5% activity was detectable after incubation at 70°C (data not shown). While this thermostability is somewhat less than that reported for purified *E. coli* AppA phytase, it suggests that ground transgenic seed meal may reduce PA levels in seed meal, especially during a pre-feed soaking. Transgenic seed phytase conserved at least two other characteristics of the native *E. coli* phytase. The pH and temperature optima of pH 4.5 and of 55°C reported for the purified *E. coli* enzyme (Greiner *et al*., 1993) agree well with the values of the

Table 1. Phytase, phosphate and phytic acid levels in transgenic *Arabidopsis* seeds

	Biovar Columbia (progenitor)	Vacuolar phytase lines		
		GLPH7–5 (5)	GLPH7–5 (13)	GLPH7–5 (10)
Phytase activity[a]	2 ± 3 (n = 4)	1611 ± 112 (n = 6)	1171 ± 46 (n = 2)	664 ± 63 (n = 4)
Free PO_4 (mg g seed^{-1})[b]	0.7 ± 0.05 (n = 4)	2.8 ± 1.2 (n = 4)	2.6 ± 0.9 (n = 4)	2.5 ± 1.0 (n = 4)
PA (mg g seed^{-1})[b]	32.5 ± 3.9 (n = 4)	11.5 ± 3.0 (n = 4)	11.3 ± 1.0 (n = 4)	13 ± 3.0 (n = 4)

PO_4, phosphate; PA, phytic acid.
[a] Units = µmol phosphate min^{-1} g seeds^{-1} released from phytate at 37°C.
[b] mg elemental P in PO_4 or in PA per g of seeds.

https://doi.org/10.1079/SSR200185 Published online by Cambridge University Press

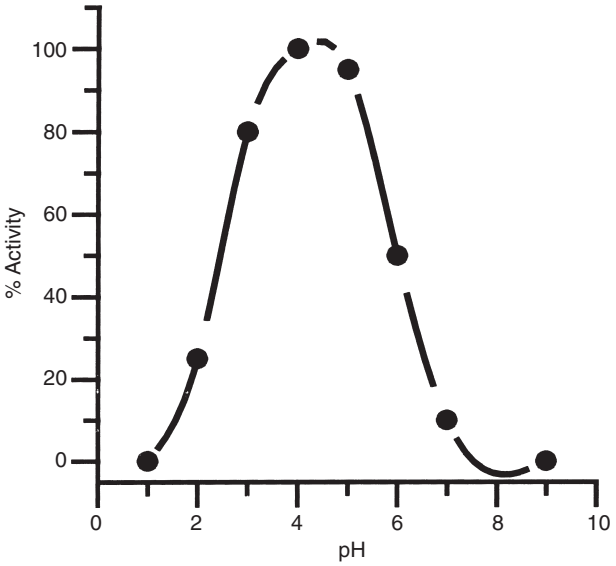


Figure 4. pH dependence of *E. coli* phytase expressed in transgenic *Arabidopsis*. The activity at pH 4.0 was taken as 100% (0.49 µmol P_i min^{-1} g $protein^{-1}$) using sodium phytate as the substrate. Buffers used were: glycine/HCl (pH 1–3), Na acetate/NaOH (pH 4–5), Tris/H-acetate (pH 6–7) and Tris/HCl (pH 7–9).

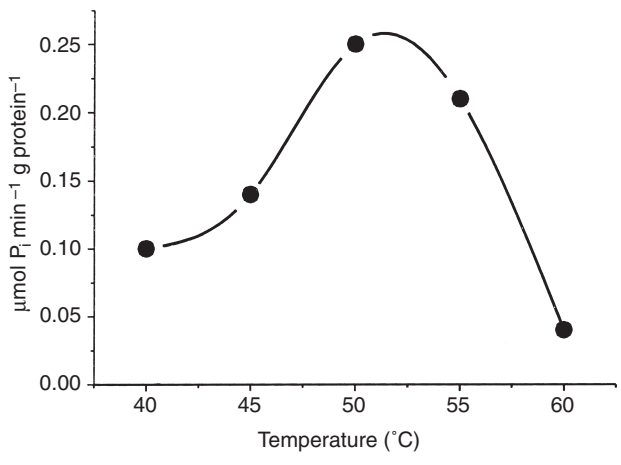


Figure 5. Temperature dependence of *E. coli* phytase expressed in transgenic *Arabidopsis*. Phytase activity in crude seed extracts was assayed for 30 min at the indicated temperatures (see Materials and methods).

recombinant enzyme assayed in crude seed extracts (Figs 4 and 5).

Recently, Brinch-Pederson *et al*. (2000) reported the generation of transgenic wheat carrying an *Aspergillus* phytase. They concluded that the highest-expressing wheat line will be adequate to ensure sufficient phytase activity for an optimal digestion of the PA in wheat-based as well as compound feed.

Demonstrating that an embryo-expressed bacterial phytase can lower PA levels in *Arabidopsis* gives impetus to expressing phytases in other plant systems. We are currently attempting to express phytase in soybean.

Acknowledgement

This work was supported by the Missouri and Illinois Agricultural Experimental Stations and by the Illinois-Missouri Biotechnology Association grant no. 95–34346–1795.

References

AOAC (Association of Official Analytical Chemists) (1990) *Official methods of analysis* (15th edition), pp. 85–88 and 800–801. Arlington, Virginia, Association of Official Analytical Chemists.

Bent, A.F. and Clough, S.J. (1998) *Agrobacterium* germ-line transformation: Transformation of *Arabidopsis* with tissue culture. pp. 22–34 *in* Gelvin, S.B.; Schilperoort, R.A. (Eds) *Plant molecular biology manual*. Dordrecht, The Netherlands, Kluwer Academic.

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research* **12,** 8711–8721.

Brinch-Pederson, H., Olesen, A., Rasmussen, S.K. and Holm, P.B. (2000) Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an *Aspergillus* phytase. *Molecular Breeding* **6**, 195–206.

Cho, M.J., Widholm, J.M. and Vodkin, L.O. (1995) Cassettes for seed-specific expression tested in transformed embryogenic cultures of soybean. *Plant Molecular Biology Reporter* **13,** 255–269.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16**, 735–743.

Coello, P., Sassen, A., Haywood, V., Davis, K.R. and Walker, J.C. (1999) Biochemical characterization and expression of RLK4, a receptor-like kinase from *Arabidopsis thaliana*. *Plant Science* **142,** 83–91.

Cromwell, G.L. and Coffey, R.D. (1991) Phosphorus – a key essential nutrient, yet possible major pollutant – its central role in animal nutrition. pp. 135–145 *in* Lyons, T.P. (Ed.) *Biotechnology in the feed industry*. Nicholasville, Kentucky, Alltech Technical Publishing Co.

Dassa, J., Marck, C. and Boquet, P. (1990) The complete nucleotide sequence of the *Escherichia coli* gene *appA* reveals significant homology between pH 2.5 acid phosphatase and glucose 1-phosphatase. *Journal of Bacteriology* **172**, 5497–5500.

Elliott, S., Chang, C.W., Schweingruber, M.E., Schaller, J., Rickli, E.E. and Carbon, J. (1986) Isolation and characterization of the structural gene for secreted acid phosphatase from *Schizosaccharomyces pombe*. *Journal of Biological Chemistry* **261**, 2936–2941.

Engelen, A.J., Van der Heeft, F.C., Randsdorp, P.H.G. and Smit, E.L.C. (1994) Simple and rapid determination of phytase activity. *Journal of AOAC International* **77**, 760–764.

Ertl, D.S., Young, K.A. and Raboy, V. (1998) Plant genetic approaches to phosphorous management in agricultural production. *Journal of Environmental Quality* **27**, 299–304.

Gabard, K.A. and Jones, R.L. (1986) Localization of phytase and acid phosphatase isoenzymes in aleurone layers of barley. *Physiologia Plantarum* **67**, 182–192.

Greiner, R., Konietzny, U. and Jany, K.D. (1993) Purification and characterization of two phytases from *Escherichia coli*. *Archives of Biochemistry and Biophysics* **303**, 107–113.

Honig, D.H. and Wolf, W.J. (1991) Phytate–mineral–protein composition of soybeans: gel filtration studies of soybean meal extracts. *Journal of Agricultural and Food Chemistry* **39**, 1037–1042.

Liu, J., Bollinger, D.W., LeDoux, D.R., Ellersieck, M.R. and Veum, T.L. (1997) Soaking increases the efficacy of supplemental microbial phytase in a low-phosphorous corn–soybean meal diet for growing pigs. *Journal of Animal Science* **75**, 1292–1298.

Lobo, P. (1999) Feed potential for layers is up – others down. *Feed Management* **50** (8), 6–17.

Lott, J.N.A., Ockenden, I., Raboy, V. and Batten, G.D. (2000) Phytic acid and phosphorus in crop seeds and fruits: a global estimate. *Seed Science Research* **10**, 11–33.

Maga, J.A. (1982) Phytate: Its chemistry, occurrence, food interactions, nutritional significance and methods of analysis. *Journal of Agricultural and Food Chemistry* **30**, 1–9.

Monma, M., Sugimoto, T., Hashizume, K. and Saio, K. (1992) Biogenesis of protein bodies in embryonic axes of soybean seeds (*Glycine max* cv. Enrei). *Bioscience, Biotechnology and Biochemistry* **56**,1036–1040.

Philip, R., Darnowski, D.W., Maughan, P.J. and Vodkin, L.O. (2001) Processing and localization of bovine β-casein expressed in transgenic soybean seeds under control of a soybean lectin expression cassette. *Plant Science* **161** (2), 323–335.

Raboy, V., Gerbasi, P.F., Young, K.A., Stoneberg, S.D., Pickett, S.G., Bauman, A.T., Murthy, P.P.N., Sheridan, W.F. and Ertl, D.S. (2000) Origin and seed phenotype of maize low phytic acid 1–1 and low phytic acid 2–1. *Plant Physiology* **124**, 355–368.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning: A laboratory manual* (2nd edition). Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press.

Wade, H.E. and Morgan, D.M. (1953) Detection of phosphate esters on paper chromatograms. *Nature* **171**, 529–530.

Received 13 February 2001
accepted after revision 14 July 2001

Vegetable Seed Production, 2nd Edition

R A T George, *former senior lecturer, University of Bath, UK and consultant to UN/FAO, Italy*

November 1999 **336 pages** **Hardback**
ISBN 0 85199 336 2
£49.95 (US$90.00)

Readership: Academics, researchers and students of horticulture, agronomy and seed science. Also growers, extension and advisory agents involved with vegetable seed production.

Successful seed supplies are essential for maintaining adequate vegetable production and availability. Vegetables are vital food security for many subsistence farmers and their dependants in developing countries. In developed countries, vegetable production industries are placing increased emphasis on a supply of high quality seeds.

- This second edition provides a full update since the previous edition (Longmans, 1985)
- Includes examples of vegetable seed projects in developed and developing countries
- Provides detailed descriptions of vegetable seed production arranged in taxonomic families
- Lists organizations concerned with vegetable seed quality, research and development
- Is essential reading for university students, technicians and practitioners of vegetable seed production in both developed and developing countries.

Contents: