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ORIGINAL RESEARCH PAPER

Phytase expression in transgenic soybeans: stable transformation with a vector-less construct

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Abstract A minimal linear gene cassette (35S-phytase gene-nos) with T-DNA borders was acquired by PCR and directly introduced into soybean through the pollen tube pathway. A total of 13% of T_1 plants were positive for *phyA* by specific PCR. Southern blot analyses showed that *phyA* insertions were harbored stably in T_2 progeny. Phytase expression level increased 2.5-fold over a 6-week period; its highest activity was 150 U/mg protein, compared to 56 U/mg protein in untransformed controls. Activity of phytase increased to 125 FTU/kg in T_3 transgenic seeds as compared to 64 FTU/kg in wild-type plants.

Keywords Pollen tube pathway transformation · Linear DNA · Phytase gene · Transgenic soybean

Introduction

Although it has adequate phosphate and protein content to meet animal and human growth requirements, up to 80% of total phosphorus in soybean is contained in phytate during seed maturation (Li et al. 1997) and phytate protein complex accounts for approximately 1% of the total weight in dry seeds (Hegeman and Grabau 2001). Phytate is largely indigestible by monogastric animals because they have no or limited phytase activity in their digestive tracts. The widespread use of soybean meal in livestock feed leads to serious environmental consequences. Introducing phytase genes into soybean meal can improve the bioavailability of phosphates and proteins and reduce phytic acid excretion, providing a less expensive alternative to phytase supplementation.

At present, in almost all processes of plant transformation, dominant selectable markers are used to select transgenic cells. However, this methodology has raised public concerns that the markers will be transferred to other organisms (Nap et al. 2003; Thompson 2000), although there is no evidence suggesting that the currently used antibiotic resistance markers pose any risks to humans, animals or the environment (Miki and McHugh 2004). In addition, routine transformation integrates vector backbone sequences into the genome that could lead to undesirable effects in situ (Kononov et al. 1997; Tingay et al. 1997), such as transgene rearrangements and multiple copies. It is common practice to remove all vector sequences before microinjecting foreign DNA into animal eggs or embryos (Palmiter and Brinster 1986).

The technique of pollen tube pathway transformation has been applied since 1978 (Wang et al. 2004; Luo and Wu 1989). Bt cottons produced by this technique have been commercially available since

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1996. This tissue culture-independent procedure, which is more suitable for crops like soybean with low regeneration rates (Franklin et al. 2004), is simple, rapid and efficient.

This study describes the production of transgenic soybean expressing phytase using minimal linear transgene constructs that lack selectable markers and vector backbone sequences ("clean" DNA). This is the first report using minimal linear DNA fragment technology for the production of transgenic plants.

Materials and methods

Gene constructs

A full length phytase gene (GenBank accession no. ***DQ192035***) was amplified from the strain *Aspergillus awamori* 3.324 by PCR. Phytase gene (*PhyA*) open reading frame was generated using the 5′ end primer 5′-GTTCTAGAGCCATGGGTGTCTCTGCCGTTCTACTTCCTTTGTACCTCCTGTCCGGAAGTCACCTCCGGACTGGCAG-3′ and the 3′ end primer 5′-GCCACGTGCTAAGCAAAACACTCCGCCCAATG-3′. A synthetic Kozak sequence GCCATGG for initiation of translation was placed right upstream of the codon beginning region, and the *Xba*l and *Sac*I restriction sites were introduced into the 5′ and 3′ ends, respectively. The *Sac*I restriction site in *phyA* was modified by PCR (GAGCTC changed to GAACTC). *Xba*I- and *Sac*I-digested *PhyA* was subcloned into the pBI121 (CLONTECH) vector to obtain the expression construct pBI121-*phyA*.

The minimal transgene expression cassette was obtained (Fig. 1) from the above plasmid by PCR using the primer 1: 5′-GTTTACCCGCCAATATATCCTGTCATCTAGAGCCATGGGTGTCTCTGCCGTTC-3′; and primer 6: 5′-TGGCAGGATATATTGTGGTGTAAACA-3′. PCR amplification products were separated on 1% agarose gel and then purified using the Qiaex II agarose gel extraction kit (Qiagen).

Plant material and transformation

Transformation experiments were conducted with Chinese soybean cultivar genotypes Liaodou13, Liaodou14 and Tiefeng29, which were all kindly provided by Liaoning Academy of Agricultural Sciences in China. All experiments were performed on fresh flowers such that the height of the corolla was greater than that of the calyx. Two wing petals and one keel petal were removed to expose the stigmas of soybean flowers. The style and the top of the ovary were cut off, and the exposed ovary was dipped into 7 µl of the gene construct DNA solution (300 µg/ml). Treated flowers were tagged, and untreated flowers and buds at the same node were removed. The pods that developed from the treated flowers were harvested individually. Seeds derived from plants treated with 0.1 × SSC (3 M NaCl, 0.3 M sodium citrate, 1 mM EDTA) were harvested as negative controls.

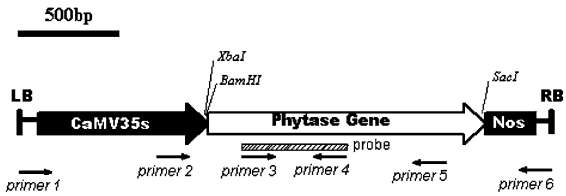

Fig. 1 Schematic maps of the linear construct used in transformation experiments The components contained the CAMV 35S promoter, phytase gene and *nos* terminator, with T-DNA left and right borders (LB and RB). Amplification strategy and restriction maps are shown in scale. Primer1 and 6 used for production of linear construct; primer2, 3 and 4 used in PCR screening; primer3 and 4 used for probe production; primer 2 and 5 used in the RT-PCR strategy

Identification of transgenic plants by PCR

Genomic DNA was extracted from young leaf tissues using a modified CTAB method (Reichandt and Rogers 1994) for PCR analysis. PCR amplifications for detection of the minimal *phyA* expression cassette were achieved with the primer 4 (5′-GAGAGACACGCCAGACAAG-3′) spanning bases 741–759 of *phyA* in combination with either primer 3 (5′-CCAAGGGCAAGAAATACTCC-3′) or primer 2 (5′-CCACTATCCTTCGCAAGACC-3′), which correspond to bases 272–291 of *phyA* and 773–792 of CaMV 35S promoter, respectively (Fig. 1). The expected amplification fragments were 488 bp and 850 bp long.

Southern blot analysis

Genomic DNA was isolated from young leaves of transgenic and untransformed control plants using

Plant DNA Extraction Kit (Roche). Integration of the phytase transgene was analyzed by digesting genomic DNA ($\sim$20 μg) with appropriate restriction enzymes. The digested DNA was separated on a 1% agarose gel and transferred onto Hybond N^+ membranes (Amersham Biosciences). The probe (Fig. 1) for *phyA* was generated by PCR using primer 3 and primer 4, the fragment was purified using the Qiaex II agarose gel extraction kit (Qiagen). Hybridization was performed at 42°C using ECL blotting kit following the manufacturer's instructions (Pharmacia, Germany).

RNA isolation and RT-PCR

RNA was isolated from leaves of 4-week-old plants using Trizol kit (GIBCO) following the manufacturer's instructions, and cDNA was synthesized from 2 μg RNA with the parameters of AMV reverse transcriptase along with Random 9mers (RNA PCR Kit Ver.2.1, TaKaRa). PCR on cDNA was performed using primers 3 and primers 5 (5′-ACAGCCATGCAGCGGAACAA-3′, corresponding to the region of 1292–1311 bp of *phyA*) (Fig. 1). The amplified fragments were 1040 bp long. Actin gene was also amplified as a native control using the forward primer (5′-CTCAACCCAAAGGTCAACAG-3′) and reverse primer (5′-ACCATCACCAGAGTCCAATA-3′).

Phytase activity assay

Total proteins were extracted from leaves (1 mg fresh sample) using 5 ml acetate buffer (50 mM sodium acetate, pH 5.5, 10 mM $CaCl_2$, 1 mM PMSF). The homogenate was centrifuged at 12,000*g* for 20 min at 4°C, the supernatant was collected, and total protein concentration was determined by the Bradford method. Phytase activity was assayed using the molybdate-blue reaction method (Murphy and Riley 1962) and measured at 820 nm. Phytase activities were expressed as Units per mg total protein.

Mature seeds were milled and protein was extracted from 0.25 g flour by adding 2.5 ml 50 mM sodium acetate buffer (pH 5.5), including 10 mM $CaCl_2$, 1 mM PMSF and Tween 20 (100 mg/l). The suspension was vortexed gently for 30 min at 4°C and centrifuged at 5,000*g* for 10 min. The supernatant was collected and assayed for phytase activity as Unit per kg seed.

Enzyme thermal stability

The thermal stabilities of the crude extracts from transgenic plants were compared with untransformed soybeans. All samples were pre-incubated for 10 min 37–90°C in a reaction mixture without sodium phytate, and allowed to cool to room temperature. Phytase activity was then assayed at 37°C, and pH 5.5.

Immunodetection of phytase

Total leaf protein (10 μg) was separated on 10% SDS-PAGE and transferred to nitrocellulose using a semi-dry blot apparatus (Bio-Rad). Chicken polyclonal antiserum against the *A. awamori* phytase was used as the primary antibody, with rabbit anti-chicken IgG peroxidase-conjugated (Sigma) as secondary antibody. Proteins were visualised by enhanced chemiluminescence (ECL; Amersham), processing and washing the membranes according to the instructions supplied with the ECL reagents.

Results

Transformation of soybean with *phy A*

Fertilization occurred approximately 6–8 h following self-pollination at the soybean ovary (Carlson and Lersten 1987). An overall success rate of 21% was achieved from 59 pods harvested from 279 flowers treated with *phy A* (Table 1). All T_0 seeds were grown in a greenhouse. Because of lacking selectable marker gene in transgenic construct, the selection process was elided, PCR identification is used for transgenic screening. A total of 13 out of 99 T_1 plants contained the gene cassette as evidenced by specific PCR amplifications of the 488 bp and 850 bp fragments (Fig. 2). PCR-positive seedlings were further confirmed by PCR-Southern, dot blot and PCR product sequencing (data not shown) and resulted in a transformation frequency of 13% of the germinated T_1 plants. Of 473 T_2 individuals determined by PCR using primer 3 and primer 4, 102 plants showed PCR-positive amplifications (Table 1). Mendelian segregation was not observed in the T_2 generation. One plausible reason might be that nearly half of seeds did not germinate, PCR detection could

Table 1 Summary of ovary wound dipping transformation experiments in soybean

Recipient parent	No. treated flowers	No. pod set	No. seed set	No. T_1 seedings	No. PCR positive	No. T_2 plants	No. PCR positive
Liaodou13	54	9	27	12	2	14	6
Liaodou14	175	42	115	75	9	432	95
Tiefeng29	50	8	20	12	2	27	1
Total	279	59	162	99	13	473	102

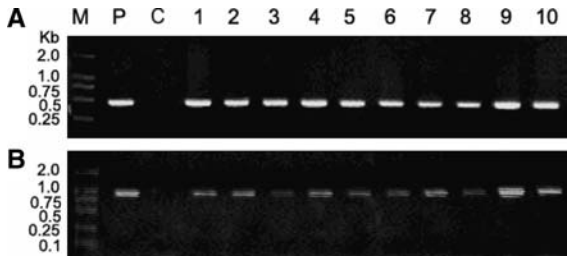

Fig. 2 PCR analysis of T_0 transgenic soybean plants. Primer 3 plus Primer 4A and combinations of Primer 2 plus Primer 4B were used for identification of the *phyA* expression cassette, PCR products were 488 bp and 850 bp respectively. Lane M, DL2000; Lane P, plasmid pBI121-phyA; Lane C, untransformed plant; Lane 1–10, T_0 transgenic plants

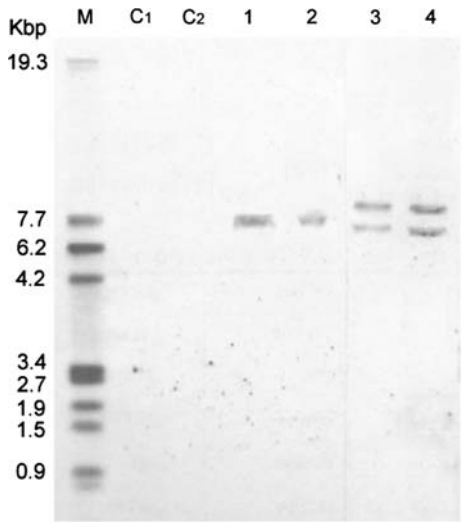

Fig. 3 Southern blot analysis of T_3 transgenic soybeans. Genomic DNA (20 μg) was digested with *Xba*I (single-cut within linear transgenic constructs); blots were hybridized with 488 bp PCR amplification products of *phyA* (shown in Fig. 1). Lane M, λ-*Eco*T14 digest; Lane C1 and C2, untransformed controls; Lane 1 and 2, transgenic line L14-19-1; Lane 3 and 4, transgenic line L14-11-2

not be carried on. In addition, our genomic DNA preparations used for PCR detection might be not reach the purity required for PCR analysis. Phytase assays were detected in the 95 T_2 PCR-positive plants from genotype Liaodou14; L14-11-2 and L14-19-1 lines showed higher phytase activities than the others (data not show) and were selected for further analysis.

Progeny and transgene inheritance

In order to investigate the stability of transgene expression, T_3 individuals derived from line L14-11-2 and line L14-19-1 were investigated by Southern blot analysis. Genomic DNA was digested with *Xba*I that cut once within the gene cassettes (Fig. 1); PCR product that was composed of the partial phytase gene (bp 272–759 of *phyA*) was used as a probe. Low copy number insertions were detected in our experiments. Plants from the L14-19-1 line showed only a single hybridizing band; plants from the L14-11-2 line showed two (Fig. 3). No signal could be detected in the control plants. All hybridizing fragments had much longer lengths than the expression cassette; these fragments presumably represent no truncation or partial deletion. Recently, similar studies (Fu et al. 2000; Loc et al. 2002) reported that minimal linear transgene constructs lacking vector backbone sequences to transform rice with marker genes by particle bombardment successfully produced predominantly 'simple' integration events (one or two bands on Southern blots) and a low frequency of transgene rearrangements, compared to supercoiled and linearized whole plasmid DNA.

Heterologous transgene expression

RNA from T_3 transgenic soybeans (lines L14-11-2 and L14-19-1) was subjected to RT-PCR to test for

the presence of *phyA* transcripts. The 1040 bp *phyA* transcription fragment was detected in the most of T_3 transgenic plants but was absent in the non-transgenic controls (Fig. 4).

Phytase assay in transgenic soybean leaves and seeds

T_3 progeny from lines L14-11-2 and L14-19-1 were used to analyze phytase accumulation in the leaves during plant growth (Fig. 5) and in the mature seeds. Leaf samples were collected each week (from 2 to 7 weeks) in the greenhouse for phytase activity assay. Phytase levels increased from week 2 to week 4 and then remained at a stable level for the following 2 weeks, declining slightly during week 7. Phytase activities in plants from line L14-19-1 were higher than those from line L14-11-2 from weeks 2 to 6. The highest expression level was observed in line L14-19-1 during week 4 (150 U/mg protein), about 3 times higher than the untransformed control (56 U/mg protein). Because the plants were grown in the greenhouse in winter, senescence of the soybean leaves occurred in advance during week 7, and phytase levels decreased slightly. Based on the amount of enzyme per soluble protein, the phytase level increased approximately 2.5-fold on average in a 6-week period.

In the mature seeds ($n = 3$), transgenics from L14-11-2-1 exhibited a substantially higher phytase activity (125 U/kg on average) than untransformed control (64 U/kg). Transgenic seeds displayed a 100% increase in phytase activity compared to wild-type. It is apparent that the increased phytase activity stably accumulated in the transgenic soybeans.

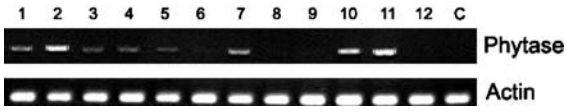

Fig. 4 RT-PCR analysis of *phyA* transcripts in T_3 plants. Primers 3 and 5 were used for cDNA amplification. The product fragments were 1040 bp long. Lane 1–6, transgenic line L14-19-1; Lane 7–12, transgenic line L14-11-2 ; Lane C, untransformed plant. The actin gene was used as a native control to verify a similar amount of cDNA template

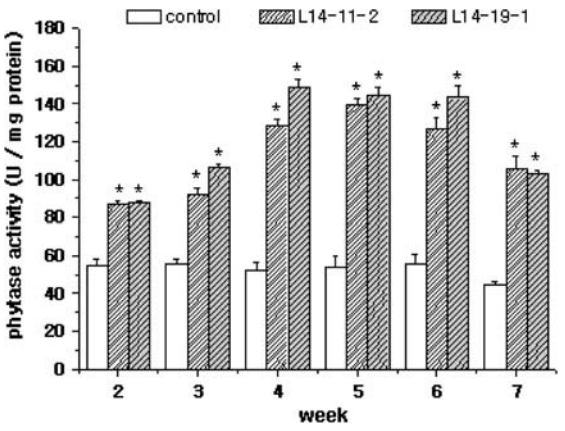

Fig. 5 Phytase activities in T_3 transgenic soybean leaves. T_3 transgenic lines from L14-11-2, L14-19-1 and untransformed control were used to analyze phytase accumulation during plant growth (from 2 to 7 weeks). Leaf samples were collected each week for phytase activity assay (per mg total protein). $^*P < 0.05$, Student's *t*-test. Bars on top of each histogram represent the standard error

Enzyme thermal stability in crude extract of soybean seeds

The temperature stability of the plant-synthesized recombinant phytase in T_3 transgenic soybeans was compared with the untransformed control after incubation at temperatures ranging from 37°C to 90°C for 10 min (Fig. 6). Only a small decrease in activity in all samples was detected when the temperature reached 55°C. Upon incubation at 60°C, 65°C and 70°C for 10 min, 70%, 40% and 10% of the activity, respectively, remained for crude extracts of transgenic seeds,

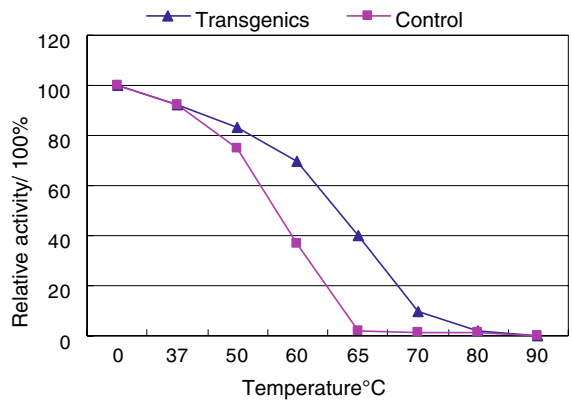

Fig. 6 Thermal stability of the phytase from the transgenic soybean seeds. All samples from transgenic seeds and untransformed control were pre-incubated for 10 min at temperatures ranging from 37°C to 90°C, and allowed to cool to room temperature. Phytase activity was assayed at pH 5.5 and 37°C

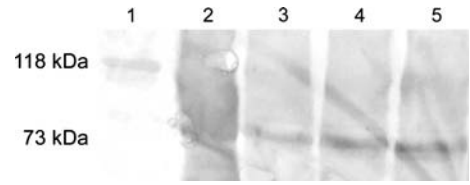

Fig. 7 Immunoblot analysis of recombinant phytase. Protein samples from purified fungal phytase and recombinant phytase. Lane 1, fungal phytase; Lane 2–5, recombinant phytase. Polyclonal antibody raised against purified A.awomori phytase was used for immunodetection. The chemiluminescent detection protocol using peroxidase-conjugated secondary antibody was used to visualize protein bands

whereas no activity was regained in untransformed control. Transgenic lines showed greater stability at the high temperature than did the control.

Expression of Phytase in transgenic soybean

The molecular mass of the mature fungal phytase based on the predicted amino acid sequence is 52 kDa. The reported molecular mass of 118 kDa (Wang et al. 2006) reflects the extensive glycosylation of the enzyme. Glycosylation is required for enzyme stability and/or activity. To determine the mass of the recombinant phytase synthesized in soybeans and to verify that it was glycosylated, recombinant phytase was compared with native fungal phytase by immunoblot analysis using polyclonal antiserum to *A. awamori* phytase (Fig. 7). Chemiluminescent detection of protein bands showed that the recombinant phytase migrated with an apparent molecular mass of approximately 73 kDa, similar to the size observed for recombinant phytase produced in tobacco (Pen et al. 1993). The apparent molecular mass of the recombinant phytase was higher than predicted by amino acid composition, which provides evidence that the recombinant enzyme is glycosylated.

Discussion

The period of exogenous DNA transformation by pollen tube pathway transgenic technology should be limited to the time between fusion of the sperm and egg and division of the zygote. In soybeans, the ripened pollen tube enters into the embryo sac 6 h after self-pollination when the height of the corolla is greater than that of the calyx. At 28 h after self-pollination, the zygote begins the first mitotic division. Thus, 6–28 h after pollination should be the time for exogenous DNA transformation in soybeans via the pollen tube pathway (Shen 1983). Furthermore, the mechanism of transformation holds that exogenous DNA enters into the embryo sacs through the interface connecting the stigmas and embryo sacs (Gong et al. 1988). Cutting off all of the style and the top of the ovary shortened the entrance pathway and greatly improved the capacity of the target DNA to enter into the embryo sacs (Wang and Shen 2006). In addition, this technique could be used to introduce total exogenous genomic DNA or plasmid DNA into plants, and it is also practical to introduce linear DNA fragments composed of only the target gene, flanking regulation elements and T-DNA border repeats into plants directly. In our experiment, a minimal linear transgenic construct was introduced into soybeans using this method and resulted in the *Aspergillus* phytase gene being inherited and expressed in progeny. The transformation efficiency of 13% by PCR screening was higher than that previously reported in soybeans using the same pathway, which was estimated as 3.8% (Yang et al. 2002).

A phytase that is stable at high temperature during the pelleting process is desirable for animal feed applications (Hamada et al. 2004). In addition, high temperature is necessary for soybean to inactivate endogenous trypsin inhibitors and other antinutrient proteins (Ponstein et al. 2002). Recombinant phytase in transgenic seeds showed a relatively small increase in thermostability than the untransformed control, but it did not appear sufficient to withstand processing temperatures. Thermostable phytase expression in soybean is one of the new current strategies for retention of phytase activity in feed after consumption by the monogastric animal.

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