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RESEARCH ARTICLE

Construction of a new plant expression vector and the development of maize germplasm expressing the *Aspergillus ficuum* phytase gene *PhyA2*

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Abstract Phytases, which belong to a special category of orthophosphoric monoester phosphohydrolases, degrade inositol hexaphosphate to produce lower-grade inositol phosphate derivatives and inorganic phosphate. Thus, phytases may improve phosphorus utilization, eliminate the anti-nutrient properties of phytic acid, and mitigate environmental pollution due to phosphorus contamination. In this study, we constructed a new root-specific expression vector by inserting the *Aspergillus ficuum* phytase gene *PhyA2* into pCAMBIA3301-ZmGLU1P-Nos. The subsequent molecular analysis confirmed that six T_4 generation transgenic plants carried and expressed *PhyA2*. A quantitative real-time PCR analysis indicated *PhyA2* was highly expressed in the transgenic roots. Additionally, the phytase activity was 10.9-fold higher in the transgenic roots (peak activity of 5.432 U/g) than in the control roots. Moreover, compared with the control rhizosphere, the organic phosphorus content in the rhizosphere of the transgenic plants decreased significantly (by up to 5.21 mg/kg). An agronomic trait analysis indicated that *PhyA2* expression can increase maize seed weight by up to 25.8 g. Therefore, the integration of *PhyA2* into the maize genome can enhance the ability of maize plants to use the phosphorus compounds in soil, while also improving the plant growth status and increasing the seed yield.

Keywords *Aspergillus ficuum* · Phytase gene · Expression vector · Maize

Abbreviations

6-BA	6-Benzylaminopurine
Bar	Herbicide
Carb	Carbenicillin
Cef	Cefradine
IBA	1 h-Indole-3-butanoic acid
Kan	Kanamycin
KT	Kinetin
NAA	1-Naphthaleneacetic acid

Introduction

As one of the three most important crops, maize is widely used, including as food, feed, and industrial raw materials (Wu et al. 2014). Early maize producers used traditional crop breeding techniques to develop and cultivate many excellent maize varieties. Recently, genetic engineering and transgenic techniques have been applied to transfer genes associated with specific functions into maize inbred lines to enhance crop

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quality and specific plant traits. Advances in molecular breeding research have provided researchers with new options for the genetic improvement of maize.

Phosphorus is one of the nutrients essential for maize growth and development. Soil organic phosphorus is an important source of phosphorus nutrients for maize (Sareh et al. 2018). Organic phosphorus represents approximately 20–80% of the total phosphorus content in soil, with more than 50% of the organic phosphorus existing as phytate phosphorus, which is difficult for plants to absorb and use (Enqing et al. 2018). Phytate phosphorus is also an important form of phosphorus in maize plants, especially in seeds, wherein it accounts for about 70% of the total phosphorus content. However, the digestive tract of monogastric animals lacks enzymes to hydrolyze phytate phosphorus, resulting in its excretion into the environment in feces. This not only decreases the phosphorus utilization by animals, it also increases environmental pollution due to organic phosphorus (Yan 2012). Additionally, phytate phosphorus can chelate other metal ions to form compounds that are relatively insoluble. Therefore, phytate phosphorus is also known as an anti-nutrient factor. Phytases are a class of enzymes that can catalyze the hydrolysis of phytate phosphorus and release inorganic phosphate and inositol that can be passively absorbed and used by plants (Valeeva et al. 2018).

In this study, the *PhyA2* gene, which encodes a phytase, was cloned from *Aspergillus ficuum* and used to construct a new plant expression vector containing a root-specific promoter. By applying genetic engineering technology, the recombinant plasmid was inserted into a maize inbred line. The root-specific expression of *PhyA2* increased the phytase content and activity to improve the degradation of the organic phosphorus in soil, enabling the transgenic maize plants to absorb more phosphorus and efficiently degrade phytic acid salt. The results of this study may be useful for producing new maize germplasm that can efficiently use phosphorus to increase the crop yield.

Materials and methods

Experimental strains, plasmid vector, and plant receptor material

Aspergillus ficuum was purchased from the Institute of Microbiology, Chinese Academy of Sciences. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* EHA105 cells, the modified plant expression vector pCAMBIA3301-ZmGLU1P-Nos, and the germinal callus cells of maize inbred line GSH9901 were provided by the Key Laboratory of Crop Molecular Breeding in Jilin Province, China.

Media

Murashige and Skoog (MS) liquid medium (pH 5.8) was used as the *A. tumefaciens* infection solution. Differentiation medium 1 (MS1) comprised MS liquid medium supplemented with 2 mL 6-BA, 1 mL NAA, 30 g/L sucrose, and 7.5 g agar (pH 5.8). Differentiation medium 2 (MS2) comprised MS1 supplemented with 50 mg/L Kan, 400 mg/L Carb, and 600 mg/L Bar (pH 5.8). Screening rooting medium (MS3) comprised MS liquid medium supplemented with 1 mL NAA, 50 mg/L Kan, 400 mg/L Carb, 600 mg/L Bar, 30 g/L sucrose, and 7.5 g agar (pH 5.8).

Preparation of the maize embryo and induction of the embryogenic callus

Young maize ears (10–13 days old) were selected. The outer bract was removed, leaving a layer of leaves, after which the maize ears were washed with 75% ethanol for 15 min. The leaves were peeled off and the palpus was removed before the maize ears were disinfected with 0.5% NaClO solution for 10 min and then rinsed with sterile water 3–4 times. In an ultra-clean workbench, a scalpel was used to cut the maize kernels. After removing the seed coat and endosperm, the young embryo was transferred to N6 medium for a 15-day incubation at 25 °C in darkness.

Cloning of *PhyA2* from *Aspergillus ficuum*

The *A. ficuum PhyA* sequence (AF537344.1) available in the GenBank database was used to design gene-specific primers (S and AS) with the Primer Premier 5.0 program. The primers included *Xba*I and *Hind*III restriction enzyme sites. Details regarding the primer sequences are provided in Table 1.

Total RNA was extracted from *A. ficuum* using a commercial RNAiso plus extraction kit (Bao Bioengineering (Dalian) Co., Ltd., Economic and Technological Development Zone, Dalian City, Liaoning Province, China) and then reverse transcribed to

cDNA. The cDNA was used as the template for a PCR amplification of the target gene (*PhyA2*) with primers S and AS. The PCR conditions are listed in Table 2. The PCR product was analyzed by agarose gel electrophoresis. After purifying the amplified *PhyA2* fragment from the gel, it was inserted into the pMD18-T vector. The resulting pMD18-T-PhyA2 recombinant plasmid was sequenced to confirm the accuracy of the *PhyA2* sequence.

Construction of a new plant expression vector

The pMD18-T-PhyA2 plasmid and the modified plant expression vector pCAMBIA3301-ZmGLU1P-Nos were digested with *Xba*I and *Hind*III and then analyzed by agarose gel electrophoresis. The *PhyA2* gene fragment and the linearized plant expression vector sequence were recovered and ligated with T_4 DNA ligase. The resulting new plant expression vector was analyzed by a PCR amplification and a restriction enzyme digestion to confirm it was constructed correctly. The process involved in constructing the new plant expression vector is presented in Fig. 1.

Transformation of maize with the plant expression vector

The *PhyA2* gene was inserted into maize inbred line GSH9901 embryonic calli via *A. tumefaciens*-mediated transformation. The optimized infection and transformation conditions were as follows: 25–30 min for the *A. tumefaciens* infection of calli; the bacterial culture was used after reaching an optical density at 600 nm (OD_{600}) of 0.6–0.7; and 2–3 days for the co-culture required for the *A. tumefaciens*-mediated transformation. After screening for herbicide (Glufosinate) resistance, calli were differentiated. When the herbicide-resistant calli on the differentiation medium 1 grew to 3–5 cm with 3–4 leaves, the seedlings were transferred to the rooting medium. Approximately 15–25 days later, 4–6 robust primary roots and many fibrous roots were detected. The seedlings were incubated at 23–26 °C for 2–3 days, after which they were transplanted to pots containing sterilized soil.

Insertion of the plant expression vector into maize

Agrobacterium tumefaciens culturing

Agar-solidified YEB medium supplemented with 50 mg/L Kan was inoculated with *A. tumefaciens* cells containing the recombinant plant expression vector carrying *PhyA2*. The culture was incubated at 28 °C for 2 days. An individual colony was added to a test tube containing 5 mL YEP/Kan medium. The culture was oscillated (180 rpm) overnight at 28 °C. A 3-mL aliquot of the overnight culture was added to 50 mL YEP/Kan medium, after which the culture was incubated at 28 °C, with shaking at 220 rpm. When the OD_{600} of the bacterial culture reached approximately 0.7, the culture was centrifuged at 5000 rcf for 20 min at room temperature to collect the bacteria. The bacterial cells were resuspended in the same volume of MS liquid medium.

Callus transformation and co-culturing

The callus was added to 50 mL infection solution in a sterile culture bottle, which was then shaken for 6 min. The infected callus was removed from the bottle, placed on sterile filter paper to absorb the excess infection solution, and then transferred to the co-culture medium for a 2-day incubation in darkness.

Screening and culturing

After the co-culture step, the embryogenic callus exhibiting healthy growth (e.g., soft and pale yellow) was transferred to the screening medium for an incubation at 28 °C under light. The medium was refreshed every 15 days.

Table 1 Primer sequences

The underlined part is the restriction enzyme digestion site

Name	Primer sequences
PhyA2-S	TTTAAGCTTACTGGCAGTCCCCGCCTCGAGAAATC
PhyA2-AS	TTTTCTAGACTAAGCAAAACACTCCGCCCAAT

Table 2 PCR amplification procedure

Target gene	Predegeneration	Degeneration	Annealing	Extend	Cycle number	posterior extension
PhyA2	94 °C 5 min	94 °C 55 s	58 °C 55 s	72 °C 80 s	32	72 °C 10 min

Rooting

After the adventitious buds grew to 2–3 cm, they were transferred to the rooting medium. After 1 week, adventitious roots were detected, enabling the transfer of plants to soil.

Detection of *PhyA2* in maize plants

PCR analysis of transgenic plants

Genomic DNA was extracted from herbicide-resistant T_3 generation maize plants with a NuClean Plant Genomic DNA kit (Changchun Feikai Biological Co., Ltd., Economic Development Zone, Changchun City, Jilin Province). The maize genomic DNA was used as a template for a PCR with primers S and AS. The plant expression vector carrying *PhyA2* was used as the positive control, whereas the genomic DNA from untransformed maize plants served as the negative control. The PCR conditions are listed in Table 2. The PCR products were analyzed by agarose gel electrophoresis. The T_3 generation maize plants that were confirmed by PCR to be transgenic were self-pollinated to produce seeds. The T_3 generation seeds were germinated to obtain T_4 generation transgenic maize plants, which were verified by PCR.

Southern hybridization analysis of transgenic plants

Large quantities of genomic DNA were extracted from the T_4 generation transgenic maize plants and untransformed maize plants (positive control) according to a modified CTAB method (Abdel and Osman 2017). A Southern blot analysis was completed with purified *PhyA2* as the probe as well as the plant expression vector carrying *PhyA2* and the genomic DNA from untransformed maize plants as the positive and negative controls, respectively. Specifically, 10–15 μg maize genomic DNA was digested with *Hind*III for 16 h. The digested DNA was separated in a 0.8% agarose gel and then transferred to a nitrocellulose membrane in a 20× SSC solution. The membrane was then incubated at 80 °C for 2 h, after which the DNA was hybridized with the probe.

Reverse transcription PCR analysis of transgenic plants

Total RNA was extracted from the roots of transgenic maize plants and reverse transcribed to cDNA. Total RNA was also extracted from the roots of untransformed maize plants and reverse transcribed (negative control). The cDNA samples for the transgenic and control maize plants were used as the templates for a PCR amplification of *PhyA2* with primers S and AS (Table 1). The maize *EF-1α* gene, which was used as the internal reference control, was amplified by PCR with primers P1 and P2 to produce a 213-bp fragment (Table 3). The PCR conditions are listed in Table 2.

Quantitative real-time PCR analysis of transgenic plants

Total RNA was extracted from the roots, stems, and leaves of untransformed (control) and transgenic maize plants and reverse transcribed to produce cDNA for a quantitative real-time (qRT)-PCR analysis of *PhyA2* expression. The maize beta-actin gene (GenBank accession number: nm_001252731.2) was selected as the internal reference control. Primers QFACT and QRACT (Table 4) were designed based on the *PhyA2* sequence. The qRT-PCR analysis was completed with the SYBR Green fluorescent dye. The reaction conditions are listed in Table 5. Each sample was analyzed with three replicates. The relative *PhyA2* expression levels in the different maize tissues were calculated according to the $2^{-\Delta\Delta Ct}$ method (Yueai et al. 2014).

Preparation of the phosphorus concentration standard curve

A phosphorus concentration standard curve was prepared as previously described (Safety certificate 2009). Additionally, a published method for analyzing

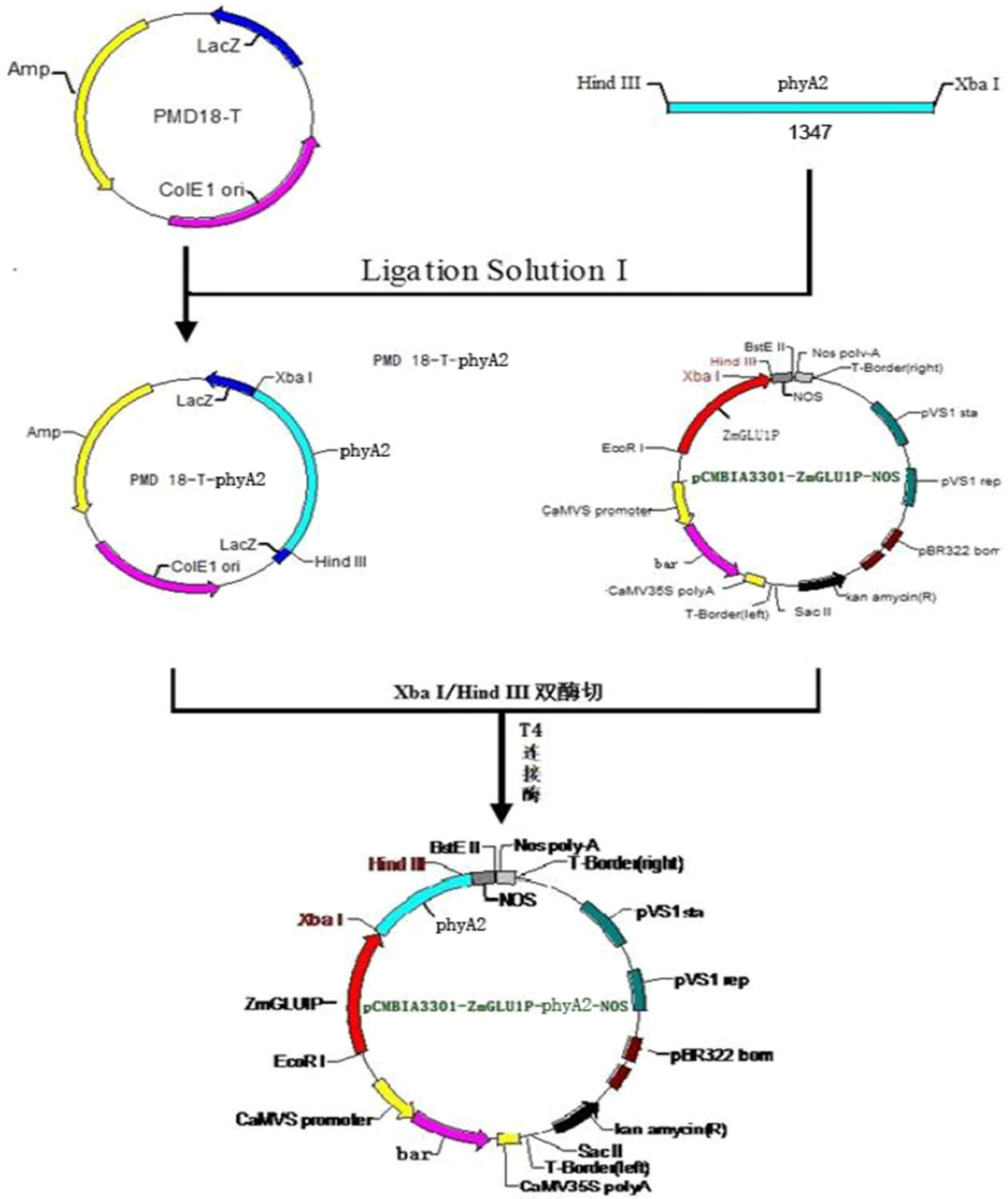

Fig. 1 The process of the construction of plant expression vector

plant root tissue phytase (Zhang 2009) was modified to examine the T_4 generation transgenic maize plants. Specifically, 2 g root tissue was ground to a homogenate, which was then mixed with 8.0 mL extraction solution (0.25 mol/L sodium acetate, 0.05% Triton X-100, and 0.05% bovine serum

Table 3 Primer sequences

Name	Primer sequences
EF-1α-P1	GCTTCACGTCCCAGGTCATC
EF-1α-P2	TAGGCTTGGTGGGTATCATC

Table 4 Primer sequences

Name	Primer sequences
PhyA2-QFACT	ATCTTGACTGAGCGTGTTATTCC
PhyA2-QRACT	GCTGGTCCTGGCTGTCTCC

albumin). The resulting solution underwent an ultrasonication for 15 min at 37 °C in a water bath, after which it was centrifuged at 12,000 rcf.

Determination of the phytase activity in transgenic plant root tissue

Phytase activity was assessed in three parallel experiments as previously described (Bouchra et al. 2018; Bekalu et al. 2017). The root tissue phytase activity [i.e., 1 unit (U)] was defined as the amount of enzyme required to release 1 μmol inorganic phosphorus from 0.10 mmol/L sodium phytate solution in 1 min at 37 °C and pH 5.5. The enzyme activity was expressed as U/g.

Analysis of the phosphorus in the rhizosphere of transgenic plants

The T_4 generation transgenic maize plants verified by the Southern blot analysis were grown in soil for an analysis of the organic phosphorus content in the rhizosphere (i.e., soil up to 20 cm deep and within 5 cm of the main root). Soil samples collected before the transgenic maize were planted were used as controls.

Analysis of the agronomic characteristics of transgenic maize plants

Three replicates of the T_3 and T_4 generation transgenic maize plants were grown in an experimental field at Jilin Agricultural University to evaluate the main agronomic traits. At maturity, plant height and other yield-related characteristics were analyzed.

Results

Construction and detection of a new plant expression vector

The results of an agarose gel electrophoresis (Fig. 2) revealed the *PhyA2* fragment amplified by PCR was approximately 1400 bp long, which was the expected size. The new plant expression vector was digested with *Xba*I and *Hind*III and the resulting fragments were examined in an agarose gel (Fig. 3). Two fragments were obtained following the enzymatic digestion, one of which was about 1400 bp, as expected for *PhyA2*. Thus, the PCR and restriction enzyme digestion confirmed that the new plant expression vector (pCAMBIA3301-ZmGLU1P-PhyA2-Nos) was constructed correctly.

Agrobacterium tumefaciens-mediated genetic transformation of the maize callus

An *A. tumefaciens*-mediated method was used to transform a maize inbred line with *PhyA2*. Six herbicide-resistant T_4 generation maize plants were obtained. The specific transformation process is presented in Fig. 4.

PCR detection of *PhyA2* in transgenic maize plants

Genomic DNA was extracted from the leaves of transgenic maize plants for a PCR analysis to confirm the insertion of *PhyA2*. The PCR product was examined by 1% agarose gel electrophoresis. The

Table 5 PCR amplification procedure

Target gene	Predegeneration	Degeneration	Annealing	Extend	Cycle number
Q-PhyA2	94 °C 30 s	94 °C 10 s	55 °C 40 s	72 °C 20 s	40

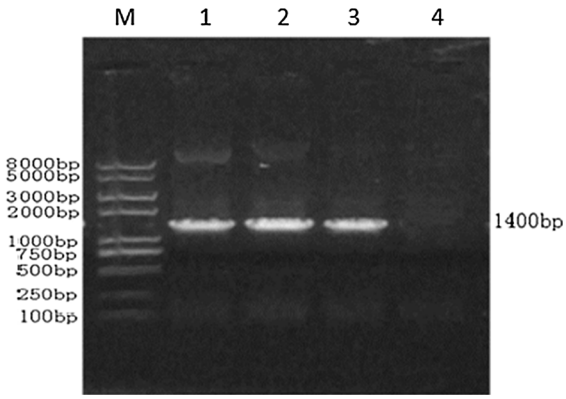

Fig. 2 The results of PCR identification. M: DL 8000 DNA Marker; 1–3: PCR products; 4: Contrast

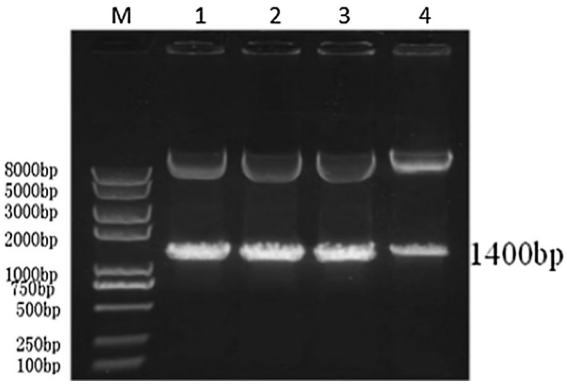

Fig. 3 Restriction enzyme digestion identification. M: DL8000 DNA Marker; 1–4: Digestion products

transgenic plants that produced the amplicon of the expected size were retained and self-pollinated to obtain T_3 and T_4 generation transgenic plants (Figs. 5 and 6).

Southern blot analysis of *PhyA2* in transgenic maize plants

The T_4 generation maize plants that tested positive for the presence of *PhyA2* in the PCR assay were selected for a Southern blot. Their genomic DNA was extracted according to a modified CTAB method. The maize genome contains multiple *Hind*III restriction enzyme sites, whereas the *PhyA2* fragment contains a single *Hind*III site at one end. Accordingly, *Hind*III was appropriate for the digestion of the maize genomic DNA to verify that *PhyA2* had been integrated into the genome of transgenic plants. The Southern blot (Fig. 7) revealed single-copy hybridization bands for two plants, whereas bands were undetectable for the untransformed control plants. Thus, the integration of *PhyA2* into the maize genome was confirmed.

Reverse transcription PCR identification of transgenic maize plants

The *PhyA2* and *EF-1α* gene sequences were amplified by PCR using cDNA prepared from the total RNA extracted from maize roots. The expected *PhyA2* and *EF-1α* sequences were amplified for the transgenic plants, whereas only the *EF-1α* sequence was amplified for the untransformed plants (Fig. 8). Thus, the reverse transcription PCR results confirmed that *PhyA2* was expressed in the roots of the transgenic maize plants.

Quantitative real-time PCR detection of transgenic lines

A qRT-PCR assay was performed to analyze the *PhyA2* expression levels in the young leaves, roots, and stems of T_4 generation transgenic plants verified by PCR and Southern blotting. The *PhyA2* expression levels were calculated relative to the expression of the maize beta-actin gene. The qRT-PCR data indicated that *PhyA2* was more highly expressed in the transgenic maize plants than in the control plants (Fig. 9). Specifically, the *PhyA2* expression level was 3.75-times higher in the transgenic maize roots than in the control roots. Additionally, the mean *PhyA2* expression levels in the transgenic stems and leaves were respectively 1.42-times and 1.84-times higher than in the corresponding control tissues. Thus, *PhyA2* was specifically expressed in the transgenic maize roots.

Determination of the phytase activity in the transgenic maize roots

Phosphorus concentration standard curve

A phosphorus concentration standard curve was prepared with the phosphorus concentration on the abscissa and the absorbance on the ordinate (Fig. 10). The linear regression equation was $y = 1.026x + 0.0053$, $R^2 = 0.9996$, reflecting the relatively linear relationship between the phosphorus concentration and absorbance.

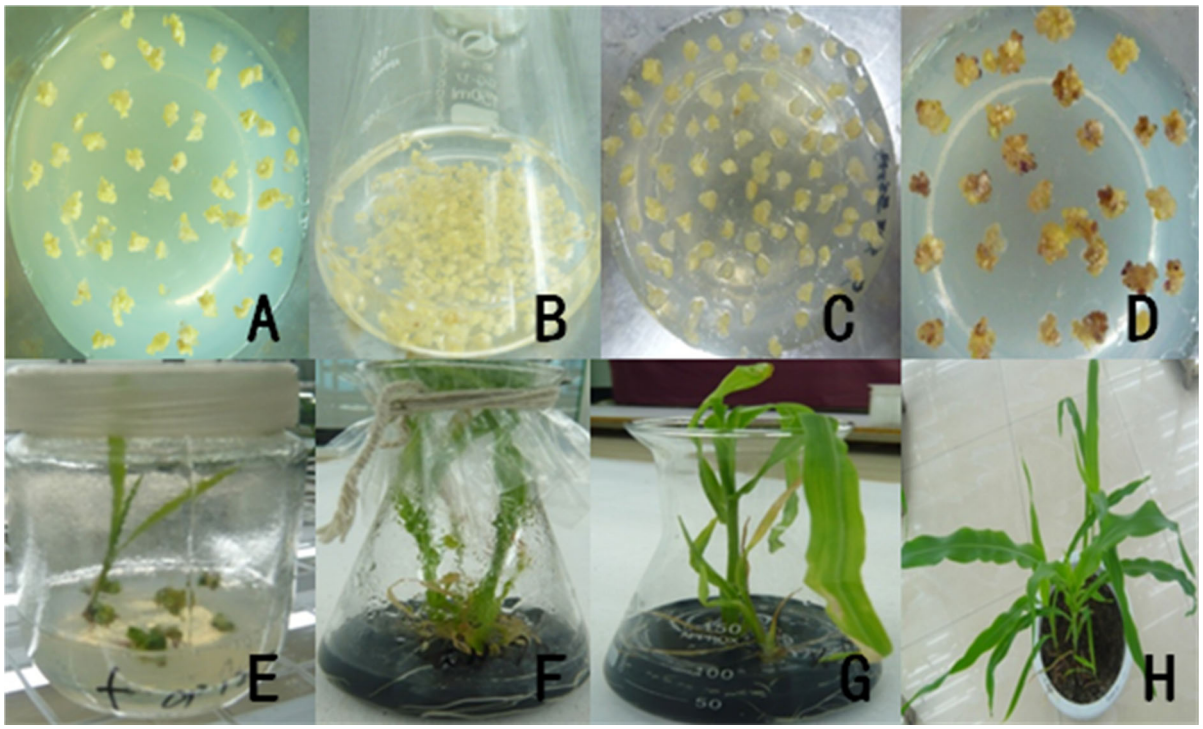

Fig. 4 Transforming into maize embryogenic callus via *Agrobacterium tumefaciens*-mediated method. (**a**) Preincubate of maize embryogenic callus. (**b**) Agrobacterium infection. (**c**) Co-cultivation. (**d**) Screening culture. (**e**) Differentiation culture. (**f**) Rooting culture. (**g**) Exercising plants. (**h**) Plants regeneration

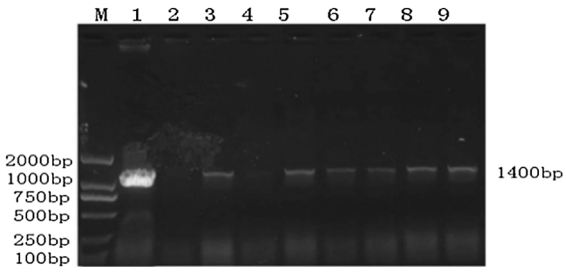

Fig. 5 PCR detection of T_3 transgenic plants with PhyA2. M: DL2000 DNA Marker; 1: Positive control; 2: Non-transformed plant

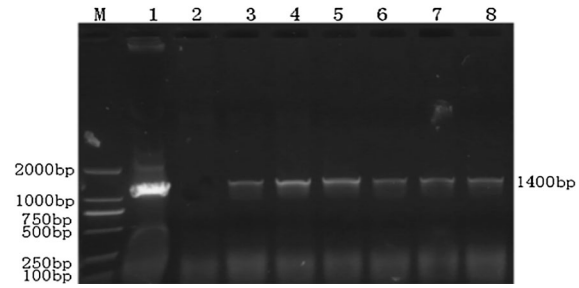

Fig. 6 PCR detection of T_4 transgenic plants with phyA2. M: DL2000 DNA Marker; 1: positive control; 2: non-transformed plant; 3–8: transgenic plants

Phytase activity analysis of transgenic maize plants

The phytase activity in the T_4 transgenic maize roots was compared with that in the roots of untransformed plants. The phytase activity was significantly ($P < 0.05$) higher in the transgenic roots than in the control roots (Table 6). On average, the phytase activity was 10.9-fold higher in the transgenic roots than in the control roots. The increased phytase activity of the transgenic roots ranged from 17.6-fold (T4-3-13) and 4.8-fold (T4-33-12) higher than that in the control roots. These results implied that *PhyA2* was correctly incorporated into the genome and was being expressed in the roots of the transgenic maize plants. Moreover, the phytase produced in the transgenic plants appeared to be highly active.

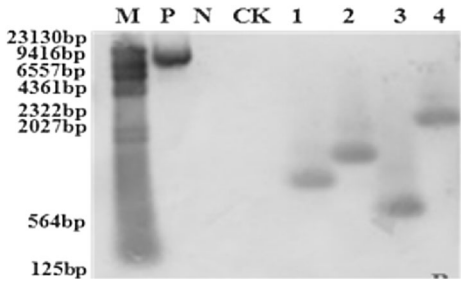

Fig. 7 Southern hybridization detection of T4 transgenic plant B: T4 generation of expression vector transgenic plants Southern hybridization detection; C: T4 generation infecting vector transgenic plants Southern hybridization detection; M: λ Hind III DNA Marker; P: Positive control; N: Water; CK: Negative control; 1–4: Positive plants

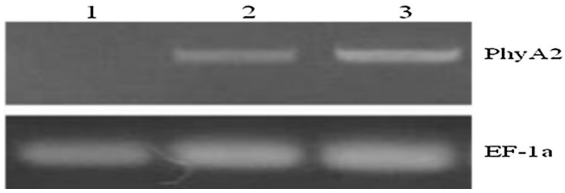

Fig. 8 RT-PCR analysis of T_4 transgenic plants. 1: Non-transformed plant; 2–3: Transgenic plants

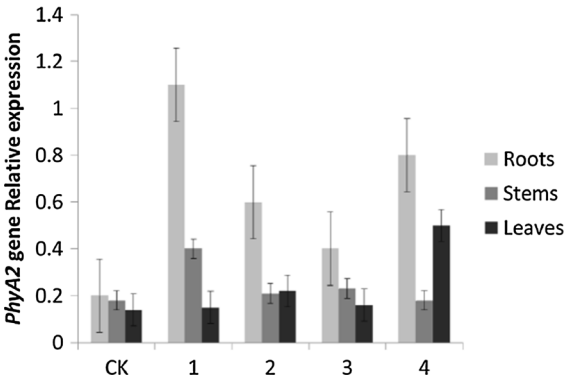

Fig. 9 Relative expression amount of PhyA2 gene. CK. Untransformed plants; 1–4: transformed plants

Analysis of the phosphorus content in the rhizosphere of the transgenic maize plants

A comparison of the rhizospheres of the transgenic and untransformed control maize plants revealed that the organic phosphorus content was 4.79-fold lower in the rhizosphere of the transgenic maize plants than in the control rhizosphere (Table 7). Thus, the expression of a phytase gene in maize roots appears to affect the soil organic phosphorus content.

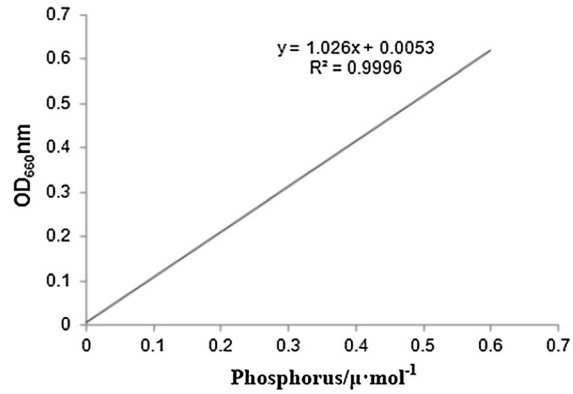

Fig. 10 Phosphorus standard curve

Agronomic characteristics of transgenic maize plants

Phenological growth stages of transgenic maize

There were no significant differences in the phenological growth stages of the transgenic (e.g., 35-15, 36-10, and 44-18) and control maize plants (Table 8). However, the tasseling stage occurred 3 days earlier in the transgenic plants than in the control plants. Additionally, the 6-13 transgenic plants reached maturity 4 days later than the controls.

Analysis of the plant height and seed weight of the transgenic maize

The ear length and thickness were similar in the transgenic and control plants (Table 9), but the effects of natural factors on the transgenic maize plants remain to be assessed. Additionally, although the height of individual transgenic maize plants differed from the mean height of control plants by 2–3 cm, this difference was not significant. Accordingly, *PhyA2* expression does not appear to affect maize plant height. Moreover, the mean 100-grain weights of the 6-13 and 13-7 transgenic plants were lower than that of the control plants. In contrast, the mean 100-grain weight of the other transgenic maize plants was 0.56 g higher than that of the control plants. Therefore, *PhyA2* expression may influence maize seed weights.

Table 6 Phytase activity analysis of root system of transgenic plants

Plant number	Phytase activity (U/g)	Plant number	Phytase activity (U/g)
T_4-3-13	5.432 aA	T_4-14-1	2.983 gF
T_4-4-20	4.349 bB	T_4-14-3	2.374 hG
T_4-5-4	4.253 cC	T_4-16-13	2.417 hG
T_4-6-13	4.172 dD	T_4-28-19	2.214 iH
T_4-13–7	3.985 eE	T_4-33-12	1.510 jI
T_4-13-11	3.032 fF	CK	0.307 kJ

T_4-3-13 comes from T_3-7-1 of T_3 generation plants. T_4-4-20 comes from T_3-5-2 of T_3 generation plants. T_4-5-4 comes from T_3-8-3 plants of T_3 generation. T_4-6-13 comes from T_3-9-4 of T_3 generation plants. T_4-13-7 and T_4-13-11 came from the same T_3 plant T_3-16-7. T_4-14-1 and T_4-14-3 came from the same T_3 plant T_3-20-3. T_4-16-13 comes from T_3-22-6 of T_3 generation plants. T_4-28-19 comes from T_3-23-4 of T3 generation plants. T_4-33-12 comes from T_3-27-9 of T_3 generation plants. The 5% level is significantly indicated by lowercase letters, and the 1% level is significantly indicated by uppercase letters

Table 7 Analysis of phosphorus content in rhizosphere soil of transgenic plants

Plant number	Total soil phosphorus before planting (mg kg^{-1})	Soil organic phosphorus content before planting (mg kg^{-1})	Total soil phosphorus content after planting (mg kg^{-1})	Soil organic phosphorus content after planting (mg kg^{-1})
T_4-4-8	314.47	53.46	309.66	49.32**
T_4-4-9	263.94	44.87	255.71*	42.59*
T_4-5-14	282.29	47.99	275.38*	43.87**
T_4-6-8	331.29	56.32	320.45**	51.11**
T_4-8-7	402.59	68.44	392.55**	64.63*
T_4-13-1	213.94	36.37	204.86**	33.46*
T_4-15-8	275.35	46.81	264.49**	45.71*
T_4-19-13	292.00	49.64	281.33**	45.36**
T_4-22-3	326.06	55.43	317.84*	52.18*
T_4-25-2	361.70	61.49	352.73*	59.02*
CK_1	349.70	59.45	343.83	598.76
CK_2	376.54	62.73	371.62	62.86

Note: Each set of data in the graph is the mean ± standard deviation of three replicates of three sites in each plant. "*, **" represents the difference in significance between the transgenic positive plants and the untransformed plants, respectively. "*" stands for $0.01 < P < 0.05$ for significant difference; "**" stands for $0.001 < P < 0.01$ for significant difference

Discussion

Phytase was initially identified in 1915, and its considerable ability to degrade phytic acid was subsequently confirmed by Anderson (1941). The rapid development of biotechnology has enabled researchers to investigate the effects of the ectopic expression of phytase genes in transgenic plants. For example, Abul et al. (1999) isolated a *PhyA* gene from *A. ficuum*, and successfully expressed it as a recombinant gene in tobacco leaves. Although the molecular weight and optimal pH was lower for the expressed product than for the natural phytase, the biological characteristics were otherwise identical. The *PhyA2* gene was successfully cloned from *A. ficuum* cDNA in this study.

In terms of molecular biology, a vector can be used to transfer a target gene into the receptor material and subsequently control the expression of the target gene (Chen 1994). A good plant expression vector is important for the successful transformation of plants with exogenous DNA material. Different types of regulatory elements in plant expression vectors can directly affect the expression of recombinant phytase

Table 8 Observation values of phenological period and growth period

Strain	Seeding stage/days from sowing	Tasseling stage/days from sowing	Spinning stage/days from sowing	Mature stage/days from sowing
GSH9901-3-13	13	75	83	144
GSH9901-4-20	14	76	83	141
GSH9901-5-2	14	74	82	144
GSH9901-5-4	14	74	83	143
GSH9901-6-13	14	75	82	145
GSH9901-13-7	13	75	81	144
GSH9901-13-11	13	73	83	140
GSH9901-14-1	13	76	84	142
GSH9901-14-3	13	77	83	141
GSH9901-16-13	13	77	82	141
GSH9901-28-19	13	74	82	141
GSH9901-33-12	13	74	83	142
GSH9901-35-15	13	75	81	139
GSH9901-36-10	12	72	80	140
GSH9901-36-12	12	73	81	140
GSH9901-37-15	13	73	81	140
GSH9901-44-18	12	72	80	139
GSH9901-CK	13	75	82	141

genes in plants. For example, the *ZmGLU1* promoter may be used for the root-specific expression of a transgene. In the current study, *PhyA2* was inserted into a plant expression vector containing the *ZmGLU1* promoter for the root-specific expression of the phytase gene. This study was similar to that conducted by Cui (2007).

Our gene expression analyses confirmed that the inserted *PhyA2* gene was expressed in the T_3 and T_4 generation transgenic maize plants. Additionally, the *PhyA2* expression level in the transgenic maize plants was higher in the roots than in the leaves. The observed phytase activities in this study were consistent with those determined by Zhang et al. (2010). Specifically, the phytase produced in the transgenic maize roots was able to degrade sodium phytate; however, the relatively low phytase activity in this study may have been due to the instability of *PhyA2*, resulting in impure transgenic maize plants. Li et al. (2009) reported similar results for their investigation of transgenic soybean plants expressing a phytase gene.

William et al. (1990) were the first to produce fertile transgenic maize plants, making it possible to improve maize traits via methods commonly used for biotechnology research. Our analysis of soil phosphorus levels revealed a decrease in the organophosphorus content in the rhizosphere of transgenic plants relative to the content in the control rhizosphere. This decrease may reflect the degradation and uptake of organophosphorus by the transgenic plants expressing *PhyA2*; however, the phosphorus use efficiency was relatively low. Song et al. (2008) explored the effects of phytase on soil organophosphorus contents. Their results indicated that increases in the abundance of active or moderately active organophosphorus compounds in the soil may be related to increases in phosphatase activities. Moreover, the phosphatase activity may decrease with increases in moderately or highly stable organophosphorus compounds in the soil.

In this study, analyses of phenological growth stages revealed a lack of significant differences between the *PhyA2*-expressing transgenic maize plants and the control plants. Additionally, there were no significant differences in the physiological indices of the transgenic and control maize plants, possibly because the plants were not exposed to adverse environmental conditions. Furthermore, *PhyA2*

Table 9 Field agronomic shape survey and overplanting

Strain	Average lodging rate (%)	The average bald tip (cm)	Plant height (cm)	Ear length (cm)	Ear diameter (cm)	Kernels per row	Kernel rows	100-grain weight (g)
GSH9901-3-13	8	0.6	154 aA	14.5	3.34	34	14	25.8 aA
GSH9901-4-20	5	0.8	154 aA	14.5	3.28	29	14	25.8 bA
GSH9901-5-2	3	0.25	153 aA	14.9	3.33	31	14	25.6 cB
GSH9901-5-4	5	0.65	153 aA	14.8	3.36	33	14	25.5 dC
GSH9901-6-13	5	0.8	153 aA	14.3	3.25	28	14	25.5 dD
GSH9901-13-7	5	0.5	153 aA	14.5	3.26	33	14	25.4 eE
GSH9901-13-11	2	1.3	152 aA	15.2	3.55	33	14	25.4 eE
GSH9901-14-1	6	0.83	152 aA	15.8	3.58	32	14	25.4 eF
GSH9901-14-3	5	1.1	152 aA	15.0	3.63	34	14	25.4 eG
GSH9901-16-13	3	0.85	152 aA	15.8	3.68	33	14	25.3 eH
GSH9901-28-19	5	0.6	152 aA	15.2	3.66	31	14	25.3 eI
GSH9901-33-12	5	1.2	152 aA	15.5	3.65	30	14	25.2 fJ
GSH9901-35-15	2	1.4	152 aA	14.8	3.59	32	14	25.0 gK
GSH9901-36-10	5	1.0	151 aA	14.5	3.53	32	14	24.9 hL
GSH9901-36-12	2	1.7	151 aA	14.6	3.63	29	14	24.9 iM
GSH9901-37-15	2	1.1	150 aA	14.2	3.65	28	14	24.7 kO
GSH9901-44-18	1	1.5	155 aA	14.9	3.85	32	14	24.6 iP
GSH9901-CK	2	0.75	155 aA	15.8	3.63	33	14	24.8 jN

The 5% level is significantly indicated by lowercase letters, and the 1% level is significantly indicated by uppercase letters

expression did not significantly influence plant height. However, there were some differences in the mean 100-grain weight, with higher weights in the transgenic plants than in the controls, except for 6-13 and 13-7. These findings suggest that maize plants expressing a phytase gene may be able to take up organic phosphorus from the soil and transfer it to the developing ears.

In summary, compared with the control maize inbred line GSH9901, the introduction of *PhyA2* into maize can positively affect the soil phosphorus use efficiency, plant growth state, and seed yield. Therefore, *PhyA2* may be useful for improving maize yield traits and phosphorus use efficiency.

Authors' contributions Peng Jiao and Wen-Ya Yuan designed and performed the experiments. Han-Dan Zhao, Jing Qu, Pi-Wu Wang analyzed the data, Peng Jiao and Wen-Ya Yuan wrote the paper, Shu-Yan Guan and Yi-Yong Ma full guided the experiments and the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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