Title: The introduction of a phytase gene from *Bacillus subtilis* improved the growth performance of transgenic tobacco

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ABSTRACT

Phytate, the main form of phosphorus storage in plant seeds, is well known to be an anti-nutrient and a major source of phosphorus pollution in animal manure. To improve phosphorus bio-availability, we introduced a recently characterized phytase from *Bacillus subtilis* into the cytoplasm of tobacco cells. Although the introduction of acid fungal phytase from *Aspergillus niger* in previous studies did not result in any phenotypic changes in tobacco, here we show that a tobacco line transformed with a neutral phytase exhibited phenotypic changes in flowering, seed development and response to phosphate deficiency. The transgenic line showed an increase in flower and fruit numbers, small seed syndrome, lower seed IP6/IP5 ratio, and enhanced growth under phosphate-starvation conditions compared with the wild type. The results suggest that the over-expression of *Bacillus* phytase in the cytoplasm of tobacco cells shifts the equilibrium of the inositol phosphate biosynthesis pathway, thereby making more phosphate available for primary metabolism. The approach presented here can be applied as a strategy for boosting productivity in agriculture and horticulture.

Keywords: *Bacillus*, inositol phosphate, phosphorus, phytase, phytic acid

INTRODUCTION

Phytins are the major organic phosphate in plants, which accumulate at late stages of a plant's life and return to the soil if they are undigested by animals or humans. In some cases, phytate content can constitute 20-50% of the total organic P in the soil (1). Phytases (EC 3.1.3.8 and EC 3.1.3.26) are enzymes that hydrolyze phosphate ions from phytic acid (*myo*-inositol 1,2,3,4,5,6 hexakisphosphate, IP6) sequentially to inositiol phosphates. In the soil, microbial phytases and various phosphatases from microorganisms and plant roots act together to recycle phytins. Hence, phytase genes from various microbial organisms have been targets for uses in biotechnology, mainly in the animal feed industry or to improve the bio-availability of phosphate locked in the phytins (2, 3). Many benefits have resulted from these attempts. First, phytase facilitates the desorption of cationic minerals (mainly Fe^{2+}, Zn^{2+}, Ca^{2+}) from phytic acid to enhance the nutritional values of crop-based animal feedstuffs. Second, the phosphate released from phytins by phytase can be taken up by monogastric animals; this also helps to minimize phosphorus contamination in river and underground water. Third, the over-expression of phytase genes in crops could bypass enzyme purification procedures if used directly as animal feeds.

Phosphate ion is a major limiting factor in plant growth and development: the organic phosphates in the forms of phytins cannot be taken up by root from the soil. Most phytins accumulate in seed endosperm during seed development, and the plant phytases are induced only at the early stages of seed germination. Relatively low phytate content as well as low phytase activity is detected in plant tissues during their vegetative stages.

Earlier work on transforming phytase genes from fungi and bacteria to plants included transit peptides that could direct the transgene's product to apoplastic locations to avoid complications on phosphate metabolism inside the plant cells (4). These approaches were logical because most of the phytases isolated from microorganisms function optimally at an acidic pH range (pH 2–6) and possess a wide spectrum of substrate specificity. A recent report on the transformation of a fungal phytase gene in *Arabidopsis* found that excreted phytase from roots would facilitate plant growth on organic phosphates, indicating that the microbial phytases could be incorporated directly in plants to enhance plant growth (5). Others have tried to target and express these phytase genes in cotyledons or in seed endosperm, because these organs are fed to animals (2, 6). To address the problem of mineral malnutrition arising from phytic acid in plant sources, some low phytic acid (*lpa*) mutants were isolated from wheat, maize, and legumes. These mutants were shown to have defects in phytate biosynthesis, which resulted in the lower phytate contents in their seeds (7). Products made from these mutant crops may have a great impact on the food industry in the future. Recently, several phytases isolated from bacilli were shown to possess unique characteristics in reaction mechanisms, substrate specificity, thermo-stability and, more importantly, they showed activity at neutral pH (8-10). The properties of these enzymes allow us to examine the effects of introducing a neutral phytase into the cytoplasm of plant cells. Our results show that the expression of *Bacillus* phytase activity in transgenic tobacco plants could probably enhance phosphate utilization inside the plants' cells, leading to a better performance in plant growth, especially under phosphate-deficient conditions.

MATERIALS AND METHODS

Materials

Tobacco (*Nicotiana tabacum*) variety 'GeXin No1' was obtained from the Shanghai Institute of Plant Physiology and Ecology. All enzymes used for molecular biology were obtained from Roche Diagnostics (Hong Kong, China) or Promega (Hong Kong, China) and all other chemicals were purchased from Sigma (St Louis, USA).

Generation of transgenic tobacco

The construction of the plant expression vector is shown in Figure 1A. The phytase gene from *Bacillus subtilis* strain 168 (10) was cloned into the pBI221 vector (Clontech laboratories, Inc., California, USA) between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase terminator. The expression cassette was then cloned into the binary vector pCAMBIA 1300 (11) which bears a hygromycin-resistant gene, resulting in the expression construct pCX-168phyA (Figure 1A). The vector was then introduced into tobacco by an *Agrobacterium tumefaciens* mediated transformation protocol (12-13). Transgenic plants were first selected by using hygromycin and then screened by PCR. Genomic DNA from plant leaves was prepared (14) and used as the template for PCR screening. The forward and reverse primers for the hygromycin resistance gene were 5'-CTACAAAGATCGTTATGTTTATCGGCA and 5'-AGACCAATGCGGAGCATAT ACG, and that for the phytase gene were 5'-ATGGCTCATTATGTGAATGAGGAA CAT and 5'-CTAGCCGTCAGAACGGTCTT, respectively.

Characterization of the transgenic lines

Genomic DNA and total RNA extracted from plant leaves were used for Southern blotting and northern blotting (15), respectively. Radiolabelled cDNA of the *168phyA* gene excluding the bacterial signal peptide was used as a probe for hybridization. For western blotting and ELISA, a polyclonal anti-*Bacillus* phytase antiserum was raised in rabbits by immunization with purified *168phyA*-encoding phytase that is over-expressed in *Bacillus subtilis* (10). The polyclonal antibodies were pre-adsorbed with the wild-type tobacco before use. A sandwich ELISA for quantification of phytase protein in plant extracts was set up by using the pre-absorbed antiserum as capturing antibodies and the protein G purified and biotinylated antibodies as detection antibodies. *Bacillus* phytase produced by the prophage expression system (10) was purified and employed as a standard.

HPLC analysis

To evaluate the phytase activity in the plant extract, 200 μg of plant proteins were incubated with 400 μg IP6 (Sigma, P8810) at 37 °C. After 4, 6 and 8 h, 1 volume of 0.05 M HCl was added to stop the enzyme action. Inositol phosphates (IP6, IP5, IP4, IP3) were then purified by anion exchange chromatography (Bio-Rad AG-1X8 column). The eluted samples were then freeze-dried and resuspended in mobile phase (50% (v/v) methanol, 0.1% (v/v) formic acid, 1.5% (v/v) tetrabutylammonium hydroxide, and 0.05 M EDTA] and injected into a C18 column (Alltech Alltima C18) for inositol phosphate determination (16). The respective peaks of IP6 and IP5 were measured by a

refractive index detector (Shimadzu R1D-10A, Shimadzu Corporation, Japan). To calculate the IP6 and IP5 content in the seeds, 1.0 g of seeds was ground in 10 ml 0.5M HCl, and the released inositol phosphates were analysed by HPLC as described above.

Growth rate of seedlings under low phosphate conditions

Germinated seedlings were first grown in modified MS medium (sucrose concentration was reduced to 10 g/l) (17) or agar with or without 1 mM phosphate (KH_2PO_4) for 10 or 20 days and then transferred to modified MS medium or agar with various levels of phosphate for 20 or 30 days. The plants were then dried and weighed individually.

RESULT

Generation of transgenic tobacco

To examine the physiological effects of a neutral phytase on plant metabolism, a plant expression vector that expresses a phytase from *Bacillus subtilis* strain 168 (168phyA) was created. The 168phyA gene, with its native signal peptide excluded, was subcloned into the binary vector pCAMBIA1300 to generate the recombinant clone pCX-168phyA (Figure 1A). The vector was transformed into tobacco and four T0 plants, named 41, 42, 43, 44, were produced. Genomic DNA isolated from these plants was shown by polymerase chain reaction (PCR) screening to carry both hygromycin-B-resistant gene (Figure 1B) and the 168phyA gene (Figure 1C), respectively. Seeds collected from these lines were then germinated and selected by using hygromycin B. A T1 line (lines 42) showed hydromycin B resistance at a 3:1 segregation ratio. The presence of the phytase gene in this T1 line was again confirmed by PCR (data not shown). Genomic DNA isolated from these T1 lines was used for Southern blotting for the determination of gene copy number. Specific bands were detected in the transgenic line but not in the control lines. As shown in Figure 1D, T1 plants generated from lines 42 carried a single copy of the transgene. Subsequently, two T1 lines (42-1 and 42-3) were found to be homozygous by segregation analysis.

Expression of phytase in the transgenic lines

The expression of the phytase gene was confirmed at the mRNA and protein levels. Analysis of northern blots using the 168phyA cDNA as a probe indicated constitutive

expression of the gene in plant leaves (Figure 2A). Protein expression of the phytase in transgenic plants was examined by a specific antiserum raised against *Bacillus* phytase both qualitatively (western blot) or quantitatively (Sandwich enzyme-linked immunosorbent assay (ELISA)). A protein band of approximately 45 kDa was detected in the F1 samples of the transgenic lines (Figure 2B) but not in the control samples, which is very close to the calculated molecular mass of 44 kDa. The results of ELISA showed that various levels of phytase (0.05-0.1% (w/w) soluble plant proteins) were available in roots, leaves and flowers. Because plant extracts contained high levels of free phosphate ions and chlorophyll, which seriously interfered with the accuracy of the conventional phytase assay, an indirect high-performance liquid chromatography (HPLC) method was adopted to compare the endogenous phytase activities. As shown in Figure 3A, the leaf extracts from line 42 (n=4) yielded a lower IP6/IP5 ratio when compared with that from the controls, indicating that the transgenic plants had higher endogenous phytase activity than the control plants.

Growth characteristics of the transgenic lines

When we grew the transgenic plants in the greenhouse with wild-type controls, more flowers and fruits were grown on the transgenic plants than on the controls (Figure 4A). For both lines, around 62–63% of flowers yielded tobacco fruits, but the average mass of fruit collected from the transgenic lines (0.222 g per fruit) was less than that collected from the controls (0.289 g per fruit) (Table 1).

Seeds of transgenic tobacco are irregular in size and have a shifted IP profile

A phenotype of smaller seed sizes could be observed in the transgenic lines under a microscope (Figure 4B). Scars could be seen in the small seeds (Figure 4C). On average, 18.6% of seeds harvested from the transgenic lines were smaller than the normal seeds, whereas only 2.2% of seeds harvested from the control line exhibited this phenotype. To examine the levels of various inositol phosphates in the seeds, tobacco seeds collected from the plants were ground and the inositol phosphates extracted. HPLC indicated increased levels of inositol pentaphosphate (IP5) and inositol tetraphosphate (IP4) in the transgenic seeds but not in the control seeds (Figure 3B).

Seedlings of the transgenic lines grow better under phosphate-deficient conditions

The germinating ability of the seeds was tested. Large seeds could germinate whereas very tiny seeds could not. The ability of the transgenic seedlings to grow under conditions of low phosphate was compared with that of the control line. The transgenic lines 42 attained higher dry masses than the control line under these conditions (Figure 5A). Similar results were obtained when transgenic line 42 was grown on medium**.** It is statistically significant that the transgenic line grew bigger than the control line, especially when the availability of phosphate was limited. In another experiment (Figure 5B), the root and shoot mass of each plant were determined individually. After the plants were transferred to a medium deficient in phosphate, both the root and shoot of the transgenic line grew bigger, to a comparable extent, than the control line. Therefore the shoot/root (S/R) ratios for both lines were very close to each other (2.72–2.90), and were considerably smaller than the S/R ratios (9.73–9.94) obtained when both lines were transferred to a medium with 1 mM phosphate.

DISCUSSION

Properties of the Bacillus phytases

According to the reaction mechanisms, phytases from various species fall into three categories: (1) histidine acid phosphatase; (2) purple acid phosphatase; and (3) β-propeller phytase. Type 1 phytases from fungi (18), *Escherichia coli* (19), rat and plant species (20) share a conserved active site motif, RHGXRXP, unique to this class of enzyme. Some of the phytases isolated from these species, including the *phyA* from *Aspergillus niger*, have been extensively exploited for feed additive uses with some success (3, 6, 21). When *phyA* was transformed to plants, in all cases phytase activity could only be detected when it was targeted to the apoplastic locations (4); no effect on growth rate or plant morphology was observed (2, 22). It is speculated that PhyA must first go through the secretion pathway in the plant's cells for activation, otherwise the enzyme simply cannot function in the cytosol at neutral pH.

Type 2 purple acid phosphatases, also known as the metallophosphoesterases, are mainly secreted by plant roots as a normal response to phosphate starvation, which enables plants to acquire phosphates from the soil. Generally this type of phytase acts non-specifically on IP6, but also on a wide range of organic phosphates including physiologically important phosphonucleotides (23). Both type 1 and type 2 phytases, with a few exceptions, generally work at acidic pH or in extracellular environments (such as in acidic soil colloidal solution, apoplast and digestive tract).

Type 3 phytases are unique to *Bacillus*. They possess the most specific substrate range and can work properly under neutral pH (9, 10). These properties make them

suitable candidates for cytosolic expression so as to examine whether they can perturb phytic acid biosynthesis inside plant cells. A recent report showed that exogenous *Bacillus* phytases could promote the growth of maize seedlings when phytate was made the sole source for phosphorus in the growth medium. These *Bacillus* strains could possibly make symbiotic associations with plant roots and help to mobilize phosphate from soil phytate for root uptake (24).

Evidence of the transgene function in vivo

The construct for tobacco transformation in this study was designed to express *Bacillus* phytase in the cell cytoplasm. Our results indicated that the phytase gene from *Bacillus* was successfully incorporated into the tobacco genome in line 42 (Southern blot analysis), being transcribed (northern blot analysis) and translated (western blot analysis). In addition, phytase activity on IP6 was also elevated in leaf extracts of transgenic line 42. The *in planta* function of the phytase transgenes could be reflected by the phenotypic changes (Table 1). β-propeller *Bacillus* phytases require Ca^{2+} as enzyme activator, and also Ca-phytate as substrate. According to the *in vitro* studies of *Bacillus* phytase (9), 10 μM of Ca^{2+} should be sufficient to drive the enzyme to about 50% of its full velocity, and that higher Ca^{2+} concentrations could lead to inhibitory effects. As the Ca^{2+} level in plant cytoplasm is in the micromolar range, it is expected that *Bacillus* phytase could function properly in the cytoplasm to hydrolyse IP6, IP5 and IP4 in the transgenic lines.

Responses of transgenic plants under phosphate-starvation conditions

Phosphate ions are important for the energy metabolism in plants (adenine-triphospho-nucleotides), carbohydrate metabolism (sugar phosphates), nucleic acid metabolism and membrane phospholipid metabolism. Phosphate ions are highly mobile among subcellular compartments. The antiports of phosphate and sugar-phosphate between subcellular compartments are well documented; up to 80% of free phosphate can be stored in the vacuole of a plant cell to regulate phosphate homeostasis in the cytosol (25). When plants are subjected to phosphate starvation, cytosolic phosphate content can go down from the 10 mM to sub-micromolar range. During vegetative growth 'excess phosphate' could be converted into relatively immobilized forms like IP3–IP6 in most of the plant cells. It is not known whether these phosphorylated inositols are metabolized during vegetative growth. So far, only one phytase from maize has been found to be induced in the root endodermis under phosphate-starvation conditions (26). Our results indicate that crude enzyme extracts from wild-type tobacco leaves demonstrate certain phytase activity in *in vitro* assay. Under phosphate-deficient conditions, plants switch on the phosphate-deficiency signal transduction pathway, as exemplified by the induction of root elongation, resulting in the decrease in S/R ratio, secretion of acid phosphatase to soil from root, and the expression of the high-affinity phosphate transporter genes in roots and leaves. The sense of phosphate deficiency is likely initiated from the shoot and is mainly dependent on the phosphate status in leaves (27, 28). Adequate levels of phosphate (5–20 mM) in the cytoplasm of plant leaves is a prerequisite for plant growth because it is required for the exchange of sugar-phosphates from the chloroplasts in the source leaves, and subsequently for the export of sugars to the sinks. The development of young leaves is seriously hampered under phosphate-starvation conditions. It is more

difficult to induce phosphorus deficiency in mature plants because phosphate is highly mobile. Therefore we conducted phosphate-starvation experiments on seedlings. Wild type and transgenic seedlings showed a similar decrease in S/R ratio, a typical response to phosphate starvation; however, the seedlings of transgenic line 42 clearly out-grew wild-type seedlings by nearly 50% when they were completely deprived of phosphate in the medium. Under our experimental conditions, the difference in growth could be attributed to the function of the phytase transgene.

Relationships with the low phytic acid mutants

Breeders have been able to isolate single-locus mutations with the lpa phenotype from various plant species by chemical and physical mutation methods. The defects in certain enzymes of the phytic acid synthesis pathway in these mutants resulted in low phytic acid content in seeds (29). The mutations did not affect the vegetative growth but resulted in reduced grain yields. In wild type plants, most of the phosphorus will be channeled to seed storage (in the form of phytins) during the late stages of development. In two low-phytate mutants isolated from *Zea*, total phosphate content of seeds remained unchanged but a reduction in seed dry mass was observed (7). The shift of phosphate homeostasis in the *lpa* mutants will affect seed development, especially in starchy grains, as high phosphate concentration is likely to inhibit key enzymes for starch synthesis, such as ADP-glucose-pyrophosphorylase (29, 30); alternatively, high cytosolic phosphate concentrations could inhibit sugar- phosphate intake to endosperm amyloplasts during the grain-filling process. In this study, the constitutive expression of *Bacillus* phytase in the transgenic tobacco did not lead to notable adverse effects on plant growth and

development. Instead, we present evidence that some aspects of tobacco reproductive growth are enhanced. We observe that the seed development of the transgenic line is abnormal: about 20% of the transgenic seeds are less than half the normal size, but these small seeds (apart from extremely small ones) could germinate well in culture media and develop into healthy plants. We speculate that the expression of the phytase transgene during seed development in tobacco would not affect embryo formation but would affect the starch deposition because of the high phosphate levels in the cytoplasm of the zygotes. In fact, in the transgenic seeds, we constantly found lower values in the IP6/IP5 ratio, an indicator of more phytase activity during seed development. Although we do not have sufficient data to compare the *lpa* mutants and our transgenic plants, these two approaches can obviously lead to similar phenotypic changes during seed development. The notion that IP4, IP5 and IP6 are not essential for the vegetative growth of plants is likely to be established by these studies.

Potential implications

Many developmental, hormonal and environmental factors regulate the switch from vegetative growth to reproductive growth in plants. Although nitrogen is more critical for vegetative growth, phosphorus availability is regarded as the major nutritional requirement for flowering and fruiting. In horticulture, both inorganic and organic phosphate fertilizers are generally applied to culture pots to promote the full bloom of flowers. Perhaps it is premature to conclude the phytase transgene functions to promote flowering. The phenotypic changes in the transgenic lines certainly prompt us to pursue this further. Based on our results, we propose that *Bacillus* phytase transgene in tobacco

serves to shift the equilibrium of the inositol phosphate biosynthesis pathway in plant cells, thereby supplying more phosphate for primary metabolism. The consequence of this shift will enable plants to perform better under phosphate-starvation conditions, and promote reproductive growth under normal growing conditions. The results in this study could also lead to an alternative approach to generate low phytate grains and help to alleviate the problems associated with phytic acid in general. The application potential of this novel approach can be further broadened by site-directed expression of the phytase in various plant tissues under the control of various specific promoters.

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FIGURE LEGENDS

Fig 1. Generation of transgenic tobacco. (A) Expression construct pCX-168PhyA. CaM, CaMV 35S promoter; NOS, nos terminator; polyA, CaMV35S poly A signal; T, T-border. (B) PCR screening of hygromycin resistance gene. PCR products were amplified from tobacco transformed with the vector pCAMBIA 1300 and the expression construct pCX-168PhyA but not from the wild type. (C) PCR screening of 168PhyA gene. PCR product of *168PhyA* was only generated from plants transformed with pCX-168PhyA but not from that transformed with pCAMBIA1300. (D) Southern blot analysis of the T1 lines 42-1, compared with the wild type (Wt). Genomic DNA was digested with HindIII.

Fig 2. Expression of *Bacillus* phytase in the transgenic lines. (A) Northern blot analysis of leaves of transgenic plants with expression of *Bacillus* phytase, compared with the wild type. (B) Western blot analysis of leaves of transgenic plants with expression of *Bacillus* phytase, compared with the wild type.

Fig 3. HPLC analysis of inosital phosphates. (A) Phytase activity in the leaf extracts. Immediately mixed with the leaf extract, the IP6/IP5 ratio of the phytic acid substrate was 3.61±0.14 (n=4). During the incubation, IP6 was gradually broken down into lower inositol phosphates (IP5, IP4 and IP3) and therefore the IP6/IP5 ratio decreased with time. (B) Inosital phosphates in the seeds (n=6).

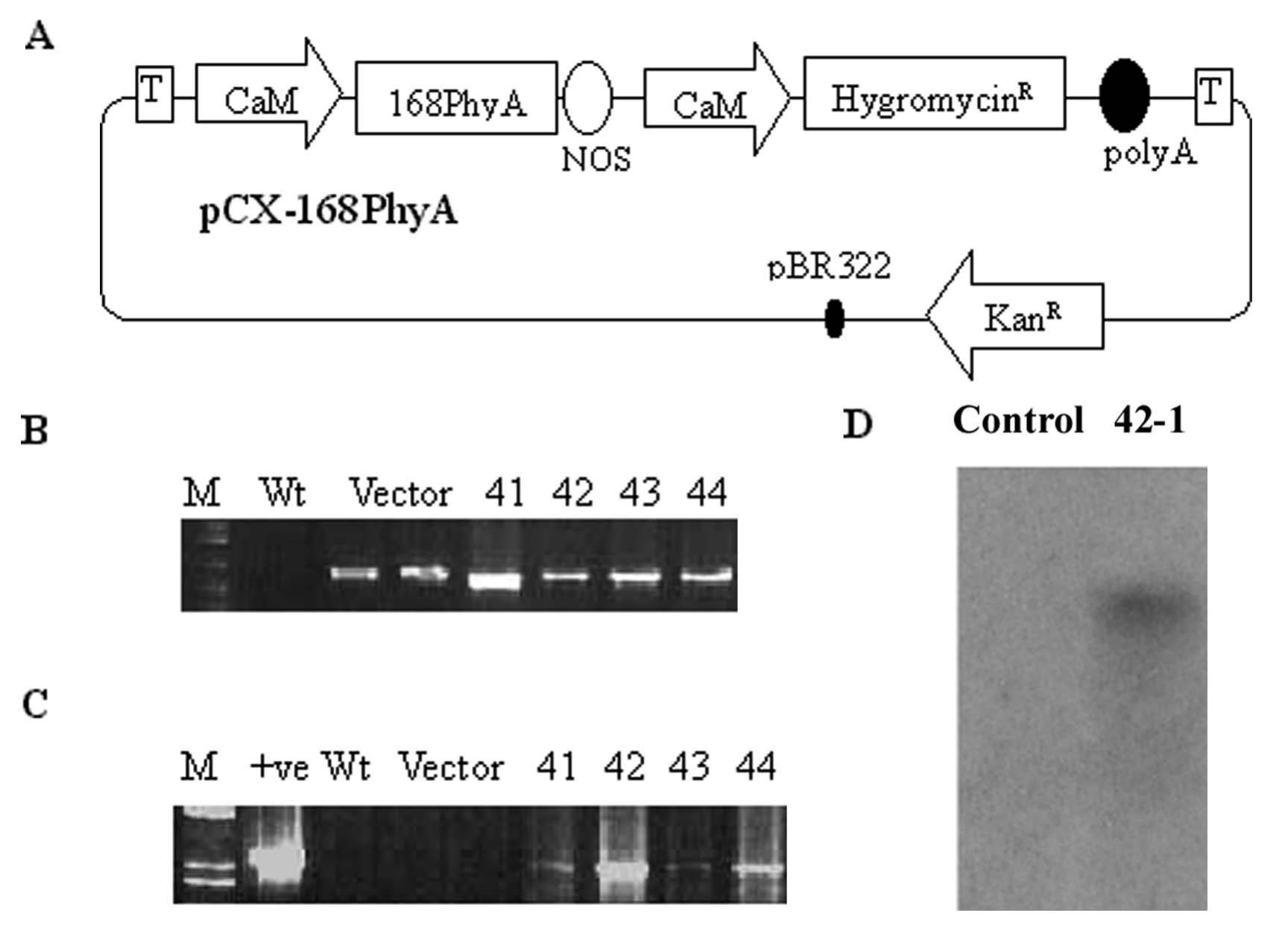
Fig 4. (A) Flower morphology. (B) and (C) Seed morphology.

Fig 5. Growth of seedlings under phosphate-deficient conditions. (A) Growth of seedlings on agar. Seeds were sown in 20 ml modified MS agar with 1 mM phosphate for 20 days; 18 seedlings were then transferred to modified MS agar with various phosphate concentrations (0.01 mM, or 0.1 mM). The seedlings were then grown for 30 days before determination of dry mass. (B) S/R ratio. Seeds were sown in MS0 medium with 1 mM phosphate for 20 days and then transferred to medium deficient in phosphate for 20 days. Fifty plants from each line were grown in ten flasks and the dry mass of the root and the shoot of each plant were determined individually.

Table 1. Phenotypic changes between the wildtype and the transgenic line.

Parameter	WT	Transgenic Line	Difference
Average no. of flower buds/plant	114.7±20.9	160.5±26.4	+39.9%
Average no. of fruits/plant	71.7±11.0	101.5±17.6	+41.5%
% fruit/flowers	62.5%	63.2%	+1.1%
Average weight per fruit (g)	0.289	0.222	-23%
Total weight of fruit per plant (g)	20.7	22.5	+8.7%
Percentage of small seeds	2.2±0.4	18.6±0.6	N.A.
IP6/IP5 ratio of the seeds	25.5±1.3	8.7±1.3	N.A.

Tobacco plants were grown in the greenhouse in 2L pots. Results were determined on fully senescent plants. Data represent the mean ± s. e. of determinations on six individual plants per line. IP6/IP5 ratios of the seeds were determined by HPLC.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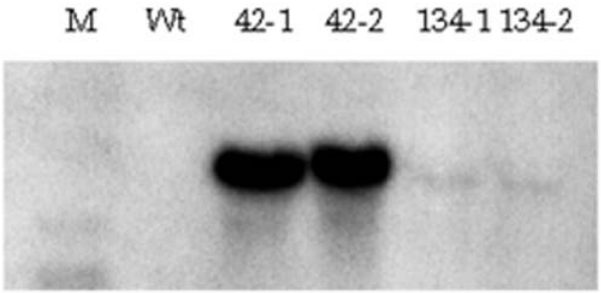
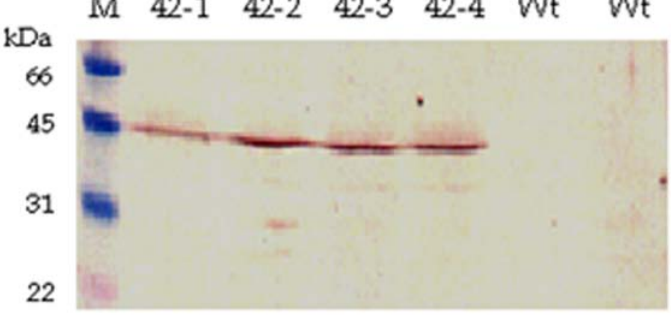
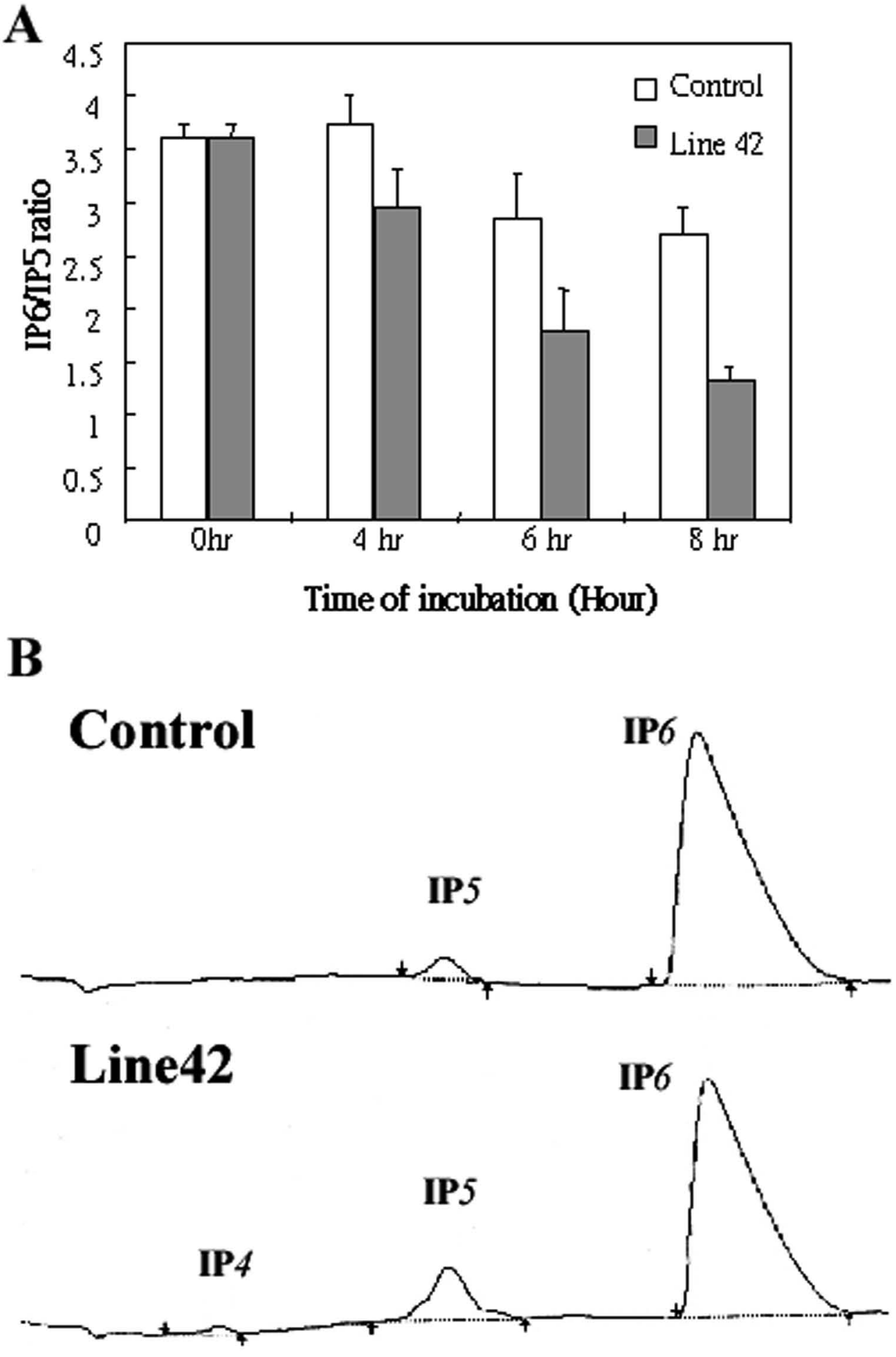
CaM
168PhyA
NOS
CaM
Hygromycin^R
polyA
T
pCX-168PhyA
pBR322
Kan^R
B
M Wt Vector 41 42 43 44
C
M +ve Wt Vector 41 42 43 44
D
Control 42-1

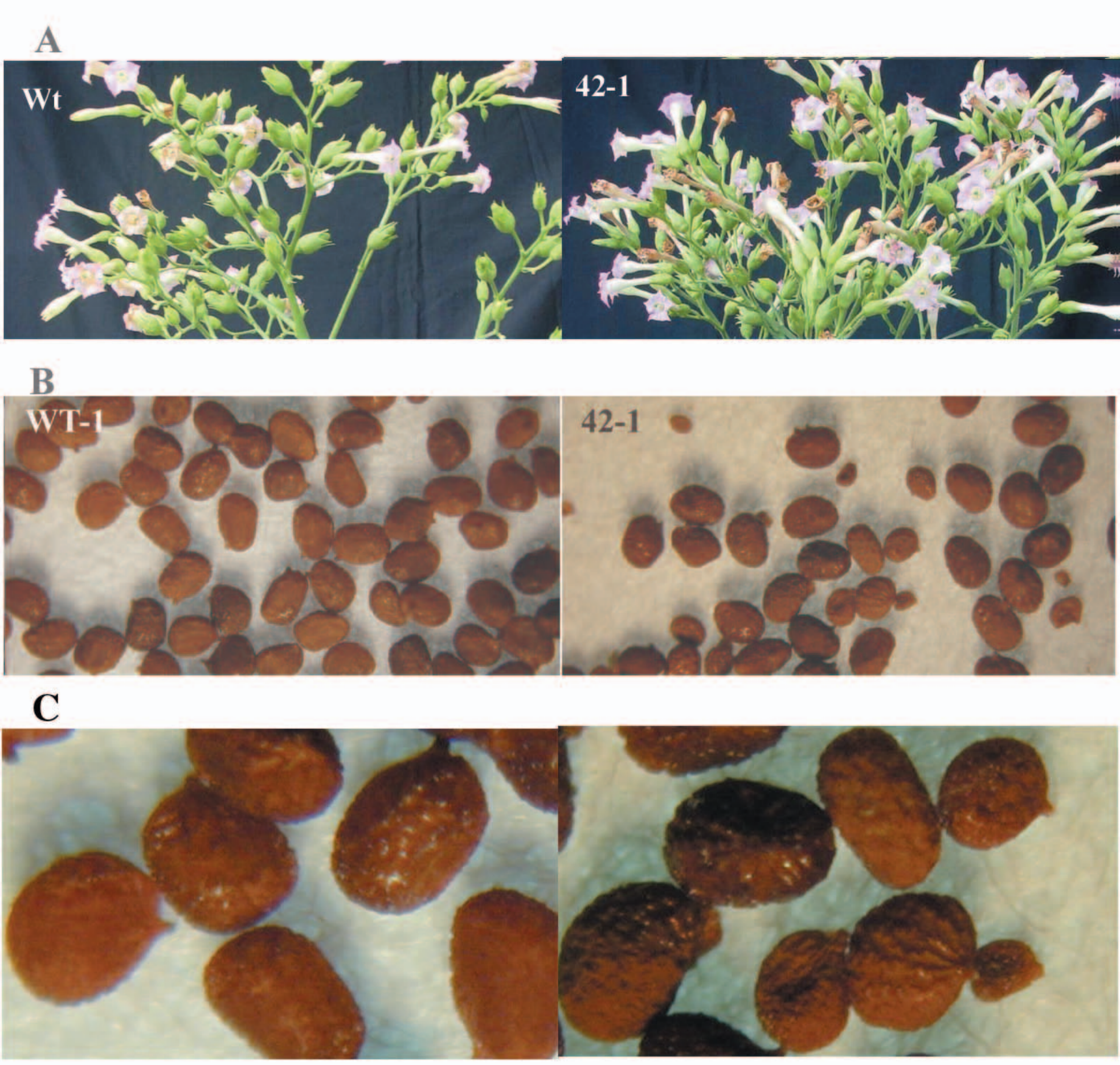
A



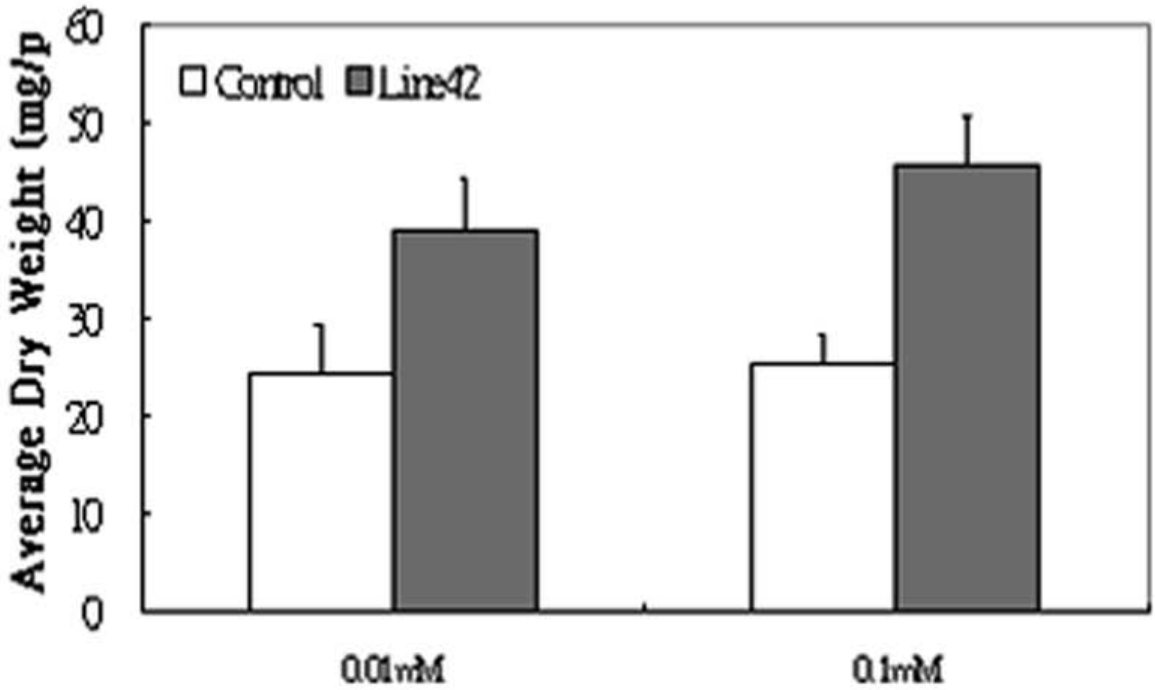
B



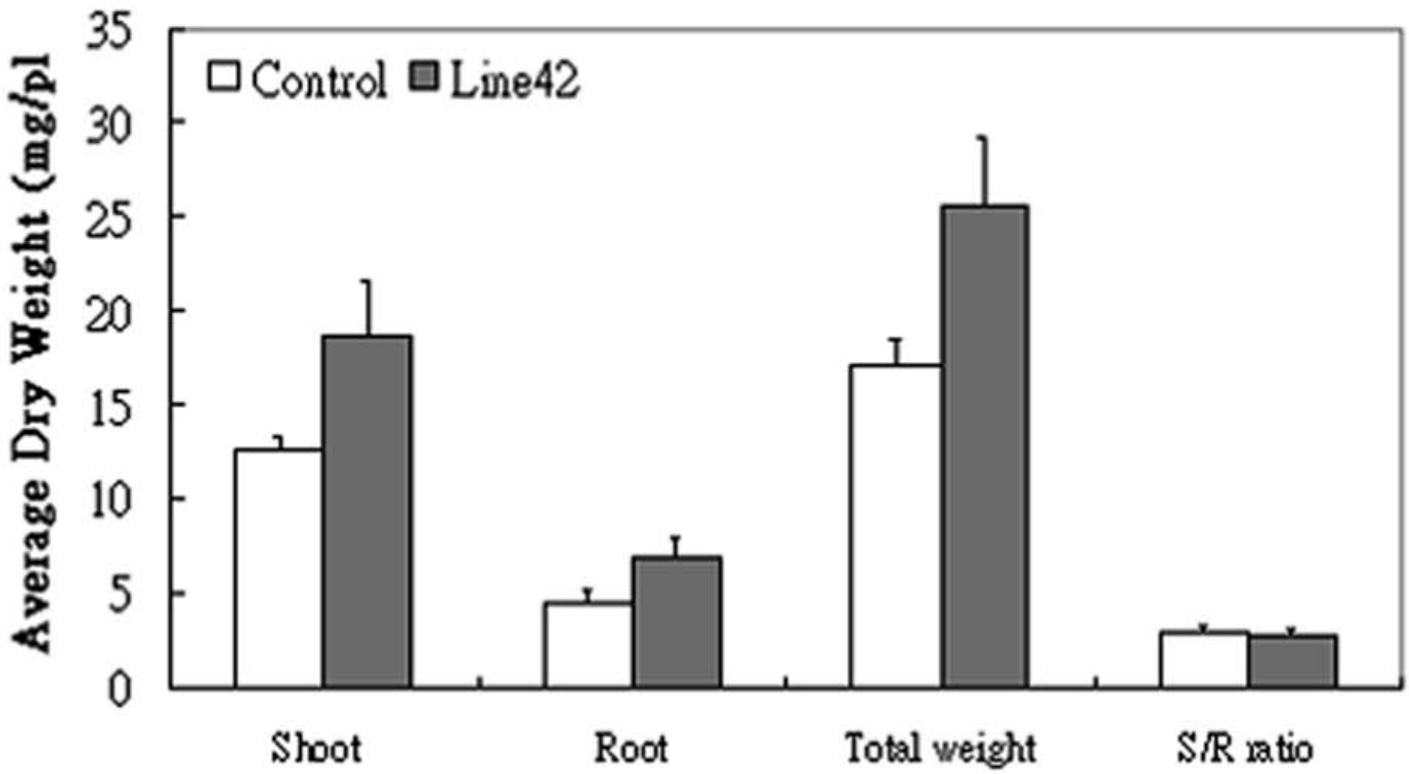

A
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
IP6/IP5 ratio
Control
Line 42
0hr
4 hr
6 hr
8 hr
Time of incubation (Hour)
B
Control
IP6
IP5
Line42
IP6
IP5
IP4


A
Wt
42-1
B
WT-1
42-1
C

A.


Average Dry Weight (mg/p
60
50
40
30
20
10
0
Control
Line42
0.01mM
0.1mM

B.


Average Dry Weight (mg/pl
35
30
25
20
15
10
5
0
Control
Line42
Shoot
Root
Total weight
S/R ratio