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Expression of Isopentenyl Transferase Gene Controlled by Seed-Specific Lectin Promoter in Transgenic Tobacco Influences Seed Development

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Abstract The bacterial isopentenyl transferase (*ipt*) gene involved in cytokinin biosynthesis was fused with a seed-specific lectin promoter from soybean and introduced into tobacco. Under the control of the lectin promoter, the expression of the *ipt* gene increased cytokinin levels and promoted cell division in the embryo in transgenic tobacco seeds. Compared with controls, the number of plerome cell layers and the cell number of cotyledons and pleromes were significantly increased from 16 DAF (days after flowering); the embryo diameter of transgenic tobacco was enlarged at 16, 19, and 21 DAF (16.1%, 12.7%, and 13.9% increase, respectively). Furthermore, the soluble protein content of the transgenic mature seeds was increased by 9.8–22.2% and the dry weight of transgenic tobacco seeds was increased by 8.8–21.8% compared with that of controls. The transgenic tobacco seedlings also grew quickly and a greater increase in fresh weight compared with controls was observed at 20 and 35 days after germination (average 14% and 8% increase above controls, respectively).

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Introduction

Cytokinins, which are involved in a wide range of physiologic and metabolic processes of plants, promote cell division (Skoog 1957), stimulate flowering and seed and fruit development, and inhibit root cell prolongation (Cary and others 1995). Cytokinins also have important regulative roles in inducing the differentiation of buds and roots, delaying leaf senescence (Schmulling 1997), and directing the transportation of nutrients (Davies 1995). Most previous studies on cytokinin actions are based on exogenous application, but there are many possible problems such as updating, transportation, and catabolism of exogenous cytokinins, that make the research difficult to figure out. With the development of molecular biology and plant transgenic technology, it has become feasible to study the function and mechanism of cytokinins in *planta* by genetic engineering. One of the new approaches for studying the regulative roles of cytokinins has been provided by identification of the isopentenyl transferase (*ipt*) gene that is responsible for cytokinin biosynthesis from *Agrobacterium tumefaciens*.

Originally, *ipt* gene expression was controlled by its native promoter or other constitutive promoters such as cauliflower mosaic virus (CaMV) 35S, which resulted in extreme overproduction of cytokinins, and consequently blocked root growth and regeneration of the whole plant (Klee and others 1987; Smigocki and Owens 1988; Wang and others 1997). Some environmental promoters such as the heat-shock promoter were also used in cytokinin

studies. However, the *ipt* gene in these plants is expressed in every tissue and organ after induction and the normal growth of plants is affected (Medford and others 1989; Ainley and others 1993; Schmulling and others 1989). Therefore, it is difficult to evaluate the cytokinin function in the normal physiologic circumstances of plants.

In recent years, more promoters from eukaryotes have been cloned, which accelerates the research of cytokinin genetic engineering. Controlling *ipt* gene expression with tissue- or developmental-specific promoters, the site, time, and level of cytokinin expression can be regulated according to the different intentions. A fruit-specific promoter 2A11 was fused with the *ipt* gene and introduced into tomato and the expression of the *2A11-ipt* gene was confined to the fruit, which led to significantly increased levels of total and soluble solids in the fruit but the transgenic tomato developed normally compared with controls (Martineau and others 1995). Under the control of the auxin-induced promoter, the tissue- and organ-specific overexpression of the *ipt* gene produced a number of morphologic and physiologic changes and it was observed that leaves displayed higher chlorophyll content and the intact and detached leaves stayed green longer than that of control plants (Li and others 1992). A wound-induced promoter PI-IIK was also used in this kind of experiment, and it was observed that the expression of the *PI-IIK-ipt*-fused gene in transgenic tobacco resulted in remarkedly delayed leaf senescence (Smigocki and others 1993).

Besides overexpression of the *ipt* gene, upregulation of the genes encoding for enzymes that catalyze cytokinin breakdown, cytokinin oxidase/dehydrogenase (CKX), has resulted in a cytokinin-deficient phenotype (Werner and others 2003). Furthermore, cytokinin receptor mutants have also been shown to have a cytokinin-deficient phenotype in a recent report (Riefler and others 2006). These provide an alternative approach to study the cytokinin actions by loss-of-function experiments.

Although some studies on the physiologic function of cytokinins have been completed by using the specific promoters, little is known about the cytologic effects of cytokinins on embryo and seed development. Using a pea storage protein vicilin promoter to direct *ipt* gene expression, we have obtained a transgenic tobacco in which the cytokinin levels have specifically increased in the developing embryo, which led to an increase in the dry weight of the mature seed (Ma and others 2002). It is interesting to know if the different seed-specific promoters with different temporal and strength characteristics will affect cytokinin biosynthesis and then the final seed development. These data may help to regulate crop seed production in agriculture. The promoter for the soybean storage protein lectin contains regulatory DNA sequences, which specify seed-specific expression in dicotyledonous plants, including soybean (Cho and others 1995), tobacco (Philip and others 1998), cotton (Townsend and Llewellyn 2002), and *Arabidopsis thaliana* (Darnowski and Vodkin 2002). In the transgenic tobacco, the lectin promoter directs *GUS* gene expression in the embryo of developing seeds 12-21 days after flowering (DAF) and the GUS protein is finally located in the protein bodies (Philip and others 1998). In this report, the *ipt* gene was fused with the lectin promoter and the chimeric *lectin-ipt* gene was introduced into tobacco. The influence of *ipt* gene expression controlled by the lectin promoter on seed development was analyzed from physiologic and cytologic aspects.

Materials and Methods

Plant Material

Nicotiana tabacum L. (cv. Wisconsin 38) was used as control. Flowers of tobacco were tagged at the emergence of petals. Capsules at intervals after flowering and mature seeds were harvested for the physiologic and cytologic analyses. The segregation of the integrated gene in the progeny was tested for kanamycin resistance by germinating seeds on agar medium containing kanamycin (150 μg mL^{-1}).

Plasmid Construction and Transformation of Tobacco

The soybean lectin promoter (Cho and others 1995) was cut by *Nco*I-*Eco*RI and inserted into the *ipt* 5′ flanking region that had created a *Nco*I site in the ATG start codon (Ma and others 1998). The fused *lectin-ipt* gene was then cut by *Eco*RI-*Hin*dIII and inserted into the pGA492 expression vector (An 1985). Transformation followed the protocol of Ma and others (1998). Briefly, tobacco leaves were cut into 0.5-mm^2 pieces and placed in *Agrobacterium* (LBA4404) solution (OD_{600} = 0.3–0.5) for 8 min. Infected leaf pieces were transferred to MS agar plates without antibiotics and left at 26°C for 48 h, then transferred to shooting medium (MS + 6-BA 1 mg L^{-1} + NAA 0.2 mg L^{-1}) containing cefotaxime (500 μg mL^{-1}) and the antibiotic for selection (kanamycin 100 μg ml^{-1}). The regenerating shoots were transferred to MS agar-containing cefotaxime (500 μg ml^{-1}) and antibiotic, and the roots formed in 7-10 days.

Polymerase Chain Reaction Analysis

DNA was isolated from young tobacco leaves as described by Edwards and others (1991). Polymerase chain reaction (PCR) amplification in a 50-μl volume contained 5 μl 10 × PCR buffer, 1 μM primers, 0.2 mM of each dNTP, and 2 U

Taq DNA polymerase. There were 35 cycles in which parameters were 95°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min, and the final extension was at 72°C for 10 min. Specific primers for the *ipt* coding region used were 5′ primer: 5′-GGACCTGCATCTAATTTTCG-3′ and 3′ primer: 5′-TTTCAGAATGGGCCTCAGC-3′. PCR products were analyzed on a 0.8% agarose gel.

RNA Gel Blot and Cytokinin Analyses

Total RNA was isolated from tobacco seeds at 19 DAF using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's instructions. Ten micrograms of each total RNA sample was electrophoresed on formaldehyde 1.4% (W/V) agarose gels. RNA was blotted onto a Hybond-N+ membrane (Amersham) using a standard transfer procedure (Sambrook and others 1989). The blots were hybridized at 42°C with 6 × SSC, 5 × Denhardt, 0.5% SDS, and 100 μg ml^{-1} salmon sperm DNA with 50% formamide and washed with 0.1 × SSC plus 0.1% SDS at 65°C. Probes were ^{32}P-labeled using a Ready-to-Go DNA Labeling Kit (Amersham). RNA hybridization signals were normalized against signals derived from a soybean 18S rRNA probe.

Cytokinin levels of tobacco seeds were determined by ELISA following the methods described by Ma and others (2002).

Determination of Seed Dry Weight, Germination, and Seedling Growth

Dry weight of mature tobacco seeds was determined as hundred-weight (weight of 100 seeds). Seeds were put into dishes with wet Whatman paper and germinated at 26°C, and the seedling fresh weight was determined at 20 and 35 days after germination.

Analysis of Seed Soluble Protein

Tobacco seed soluble protein was extracted using phosphate buffer (0.2 M Na_2HPO_4-NaH_2PO_4, pH 7.0). Protein concentrations were determined by the Bradford assay (Bradford 1976). Under the above conditions, the absorbance of bovine serum albumin (BSA) of different concentrations was used as a standard curve.

Embryo Staging and Microscope Analysis

Tobacco seeds from different development stages were cleaned with distilled water and then fixed with FAA [formalin : glacial acetic acid : 50% methanol (5: 5: 95)] (Barker and others 1988). Fixed seeds were dehydrated through a graded ethanol and xylene series to 100% and embedded in paraffin at 56–58°C for about 24 h. Paraffin blocks with tobacco seeds were trimmed and thin longitudinal sections (10 μm) were serially produced using a Reichert Jung microtome. For anatomical observation, sections were deparaffinized in xylene, rehydrated through a graded water-ethanol series, and double-stained with 1.0% Safranin (w/v in 50% ethanol) and 1.0% Fast Green (w/v in 95% ethanol). After staining, sections were dehydrated through a graded series of ethanol plus xylene and sealed with Canada resin dissolved in xylene. Sections were examined and photographed under bright-field optics with an Olympus microscope (Melville, NY).

Embryo Phenotype Analysis

The paraffin slices of tobacco seeds near the center of axial orientation were selected. The diameter of embryos was determined at the hypocotyl under the plumule meristem. Embryo length was determined from the top of the cotyledon to the terminal of the radical cap. The number of plerome cell layers and the cell numbers of the endosperm, cotyledon, and plerome at every 10^4-μm^2 area were counted.

Embryo Cell Mitotic Index Analysis

Fresh collected seeds from different developing stages were fixed in Carnoy fixative solution (1 part glacial acid plus 3 parts 96% ethanol) for 10 h. The embryo was isolated from seeds with minute needles under a dissection microscope after transfer of the fixed material into 45% acetic acid. The embryo samples were treated with 1 M HCl for 10 min and washed with distilled water. The embryos were then stained with aceto-orcein solution (1% orcein in 60:40 v/v acetic acid:lactic acid). The cell number was determined in squash preparations by counting the nuclei, and the number of cells in mitosis was determined by counting the nuclei showing late prophase to early telophase. The mitotic index was expressed as number of cells in mitosis in 100 cells observed.

Results

Obtaining Transgenic Tobacco Plants and PCR Analysis

Twenty-nine lines of transformed tobacco plants were obtained after infection with *Agrobacterium tumefaciens*

and named C-1, C-2, C-3, …, C-28, C-29, respectively. DNA of tobacco leaves was isolated and *ipt* gene-specific primers were used for PCR analysis. A 504-bp band was detected in transgenic tobacco plants (Figure 1), but not in control tobacco, indicating that the *lectin-ipt* gene was introduced into the plants. The inheritance of the *lectin-ipt* gene with kanamycin resistance was further analyzed in self-fertilized progeny of the transgenic tobacco. Resistance versus sensitivity to kanamycin segregated in a ratio close to 3:1 in the T1 progeny of lines C-1, C-2, C-5, C-14, C-15, and C-21, indicating that probably one copy of T-DNA was integrated into the genome of these plants (data not shown). Compared with controls, the growth, morphology, and flowering of transgenic tobacco did not appear significantly different. The seeds from different developmental stages after flowering were collected for further analysis.

Lectin-ipt Gene Expression and Its Effects on Cytokinin Accumulation in Seed

To examine the expression of the *lectin-ipt* gene in the transgenic plants, RNA gel blot analysis was performed using total RNA from 19-DAF seeds. A transcript of the *ipt* gene was detected in all transgenic lines, but not in the wild type of Wisconsin 38 (Figure 2). Various transgenic lines had different expression levels; C-2 showed the highest level, whereas C-1 showed the lowest level.

Coinciding with the *lectin-ipt* gene expression, seed cytokinin levels were increased by 156-308% in the different transgenic lines compared with control (Table 1). The increase of cytokinin levels in the different transgenic plants was approximately proportional to the *ipt* mRNA levels. These results demonstrated that the expression of the *lectin-ipt* gene resulted in a strong elevation of seed cytokinin levels.

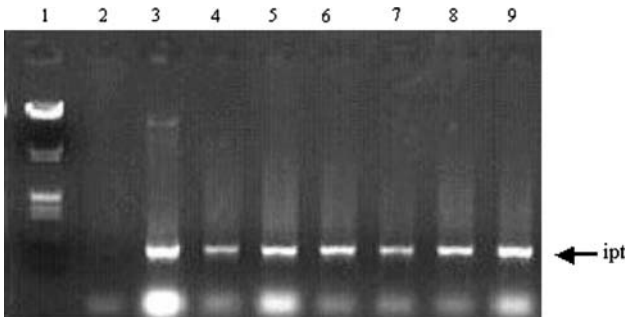

Fig. 1 PCR analysis of the different transgenic tobacco lines. Lane 1: λ DNA *Hin*dIII/*Eco*RI marker; lane 2: Wisconsin 38 (control tobacco); lane 3: transgene-construct-carrying plasmid (positive control); lanes 4-9: transgenic tobacco line C-1, C-2, C-5, C-14, C-15, and C-21

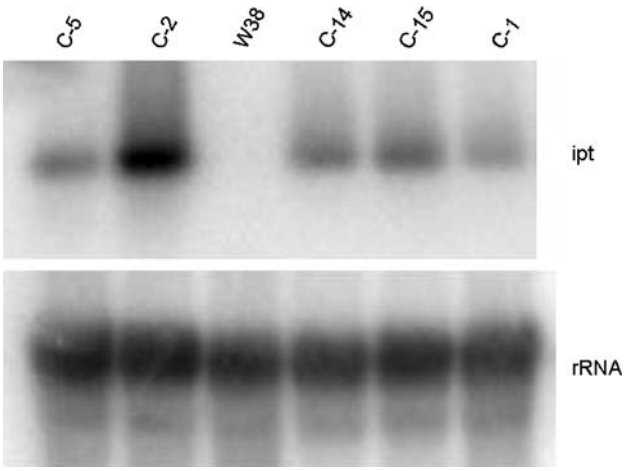

Fig. 2 RNA gel blot analysis of *lectin-ipt* gene expression in the seeds of various transgenic tobacco lines. Hybridization with an 18S rDNA probe has been included to confirm that the RNA preparations are undegraded and to serve as an internal control for variations in gel loading and blotting

Effects of *Lectin-ipt* Gene Expression on Embryo Phenotype

From 10 DAF, the embryo developed through the heart, torpedo, and cotyledon stages. At 13 DAF, the cotyledon, plerome, plumule axis, and radical were observed in the small embryo. Because none of the phenotypes tested was observed before 16 DAF, only later time points were included for further phenotypic descriptions.

At 16 DAF, the control embryos had 4-5 plerome cell layers, whereas the plerome in the *lectin-ipt* embryos was made up of 5-7 cell layers. At 19 DAF, the *lectin-ipt* embryos had 6-8 layers of plerome cells, which was 20-35% above that of controls. The number of plerome cell layers in the *lectin-ipt* embryos was increased by up to 40-50% at 21 DAF compared with controls (Table 2).

The diameter and length of transgenic and control embryos were measured (Table 3). No significant differences in diameter or length between the *lectin-ipt* and control embryos were observed at 13 DAF, which was consistent with the unchanged number of plerome cell layers at the same developmental stage of the embryos. From 16 to 21 DAF, the diameter of the *lectin-ipt* embryos was significantly larger compared with that of control embryos, whereas the length of the *lectin-ipt* embryos was similar with that of controls.

Effects of *Lectin-ipt* Gene Expression on the Cell Number and Cell Division of Seed

It is interesting to know whether the increasing embryo size in the transgenic seeds has any link with cell activity, and

Table 1 Cytokinin levels in the developing seeds of different tobacco lines (ng zeatin plus zeatin riboside/g tissue)

Plant	W38	C-1	C-2	C-5	C-14	C-15
Cytokinin levels	64 ± 9.5	164 ± 12.2***	261 ± 16.7***	221 ± 26.7***	184 ± 19.7***	196 ± 18.1***
% of control	100	156	308	245	188	206

Data are the mean of three replicates with 6–8 capsules per replicate. Values are expressed as mean ± SD, $n = 3$. Probability values between the control and transgenic tobacco were estimated by Student's t test and significant difference at the $p_{0.001}$ level between control and transgenic plants is marked by ***

Table 2 The number of plerome cell layers in tobacco seeds

Plants	W38	C-1	C-2	C-5	C-15
16 DAF	4.3 ± 0.7	5.6 ± 0.7***	5.9 ± 0.6***	5.8 ± 0.6***	6.6 ± 0.9***
19 DAF	5.3 ± 0.6	6.4 ± 1.0***	6.8 ± 0.9***	5.9 ± 0.6***	6.1 ± 0.7***
21 DAF	5.5 ± 0.6	7.3 ± 1.2***	7.8 ± 0.9***	7.5 ± 1.2***	7.5 ± 0.9***

Each value is the mean of 30 independent seeds plus SD. Probability values were estimated by Student's t test and significant differences at the $p_{0.001}$ level between control and transgenic plants are marked by ***

the total cell number in the different sections of the developing seeds was analyzed. Compared with controls, the cell numbers of the endosperm, cotyledon, and plerome in the transgenic embryos were all increased significantly. At 16 DAF, the cell numbers of the endosperm, cotyledon, and plerome were increased by up to 8.3%, 31.8%, and 29.5%, respectively; at 19 DAF, these three parameters were 14.4%, 29.2% and 30.3%, respectively; and at 21 DAF, these three parameters were 14.9%, 25.6%, and 23.0%, respectively (Table 4). Overall, the embryo, including the cotyledon and plerome, experienced a greater increase in cell number than the endosperm, indicating that the *lectin-ipt* gene promoted cell division, particularly in the embryo during seed development.

To test whether the increase in cell number in the transgenic embryo came from increased cell division activity, the mitotic index was determined. The data showed that the mitotic index slowly decreased from 16 to 21 DAF in control embryos, reflecting that embryo development had reached maturity. However, the expression of the *lectin-ipt* gene promoted a higher mitotic index compared to controls (Table 5), particularly at 16 and 19 DAF, which showed up to a 13% increase. The difference in the mitotic index between the transgenic and control was less obvious at 21 DAF.

Changes in Seed Soluble Protein Content and Dry Weight

The soluble protein content of mature seeds was analyzed (Table 6). There was a 9.8-22.2% increase in the transgenic tobacco mature seeds compared with that of controls. Statistical analysis using Student's t test showed that C-1 and C-2 had a significant increase at the $p_{0.001}$ level, C-14 and C-15 at the $p_{0.01}$ level, and C-5 and C-15 at the $p_{0.05}$ level.

Seed soluble protein content at different developmental stages was further analyzed (Table 7). From 16 DAF, there was a significant increase of soluble protein

Table 3 Effects of *lectin-ipt* gene expression on diameter (μm) and length (μm) of tobacco embryos at different developmental stages

Plants	16 DAF		19 DAF		21 DAF	
	Diameter	Length	Diameter	Length	Diameter	Length
W38	183 ± 24	522 ± 47	216 ± 27	580 ± 43	215 ± 24	594 ± 42
C-1	219 ± 28***	525 ± 52	243 ± 25***	582 ± 50	248 ± 30***	597 ± 53
C-2	212 ± 25***	518 ± 49	245 ± 32***	586 ± 48	250 ± 32***	595 ± 49
C-5	214 ± 24***	515 ± 53	239 ± 37**	592 ± 55	242 ± 20***	602 ± 52
C-14	207 ± 21***	520 ± 42	243 ± 34**	584 ± 49	240 ± 28***	593 ± 38
C-15	210 ± 26***	526 ± 49	247 ± 31***	587 ± 47	244 ± 32***	596 ± 42

Each value is the mean of 30 independent seeds plus SD. Probability values between the control and transgenic tobacco were estimated by Student's t test and significant differences at the $p_{0.01}$ and $p_{0.001}$ levels between control and transgenic plants in the same column are marked by ** and ***, respectively

Table 4 Effects of *lectin-ipt* gene expression on the cell number of the endosperm, cotyledon, and plerome at different developmental stages

Plant	Endosperm			Cotyledon			Plerome		
	16 DAF	19DAF	21DAF	16DAF	19DAF	21DAF	16DAF	19DAF	21DAF
Control	10.8 ± 2.3	12.5 ± 2.5	12.1 ± 1.8	33.3 ± 5.2	38.7 ± 4.6	38.3 ± 3.4	66.1 ± 9.6	55.4 ± 7.4	52.2 ± 6.3
C-1	11.4 ± 2.2	14.2 ± 2.2**	13.9 ± 2.7**	40.7 ± 4.0***	47.4 ± 4.8***	43.9 ± 4.7***	82.0 ± 10.1***	67.1 ± 7.7***	63.0 ± 6.9***
C-2	11.3 ± 1.4	14.3 ± 2.3**	13.8 ± 2.4**	42.0 ± 4.5***	52.1 ± 5.1***	46.9 ± 4.9***	83.1 ± 9.61***	68.8 ± 7.3***	63.4 ± 6.5***
C-5	11.7 ± 1.5	14.0 ± 2.0*	13.7 ± 2.2**	43.9 ± 4.8***	50.0 ± 3.4***	48.1 ± 4.7***	85.6 ± 10.1***	72.2 ± 7.6***	64.2 ± 5.9***

Each value is the mean of 30 independent seeds plus SD. Probability values between the control and transgenic tobacco were estimated by Student's *t* test and significant differences at the $p_{0.05}$, $p_{0.01}$, and $p_{0.001}$ levels between control and transgenic plants in the same column are marked by *, **, and ***, respectively

Table 5 Effects of *lectin-ipt* gene expression on the mitotic index of tobacco embryos at different developmental stages

Plants	16 DAF	19 DAF	21 DAF
W38	4.02 ± 0.412	3.94 ± 0.444	3.70 ± 0.418
C-1	4.51 ± 0.387***	4.30 ± 0.363**	3.93 ± 0.411*
C-2	4.61 ± 0.512***	4.46 ± 0.401***	4.01 ± 0.402**
C-5	4.55 ± 0.456***	4.42 ± 0.412***	3.96 ± 0.318**
C-14	4.47 ± 0.406***	4.39 ± 0.389***	3.91 ± 0.343*
C-15	4.54 ± 0.424***	4.29 ± 0.399**	3.89 ± 0.284*

Thirty independent embryos were analyzed and about 100 cells were counted for each embryo. Mitotic indices were expressed as number of cells in mitosis in 100 cells observed. Values are expressed as mean ± SD, $n = 30$. Probability values between the control and transgenic tobacco were estimated by Student's *t* test and significant differences at the $p_{0.05}$, $p_{0.01}$, and $p_{0.001}$ levels between control and transgenic plants in the same column are marked by *, **, and ***, respectively

content in the *lectin-ipt* seeds compared with that in controls (11.9–25.0%, 23.4–41.3%, and 11.6–25.0% increase at 16, 19, and 21 DAF, respectively). These results confirmed that actions of the *lectin-ipt* gene promoted the accumulation of storage protein in tobacco seeds. As reported before, the lectin promoter was sufficient to direct the attached protein to the protein bodies

Table 6 Effects of *lectin-ipt* gene expression on soluble protein content in mature tobacco seeds

Plant	Soluble protein content (μg/mg seed)	% of control
Control	55.01 ± 2.23	100
C-1	66.55 ± 3.70***	121.0
C-2	67.22 ± 2.97***	122.2
C-5	60.41 ± 3.58*	109.8
C14	60.71 ± 2.67**	110.4
C-15	60.42 ± 3.09*	109.8
C-21	62.97 ± 3.72**	114.5

Each value is the mean of five independent samples plus SD. Probability values between the control and transgenic tobacco were estimated by Student's *t* test and significant differences at the $p_{0.05}$, $p_{0.01}$, and $p_{0.001}$ levels between control and transgenic plants in the same column are marked by *, **, and ***, respectively

Table 7 Changes in seed soluble protein content at different seed development stages

Plant	Soluble protein content (μg/mg seed)		
	16 DAF	19 DAF	21 DAF
Control	10.09 ± 0.47	20.35 ± 0.94	46.59 ± 1.70
C-1	11.29 ± 0.72*	25.11 ± 0.85***	52.01 ± 2.84**
C-2	11.99 ± 0.83**	28.60 ± 1.81***	58.24 ± 2.78***
C-5	11.22 ± 0.58**	26.73 ± 1.61***	51.89 ± 2.55**
C14	12.61 ± 0.52***	28.54 ± 0.72***	55.21 ± 2.28***
C-21	12.09 ± 0.49***	28.75 ± 1.03***	53.84 ± 2.11***

Each value is the mean of five independent samples plus SD. Probability values between the control and transgenic tobacco were estimated by Student's *t* test and significant differences at the $p_{0.05}$, $p_{0.01}$, and $p_{0.001}$ levels between control and transgenic plants in the same column are marked by *, **, and ***, respectively

during seed development of tobacco (Philip and others 1998). This is consistent with our results.

The hundred-weight of tobacco mature seeds was determined (Table 8). Compared with controls, the dry weight of the *lectin-ipt* tobacco seeds was increased by 8.8–21.8%, with the significant differences at the $p_{0.001}$ level analyzed by Student's *t* test.

Table 8 Effects of *lectin-ipt* gene expression on tobacco seed dry weight

Plant	Dry weight (mg/100 seed)	% of control
Control	8.89 ± 0.22	100
C-1	10.01 ± 0.15***	112.6
C-2	10.83 ± 0.28***	121.8
C-5	10.06 ± 0.18***	113.2
C14	9.77 ± 0.15***	112.1
C-15	9.67 ± 0.43***	108.8
C-21	10.58 ± 0.36***	119.0

Each value is the mean of 10 independent samples plus SD. Probability values between the control and transgenic tobacco were estimated by Student's *t* test and significant differences at the $p_{0.001}$ level between control and transgenic plants in the same column are marked by ***

Table 9 Effects of *lectin-ipt* gene expression on tobacco seedling growth

Plant	20 DAG		35 DAG	
	FW (mg/seedling)	% of control	FW (mg/seedling)	% of control
Control	25.0 ± 2.06	100	44.2 ± 3.32	100
C-1	27.5 ± 2.13*	110.0	46.5 ± 3.06	105.2
C-2	28.6 ± 2.26**	114.4	48.3 ± 3.32*	109.3
C-5	28.5 ± 2.16**	114.0	47.8 ± 2.65*	107.4
C14	29.4 ± 2.20***	117.6	48.8 ± 2.92**	110.4
C-15	28.8 ± 2.25**	115.2	47.5 ± 3.31*	107.5

Fresh weight (FW) of seedlings was measured 20 and 35 days after germination (DAG), respectively. Each value is the mean of 10 independent samples with 20 seedlings per sample. Probability values between the control and transgenic tobacco were estimated by Student's *t* test and significant differences at the $p_{0.05}$, $p_{0.01}$, and $p_{0.001}$ levels between control and transgenic plants in the same column are marked by *, **, and ***, respectively

Effects of *Lectin-ipt* Gene Expression on Seedling Growth

Seedling growth of tobacco was analyzed to see whether the higher cytokinin biosynthetic gene activity affected subsequent seedling morphology. The *lectin-ipt* seeds germinated 2–3 days earlier and grew faster during the first 35 days after germination (DAG) compared with controls. The fresh weight of the *lectin-ipt* tobacco seedlings was increased up to 10.0–17.6% at 20 DAG compared with controls, with a significant difference analyzed by Student's *t* test; this increase was decreased to 5.2–10.4% at 35 DAG (Table 9). These results demonstrated that the *lectin-ipt* gene promoted nutrition accumulation in mature seeds and subsequently increased seedling growth, particularly at the early stage, which was consistent with the results from transgenic tobacco expressing the *vicilin-ipt* gene (Ma and others 2002).

Discussion

Cytokinins are thought to play an important role in seed development. This has been demonstrated by seed-specific expression of a heterologous *vicilin-ipt* gene in transgenic tobacco in our previous work (Ma and others 2002). Considering the importance of seed production in agriculture, it is interesting to know if the different seed-specific promoters will have different effects and mechanisms of cytokinin actions on seed development. Therefore, we have used the lectin promoter from soybean (Cho and others 1995), which may have a stronger and earlier expression pattern than that of the vicilin promoter in directing seed-specific expression, to investigate the cytokinin effects on seed development.

Under the control of the lectin promoter, the *ipt* gene was specifically expressed in transgenic tobacco seeds and expression levels varied in the different lines (Figure 2). Coinciding with *lectin-ipt* gene expression, endogenous cytokinin levels were up to two- to threefold higher in transgenic seeds at 19 DAF compared with controls (Table 1). The increase of cytokinin levels in the different transgenic lines was approximately proportional to the *ipt* mRNA levels; line C-2 showed the highest *ipt* mRNA and cytokinin levels, whereas C-1 showed the lowest levels.

Our results indicate that seed-specific expression of the *lectin-ipt* gene increases cytokinin accumulation and promotes cell division in the developing embryo, reflected by the higher mitotic index and greater cell number in the transgenic embryos (Tables 4 and 5). Consistent with these results, the *lectin-ipt* embryos contained 30–50% more layers of plerome cells (Table 2), and the diameter of the *lectin-ipt* embryos was significantly larger (12–20%) than that of controls in the different transgenic lines (Table 3). As a consequence of increased cell division in the embryo, accumulation of nutrients in the *lectin-ipt* seeds is promoted (Table 7), which leads to the higher protein content (Table 6) and dry weight (Table 8) in the mature seeds. Furthermore, the expression of the *lectin-ipt* gene promoted faster growth of transgenic seedlings (Table 9).

Our previous results demonstrated that seed-specific expression of the *vicilin-ipt* gene led to increasing cytokinin levels in the seed, which in turn stimulated embryo growth and protein accumulation in seed development (Ma and others 2002). Our analyses in embryo morphology, seed dry weight, protein content, and seedling growth of the *lectin-ipt* seeds indicated very similar actions to that of the *vicilin-ipt* gene. Furthermore, the actions of the *lectin-ipt* gene on embryo development were linked with its effects on promoting cell division. Moreover, the effects of the *lectin-ipt* gene are likely stronger than that of the *vicilin-ipt* gene. This is reflected by the greater increase in the hundred-weight and soluble protein of the *lectin-ipt* seeds compared with that of the *vicilin-ipt* seeds. This is most probably due to the higher cytokinin accumulation in the *lectin-ipt* seeds, which experienced a 156–308% increase in cytokinin levels compared with control tobacco, whereas *vicilin-ipt* seeds experienced only a 133–267% increase (Ma and others 2002). It will be very interesting to test these two genes in the important crops with large seeds, such as soybean and pea.

The lectin promoter has been reported to direct *GUS* gene expression in the 12–21-DAF developing seeds in transgenic tobacco (Philip and others 1998), which is earlier than the *vicilin-ipt* gene that we used previously (Ma and others 2002). However, the expression of the *lectin-ipt*

gene was detectable only at 16 DAF, which led to the remarkable cytokinin accumulation. This may be due to the highly sensitive GUS staining method which detects very marginal increases. However, for the cytokinin analysis procedure, a very small increase in cytokinin levels will be obscured by normal fluctuations. Furthermore, the small variation in cytokinin biosynthesis is likely to have little influence on seed development. Using a promoter (other than the seed storage protein) with an earlier expression pattern fused with the *ipt* gene will be valuable to test cytokinin actions in the initiating stage of embryo development.

Different promoters have been used to direct cytokinin gene expression in sink tissues. Fruit-specific expression of the *ipt* gene has been achieved in tomato fruit through the use of a fruit-specific promoter 2A11 fused to the *ipt* gene. Transgenic tomato with the *2A11-ipt* gene was phenotypically normal but the levels of total and soluble solids in the transgenic fruits were significantly increased (Martineau and others 1995). The AGPase S1 promoter has been used to direct *ipt* gene expression in sink tissues of leaves and fruits. The results, however, showed little influence on leaf and fruit development except to delay leaf senescence (Luo and others 2005). Another seed-specific promoter, 2S albumin AT2S1 from *Arabidopsis thaliana*, was used to direct *ipt* gene expression in canola (*Brassica napus* L.) and tobacco. Compared with controls, the average number of capsules of tobacco and siliques of canola in the *AT2S1-ipt* plants was 82.6% and 24.8% higher, respectively (Roeckel and others 1997). Combined with the data from the *vicilin-ipt* and *lectin-ipt* genes, therefore, it is reasonable to suggest that the seed- and fruit-specific expression of the *ipt* gene are the preferable systems to leaf in the investigation of cytokinin effects and also may be more useful in agriculture.

Seed production is very important in agriculture. However, not much is known about the factors regulating embryogenesis and subsequent seed development. Recently, several reports have shown strong evidence for the role of cytokinins in this process. By overexpression of the cytokinin oxidase/dehydrogenase (*CKX*) gene in *Arabidopsis*, the cytokinin levels were strongly reduced. This led to a strong reduction in seed set (up 80% decrease) but much larger seeds (approximately two times the wild-type weight). A similar enlargement was observed for transgenic embryos; this was attributable to increases in both cell number and cell size (Werner and others 2003). Similarly, a mutant named *Gn1a* was shown to reduce the expression of the *CKX* gene and increase cytokinin accumulation in inflorescence meristems in rice. This mutant had greater numbers of reproductive organs and higher grain numbers, which resulted in enhanced grain yield (Ashikari and others 2005). In another report, loss-of-function mutants of cytokinin receptors in *Arabidopsis* produced mutant seeds that were twice the size of wild-type seeds, although the cytokinin content was increased in the mutants. Genetic analysis indicated a cytokinin-dependent endospermal and/or maternal control of embryo size (Riefler and others 2006). Combined with our data, this strongly suggests that cytokinins have a pivotal role in seed development. However, the detailed mechanism behind the cytokinin regulation is complex and warrants a case-by-case investigation. In cytokinin receptor mutants, the increase of seed size is probably a direct consequence of loss-of-receptor functions and its role in growth control. Cytokinin levels seem to have limited roles in this case. However, via the regulation of the *CKX* gene, the increase in seed size is absolutely linked to cytokinin levels. Besides, increased cytokinin levels by downregulation of the *CKX* gene has lead to increased seed set instead of seed size in rice. We propose that different mechanisms may act in these processes; some may be linked with cytokinin receptors and some with cytokinin biosynthesis. This warrants further investigation. Using the specific promoter to confine the cytokinin actions in the defined tissues may be important to elucidate the mechanism of cytokinin regulation.

In conclusion, under the control of the seed-specific lectin promoter from soybean, the expression of the *ipt* gene had increased cytokinin levels and promoted cell division of tobacco embryos, which resulted in increases of plerome cell layers, the cell number in cotyledons and pleromes, and subsequently acceleration in seed development. As a result, the enlargement of the embryo provided a larger storage capacity for nutrients, and more nutrients (such as seed soluble protein) were transported to the developing seeds. The seed dry weight and the seedling growth of the *lectin-ipt* tobacco were increased significantly compared with that of controls. The lectin promoters offer a potential means to target cytokinin gene expression to the developing seeds of tobacco or other dicotyledonous plants that will be useful in agriculture.

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