Interactive Effects of Cytokinins, Light, and Sucrose on the Phenotypes and the Syntheses of Anthocyanins and Lignins in Cytokinin Overproducing Transgenic *Arabidopsis*

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ABSTRACT

Elevated cytokinins levels *in vivo* resulted in both enlarged and retarded growth phenotypes. Cytokinin levels were similar in both phenotypic groups. The ratio of the two phenotypes could be altered by changing the sucrose concentration in MS medium or the light intensity. Increased cytokinins in ipt:: GUS activated *Arabidopsis* induced the syntheses of anthocyanins and lignins, while light speeded up the anthocyanin accumulation. The endogenous increase in cytokinins and exogenous addition of sucrose in MS medium caused similar effects on the formation of anthocyanins and lignins in shoot, especially in the shoot apical meristem. Cytokinins induced *CHS* transcription in ipt:::GUS-activated *Arabidopsis* seedlings in the vegetative growth period; the transcription of *CHS* was low in *ipt* inactive plants and in wild-type *Columbia*. Cytokinins were capable of influencing either the synthesis or distribution of chlorophylls, but they did not alter *cab* and *rbc*S transcriptions in light-grown seedlings.

Key words: Interactive effects; Cytokinins; Light; ipt:::GUS-activated *Arabidopsis*; Anthocyanins; Lignins

INDRODUCTION

Growth and development in higher plants are the results of a combination of internal genetic events and external stimuli. One of the most important external stimuli is light. One of the earliest developmental events during embryogenesis is the initiation of the shoot and root meristems. In the shoot meristem, cytokinins and light cause similar effects, such as cotyledon expansion, leaf development, and induction of light-inducible gene expressions (Flores and Tobin 1986). The exogenous application of cytokinins has been shown to promote chloroplast

development in etiolated cucumber cotyledons (Fletcher and McCullagh 1971). An increased synthesis of chlorophyll-*a/b*-binding proteins by cytokinins was identified in a tobacco cell line (Teyssandier de la Serve and others 1985). The normal dark-adaptation response of *Lemna gibba* light-grown plants moved to the dark involved decreased levels of *cab* and *rbcS* mRNA, which was diminished in the presence of kinetin or benzyladenine (Tobin and Tukaly 1982; Flores and Tobin 1986). *Arabidopsis* seedlings grown in total darkness but in the presence of cytokinins had de-etiolated phenotypes, such as short hypocotyls, expanded cotyledons, high levels of chloroplasts, and light-regulated proteins (Chory and others 1991). The *amp1* mutant, with sixfold higher endogenous cytokinins than the wild type, showed increased levels of light-regulated gene expressions (*cab*, *rbcS*, and *CHS*) in dark conditions (Chin-Atkins and others 1996). The addition of cytokinins caused hp-*2* mutant phenotype alterations, such as shorter hypocotyls, apical hook opening, and cotyledon expansion. However, the increased anthocyanin accumulation was observed only in light-grown seedlings, not in dark-grown seedlings. Furthermore, evidence has shown that, in the presence of cytokinins, the *hp-2* mutant appears to be significantly enhanced in *CHS* mRNA only in light, not in dark-grown conditions (Mustilli and others 1999).

Sucrose inhibited de-etiolation in far-red light (Barnes and others 1996; Dijkwel and others 1997) and induced *CHS* activation (Koch 1996). The above evidence indicates that, somehow, exogenous cytokinins, light, and sucrose had exhibited cross-talk with regard to de-etiolated phenotype alteration and photo-regulated gene expression. However, understanding the cross-talk between light, sucrose, and endogenous increases of cytokinins *in vivo* is still limited. Chin-Atkins and others (1996) hypothesized that light and cytokinins likely act independently of one another to elicit downstream light-regulated responses. Whether cytokinins act as direct or indirect regulators on the development of chloroplasts and the regulation of light-activated gene expression remains to be determined.

To advance our understanding in these areas, in the present work, we investigated the interactions among light, sucrose, and cytokinins with cytokinin overproducing *Arabidopsis* transgenic lines, which harbor an ipt:::GUS transcriptional fused gene downstream of a pOp promoter. The pOp promoter was activated by 35S-LhG4 transcriptional factor, which was expressed independently in another transgenic event. The ipt:::GUS gene transcription was activated by crossing the pOp-ipt-GUS plant with a 35S-LhG4 plant, as shown in Figure 1A (Moore and others 1998).

MATERIALS AND METHODS

Plant Culture

Arabidopsis seedlings were grown on MS (1×) medium. Seeds were sowed on the surface of MS medium with sucrose (1%, 2%, 3%, 5%, 8%) and kept at 4°–8°C for 2 days, before they were transferred to a temperature of 22°C and a light cycle of 16 h light/8 h dark. Light intensity of 45–60 μmol m^{-2} s^{-1} was used in some experiments as treatment, designated Light-1, and 150–180 μmol m^{-2} s^{-1} in other experiments, designated Light-2.

Anthocyanin Analysis

Around 50 mg of seedlings were pooled, placed in 15 ml extraction solution (propanol:HCl:H2O = 18:1:81), boiled for 1.5 min, and then incubated at 25°C for 24 h in the dark. The extracts were centrifuged for 40 min at 5000 rpm (Beckman J2-HS). Absorbance was measured at 535 nm and 650 nm, and the corrected value was taken by the formula of $A_{535}-2.2A_{650}$.

Lignin Analysis

Around 150 mg of fresh seedlings grown in Light-1 conditions were placed in 85% ethanol for 12 h to remove chlorophylls. The seedlings were then dried at 50°–60°C. The dry seedlings were incubated in 2 N NaOH for 1 h at 25°C (50 μl NaOH/mg seedling dry weight), then 500 μl 25% acetylbromide in CH3COOH was added, and the mixture was incubated for another 30 min at 70°C. The reaction was rapidly cooled to room temperature (RT) and diluted with 2 N NaOH to 1 ml final volume. Before measurement of lignin, 50 μl 7.5 M hydroxyalanine was added to remove bromine and polybromide. Absorbance was measured at 280 nm. Lignin content was calculated using the following formula

$$\text{Lignin content} = [\text{OD}_{280\text{nm}}/20 \times 100]/[\varepsilon \times \text{final dry weight(g)}]$$

(Coefficient ε of lignin standard=24)

Lignin staining was performed with the seedlings growing in Light-1 conditions. The fresh seedlings were stained in phloroglucinol/6 N HCl (1/100, v/v) for 3–5 min. After staining, the materials were fixed in glycerol/6 N HCl (1:1, v/v) for 2–3 min.

Extraction and Purification of Cytokinins for HP LC/MS Analysis

The method for cytokinin extraction and purification was briefly described as: the internal standard–free bases and phosphates were added prior to the homogenization of the *Arabidopsis* seedlings (around 100 mg). Samples were kept in the freezer overnight, then centrifuged at 13,000 rpm for 10–15 min at 4°C. The re-extraction was done by adding 80% MeOH and shaking for 1 h at 4°C; it was then centrifuged again as above. Supernatant was first loaded on the C18 cartridges; then the elution was adjusted to pH 6.5 before being loaded a DEAE Sephadex A25 column for separation of the free bases or phosphates, respectively. The separated free bases and phosphates were analyzed by PHLC/MS. Twenty-two kinds of cytokinins were analyzed as total cytokinins presented here (Z, cZ, DHZ, ip, ZR, cZR, DHZR, iPR, ZRP, cZRP, DHZRP, iPRP, ZOG, ZROG, DHZROG, ZOX, Z7G, DHZ7G-1, ZHZ7G-2, iP7G, Z9G, DHZ9G).

Analyses of Light-inducible Gene Transcriptions by RT-PCR

After the seeds germinated and seedlings were grown under Light-1 conditions for 6 days, 12 days, 20 days, and 30 days, respectively, approximately 50–100 mg of seedlings were harvested for isolating total RNA by TRIZOL reagent. Reverse transcription (RT) was performed immediately after the isolation following the standardized reaction. Briefly, 5 μg of total RNA in 2 μl Oligo dT (5 pmol/μl) and 20 μl distilled water were mixed and incubated at 70°C for 10 min; then, 8 μl of 5× RT buffer, 4 μl DTT (10 mM), and 2 μl dNTP (10 mM) were added; the mixture was incubated at 42°C for 2 min; finally 2 μl Superscript II RT (200 U/μl) was added, and the reaction was performed at 42°C for 50 min and stopped by incubation at 70°C for 15 min.

The polymerase chain reactions (PCR) were performed in 50 μl volume, which included 2 μl of the above RT reaction solution, 3 μl (5 pmol/μl) gene-specific primers, 3 μl dNTPs (5 mM), 5 μl 10× PCR buffer, and 31 μl ddH$_2$O. The mixture was preheated at 94°C for 5 min before 3 U of Taq DNA polymerase was added. The PCR cycle setting was 94°C for 45 s, annealing temperature (different for each gene) for 45 s, and 72°C for 90 s. Reactions were tested at 20, 25, 30, 35, and 40 cycles to find the cycle number that gave a semi-saturated PCR. Finally, PCR reactions were run with that cycle number. Actin-specific primers were 5′TTCCTCA ATCTCATCTTCTTCC 3′ and 5′GACCTGCCTCATCA TACTCG 3′; chalcone synthase (CHS)-specific
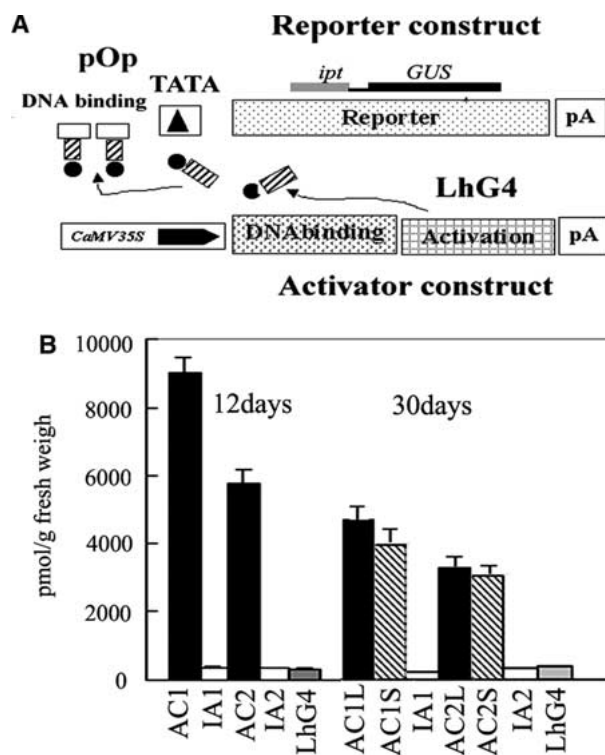

Figure 1. *ipt*/*GUS* activation by the pOp/LhG4 system. **A.** pOp/LhG4 transcription system. **B.** cytokinin content in pOp-ipt-GUS-activated *Arabidopsis*. AC1 and AC2 are two independent lines of pOp-ipt-GUS-activated *Arabidopsis*; L and S after line name (for example, AC1L) stand for large and small phenotype, respectively; IA1 and IA2 are inactive isolines of AC1 and AC2, respectively; LhG4: CaMV35S-LhG4 activator *Arabidopsis*. Around 100 mg fresh weight was used for cytokinin measurements. Bars in the graph are the mean of two independent measurements. Error bars: SE.

primers were 5′GCTCTAGACACCCACCTTGACTCC CTCG 3′ and 5′AACTGCAGCCAAACAAGACACCC CACTC 3′; *cab* 5′ATGGGCTATGCTCGCTGTTC 3′ and 5′CCGCCCGTTCTTGATCT 3′, and rboS 5′GCTCTCTTCCGCTAC 3′ and 5′GACTTGACGGGT GTT 3′. The transcription level of each gene was the average of four set PCR measurements, with templates from seedlings cultured in two independent batches; two PCR reactions were performed for each RT reaction. The quantity of DNA in the gel was detected by the Kodak image program system. Agarose electrophoresis was performed using 10 μl of the above-mentioned PCR reactions.

RESULTS

ipt:::GUS-fused Gene Activation by CaMV 35S-LhG4

Lines of pOp-ipt-GUS transgenic *Arabidodpsis* were crossed with an activator CaMV35S-LhG4 line (Fig-
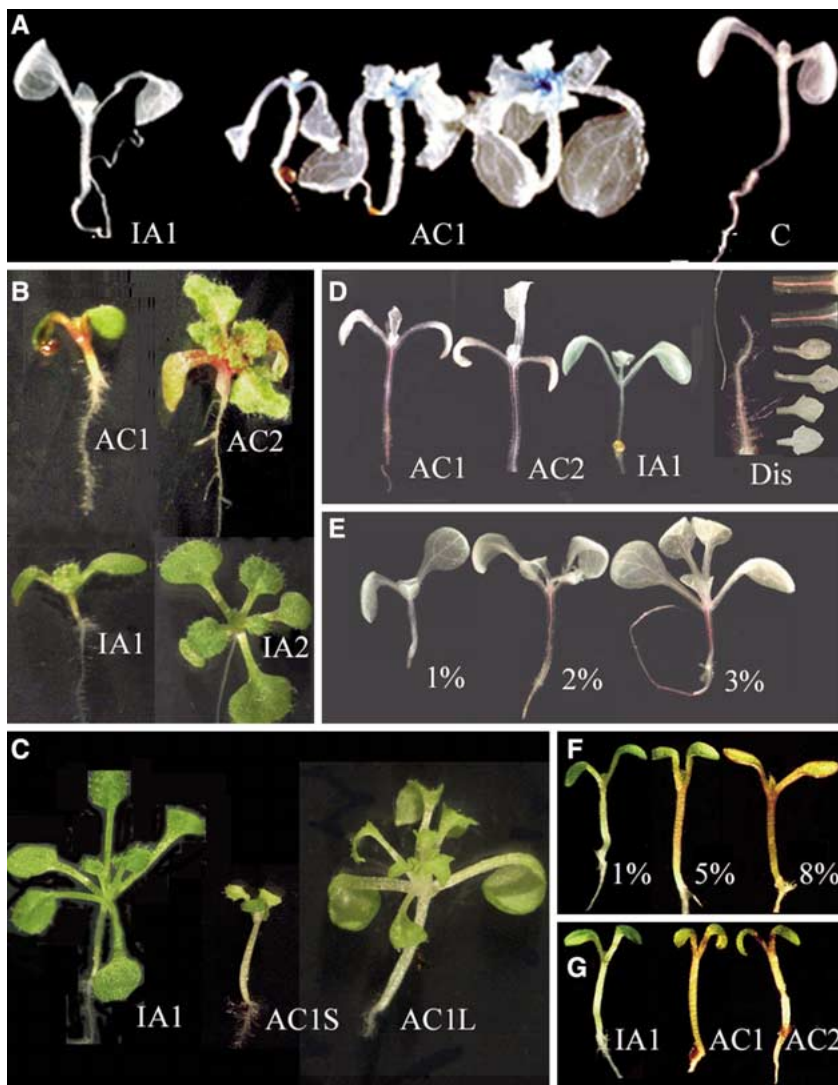
Figure 2. Effects of endogenously increased cytokinins, light, and sucrose on morphology and accumulation of lignin and anthocyanin. See Figure 1 for most of the plant labels; C in A, *Columbia*; Dis in D, dissected plant; 1%, 2%, 3%, 5%, and 8% in E and F represent the amount of sucrose in the medium used to grow the plants-*Columbia*. **A.** GUS staining in ipt:::-GUS-activated and inactive *Arabidopsis*. The three activated plants shown were grown for 1, 2, or 3 week, respectively. **B.** Light intensity stimulated anthocyanin accumulation in cytokinin-overproducing *Arabidopsis*. Seedlings were cultured under Light-2 conditions (150–180 μmol m^{-2} s^{-1}) for 1 week (AC1, IA1) or 2 weeks (AC2, IA2). **C.** morphology variation ipt:::GUS-activated *Arabidopsis*. Seedlings were grown in tissue culture conditions for 3 weeks. **D and E.** Lignin accumulation by endogenously increased cytokinin and higher sucrose concentration in culture medium. Seedlings were grown for 10 days. **Insert (Dis)** showing separated organs of ipt:::GUS-activated *Arabidopsis*. **F and G.** Anthocyanin synthesis by endogenously increased cytokinin and higher sucrose concentration in culture medium.

ure1A). The seeds from the crosses were germinated, and GUS staining showed that the ipt:::GUS-fusion gene was activated (Figure 2A). Two single-locus lines were selected from 25 independent lines of ipt:::GUS-activated transgenic *Arabidopsis*, and these were used to analyze their cytokinin levels by high performance liquid chromatography mass spectroscopy (HPLC/MS) at the stages of 12 days and 30 days in MS medium. The content of total cytokinins in ipt:::GUS-activated transgenic *Arabidopsis* was 20–25-fold higher than their inactive reporters when the seedlings were 12 days old, and 10–15-fold higher than their inactive reporters when the seedlings were 30 days old in both enlarged phenotypes (L) and retarded growth phenotypes (S) (Figure1B). These results showed that the pOp promoter was activated in the crosses, both *ipt* and *GUS* were correctly transcribed, and functional protein was produced for both ipt and GUS enzymatic activities.

Morphological Characteristics of Cytokinin-overproducing *Arabidopsis*

The ipt:::GUS-activated transgenic *Arabidopsis* showed a number of visibly altered phenotypes similar to all *ipt*-overexpressing transgenic plants reported previously, such as root inhibition, bushy growth, reduced apical dominance, serrated leaves, no flowers, or few sterile flowers in soil (Smigocki and Owens 1989; Schmülling and others 1989; Li and others 1992; Hewelt and others 1994). Besides these abnormalities, we have observed what we called ''enlarged'' and ''growth-retarded'' phenotypes in MS medium (Figure 2C). The enlarged phenotype (L) included a severe inhibition in root development, a longer and larger shoot, enlarged shoot apical meristems, and serrated leaves. The growth-retarded phenotype (S) displayed a high degree of dwarfism. The seedlings of S phenotype

Table 1.   Morphological and Physiological Characteristics of ipt-GUS-Activated Seedlings

Plant	Number of leaves	Hypocotyl length (mm)	Hypocotyl diameter (mm)	Hypocotyl tip (mm)	Wet weight of 10 seedlings (mg)	Chlorophyll (μg/g)
AC1L	8–12	6.0 ± 0.5	0.5 ± 0.1	(0.8 ± 0.3) × (1.0 ± 0.4)	139.3 ± 15	710 ± 35
AC1S	5–7	2.5 ± 0.2	0.25 ± 0.1	(0.3 ± 0.1) × (0.3 ± 0.1)	55.40 ± 10	570 ± 32.3
IA1	6–8	3.8 ± 0.1	0.3 ± 0.1	(0.3 ± 0.1) × (0.3 ± 0.1)	60.2 ± 12	408 ± 28.5
C	6–8	3.8 ± 0.1	0.3 ± 0.1	(0.3 ± 0.1) × (0.3 ± 0.1)	64.1 ± 13	353 ± 25

AC1L: activated pOp-ipt-GUS Arabidopsis line AC1 large phenotype; AC1S: AC1 small phenotype; IA1: inactive isoline of AC1; C: wild-type Arabidopsis control. Each measurement was taken from 3 weeks of 10 old seedlings and data in the table are means of the three repeat measurements. The variety was SE.

developed more slowly with smaller leaves and shorter shoots than the L phenotype (Table 1). The colors of the leaves in the S phenotype were variable: the older leaves and the edges and tips of young leaves were green, whereas some newly initiated leaves were pale yellow. As the new leaves aged, they gradually became green. The roots in the S phenotype grew faster at later stages compared to the L phenotype (Figure 4). However, the chlorophyll content in cytokinin-overproducing ipt:::GUS-activated *Arabidopsis* (enlarged and retarded growth phenotypes) was higher than the inactive reporters (Table1). When the seedlings were cultured in MS medium supplemented with 1% sucrose under the culture conditions of Light-1, the ratio of the S phenotypes in the whole seedlings varied from 5% to 20% from line to line (Figure 3).

The Effects of Environmental Factors on the Phenotypes of Cytokinin-overproducing *Arabidopsis*

Light and sucrose are among the most important stimuli for plant development. In our experiment, the ratio between L and S phenotypes depended on the carbohydrate status of the medium and the light intensity. When seedlings were grown on MS medium supplemented with 1% sucrose, 5%–20% of them developed as S phenotype. The ratio of S phenotype was up to 40% when there was 2% sucrose in the MS medium. When light intensity was increased from 45–60 μmol m^{-2} s^{-1} to 145–180 μmol m^{-2} s^{-1}, the percentage of S phenotype was increased from 5%–20% to approximately 60% (Figure 3).

Anthocyanin Accumulation in ipt:::GUS-activated Transgenic *Arabidopsis*

When the seedlings were cultured under Light-1 conditions, a deeper red color in the shoot (especially in the shoot apical meristem) was observed in ipt:::GUS-activated transgenic lines (AC1, AC2)
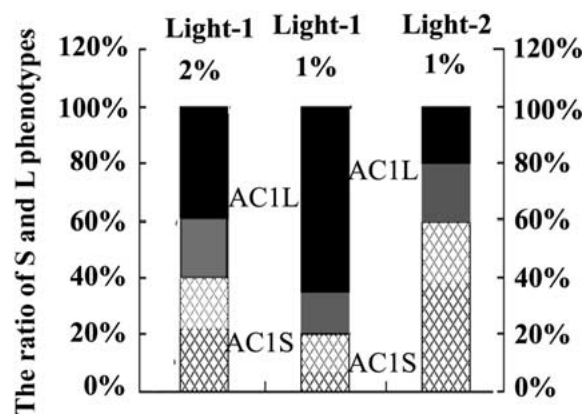

Figure 3.   The effects of environmental factors on the phenotypes of cytokinin-overproducing *Arabidopsis*. Label interpretation is the same as in Figure 2. 1%, 2% represent the sucrose content in MS medium. Light-1, light intensity 45–60 μmol m^{-2} s^{-1} 16 h light/8 h dark photoperiod. Light-2, light intensity 150–180 μmol m^{-2} s^{-1}, 16 h light/8 h dark photoperiod. Fifty seedlings were counted for each line. Ten independent lines were examined. The experiment was performed twice. The gray bars indicate the variety of phenotypes from line to line.

than in their corresponding inactive lines (IA1, IA2), indicating that increased cytokinins *in vivo* caused more anthocyanin accumulation in shoots (Figure 2G). Quantitative analysis showed higher anthocyanin content in ipt:::GUS-activated lines than in their inactive lines (Figure 5). The increased anthocyanin levels gradually decreased as the cultivation time was prolonged (Figure 5A).

Light-stimulated Anthocynin Synthesis in ipt:::GUS-activated *Arabidopsis*

Light was a sensitive stimulus for anthocyanin accumulation in cytokinin-overproducing *Arabidopsis*. When the ipt:::GUS-activated seedlings (AC1, AC2) were cultivated under strong light conditions (Light-2), more anthocyanins were accumulated around the areas of the shoot meristem than in their
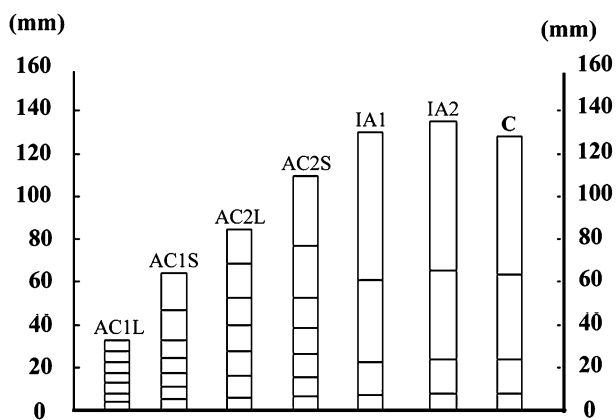
Figure 4. Root development in ipt:::GUS-activated *Arabidopsis*. Seedlings were cultured in tissue culture conditions for 1 month. The measurements were the average of 10 seedlings. See Figure 2 for explanation of labels. Each section in the columns represents root length change over a 4-day period. The time starts from the bottom of the column and continues to the top. The experiment was performed three times. Sixteen days of growth were shown for the controls IA1, IA2, and C, while 28 days of growth were shown for all activated seedlings.

isolines of IA1 and IA2 (Figure 2B). From the quantitative analysis, the anthocyanin content in the ipt:::GUS-activated seedlings (AC1, AC2) was almost twofold that of the corresponding inactive lines (IA1) when the seedlings were at the stages of 7 days (Light-1 and Light-2) and 14 days (Light-2); a smaller increase was observed when the seedlings were at the stage of 14 days in Light-1 conditions. In comparison to the activated seedlings, light was not a sensitive stimulus for inducing the inactive lines (IAI and IA2) to accumulate anthocyanins. The anthocyanin content was slightly increased in Light-2 compared to Light-1 when the inactive seedlings (IA1 and IA2) were at the stage of 7 days. However, when the inactive seedlings (IA1 and IA2) were at the stage of 14 days, the anthocyanin content was almost the same in Light-1 and Light-2 conditions (Figure 5B).

Sucrose Promoted the Accumulation of Anthocyanins in Wild-type *Arabidopsis*

Wild-type *Columbia* was cultivated on MS medium supplemented with 1%, 5%, and 8% sucrose for 2 weeks under Light-1 conditions. The amount of anthocyanins was visibly increased as the sucrose content increased in MS medium (Figure 2F). In the first week, anthocyanin content in the seedlings grown in 8% sucrose was almost fourfold that of the seedlings grown in 1% sucrose (Figure 5A).

Anthocyanin content decreased in the second week among seedlings grown in 5% and 8% sucrose; however, it slightly increased at this time in seedlings grown in 1% sucrose (Figure 5A).

Lignin Accumulation in ipt:::GUS-activated Transgenic *Arabidopsis*

Increased lignins were found in cytokinin-overproducing *Arabidopsis*. Lignin staining showed that more lignins were distributed in the vascular tissue of hypocotyls, shoot apex, cotyledon veins, and the primary roots of the ipt:::GUS-activated seedlings (Figure 2D). Lignin content in ipt:::GUS-activated lines was approximate 1.5-fold that of the inactive controls (Figure 6). The subsequent experiments showed that higher sucrose in the MS medium induced lignin synthesis in the shoots of wild-type *Columbia* (Figure 2E and Figure 6).

Transcription of Light-sensitive Genes in ipt:::GUS-activated *Arabidopsis*

Several light-sensitive genes, such as the major chlorophyll a/b binding polypeptide of the light-harvesting complex (*cab*), the small subunit of ribulose-1,5-biphosphate carboxlase/oxygenase (*rbc*S), and the rate-limiting enzyme of anthocyanins (*CHS*), have been analyzed for their steady-state mRNA levels by RT-PCR in cytokinin-overproducing activated *Arabidopsis*. The analyses were performed at four different seedling developmental stages: 6 days, 12 days, 20 days, and 30 days under Light-1 conditions. In control plants of wild-type *Columbia*, the higher transcription of *cab* was found at 20 days, whereas that of *rbc*S was detected at 30 days, although levels at 20 days were also high (Figure 7 B). High transcription of *CHS* was detected at both 6 days and 30 days. The increased endogenous cytokinins did not elevate the transcription of *cab* and *rbc*S genes in any of the four developmental stages (Figure 7C and 7D). The elevated mRNA level of *CHS* by the increased cytokinin induction was found at the stages of 12 days and 20 days, when the message RNA level in wild-type *Columbia* was low (Figure 7A).

DISCUSSION

Cytokinin Effects on Plant Development Are Modulated by Light and Sucrose

Generally, cytokinin-overproducing plants have a range of abnormalities (Smigocki and Owens 1989; Schmülling and others 1989; Hewelt and others 1994; Li and others 1992). High cytokinin in
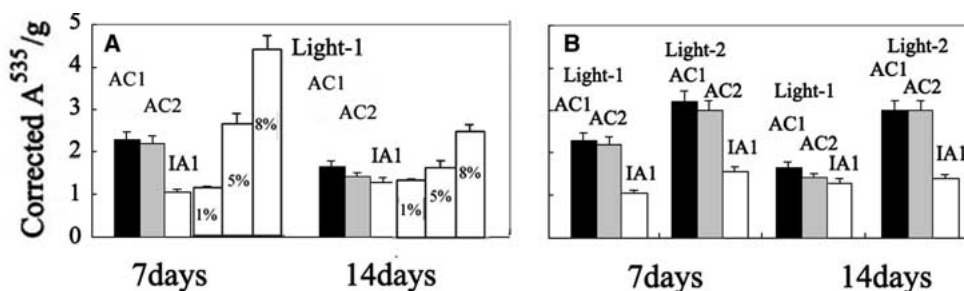
Figure 5. Anthocyanin quantification in ipt:::GUS-activated and wild-type *Arabidopsis*. Label interpretation is the same as in Figure 2. 1%, 5%, 8% represent the amount of sucrose in the medium used to grow the plants—*Columbia*. **A.** Comparison of the effects of overproducing cytokinin and higher concentration of sucrose. **B.** Combined effects of cytokinin overproduction and light. Light-1, light intensity 45–60 μmol m^{-2} s^{-1}16 h light /8 h dark photoperiod. Light-2, light intensity 150–180 μmol m^{-2} s^{-1}, 16 h light/8 h dark photoperiod. Bars in the graphs are the means of three independent experiments. Error bars: SE.

transgenic plants caused an increased stem diameter by increasing the size of cortical and epidermal cells (Li and others 1992; Hewelt and others 1994). *ipt* transgenic *Arabidopsis* under the control of a heat-shock promoter also showed increased stem diameter and enlarged pith after daily heat shocks for 2 weeks (Rupp and others 1999). In contrast to the increased cytokinins *in vivo*, the CKX transgenic *Arabidopsis* with 30%–45% of wild-type cytokinin concentration showed retarded shoot development with reduced shoot apical meristem and enhanced root growth (Eckardt 2003). In our current experiment, the cytokinin level was manipulated by an *ipt* gene that was activated by the pOp/LhG4 system through crossing the reporter line harboring pOp-ipt-GUS with the activator line expressing LhG4, so that cytokinins increased in plants noninvasively. This made it easier to observe the effects of cytokinins on plant growth. The enlarged and retarded phenotypes were obtained in the same cytokinin-overproducing transgenic lines. Both phenotypes had similar amounts of increased cytokinins *in vivo* (Figure 1B). Another interesting point is that the ratio of these two phenotypes changes with the sucrose concentration in the MS medium and the changes in light intensity. Thus, the size of the shoot and the length of the root do not simply correspond to the cytokinin content *in vivo*. The effect of cytokinins on plant development is modulated by environment stimuli, such as light and sucrose.

The evidence that light was essential for the induction of anthocyanin synthesis by cytokinins came from the observations of the *hp-2* mutation, in which cytokinin induction of anthocyanin biosynthesis was observed in light-grown but not in dark-grown seedlings, although short hypocotyls, apical hook opening, and cotyledon expansion were noticed in both conditions (Mustilli and others 1999).
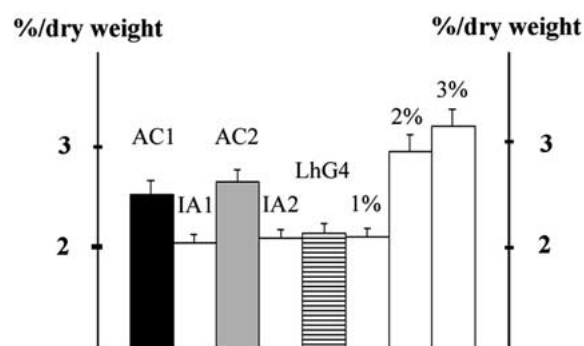

Figure 6. Lignin quantification in ipt:::GUS transgenic and wild type *Arabidopsis*. Label interpretation is the same as in Figure 1 and Figure 2. 1%, 2%, 3% represent the amount of sucrose in the medium used to grow the plants—*Columbia*. Bars in the graphs are the mean of two independent measurements. Error bars: SE.

In the present work, stronger light caused more anthocyanin accumulation in cytokinin-overproducing seedlings but not in the wild-type control, indicating that the signal of light for anthocyanin synthesis is modulated by cytokinin.

Sucrose Can Cause Effects on Anthocyanin and Lignin Content that Are Similar to Cytokinin Effects

Anthocyanins are pigmented flavonoids that are responsible for most of the red, pink, purple, and blue colors in plants. Chalcone synthase is considered to be the rate-limiting enzyme in flavonoid synthesis, which catalyzes the formation of naringenin chalcone by condensing 4-coumaroyl-CoA with three molecules of malonyl-CoA, and then forms anthocyanins (Dong and others 1991; Shirley and others 1992). Lignins constitute a group of heterogeneous phenylpropane polymers in plants,
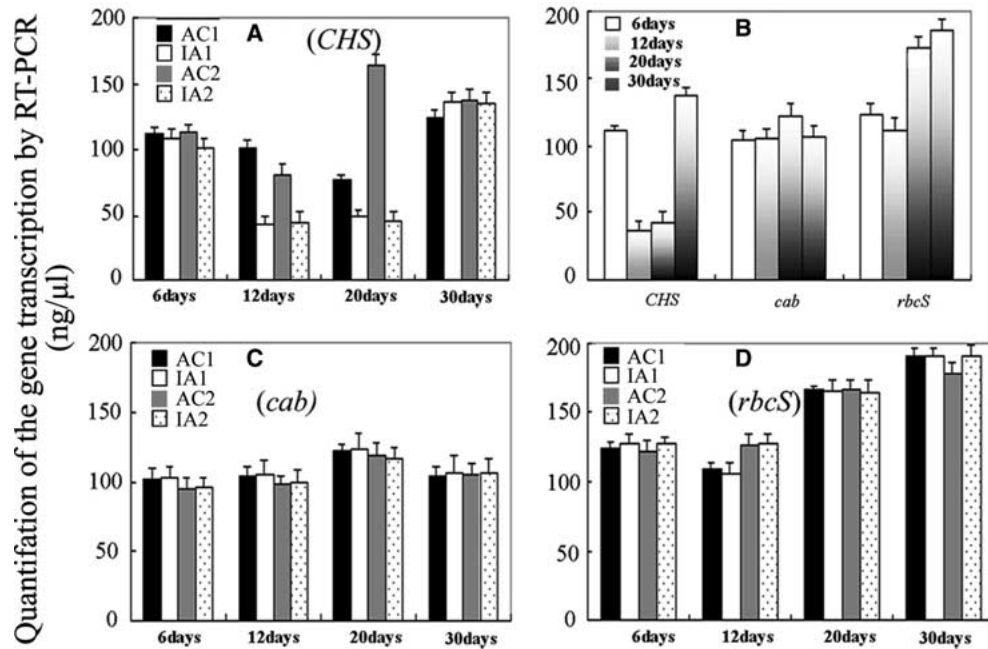
Figure 7. Cytokinins induce transcription of light-sensitive genes in ipt:::GUS-activated *Arabidopsis*. **A, C, D.** Steady-state mRNA levels of *CHS*, *cab*, and *rbcS* in transgenic lines. **B.** Steady-state mRNA levels of *CHS*, *cab*, and *rbcS* in wild-type *Columbia*. Polymerase chain reactions were semi-saturated PCR. The transcription level of each gene was the average of four set PCR measurements, with templates from seedlings cultured in two independent batches. Error bars: SE.

and these are important constituents of cell walls in supporting and conducting tissues (for example, xylem cells). In plants, glucose is the source molecule for numerous phenolic compounds, for instance, cinnamic acid and coumaric acid, which are the substrates of both lignins and anthocyanins (Dey and Harborne 1997). In our experiment, elevated cytokinin levels in ipt:::GUS-activated *Arabidopsis* and higher concentrations of sucrose in MS medium increased the accumulation of both anthocyanins and lignins; and the locations of accumulation are similar for both cytokinin and sucrose effects. Given these results, we suggest that the syntheses of lignin and anthocyanin by cytokinin and sucrose are merged at certain point of the synthesis pathways. The role of increased cytokinins may be strengthening the ability to accumulate sucrose, and sucrose can be readily converted to glucose, which may be the initial substrate for lignin and anthocyanin synthesis or a source for cell division thereby causing the enlarged shoot or shoot apical apex.

Cytokinins Induced *CHS* Transcription in ipt:::GUS-activated *Arabidopsis* Seedlings in the Vegetative Growth Period

Cytokinins caused an increase in anthocyanin accumulation in tissue culture and in parts of intact plants by treatment with exogenous cytokinins (Ozeki and Komamine 1981; Constabel and others 1971; Pecket and Bassim 1974). High-level cytokinin *amp1* mutant seedlings have been shown to cause an increased *CHS* expression (Amy and others 1996). In our experiment, *CHS* expression in the control plants was high at 6 days, dropped to low levels at 12 and 20 days, and reached the highest level at 30 days (Figure 7). In the activated lines with increased cytokinin levels, *CHS* expression was higher than in the controls. At 20 days, *CHS* expression in the activated lines was almost as high as the expression level seen at 30 days. Theses results suggest that there is an upper limit of *CHS* expression, and at 30 days the expression had already reached this limit in the control plants, so that the effect of cytokinins became invisible. On the MS medium, the inactive *Arabidopsis* showed similar phenotypes to wild-type *Columbia* and initiated true leaves around 6 ± 1 days and started to initiate inflorescent shoots at 22 ± 2 days. The activated *Arabidopsis* of pOp-ipt-GUS initiated their true leaves around 5 ± 1 days, and it started to initiate inflorescent shoots at 26 ± 3 days, which was slower than in their inactive ones. Cytokinin affects *CHS* expression in stages, in which the inactive plants are in vegetative growth and the *CHS* expression is low (Figure 7B). Alternatively, *CHS* expression could be modulated directly by

glucose, which is one of the substrates for anthocyanin synthesis, and indirectly by providing more glucose at the stages of vegetative growth.

Cytokinins Did Not Alter *cab* and *rbc*S Transcriptions in Light-grown Seedlings

Even though the chlorophyll content in cytokinin-overproducing ipt:::GUS-activated *Arabidopsis* (enlarged and retarded growth phenotypes) was higher than in their inactive reporters (Table1), the colors of the leaves in the S phenotype were non-uniformly distributed between the old leaves and young leaves. The endogenously increased cytokinins in our experiment did not alter the transcript levels of *cab* and *rbc*S at any of the four stages tested under light conditions (Figure 7CD).

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