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Cytokinin-induced Transcriptional Activation of NADPH-protochlorophyllide Oxidoreductase Gene in Cucumber

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Pretreatment of cucumber cotyledons with cytokinins such as benzyladenine (BA) in darkness increases the amount of chlorophyll (Chl) formed on subsequent exposure to light. We previously showed that in etiolated cotyledons of cucumber (*Cucumis sativus*), BA remarkably increased the level of mRNA of NADPH-protochlorophyllide oxidoreductase (POR, EC1.3.1.33), which catalyzes the photoreduction of protochlorophyllide *a* to chlorophyllide *a*. In this study, we examined the regulatory mechanism of the BA-induced expression of the *POR* gene in more detail. In etiolated cotyledons of cucumber, the levels of *POR* mRNA and its protein were remarkably increased by BA treatment dose- and time-dependently. The increase was more marked in the mRNA than the protein. The BA-induced increase in the mRNA was repressed by treatment with cycloheximide. A run-on assay showed that BA increases the *POR* mRNA at least at the transcriptional level. Moreover, a transient reporter assay of the 5′-upstream region of the *POR* gene reveled this region to contain a *cis*-acting element(s) for cytokinin response. These results indicated that the BA-induced increase in the mRNA is mainly dependent on transcriptional activation, which accompanies *de novo* protein synthesis in cytoplasm.

Key words: Chlorophyll synthesis — Cucumber — *Cucumis sativus* — Cytokinin — Gene expression — NADPH-protochlorophyllide oxidoreductase

Cytokinins are phytohormones that play a crucial role in plant growth and development, including cell division, shoot formation and photomorphogenic development (Schmulling *et al.* 1997). Stimulation of greening is one of the effects of cytokinins. This effect is closely related with stimulation of chloroplast development and Chl synthesis by cytokinins. Chory *et al.* (1991) reported that cytokinin treatment in darkness resulted in chloroplast development and the expression of light-regulated genes. The expression of some nuclear genes encoding plastid protein has been reported to be stimulated by cytokinin treatment (Schmulling *et al.* 1997). Among these genes, Chl *a/b* binding protein and small subunit of ribulose 1, 5-bisphosphate carboxylase genes have been well studied for the effects of cytokinins on their expression. Both genes are positively regulated by cytokinins (Ohya and Suzuki 1991, Lerbs *et al.* 1984, Abdelghani *et al.* 1991, Flores and Tobin 1986, 1988). Flores and Tobin (1986 and 1988) reported that cytokinin did not alter the transcriptional activity of *Lemna gibba* Chl *a/b* binding protein and small subunit of ribulose 1, 5-bisphosphate carboxylase genes and indicated that post-transcriptional processes were important in the regulation of the two mRNAs by cytokinin.

Chl synthesis is upregulated on treatment with cytokinins in cucumber (Fletcher and McCullagh 1971), pumpkin (Knypl 1969) and wheat (Beevers *et al.* 1970). The increase has been suggested to be due to elevated levels of 5-aminolevulinic acid (ALA), because ALA synthesis is a rate-limiting step in the Chl biosynthetic pathway (Fletcher and McCullagh 1971). Indeed, the activity to synthesize ALA was increased by BA treatment in cucumber (Masuda *et al.* 1995). Masuda *et al.* (1995) reported that the activity and mRNA levels of glutamyl-tRNA reductase were increased by BA treatment, and indicated that the stimulation of ALA synthesis by BA was caused by an increase in glutamyl-tRNA reductase. However, the effects of cytokinins on the regulation of other steps in the Chl biosynthetic pathway have been less-well investigated.

Previously, we showed that in etiolated cotyledons of cucumber, the expression of a gene encoding NADPH-protochlorophyllide oxidoreductase (POR) which catalyzes the photoreduction of protochlorophyllide *a* to chlorophyllide *a* in the biosynthesis of Chl (Apel 1980) is regulated by various phytohormones such as BA, gibberellin (GA) and abscisic acid (ABA) (Kuroda *et al.* 1996). In particular, BA markedly increased the level of *POR* mRNA. We suggested that in addition to glutamyl-tRNA reductase, POR played an impor-

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Abbreviations: ABA, abscisic acid; ALA, 5-aminolevulinic acid; BA, benzyladenine; Chl, chlorophyll; CP, chloramphenicol; CH, cycloheximide; GA, gibberellin; GUS, β-Glucuronidase; LUC, luciferase; POR, NADPH-protochlorophyllide oxidoreductase.

tant role in cytokinin-regulated Chl biosynthesis. Recently, the stimulation of *POR* gene expression by BA was also reported in etiolated *Lupinus luteus.* (Kusnetsov 1998).

In spite of the many reports on the physiological effects of cytokinins on greening, the molecular mechanism involved is little understood. In this study, to understand the regulatory mechanism of controlling cytokinin-induced expression of *POR*, we examined the effects of cytokinin (BA) on the expression in cucumber. The results showed that BA increased the levels of both the mRNA and protein in etiolated cotyledons, and the increase was accompanied by a *de novo* protein synthesis in cytoplasm. Run-on and promoter assays showed that BA regulates the expression of cucumber *POR* gene at the transcriptional level via the 5′-upstream region of the gene.

Materials and Methods

Plant materials and growth conditions

Seeds of cucumber (*Cucumis sativus*, cv. Aonagaijibai) were germinated on wet vermiculite at 27 C. For cytokinin treatment, cotyledons excised from 4-day-old etiolated seedlings were preincubated for 16 hr in a petri dish containing 2 ml of water in darkness. The cotyledons were then transferred to another petri dish containing 2 ml of BA solution (0 to 100 μM) and incubated for 12 hr in darkness. For run-on assay, 5-day-old etiolated seedlings were sprayed with BA (100 μM) and then incubated for 8 hr in darkness. For inhibitor treatment, the cotyledons pretreated with water were transferred to another petri dish containing 2 ml of BA solution (10 μM) with or without cycloheximide (100 μM) or chloramphenicol (100 μM) and incubated for 12 hr in darkness.

RNA isolation and Northern blot analysis

Isolation of total RNA and Northern blot analysis were performed as described previously (Kuroda *et al.* 1995). The hybridization was analyzed with a Bio-image Analyzer BAS2000 (Fuji Film Co.).

Isolation of total protein and Western blot analysis

Five pairs of cotyledons were homogenized in 1 ml of extraction buffer containing 50 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol and 2% SDS. The homogenate was heated at 100 C for 5 min and then centrifuged at 13,000 g for 10 min at 4 C. The supernatant was used for Western blot analysis. The protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The total protein (50 μg/lane) was separated by SDS-PAGE as described by Laemmli (1970), and then electroblotted onto a nitrocellulose membrane. The membrane was incubated with antibody raised against cucumber POR (Yoshida *et al.*, 1995), and subsequently treated with alkaline phosphatase-conjugated goat antibody against rabbit IgG. Quantitation of the protein level was carried out using NIH-image software.

Nuclear isolation and run-on transcription

Nuclei were isolated from cotyledons treated with BA (100 μM) or water according to the method of Flores and Tobin (1988) with the following modifications. The crude nucleic suspension was layered on the top of a discontinuous gradient consisting of 80% sucrose (W/V), and 80 and 40% (V/V) Percoll (Amersham-Pharmacia Biotech). The purified nuclei were collected from the interface between the 80% sucrose and 80% Percoll after centrifugation and then washed with the nucleic wash buffer. The final purified nuclei were suspended in the nucleic resuspension buffer. The nuclear DNA content was determined according to Barton (1968).

About 5×10^6 nuclei estimated from the DNA content were incubated in a reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM KCl, 5 mM DTT, 500 unit of RNasin (Promega), and 0.5 mM each of ATP, CTP, GTP, and [^{32}P]-UTP in a total volume of 500 μl) at 27 C for 20 min with gentle shaking. The solution was treated with 10 μl of RNase-free DNase I (Amersham-Pharmacia Biotech) at 27 C for 10 min. Then an equal volume of stop solution (20 mM Tris-HCl, pH 7.4, 10 mM EDTA and 2% SDS and 200 μg/ml proteinase K) was added and the mixture was incubated at 42 C for 30 min. Labeled RNA was extracted with an equal volume of phenol: chloroform: isoamylalcohol (25 : 24 : 1) and precipitated by ethanol. Unincorporated [^{32}P]-UTP was removed using Probe Quant G-50 Micro Columns (Amersham-Pharmacia Biotech). The labeled RNA was hybridized to 10 μg of *POR* cDNA in pCR II vector (Invitrogen) blotted to a nylon membrane at 65 C for 48 hr. The hybridized membrane was washed two times with 0.2X SSC/0.1% SDS at 65 C for 30 min. The hybridization was analyzed with a Bio-image Analyzer BAS 2000 (Fuji film Co. Japan).

Construction and screening of a genome library

Genome DNA was extracted from mature leaves of cucumber as described by Murray and Tompson (1980). A genome library was constructed from partially digested *Sau3A* fragments of the DNA. Fragments of 10-20 kb purified by centrifugation with a NaCl gradient were cloned into a *Bam*H I site of the λDASH II vector (Stratagene) and packaged using Giga Pack Gold (Stratagene) as instructed by the manufacturer. Approximately 1×10^6 plaques of the cucumber genome library were screened with the cucumber *POR* cDNA as a probe according to Maniatis *et al.* (1982) at low stringency hybridization (hybridization: 50 C for 16 hr, washing: 2×SSC/0.1% SDS for 30 min at 50 C). The hybridization was analyzed with a Bio-image Analyzer BAS 2000 (Fuji Film Co.). Coding regions of *POR* genes in positive clones were amplified by PCR and sequenced. A 2.5-Kb *Sac*I-*Eco*RI fragment of one of the positive clones, clone 8, suspected to contain the promoter region of the *POR* gene, was subcloned into pUC 118 vector and sequenced. Sequencing in all experiments was carried out on both strands using SequiTherm Long-Read Cycle Sequencing Kit-LC (Epicenture Technologies) with a LI-COR model 4000L DNA sequencer (LI-COR, Inc.).

Primer extension

Primer extension analysis was performed as described by Lee *et al* (1989) with total RNA from BA-treated cotyledons and [γ-32p] ATP-labeled synthetic oligonucleotide (5′-AGAGCAGGGGAAACCAATGA-3′). The sequencing reaction was performed with the same oligonucleotide and *Bca*BEST sequencing kit (Takara Shuzo Co., Japan). The dried gel was analyzed with the BAS2000 Image Analyzer (Fuji Film Co.).

Construction of a chimeric gene and transient expression analysis

To construct the *POR*-β-Glucuronidase (GUS) fusion gene, the region from −2283 to +76 from the transcription start site (T) in one of the *POR* genes (clone 8) was amplified by PCR and replaced with 35S promoter in pBI 221.2 vector. This construct contained 2,283 bp of promoter region, 62 bp of 5′-untranslated region and 14 bp of translated region (Fig. 4A). Plasmid DNAs were purified on Qiagen tips 100 (Qiagen, Hiden, Germany).

Six cotyledons were excised from the 4-day-old etiolated seedlings, incubated for 16 hr in a 6-cm petri dish containing 0.5 ml of 10 mM sodium phosphate buffer (pH 7.0) in darkness at 27 C, and used for particle bombardment. Plasmid DNA containing 9 μg of *POR-GUS* and 3 μg of *35S*-luciferase (*LUC*) was precipitated onto 3 mg of gold particles and suspended with 300 μl of ethanol. Twenty micro liters of the suspension was used to bombard the cotyledons with a particle gun apparatus (Takeuchi *et al*. 1992) under a green safety light. The bombarded cotyledons were transferred to a new petri dish containing 0.5 ml of 10 μM sodium phosphate buffer (pH 7.0) with or without 10 mM BA and incubated for 24 hr at 27 C in darkness. The cotyledons were homogenized with 3 ml of Reporter lysis buffer (Promega) and the homogenate was centrifuged at 13,000 rpm for 10 min at 4 C. GUS and LUC assays were performed according to Jefferson *et al*. (1987) and the manufacturer's protocol (Promega), respectively. All GUS activities were normalized to the corresponding LUC activity.

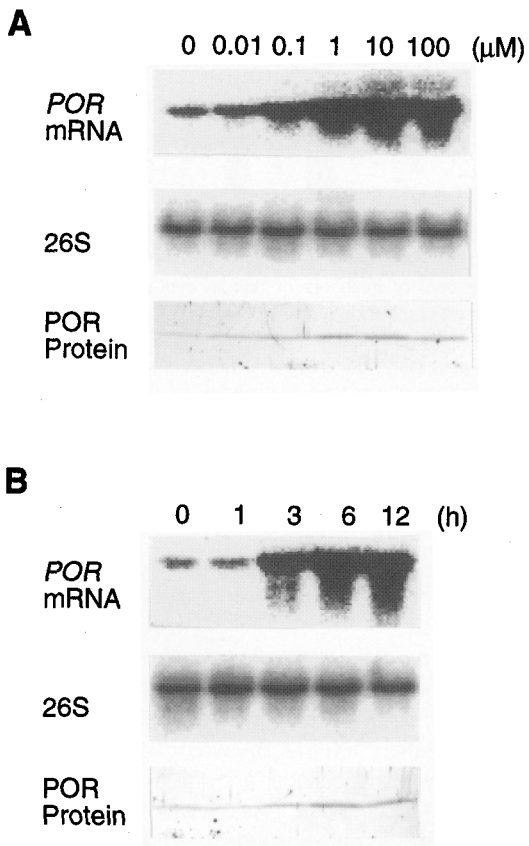

Fig. 1. Effects of BA on the levels of *POR* mRNA and its protein. Total RNA and total protein were isolated from the BA-treated cotyledons and used for Northern or Western analysis. A, Effects of BA concentration on the level of mRNA and protein. Etiolated cotyledons were pretreated with water for 16 hr and then treated with 0 to 100 μM BA solutions for 12 hr in darkness. B, Effect of treatment time on the levels of mRNA and protein. The pretreated etiolated cotyledons were treated with a 1 μM BA solution for 12 hr in darkness. As a loading control of Northern analysis, the total RNA was hybridized with the 26S rRNA gene.

Results

Effects of BA on the levels of POR *mRNA and* POR *protein*

Previously, we reported that the level of *POR* mRNA increased when etiolated cotyledons were treated with 100 μM BA for 12 hr in the dark (Kuroda *et al*. 1996). However, little is known about the expression profiles of the *POR* gene in BA-treated cotyledons. Therefore, we examined the dependence on BA concentration and treatment time of the levels of mRNA and protein for POR. The levels of the mRNA were slightly increased by 0.1 μM BA and remarkably increased by more than 1 μM BA (Fig. 1A). The levels reached a maximum at 10 μM and slightly decreased at 100 μM. The level at 10 μM was about 8-fold that of the water control. When the etiolated cotyledons of cucumber were treated with 1 μM BA solution for 12 hr in darkness, the levels of *POR* mRNA increased with time from 3 hr after treatment (Fig. 1B). The level in the 12-hr treated cotyledons was about 8-fold that at 0 hr. The profile of the changes in the level of POR protein paralleled the expression profile of *POR* mRNA (Fig. 1A, B). However, the maximum level of the protein was about 2 times higher than that in untreated cotyledons, indicating that the level of POR protein is controlled at transcriptional and posttranscriptional levels.

Treatment with inhibitors of protein synthesis

To examine the effects of inhibitors of protein synthesis on the expression of the *POR* gene, excised cotyledons from 4-day-old etiolated cucumber were treated with BA (10 μM) with or without cycloheximide and chloramphenicol for 12 hr. BA increased the levels of *POR* mRNA as described above and this increase was inhibited more than 90% by treatment with cycloheximide but not inhibited by chloramphenicol (Fig. 2). Treatments with only cycloheximide or chloramphenicol did not affect the expression. These results indicate that the increase in the levels of *POR* mRNA induced by BA

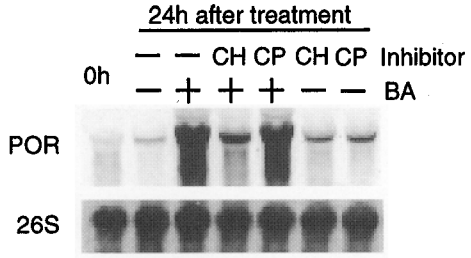

Fig. 2. Effects of inhibitors of protein synthesis on the BA-induced expression of the *POR* gene. Cotyledons excised from 4 day-old etiolated seedlings were incubated for 16 hr in a petri dish containing 2 ml of water in darkness. The cotyledons were then transferred to another petri dish containing 2 ml of solution of BA (10 μM) with or without chloramphenicol (CP) and cycloheximide (CH) and incubated for 24 hr in darkness. The levels of *POR* mRNA were analyzed by Northern blot analysis.

requires *de novo* protein synthesis in cytoplasm.

Run-on assay

To examine the transcriptional activation of the *POR* gene by BA, a run-on assay with isolated nuclei was carried out. In this assay, to obtain a quantity of cotyledons large enough for nucleic isolation, 100 μM BA was directly sprayed onto etiolated 5-day-old seedlings, and this was followed by an 8-hr incubation in darkness. As shown in Figure 3, the level of *POR* transcripts in nuclei isolated from BA-treated cotyledons was about 4 fold the control value. This result clearly shows that BA activates transcription of the *POR* gene.

Isolation of a 5′-upstream promoter region of the POR *gene and transient expression analysis*

Then, to clarify whether the 5′-upstream region of the *POR* gene was responsible for the BA-induced increase in the mRNA, we performed transient promoter analysis. To obtain the 5′-upstream region, the cucumber genomic library was screened and 17 positive clones were obtained. By physical mapping and sequence analysis, we found that all

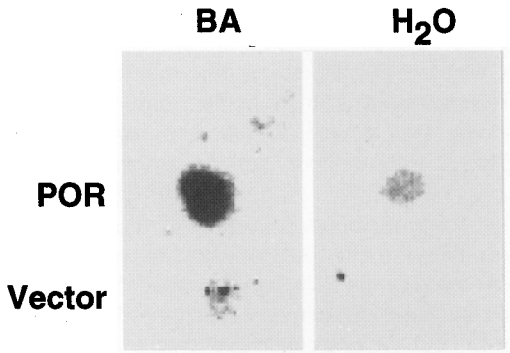

Fig. 3. Nuclear run-on transcription of the *POR* gene. Five-day-old dark-grown cucumber seedlings were sprayed with a 100 μM BA solution or water and incubated for 8 hr in darkness. Nuclei were isolated from the cotyledons of cytokinin-treated seedlings and used for run-on analysis. Radiolabeled RNA isolated from nuclei were hybridized with dot-blotted *POR* cDNA in pCR II vector.

of the positive clones comprised the same type of *POR* gene. Thus, one of the clones (clone 8) was used for the subsequent analysis. A 2.5-kb *Sac*I-*Eco*RI fragment containing the 5′-region was subcloned into pUC 118 vector and sequenced (accession no. AB024081). The transcription start site of the *POR* gene was determined by primer extension analysis using total RNA from BA-treated cotyledons. Two major primer extension products were detected. A major transcription start site, which is designated +1 in the base numbering, was mapped to T located 62 nucleotides from the ATG start codon. The fragment contained the sequence from −2,283 bp to +321 bp. A 130-bp intron

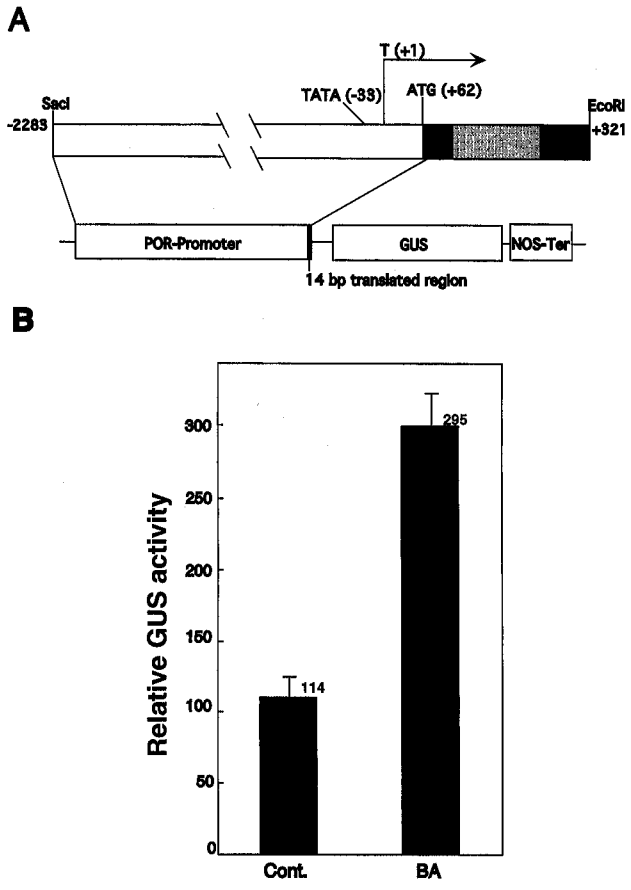

Fig. 4. Transient expression analysis of the *POR* gene. A, Structure of the *POR-GUS* gene fusion construct. T indicates transcription start site and is designated +1. Exons and introns are indicated by black and gray boxes, respectively. The −2,283 to +76 region was fused in frame to the *GUS* gene. This construct contained 2,283 bp of promoter region, 62 bp of 5′ untranslated region and 14 bp of translated region. B, Transient *GUS* gene expression after particle bombardment with the *POR-GUS* gene fusion construct. The *GUS* reporter gene fused to the POR 5′ upstream region and *35S-LUC* were introduced to cotyledons by particle gun in darkness. The bombarded cotyledons were treated with BA for 24 hr, and GUS and LUC activities were measured. The activity of GUS was normalized to the corresponding LUC activity. Each value represents the average of three shots. Error bars indicate SE values.

was located in the transcribed region. A putative TATA box was located at position −33. In the 5′-region, we found putative *cis*-acting elements for GA (Gubler *et al.* 1995), ABA (Abe *et al.* 1997) and ethylene (Montagomery *et al.* 1993) by motif search for the *cis*-acting elements with the PLACE program (Higo *et al.* 1998).

The obtained 5′-upstream region of the *POR* gene was fused to the *GUS* reporter gene (Fig. 4A). This *POR-GUS* chimeric construct was mixed with *35S-LUC* as an internal control, adsorbed to gold particles, and then introduced into cotyledons in the dark with a particle gun apparatus. After bombardment, the cotyledons were treated with 10 μM BA solution, and incubated for 24 hr in darkness. The GUS activity relative to LUC activity in BA-treated cotyledons was about 3-fold that of control cotyledons (Fig. 4B), showing that this 5′-region contains a *cis*-element(s) for BA.

Discussion

Previously, we showed that treatment with cytokinin increased the levels of *POR* mRNA in etiolated cucumber cotyledons, and suggested that the increase of *POR* mRNA in darkness was one of the most important steps in the stimulation of Chl synthesis by pretreatment with cytokinins (Kuroda *et al.* 1996). However, little is known about the mode of action of cytokinin. In this study, we further investigated the action of BA on the expression of POR in cucumber.

BA treatment increased the levels of *POR* mRNA about 8-fold, but those of *POR* protein only about 2-fold, suggesting translational and/or post-translational regulation of the expression of the *POR* gene. A limited supply of protochlorophyllide or NADPH, substrates of POR, with which it forms a ternary complex, may have limited the accumulation of stable POR protein (Oliver and Griffith 1982). In this context, it is interesting to note that the level of protochlorophyllide *a* regeneration is also increased 2-fold by BA-treatment (Lew and Tsuji 1982) in cucumber, which is consistent with the increase in the levels of POR protein in this study.

The present study showed that the enhancement of *POR* gene by BA required *de novo* protein synthesis in the cytoplasm. Recently, it has been suggested that cytokinin-induced response regulator homologs are involved in cytokinin signal transduction (Brandstatter and Kieber, 1998, Sakakibara *et al.* 1998, Taniguchi *et al.* 1998). It is possible that cytokinins regulate the expression of the *POR* gene through such homologs, although none have been identified yet in cucumber.

Run-on assay clearly revealed that the increase in the levels of the mRNA on treatment with BA was caused by the increase in the transcriptional activity. However, the difference in the extent of the increase between the mRNA level (8-fold) and transcription activity (3 to 4-fold) suggests that other factors affect the levels of mRNA, e.g., stabilization of mRNA by BA.

Transient expression assay indicated that the 5′-upstream region was responsible for the transcriptional activation of the *POR* gene by BA, suggesting that this region contains a *cis*-acting element(s) for cytokinin. Although numerous cytokinin-regulated genes have been reported in plants to date, few cytokinin-specific *cis*-acting elements and trans-acting factors have been identified (Schmulling *et al.* 1997). Recently, Jin *et al.* (1998) reported that the region from −382 to −67 in the hydroxypyruvate reductase gene in cucumber contained two cytokinin-dependent protein-protected sequences. One sequence contains an *as-1* TGACG motif, but the function of the other is unknown. In the 5′-region of *POR*, we found sequences similar to the sequence (5′-AAGATTGATTGAG-3′) in the 5′-region of hydroxypyruvate reductase gene. Further analysis is required to determine whether these similar regions are involved in the cytokinin-dependent expression of *POR*. In addition, it is important to examine whether the *cis-acting* elements for cytokinin are located in other regions such as the 3′-region of the gene.

A motif search for the *cis*-acting elements in the 5′-region revealed the presence of putative *cis*-acting elements for GA (Gubler *et al.* 1995), ABA (Abe *et al.* 1997) and ethylene (Montagomery *et al.* 1993). Previously, we reported that the level of *POR* mRNA was increased by GA and decreased by abscisic acid (Kuroda *et al.* 1996). From these results, it seems likely that GA and ABA also control the expression of the *POR* gene at the transcriptional level, although no direct evidence of this is available.

Although the genes involved in the synthesis of Chl have been cloned in many plants and their expression has been analyzed (Reinbothe and Reinbothe 1996), few studies of the regulatory mechanism controlling the expression have been made at the transcriptional level. We emphasize that this is the first report of a promoter analysis on the genes involved in Chl synthesis in higher plants. Distinctly light-regulated *POR* genes have been cloned from *A. thaliana* (Armstrong *et al.* 1995, Oosawa *et al.* 2000) and barley (Holtorf *et al.* 1995), and the two types of *POR* cDNA were also cloned from gymnosperms (Forreiter and Apel, 1993), suggesting that they widely exist in higher plants. However, the gene in cucumber is present in only a single copy (Kuroda *et al.* 2000, Fusada *et al.* 2000). Therefore, the expression of the *POR* gene in cucumber will be a potential model for the study of the mechanism of the cytokinin-regulated gene expression.

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