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Cytokinin Stimulates Expression of the Chloroplast ATP Synthase VI Subunit Gene (*atpI*)

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To investigate the signal transduction pathway of cytokinin, we incubated seven-day-old maize leaves in distilled water with or without 0.1 mM benzylaminopurine (BAP). Several gene fragments were either induced or repressed by this exogenous treatment, and were then isolated by differential display-polymerase chain reaction (DD-PCR). One fragment (IBC4) had significant sequence homology with the N-terminal portion of the chloroplast ATP synthase IV subunit gene (*atpI*) of higher plants. After the stimulated expression of IBC4 by BAP was confirmed using orthern blot hybridization, we isolated the full-length gene, including IBC4, from the chloroplast genome. We verified it as an *atpI* gene, using BLAST search analysis. This *atpI* gene, designated as *Zm-atpI* (*Zea mays-atpI*), is 744 nucleotides long and encodes 247 amino acids. Its deduced amino acid sequence does not vary among maize cultivars, and it shares >90% identity with the *atpI* genes from *Oryza sativa, Triticum aestivum, Nicotiana tabacum, Spinacia oleracea,* and *Pisum sativum*. We also confirmed, by northern blot analysis, that expression of *Zm-atpI* transcripts was significantly stimulated by the exogenous application of BAP.

Keywords: ATP synthase IV subunit gene, *atpI*, cytokinin, *Zea mays*

Cytokinins are purine derivatives that have been implicated in many aspects of plant growth and development, including cell division, shoot formation, senescence, and photomorphogenesis (Bayliss, 1985; Westhoff et al., 1985; Binns and Thomashow, 1988; Nagata et al., 1994). These roles have been identified in studies using either exogenous applications or altered levels of endogenous cytokinins in transgenic plants (Binns and Thomashow, 1988). Victor et al. (1999) have described a two-component system in plants that consists of cyclin-dependent protein kinases (CDKs) and cyclins that are required for inducing the primary mechanisms of cytokinin perception and signal transduction. However, the complete signaling pathway leading to induction of various physiological effects by cytokinins has not been elucidated. An initial investigation would include ascertaining the genes related to complete signal transduction.

The F_1F_0-type ATP synthase is found in the thylakoid membranes of chloroplasts, where it functions to convert the free energy of the proton motive force into the chemical energy source, ATP (Capaldi et al., 1994; Engelbrecht and Junge, 1997). This large enzyme complex comprises two major parts: water-soluble CF_1 and membrane-embedded CF_0. The former is composed of five different subunits (α, β, γ, δ, and ε), with a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ (Moroney et al., 1983). CF_0 contains four different subunits, I, II, III, and IV, with a stoichiometry of $I_2II_2III_{6\text{-}12}IV$ (Fromme et al., 1987; Grotjohann and Graber, 1990). The α, β, and ε subunits of CF_1 and the I, III, and IV subunits of CF_0 are encoded in the chloroplast genome (Nelson et al., 1980). In contrast, the γ and δ subunits of CF_1 and the II subunit of CF_0 are encoded in the nuclear genome, then synthesized on cytosolic ribosomes as precursor proteins and imported into the chloroplast (Shinohara et al., 1988; Herrmann et al., 1991). Many of the plastid protein genes encoded in the nuclear and chloroplast genomes are transcriptionally and/or translationally regulated by such cell-internal and -external factors as light, plant hormones, diurnal changes, or the particular type of plastid or tissue (Oelmüller et al., 1995; Bolle et al., 1996; Kusmetsor et al., 1996). Victor et al. (1999) have reported that expression of the *atpC* (γ subunit), *atpD* (δ subunit), and *atpG* (II subunit) genes is regulated by light and by cytokinin.

In this study, we report the stimulation of *Zm-atpI* gene expression by cytokinin and the full-length sequence of the gene.

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Abbreviations: *atpI*, ATP synthase IV subunit gene; BAP, benzylaminopurine; DD-PCR, differential display-polymerase chain reaction.

MATERIALS AND METHODS

Plant Material and Growing Conditions

We purchased seeds of maize (*Zea mays* L. cv. Golden Cross Bantam) from the Sakada Seed Co. (Japan). In preparation for our analyses, the seeds were imbibed at 28°C for 16 h, then germinated in the dark at 28°C for 7 d. All leaf manipulations of the dark-treated leaves were performed in complete darkness, but if required, safe illumination was provided from a dim-green light. To isolate the chloroplasts, we grew the plants in a 28°C growth chamber under a 16/8 h light/dark cycle.

Differential Display-Polymerase Chain Reaction (DD-PCR)

Seven-day-old etiolated leaves were soaked in distilled water for 14 to 18 h to eliminate endogenous cytokinin. They were then incubated in the dark in distilled water either with 0.1 mM BAP or without (control) for 4 h. Total RNA was isolated from the both the BAP- and the control leaves, and was used as template in a differential display-polymerase chain reaction (DD-PCR; Glisin et al., 1974). Following the protocol of the manufacturer (Genehunter, USA), we first synthesized the cDNA using the $T_{12}MG$ (5′-TTTTTTTTTTTTMG-3′) primer, then cycled by using the H-AP primer (Numbers 1 to 8), the H-$T_{12}MG$ primer (5′-GMAAAAAAAAAAA-3′), and ^{32}P-labeled dCTP (3000 mCi/mmol, Amersham). Synthesis conditions were 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s. The PCR products were separated on a 6% polyacrylamide gel and exposed on X-ray film (Kodak BioMax, USA). Signals that were either induced or repressed by the application of BAP were eluted from the dried gel, reamplified by PCR, and cloned into the pGEM-T easy vector (Promega, USA).

Chloroplast DNA Extraction

Chloroplast DNA was isolated according to the method of Bookjans et al. (1984). Leaves (20 g) were homogenized in 50 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 10 mM β-mercaptoethanol, 0.1% BSA, and 1.25 M NaCl. The homogenate was filtered through four layers of Miracloth (Calbiochem, USA) and centrifuged at 1,500*g* for 5 min. After adding 5 mL of 1% SDS, 4% N-laurylsarcosine, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), and 50 μL/mL proteinase K, we resuspended the pellets in 5 mL of 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0), and incubated them with shaking at room temperature for 5 h. Chloroplast lysate was extracted with phenol:chloroform:isoamylalcohol (25:24:1), and precipitated with ethanol. The isolated chloroplast DNA was identified through restriction-enzyme digestion.

Southern and Northern Blot Analyses

Following digestion with EcoRI and HindIII, the chloroplast DNA was electrophoresed on 0.7% agarose gel and transferred to Hybond-N^+ membranes (Amersham, USA), as described by Sambrook et al. (1989). The blots were prehybridized, then hybridized with a ^{32}P-labeled DNA probe that was prepared with the Ladderman™ labeling kit (TaKaRa, Japan). After hybridization, the filters were washed at 65°C for 15 min in 2X SSC (containing 0.1% SDS), and then were exposed on X-ray film.

For the northern blot analysis, we prepared the plant materials as described for our DD-PCR. Isolated total RNA (20 μg) was electrophoresed on a 1.2% agarose/2.2 M formaldehyde gel, then transferred to Hybond-N^+ membranes. Conditions for hybridization and washing were the same as with our Southern blot analysis.

RESULTS AND DISCUSSION

Increased Cloning of a DD-PCR Product (IBC4) Resulting from Exogenous Cytokinin

DD-PCR was performed with total RNA from seven-day-old etiolated maize leaves. When incubation occurred in the presence of 0.1 mM BAP, the level of one of the PCR products was remarkably increased by this exogenous cytokinin (Fig. 1A). Designated as IBC4, this product was then directly eluted from the 6% gel, reamplified by PCR, and cloned in the pGEM-T vector. The reamplified IBC4 was about 520 bp in size (Fig. 1B). A BLASTX search of IBC4 showed that the highest sequence homology was with the N-terminal part of the chloroplast ATP synthase IV subunit (*atpI*). To confirm the result of the first DD-PCR, a northern blot analysis was carried out using ^{32}P-labeled IBC4 as a probe. Here, we detected a strong signal that was stimulated by the exogenous application of BAP (data not shown).

Isolation of the Gene Including IBC4 from Chloroplast DNA

To identify the gene that includes IBC4, we isolated chloroplast DNA and digested it with HindIII and EcoRI.

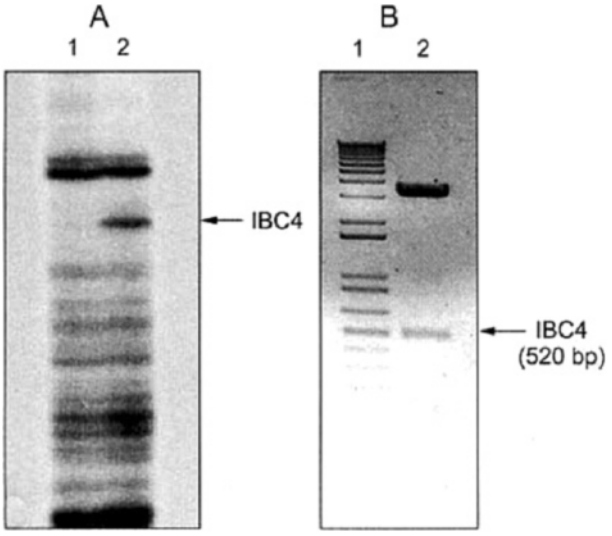

Figure 1. Cloning of a DD-PCR product, IBC4. **A.** DD-PCR was performed using the total RNAs from seven-day-old etiolated maize leaves incubated in distilled water with (Lane 2) or without (Lane 1) 0.1 mM BAP. The PCR products were separated on a 6% acrylamide gel by electrophoresis, and exposed on X-ray film. One of the PCR products, IBC4, remarkably increased when treated exogenously with 0.1 mM BAP (Lane 2). **B.** The pGEM-T vector containing IBC4 was digested with EcoRI. 1, BRL's 1-kb ladder; 2, EcoRI digestion of pGEM-T vector containing IBC4.

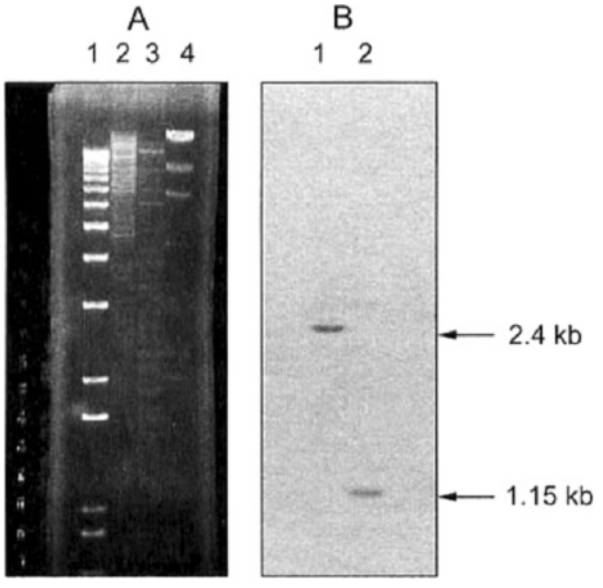

Figure 2. Southern blot analysis for the gene including IBC4. **A.** Twenty µg of maize chloroplast DNA was digested with HindIII (Lane 2) or EcoRI (Lane 3), and was electrophoresed on a 0.6% agarose gel. 1, BRL's 1-kb ladder; 4, λ-HindIII digested size marker. **B.** chloroplast DNA digested with HindIII (Lane 1) or EcoRI (Lane 2) was blotted onto a nylon membrane, and hybridized with ^{32}P-labeled IBC4.

For our Southern blot analysis, the 2.4-kb HindIII fragment and the 1.15-kb EcoRI fragment were hybridized with a ^{32}P-labeled IBC4 probe (Fig. 2, A and B). Both fragments were eluted from the agarose gel, cloned into pBluescript SK, and bi-directionally sequenced. Using a BLASTX search of the EcoRI fragment, we determined that the EcoRI fragment covered the full coding region of the *atpI* gene as well as a 40-bp region upstream from its translation initiation site. To obtain this longer promoter region, we further digested the 2.4-kb HindIII fragment with EcoRI. A 199-bp EcoRI fragment, equivalent to the continuous region of the 5 end of the 1.15-kb EcoRI fragment, was subcloned and sequenced.

Based on the sequencing results for both fragments, we identified the genomic sequence of this maize *atpI* gene and named it *Zm-atpI* (*Zea mays-atpI*). This gene is 744 nucleotides long, encodes 247 amino acids, and contains five predicted transmembrane domains (Fig. 3). Its deduced amino acid sequences show >90% identities with those from *Oryza sativa, Triticum aestivum, Nicotiana tabacum, Spinacia oleracea,* and *Pisum sativum*. The core promoter region, located 125 bp upstream from an ORF of the *Zm-atpI* gene, consists of 32 nucleotides. A potential ribosome binding site (**GAGG**) was found 5 bp upstream from the translation initiation codon for *Zm-atpI* (Fig. 3).

Stimulation of *Zm-atpI* Expression by Cytokinin

The level of *Zm-atpI* transcripts was markedly increased by exogenous applications of BAP (Fig. 4), and significant northern signals were detected in two regions -- at approximately 4.6 kb and 3.4 kb. Several minor signals also were detected (Fig. 4).

Hudson et al. (1987) have reported the order of gene-clustering on the chloroplast genome as: *rps2* (small ribosomal subunit protein 2 gene), *atpI*, *atpH*, *atpF*, and *atpA*. These genes are then co-transcribed from two promoter regions for *rpsI* or *atpH* and, finally, are processed. Many other gene clusters (e.g., *rrn* operons, *atp* clusters, *trn* clusters, *psb* clusters, and ribosomal protein gene clusters) are also encoded in the chloroplast genome, co-transcribed, and post-transcriptionally modified (Deno et al., 1984; Ohme et al., 1985; Westhoff, 1985; Westhoff et al., 1985; Cozens et al., 1986; Tanaka et al., 1986). Therefore, the multiple transcripts shown in Figure 4 might have been generated by two different polycistronic transcripts, with multiple processing of their primary transcripts.

We analyzed the levels of *atpH* transcripts to further confirm the stimulation of *Zm-atpI* expression by cyto-

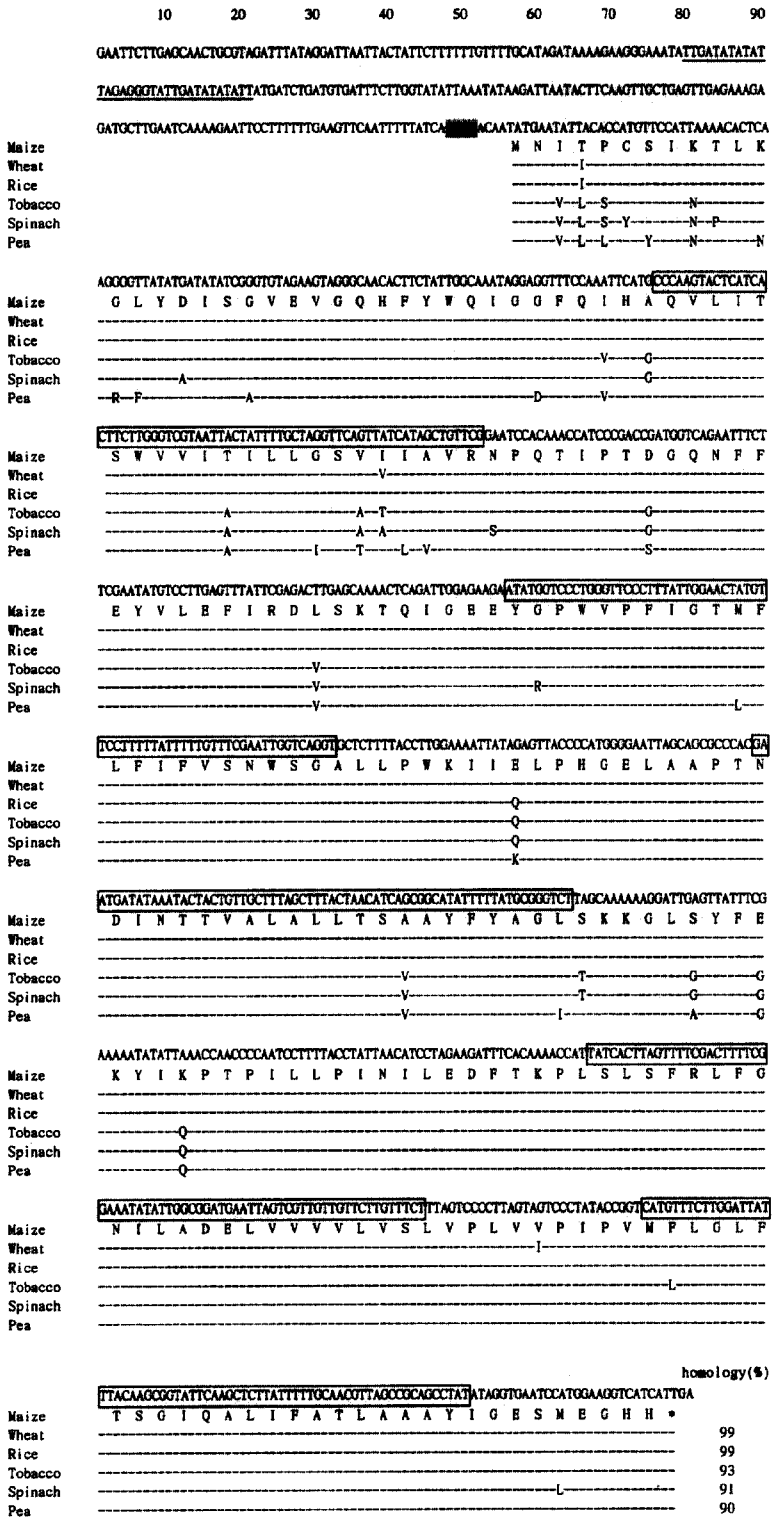

Figure 3. Nucleotide and deduced amino acid sequences of the *Zm-atpI* gene. The 1.15-kb and 199-bp EcoRI fragments (see "Material and Methods") were cloned, analyzed by nucleotide sequencing, and searched with BLASTX. The promoter region of the *Zm-atpI* gene is underlined. is the potential ribosome binding site. *indicates the translation stop codon. Boxes represent five predicted transmembrane domains. The *atpI* genes from maize, wheat, rice, tobacco, spinach, and pea were deposited under GenBank accession numbers X52270.1, AB027572.1, X15901, Z00044.1, X03775, and X05917, respectively.

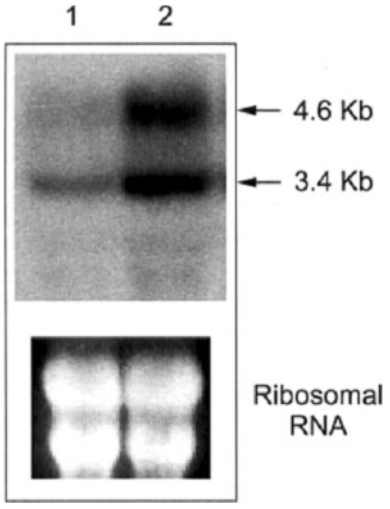

Figure 4. Effect of exogenous BAP on expression of the *Zm-atpI* gene. Total RNA was isolated from maize leaves incubated in distilled water with (Lane 2) or without (Lane 1) 0.1 mM BAP. Twenty μg of total RNA was electrophoresed on a 1% formaldehyde/agarose gel, and hybridized using ^{32}P-labeled *Zm-atpI* as a probe (upper). Ethidium-bromide staining of the gel prior to transfer indicates equal loading of RNA (lower).

kinin. As expected, the northern signal corresponding with *atpH* was similar to that for *atpI*. Likewise, the amount of *atpH* mRNA was remarkably increased by exogenous BAP treatment (data not shown).

According to Victor et al. (1999), expression of the *atpC*, *atpD*, and *atpG* genes, which exist in the nuclear genome, is transcriptionally increased by cytokinin, light, or the developmental stage of the plastids. The mRNA level of an *atp* cluster from spinach was reported to be increased by a light signal (Stollar and Hollingsworth, 1994); we also observed this in the northern blot analyses for *Zm-atpI* and *Zm-atpH*. In this study, we demonstrated that the expression of *Zm-atpI* gene was stimulated not only by cytokinin but also by light. Therefore, we suggest that the transcription of chloroplast ATP-synthase subunit genes is coordinated by light and cytokinin signals, even though the regulating gene for each signal is located in different cell compartments, i.e., the chloroplast and the nuclear genomes. These signal transduction pathways should be studied further as they relate to the stimulated expression of the *Zm-atpI* gene by cytokinin.

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