

Two Genes with Similarity to Bacterial Response Regulators Are Rapidly and Specifically Induced by Cytokinin in Arabidopsis

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Cytokinins are central regulators of plant growth and development, but little is known about their mode of action. By using differential display, we identified a gene, *IBC6* (for induced by cytokinin), from etiolated Arabidopsis seedlings, that is induced rapidly by cytokinin. The steady state level of *IBC6* mRNA was elevated within 10 min by the exogenous application of cytokinin, and this induction did not require de novo protein synthesis. *IBC6* was not induced by other plant hormones or by light. A second Arabidopsis gene with a sequence highly similar to *IBC6* was identified. This *IBC7* gene also was induced by cytokinin, although with somewhat slower kinetics and to a lesser extent. The pattern of expression of the two genes was similar, with higher expression in leaves, rachises, and flowers and lower transcript levels in roots and siliques. Sequence analysis revealed that *IBC6* and *IBC7* are similar to the receiver domain of bacterial two-component response regulators. This homology, coupled with previously published work on the CKI1 histidine kinase homolog, suggests that these proteins may play a role in early cytokinin signaling.

INTRODUCTION

Cytokinins, which are *N*⁶-substituted adenine derivatives, were first identified as factors that promoted division and sustained growth of tobacco pith cells in vitro (Miller et al., 1955, 1956). Cytokinins have been implicated in many aspects of plant growth and development, including cell division, shoot initiation and growth, leaf senescence, and photomorphogenic development (Binns and Thomashow, 1988; Mok and Mok, 1994). These roles for cytokinin are derived from numerous studies using exogenous application of cytokinins and alterations of endogenous cytokinin levels using transgenes and by the correlation of endogenous levels with various developmental processes (Binns and Thomashow, 1988; Mok and Mok, 1994).

Very little is known about the mechanisms underlying cytokinin perception and signaling. One likely cytokinin signaling element recently was identified by its ability, when overexpressed, to confer cytokinin-independent growth to Arabidopsis cells in culture (Kakimoto, 1996). This *CKI1* (for cytokinin independent) gene contains sequences similar to both the histidine kinase and the response regulator domains of bacterial two-component systems. In addition to

these domains, CKI1 has a unique N-terminal extension that contains two predicted transmembrane domains. It has been suggested that CKI1 might be a cytokinin receptor, with the inferred extracellular domain acting as the site of cytokinin binding (Kakimoto, 1996).

Two-component regulators are the major route by which bacteria sense and respond to various environmental cues (Stock et al., 1990; Hoch and Silhavy, 1995). The two components consist of a sensor and an associated response regulator. The sensor is responsible for perceiving the signal (the input domain), which induces autophosphorylation of the histidine kinase domain. This phosphate is then transferred to a conserved aspartate residue on the receiver domain of the cognate response regulator, which in turn regulates the activity of an output domain. The first identified histidine kinase homolog in eukaryotes was the receptor for the plant hormone ethylene, ETR1 (for ethylene resistant; Chang et al., 1993; Hua et al., 1995; Schaller and Bleecker, 1995). Genes similar to two-component elements also have been identified in other eukaryotes, including tomato, *Dictyostelium discoideum*, *Schizosaccharomyces pombe*, *Neurospora crassa*, and *Saccharomyces cerevisiae*, as well as the plastid genome of eukaryotic algae (Ota and Varshavsky, 1993; Reith and Munholland, 1993; Maeda et al., 1994; Wilkinson et al., 1995; Alex et al., 1996; Schuster et al., 1996; Wang et al., 1996; Zhou et al., 1996). In most of the eukaryotic homologs, including ETR1 and CKI1 from Arabidopsis, the putative histidine kinase domain is fused to a

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response regulator receiver domain at the C terminus. These proteins have been referred to as hybrid kinases. One exception is *ERS* (for ethylene response sensor), a member of the Arabidopsis *ETR* gene family, which is comprised solely of a histidine kinase and a putative ethylene binding domain.

Several proteins with similarity to the receiver domain of response regulators that are not fused to histidine kinase domains also have been identified in eukaryotes, including MCS4 from *S. pombe*, RegA from *D. discoideum*, and Ssk1 and Skn7 from *S. cerevisiae* (Brown et al., 1994; Maeda et al., 1994; Shaulsky et al., 1996; Shieh et al., 1997). In addition to the receiver domain, Skn7 also contains a DNA binding and transcription activation domain, which is analogous to that found in most bacterial response regulators. RegA is unique in that it contains a domain similar to cyclic nucleotide phosphodiesterases fused at the N terminus. In several cases, elements of a MAP kinase cascade have been identified that act downstream of the histidine kinase (Kieber et al., 1993; Maeda et al., 1994; Shieh et al., 1997).

The yeast osmosensing pathway is the best understood of these signaling systems. It involves a multistep phosphorelay in which the transmembrane Sln1p protein first autophosphorylates on a His residue; the phosphate is then transferred to an Asp on Sln1p, then to a His residue on a novel protein called Ypd1p, and finally to an Asp residue on Ssk1p, which is a protein with homology to response regulators (Maeda et al., 1994; Posas et al., 1996). Ssk1p then activates elements of a MAP kinase cascade (Maeda et al., 1994; Shieh et al., 1997).

We have been characterizing the signaling pathway leading from cytokinin perception to the induction of ethylene biosynthesis in etiolated Arabidopsis seedlings. A number of mutants that fail to induce ethylene in response to cytokinin have been identified, including a disruption of the gene encoding one isoform of 1-aminocyclopropane-1-carboxylate (ACC) synthase, the first committed step in ethylene biosynthesis (Vogel et al., 1998a, 1998b). To analyze further this signaling pathway, we set out to isolate Arabidopsis genes that are specifically and rapidly induced by cytokinin in etiolated seedlings by using differential display (Liang and Pardee, 1992). A number of research groups have isolated genes whose expression is responsive to cytokinin (Chen et al., 1993; Crowell and Amasino, 1994), but these genes are generally also induced by other stimuli, most notably light and auxin, and are induced only after a relatively long lag period (>1 hr).

Here, we describe the analysis of two homologous genes, *IBC6* and *IBC7* (for induced by cytokinin), that are induced within minutes after exposure to exogenous cytokinin. This rapid induction, coupled with the insensitivity of induction to cycloheximide and the specificity of the induction for cytokinins, suggests that *IBC6* and *IBC7* may be cytokinin primary-response genes. The sequences of *IBC6* and *IBC7* are similar to two-component bacterial response regulators. These results suggest that *IBC6* and *IBC7* may be involved in an early step of cytokinin responsiveness.

RESULTS

Isolation of Cytokinin-Induced Clones

We isolated total RNA from the aerial portion of etiolated Arabidopsis seedlings that were treated with either 50 μ M cytokinin (benzyladenine [BA]) or a buffer alone (control) for 2 hr and subjected these samples to differential display analysis. Thirty-two clones were identified in our primary screens for transcripts that were more highly expressed after the 2-hr BA treatment. Analysis of 10 of these clones revealed that two were inducible by ethylene, one of which was identical to several Arabidopsis expressed sequence tag (EST) clones (Newman et al., 1994) that encode a novel ACC oxidase isoform (data not shown). ACC oxidase catalyzes the final step in ethylene biosynthesis (Yang and Hoffman, 1984; Kende, 1989). A distinct ACC oxidase isoform was identified previously in Arabidopsis that is also induced by ethylene (Gomez-Lim et al., 1993). These ethylene-induced clones were most likely identified because ethylene is produced in response to applied cytokinin in etiolated Arabidopsis seedlings via a post-transcriptional modification of one isoform of the key ethylene biosynthetic enzyme ACC synthase (Vogel et al., 1998a). One of the remaining clones, *IBC6*, retested as rapidly inducible by cytokinin, but not by ethylene, and is the focus of this report.

IBC6 Is Homologous to Bacterial Response Regulators

A set of overlapping *IBC6* cDNA clones was identified from the Arabidopsis EST database (Newman et al., 1994). Sequence analysis of these EST clones revealed a single large open reading frame of 552 bp with a 59-nucleotide 5' untranslated region and a 267-nucleotide 3' untranslated region, which is in agreement with the size predicted from RNA gel blot analysis. The predicted initiation codon is within a sequence context that is favorable for translation in plants (Lükte et al., 1987; Joshi et al., 1997). The *IBC6* gene encodes a predicted protein of 20.1 kD with a pI of 4.8 (Figure 1A). Searches of GenBank revealed that the predicted *IBC6* amino acid sequence is most similar to bacterial two-component response regulators (Figure 1C). Overall, the *IBC6* amino acid sequence is 25% identical and 76% similar to the bacterial response regulator CheY. The three highly conserved residues of response regulators are all conserved in *IBC6*, including the putative phosphorylation site at Asp-87 (Figure 1C). There is also weak similarity (20% amino acid identity) to the domain of the Arabidopsis ethylene receptor ETR1 that is homologous to the receiver domain of response regulators and weaker similarity to the putative receiver domain present on CKI1.

A second gene, *IBC7*, was identified from a set of overlapping clones in the Arabidopsis EST database. The longest of these contains an open reading frame of 1055 bases, which is in good agreement with the size predicted from RNA gel

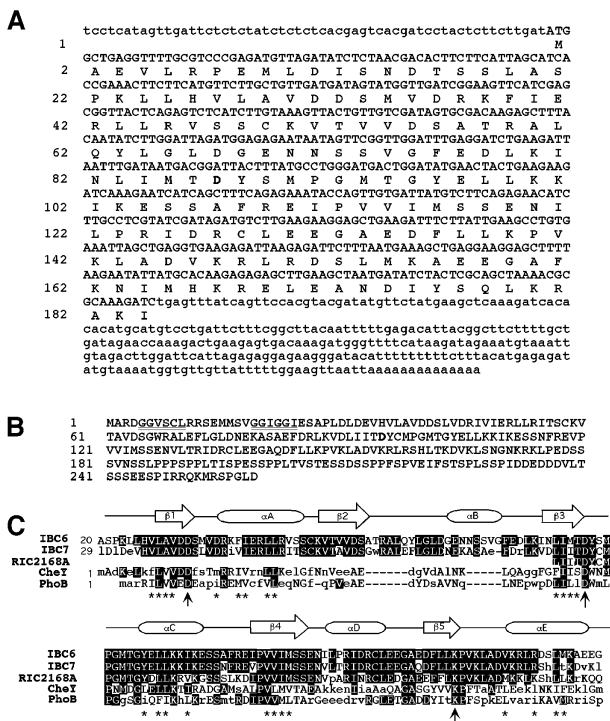


Figure 1. *IBC6* and *IBC7* Encode Proteins Similar to Bacterial Response Regulators.

(A) The cDNA and deduced amino acid sequence of *IBC6* (GenBank accession number AF057281). The boldface D residue indicates the predicted aspartate phosphorylation site.

(B) The deduced amino acid sequence of *IBC7* (GenBank accession number AF057282). The Asp that is the predicted site of phosphorylation is indicated in boldface, and the double underline indicates a potential myristylation site.

(C) Deduced amino acid sequence of *IBC6* and *IBC7* aligned with the conserved regions of various response regulators: CheY from *Escherichia coli* (Mutoh and Simon, 1986); PhoB from *E. coli* (Makino et al., 1986); and the rice EST RIC2168A (GenBank accession number D24560). The secondary structure of CheY is indicated above the sequence: arrows represent β sheets, ovals represent α helices, and lines represent loop regions (from A.M. Stock et al., 1989; J.B. Stock et al., 1989). Residues marked with an asterisk correspond to those forming the hydrophobic core of CheY, and invariant residues are indicated by black arrows, including the predicted Asp phosphorylation site of *IBC6* and *IBC7* in $\beta 3$. The sequence of *IBC6* is given in all capital letters. Capital letters in other sequences indicate identity or similarity with *IBC6* (score of >0.2 as defined by Schwartz and Dayhoff [1978]), with identical residues blocked in black. Dashes represent gaps in the sequence homology.

blot analysis (1.3 kb) after inclusion of the poly(A) tail. The start codon is in a context favorable for initiation in plants (Lükte et al., 1987; Joshi et al., 1997). However, the open reading frame continues to the 5' end of the longest cDNA, and thus, it is possible that the protein is slightly longer than

that predicted from our sequence. The predicted *IBC7* protein is 72% identical and 91% similar to *IBC6* across the response regulator domain (Figure 1C). In addition to the putative response regulator domain, the predicted *IBC7* amino acid sequence contains a 10-kD C-terminal extension that has a Ser/Pro-rich stretch (32 Ser/Pro of 52 total residues) followed by a short acidic domain (Figure 1B). This domain of *IBC7* does not share high primary sequence homology to any other sequences in GenBank and is not present in *IBC6*. *IBC7* also contains two potential myristylation sites near the N terminus.

A cDNA clone (RIC2168A) was identified in the rice EST database whose sequence is 69% identical and 95% similar to the *IBC6* amino acid sequence (Figure 1C). This rice gene also possesses a C-terminal extension after the receiver domain, although it is not similar to the sequence of the *IBC7* extension (data not shown). The highly conserved residues of response regulators are also present in RIC2168A, including the putative phosphorylation site at Asp-87 (Figure 1C).

DNA gel blot analysis was used to determine the number of related *IBC* genes in the Arabidopsis genome. Hybridization with either *IBC6* or *IBC7* full-length cDNA probes at low stringency revealed that these are both single copy genes in Arabidopsis (Figure 2). The two bands observed in the EcoRI digest for the *IBC7* probe are the result of a single EcoRI site

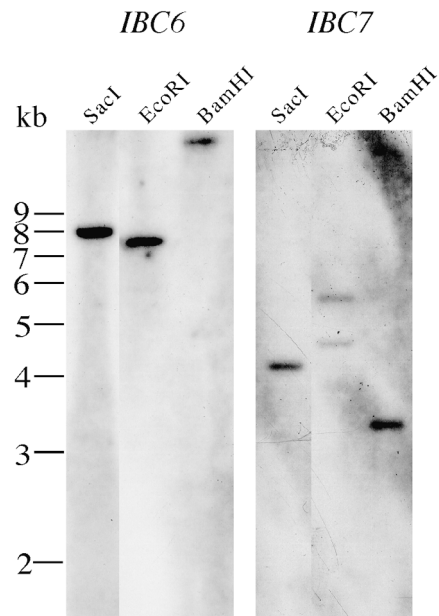


Figure 2. DNA Gel Blot Analysis of *IBC6* and *IBC7*.

Five micrograms of Arabidopsis genomic DNA (ecotype Wassilewskija) was digested with the indicated restriction enzyme, blotted, and probed with either an *IBC6* or *IBC7* cDNA, as indicated. The blot was washed at low stringency, as described in Methods. The position of molecular length standards is indicated at left.

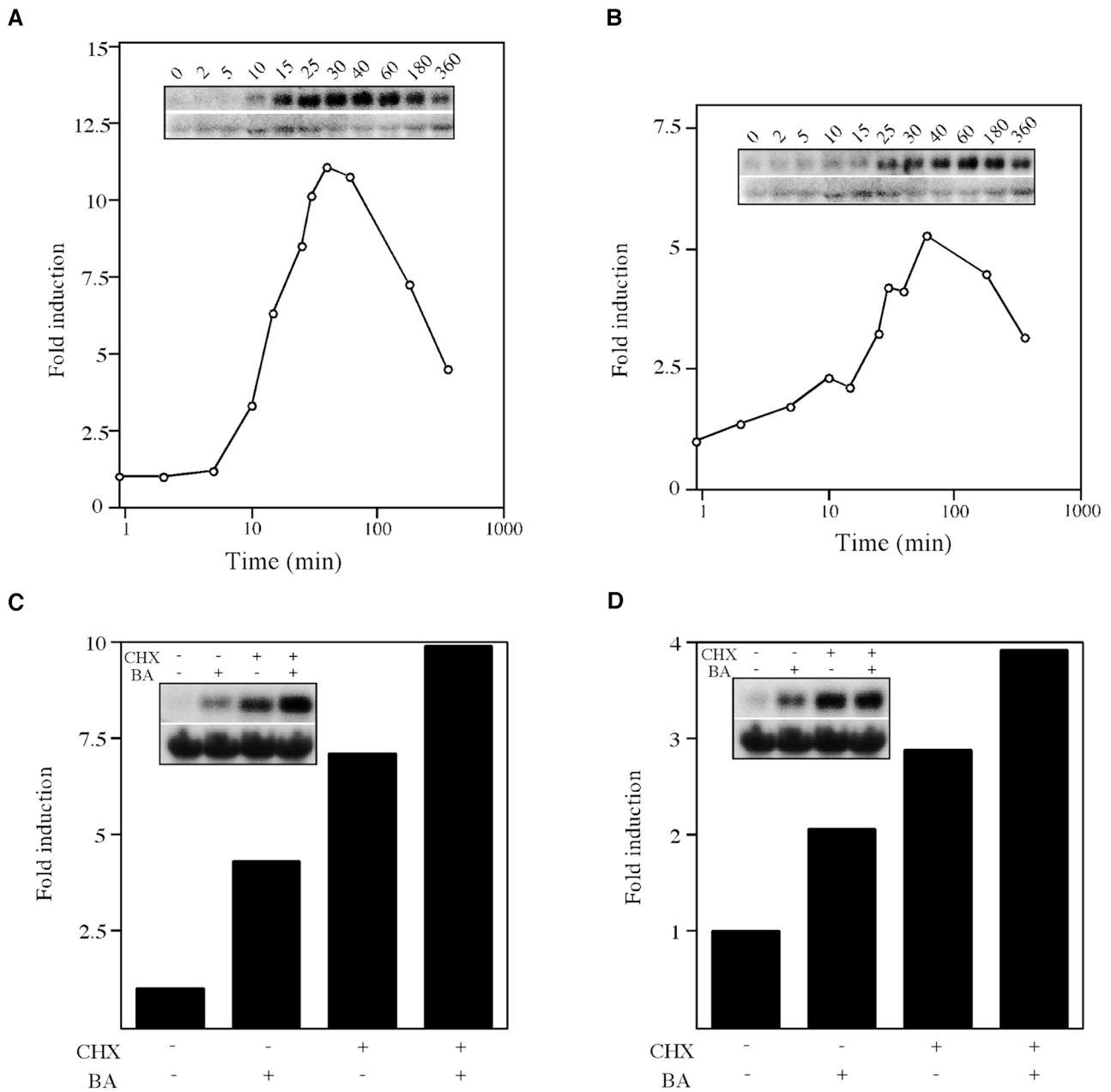


Figure 3. Kinetics of *IBC6* and *IBC7* Induction.

(A) and (B) RNA gel blot analysis of 15 μ g of total RNA from 3-day-old etiolated seedlings treated with 5 μ M BA for various times (time in minutes is indicated above each lane) and hybridized with either an (A) *IBC6*, (B) *IBC7*, or β -tubulin (bottom of each inset) probe.

(C) and (D) Effect of the protein synthesis inhibitor cycloheximide on *IBC6* and *IBC7* induction. Total RNA was isolated from 3-day-old etiolated seedlings that were pretreated with 50 μ M cycloheximide (CHX) or buffer alone for 30 min, then treated with 5 μ M BA or buffer alone for 30 min, indicated with + or -, respectively. Blots were hybridized with an (C) *IBC6*, (D) *IBC7*, or β -tubulin (bottom of each inset) probe.

The signals were quantified with a PhosphorImager and normalized to the β -tubulin loading control. The expression level in the control (no added BA) was set to an arbitrary value of 1.0, and all other time points were normalized to it (fold induction). The original images of the blots are shown in the insets.

present in the coding region of this gene. Somewhat surprisingly, there was almost no cross-hybridization between *IBC6* and *IBC7* in this analysis. However, close inspection of the DNA sequences showed that there is almost no DNA sequence similarity to *IBC7* outside of the coding region of *IBC6*, and this 550-bp region is interrupted by three introns (I. Brandstatter and J.J. Kieber, unpublished observations). Thus, even though the DNA sequences over this region are 67% identical, the presence of these three introns may disrupt the cross-hybridization between the cDNA probes and the genomic sequences.

Characterization of *IBC6* and *IBC7* Expression

We analyzed further the expression of the *IBC* genes by RNA gel blot analysis. The steady state level of *IBC6* mRNA in etiolated seedlings first increased above the basal level within 10 min of cytokinin application, rose to maximal levels within 40 min, and then declined to fourfold above the uninduced level 6 hr after the application of cytokinin (Figure 3A). The maximal induction was at least 10-fold above the basal level in etiolated seedlings. This rapid response indicates that *IBC6* may be a cytokinin primary-response gene. *IBC7* mRNA levels were also elevated by cytokinin, but the kinetics of induction were slower, and the maximum extent of induction was only approximately fivefold (Figure 3B). Pretreatment of seedlings with the protein synthesis inhibitor cycloheximide did not eliminate cytokinin induction of *IBC6* or *IBC7* (Figures 3C and 3D), which indicated that de novo protein synthesis was not required. In fact, cycloheximide itself actually induces these genes, which was also observed for the auxin primary response genes *PS-IAA* (for *Pisum sativum*-indoleacetic acid induced) and *SAUR* (for small auxin up RNA; Theologis et al., 1985; Franco et al., 1990).

We analyzed a dose-response of *IBC6* and *IBC7* gene expression by treating leaves of adult plants with various concentrations of BA for 50 min (Figure 4). The expression of *IBC6* was maximal at 1 μ M and declined sharply at slightly higher and lower concentrations (Figure 4A). The expression of *IBC7* was maximal at 20 μ M and decreased at higher concentrations (Figure 4B). However, both genes remained induced at concentrations as high as 50 μ M. This is consistent with a physiologically relevant concentration.

We tested the effect of other plant hormones and light on the steady state level of *IBC6* mRNA in both etiolated seedlings and adult leaves (Figure 5). *IBC6* was not rapidly induced by any of the other plant hormones that we tested, including gibberellic acid, abscisic acid, ethylene, and auxin (Figure 5); nor was it induced by light. This is in contrast to other cytokinin-regulated genes that have been identified (Crowell and Amasino, 1994) and indicates that induction of *IBC6* is specific for cytokinin. However, upon prolonged treatment with the plant hormone abscisic acid (2 hr), *IBC6* mRNA levels were elevated slightly in etiolated seedlings, perhaps due to alterations in endogenous cytokinin levels or to sensitivity.

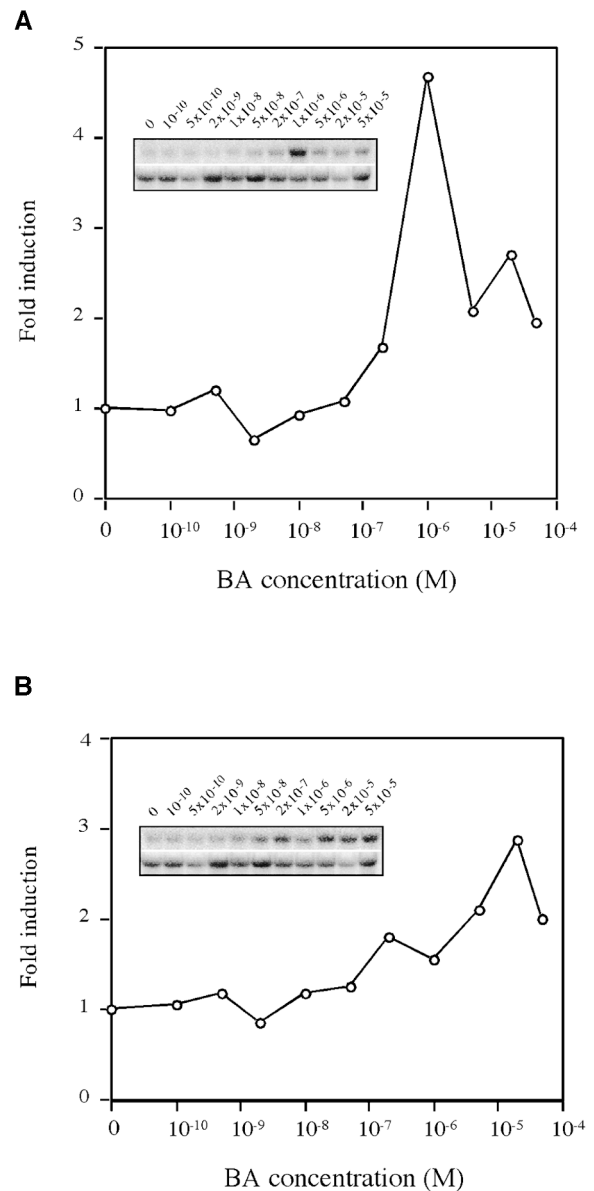


Figure 4. Dose-Response Analysis.

Total RNA was isolated from adult leaves treated with various concentrations (molarity indicated above the lanes) of BA. Fifteen micrograms of RNA was separated by agarose gel electrophoresis and blotted to a nylon membrane.

(A) and (B) Blots were hybridized with an (A) *IBC6*, (B) *IBC7*, or β -tubulin (bottom of each inset) probe. This dose-response analysis was repeated three times, with essentially the same results. The signals were quantified as described in the legend to Figure 3.

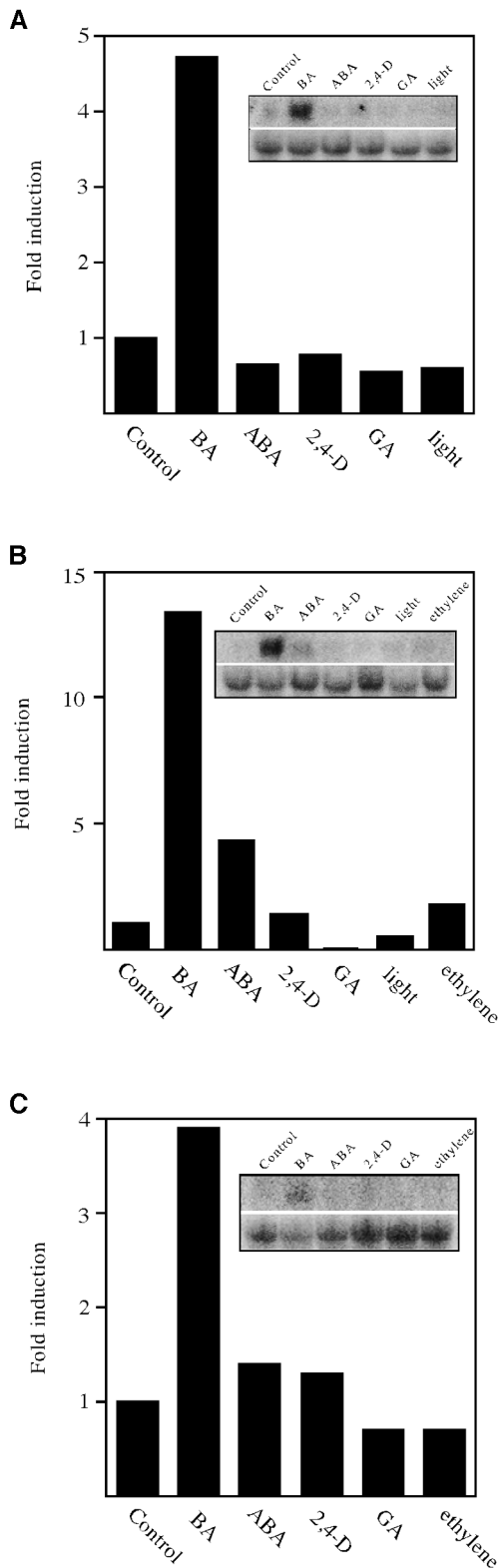


Figure 5. Specificity of *IBC6* Induction.

Analysis of Induction by Different Cytokinins

We examined the induction of *IBC6* (Figure 6A) and *IBC7* (Figure 6B) in adult *Arabidopsis* leaves treated for 40 min with 5 μ M various cytokinins. Of the adenine cytokinins, BA caused the highest induction of both *IBC6* and *IBC7*, and the induction of *IBC6* by zeatin and dihydrozeatin was approximately half that of BA. The induction by 2iP and kinetin was only slightly higher than that of adenine, which structurally is related to cytokinins but acts only as a weak cytokinin in bioassays (Skoog et al., 1967). Phenylurea cytokinins also induced *IBC6* and *IBC7*, and there was a rough correlation between the level of induction and the strength of the cytokinins in bioassays (Shudo, 1994). Diphenylurea, a very weak cytokinin in most bioassays, had the weakest effect on *IBC* gene expression, but the active urea cytokinins thidiazuron (*N*-phenyl-*N'*-1,2,3-thiazol-5-urea) and forchlorofenuron (*N*-[2-chloro-4-pyridyl]-*N*-phenylurea) were strong inducers.

Spatial Pattern of Expression

To begin to understand the function of these genes, we examined their expression in response to cytokinin in various tissues (Figure 7). We treated buds and newly opened flowers, leaves from 2-week-old plants, fully expanded siliques, soil-grown roots, and the stems of young inflorescences (rachises) with 5 μ M BA or with a DMSO control for 50 min. *IBC6* was most highly expressed in adult leaves, flowers, and stems and at a lower level in roots and siliques in response to exogenous cytokinin (Figure 7A). *IBC7* displayed a similar pattern of expression but generally had a higher basal level of expression, especially in the rachis (Figure 7B). *IBC7* also showed slightly elevated expression relative to *IBC6* in response to BA in siliques.

DISCUSSION

We describe the isolation and characterization of a pair of homologous genes that are rapidly and specifically induced by exogenous cytokinins. The sequences of these *IBC* genes are similar to bacterial response regulators. In addition,

(A) to (C) Fifteen micrograms of total RNA from 3-day-old etiolated seedlings (A) and (B) or fully expanded adult leaves (C) was analyzed by RNA gel blotting. Tissue was treated for 15 min (A) and (C) or 2 hr (B) with a DMSO control, 5 μ M cytokinin (BA), 20 μ M abscisic acid (ABA), 50 μ M auxin (2,4-D), 50 μ M gibberellin (GA), or 10 ppm ethylene or were placed 30 cm from a fluorescent light source. The blots were hybridized with an *IBC6* (top of each inset) or β -tubulin (bottom of each inset) probe. The signals were quantified as described in the legend to Figure 3.

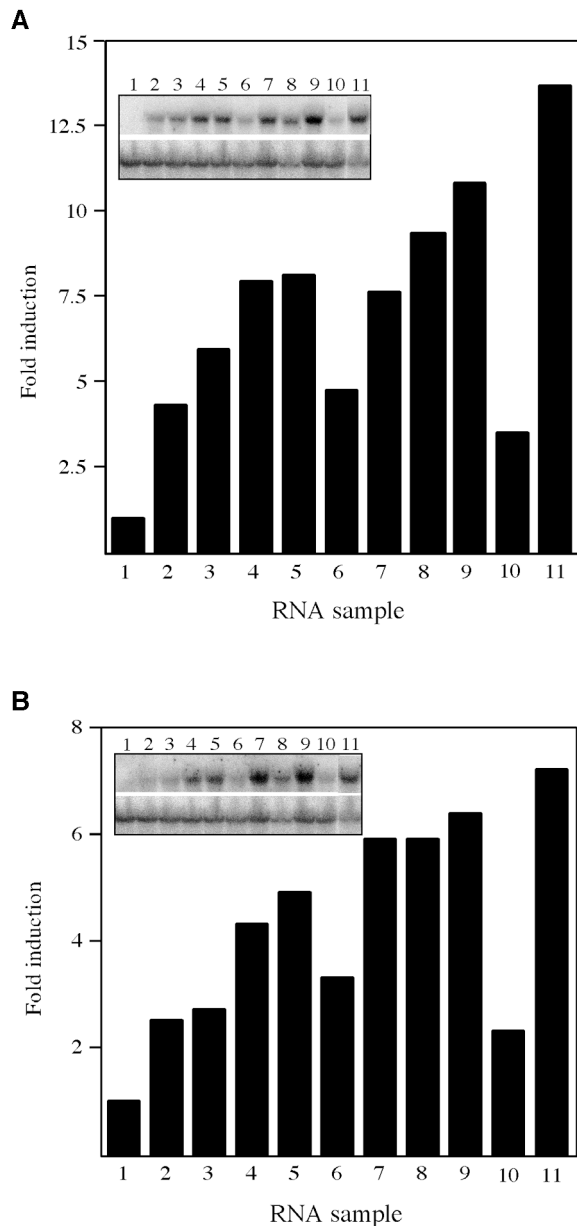


Figure 6. *IBC6* and *IBC7* Induction by Different Cytokinins.

(A) and (B) RNA gel blots of total RNA (15 μ g) from leaves of 2-week-old plants treated with MS salts containing 5 μ M of the indicated cytokinin for 50 min are shown. The RNA is from seedlings treated with DMSO (lanes 1), 2-isopentyladenine (lanes 2), kinetin (lanes 3), zeatin (lanes 4), dihydrozeatin (lanes 5), diphenylurea (lanes 6), thidiazuron (lanes 7), *N*-benzyl-9(2-tetrahydropyranol) adenine (lanes 8), *N*-(2-chloro-4-pyridyl)-*N*-phenylurea (lanes 9), adenine (lanes 10), or BA (lanes 11). The blots were hybridized with an (A) *IBC6*, (B) *IBC7*, or β -tubulin (bottom of each inset) probe. The signals were quantified as described in the legend to Figure 3.

tion to providing excellent molecular markers for early events in cytokinin signaling, their homology to response regulators suggests that these proteins may be involved directly in cytokinin signal transduction. The identification of a gene that is very similar to *IBC6* in rice, which is evolutionarily distant from Arabidopsis, suggests that the *IBC* genes may be part of a conserved gene family in higher plants.

The similarity of *IBC6* to bacterial response regulators, coupled with its rapid and specific induction by cytokinin, suggests that *IBC6* may be involved in an early step in cytokinin signal transduction. The homology of *IBC6* to response regulators is intriguing given recent work that implicates a two-component histidine kinase (CKI1) in cytokinin signaling (Kakimoto, 1996). *IBC6* may be the downstream partner of CKI1, and these two proteins may act as a receptor (sensor) and transducer of the cytokinin signal. In bacterial systems, the first gene upregulated by perception of the signal is often the response regulator. Thus, we propose that *IBC6* acts in the cytokinin signaling pathway and is upregulated in response to elevated cytokinin levels. This upregulation would result in a self-amplifying, autoregulatory loop that would increase the amplitude of a response to a given concentration of hormone. Further testing of this model awaits disruption of *IBC6* function in vivo and studies of the interaction between *IBC6* and CKI1.

Alternatively, *IBC6* and/or *IBC7* may be involved in light signaling. Cytokinins have been linked to light responsiveness: a number of light-regulated genes are induced by cytokinin (Crowell and Amasino, 1994), and cytokinin can induce deetiolation in Arabidopsis seedlings (Chory et al., 1994). Perhaps the *IBC* genes provide the integration between light and cytokinin signaling by acting downstream of the phytochrome photoreceptors, which have been shown to possess a C-terminal domain similar to that of bacterial histidine kinases. However, histidine kinase activity has not been demonstrated in higher plant phytochromes (Wong et al., 1986, 1989; Schneider-Poetsch et al., 1991; Quail et al., 1995; Yeh et al., 1997).

The induction of *IBC6* by cytokinin is similar to the induction kinetics of the *PS-IAA* and *SAUR* genes in response to auxin (Theologis et al., 1985; McClure and Guilfoyle, 1987). These genes are auxin primary-response genes and encode short-lived, nuclear-localized proteins (Abel et al., 1994). The rapid response to cytokinin indicates that *IBC6* may be a cytokinin primary-response gene. Furthermore, like the *PS-IAA* and *SAUR* genes, *IBC6* and *IBC7* are induced by the protein synthesis inhibitor cycloheximide (Theologis et al., 1985; Franco et al., 1990). This induction by cycloheximide suggests that either the transcription of these genes is repressed or the mRNAs are degraded rapidly by a short-lived protein.

IBC7 generally displays a higher basal level of expression and is not induced as rapidly as *IBC6*. *IBC7* may be involved in constitutive cytokinin responses, or perhaps it is responsible for the initial perception of cytokinin and then *IBC6* amplifies this signal. Overall, *IBC7* is extremely similar in its primary amino acid sequence to *IBC6*. The main difference is the

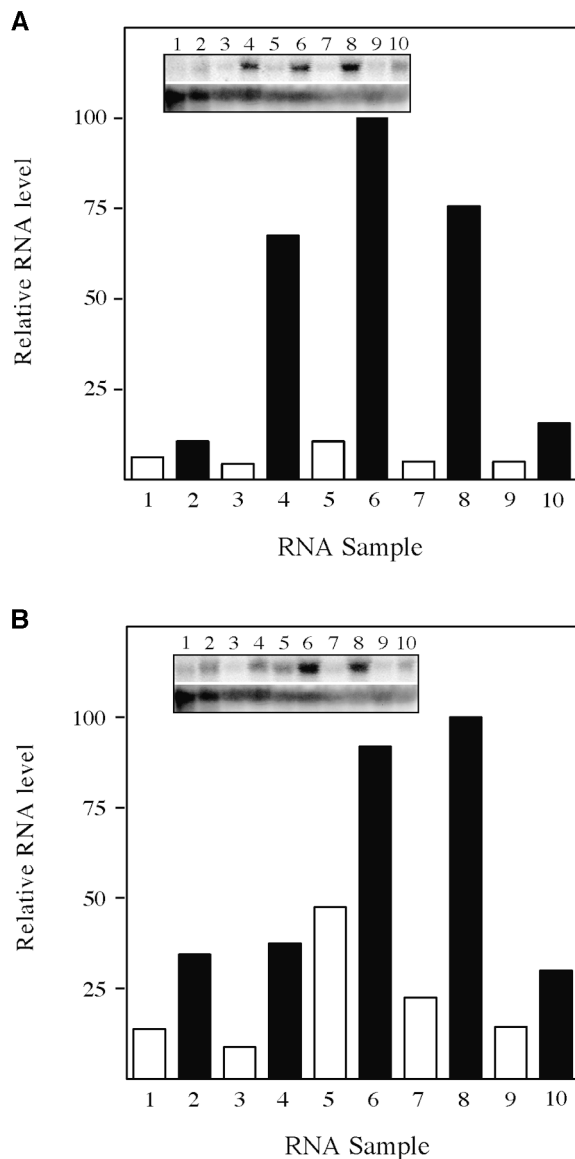


Figure 7. Tissue-Specific Expression of *IBC6* and *IBC7*.

(A) and (B) RNA was extracted from various tissue treated with either 5 μ M BA (lanes 2, 4, 6, 8, and 10) or a buffer control (lanes 1, 3, 5, 7, and 9) for 50 min. Fifteen micrograms of total RNA was separated by agarose gel electrophoresis, blotted to nylon, and hybridized with an (A) *IBC6*, (B) *IBC7*, or 18S rDNA (bottom of each inset) probe as a loading control. The tissues analyzed were fully expanded siliques (lanes 1 and 2), buds and young flowers (lanes 3 and 4), stems of young inflorescences (rachises; lanes 5 and 6), leaves from 2-week-old plants (lanes 7 and 8), and soil-grown roots (lanes 9 and 10). The expression level in each tissue was normalized to the 18S loading control, the highest signal was set to 100%, and all other time points were normalized to it.

presence of a C-terminal extension on *IBC7*. This C-terminal extension of *IBC7* is acidic, which is a feature found in many transcriptional activators. Many bacterial response regulators are transcription factors, raising the intriguing possibility that *IBC7* may directly regulate gene expression.

The spatial pattern of expression of *IBC6* and *IBC7* displays some subtle differences that, coupled with the distinct induction kinetics, may reflect slight differences in function. The ubiquitous expression of these genes may reflect the multitude of growth and developmental processes that involve cytokinin. Elevation of endogenous cytokinins via transgenes results in alterations in root, leaf, and stem growth and development (Smigocki and Owens, 1988; Medford et al., 1989; Schmülling et al., 1989; Smart et al., 1991; Smigocki, 1991; Li et al., 1992; Ainley and Key, 1993).

Cytokinins may increase the steady state level of these genes by increasing the rate of transcription, by increasing the half-life of the mRNA, or by a combination of both mechanisms. Direct measurements of transcription rates by using nuclear run-on assays and pulse-chase studies should distinguish among these possibilities. In either case, the *cis*-acting sequences responsible for this regulation can be delineated and should provide a starting point in the elucidation of the elements involved in controlling the expression of these genes.

The extremely rapid induction of these genes and the persistence of induction in the presence of an inhibitor of protein synthesis indicate that the cytokinin signal is transmitted via preexisting components. Analysis of signaling factors required for this induction should help to elucidate early events in cytokinin signaling.

METHODS

Plant Material

The *Arabidopsis thaliana* ecotype Wassilewskija (WS) was used in this study. The *Arabidopsis* expressed sequence tag (EST) cDNA clones were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus). Plants were grown in Metro-Mix 200 (Grace, Boca Raton, FL) under continuous illumination with fluorescent lights at 23°C. All phytohormones were obtained from Gibco BRL (Gaithersburg, MD), except for thidiazuron, which was obtained from Sigma (St. Louis, MO). Etiolated seedlings were grown as follows: seeds were surface sterilized for 8 min in 5% sodium hypochlorite (Clorox)/0.01% Tween 20 and rinsed five times with sterile water. The sterile seeds were then resuspended in 0.8% low-melting-point agarose and plated on 0.8% agar containing Murashige and Skoog (MS) salts (Gibco) supplemented with 2% sucrose (MS agar). Seeds were incubated at 4°C for 4 days and then transferred to the dark and incubated at 23°C for 3 days.

Differential Display

RNA for use in differential display was prepared as follows: the roots of 3-day-old etiolated seedlings were detached, and the aerial por-

tions were incubated overnight in the dark in liquid MS medium at 23°C in an attempt to deplete endogenous cytokinins. The tissue was then transferred to liquid MS media containing 50 μ M benzyladenine (BA) or an equal volume of DMSO (control) and incubated for 2 hr at 23°C. Total RNA was isolated by extraction with phenol-chloroform (Ausubel et al., 1987) and treated with RNase-free DNase (Boehringer Mannheim, Indianapolis, IN) to remove residual DNA. Differential display comparing BA-treated and control seedlings of these two RNA populations was performed in duplicate, as described by Liang and Pardee (1992), by using either random 10-mers (Operon, Alameda, CA) or random 13-mers (GenHunter, Nashville, TN) and α -³²P-dATP. The ³²P-labeled products were separated on an 8% denaturing SDS-PAGE gel and visualized by autoradiography. Bands that appeared to be more intense in lanes derived from the BA-treated seedlings were excised from the polyacrylamide gel (Liang and Pardee, 1992), reamplified with the appropriate primers, and cloned into the pCR2.1 vector, as described by the manufacturer (Invitrogen, Carlsbad, CA).

DNA Manipulations

IBC6 and *IBC7* were sequenced at the automated sequencing facilities of the University of Illinois at Chicago by using a series of gene-specific oligonucleotide primers. All regions were sequenced on both strands. All other DNA manipulations were essentially as described by Ausubel et al. (1987).

DNA Gel Blot Analysis

Genomic Arabidopsis DNA (from ecotype WS) was prepared using genomic Qiaprep columns, as described by the manufacturer (Qiagen, Chatsworth, CA). Five micrograms of DNA was digested with the appropriate enzyme, and the products were separated on an 0.8% agarose gel. The DNA was blotted to Hybond N⁺ membrane (Amersham, Arlington Heights, IL) by using alkaline transfer (Ausubel et al., 1987). The blot was then hybridized to a full-length *IBC6* or *IBC7* cDNA probe prepared by random hexamer labeling (Ausubel et al., 1987) in low-stringency hybridization buffer (40% formamide, 6 \times SSC [1 \times SSC is 0.15 M NaCl, 0.015 M sodium citrate], 0.1% SDS, 0.1% nonfat dry milk, and 100 μ g/mL sheared denatured salmon testes DNA) at 37°C for 14 hr. The blot was then washed twice in 2 \times SSC and 0.1% SDS for 15 min at room temperature and twice in 1 \times SSC and 0.1% SDS at 42°C for 20 min. Autoradiography was performed at -80°C by using Kodak XAR-5 film and an intensifying screen for 48 hr.

RNA Gel Blot Analysis

Seedlings were grown (~2000 per 100-mm plate) on MS agar for 3 days as described above, except that sterile filter paper was placed on the top of the agar. For hormone treatments, seedlings were submerged in 10 mL of liquid MS containing the indicated concentration of each hormone (controls were submerged in MS alone containing an equal volume of DMSO), and the seedlings were harvested at the indicated times. For the cycloheximide treatment, seedlings were exposed to 50 μ M cycloheximide for 30 min before treatment with BA. Adult tissues were excised and incubated with rocking in liquid MS medium containing either the indicated concentration of cytokinin or an equal volume of DMSO as a control. Total RNA was prepared by extraction with phenol-chloroform, fractionated on a glyoxyl-agarose

gel, transferred to a Hybond N membrane (Amersham), and hybridized with random-hexamer-labeled DNA probes, as described by Ausubel et al. (1987).

A full-length cDNA fragment was used as the *IBC6* probe, which was obtained by polymerase chain reaction by using two *IBC6*-specific primers (IB16, 5'-aagatccATGGCTGAGGTTTTCGT-3'; IB19, 5'-aagaattcgCAGGACATGCATGTGTGTG-3'; homology to *IBC6* indicated by capital letters) with cDNA derived from cytokinin-treated seedlings as a template. The *IBC7* probe was a 390-bp fragment derived from the 3' portion of the gene obtained by polymerase chain reaction with oligonucleotide primers (Gag4, 5'-aagatccGTGGAGCGCGAGAGATT-3'; Gag5, 5'-CGGGAAGATTCTAGTCCG-3') by using cDNA from cytokinin-treated seedlings as a template. Both were shown to be gene specific under our hybridization and washing conditions by dot-blot analysis using purified clones as substrates (data not shown). Replica blots were probed with either β -tubulin or an Arabidopsis 18S rDNA clone. The β -tubulin and rDNA fragments used as loading controls were obtained by amplifying the genes from Arabidopsis genomic DNA by using oligonucleotide primers (JVTF, 5'-GAGATTCTTACATCCAGGG-3'; JVTR, 5'-CATCTCGTCCATCCCTCAC-3' for β -tubulin and 18S 3', 5'-GGTATCGACAATGATCCTTC-3'; 18S 5', 5'-CCTGGTGTATCCTGCCAG-3' for the 18S rDNA). Signals were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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