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Changes in expression of two cytokinin-repressed genes, *CR9* and *CR20*, in relation to aging, greening and wounding in cucumber

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Abstract. The expression of two cytokinin-repressed genes, *CR9* and *CR20*, was investigated in cucumber (*Cucumis sativus* L.). In excised cotyledons, the level of the *CR20* transcript markedly decreased in the early periods of treatment with N^6-benzyladenine (BA), i.e. well before BA-induced expansion of cotyledons was observed. The repression of *CR20* was BA-dose dependent and highly specific for cytokinins. These features were similar to those of *CR9* reported previously (H. Teramoto et al., 1994, Planta **193**, 573–579). Furthermore, levels of the *CR9* and *CR20* transcripts decreased during the early phase of greening and soon after wounding of cotyledons. The levels were much lower in young leaves than in mature or senescent leaves. Because cytokinins are thought to control greening and aging of leaves, and to mediate response to wounding, the expression of these two genes could be closely related to the action of such hormones. However, there were some differences between the expression of *CR9* and *CR20*, i.e. the pattern of diurnal changes and the transcript levels in roots. Therefore, some other factors in addition to cytokinins appear to differentially affect the expression of these two genes. Several transcripts of *CR20* of different lengths (0.8–2.3 kb) were detected by Northern blot analysis. No long open reading frames could be detected in two CR20 cDNAs with different structures.

Key words: cDNA (for cytokinin-repressed mRNA) – *Cucumis* – Cytokinin – Gene expression

Introduction

Cytokinins are involved in the control of various phases of development of plants. However, the molecular mechanism of action of these hormones remains unknown. For the study of the mechanism, it may be important to investigate the genes whose expression is rapidly changed by cytokinins. The expression of a number of genes appears to be modulated by cytokinins in various plant materials (for review, see Chen et al. 1993). For example, cytokinins increase the extent of light-induced increases in RNAs for chloroplast proteins, such as light-harvesting chlorophyll *a/b*-protein and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Teyssendier de la Serve et al. 1985; Flores and Tobin 1986, 1988; Ohya and Suzuki 1991). N^6-Benzyladenine (BA) stimulates light induction of nitrate reductase in excised barley leaves in the presence of nitrate (Lu et al. 1990, 1992). Cytokinins also affect the expression of phospho*enol*pyruvate carboxylase (Schmitt and Piepenbrock 1992; Sugiharto et al. 1992) and the apoprotein of phytochrome (Cotton et al. 1990). However, most authors observed changes after long periods of cytokinin treatment or modification by cytokinins of the effects of other factors such as light and inducers. Therefore, it is difficult to relate such changes to the primary action of this class of hormone. Several cDNAs for mRNAs that show fairly rapid increase or decrease in response to treatment with cytokinins have been isolated by differential screening from cultured cells of soybean (Crowell et al. 1990; Crowell and Amasino 1991) and tobacco (Dominov et al. 1992), but their expression is modulated by auxins as well as, or better than, by cytokinins. Very recently, Sano and Youssefian (1994) reported that cytokinin specifically increases levels of transcript encoding *wpk4*, wheat protein-kinase homolog, in wheat seedlings.

In a previous report, we described a cDNA for a cytokinin-repressed gene (*CR9*) in cucumber, which was isolated by differential screening (Teramoto et al. 1994). In excised cotyledons of cucumber, the level of the *CR9* transcript markedly decreased within 4 h of BA treatment well before the BA-induced expansion of cotyledons. The repression was BA-dose dependent, and highly specific for cytokinins (Teramoto et al. 1994). In this report, we describe a cDNA for *CR20*, another gene whose expression was similarly repressed by cytokinins in the same experimental system, and compare it with *CR9* from various aspects.

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Abbreviations: BA = benzyladenine; 16 L/8 D = cycle of 16 h light and 8 h darkness

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Cotyledons of cucumber and of other dicotyledonous plants have been used for studies of the roles of cytokinins in various physiological phenomena, such as cotyledon expansion (Gordon and Letham 1975; Huff and Ross 1975; Bewli and Witham 1976; Longo et al. 1978; Naito et al. 1980; Thomas et al. 1980; Haru et al. 1982; Galli 1984), greening (Fletcher and McCullagh 1971; Fletcher et al. 1973; Uheda and Kuraishi 1977), and response to wounding (Crane and Ross 1986; Ohya and Suzuki 1988). In this study, we investigated changes in the expression of *CR9* and *CR20* that were associated with these phenomena in cucumber. We also studied the expression of these genes in various organs of cucumber. In particular, levels of the transcripts were examined in relation to aging of leaves because cytokinins are thought to regulate the growth and senescence of leaves (for review, see Hall 1973).

Reimmann and Dudler (1993) have described a rice gene, *lir1*, which is structurally homologous to our *CR9*, and shown that it is controlled by light and a circadian clock. We report here that the level of the *CR9* transcript also increased in the light phase and decreased in the dark phase in cotyledons of cucumber seedlings. By contrast, the *CR20* transcript accumulated in the dark phase. Moreover, other slight differences between the expression of *CR9* and *CR20* were also noted, suggesting that other factors in addition to cytokinins differentially affect the expression of these genes.

Materials and methods

Treatment of excised cucumber cotyledons with cytokinins. Excised cotyledons of cucumber (*Cucumis sativus* L. cv. Aonagajibai) were treated with cytokinins as described previously (Teramoto et al. 1994). Cotyledons were excised from seedlings which were germinated for 5 d at 28 °C in darkness. The cotyledons were incubated for 18 h on filter paper (No. 2; Advantec, Tokyo, Japan) wetted with 1.6 mL of water in a 5.5-cm Petri dish at 28 °C in darkness. Following this pretreatment with water, various treatments were performed. In time-course experiments, 0.4 mL of a 50-μM solution of BA was added to the Petri dish containing water-pretreated cotyledons (final concentration of BA, 10 μM) and incubated another 23 h under the same conditions. Samples were harvested at various periods after the addition of BA and stored at − 80 °C until use. In dose-response experiments, 0.4 mL of 0.05, 0.5, 5 or 50 μM BA was added. In experiments to compare the effects of isopentenyladenine, *trans*-zeatin, adenine and 2,4-dichlorophenoxyacetic acid, the final concentration of each compound was 10 μM. Samples were harvested after a 4-h treatment with the various compounds. All manipulations were performed under a dim green safelight.

Wounding of cotyledons. Cotyledons excised from etiolated cucumber seedlings were incubated with water for 18 h in darkness, then they were cut into two pieces, and incubated another 23 h under the same conditions. Samples were harvested at various periods after the first excision and the second cutting, and they were stored at − 80 °C until use.

Greening of cotyledons. Cucumber seedlings were germinated for 5 d at 28 °C in darkness, and then transferred to light. Alternatively, cotyledons were excised from the etiolated seedlings, incubated with water for 18 h in darkness in the same way as described for treatment with cytokinins, and then transferred to light. In either case, samples were illuminated with white fluorescent lamps (43 $\mu mol \cdot m^{-2} \cdot s^{-1}$; 400–700 nm). Cotyledons were harvested at various periods after transfer to light and stored at − 80 °C until use.

Various organs of cucumber plants. Cucumber plants were grown in a growth chamber under a cycle of 16 h light and 8 h darkness (16 L/8 D) at 28 °C. Cotyledons, hypocotyls and roots were harvested from 7-d-old seedlings, cotyledons of which had just opened fully. Apical buds were harvested from one-month-old plants, the third leaves of which had just opened. Leaves of three different ages (young, mature and senescent) were harvested from four-month-old plants. "Young leaves" were the upper pale-green leaves that had just opened and were about 3 cm in length. "Mature leaves" were the middle fully-green leaves of 5–10 cm in length. "Senescent leaves" were the lower leaves that were turning yellow. Etiolated cotyledons were harvested from seedlings which were germinated for 5 d at 28 °C in darkness. Samples were stored at − 80 °C until use.

Experiments to examine diurnal changes. Cucumber seedlings were grown in a growth chamber under a 15 L/9 D cycle at 28 °C for 8 d. Cotyledons were harvested at 3-h intervals for 24 h on the eighth day and stored at − 80 °C until use.

Total-RNA preparation. Total RNA was prepared by the method of Chomczynski and Sacchi (1987) with some modifications. Cotyledons were homogenized with a Potter-Elvehjem-type glass homogenizer in GT buffer (4 M guanidine thiocyanate; 25 mM sodium citrate; 0.5% sarcosyl; 2% 2-mercaptoethanol) with an equal volume of phenol:chloroform:isoamyl alcohol (75:24:1, by vol.). The homogenate was centrifuged at 18 600·*g* for 10 min at room temperature. The aqueous phase was mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, by vol.) and centrifuged again. The extraction with phenol:chloroform:isoamyl alcohol (25:24:1, by vol.) was repeated, and the aqueous fraction was then extracted with chloroform:isoamyl alcohol (24:1, v/v). The aqueous phase was mixed with one-tenth volume of 2 M sodium acetate (pH 4.0), one volume of water-saturated phenol and two-tenth volume of chloroform:isoamyl alcohol (24:1, v/v). The mixture was centrifuged at 18 600·*g* for 10 min at room temperature. The aqueous phase was mixed with six-tenth volume of isopropanol, and then centrifuged at 18 600·*g* for 10 min at room temperature. The precipitate was dissolved in SDS buffer (50 mM Tris-HCl, pH 9.0; 0.1% SDS), and reprecipitated with ethanol.

Northern blots. Northern blot analysis was performed as described by Teramoto et al. (1994) with slight modifications. Total RNA was electrophoresed on an agarose-formaldehyde gel, and transferred to a nylon membrane (PALL, New York, USA). The RNA was cross-linked to the membrane by exposure to UV irradiation (using a Funa-UV-Linker; Funakoshi, Tokyo, Japan). The membrane was prehybridized at 42 °C for 4 h in a prehybridization solution consisting of 50% formamide, 5 × Denhardt's solution (1 × Denhardt's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin), 5 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, pH 7.4 and 1.25 mM EDTA), 100 $\mu g \cdot mL^{-1}$ denatured salmon sperm DNA and 0.5% SDS. Probes labeled with ^{32}P were prepared from the cDNA inserts using a random-primed DNA labeling kit (Boehringer Mannheim, Germany). Hybridization was performed with ^{32}P-labeled probes in the prehybridization solution for 18 h at 42 °C. The membrane was washed twice at 42 °C in 1 × SSC plus 0.1% SDS for 20 min each, and then in 0.2 × SSC plus 0.1% SDS for 20 min.

Genomic DNA preparation. Genomic DNA was prepared from cucumber seedlings as described by Ausubel et al. (1991a) with some modifications. Seedlings were germinated for 5 d in darkness. The seedlings were ground in liquid nitrogen. The resultant powder was suspended in extraction buffer (100 mM Tris-HCl, pH 8.0; 100 mM EDTA; 250 mM NaCl), and sarcosyl was added to a final concentration of 1% (w/v). The mixture was incubated at 55 °C overnight, and then centrifuged at 4000·*g* for 10 min at 4 °C. The supernatant was mixed with an equal volume of phenol:chloroform:isoamyl alcohol

(25:24:1, by vol.), and then centrifuged at 3000·*g* for 10 min at room temperature. The aqueous fraction was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, by vol.). Nucleic acid in the aqueous phase was precipitated with six-tenth volume of isopropanol, and then dissolved in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). DNA was purified by centrifugation on a cesium-chloride density gradient (Ausubel et al. 1991a).

Southern blots. Genomic DNA was digested with six restriction enzymes (*Eco*RI, *Hind*III, *Bam*HI, *Apa*I, *Kpn*I and *Xho*I), and electrophoresed on an agarose gel, and transferred to a nylon membrane (PALL) as described by Ausubel et al. (1991b). Prehybridization, hybridization and washing were carried out as described above for Northern blots.

Sequencing of DNA. For nucleotide sequencing, restriction fragments of the inserts were subcloned into the Bluescript plasmid (Stratagene, La Jolla, Calif., USA). Sequencing was performed by the dideoxy-chain-termination method (Sambrook et al. 1989) using a DNA sequencer (373A; ABI, Foster, Calif., USA) in accordance with the manufacturer's instructions.

Results

Changes in levels of the CR20 transcript during incubation of excised cucumber cotyledons with BA in darkness. In a previous study (Teramoto et al. 1994), we isolated cDNAs for cytokinin-repressed genes from a cDNA library of cucumber cotyledons by differential screening. CR20 is one of these cDNAs. Its corresponding gene is designated as *CR20*. Figure 1A shows the time course of changes in the *CR20* transcript during 23 h of incubation of excised cucumber cotyledons with BA in darkness. Several bands hybridizing with CR20 cDNA were detected by Northern blot analysis. The 1.4-kb band was visualized reproducibly as a major band. The relative intensities of the other bands, e.g. those of 2.3 kb or 0.8 kb, varied among several experiments. The level of the *CR20* transcript decreased 2–4 h after application of BA if we consider the 1.4-kb RNA or total hybridizing bands. This decrease was faster than that of the level of the transcript of *CR9*, another cytokinin-repressed gene that was reported previously (Teramoto et al. 1994, as shown in Fig. 1B). This decrease preceded the increase in fresh weight of cotyledons, which occurred from 4 to 24 h after the application of BA. The same samples of RNA as used for the above experiments were analyzed by Northern hybridization, using CR9 cDNA and NC1 cDNA as probes. The *CR20* transcript remained at a low level after repression by treatment with BA (Fig. 1A), while the level of the *CR9* transcript increased at 23 h (Fig. 1B). Figure 1C shows results of a control experiment. NC1 is a cDNA for an mRNA that was relatively abundant and showed no change in level between 0 and 4 h of treatment with BA when examined by differential hybridization. Northern blot analysis showed that the level of the NC1-hybridizing RNA did not change during 23 h of treatment with BA. The sequence of NC1 cDNA was not determined.

N^6-Benzyladenine-dose response and cytokinin specificity of the repression of the CR20 gene. In order to investigate the effect of different doses of BA on the repression of the *CR20* gene, excised cotyledons were incubated

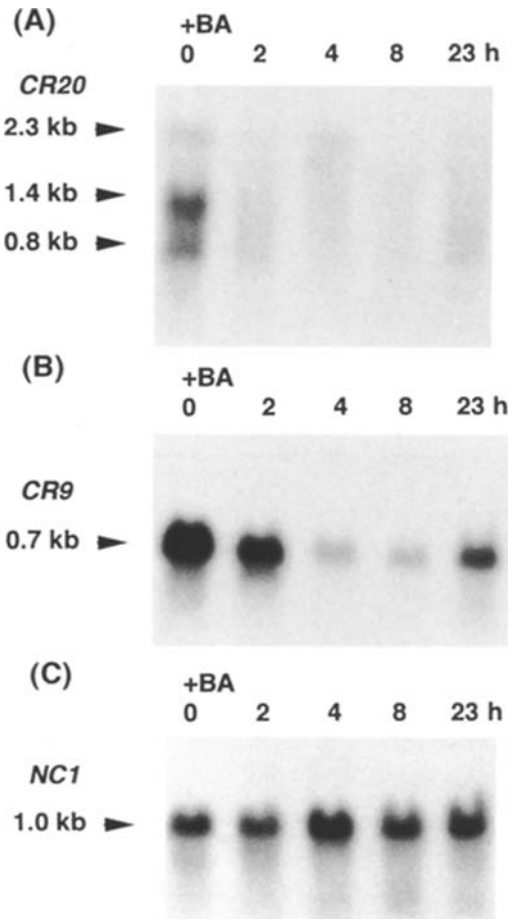

Fig. 1A–C. Changes in levels of *CR20* (**A**), *CR9* (**B**) and *NC1* (**C**) transcripts in cucumber cotyledons during dark incubation with BA. Excised cotyledons were incubated with water for 18 h and then with 10 μM BA for 0, 2, 4, 8 and 23 h at 28 °C in darkness throughout. Twenty micrograms of total RNA, extracted from cotyledons of each sample, was loaded on the gel, electrophoresed and analyzed by Northern hybridization with ^{32}P-labeled CR20, CR9 and NC1 cDNAs as probes. CR20 and CR9 are cDNAs for cytokinin-repressed genes. NC1 is a cDNA for an RNA whose amount is not changed by treatment with BA. Several bands of CR20-hybridizing RNA were detected, and three distinct bands are indicated by *arrowheads* with their estimated lengths

for 4 h in darkness with various concentrations of BA (10^{-8}–10^{-5} M) after dark pretreatment with water for 18 h. Total RNA was prepared from each sample, and analyzed by Northern hybridization with CR20 cDNA as a probe. The higher concentrations of BA strongly repressed the total level of the *CR20* transcript (Fig. 2A).

In order to examine the cytokinin specificity of repression of the *CR20* gene, effects of various compounds on expression of *CR20* were tested in the same manner as described above (Fig. 2B). The total level of the *CR20* transcript was similarly reduced by treatments with three different cytokinins, namely, BA, isopentenyladenine and *trans*-zeatin, but was not reduced by adenine, a cytokinin analogue without activity, or by 2,4-dichlorophenoxyacetic acid, an auxin. All compounds were tested at a concentration of 10 μM.

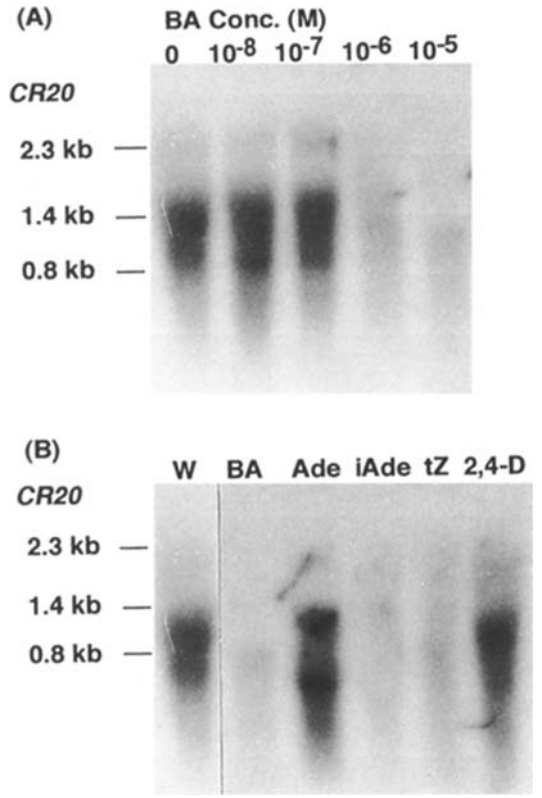

Fig. 2A, B. The BA-dose response of the repression of the *CR20* gene (**A**) and specificity of the repression to cytokinins (**B**). **A** Cotyledons were treated with 0, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M BA for 4 h in darkness after an 18-h preincubation with water. **B** Cotyledons were treated with water (*W*), BA, adenine (*Ade*), isopentenyladenine (*iAde*), *trans*-zeatin (*tZ*) and 2,4-dichlorophenoxyacetic acid (*2,4-D*). All compounds tested were used at a concentration of 10^{-5} M. Other conditions of incubation were the same as described for **A**. Northern blot analysis was carried out as described in the legend to Fig. 1

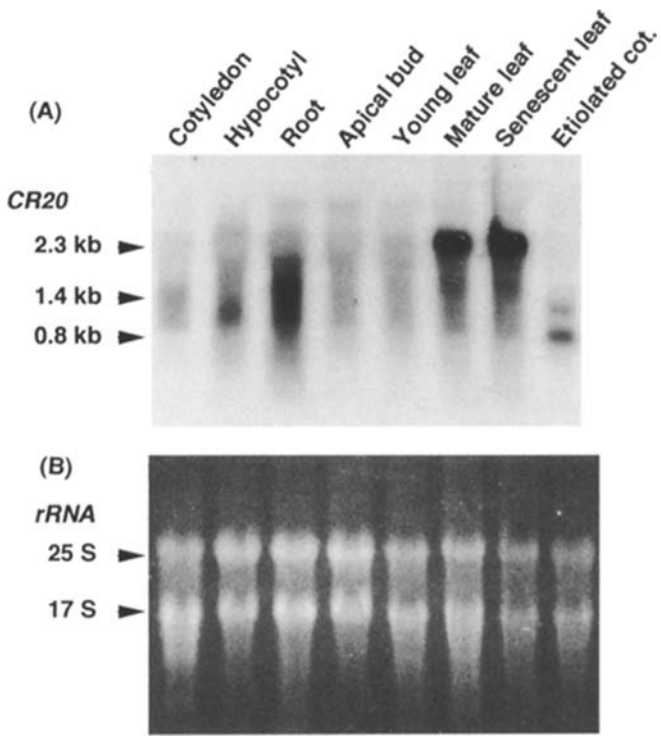

Fig. 3A, B. Expression of the *CR20* gene in various organs of cucumber. **A** Cucumber plants were grown in a growth chamber under a 16 L/8 D cycle at 28 °C. Cotyledons, hypocotyls and roots were harvested from 7-d-old seedlings, apical buds from one-month-old plants, and leaves of three different ages (young, mature and senescent) from four-month-old plants. Etiolated cotyledons were obtained from seedlings germinated for 5 d in darkness. Northern blot analysis was carried out as described in the legend to Fig. 1. **B** The RNA on the gel in **A** was detected with ethidium bromide

The dependence on the dose of BA and the cytokinin specificity of the repression of the *CR20* gene were almost the same as results for the *CR9* gene that were reported previously (Teramoto et al. 1994).

Different transcripts that hybridize with CR20 cDNA and the patterns of their expression in various organs of cucumber plants. Figure 3 shows levels of the *CR20* transcript in various organs, as determined by Northern blot analysis. Cotyledons, hypocotyls and roots were harvested from light-grown seedlings. Apical buds, young, mature and senescent leaves were harvested from light-grown plants. Etiolated cotyledons were harvested from dark-grown seedlings. Total RNA was prepared from each sample, and analyzed by Northern hybridization with CR20 cDNA as a probe (Fig. 3A). The results provided a clue to the question of whether or not the various transcripts of *CR20* resulted from degradation during preparation of the RNA. Products of the degradation of rRNAs from each sample were nearly undetectable, and the patterns of bands of rRNAs were almost identical (Fig. 3B). However, several bands of CR20-hybridizing RNA were detected by Northern blot analysis, and different patterns of bands were obtained from the various organs (Fig. 3A). These results suggest that the diversity of the *CR20* transcripts is a reflection of the situation within the plant rather than an artifact. As shown in Figure 3A, levels of all transcripts (0.8–2.3 kb) of *CR20* were low in cotyledons, hypocotyls, apical buds and young leaves. The total level of the *CR20* transcript was relatively high in roots, and the highest in mature and senescent leaves. The *CR20* transcripts of less than 1.4 kb were relatively abundant in cotyledons and hypocotyls, while those of more than 1.4 kb accumulated in mature and senescent leaves, although they did not yield completely identical patterns in two independent experiments.

Two CR20 cDNAs with different structures. We isolated five other CR20 cDNAs by screening a cDNA library from excised cucumber cotyledons using the CR20 cDNA (1.0 kb), which had been first isolated by differential screening, as a probe. Two of them were 1.8 kb, one was 1.0 kb, and the remaining two were 0.3 kb long. We sequenced the first cDNA, designated CR20 (1.0 kb) and the other long cDNA, designated CR20L (1.8 kb). Figure 4 shows the restriction maps of both cDNAs. The sequence of CR20L is identical to that of CR20 except that CR20L contains a long inserted sequence (0.7 kb) and is a little longer at each end. This shows that there are at least two types of *CR20* transcript that differ in structure.

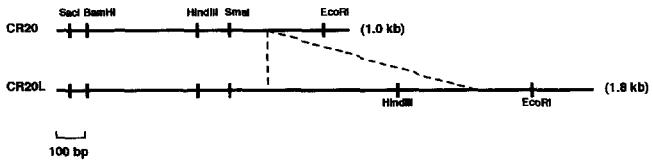

Fig. 4. Restriction maps of two different CR20 cDNAs. Two independent CR20 cDNAs, CR20 (1.0 kb) and CR20L (1.8 kb), were sequenced. The sequence of CR20L is identical to that of CR20 except that CR20L contains an insert of 0.7 kb and is a little longer at each end

They are either the results of differential processing of the same pretranscript, or they are transcribed from different *CR20* genes. Amino-acid sequences were deduced from the nucleotide sequences of both cDNAs, but they involved many termination codons and no long open reading frame. The nucleotide sequences showed no significant homology to any sequences in the GenBank and EMBL data bases.

Southern blot analysis of genomic DNA with CR20 cDNA and CR9 cDNA. Genomic DNA was prepared from etiolated seedlings of cucumber. The DNA was digested with six restriction enzymes (*Eco*RI, *Hind*III, *Bam*HI, *Apa*I, *Kpn*I and *Xho*I), and each digest was analyzed by Southern hybridization. An *Eco*RI fragment of CR20 cDNA was used as the probe (Fig. 5A). Thus, the probe contained *Hind*III and *Bam*HI sites, but none of the other four sites. Several bands hybridized with CR20 cDNA among fragments generated by *Eco*RI, *Hind*III and *Bam*HI, but there were two bands in the case of digestion with *Apa*I, *Kpn*I and *Xho*I. This result suggests that the *CR20* gene does not construct a large and complex gene family.

Fragments of genomic DNA generated with six restriction enzymes were hybridized with CR9 cDNA (Fig. 5B). One or two strong bands and a few weak bands were detected in each case. This result suggests that the coding region of *CR9* mRNA is separated by a few introns and/or that there are two or more *CR9* genes.

Expression of the CR9 gene in various organs of cucumber. Figure 6 shows levels of the *CR9* mRNA in various organs, as determined by Northern blot analysis. Samples of RNA were the same as described above for hybridization with CR20 cDNA (Fig. 3). In 7-d-old seedlings, level of the *CR9* transcript was much higher in cotyledons than in hypocotyls (very low) and roots (undetectable). In four-month-old plants, level of the transcript was high in mature and senescent leaves, but very low in young leaves. The transcript was nearly undetectable in apical buds.

Effects of excision on levels of the CR9 and CR20 transcripts in cotyledons. In experiments to examine changes in gene expression induced by BA, excised cotyledons were incubated with water for 18 h before application of BA. This pretreatment should eliminate the effects of wounding caused by excision. The effects of excision on levels of the *CR9* (Fig. 7A) and *CR20* transcripts (Fig. 7B) were examined by Northern blot analysis. Cotyledons were excised from etiolated seedlings, incubated with water for

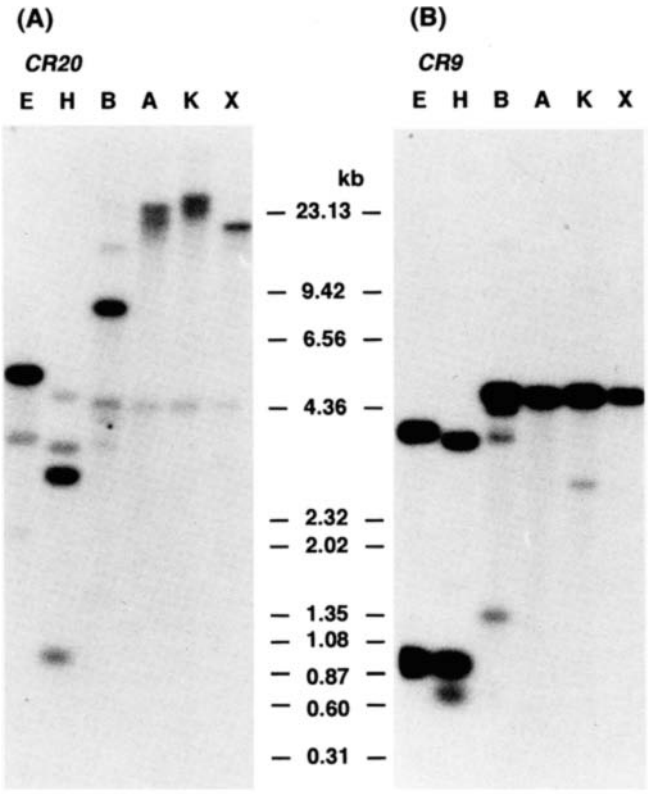

Fig. 5A, B. Southern blot analysis of genomic DNA probed with CR20 (**A**) and CR9 (**B**) cDNAs. Genomic DNA was prepared from cucumber seedlings. The DNA was digested with six restriction enzymes (*E*: *Eco*RI, *H*: *Hind*III, *B*: *Bam*HI, *A*: *Apa*I, *K*: *Kpn*I, *X*: *Xho*I), and 2 μg of DNA per digest were electrophoresed on an agarose gel, and analyzed by Southern hybridization with CR20 (**A**) and CR9 (**B**) cDNAs as probes. The positions of molecular markers are indicated between the two panels

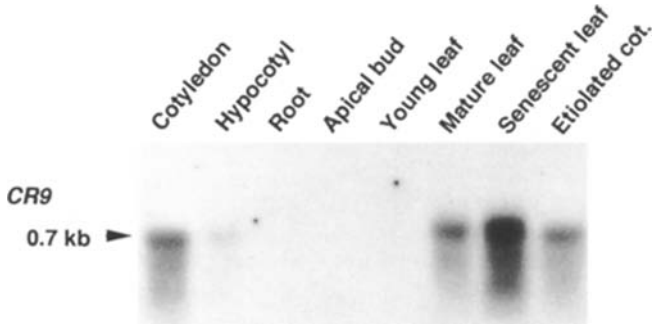

Fig. 6. Expression of the *CR9* gene in various organs of cucumber. The RNA samples were the same as those described in the legend to Fig. 3, and Northern hybridization was carried out using CR9 cDNA as a probe as described in the legend to Fig. 1

up to 18 h in darkness, and then they were cut into two pieces that were incubated in darkness for up to 23 h. The level of the *CR9* transcript decreased 2 h after the first excision, and then it increased up to 18 h. It decreased a little 4 h after the second cutting, and increased again from 8 to 23 h. The total level of the *CR20* transcript was relatively low in etiolated cotyledons. It decreased 1 h

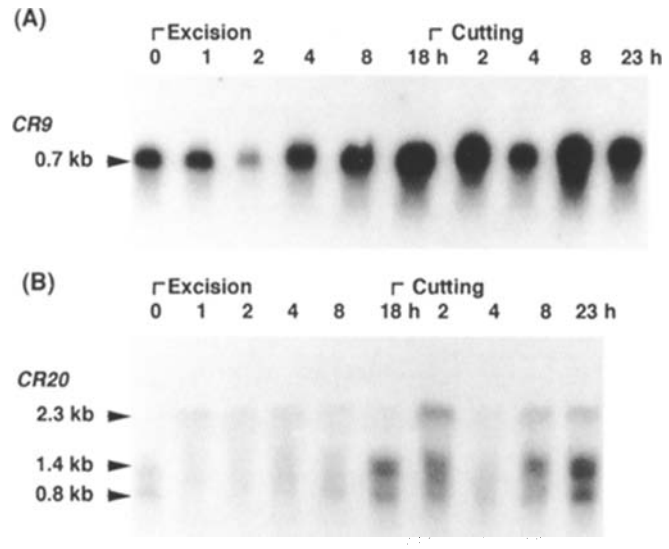

Fig. 7A, B. Effects of excision on levels of the *CR9* (**A**) and *CR20* (**B**) transcripts in cotyledons. Cotyledons were excised from etiolated seedlings and incubated with water for 18 h in darkness. After that, cotyledons were cut into two pieces, and incubated for a further 23 h in darkness. Samples were harvested at the indicated times, and analyzed by Northern hybridization with CR9 (**A**) and CR20 (**B**) cDNAs as probes as described in the legend to Fig. 1

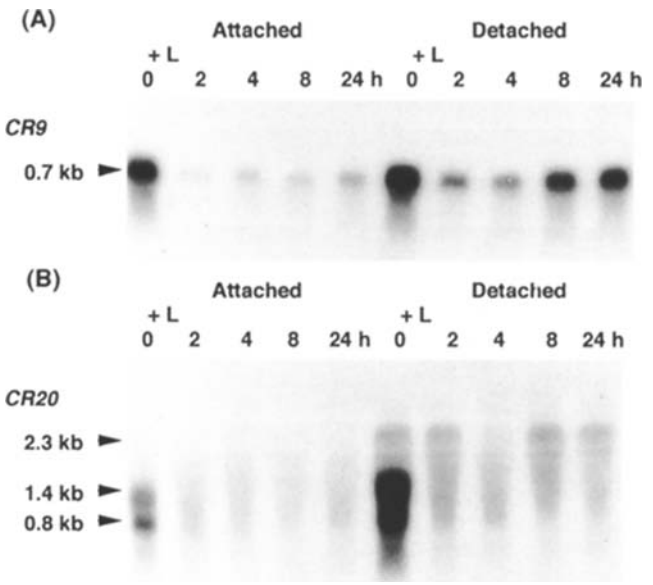

Fig. 8A, B. Changes in levels of the *CR9* (**A**) and *CR20* (**B**) transcripts during greening of cotyledons. Seedlings were germinated for 5 d in darkness, and then transferred to light (*Attached*). Cotyledons were excised from 5-d-old etiolated seedlings, incubated with water for 18 h in darkness, and then transferred to light (*Detached*). Total RNA was prepared from cotyledons that had been illuminated for 0, 2, 4, 8 and 24 h, and analyzed by Northern hybridization with CR9 (**A**) and CR20 (**B**) cDNAs as probes as described in the legend to Fig. 1

after excision, and it increased again. The level at 18 h was even higher than the initial level. The total level of the *CR20* transcript decreased again 4 h after the second cutting, and then it increased from 8 to 23 h. The patterns of changes in levels of these transcripts by wounding differed slightly between two independent experiments, but the temporary repression by wounding was quite reproducible.

Changes in levels of the CR9 and CR20 transcripts during greening. Figure 8 shows changes in levels of the *CR9* and *CR20* transcripts during greening of cucumber cotyledons. Two types of experiment were performed. Seedlings were germinated for 5 d in darkness, and then transferred to light (Fig. 8, Attached). Alternatively, cotyledons were excised from the etiolated seedlings, incubated with water for 18 h in darkness, and then transferred to light (Fig. 8, Detached). The level of the *CR9* transcript decreased 2 h after the onset of illumination in both systems. Then it increased from 4 to 24 h. The level decreased more markedly in attached cotyledons than in detached ones. The total level of the *CR20* transcript also decreased 2 h after the onset of illumination. However, the *CR20* transcript remained at a low level for up to 24 h in the light.

Diurnal changes in levels of the CR9 and CR20 transcripts. In order to investigate the diurnal changes in the expression of the two *CR* genes, cucumber seedlings were grown in a growth chamber under a 15 L/9 D cycle for 8 d. Changes in levels of the transcripts during a 24-h period on day 8 were examined by Northern blot analysis (Fig. 9). The level of the *CR9* transcript increased in the light phase, reaching a maximum 12 h after the onset of

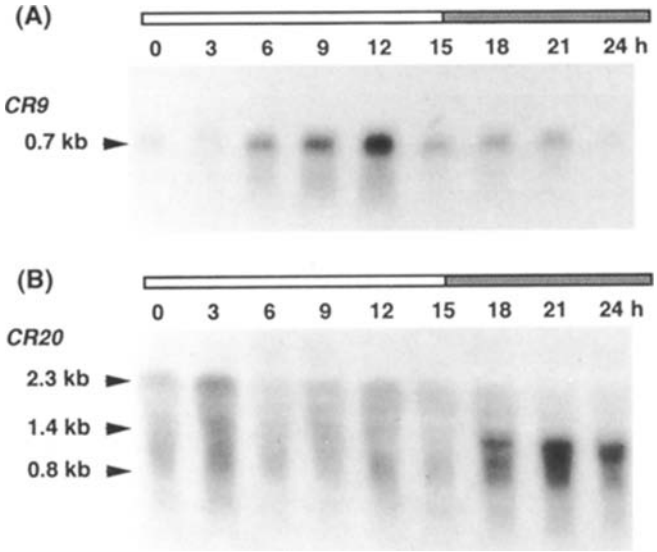

Fig. 9A, B. Diurnal changes in the levels of *CR9* (**A**) and *CR20* (**B**) transcripts in cotyledons. Seedlings were grown in a growth chamber under a 15 L/9 D cycle for 8 d. On the eighth day, cotyledons were harvested 0, 3, 6, 9, 12, 15, 18, 21 and 24 h after the light had been turned on (at 15 h, the light was turned off). Total RNA was prepared from each sample, and analyzed by Northern hybridization with CR9 (**A**) and CR20 (**B**) cDNAs as probes as described in the legend to Fig. 1. □, Light; ■, darkness

illumination. Then it decreased within the next 3 h, and remained at a low level in the dark phase. By contrast, the total level of the *CR20* transcript was low in the

light phase, and high in the dark phase. The level changed rapidly after the light was turned on and turned off.

Discussion

A second cytokinin-repressed gene CR20. In a previous paper, we reported a cytokinin-repressed gene, *CR9* (Teramoto et al. 1994). In the present paper, another such gene, *CR20*, is described. In excised cotyledons of cucumber, the level of the *CR20* transcript decreased drastically from 2 to 4 h after the application of BA in darkness (Fig. 1). This repression was BA-dose dependent, and highly specific for cytokinins (Fig. 2) as was the case for repression of *CR9* reported previously (Teramoto et al. 1994). At present, it is not clear whether the rapid decrease in the two *CR* transcripts is due to repression of transcription or degradation of their RNAs.

Diversity of CR20 transcripts. Northern blot analysis revealed various transcripts (from 0.8 to 2.3 kb in length) of *CR20* in cucumber. There would be little contribution of degradation products generated during preparation of RNA to the observed diversity of RNA bands, as indicated by the fact that (i) degradation of rRNAs was not detected, and (ii) the patterns of bands on Northern blots differed among different organs, while those of rRNAs were identical (Fig. 3). This diversity of *CR20* transcripts could be due to (i) different members of a gene family, (ii) alternative splicing, and/or (iii) degradation of the RNAs in the intact plants. Genomic Southern blot analysis (Fig. 5A) suggested that at least two *CR20* genes exist in the cucumber genome, but the number of the genes is clearly much smaller than that of the transcripts. We sequenced two independent *CR20* cDNAs of 1.0 kb and 1.8 kb in length, respectively. The sequence of the 1.8 kb cDNA contains a long insert within the same sequence as that of the 1.0 kb cDNA (Fig. 4). This result suggests that the 1.8 kb cDNA and 1.0 kb cDNA correspond to the longer and shorter transcripts, respectively. The latter would be derived from the gene for the longer transcript by alternative splicing, or from another gene, that does not have the inserted sequence. Because there were a number of bands of *CR20* transcripts on Northern blots, the hypothesis that a gene family exists and/or alternative splicing occurs fails adequately to explain the diversity. Therefore, some RNAs might be degradation products of a longer *CR20* RNA that accumulate in the plant. More information about the diverse *CR20* transcripts is needed. Analysis of other CR20 cDNAs and genomic clones is now in progress.

Changes in levels of CR9 and CR20 transcripts in relation to aging, greening and wounding. Cytokinin is thought to play an important role for growth and senescense of leaves. In various plant species, exogenously applied cytokinins delay senescence of leaves (for review, see Hall 1973). It has also been known for a number of plants that levels of endogenous cytokinins are high in young leaves and decrease with the progress of aging (Hewett and Wareing 1973; Henson and Wareing 1976; Singh et al. 1992; Ulvskov et al. 1992). Both *CR9* and *CR20* transcripts accumulated in mature and senescent leaves, while their levels were quite low in young leaves and apical buds, the *CR9* transcript being almost undetectable in apical buds (Figs. 3, 6). The levels of the *CR* transcripts in leaves seem to be correlated with the activity of endogenous cytokinins.

Cotyledons of cucumber have been used for studies of roles of cytokinins in greening. When cotyledons excised from etiolated cucumber seedlings are exposed to light, chlorophyll accumulation starts after a lag period of 1–2 h. Preincubation with a cytokinin in darkness eliminates the lag period (Fletcher and McCullagh 1971; Fletcher et al. 1973). Uheda and Kuraishi (1977) observed the same effect of cytokinins on greening of squash cotyledons, and showed that cytokinin activity in excised squash cotyledons increased rapidly 1–2 h after the onset of illumination, and then decreased rapidly for another hour. This temporary increase in cytokinin activity occurred during the lag period that precedes the accumulation of chlorophyll, suggesting that cytokinin is involved in the early phase of chlorophyll formation. When cotyledons excised from, or attached to, etiolated cucumber seedlings were exposed to light, the levels of the *CR9* and *CR20* transcripts decreased rapidly within 2 h after the onset of illumination (Fig. 8). Therefore, the decrease in the two *CR* transcripts seems to correlate with an increase in activity of endogenous cytokinins induced by illumination.

When cotyledons excised from etiolated seedlings were incubated with water in darkness, levels of the *CR9* and *CR20* transcripts decreased temporarily in the early phase of the incubation (Fig. 7). Cutting of cotyledons after the incubation with water again caused decreases in levels of the *CR9* and *CR20* transcripts. Such decreases seem to be effects of wounding. In this system, levels of endogenous cytokinins have not been measured, but Crane and Ross (1986) observed that wounding and cytokinin treatment cause the same phenomena, and suggested that wounding enhances the activity of endogenous cytokinins. Mitchell and van Staden (1983) showed a positive correlation between wounding and endogenous levels of cytokinins in potato tubers.

The expression of two cytokinin-repressed genes, *CR9* and *CR20*, changed similarly in relation to several phenomena in cucumber, such as growth and senescence of leaves, the early phases of greening and wounding, during which the levels of endogenous cytokinins seem to change, although we did not measure the cytokinin levels. Thus, it is possible that these two genes are regulated by endogenous cytokinins and the gene products may be involved in the early steps of signal-transduction pathway of cytokinins, which affect various aspects of physiological events. However, it cannot be ruled out that the *CR* genes directly respond to other factors, such as light and wounding.

Differential expression of CR9 and CR20 under various conditions. Although *CR9* and *CR20* were repressed by cytokinins, there were some differences between their expressions under various conditions. (i) Diurnal changes in the levels of the *CR9* and *CR20* transcripts were the opposite of one another: the *CR9* transcript accumulated in the light phase, while those of *CR20* did so in the dark

phase (Fig. 9). (ii) A relatively high level of the *CR20* transcript was found in roots, while the *CR9* transcript was not detectable there (Figs. 3, 6). (iii) Levels of the *CR9* transcript decreased temporarily during treatment with cytokinins or greening, while the *CR20* transcript remained at a low level after an initial decrease (Figs. 1, 8). (iv) The expression of *CR9* indicated higher organ-specificity, while the *CR20* transcript was detected at some level in all organs tested (Figs. 3, 6). These results reveal that the two genes have different properties in terms of their regulation and, perhaps their function. Other factors, in addition to cytokinins, differentially affect the expression of these two genes.

Circadian control of the expression of CR9. The predicted polypeptide sequence of *CR9* exhibits homology to that of *lir1*, a light-induced gene in rice reported by Reimmann and Dudler (1993). They showed the diurnal changes in level of the *lir1* mRNA in rice seedlings: it increased in the light and decreased in darkness. They also showed that the oscillation of the abundance of its transcript persisted after plants are transferred to continuous light or darkness, suggesting that the expression of *lir1* is controlled by a circadian clock. The level of the *CR9* transcript in cucumber cotyledons also showed diurnal fluctuations when the seedlings were grown under a light/dark cycle (Fig. 9). However, the *CR9* transcript accumulated in etiolated cucumber cotyledons, while the *lir1* transcript was not found in dark-grown rice seedlings. This discrepancy may be due to the difference in organs: cotyledons are storage organs and seedlings consist of growing organs. If the expression of *CR9* is controlled by a circadian clock, the decrease in level of the *CR9* transcript caused by BA or light may be the result of resetting of the clock, and the increase after the rapid decrease during these treatments may represent a phase of circadian rhythmic changes by the clock. It has been shown that greening is under a circadian control in pea (Otto et al. 1992) and barley seedlings (Beator et al. 1992; Beator and Kloppstech 1993), and the clock can be reset by heat-shock treatments.

Further information about the functions and the regulation of expression of the cytokinin-repressed genes is required for the understanding of their involvement in the molecular mechanism leading to the various physiological effects of cytokinins.

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