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Cloning cDNAs for Genes Preferentially Expressed during Fruit Growth in Cucumber

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ABSTRACT. **A cDNA library was constructed from poly(A)$^+$RNA extracted from pollinated fruit of 'PMR-142' cucumber (*Cucumis sativus* L.). Subtraction hybridization was made between the cDNAs and poly(A)$^+$RNA from unpollinated fruit to isolate cDNA clones that corresponded to the genes preferentially expressed in the pollinated fruit. We isolated three cDNAs, which were 756, 826, and 998 nucleotides long and designated Csf1, Csf2, and Csf3, respectively. When fruit growth was triggered by pollination, auxin treatment and natural parthenocarpy, Csf2 was always expressed. Time course of expression of the Csf2 gene was nearly parallel to that of the fruit growth. Nucleotide sequences of the Csf cDNAs were fully determined. Homology of the deduced amino acid sequence for Csf1 showed 75% identity with a pea extensin. Only 37%, 33%, and 26% homology was found between Csf2 and bell pepper CaSn-2, tobacco FB7-4, and opium poppy gMLP15, respectively. The Csf3 sequence showed 68% identity with the large subunit of 60S ribosomal protein L3 of *Arabidopsis thaliana*.**

Physiological studies have shown that fruit growth is regulated by plant hormones and sugar metabolism (Hedden and Hoad, 1985). Genes whose expression is positively correlated with fruit growth have been reported from several species including tomato (*Lycopersicon esculentum* Mill.) (Pear et al., 1989; Salts et al., 1991; Tieman and Handa, 1996), peach (*Prunus persica* Batsch.) (Callahan et al., 1993), kiwifruit [*Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa*] (Ledger and Gardner, 1994), and apple (*Malus pumila* Miller var. *domestica* Schneid.) (Dong et al., 1997). However, roles of these genes in the regulation of fruit growth are still unknown.

In addition to triggering fruit growth, pollination induces ethylene synthesis and many other biochemical responses (Wang et al., 1996). Therefore, some of the genes expressed after pollination may not be directly related to fruit growth. Our strategy to isolate the specific genes regulating fruit growth was to identify genes which are activated in fruit whose growth was triggered by each of several different factors. Unpollinated cucumber flowers are responsive to auxin for induction of parthenocarpic fruit growth (Takeno et al., 1992). The cultivar ('PMR-142') used in the present study, also has the potential to set fruit parthenocarpically from flowers formed at higher nodes (Takeno and Ise, 1992). Therefore, fruit growth in this cucumber genotype can be induced by three different factors, pollination, auxin treatment and natural parthenocarpic potential. In the present work, we used subtractive hybridization to clone cDNAs expressed during fruit growth of cucumber.

Materials and Methods

PLANT MATERIALS. 'PMR-142' cucumber was used for all experiments. The seeds were planted in unglazed pots (21 cm in diameter) filled with soil, and the plants were grown in a glasshouse under natural conditions from May to July. The female flowers formed on the 14th and lower nodes and those on the 26th and higher nodes were used. The former has a low potential to set parthenocarpic fruit and the latter has a high potential to do so (Takeno and Ise, 1992).

To induce male flowers in this gynoecious cultivar, a 50% acetone solution containing 2.9×10^{-4} M gibberellin A_3 and an aqueous solution of 1.6×10^{-3} M aminoethoxyvinyl glycine were sprayed on leaves once a week. Female flowers were covered with paper bags the day before anthesis, and were either artificially pollinated the next morning or left unpollinated. Ten microliters of a 50% acetone solution containing 5.4×10^{-3} M naphthaleneacetic acid (NAA) (Sigma Chemical Co., St. Louis) was applied once to ovaries of some unpollinated flowers on the day of anthesis.

Fruit were harvested at 0, 1, 2, 3, 5, or 7 d after anthesis depending on the experiment. After measuring length and fresh weight, the fruit were frozen in liquid nitrogen. The fruit length and fresh weight were shown as means of 5 to 37 fruit with SE. Roots, stem tips including young leaf primordia, mature leaves at nodes 6 to 10, and stem segments between nodes 6 and 10 were also harvested.

ISOLATION OF POLY(A)$^+$RNA. To extract total RNA, plant material (10 g) was homogenized in 50 mL water-saturated phenol and 50 mL 0.2 M Tris-HCl buffer (pH 9.0) containing 0.2 M LiCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecylsulfate (SDS) and 1% 2-mercaptoethanol. The homogenate was centrifuged at 5,000 g_n for 5 min to collect the aqueous phase. The aqueous phase was added with an equal volume of chloroform, and the mixture was shaken vigorously and then centrifuged at 5,000 g_n for 5 min. The aqueous phase (≈55 mL) was combined with NaCl and cetyltrimethylammonium bromide (CTAB) to a final concentration of 0.7 M and 1.0%, respectively and an equal volume of chloroform. The mixture was shaken vigorously, then centrifuged at 5,000 g_n for 5 min. An equal volume of 2-propanol and 1/300-volume of acetic acid were added to the aqueous phase, the mixture was incubated at room temperature for 1 h, and the precipitate was collected at 10,000 g_n for 10 min. The precipitate was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA (TE) and added with LiCl to a final concentration of 2 M. The mixture was placed on ice for 1 h, then centrifuged at 10,000 g_n for 5 min. The precipitate was washed with ethanol and dissolved in TE to be used as total RNA. Poly(A)$^+$RNA was isolated from the total RNA by oligo(dT)-cellulose, Type 7 (Pharmacia Biotech, Uppsala, Sweden) chromatography.

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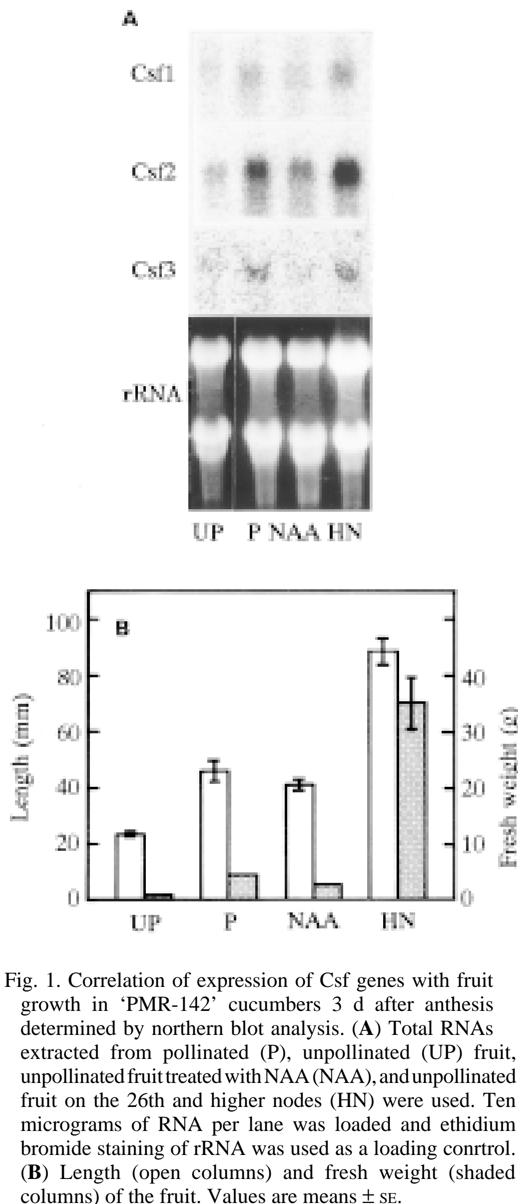

Fig. 1. Correlation of expression of Csf genes with fruit growth in 'PMR-142' cucumbers 3 d after anthesis determined by northern blot analysis. (**A**) Total RNAs extracted from pollinated (P), unpollinated (UP) fruit, unpollinated fruit treated with NAA (NAA), and unpollinated fruit on the 26th and higher nodes (HN) were used. Ten micrograms of RNA per lane was loaded and ethidium bromide staining of rRNA was used as a loading conrtrol. (**B**) Length (open columns) and fresh weight (shaded columns) of the fruit. Values are means ± SE.

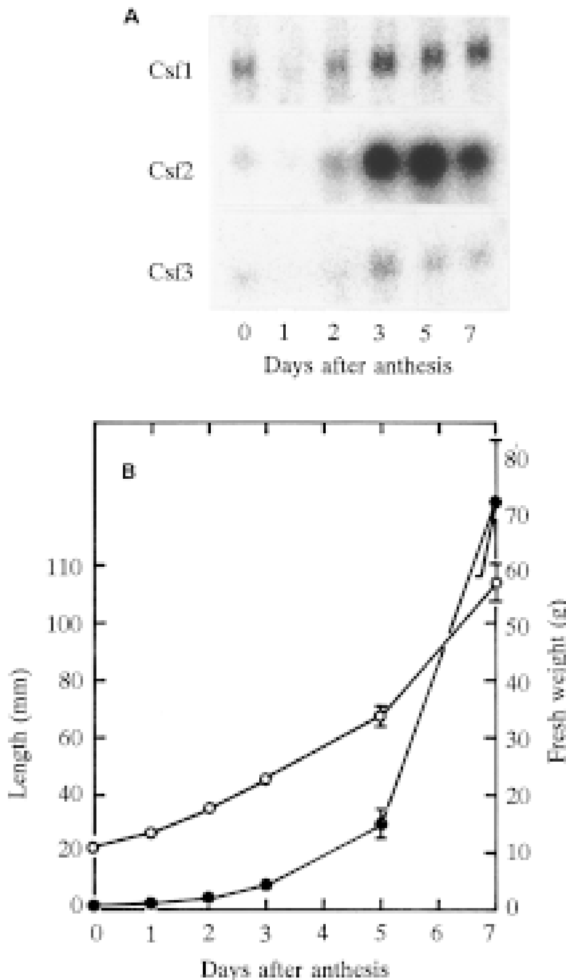

Fig. 2. Time course of fruit growth and expression of Csf genes in 'PMR-142' cucumbers. (**A**) Changes in expression of genes corresponding to Csf probes at different days after anthesis. (**B**) Changes in length (open circles) and fresh weight (closed circles) of pollinated fruit after anthesis. Values are means ± SE. Some SE

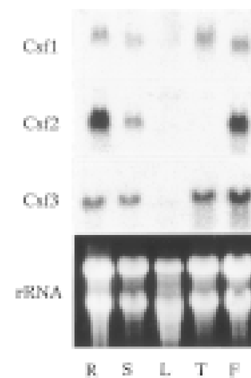

Fig. 3. Expression of Csf genes in roots (R), stem between the 6th node and 10th node (S), mature leaves attached to the 6th to 10th nodes (L), stem tip including young leaf primordia (T), and 3-d-old pollinated fruit (F) of 'PMR-142' cucumber.

SUBTRACTION HYBRIDIZATION. The cDNA Synthesis Kit (Amersham Japan, Tokyo) was used to synthesize cDNA from poly(A)$^+$RNA (5 μg obtained from pollinated fruit 3 d after anthesis. Poly(A)$^+$RNA (10 μg from unpollinated fruit 3 d after anthesis was hybridized with the first-strand cDNAs and the common messages were cross-linked by the method of Hampson et al. (1992). The single-stranded subtraction product cDNAs were labeled with [^{32}P]deoxycytidine 5'-triphosphate (dCTP) using Multiprime DNA Labeling System (Amersham Japan).

CONSTRUCTION OF A CDNA LIBRARY AND COLONY HYBRIDIZATION. A cDNA library was constructed with poly(A)+RNA isolated from 3-d-old pollinated fruit in the vector pBluescript SK(-) (Strategene, La Jolla, Calif.) according to the method of Mori et al. (1991). About 3,000 colonies of transformed *Escherichia coli* DH5 α were blotted onto a nitrocellulose filter, and probed with ^{32}P-labeled subtraction product cDNAs. Hybridization was carried out in a mixture of 0.9 M NaCl, 60 mM NaH_2PO_4 and 7.5 mM EDTA, pH 7.4 (6× SSPE), 50% formamide, 5% Irish Cream (R. and A. Bailey & Co., Dublin, Ireland), 0.5% SDS and 100 μg·mL^{-1} denatured salmon sperm DNA at 42 °C for 14 h. The filter was washed twice with 2× SSPE containing 0.1% SDS at 65 °C for 30 min each. The hybridizing colonies were visualized by Imaging Plate BAS III (Fuji Film, Tokyo).

NORTHERN BLOT ANALYSIS. Total RNA (10 μg was subjected to electrophoresis on 1.0% agarose gels in the presence of 0.66 M formaldehyde, and transferred by blotting to a Hybond-N$^+$ membrane (Amersham Japan). To demonstrate equal loading of samples, the agarose gels were stained with ethidium bromide to detect rRNA. The membrane was probed with [^{32}P]-labeled cDNAs. Hybridization was carried out at 42 °C overnight in the same manner as for the colony hybridization.

SEQUENCE OF CDNA. The plasmids containing cDNA inserts were digested with *Hinc*II and *Cla*I and subcloned into pBluescript SK(-). Nucleotide sequences were determined using a DNA-sequencer (LI-4000; LI-COR Inc., Lincoln, Neb.) and the TaKaRa Cycle-sequencing Kit with IRD_{41}T3primer (LI-COR). Homology of each cDNA was determined by searching the GenomeNet Kyoto-Center NR-AA database.

Results

CLONING AND EXPRESSION OF THREE FRUIT-DERIVED CDNAS. Colony hybridization of the cDNA library for pollinated fruit with the subtraction product cDNAs provided 20 positive clones. These cDNAs are hereafter called Csf (*Cucumis sativus* fruit) cDNAs. When the Csf cDNAs were used to probe total RNA from unpollinated and pollinated fruit, three of the Csf clones detected more abundant mRNAs in the pollinated fruit than in the unpollinated ones (Fig. 1A, UP and P). These clones were designated Csf1,

Fig. 4. Alignment of deduced amino acid sequence of Csf1 from 'PMR-142' cucumbers with a homologous sequence, pea extensin. The amino acids are given in single letter code. Identical amino acids in Csf1 and pea extensin are boxed.

Csf2, and Csf3, and the estimated length of the corresponding mRNAs was 800, 800, and 1,400 nucleotides, respectively, based on northern blot analyses with two major ribosomal RNAs as molecular markers.

Ovaries of flowers formed below the 15th node averaged 22.7 ± 0.32 mm long and 0.68 ± 0.04 g fresh weight on the day of anthesis. Pollinated flowers and parthenocarpic unpollinated or NAA-treated flowers showed substantial growth 3 d after anthesis (Fig. 1B). Parthenocarpic fruit on the higher nodes were larger than others because the ovaries at higher nodes were larger at anthesis (Takeno and Ise, 1992).

The expression of three Csf genes in the naturally parthenocarpic fruit was as high or higher than that in the pollinated fruit (Fig. 1A). The expression of Csf2 in the NAA-treated fruit was almost the same as that in the pollinated fruit, whereas Csf1 and Csf3 were expressed at barely detectable levels in the NAA-treated fruit compared with the pollinated fruit.

Over a 7-d time period, the level of mRNA for Csf2 increased from d 2 after pollination (Fig. 2A). An appreciable level of mRNA for Csf1 was detected even on the day of anthesis, and the highest level of mRNA for Csf3 was detected on day 3 after anthesis. During the first 7 d after anthesis, length and fresh weight of pollinated fruit increased (Fig. 2B).

Expression for all three Csf genes was not restricted to fruit but was undetectable in leaves (Fig. 3). Expression of Csf2 was not detected in stem tips, but was as high in roots as in fruit, and somewhat less in stem. Expression of Csf3 was roughly equivalent in roots, stems and stem tips, but it was lower than in fruit.

Nucleotide sequences of Csf cDNAs. The Csf1 cDNA consisted of 756 bp and contained an open reading frame of 399 bp. The deduced amino acid sequence for Csf1 showed 75% identity with an extensin isolated from pea (*Pisum sativum* L.) (Fig. 4).

The Csf2 cDNA consisted of 826 bp and contained an open reading frame of 453 bp. Sequence homology was found with Sn-2 of bell pepper (*Capsicum annuum* L. var. *annuum*), FB7-4 of tobacco (*Nicotiana tabacum* L.), and gMLP15 of opium poppy (*Papaver somniferum* L.) at 37%, 33%, and 26%, respectively (Fig. 5).

No initiation codon was found in Csf3 cDNA, and it consisted of 998 bp although the length of Csf3 mRNA in the northern blot analysis was ≈1,400 nucleotides. The deduced amino acid sequence for Csf3 showed 68% identity with about two-thirds of the C-terminal amino acid sequence for a large subunit of 60S ribosomal protein L3 isolated from *Arabidopsis thaliana* (L.) Heynh. (Fig. 6).

Discussion

We cloned three cDNAs for genes whose expression was induced in fruit of cucumber when growth was triggered by pollination. Among these cDNAs, Csf2 was always expressed when fruit growth was triggered not only by pollination but also by auxin treatment or natural parthenocarpy. The time course of expression of the Csf genes was nearly parallel to that of fruit growth. However, the expression of Csf1 was detected even at anthesis, and the most abundant expression of Csf3 was on day 3. These results suggest that the role of Csf2 in the fruit growth is more important than that of Csf1 and Csf3.

Since the three Csf genes were present in fruit of other two cucumber cultivars when growth was induced (data not shown), the Csf genes may be common in this species.

Csf1 and Csf3 were expressed in actively growing tissues and may represent extensin and a fragment of a gene encoding a ribosomal protein, respectively. The genes for extensin and ribo-

Fig. 5. Alignment of the deduced amino acid sequence of Csf2 from 'PMR-142' cucumbers with other homologous sequences, bell pepper CaSn-2, tobacco FB7-4, and opium poppy gMLP15. The amino acids are given in single letter code. Identical amino acids in Csf2 and others are boxed.

Fig. 6. Alignment of the deduced amino acid sequence of Csf3 from ‘PMR-142’ cucumbers with a homologous sequence, *Arabidopsis thaliana* 60S ribosomal protein (60SRPL3). The amino acids are given in single letter code. Identical amino acids in Csf2 and 60SRPL3 are boxed.

somal protein are expressed in roots and stems of other species, but not in their mature leaves (Marty and Meyer, 1992; Showalter et al., 1992; Stafstrom and Sussex, 1992). The accumulation of mRNAs for extensins and ribosomal proteins is correlated with the growth process. It is possible, therefore, that Csf1 and Csf3 play some role in the regulation of fruit growth in cucumber.

The homology between Csf2 and Sn-2, FB7-4 and gMLP15 is not high, and therefore, Csf2 may encode an unreported protein. The Sn-2 cDNA was cloned from mature fruit of bell pepper (Pozueta-Romero et al., 1995). The FB7-4 gene was expressed during flower formation in tobacco (Neale et al., 1990). However, functions of these genes are unknown. It was reported that gMLP15 was a major latex protein of opium poppy (Nessler et al., 1990). Therefore, the function of Csf2 cannot be predicted.

We have previously reported that fruit growth of eggplant (*Solanum melongena* L.) and melon (*Cucumis melo* L.) is initiated by accumulation of sugars in ovary cells through activation of sugar-metabolizing enzymes (Lee et al., 1997b, 1997c), and these enzymes are activated by auxin (Lee et al., 1997a). Although genes encoding enzyme proteins relating to sugar metabolism may be expressed during fruit growth, the Csf genes isolated from cucumber did not show homology with known genes that relate to sugar metabolism. Characterization of the Csf genes, especially Csf2, may provide new insight into the study of fruit growth regulation.

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