Journal of Plant Biology, December 2001, 44(4) : 199-204

A Translation Elongation Factor 1A (*CaEF1A*) Gene from Hot Pepper (*Capsicum annuum* L.) is Induced by the Tobacco Mosaic Virus and by Wounding

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We have identified a cDNA clone (*CaEF1A*) sharing significant homology with the protein synthesis elongation factor 1As (eEF1A) found in eukaryotes. This clone was isolated from a tobacco mosaic virus (TMV)-inoculated hot pepper (*Capsicum annuum* L.) cDNA library by differential screening. Based on the results of our Southern blot analysis using the *CaEF1A* full-length cDNA clone as a probe, we suggest that *CaEF1A* exists as part of a multigene family in the hot pepper genome. In another experiment, the mRNA of the *CaEF1A* gene was found to accumulate in ripe fruits, flowers, young and old leaves, and roots, but was not detected in immature fruits or stems. The transcript of *CaEF1A* accumulation was induced within 12 h of local wounding treatment, as well as by both avirulent and virulent TMV inoculations.

Keywords: *Capsicum annuum* L., Hot pepper, Tobacco mosaic virus, Translation elongation factor 1A

Translation elongation factor 1A (eEF1A) is an abundant and, presumably, a ubiquitously expressed protein. It binds and leads aminoacyl tRNAs to the acceptor site of the ribosome during the peptide chain elongation phase of protein synthesis. However, eEF1A appears to be involved in several cellular processes. Although it behaves as a multifunctional protein, the biological significance of those processes is not fully understood.

Several genomic or cDNA clones encoding eEF1A have been identified and characterized in a variety of eukaryotes and archaea. Among them, some species have more than two genes encoding it. For example, in *Saccharomyces cerevisiae*, genomic clones encoding the eEF1A gene were classified into two groups via Southern blot analysis (Nagata et al., 1984). Likewise, in *Xenopus laevis*, three eEF1A genes have been isolated and shown to be differentially expressed during development (Die et al., 1990). In higher eukaryotes, eEF1A typically is encoded by a multigene family, including 18 human genes (Lund et al., 1996), and 4 genes from *Arabidopsis* (Axelos et al., 1989). Several studies have provided evidence for tissue-specific expression of the gene family members (Die et al., 1990; Aguilar et al., 1991; Lee et al., 1993; Lund et al., 1996), but the importance of this regulation has not been widely investigated.

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The nucleotide sequence of *CaEF1A* reported in this paper has been submitted to the EMBL, GenBank, and DDBJ under accession number AF242732.

Recently, some interesting data have been reported for the expression of plant eEF1A genes. In potato tubers, accumulation of eEF1A transcript was induced by wounding (Morelli et al., 1994) and by low oxygen levels (Vayda et al., 1995). A barley eEF1A gene was induced by low-temperature stress (Dunn et al., 1993), while a maize eEF1A was also induced by cold treatment (Berberich et al., 1995). Furthermore, RNA levels for the eEF1A-gus chimeric genes were induced by 2,4-D treatment in transgenic tobacco plants (Axelos et al., 1989). eEF1A may also be involved in the control of cell proliferation (Roth et al., 1987; Rao and Slobin, 1988) and aging (Shepherd et al., 1989).

In this paper, we describe the isolation of a hot pepper cDNA clone, *CaEF1A*, that encodes EF1A. We also investigated whether this gene could be induced by wounding treatment or TMV inoculation.

MATERIALS AND METHODS

Plant Material and Virus Inoculation

Seeds of *Capsicum annuum* L cv. VK-1 and its F1-hybrid, Bugang, were used for germination. For viral inoculum preparation, we used a pre-chilled pestle and a mortar to grind the leaves of *Nicotiana tabacum*

Abbreviations: eEF1A, eukaryotic translation elongation factor-1A; HR, hypersensitive response; ORF, open reading frame; PR, pathogenesisrelated; TMV, tobacco mosaic virus.

L. cv. Burley, propagated with TMV-P_0, and the leaves of *C. annuum* L cv. Bugang, propagated with TMV-$P_{1.2}$, in a virus extraction buffer (50 mM phosphate buffer, pH 7.0). The crude extract was centrifuged at 8,000g for 10 min, and the supernatant was used as the viral inoculum. The fourth or fifth leaf of each greenhouse-grown plant was inoculated by rubbing on the viral inoculum with a 500-mesh carborundum (Hayashi Chemical, Japan). For the control (i.e., mock inoculation), leaves were rubbed with just the avirulent extraction buffer and the carborundum.

DNA and RNA Gel Blot Analyses

A cDNA library was constructed from poly(A)$^+$ RNA extracted from hypersensitive response (HR)-exhibiting pepper leaves 24 or 48 h after inoculation with TMV-P_0. The library was screened differentially with single-stranded cDNA probes synthesized from either the TMV-P_0-inoculated or the mock-inoculated poly(A)$^+$ RNA (Shin et al., 2001). Total RNAs were isolated from leaves according to the guanidine thiocyanate phenol chloroform extraction method (Hong et al., 1998). Genomic DNA was isolated from our hot pepper leaves (Shure et al., 1983). This DNA was digested with HindIII, XbaI (Takara, Japan), or EcoRI (Boehringer Mannheim, Germany), then fractionated on an agarose gel and transferred to nylon membranes (Schleicher and Schuell, Germany) in 10× SSC. The blotted membranes were hybridized with probes prepared by the random priming method in the presence of α-[^{32}P]-dCTP. For the RNA gel blot analysis, 20 μg of total RNA was separated on a 1.0% agarose gel containing 10% formaldehyde, then blotted to a nylon membrane. The RNA was vacuum-transferred with a Model 785 vacuum blotter (BioRad, USA) in the presence of 10× SSC. Labeled probes were prepared via PCR, in which the inserts of specific clones were amplified with T7 and T3 universal primers and specific primers of the 3'UTR region. Afterward, the unincorporated nucleotides were eliminated by using a Qiaquick Nucleotide Removal kit (Qiagen, USA). Membrane filters were then prehybridized at 65°C for 3 h in a solution of 0.25 M sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA, and 1% bovine albumin. The heat-denatured probe was mixed with fresh buffer, and hybridization was carried out at 65°C for at least 16 h. After hybridization, the filters were washed at room temperature in a solution of 2× SSC for 10 min, then in a solution of 0.2× SSC containing 0.2% SDS for 30 min. The washed filters were directly visualized with a BAS-2500 phosphorimage analyzer (Fuji Photo Film, Japan).

To examine the steady-state transcript level of *CaEF1A* in various plant organs, we also extracted total RNA from young and old leaves, flowers, stems, roots, green (immature) fruits, and red (ripe) fruits of our hot pepper plants. To investigate expression of *CaEF1A* following wounding stress, we isolated total RNA from *C. annuum* L. cv. Bugang after incising the leaves with scissors at various time points.

DNA Sequence Analysis

Plasmids were isolated according to the alkaline lysis method (Sambrook et al., 1989). Nucleotide sequencing was carried out by dye-terminator cycle sequencing performed with Thermo Sequenase (USB, USA). The reaction mixture was separated with an ABI Prism 377 DNA Sequencer (Perkin-Elmer Applied Biosystems, USA). In both cases, the chain termination reaction was initiated with T7 and T3 universal primers. We used the BLAST program of Altschul et al. (1990) to compare the deduced amino acid sequences with those from the database. Alignment of the predicted amino acid sequences was carried out from the BCM search launcher homepage (http://www.hgsc.bcm.tmc.edu/SearchLauncher/).

RESULTS AND DISCUSSION

Isolation and Sequence Analysis of *CaEF1A* cDNA

From our differential screening, we selected a cDNA clone encoding the hot pepper protein synthesis elongation factor 1A (eEF1A). Figure 1 shows the comparison of the entire deduced amino acid sequences from the various eEF1A. CaEF1A shared >90% amino acid identity with EF1A of other plants, and >70% amino acid identity with that of the nematode and *Xenopus*. This elongation factor is involved in mRNA translation. Therefore, the high level of conservation arises from the fact that EF-1A binds GTP and the aminoacyl-tRNA to bring about codon-dependent alignment of the aminoacyl-tRNA. Although CaEF1A and other elongation factor 1As had >70% amino acid homology within the coding region, the sequence homology was much lower in the non-coding regions (data not shown).

Genomic Organization and Tissue-Specific Expression Patterns of *CaEF1A*

Because eEF1A has a central role in protein synthe-

Figure 1. Sequence analysis of pepper *CaEF1A* cDNA clone. Comparison of the predicted amino acid sequence of *CaEF1A* with that of translational elongation factor 1As of tobacco (D63396), corn (D45407), nematode (M64333), and *Xenopus* (M25697). The black boxes indicate identical amino acids and the gray boxes indicate similar amino acids.

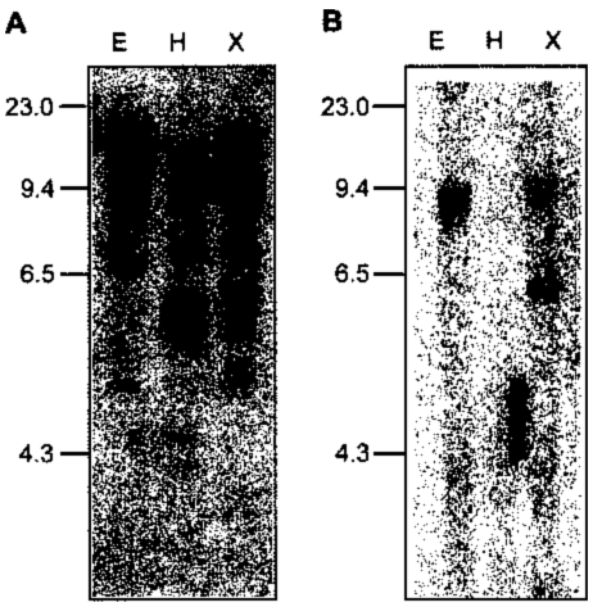

Figure 2. Genomic DNA gel blot analysis of the *CaEF1A* gene. Hot pepper genomic DNA (10 μg) was digested with EcoRI (E), HindIII (H), or XbaI (X), and separated in an agarose gel. After transfer to a nylon membrane, the blot was hybridized with α-[^{32}P]-labeled full-length *CaEF1A* cDNA probe (A) or 3'UTR region (B). The autoradiogram was visualized with a Fuji-BAS 2500 phosphorimage analyzer.

sis and cytoskeletal interactions, it is an abundant protein in eukaryotes and, therefore, is encoded by multiple genes (Carneiro et al., 1999). The genomic DNA of hot pepper was digested with restriction enzymes EcoRI, HindIII, and XbaI, and was electrophoresed on an agarose gel. To determine which of the *CaEF1A* genomic fragments corresponded to the *CaEF1A* cDNA, we prepared a full-length *CaEF1A* cDNA probe and the 3' non-coding region of the *CaEF1A*-specific probe. At least six bands of varying hybridization intensity were resolved in the XbaI digest when the full-length cDNA probe was incorporating (Fig. 2A). Using the 3'UTR specific probe, we were able to hybridize just one or two fragments (Fig. 2B). These results suggest that a *CaEF1A* homologue may exist as a multigene family in the hot pepper genome, and that the 3'UTR specific probe can detect only the *CaEF1A* clone.

In our examination of steady-state levels, we found that *CaEF1A* transcripts were abundant in flowers, roots, and ripe fruits. Although leaf transcripts were barely detectable, the levels were slightly higher in old rather than young leaves, but were virtually absent in immature fruits and stems (Fig. 3).

Induction of *CaEF1A* by Wounding Treatments

CaEF1A transcript levels were slightly increased within 12 h of incision, with the maximum being reached at 72 h post-wounding (Fig. 4). In contrast to the response from the wounded leaves, *CaEF1A* transcript was not systemically induced in upper, unwounded leaves. As a positive control, we also monitored the expression pattern of *CaPinII*, which is known to show wounding-induced expression in hot pepper (Shin et al., 2001). Although this gene is rapidly induced by both local and systemic signals of wound stress (Shin et al., 2001), the *CaEF1A* gene was induced only by local wounding, and only after 12 h.

Temporal Accumulation of CaEF1A in TMV-P_0 and TMV-$P_{1.2}$ Inoculated Leaves

To determine the temporal expression of *CaEF1A* in response to TMV-P_0 or TMV-$P_{1.2}$ infection, and com-

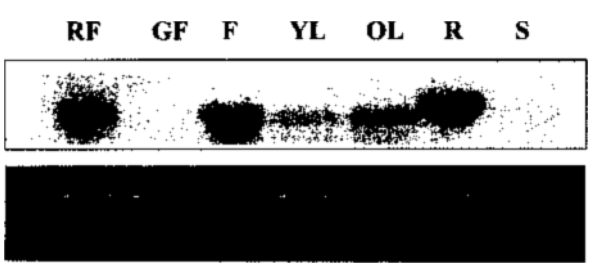

Figure 3. Organ-specific expression of the *CaEF1A* gene. Total RNAs were isolated from different organs of the hot pepper plant. RF, red fruit (ripe fruit); GF, green fruit (immature fruit); F, flower; YL, young leaves; OL, old leaves; R, root; S, stem. The blotted membrane was hybridized with the 3'UTR fragment probe. The 18S and 28S rRNAs in ethidium bromide-stained gels are shown as a loading control.

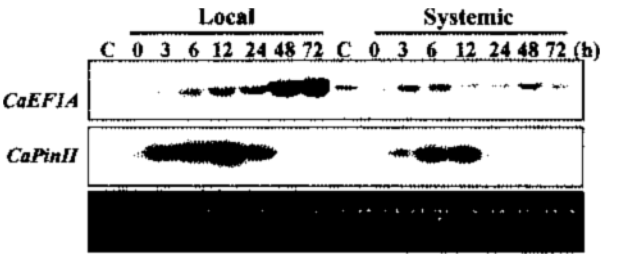

Figure 4. Expression patterns of *CaEF1A* by incision-wounding. Hot pepper leaves were incised with scissors and RNA was extracted from the incised leaves (Local) and upper unincised leaves to detect systemic induction (Systemic). The 18S and 28S rRNAs in ethidium bromide-stained gels are shown as a loading control. Ca*PinII* was used as a control for wounding treatment.

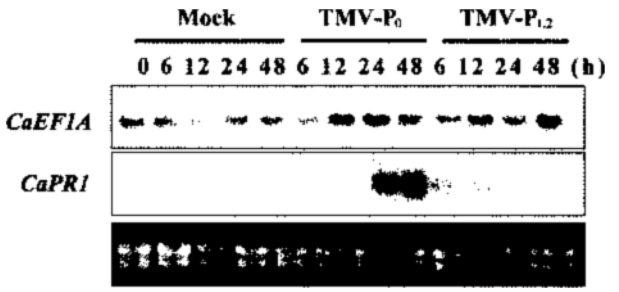

Figure 5. Expression patterns of *CaEF1A* gene upon TMV-P_0 or TMV-$P_{1.2}$ inoculation. Total RNAs were prepared from leaves inoculated with TMV-P_0 or TMV-$P_{1.2}$ at the indicated time points. As a control, leaves were mock-inoculated with phosphate buffer. RNA was separated in a formaldehyde-agarose gel and transferred onto a nylon membrane. The membrane was hybridized with a *CaEF1A*-specific probe of the 3'UTR region. Ca*PR1* was used as a control for HR.

pare it with that of other known PR proteins, we ran RNA blot analyses using a 3'UTR *CaEF1A*-specific probe. The expression pattern was monitored in resistant Bugang plants after TMV-P_0 inoculation. In addition, to investigate whether induction of the *CaEF1A* gene upon TMV-P_0 inoculation was associated with a specific disease resistance response during HR, we also monitored its expression pattern after the TMV-$P_{1.2}$ inoculation to which 'Bugang' was susceptible. Our mock inoculation treatment, with phosphate buffer and carborundum only, served to exclude expression that might have resulted from the wounding caused by simple rubbing. The transcripts corresponding to *CaEF1A* started to accumulate at 12 h, and were maintained until 24 h after inoculation with the TMV-P_0. Transcript levels then decreased after 48 h (Fig. 5). Moreover, the expression pattern of the *CaEF1A* by TMV-$P_{1.2}$ treatment was similar to that of the TMV-P_0 treatment (Fig. 5). As a positive control for HR, we monitored the expression pattern of the Ca*PR1* gene, which is known to show HR-specific expression upon TMV-inoculation in hot pepper (Park et al., 2001). Compared with Ca*PR1*, the induction time for *CaEF1A* expression was earlier upon TMV-P_0 inoculation. However, *CaEF1A* was also expressed by TMV-$P_{1.2}$ inoculation, which was not the case with Ca*PR1*. Creager et al. (1999) have suggested that two candidate host factors may be involved in TMV RNA synthesis: 1) eEF-1A, which colocalizes with the replicase complex; and 2) a subunit of eEF-3, which copurifies with the replicase. If *CaEF1A* were related to TMV RNA synthesis, it would be induced by both compatible and incompatible interactions with TMV. Our results are consistent with the prediction that the expression pattern of *CaEF1A* was not specific for the plants exhibiting a hypersensitive response to TMV-P_0 but was related to TMV pathogenesis itself.

Generally, eEF1A is widely distributed throughout the cell. Therefore, Carneiro et al. (1999) have hypothesized that its function and/or location may be regulated by different post-translational modification. Nonetheless, we have been able to demonstrate here that the *CaEF1A* gene is among the multifunctional eEF1A genes in hot pepper that is induced by both avirulent and virulent TMV inoculation. We therefore propose that this gene has a possible role in TMV replication.

ACKNOWLEDGMENTS

This work was supported by a grant (CG2113) from the Crop Functional Genomics Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology of the Republic of Korea, and by a grant from the Center for Plant Molecular Genetics and Breeding Research of the Korea Science and Engineering Foundation.

Received October 24, 2001; accepted November 28, 2001.

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