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Regulated expression of the calmodulin-related *TCH* genes in cultured *Arabidopsis* cells: Induction by calcium and heat shock

JANET BRAAM

Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77251-1892

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ABSTRACT **Expression of the calmodulin-related *TCH* genes of *Arabidopsis* is strongly and rapidly up-regulated in plants after a variety of stimuli, including touch. As an approach to investigating the mechanism(s) of *TCH* gene regulation, a manipulable cell culture system in which *TCH* gene expression is regulated has been developed. In response to increased external calcium or heat shock, TCH2, -3, and -4 mRNA levels significantly increased. Significantly, these two stimuli are known to result in cytoplasmic calcium increases, therefore implicating a role for calcium itself in the regulation of calmodulin-related genes. Further, external calcium is required for maximal heat-shock induction of expression of the *TCH* genes but not of the 70-kDa heat shock protein; therefore, there may exist at least two distinct mechanisms of heat shock induction of gene expression. Calcium ion regulation of genes encoding calcium-binding proteins may ensure the efficacy of calcium ion as a transient second messenger and the maintenance of cellular homeostasis. This possible regulatory circuit would likely be relevant not only for plant cells but also for the great variety of animal cells that transduce extracellular stimuli, such as hormones and electrical impulses, into calcium signals.**

Despite their passive appearance, plants sense and actively respond to many environmental stimuli. The long-term responses often include developmental alterations that result in phenotypes better adapted to the specific stresses of the local environment. For example, plants exposed to wind tend to be shorter and sturdier and, thus, less easily damaged by wind. It is now clear that plants possess sensory networks capable of generating genetic responses to a variety of environmental stimuli. For example, in response to touch, rain, wind, wounding, and darkness, there is a strong and rapid induction of expression of at least five specific genes in *Arabidopsis* (1). Because touch represents a common and most basic feature of several of the inductive stimuli these genes were termed the touch (*TCH*) genes. Ten to 30 min after stimulation, TCH mRNAs increase in abundance 10- to 100-fold (1). Sequence analysis indicates that the *TCH1* gene most likely encodes an *Arabidopsis* calmodulin; *TCH2* and *TCH3* encode proteins closely related to calmodulin (1). In addition, another gene with 13% amino acid divergence from *TCH1* has been isolated from *Arabidopsis* and shown to be similarly induced in expression by touch stimulation (2). Regulation of expression of this calmodulin-related gene family in *Arabidopsis* suggests that calcium ions and calmodulin-related proteins may be involved in transduction of environmental signals or in the elicitation of developmental alterations. As yet, partial sequence analysis of *TCH4* has not revealed similarities to sequences in the data bases (1).

Changes in the cytoplasmic concentration of Ca^{2+} most likely serve as a major second messenger in plants. Fluctuations in Ca^{2+} concentrations have been implicated in evoking cellular responses to a great variety of environmental stimuli (for reviews, see refs. 3–5). For example, it is possible that $[Ca^{2+}]$ acts as a second messenger after touch stimulation and evokes cellular responses, including the induction of expression of the calmodulin-related *TCH* genes. Indeed, Knight *et al.* (6) have recently shown that touch stimulation led to rapid and transient $[Ca^{2+}]$ fluxes. Therefore, a homeostatic regulatory network may exist, whereby Ca^{2+} directly or indirectly controls the expression of the genes that encode calmodulin-related proteins, thus ensuring that enough Ca^{2+}-binding proteins are produced to chelate available Ca^{2+}.

Because of the universal significance of Ca^{2+} in eukaryotic signal transduction, it is important to investigate the possibility of cytoplasmic $[Ca^{2+}]$ homeostasis through Ca^{2+}-induced calmodulin-related gene induction. If Ca^{2+} acts as a positive regulator of gene expression, we would predict that induction of *TCH* gene expression would also occur in response to other stimuli, unrelated to touch, that are known to result in cytoplasmic $[Ca^{2+}]$ increases. Precise physiological experiments with intact plants, however, are difficult because of the inability to alter the availability of external Ca^{2+}. To overcome this problem and to create a more manipulable experimental system, we sought to determine whether cultured cells are capable of faithful regulation of *TCH* gene expression. We report that *TCH* gene expression is regulated in cultured cells by two stimuli that are known to lead to increases in cytoplasmic $[Ca^{2+}]$, increased external $[Ca^{2+}]$, and heat shock. Characteristics of this regulation and the evidence for distinct mechanisms of heat shock induction of gene expression are described.

MATERIALS AND METHODS

***Arabidopsis* Root Cell Culture and Manipulations.** Sterile, 2- to 3-week-old *Arabidopsis* (Columbia) roots were finely chopped and maintained in Gamborg's B5 medium with kinetin at 0.2 mg/liter and 2,4-dichlorophenoxyacetic acid at 0.05 mg/liter to stimulate growth of callus cells. Cells were maintained at 24°C in constant light and subjected to rotary shaking at 120 rpm for 3 to 6 weeks. Fourteen to 24 hr before experimentation, cells and medium were pooled and divided equally among four to six flasks. Sterile $CaCl_2$, $MgCl_2$, or water was added to the medium as described. After the appropriate time, the medium was removed, and the cells were immediately frozen in liquid nitrogen. This procedure of harvesting cells takes ≈15 sec. To generate a sudden heat shock, an equal volume of medium at 47°C was added to the cells; the cells were then placed in a rotary shaker water bath at 37°C for the appropriate duration. Addition of an equal volume of 24°C medium to the cells had no effect on *TCH* gene expression (data not shown).

RNA Purification and Northern (RNA) Analysis. Total RNA was purified essentially as described (7), and Northern analysis was conducted as described (1).

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Abbreviations: HSF, heat shock factor; HSP70, 70-kDa heat shock protein.

RESULTS

TCH Gene Expression Is Induced in Cultured Cells by Increased External Ca^{2+}. Sudden increases in extracellular [Ca^{2+}] have been shown to result in increases of cytoplasmic [Ca^{2+}] in cultured plant and animal cells (8–10). Fig. 1 shows that cultured primary *Arabidopsis* root cells exposed to high extracellular [Ca^{2+}] for 30 min accumulate increased levels of TCH2, -3, and -4 mRNAs (lanes 10 Ca^{2+} and 100 Ca^{2+}). The TCH1 mRNA levels, however, are not significantly affected. This example of non-coordinant *TCH* gene activity is consistent with previous studies that indicated that differences may occur in the mechanisms of regulation of the different *TCH* genes (1). Expression of the gene encoding the *Arabidopsis* 70-kDa heat shock protein (HSP70) is also unaffected by increased [Ca^{2+}], further indicating that there is not a general induction of gene expression. This finding is consistent with studies showing that raising intracellular [Ca^{2+}] alone is insufficient to activate HSP70 expression (11, 12). To control for potential nonspecific effects of increased concentrations of divalent cations, cells were exposed to similar concentrations of $MgCl_2$, and, as seen in Fig. 1 (lanes 10 Mg and 100 Mg), TCH mRNA levels are unchanged. Therefore, Ca^{2+} itself, or some effect of increased [Ca^{2+}], may be sufficient for the induction of expression of the *TCH2*, *-3*, and *-4* genes.

Similar to the kinetics of *TCH* gene induction by touch stimulation in intact *Arabidopsis* plants, TCH2, -3, and -4 transcripts begin to accumulate within 10 min after Ca^{2+} addition to the medium of cultured cells (Fig. 2, lane 10′). These data are consistent with the hypothesis that Ca^{2+}, as a second messenger, is generated by mechanical stimulation and evokes an initial regulatory event that leads to increased TCH2, -3, and -4 mRNA accumulation. The significance of the slight increase in TCH1 mRNA levels in this experiment (Fig. 2, top row) is unknown because this induction is variable (for example, see Fig. 1). Strikingly, the time points

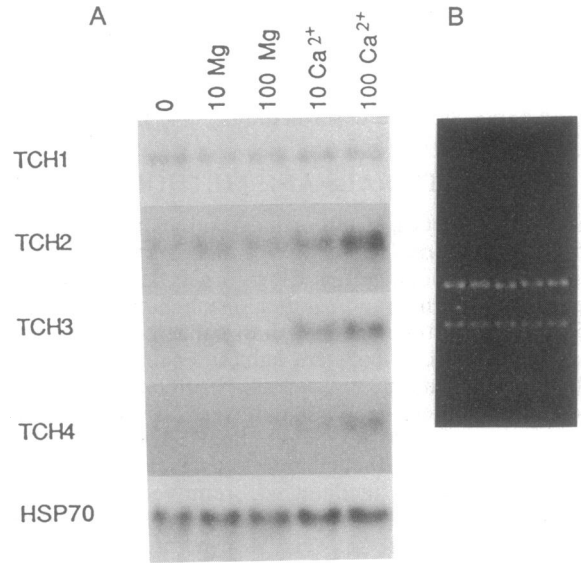

FIG. 1. Regulation of *TCH* gene expression by increased extracellular Ca^{2+}. Cells were untreated (lane 0) or exposed to 10 mM $MgCl_2$ (10 Mg), 100 mM $MgCl_2$ (100 Mg), 10 mM $CaCl_2$ (10 Ca^{2+}), or 100 mM $CaCl_2$ (100 Ca^{2+}) for 30 min. Total RNA was purified, and 2 μg of RNA was electrophoresed through formaldehyde gels. (*A*) Gels were blotted to filters, and filters were hybridized with the cDNA probes listed at left. Blank regions of photographs of the five autoradiograms were cut to show only the bands of hybridization to display results of all probes in a single composite figure. (*B*) Ethidium bromide-stained gel before blotting to show that similar amounts of RNA were loaded per lane. The two prominent bands are rRNA.

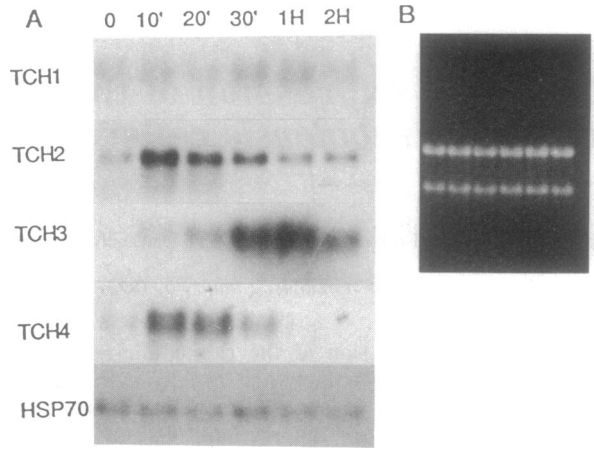

FIG. 2. Kinetics of Ca^{2+} induction of *TCH* gene expression. Cells were untreated (lane 0) or were exposed to 100 mM $CaCl_2$ for 10 min (10′), 20 min (20′), 30 min (30′), 1 hr (1H), or 2 hr (2H). (*A*) Northern blot analysis (as in Fig. 1) of RNA hybridized to the cDNA probes listed at left. (*B*) Ethidium bromide-stained gel before blotting.

at which the TCH3 and -4 mRNA transcripts reach maximum levels are virtually identical in touch-stimulated plants and Ca^{2+}-treated cultured cells. That is, TCH3 mRNAs accumulate to highest levels 30 min–1 hr after Ca^{2+} addition (Fig. 2, lanes 30′ and 1H); similarly, in plants, TCH3 transcripts are at maximal levels 30 min–1 hr after touch stimulation (1). Likewise, TCH4 expression levels are maximal after 10 min in both Ca^{2+}-induced cultured cells (Fig. 2, lane 10′) and in touch-treated plants (1). TCH2 mRNAs, however, reach peak levels more rapidly in Ca^{2+}-treated cultured cells (within 10 min, lane 10′) than in touch-stimulated plants (30 min–1 hr) (1).

The TCH transcripts also demonstrate a rapid decrease in accumulation within 1–2 hr after sustained external [Ca^{2+}] increases (Fig. 2; TCH2 and TCH4, lanes 30′ and 1H; TCH3, lane 2H), suggesting an adaptation to increased external [Ca^{2+}] and/or a negative feedback regulation of *TCH* gene expression within 2 hr.

***TCH* Gene Expression Is Induced by Heat Shock in Cultured Cells.** Another stimulus shown to result in cytoplasmic [Ca^{2+}] increases in cultured cells is heat shock (13–15). Thirty minutes after a sudden change from 24°C to 37°C, there is a significant increase in accumulation of TCH2, -3, -4 and HSP70 mRNAs and a somewhat weaker effect on the levels of TCH1 transcripts (Fig. 3, compare lanes 0 and HS). To determine whether the heat shock induction of *TCH* gene expression depends on the presence of external Ca^{2+}, EGTA, a Ca^{2+}-specific chelator, was added to the medium. The presence of EGTA lowers the magnitude of heat shock induction or abolishes the heat shock induction of *TCH* gene expression (Fig. 3, compare lanes HS and HS+EGTA). This inhibition is likely due to the specific chelation of Ca^{2+} because EGTA in the presence of additional Ca^{2+} has no detectable effect on heat shock induction (Fig. 3, lane HS+EGTA+Ca^{2+}). It is possible that the residual induction of *TCH* gene expression that occurs in the presence of EGTA is due to heat shock-induced release of Ca^{2+} from intracellular stores; alternatively an additional Ca^{2+}-independent mechanism may act to regulate *TCH* gene expression. In the absence of heat shock, EGTA alone has no effect on *TCH* gene expression (data not shown). Heat shock induction of HSP70 expression is unaffected by the presence of EGTA; therefore, the addition of EGTA to the medium does not nonspecifically inhibit gene induction. More significantly, because maximal induction of only the *TCH* genes and not of HSP70 depends on the availability of external Ca^{2+}, heat

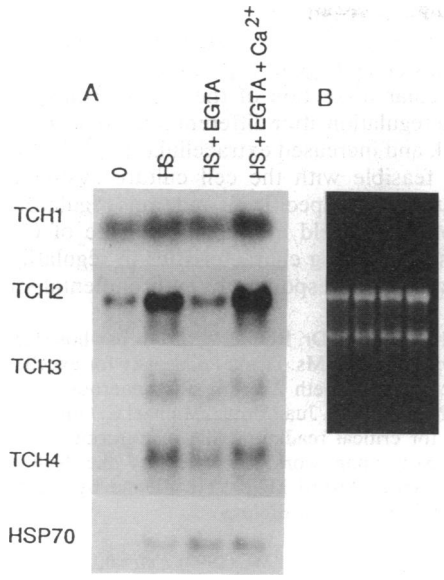

FIG. 3. Heat shock induction of the *TCH* and HSP70-encoding genes and the effects of external EGTA. Cultured cells were maintained at 24°C (lane 0) or subjected to 37°C for 30 min, as described fully in text, with no additions to the medium (HS), in the presence of 100 mM EGTA (HS+EGTA) or in the presence of 100 mM EGTA and 10 mM $CaCl_2$ (HS+EGTA+Ca^{2+}). (*A*) Northern blot analysis of total RNA (as in Fig. 2) hybridized to the cDNA probes listed at left. (*B*) Ethidium bromide-stained gel before blotting.

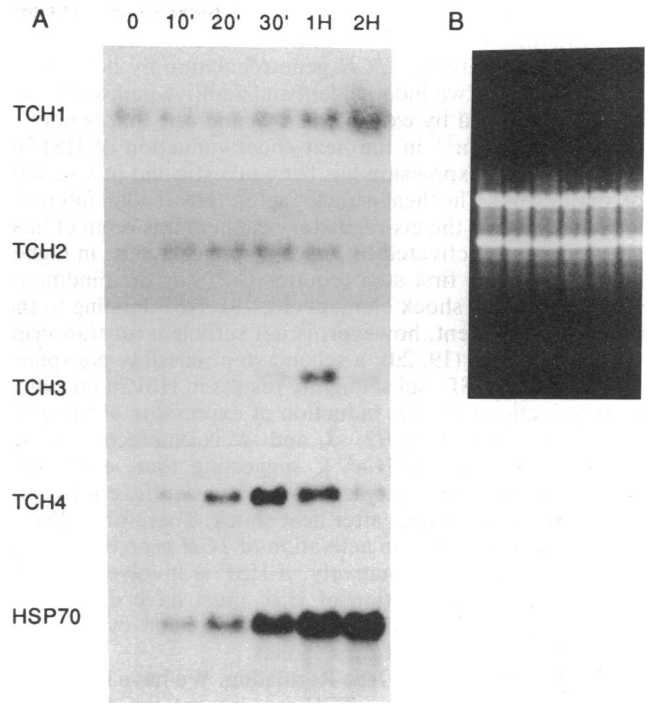

FIG. 4. Kinetics of heat shock induction of the *TCH* and HSP70-encoding genes. Cultured cells were untreated (lane 0) or subjected to heat shock, as described in text, for the times indicated. (*A*) Northern blot analysis of total RNA (as in Fig. 2) hybridized to the cDNA probes listed at left. (*B*) Ethidium bromide-stained gel before blotting.

shock induction of gene expression must result by at least two distinct mechanisms.

The kinetics of induction of expression of the *TCH* genes by heat shock are similar, but slightly delayed, relative to the time course of Ca^{2+} induction and again are distinct for the different *TCH* genes. TCH2 mRNAs reach maximal levels 10 min after heat shock stimulation and stay relatively constant for ≈1 hr (Fig. 4, lanes 10′, 20′, 30′, and 1H). Regulation of expression of TCH3 and -4 is more transient, reaching maximal accumulation of transcripts at 1 hr and 30 min respectively, after heat shock stimulation (Fig. 4, lanes 1H, 30′). These slight differences in the kinetics of *TCH* gene induction by different stimuli may indicate variation in the signals or mechanism(s) involved in *TCH* gene regulation, as described fully in the *Discussion*.

DISCUSSION

The *TCH* genes of *Arabidopsis* were isolated as a result of the increased accumulation of the corresponding mRNAs after mechanical stimulation of plants (1). Three of the five *TCH* genes encode proteins closely related to the Ca^{2+}-binding protein calmodulin. The rapid and strong induction of expression of calmodulin-related genes suggested that in response to the inductive stimuli, such as touch, there is an immediate but temporary requirement in the cells for increased synthesis of Ca^{2+}-binding proteins, perhaps due to cytoplasmic [Ca^{2+}] increases (1). Subsequently, transient cytoplasmic [Ca^{2+}] increases have been documented in touch-stimulated plants (6). It is possible, therefore, that the calmodulin-related *TCH* genes may be regulated directly or indirectly in response to an increased accumulation of cytoplasmic Ca^{2+}, the ligand for calmodulin and calmodulin-related proteins. Despite the significance of this possible regulatory circuit, the regulation of calmodulin-related gene expression by stimuli known to cause cytoplasmic [Ca^{2+}] increases has not been previously reported for any organism.

Two stimuli known to result in elevated cytoplasmic [Ca^{2+}], heat shock and increases of external Ca^{2+}, significantly induce expression of the *Arabidopsis TCH2*, *-3*, and *-4* genes. TCH1 expression, however, is not significantly induced, indicating that the requirements for TCH1 regulation are distinct. Full induction of *TCH* gene expression by heat shock requires the presence of external Ca^{2+} because the presence of EGTA in the medium significantly suppresses the induction. These results support the hypothesis that *TCH2*, *-3*, and *-4* gene expression levels respond to changes in cytoplasmic [Ca^{2+}]. Such a feedback circuitry would ensure that enough Ca^{2+}-binding proteins are synthesized to accommodate increases in cytoplasmic [Ca^{2+}], thus maintaining the effectiveness of Ca^{2+} as a transient cellular signal and ensuring that [Ca^{2+}] does not reach cytotoxic levels. The development of a cell culture system that demonstrates *TCH* gene regulation will greatly simplify dissection of the mechanisms of regulation of gene expression. For example, using specific inhibitors of calmodulin function, we will be able to determine whether calmodulin function is required for *TCH* gene regulation in response to [Ca^{2+}] increases and heat shock. In addition, direct measurements of cytoplasmic [Ca^{2+}] levels using indicators such as aequorin (16), fura-2 (17), or indo-1 (17) and manipulations of cytoplasmic [Ca^{2+}] levels can be used to more directly determine whether Ca^{2+} fluxes are necessary and/or sufficient for *TCH* gene regulation.

Distinct Mechanisms of Heat Shock Regulation of Gene Expression. The experiments described demonstrate that there are probably at least two distinct mechanisms by which heat shock regulates gene expression. Heat shock induction of HSP70 expression is unaffected by the availability of external Ca^{2+}; whereas maximal heat shock induction of the *TCH* genes is likely to occur, at least in part, by an influx of external Ca^{2+} because *TCH* gene induction is partially, but specifically, inhibited in the presence of EGTA. One possible explanation for the residual induction in the absence of available external Ca^{2+} is that heat shock may also release

Ca^{2+} from internal stores; heat shock-induced release of Ca^{2+} from internal stores has been demonstrated in other cell types (13–15). Alternatively, *TCH* gene regulation by heat shock could occur via two independent and additive pathways, one that is influenced by external [Ca^{2+}] and one that is not.

The role of Ca^{2+} in the heat shock-induction of HSP70-encoding gene expression has been investigated in a variety of organisms. The heat shock factor (HSF) that interacts specifically with the cis-regulatory element upstream of heat shock genes is activated by a two-step mechanism in higher eukaryotes. The first step requires Ca^{2+} for the binding of HSF to the heat shock element (18, 19). HSF binding to the heat shock element, however, is not sufficient for transcriptional activation (19, 20); a second step, possibly phosphorylation of the HSF, subsequently results in HSP70-encoding gene induction (19–21). Induction of expression of the gene *HSP70*, but not of *TCH2*, *-3*, and *-4*, is unaffected by the chelation of external [Ca^{2+}], suggesting that, even with external EGTA, the cytoplasmic [Ca^{2+}] is sufficient to activate HSF DNA binding after heat shock. Therefore, HSF is probably not involved in activation of *TCH* gene expression after heat shock. Alternatively, if HSF is involved in *TCH* gene regulation, activation of HSF must have distinct requirements, such as Ca^{2+} influx or greater cytoplasmic [Ca^{2+}], for activation of these genes.

Complexities of *TCH* Gene Regulation. We have found that a variety of stimuli induce *TCH* gene expression. For example, *TCH* gene expression in plants increases after touch, wind, rain, wounding, darkness, and heat shock (ref. 1, and unpublished results). Several of the inductive stimuli may be related because they share mechanical properties; however, heat shock and darkness are seemingly unrelated, nonmechanical stimuli. In addition, expression of *TCH2*, *-3*, and *-4* genes is regulated in cultured cells after heat shock and increases in external [Ca^{2+}] (Figs. 1 and 3) and cold shock (unpublished work). Although these stimuli appear unrelated, they share two features: (*i*) induction of *TCH* gene expression and (*ii*) involvement of [Ca^{2+}] changes as a response or mediator of physiological responses. These data are consistent with the hypothesis that the *TCH* genes are up-regulated in response to stimuli that generate Ca^{2+} changes.

Although expression levels of the three *TCH* genes, *TCH2*, *-3*, and *-4*, are up-regulated in response to the same stimuli (an exception being *TCH3* regulation after ethylene treatment; ref. 1), the TCH2, -3, and -4 transcripts accumulate with distinct kinetics, and each kinetic profile differs depending on the stimulus. Furthermore, the genes are differentially sensitive to the stimuli. Therefore, regulation of the different *TCH* genes likely occurs via mechanisms that are distinct or variable for each gene. In addition, for each gene either a distinct regulatory mechanism functions in response to each stimulus or, more likely, the regulatory machinery is not simply an on/off switch but functions to control strength and temporal variation in the *TCH* gene responses. This complexity of *TCH* gene regulation reflects the known complexities of Ca^{2+} as a second messenger. Magnitude, duration, spatial localization, and frequency of spiking are examples of characteristics of the Ca^{2+} signal that are thought critical for the elicitation of appropriate cellular responses to diverse stimuli (22–25). Because several of the *TCH* genes encode calmodulin-related proteins, they may be up-regulated following stimuli that evoke [Ca^{2+}] changes, as shown here, and the characteristics of the Ca^{2+} signal may control the strength and kinetics of *TCH* gene expression.

A molecular dissection of the mechanism(s) involved in *TCH* gene regulation after different inductive stimuli, such as heat shock and increased extracellular [Ca^{2+}], is now experimentally feasible with the cell culture system described. Identification of the specific cis and trans regulatory elements will undoubtedly yield insight on the role of Ca^{2+} and its variations in signaling characteristics in regulating gene expression and plant responses to environmental stimuli.

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