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Short communication

Induction of tomato stress protein mRNAs by ethephon, 2,6-dichloroisonicotinic acid and salicylate

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Abstract

To study the possible involvement of plant hormones in the synthesis of stress proteins in tomato upon inoculation with *Cladosporium fulvum*, we investigated the induction of mRNAs encoding PR proteins and ethylene biosynthesis enzymes by ethephon, 2,6-dichloroisonicotinic acid (INA) and salicylic acid (SA) by northern blot analysis. Ethephon slightly induced some but not all mRNAs encoding intra- and extracellular PR proteins. INA induced all PR protein mRNAs analysed, except for intracellular chitinase and extracellular PR-4. SA induced all PR protein mRNAs analyzed, except for intracellular chitinase and osmotin. None of the inducers affected the expression of ACC synthase mRNA, whereas all three induced ethylene-forming enzyme (EFE) mRNA.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; EFE, ethylene-forming enzyme; HR, hypersensitive response; INA, 2,6-dichloroisonicotinic acid; PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance

Plants respond to pathogen attack by activating defense responses which may act to prevent damage or nullify the pathogen. A defence response frequently investigated is the systemic acquired resistance (SAR) response, which can be invoked by one pathogen, and leads to resistance against subsequent challenge by a wide range of pathogens (reviewed by Ryals *et al.* [30]). SAR is associated with the activation of a large number of genes encoding various types of stress proteins, including pathogenesis-related (PR) proteins [4, 10, 30, 35]. In tomato, SAR can be induced by infection with the fungal pathogen *Phytophthora infestans* [4, 10]. In contrast, inoculation with an avirulent race of the fungus *Cladosporium fulvum* does not induce SAR, but is only capable of inducing local resistance to subsequent challenge with *P. infestans* [4].

Pathogen infection of tomato results in the accumulation of defence proteins, such as PR proteins [4, 6, 10, 12, 17, 32, 33]. As tobacco, tomato produces extracellular (type II) and intracellular (type I) PR proteins. These two types of tomato PR proteins are differentially induced during compatible or incompatible *C. fulvum*-tomato interactions [5, 6, 17, 32]. Incompatible interactions result in an effective host resistance reaction, which is mediated by a hypersensitive

response (HR). In both compatible and incompatible interactions, all extracellular PR proteins and their corresponding mRNAs are induced. However, the mRNAs appear two days earlier during incompatible interactions, as compared to compatible interactions [5, 21, 32]. In contrast, the intracellular PR protein mRNAs are induced with similar kinetics, and to similar levels by both compatible and incompatible interactions [5]. During HR triggered by an avirulent race of *C. fulvum*, levels of SA and ethylene in inoculated leaf tissue increase [16]. This prompted us to investigate whether these plant hormones, or the chemical INA, are involved in the coordinate induction of the PR protein mRNAs. To this end, we have assessed the ability of these compounds to induce PR protein gene expression. Furthermore, to determine whether SA-mediated gene induction acts through ethylene biosynthesis, we concomitantly studied the expression of two ethylene biosynthesis genes, ACC synthase and ethylene-forming enzyme (EFE).

In tomato, ethylene production has mainly been implicated in developmental processes, such as fruit ripening and leaf senescence [14, 24, 25]. Increased ethylene synthesis also occurs in tomato upon pathogen attack [1, 9], coinciding with expression of a wide range of defense genes [8]. However, an ethylene overproducing tomato mutant (*Epinastic*) did not synthesize PR proteins constitutively. Moreover, this mutant still responded to the ethylene releasing compound ethephon by synthesizing PR proteins [2]. Recently Lawton *et al.* [19] have postulated that, in *Arabidopsis thaliana*, ethylene-mediated responses are not essential for SAR, although ethylene seems to sensitize cells for SAR responsiveness. Salicylic acid plays a role as signal molecule in SAR [11, 26, 34]. Pathogen attack results in an increase of the cellular SA concentration, either by *de novo* synthesis or release from inactive conjugates [16, 26, 37]. SA increase in tomato coincides with induction of several defence responses [16]. SA is required for systemic gene activation during SAR [11, 34], although long distance transport of SA itself is not essential [11, 27, 30, 34]. The synthetic compound 2,6-dichloroisonicotinic acid (INA) induces resistance to different pathogens in cucumber and tobacco [23]. In tobacco and parsley, INA activates the same set of defence genes as SA, suggesting that the two compounds act in the same signaling cascade [18, 35].

In order to examine the involvement of plant hormones in gene activation during *C. fulvum*-tomato interactions, we studied the effect of exogenously applied inducers on defence gene expression. Here we report on northern blot analysis of tomato PR protein mRNAs and ethylene biosynthesizing enzyme mRNAs in response to the ethylene-releasing compound ethephon, 2,6-dichloroisonicotinic acid (INA) and salicylic acid (SA).

Plant treatments

Tomato plants (cv. Moneymaker) were treated with various inducers. Spraying leaves with 5 mM ethephon (2-chloroethylphosphonic acid) resulted in a slight epinasty; spraying with higher concentrations resulted in complete wilting. Since ethephon decomposes into ethylene, hydrochloric acid and phosphonic acid, control treatments were performed to assess the effects that are not related to ethylene action. Plants were treated either with 2 mM hydrochloric acid/2 mM phosphonic acid (control C_2), or with 2 mM phosphate buffered saline (PBS, the neutralized product of these acids, control C_3). 4 h after spraying the plants were rinsed with tap water. PBS treatment caused no symptoms, whereas treatment with 2 mM hydrochloric acid/2 mM phosphoric acid caused large numbers of tiny necrotic lesions. The level of 2 mM was chosen for the acid control (C_2) since 5 mM of the acids completely necrotized the leaves. Necrosis was not observed in plants treated with 5 mM ethephon. The discrepancy may be explained by assuming that ethephon releases hydrochloric and phosphonic acid sufficiently slowly to enable neutralization by the plant. Neutralization can probably not be achieved when such a concentration of acid is applied at once on the leaf surface. RNAs iso-

lated from acid-treated leaves were always partly degraded in comparison to the other RNA samples (data not shown).

2,6-Dichloroisonicotinic acid (INA) [23], sprayed in a formulation at a concentration of 0.5 mM (active ingredient), did not cause visible damage to the tomato leaves. The formulation compound lacking INA was sprayed onto control plants (control C_4). 4 h after spraying the leaves were rinsed with tap water.

Spraying with sodium salicylate is an efficient method for inducing PR protein gene expression in tobacco [35]. However, spraying of tomato leaves with salicylate in concentrations up to 20 mM did not result in accumulation of PR proteins in the intercellular space (data not shown), as was also found by Christ and Mösinger [4]. Therefore salicylate was applied via the transpiration stream by cutting tomato leaves at the petiole and putting them in 1 mM salicylate. This induced the accumulation of tomato PR proteins in the intercellular space without triggering symptoms (data not shown). The inactive analogue para-coumaric acid was used in a 1 mM solution as a negative control (control C_5). An additional control for all three treatments was included by spraying leaves with water (control C_6).

Tomato leaves treated with the various abiotic inducers were harvested for RNA isolation at 4 h (day 0), 24 h (day 1) or 48 h (day 2) after treatment, respectively. Tomato leaves treated with control agents C_2-C_6 were harvested for RNA isolation 1 day after treatment. Leaves of tomato genotype Cf5 infected by *C. fulvum* race 5 were harvested 10 days after inoculation, and RNA was isolated from these leaves to serve as a positive size control in the hybridizations (control C_1). The expression of tomato stress protein mRNAs was studied by northern blot analysis, using as probes tomato cDNA clones encoding intracellular and extracellular chitinases and β-1,3-glucanases [5, 32], PR-1 [32], PR-4 [21], ACC synthase [29] and ethylene-forming enzyme (EFE) [15]. A PCR fragment containing tomato cDNA encoding an intracellular PR-5 protein highly homologous to tobacco osmotin (Melchers, unpublished) was also used as probe. As a control for equal RNA loading, we used the radish rDNA probe pRG3 [13]. The results of the hybridizations with the above probes are shown in Fig. 1. Autoradiograms were scanned with a densitometer (LKB Ultroscan XL) in order to quantify the relative induction ratios caused by the various treatments. The intensity of hybridizing mRNA upon ethylene treatment was compared to controls C_2, C_3 and C_6; the intensity of hybridizing mRNA upon INA treatment was compared to controls C_4 and C_6; the intensity of hybridizing mRNA upon SA treatment was compared to controls C_5 and C_6. All the values were corrected for the relative amounts of rRNA in each lane. The results of the densitometric scanning are presented in Table 1. A ratio of 2 or higher between mRNAs in treated leaves versus control leaves was regarded as significant induction by the treatment.

Induction by ethephon

Treatment of tomato plants with ethephon resulted in rapid induction of mRNAs for EFE and extracellular PR-4 (panels F and H, day 0). The transient decrease of PR-4 mRNA on day 1 (Fig. 1, panel H) was not reproducible in repeated experiments, but rather an increase occurred at this time (data not shown). Table 1 shows that the maximal level of PR-4 mRNA was 10-fold higher than the acid control (lane C_2) and 2.7-fold higher than the neutralized salt control (lane C_3). EFE mRNA (panel F) was induced 2.8-fold in relation to both the acid control and the salt control. Of the other mRNAs, intracellular β-1,3-glucanase, intracellular chitinase and intracellular osmotin were slightly induced in relation to the water control (C_6), but not in relation to the other controls (C_2, C_3).

Studies by Conejero and co-workers and by Christ and Mösinger have demonstrated that many tomato PR proteins and their corresponding mRNAs are inducible by ethephon treatment [4, 7, 12, 28, 31]. The most probable explanation for the discrepancy with our results may be the method of application of ethephon. The other

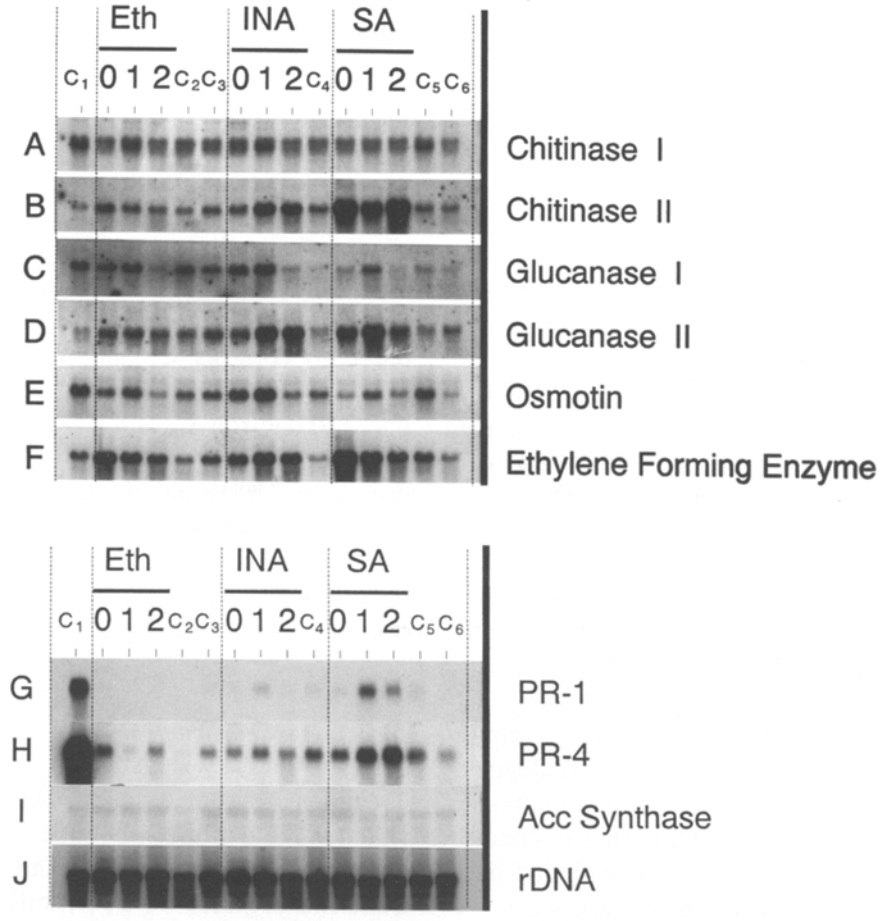

Fig. 1. Northern blot analysis of the expression of tomato PR protein mRNAs upon treatment with ethephon, INA and SA. Total RNA (15 μg), isolated from tomato leaves treated as described in the text, was electrophoresed on a denaturing agarose gel, blotted onto Hybond N^+ nylon membranes, hybridized and washed under high-stringency conditions (0.5 × SSC, 0.1% SDS, 65 °C). Four blots were made containing identical RNA samples, and subsequently hybridized with all the probes. After autoradiography, blots were deprobed in 0.1% SDS at 100 °C, and successful deprobing was checked by re-exposure prior to the next hybridization. The RNAs were loaded in the following order from left to right: control C_1, compatible *C. fulvum*-tomato interaction (race 5 on genotype Cf5) 10 days after inoculation; ethephon-treated tomato leaves, harvested at 4 h (indicated as day 0), 24 h (day 1) and 48 h (day 2) after treatment, respectively; control C_2, treatment with hydrochloric acid/ phosphonic acid, 1 day after treatment; control C_3, treatment with phosphate buffered saline, 1 day after treatment; INA-treated leaves, harvested at 4 h (day 0), 24 h (day 1) and 48 h (day 2) after treatment, respectively; control C_4, treatment with formulation compound, 1 day after treatment; SA-treated leaves, harvested at 4 h (day 0), 24 h (day 1) and 48 h (day 2) after treatment, respectively; control C_5, coumaric acid treatment, 1 day after treatment; control C_6, water treatment, 1 day after treatment. The probes used in the hybridizations were: A, intracellular type I chitinase clone CHI9 [5]; B, extracellular type II chitinase clone CHI3 [5]; C, intracellular type I β-1,3-glucanase clone GLUBAS [32]; D, extracellular type II β-1,3-glucanase clone GLUAC [32]; E, a 600 bp PCR fragment encoding tomato osmotin (L.S. Melchers, unpublished); F, ethylene forming enzyme clone pRC13 [15]; G, extracellular PR-1 clone P6 [32]; H, extracellular PR-4 clone PR-P2 [21]; I, ACC synthase clone ptACC2 [29]; J, radish 18S rDNA clone pRG3 [13].

groups applied ethephon by injecting or pricking highly concentrated solutions into the leaves. In our experiments, we sprayed low concentrations of ethephon and rinsed the leaves 4 h after treatment. It might be argued that this period of exposure was too short to allow induction of gene expression. The short exposure did however not preclude induction of EFE and PR-4 mRNAs.

Table 1. Maximum induction of tomato mRNAs by treatment with abiotic inducers in relation to control treatment[a].

	Ethephon			INA		SA	
	C_2[b]	C_3[c]	C_6[d]	C_4[e]	C_6[f]	C_5[g]	C_6[h]
Extracellular PR-1	ND	ND	ND	2.0*	ND	13*	ND
Intracellular β-1,3-glucanase (type I PR-2)	0.5	1.4	4.2*	29*	4.6*	2.0*	2.8*
Extracellular β-1,3-glucanase (type II PR-2)	1.0	1.3	1.5	6.2*	3.0*	6.2*	4.5*
Intracellular chitinase (type I PR-3)	0.9	1.7	2.3*	1.9	1.9	0.8	1.6
Extracellular chitinase (type II PR-3)	1.5	1.7	1.2	2.7*	2.3*	5.6*	6.7*
Extracellular PR-4 (type II)	10*	2.7*	2.2*	0.7	1.4	2.5*	6.2*
Intracellular osmotin (type I PR-5)	0.8	1.8	3.7*	3.0*	5.4*	0.6	2.5*
ACC Synthase	1.1	0.9	0.9	0.8	1.0	1.6	1.6
EFE	2.8*	2.8*	4.2*	6.4*	3.9*	3.5*	9.0*

[a] The hybridization signals on the autoradiograms were quantified with a densitometer. The maximum intensity obtained during the time-course of inducer treatment was divided by the intensity of the control treatment, and corrected for the relative rRNA content to obtain the induction factor.

[b] Maximum induction by ethephon in relation to control C_2, the acid treatment.

[c] Maximum induction by ethephon in relation to control C_3, the PBS treatment.

[d] Maximum induction by ethephon in relation to control C_6, the water treatment.

[e] Maximum induction by INA in relation to control C_4, the formulation treatment.

[f] Maximum induction by INA in relation to control C_6, the water treatment.

[g] Maximum induction by SA in relation to control C_5, the coumaric acid treatment.

[h] Maximum induction by SA in relation to control C_6, the water treatment.

ND: not determined, no hybridization signal detectable in controls.

* Significant induction.

Moreover, rinsing treated leaves did not prevent induction of several mRNAs by INA, applied in a similar way, as will be discussed below.

Our observation that spraying with acids (control C_2) or salts (control C_3), as compared to water treatment (control C_6), leads to higher expression of some tomato stress protein mRNAs (e.g., Fig. 1, panels C and E), implicates that the interpretation of experiments using ethephon should be done with caution. Proper controls are required before one can conclude that ethylene plays a role in ethephon-induced gene expression. This is strengthened by the finding that an ethylene overproducing tomato mutant does not synthesize PR proteins constitutively, yet responds to ethephon by synthesizing PR proteins [2], indicating that ethephon does not necessarily trigger the same responses as ethylene. This is further supported by recent evidence that induction by ethephon of defence genes, in ethylene-insensitive mutants of *Arabidopsis thaliana*, is unrelated to ethylene action but is rather caused by the (hydrochloric and phosphonic) acids released during decomposition of ethephon [19]. From our results, it seems unlikely that ethylene plays an important role as signal molecule in the induction of PR proteins during compatible or incompatible *C. fulvum*-tomato interactions. This might be tested by introducing the *Epinastic* mutation [2] into the near-isogenic tomato genotypes carrying various *Cf* resistance genes, and testing the effect of this mutation on PR protein induction and *Cf*-mediated resistance.

The observation that ACC synthase mRNA (panel I) is not induced by ethephon treatment was unexpected. The cDNA probe used (ptACC2) corresponds to an abundant ethylene-inducible mRNA in ripening tomato fruits [29]. Several studies have been reported on the expression of the ACC synthase gene family in tomato fruits, but these did not include expression studies in leaves [20, 24, 29, 39]. Apparently, ethyl-

ene inducibility of the ACC2 gene is restricted to fruit tissue. Repeating the hybridization under low-stringency conditions yielded no indication for induction of any mRNA derived from the ACC synthase gene family (data not shown). On the other hand, EFE mRNA was induced by ethephon application (panel F), suggesting autocatalytic induction of this step in the ethylene biosynthesis pathway. However, the fact that ACC synthase is not induced by any of the treatments, and the consideration that ACC synthase is the rate-limiting enzyme in ethylene biosynthesis [38], imply that none of the treatments should result in an increased ethylene production. Measurements of ethylene production upon treatments with the various abiotic agents have not been performed.

Induction by INA

INA induced all stress protein mRNAs analysed, with the exception of intracellular chitinase (panel A), extracellular PR-4 (panel H) and ACC synthase (panel I). Although the level of PR-4 mRNA slightly increased on day 1 after INA treatment, the expression level in the plants treated with the formulation compound (control C_4) was even higher. Therefore this mRNA was regarded as not induced. The level of induction of the other mRNAs in relation to the control C_4 (Table 1) varied from 2.0 (PR-1, panel G) to 29 (intracellular β-1,3-glucanase, panel C). For the PR-1 mRNA, some induction ratios could not be calculated because of the lack of detectable hybridization in control C_6. The timing of induction was different for the various mRNAs analysed. Intracellular and extracellular β-1,3-glucanase, osmotin, and EFE were induced rapidly by INA treatment (within 4 h). On the other hand the extracellular chitinase and the PR-1 mRNA were only induced 1 day after treatment. Except for the lack of induction of intracellular chitinase (panel A) and PR-4 (panel H) mRNAs in tomato, our results are analogous to those obtained with the homologous genes in tobacco, including differences in timing and level of induction among genes [35].

Induction by SA

SA induced mRNAs encoding extracellular chitinase (panel B), intra- and extracellular β-1,3-glucanase (panels C and D), EFE (panel F), extracellular PR-1 (panel G) and extracellular PR-4 (panel H). The timing of induction differed among the genes analysed. The extracellular chitinase, extracellular β-1,3-glucanase and EFE mRNAs were induced within 4 h after treatment, whereas intracellular β-1,3-glucanase, extracellular PR-1 and extracellular PR-4 mRNAs were induced one day later. The extent of induction in relation to the control treatment with coumaric acid (C_5) varied from 2.0-fold to 13-fold (Table 1). Especially the relative level of induction of mRNAs encoding extracellular PR-1, extracellular β-1,3-glucanase and extracellular chitinase was relatively high (Fig. 1, panels G, D and B, respectively). For the PR-1 mRNA, some induction ratios could not be calculated because of the lack of detectable hybridization in control C_6. The expression level of the PR-1 and PR-4 mRNAs induced by SA were rather low in comparison to the expression levels induced by *C. fulvum* infection (control C_1 lanes).In several cases the coumaric acid control (C_5) gave higher mRNA levels than the water control (C_6), as observed for the osmotin, the EFE and the PR-4 mRNAs. The fact that for these three genes, control C_3 (salt treatment) also induced higher mRNA levels than water treatment (control C_6) suggests that this induction may have been related to osmotic effects. The observation that INA and SA induce the same tomato mRNAs (except for PR-4 mRNA) is in agreement with results obtained in tobacco. The qualitative response of tomato and tobacco stress protein genes is similar, but the extent and timing of induction by the two chemicals in tomato are slightly different from tobacco [36]. These differences may be caused by differences in the method of application or in uptake of the chemicals. In parsley cells, INA and SA do not directly induce specific stress protein gene expression, but rather increase the sensitivity of cells to subsequent elicitor treatment [18]. These observations indicate that INA and SA act in the

same signaling pathway, although the results presented here are insufficient to unequivocally confirm such a relationship between the two compounds.

In tobacco, all mRNAs encoding extracellular (type II) PR proteins are strongly induced synchronously by SA and moderately by ethephon, whereas the mRNAs encoding intracellular (type I) PR proteins are strongly induced by ethephon but hardly by SA [3, 22, 35]. Clearly the intracellular and extracellular PR proteins in tomato show a more diverse regulation by the abiotic inducers tested. Intracellular chitinase was not inducible by any of the treatments, although we have previously shown that the amount of this mRNA is increased during both compatible and incompatible *C. fulvum*-tomato interactions [5]. The mRNAs encoding extracellular PR proteins (extracellular chitinase and β-1,3-glucanase, PR-1 and PR-4) all appeared to be inducible by both INA and SA, although to different extent and with different timing. Especially PR-1 mRNA was only induced late and to low levels in comparison to chitinase and β-1,3-glucanase.

All the extracellular PR protein mRNAs are induced two days earlier during incompatible *C. fulvum*-tomato interactions, as compared to compatible interactions [5, 17, 21, 32]. The relevance of elicitor recognition to this induction was demonstrated by the finding that mRNAs for extracellular (but not intracellular) chitinase and β-1,3-glucanase are induced specifically after injection of race-specific elicitors of *C. fulvum* into resistant tomato genotypes [36]. Depending on the elicitor-resistance gene combination, these elicitors cause specific necrosis, which coincides with a rapid increase of SA [16]. This may indicate that SA is involved in signalling responses of tomato towards *C. fulvum*, including the early induction of extracellular PR protein synthesis in incompatible *C. fulvum*-tomato interactions [6, 17]. However, the fact that extracellular PR-1 is one of the first PR proteins detectable in incompatible *C. fulvum*-tomato interactions [6] and our current observation that PR-1 mRNA is induced only slowly and to low levels by SA suggests that SA increase, when involved in the induction process, may not be sufficient to trigger extracellular PR-1 gene expression. In this respect, we cannot distinguish between the possibilities that either additional signals or higher endogenous SA concentrations are needed for the fast and high PR-1 mRNA induction that is normally observed. Comparing the effects of *in vitro* applied SA with those of endogenously synthesized SA remains difficult.

In conclusion, our data suggest that SA-mediated pathways could play a role in the induction of extracellular tomato PR protein synthesis by *C. fulvum*, but additional signals might be needed for rapid and high levels of induction. Further evidence could be obtained by testing PR protein induction by avirulent *C. fulvum* races on transgenic tomato plants carrying the salicylate hydroxylase (*nahG*) gene from *Pseudomonas putida* [11].

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