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Ced-9 inhibits Al-induced programmed cell death and promotes Al tolerance in tobacco

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ABSTRACT

Our previous data showed that apoptotic suppressors inhibit aluminum (Al)-induced programmed cell death (PCD) and promote Al tolerance in yeast cells, however, very little is known about the underlying mechanisms, especially in plants. Here, we show that the *Caenorhabditis elegans* apoptotic suppressor Ced-9, a Bcl-2 homologue, inhibited both the Al-induced PCD and Al-induced activity of caspase-like vacuolar processing enzyme (VPE), a crucial executioner of PCD, in tobacco. Furthermore, we show that Ced-9 significantly alleviated Al inhibition of root elongation, decreased Al accumulation in the root tip and greatly inhibited Al-induced gene expression in early response to Al, leading to enhancing the tolerance of tobacco plants to Al toxicity. Our data suggest that Ced-9 promotes Al tolerance in plants via inhibition of Al-induced PCD, indicating that conserved negative regulators of PCD are involved in integrated regulation of cell survival and Al-induced PCD by an unidentified mechanism.

Introduction

Aluminum (Al) rhizotoxicity is a major constraint that causes severe yield loss of crops in acid soils (pH $\leqslant$ 5.0). The initial and most dramatic symptom of Al toxicity is a rapid inhibition of root elongation, resulting in a damaged root system and even limited water and mineral nutrient uptake [1,2]. In recent years, there has been significant progress in understanding of the molecular mechanisms of Al toxicity and tolerance in plants [3–9]. Great efforts have been made to obtain Al-tolerant plants through genetic and biotechnological approaches [10–12].

The molecular mechanisms of Al tolerance in plants identified so far are external exclusion and internal tolerance. An Al-tolerant gene *ALMT1* (*Al-activated malate transporter*) has been isolated in some plant species [9,12,13]. The *ALMT1* genes conserved between monocots and dicots provide Al tolerance by facilitating Al-triggered excretion of the Al-chelating malate from the root tips [12–14]. However, little is known of the molecular mechanisms of internal Al tolerance, possibly due to its genetic complexity.

Increasing evidence has shown that Al-toxicity-induced programmed cell death (PCD) plays a significant role in Al tolerance of plants, animals and yeasts [15–19]. Our recently published data revealed that apoptotic suppressors such as Ced-9, Bcl-2 and PpBI-1, enhance Al tolerance in yeast cells [19]. However, it is not known whether these apoptotic suppressors enhance plant tolerance to Al toxicity by inhibition of Al-induced PCD. In addition, downstream factors involved in apoptosis such as caspases (cysteine–aspartic acid specific proteases) have been shown to act as crucial executors of apoptosis in animals [20,21]; and a vacuole-localized protease, vacuolar processing enzyme (VPE), has been shown as involved in cell death of plants [22]. Thus, it is interesting to investigate whether VPE is responsible for Al-induced PCD in plants.

In this report, we tested whether Ced-9, an apoptotic suppressor, plays a role in Al tolerance in tobacco. Our data showed that Ced-9 significantly inhibited Al-induced PCD and Al-induced expression of NtVPE-1. Further analysis showed that Ced-9 significantly alleviated Al inhibition of root elongation, decreased Al accumulation in the root tip, and inhibited Al-induced gene expression in early responses to Al. These data suggest that Ced-9 inhibits Al-induced PCD and promotes Al tolerance in plants.

Materials and methods

Constructs and plant transformation. Binary vector pBS harboring *Ced-9* was kindly provided by Dr. Horvitz (Massachusetts Institute of Technology). *Ced-9* was inserted into plant expression vector

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pBI121, and expressed under the control of the cauliflower mosaic virus 35S promoter. The resulting construct was transformed into *Agrobacterium tumefaciens* strain EHA105 and transformants were selected on LB medium supplemented with kanamycin (50 mg/L) and rifampicin (50 mg/L). The transformation of tobacco (*Nicotiana tabacum* L.) leaf discs and the regeneration of transgenic plants were performed according to previously described procedures [23].

Expression analysis of Ced-9 in transgenic tobacco lines. Southern blotting analysis was used to detect the insertional copy number. Genomic DNAs were extracted from the selected tobacco plants using a CTAB protocol [24]. HindIII-digested DNA samples were separated on 1% agarose gels, and then transferred onto nylon membranes (Hybond-N, Amersham, UK), and hybridized to ^{32}P-labelled DNA probes. Hybridization was carried out in 0.25 M NaH_2PO_4 (pH 7.2), 1 mM EDTA, 7% SDS and 1% BSA at 65 °C. Membranes were washed twice at 65 °C in washing solution 1 (2× SSC buffer, 0.1% SDS) and twice in washing solution 2 (0.1× SSC buffer, 0.1% SDS). After being washed, the membranes were exposed to a phosphor screen (Kodak storage phosphor screen; Molecular Dynamics, Krefeld, Germany) for 24 h, and the hybridized signals were captured as image files using a Typhoon 9100 scanner (Molecular Dynamics).

RT-PCR assay was conducted to detect gene expression. Total RNA was isolated from one-month-old wild-type (WT) and transgenic tobacco plants using RNeasy kit (QIAGEN, CA). cDNA synthesis was according to the manufacturer's instructions using a Reverse Transcription System (Promega). TaKaRa Taq™ (DR001B) was used in PCR amplification. PCR conditions were 95 °C for 3 min followed by 30 cycles at 95 °C for 1 min, 60 °C for 30 s and 72 °C for 1 min, and finally 72 °C for 10 min on a thermocycling machine (Hybaid). The primers used for *Ced-9* and *Actin* were as follows: *Ced-9*, 5′-ATGACACGCTGCACGGCG-3′ and 5′-CGCCTACA AGTCGAACTTCATT-3′; *Actin*, 5′-ATGGCAGACGGTGAGGATATTCA-3′ and 5′-GCCTTTGCAATCCACATCTGTTG-3′.

Al treatments. Seeds of the control and Ced-9-expressing lines were surface-sterilized with 10% sodium hypochlorite (v/v) for 10 min and rinsed with sterile double distilled H_2O, and then plated onto solid-agar MS medium supplemented with different concentrations of Al (0, 100, 300, 500 and 1000 μM, pH 4.7) in Petri dishes, and incubated at 25 ± 1 °C. After two weeks, the length of main root axis was measured.

DNA fragmentation analysis. After Al treatments, roots of the control lines (WT and *Ced-9-10*) and Ced-9-expressing lines (*Ced-9-6* and *-23*) were collected and homogenized in liquid nitrogen. DNAs were extracted by the CTAB protocol [24]. DNA samples were digested with 100 μg/mL DNase-free RNase for 1 h at 37 °C, and DNA fragments were separated by electrophoresis on a 1.8% (w/v) agarose gel, followed by visualization by ethidium bromide staining.

Semi-quantitative reverse-transcription PCR. Total RNAs were prepared from the leaves and roots of the control lines (WT and *Ced-9-10*) and Ced-9-expressed lines (*Ced-9-6, -23*) treated with or without Al, respectively. Expression levels of targeted genes were detected by semi-quantitative RT-PCR with gene specific primers such as *NtVPE-1* (5′-GGGTGGTCTCAAAGATGAGAACATTG-3′ and 5′-GTATA GAGCATCCTTGCTG-3′); *NtGDI1* (5′-TGGAGGCACTTAAATCTCCTCT GAT-3′ and 5′-GGGTTATCAGTTTCTGCCTCTGTT-3′); *NtPox* (5′-CCT GCTACAAACATCACGAA-3′ and 5′-TGAAGTCATAGAACAAGCTAAAC AA-3′); *parB* (5′-GCGATCAAAGTCCATGGTAG-3′ and 5′-TTAACCCA AGCTGGCCTG-3′); and *Actin* as a control [22]. To amplify *NtVPE-1*, 35 cycles were used, and 30 for *NtGDI1*, *NtPox*, *parB*, and *Actin*.

Hematoxylin staining. Hematoxylin staining was by a modified method as previously described [25]. Two-week-old seedlings of WT and *Ced-9-23*, treated with indicated concentrations of Al (pH 4.7), were washed for 30 min in distilled water (repeated three times) and their roots stained in 0.2% hematoxylin solution for 30 min. After this, all stained roots were washed for 30 min in distilled water, and then photographed.

Results

Generation of transgenic lines overexpressing Ced-9 in tobacco

Transgenic lines overexpressing Ced-9 were generated using agrobacterium-mediated transformation. Four lines, *Ced-9-6*, *-9*, *-10*, and *-23*, were found to contain a single insertion and four (*Ced-9-3*, *-7*, *-13*, and *-38*) had multiple insertions (Fig. 1A). The transcript analysis using RT-PCR showed that Ced-9 was expressed in three of the four lines (*Ced-9-6*, *-9* and *-23*) (Fig. 1B). Two over-expressing lines, *Ced-9-6* and *-23*, were used in further experiments below; while WT and *Ced-9-10* not expressing Ced-9 were used as negative controls.

Overexpression of Ced-9 inhibits Al-induced genomic DNA degradation

We first tested whether an apoptotic suppressor Ced-9 from *Caenorhabditis elegans* effectively blocked Al-induced PCD in plants. DNA ladder formation was thought a hallmark of cellular apoptosis [26]. Genomic DNAs were isolated from both leaves and roots of plants treated with or without Al. A clear DNA-laddering pattern was detected in the control lines (WT and *Ced-9-10*) treated with Al (100 μM for 6 h); however, this was very weak in the Ced-9-expressing lines (*Ced-9-6* and *-23*) under the same conditions (Fig. 2A). This suggests that Ced-9 effectively blocks Al-induced PCD in plants, indicating that PCD pathways are evolutionarily conserved in both plants and animals.

Ced-9 inhibits Al-induced expression of NtVPE-1 in tobacco

Recent studies have demonstrated that vacuole-localized VPE has caspase-1 activity in plants and plays a key role in plant PCD through vacuolar disruption and release of their contents [22,27,28]. To address the mechanisms by which Ced-9 inhibits Al-induced PCD in plants, we first tested whether Al affects VPE expression in the course of Al-induced PCD in plants. In the absence of Al, the transcriptional level of NtVPE-1 was not visibly changed in the time-course in leaves and roots (Fig. 2B). Interestingly, 100 μM Al obviously enhanced NtVPE-1 expression in leaf tissues, the level of which steadily increased with increased incubation time. However, NtVPE-1 expression rapidly increased to a maximum level within 3 h in roots under the same conditions. It was worth noting that Al-induced NtVPE-1 expression

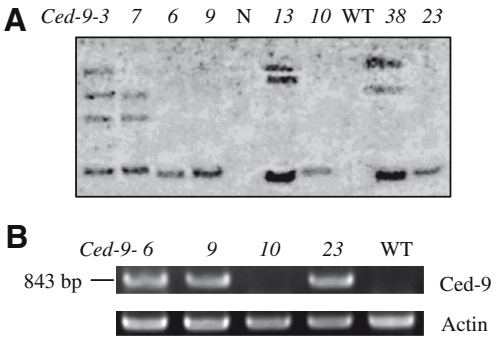

Fig. 1. Molecular identification of transgenic tobacco lines. (A) Representative Southern blotting analysis in some *Ced-9* transgenic lines. N, no loading. (B) RT-PCR analysis of *Ced-9* expression in some transgenic lines. *Ced-9* expression in some of single insertional transgenic lines was detected by RT-PCR. Actin was the internal standard.

preceded Al-induced PCD (Fig. 2A and B). Thus, it was reasonably proposed that VPE activity is required for Al-induced PCD in plants.

Next, we tested if Ced-9 could inhibit Al-induced VPE expression. As expected, Ced-9 dramatically inhibited Al-induced transcriptional level of NtVPE-1 during 18-h incubations in the leaf tissues of Ced-9-expressing lines (Fig. 2B; data not shown). In roots, Al-induced NtVPE-1 expression was also similarly inhibited by Ced-9, although Al appeared to induce its expression to a lesser extent at later stages (12–18 h) (Fig. 2B; data not shown). These results suggest that Ced-9 effectively inhibited Al-induced expression of VPE in plants. Taken together, our data indicate that Ced-9 inhibited Al-induced PCD, probably via blocking Al-induced expression level of VPE.

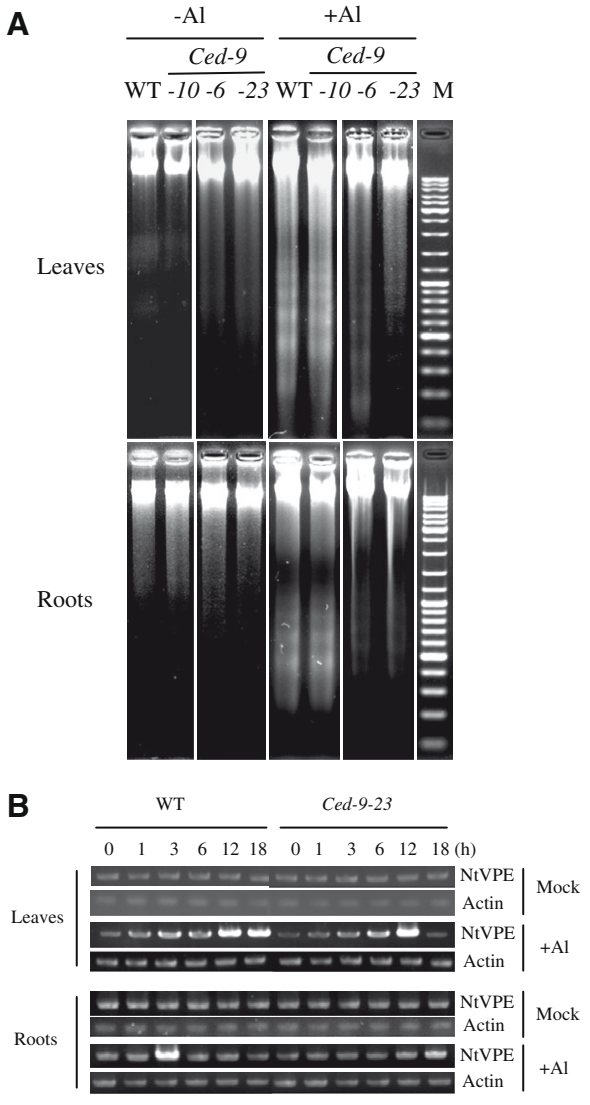

Fig. 2. Ced-9 inhibition of Al-induced cell death and Al-induced VPE expression in tobacco. (A) The control lines (WT and *Ced-9-10*) and Ced-9-expressing lines (*Ced-9-6* and *-23*) were treated for 6 h with or without 100 μM Al (pH 4.7, 0.1 mM $CaCl_2$). Roots and leaves were separately collected for DNA extraction. RNase-treated DNAs were separated by ethidium bromide-stained agarose gel. The amount of DNA loaded in each lane was equal in each treatment. (B) WT and *Ced-9-23* were treated with 100 μM $AlCl_3$ (0.1 mM $CaCl_2$, pH 4.7) for indicated times. Roots and leaves were separately collected for total RNA extraction. Semi-quantitative RT-PCR was as described in Materials and methods. Actin was the internal standard.

Ced-9 promotes the tolerance of tobacco plants to Al toxicity and reduces Al accumulation in the root tips

The initial and most dramatic symptom of Al toxicity is rapid inhibition of root elongation, and thus root relative elongation rate has served as a typical marker of levels of Al toxicity and tolerance in plants [2]. Thus, we examined whether Ced-9 enhances plant tolerance to Al toxicity by measuring the root relative elongation. After two weeks of incubation, almost all seeds in Ced-9-expressed lines (*Ced-9-6* and *-23*) and the control lines (WT and *Ced-9-10*) had germinated and developed four young leaves in the 0–1000 μM Al treatments. There was no significant difference in the germination capacity between controls and the two lines expressing Ced-9 (data not shown), indicating that both Al itself and Ced-9 do not affect seed germination under these conditions. We further tested whether Ced-9 blocks Al-induced inhibition of root elongation. Interestingly, the root length in lines expressing Ced-9 was significantly higher than in controls in the presence of Al; however, in the absence of Al, there were no visible differences in root length (Fig. 3A). These data show that Ced-9 dramatically alleviates Al-induced inhibition of root elongation, suggesting that Ced-9 can promote Al tolerance of plants.

Next, we examined Al accumulation in root tips using hematoxylin staining in WT and the *Ced-9-23* line in the presence of Al. Al accumulation levels in the WT roots increased with increased Al levels (Fig. 3B); whereas in the *Ced-9-23* line, Al accumulation was significantly less in the root tip, than in WT control (Fig. 3B).

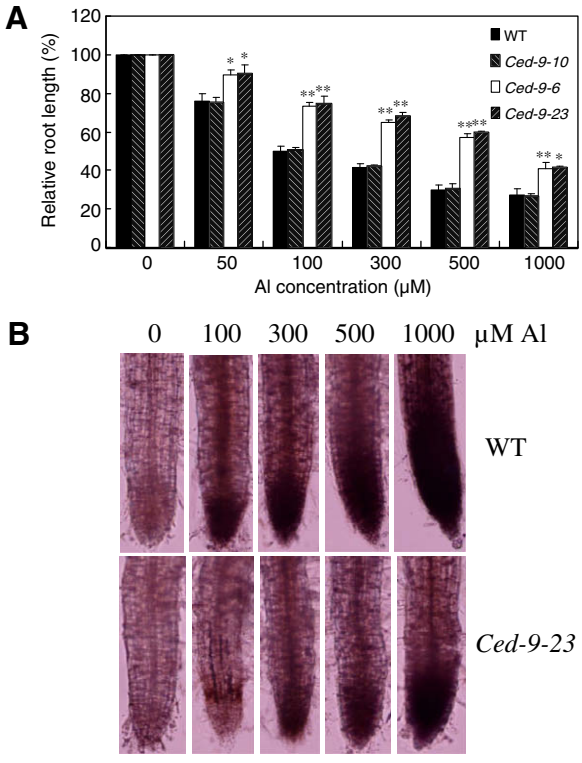

Fig. 3. Ced-9 blocks Al-induced inhibition of root elongation in tobacco and reduces Al accumulation in root tips. (A) The control lines (WT and *Ced-9-10*) and Ced-9-expressing lines (*Ced-9-6* and *-23*) were grown for two weeks in liquid MS medium supplemented with different concentrations of Al (0, 50, 100, 300, 500 and 1000 μM, respectively, 0.1 mM $CaCl_2$, pH 4.7). Means and SD for relative root lengths are shown ($n = 30$). $*$, Denotes significance at $P \leqslant 0.05$ (Student's t test), compared with WT. $**$, Denotes significance at $P \leqslant 0.01$ (Student's t test), compared with WT. (B) Seeds of WT and *Ced-9-23* were germinated on MS medium supplemented with indicated concentrations of Al (pH 4.7) for two weeks. Hematoxylin staining was as described in Materials and methods.

These data indicate that Ced-9 decreased Al accumulation in the root, possibly via inhibition of Al-induced PCD.

Ced-9 alters plant response to Al toxicity

As Ced-9 inhibition of Al-induced PCD significantly enhanced Al tolerance of plants and reduced Al accumulation in root tips, this prompted us to address whether Ced-9 affects plant response to Al toxicity. We detected expression levels of three Al-inducible early-response genes *NtGDI1, NtPox*, and *parB* [29–31] in WT and *Ced-9-23* treated with or without Al. In the absence of Al, there was no visible difference in transcriptional levels between WT and the *Ced-9-23* line. In WT treated with Al, Al greatly induced their expression levels compared with the mock in WT (Fig. 4A and B), consistent with published data [29–31]. In the Al-treated *Ced-9-23* line, interestingly, Ced-9 greatly inhibited their expression levels compared with Al-treated WT (Fig. 4A and B). These data indicate that Ced-9 alleviated the response of plants to Al toxicity, possibly via inhibition of Al-induced PCD.

Discussion

Although recent evidence has shown that Al toxicity induces cell death in plants, animals and yeasts [15–19], the molecular mechanisms underlying Al-triggering evolutionarily-conserved PCD pathways have remained elusive. It is well known that Ca^{2+} and reactive oxygen species (ROS) act as important signal molecules in triggering PCD in plants and animals. In animals, apoptotic signals cause loss of mitochondrial membrane potential through signal transduction pathways, and thereby release apoptosis-inducing factors such as cytochrome *c*, and consequently activate caspases leading to cell death [32,33]. Thus, it is very likely that Al toxicity indirectly activates plant PCD through inducing cytoplasmic Ca^{2+} increase and/or ROS generation; the latter is further supported by a recent observation that Al toxicity induced ROS generation and the release of cytochrome *c* from isolated mitochondria [34]. Our data are further evidence that Al toxicity increases VPE expression level during Al-induced PCD (Fig. 2B), implying that VPE activity plays a role in Al-induced PCD. More likely, Al toxicity indirectly induces VPE activity through Al-triggered Ca^{2+} or ROS generation.

In animals, apoptotic-suppressor Bcl-2 family members are important regulators of cell survival and cell death. It has been proposed that Bcl-2 family members function in mitochondria and endoplasmic reticulum (ER) via positively or negatively regulating the release of cytochrome *c* or Ca^{2+} [35,36]. It is well demonstrated that Ced-9 inhibits the function of Ced-4 (apoptosis protease activating factor-1) by its binding to and sequestering the latter, leading to negative regulation of cell death in *C. elegans* [37]. Thus, Ced-9 inhibited Al-induced VPE expression possibly via modulating intracellular signals such as Ca^{2+}.

Our previous data show that several apoptotic suppressors such as Bcl-2 and Ced-9 effectively promote cell survival and Al tolerance in yeast cells [19]. In the present study, we provided further evidence that Ced-9 inhibits Al-induced PCD and promotes Al tolerance in plants possibly via inhibiting Al-induced VPE activity (caspase-like VPE) (Fig. 2B). Interestingly, Ced-9 compromised the plant response to Al toxicity and significantly reduced Al accumulation in the root tip (Figs. 3B and 4). Our findings suggest that heterogeneous apoptotic suppressors effectively and negatively regulate endogenous PCD machinery in plants, leading to enhanced plant tolerance to Al through an unknown mechanism. It is likely that such negative regulation of PCD pathways leads to activating Al-tolerant mechanisms such as Al external exclusion, which in turn prevent Al accumulation in the root and restore normal root elongation. Although it was proposed that apoptotic suppressors inhibit ROS generation in chloroplasts and mitochondria [26,38], whether Ced-9 inhibits Al-elicited Ca^{2+} or ROS remains to be elucidated. Clarifying this issue will better explain the mechanisms that underlie the apoptotic-suppressor-integrated regulation of the Al-toxicity signal and PCD pathways in plants.

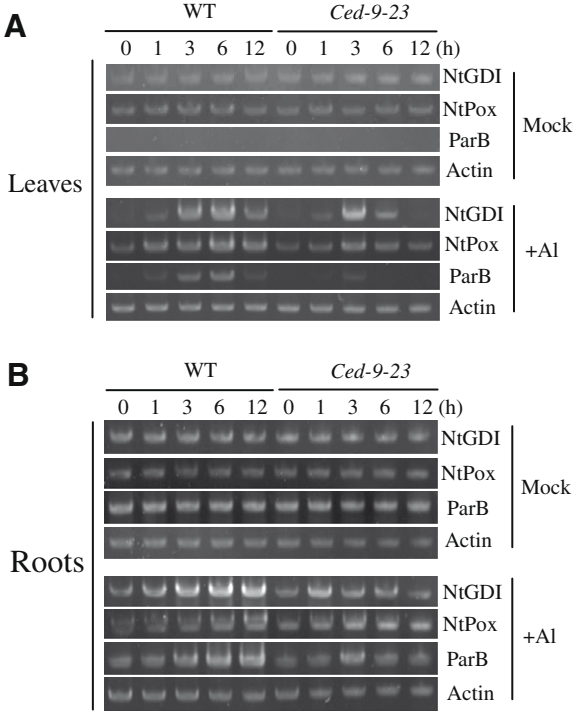

Fig. 4. Ced-9 alters the response of plants to Al toxicity. (A) Effects of Ced-9 on Al-induced genes (*NtGDI1*, *NtPox* and *parB*) in the leaves. (B) Effects of Ced-9 on Al-induced genes (*NtGDI1*, *NtPox* and *parB*) in the roots. WT and *Ced-9-23* lines were treated with 100 μM $AlCl_3$ (0.1 mM $CaCl_2$, pH 4.7) for indicated times. Roots and leaves were separately collected for total RNA extraction. Semi-quantitative RT-PCR was as described in Materials and methods. Actin was the internal standard.

Acknowledgments

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