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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

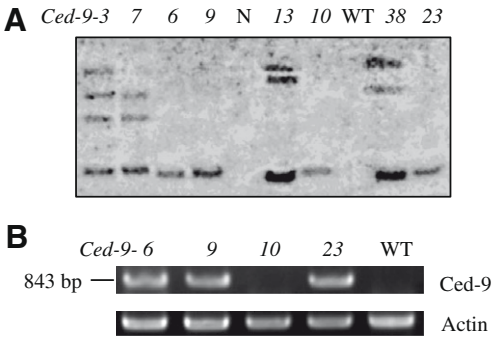

Fig. 1. Molecular identification of transgenic tobacco lines. (A) Representative Southern blotting analysis in some *Ced-9* transgenic lines. N, no loading. (B) RT-PCR analysis of *Ced-9* expression in some transgenic lines. *Ced-9* expression in some of single insertional transgenic lines was detected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