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Chronic mitochondrial calcium elevation suppresses leaf senescence

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ABSTRACT

Mitochondria Ca^{2+} overload has long been recognized as a cell death trigger. Unexpectedly, we demonstrated a signaling complex composed of Calmodulin (CaM), *Arabidopsis thaliana* Bcl-2-associated athanogene 5 (AtBAG5) and Heat-shock cognate 70 protein (Hsc70) within *Arabidopsis thaliana* mitochondria which transduces mitochondria Ca^{2+} elevations to suppress leaf senescence. Gain- and loss-of-function AtBAG5 mutant plants revealed that, mitochondria Ca^{2+} elevation significantly increase chlorophyll retention and decrease H_2O_2 level in dark-induced leaf senescence assay. Based on our findings, we proposed a molecular mechanism in which chronic mitochondria Ca^{2+} elevation reduced ROS levels and thus inhibits leaf senescence.

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1. Introduction

Ca^{2+}, as the second messenger, plays an important role in various signaling pathway from life to death [1]. Mitochondria function as signaling nodes to transduce intracellular Ca^{2+} signals [2]. When intracellular Ca^{2+} concentration rise, mitochondria take up large amounts of Ca^{2+} to avoid Ca^{2+} cytotoxicity and subsequently release Ca^{2+} via different routes [3]. However sustained mitochondria Ca^{2+} accumulation has long been recognized known as a canonical cell death trigger [4,5]. Mitochondria Ca^{2+} overload causes the prolonged opening of the mitochondrial permeability transition pore (mPTP), which leads to the release of mitochondria protein, including cytochrome c or apoptosis inducing factor into cytosol, eventually resulting in cell death [6].

Plant senescence occurs at various levels. At organ level, the most obvious phenomenon is the changes in leaf color and subsequent the death of the leaves [7]. During leaf senescence, the cells will undergo a series of changes about cellular metabolism and the cell structure will degrade in an orderly manner. Initially, the chloroplast DNA degrade and chloroplast shrinkage, followed by the catabolism of macromolecular such as nucleic acids, protein, lipid and mitochondria remain intact until the final stage of leaf senescence [8]. Once mitochondria dysfunction, it will produce much mitochondria ROS and loss its transmembrane potential. Previous studies reported that Ca^{2+}/Calmodulin and Calmodulin-binding protein maybe involved in the mechanism of plant senescence [9]. Until recently, CaM/BAG5/Hsc70 signaling complex in mitochondria have been identified to regulate leaf senescence [10].

Heat-shock 70 protein (Hsc70) is a molecular chaperone and folding catalyst that consist of a 45-kDa N terminal ATPase and a 25 kDa C-terminal substrate binding domain and plays a central role in mitochondria protein trafficking and folding process. Hsc70 is also known to play an anti-apoptotic role in eukaryotic programmed cell death (PCD) [11]. Overexpression of Hsc70 has been reported to protect against Aβ(21–35)-induced neuronal apoptosis through inhibiting both caspase-dependent and caspase-independent PCD pathways [12]. Additionally, overexpression of Hsc70 in rice protoplasts suppressed programmed cell death by maintaining membrane potential and inhibiting ROS production [13]. The BAG (Bcl-2-associated athanogene) proteins are among the major co-chaperones of Hsc70 that regulate a series of physiological process ranging from proliferation to cell death. BAG5 is a member of BAG protein family in *Arabidopsis thaliana*, which localizes in mitochondria. In the crystal structure of *Arabidopsis*

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thaliana BAG5(AtBAG5) [10], it contains two protein interaction domains: the IQ motif at the N terminal for CaM binding and BAG domain at the C terminal for Hsp70/Hsc70 binding. As described in our previous study [10], AtBAG5 overexpression plant (*OxBAG5*) exhibited early senescence phenotypes compared with *bag5-1/bag5-2* mutant plants (AtBAG5-knockout) in dark-induced senescence experiment. Ca^{2+}-saturated Calmodulin (holo-CaM) and Hsc70 bind to AtBAG5 with negative cooperativity according to the result of Isothermal Titration Calorimetry (ITC) experiment [10]. However, the specific role of mitochondria Ca^{2+} signaling in plant senescence has not been characterized clearly.

2. Materials and methods

2.1. Plant materials

The *Bag-1/Bag-2*, *OxBAG5*, IQR and SS plants were prepared as previously described [10]. T-DNA insertion alleles for AtBAG5 (SALKseq_037369 and SALKseq_205784C) were obtained from the *Arabidopsis* Biological Resource Center (ABRC), RT-PCR indicated that AtBAG5 transcription was not detected in the *bag5-1/bag5-2* mutants. *OxBAG5* plants were AtBAG5 overexpression lines. IQR mutant were mutant in IQ motif which disrupting the interaction between AtBAG5 and apo-Calmodulin. Similarly, SS mutant were mutant in BAG domain which disrupting the interaction between AtBAG5 and Hsc70.

2.2. Leaf senescence assays

The experiment were carried out by the procedure as described in previous study [10]. 5th and 6th detached rosette leaves were placed into plate, immersed in buffer containing 3 mM MES (pH 5.7) with or without 20 mM $CaCl_2$ at 22 °C for dark-induced senescence experiment. Images were taken after 0, 3 and 5 days.

2.3. Chlorophyll assays

The 5th and 6th leaves were cut from 30-day-old plants, the leaves were weighted, chlorophyll were extracted on day 0 and after 5-days dark treatment in buffer with or without 20 mM $CaCl_2$. Chlorophyll extraction and quantification were prepared following an established protocol [10].

2.4. Analysis of H_2O_2 accumulation

The 5th and 6th leaves were immersed in DAB solution (1 mg/mL, pH 3.8) overnight after 5-days dark treatment in buffer with or without $CaCl_2$ for analysis of H_2O_2 accumulation, as previously described [10].

2.5. Mitochondria calcium measurements

Rosette leaves from 4-to-5-week-old *Arabidopsis* Col-0 plants were excised and randomly allocated into the calcium treatment group (T) or the control group (C). Leaves for calcium treatment were soaked in treatment buffer (TC5) containing 100 mM KCL, 10 mM MES-KOH, pH 6.15, and 5 mM $CaCl_2$ for 5 days at 22 °C in the dark. Leaves were then transferred into treatment buffer with 5 μM Rhod-2 AM (Abcam Company, USA) and 300 μM neostigmine methyl sulfate (Sigma Aldrich, USA) for 3 h at 22 °C in the dark for dye loading. Afterwards, Rhod-2-loaded leaves were rinsed with treatment buffer and re-incubated at 22 °C in the dark for 1 h. Each leaf was transferred into 24-well plate (BD biosciences) with 1 mL of treatment buffer in each well before fluorescence measurement.

Fluorescence was monitored on a Synergy 4 Microplate Reader (BioTek Laboratories, Inc., USA) at 550 nm excitation and 582 nm emission. The control group was treated and measured following the same procedure except that no $CaCl_2$ was included into the buffer (TC0).

$[Ca^{2+}]_{mito}$ was calculated according to the following formula: $[Ca^{2+}] = K_d*(F-F_{min})/(F_{max}-F)$, where F is the fluorescence of the indicator at the experimental Ca^{2+} levels, F_{min} is the fluorescence in the absence of Ca^{2+} and F_{max} was determined by the addition of 20% ethanol and 500 mM Ca^{2+}, and F_{min} was determined by the addition of 5 mM EGTA, where the K_d of Rhod-2 was 570 nM.

3. Results

3.1. Extracellular Ca^{2+} treatment suppresses premature senescence

To investigate how mitochondrial Ca^{2+} elevation affects AtBAG5-induced leaf senescence, leaves were treated with $CaCl_2$ soaking to manipulate chronic Ca^{2+} elevation inside mitochondria for five days (Fig. 1A). Rhod-2 fluorescence dye were then used to quantify the mitochondrial Ca^{2+} levels. As we expected, extracellular Ca^{2+} treatment indeed increased mitochondrial basal Ca^{2+}

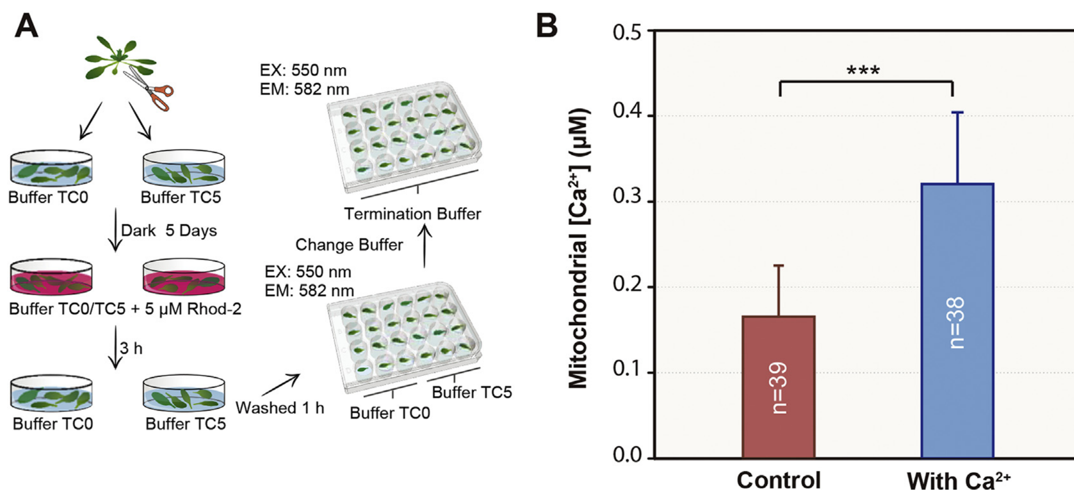

Fig. 1. Extracellular calcium treatment increases mitochondrial Ca^{2+} level. (A) Schematic representation of mitochondrial Ca^{2+} measurements for the extracellular Ca^{2+} treatment experiment. **(B)** Quantitative values of the Ca^{2+} concentration before and after Ca^{2+} treatment.

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