

DETECTION OF APOPTOSIS IN CHLOROPLASTS AND NUCLEI IN DIFFERENT GRAVITATIONAL ENVIRONMENTS

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INTRODUCTION

Plant cells either die by "accident" (traumatic cell death) or by "design" (programmed cell death; PCD). There is clear evidence that cell death during plant development and interactions with the environment involves PCD (in Gray and Johal, 1998). *K. daigremontiana* reproduces asexually by forming plantlets from leaf indentations which fall to soil and convert into adult plants. In nature, its entire plant body except leaf-plantlets senesces as consequence of floral differentiation or stressful environmental conditions. At unit gravity, PCD precedes plantlet detachment from the mother-leaf, leading to an abscission scar after plantlet fall. Earlier experiments have shown that leaf-plantlet formation and asexual reproduction increased with short duration hypergravity treatments and decreased in simulated hypogravity (Pedroso and Durzan, 1998).

The present experiments were designed to determine if and what type of cell death occurs following gravitational changes, and the sequence of events leading to it.

Our study shows that changes in gravitational environment cause a burst in nitric oxide, followed by a sequence of events that may ultimately led to programmed cell death by apoptosis.

METHODS

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In vitro cultured leaf-plantlets and leaves of *Kalanchoë daigremontiana* were exposed to hypergravity (20 to 300 xg; 15 days and 10-60 minutes, in centrifuges) in darkness, and to simulated hypogravity (2×10^{-4} xg; 15 days, in 1 r.p.m. clinostats), in darkness and under 16h light photoperiod. Drastic hypergravity treatments were chosen to ascertain that cell death was of programmed and not of traumatic origin. Controls at 1 xg were kept under the same light conditions for identical periods of time. Leaves, collected immediately after each treatment (T0) and 24h after exposure to 200 xg for 10, 30 and 60 minutes, were processed for fluorescence and confocal microscopy. *In situ* detection of DNA fragmentation in leaf sections was performed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) of DNA 3'-OH groups followed by counterstaining with 4,6-diamidino-2-phenyl indole dihydrochloride (DAPI) (Havel and Durzan, 1996). Negative (no enzyme) and positive (Dnase-I) controls performed as expected. Merged and 3D reconstruction images from sequence series of optical sections were obtained. Fresh leaves were incubated for 1h, at 25°C, with 10 μ M 4,5-diaminofluorescein diacetate for detection of nitric oxide formation after 10, 30 and 60 minutes exposure at 200 xg. Sodium nitroprusside (SNP; a nitric oxide donor) and N^G-monomethyl-L-arginine (NMMA; a nitric oxide synthase inhibitor) were used to show that nitric oxide formation was correlated with DNA fragmentation. Experiments were repeated at least 4 times; 4-6 clonal leaves were randomly collected per experiment, and 3-5 sections/leaf were used for quantifying cell death and nitric oxide formation.

RESULTS

The index of DNA fragmentation and cytological evidence showed that changes in gravitational environment can lead to programmed cell death in epidermal (mainly guard cells) and mesophyll cells. PCD was higher in hypergravity and lower in simulated hypogravity, compared to controls cultured for 15 days at 1 xg. Chloroplastial and nuclear DNA fragmentation, chloroplast redistribution, nuclear condensation, marginalization of chromatin and formation of apoptotic bodies were observed. Acute hypergravity treatments (10, 30, and 60 min) showed that a burst in nitric oxide occurred immediately after treatment (mainly in chloroplasts), and that nucleoid DNA fragmentation occurred prior to fragmentation of nuclear DNA. Movement of chloroplasts toward the nucleus was evident. Increase of treatment duration led to an increase of irreversible nuclear DNA damage (Fig. 1).

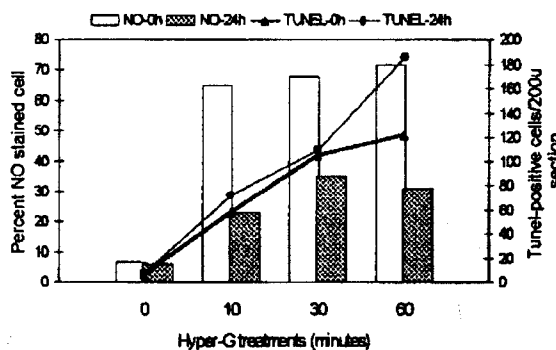


Fig. 1- Nitric oxide (NO) formation and DNA fragmentation (TUNEL-positive) in leaf cells of *K. daigremontiana*, immediately (0h) and 24h after leaf exposure to 200 xg for 10, 30 and 60 minutes. SD was lower than 7%.

Twenty four hours after the hyper-G treatments, nitric oxide levels were significantly lower, although higher than in

controls, whereas the number of TUNEL-positive cells was still increasing (Fig.1). Nitric oxide formation, DNA fragmentation, and consequently cell death, were increased at 1xg by the addition of SNP, and decreased, in hypergravity by NMMA.

DISCUSSION

Drastic hypergravity treatments induced programmed cell death by apoptosis. Changes in gravitational environment caused a burst in nitric oxide, which was followed by a sequence of events leading to chloroplast damage and degeneration, nuclear degradation and cell death by apoptosis. This sequence of events represents a senescence-like process. As earlier reported for leaf-plantlet formation and asexual reproduction (Pedroso and Durzan, 1998), apoptosis increased in hypergravity and decreased in simulated hypogravity.

Our results support the hypothesis that apoptosis (during senescence) and leaf-plantlet formation are correlated in this species, and that stressful gravitational environments trigger a "survival" process identical to that observed under natural conditions (1 xg). This study represents one of the first examples of the role of nitric oxide in response to gravitational stresses.

REFERENCES

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