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Ethylene produced by the endosperm is involved in the regulation of nucellus programmed cell death in *Sechium edule* Sw

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ABSTRACT

The nucellus is a maternal tissue that feeds the developing embryo and the secondary endosperm. During seed development the cells of the nucellus suffer a degenerative process early after fertilization as the cellular endosperm expands and accumulates reserves. Nucellar cell degeneration has been characterized as a form of developmentally programmed cell death (PCD).

In this work we analysed the role of the endosperm as main regulator of nucellus PCD. We demonstrated that endosperm produces high amount of ethylene, nitric oxide and indoleacetic acid. We examined the role of these small and diffusible signalling molecules in the regulation of nucellus PCD and we tried to elucidate how they can cooperate and regulate each other into the endosperm. We showed that ethylene acts a positive regulator of nucellus PCD and its synthesis can be in part induced by nitric oxide. High levels of IAA were detected both in the endosperm and in dying nucellus but this hormone is not directly involved in the execution of PCD.

1. Introduction

In flowering plants, the maternal tissue nucellus degenerates soon after fertilization supplying assimilates to the young embryo and to the growing endosperm. The endosperm expands and accumulates reserves while the nucellus is no longer present in mature seeds [1]. Nucellar cell degeneration has been described in many species as a form of developmentally regulated programmed cell death (PCD) being characterized by several PCD hallmarks such as chromatin condensation, profound alterations in nuclear shape, nuclear DNA fragmentation and caspase-like protease activation [2–4].

The squash *Sechium edule* Sw. represents an interesting model system to study the fate of the different tissues during seed development. In the large *S. edule* seed, autolysis of the nucellus after fertilization gradually leaves a cavity inside the ovule that is filled by both the growing endosperm and embryo. Endosperm expansion and nucellus degeneration are two synchronous events: the extent of nucellar degeneration follows the progression of endosperm growth and, during the whole process, the shape of the nucellar margin exactly matches that of the expanding endosperm.

The fine temporal and spatial regulation of this phenomenon requires strict coordination between maternal tissues degeneration and endosperm expansion. It is reasonable to hypothesise an active role for endosperm, however the signals responsible for the induction and the regulation of this degenerative process are largely unknown.

An integrated network of signals able to guarantee optimal communication between the tissues should be operational, in order to ensure the regulation and the correct execution of the whole process. The communication between the two tissues should rely on small and diffusible molecules, which can be synthesised in a tissue and translocated to another. Ethylene and nitric oxide (NO) having simple structures, small dimensions, and high diffusibility in aqueous and lipid environments, are key signalling molecules in the regulation of many plant processes [5–7] and might be good candidates for the role of messengers also in the communication between endosperm and other seed tissues.

Ethylene regulates a variety of plant processes from germination and growth to ripening and senescence (for review [8]). Ethylene has been implicated in stress response and in the regulation of programmed cell death, including aerenchyma and xylem formation, epidermal PCD above emerging roots, leaf and petal senescence, and PCD of seed tissues like endosperm (reviewed in [9]).

Abbreviations: c-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxide; DAF-2DA, diaminofluorescein-2 diacetate; DAPI, 4,6-diamidino-2-25 phenylindole; DTT, dithiothreitol; IAA, indoleacetic acid; L-NAME, N-nitro-L-arginine methyl ester; NAA, 1-naphthaleneacetic acid; NBD, 2,5-norbornadiene; PCD, programmed cell death; SNP, sodium nitroprusside; TUNEL, terminal dUTP nick-end labelling.

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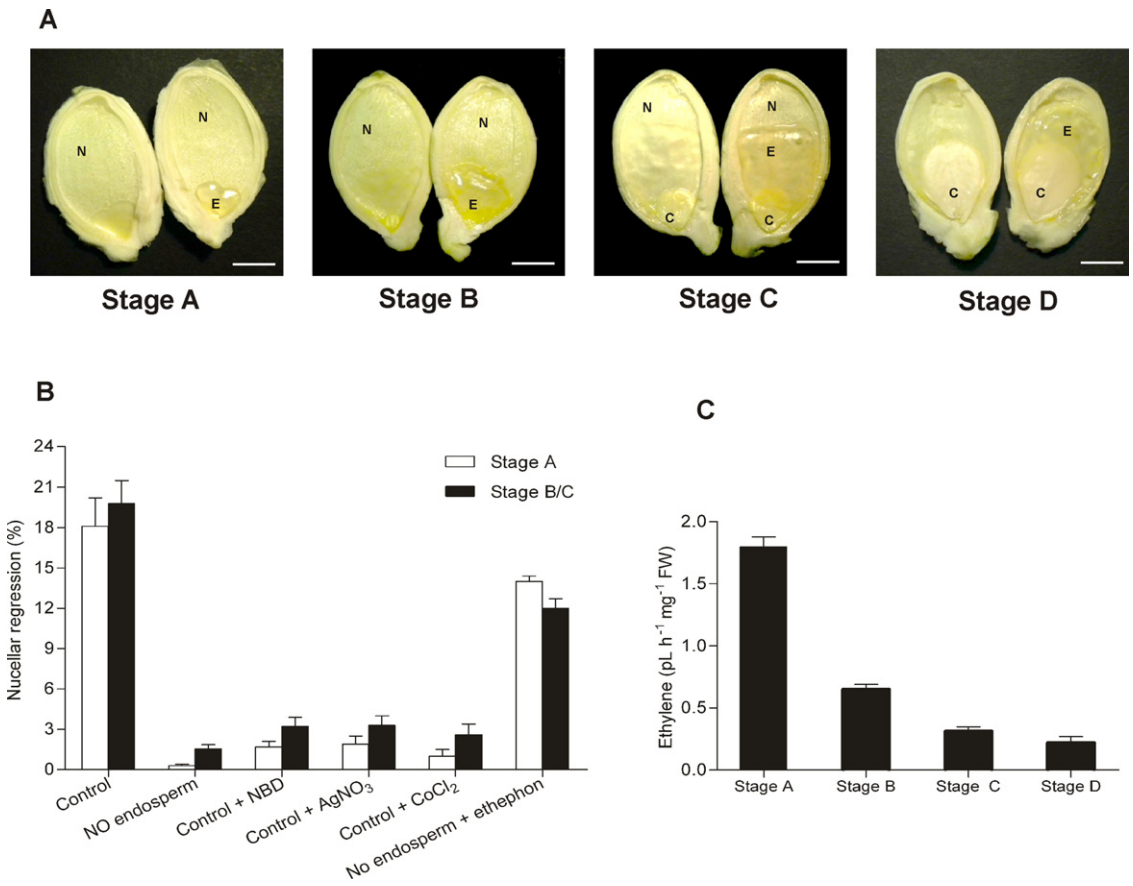

Fig. 1. (A) Stages of *Sechium edule* seed development, based on endosperm expansion. As the endosperm (E) expands and the embryo cotyledons (C) grow, nucellus (N) undergoes autolysis and gradually leaves space into the seed cavity. In stage D seeds nucellus is no more present and the underlying integuments are visible. Bars = 1 cm. (B) Nucellus degeneration measured *in vivo* as percentage of length shrinkage in 48 h. Seeds were longitudinally cut with a scalpel while still attached to the fruit and treated with 50 μL L^{-1} NBD, 15 μM $AgNO_3$ (ethylene perception inhibitors, both layered on the nucellus) and with 25 μM $CoCl_2$ (ethylene synthesis inhibitor, injected into the endosperm). The treatment with 100 μM ethephon, was made with a small piece of cotton wool placed in the cavity left after the removal of the endosperm. (C) Ethylene produced by the endosperm at different stages of development, expressed as pL h^{-1} mg^{-1} of fresh weight.

Gaseous and diffusible nitric oxide is often involved in the same processes regulated by ethylene, from seed germination and dormancy, to xylogenesis, flowering, fruit ripening and many cases of PCD [10–12]. NO, interplaying with ROS and hormones is a fundamental part of many signal transduction pathways [13], regulating enzyme activity by S-nitrosylation [14] and gene expression [15,16]. Cross-talk between nitric oxide, ROS, ethylene and other hormones is essential for the execution of PCD in many systems [17–19] and recently a major role of nitric oxide in PCD of the nucellus has been demonstrated [20].

The aim of this work was to investigate the role of endosperm as inducer and regulator of nucellus PCD. By using pharmacological and biochemical approaches, we analysed the importance of ethylene and NO produced by the endosperm as signalling molecules for nucellus degeneration. Moreover we tried to elucidate the interrelationship between NO, ethylene and other hormones like auxin in order to understand the ability of these molecules to cooperate, and to act synergistically in the induction of PCD in the nucellar tissue.

2. Materials and methods

2.1. Plant material and treatments

Plants of *S. edule* Sw. (Cucurbitaceae) were grown in the field in 2010 and 2011 from March to November and fruits harvested starting from the end of September.

Seeds were classified into four different stages based on endosperm growth (Fig. 1A): stage A (very small endosperm, length is 1/6 of the seed), stage B (endosperm length is 1/3 of the seed), stage C (it occupies more than half of the seed), and stage D (endosperm has almost filled the seed).

For *in vitro* treatments, seeds were cut longitudinally and the tissues were gently removed to be treated or to be stored at −80 °C for further analysis. For *in vivo* treatments, fruits were cut longitudinally to expose the big seed, which was gently cut on one side with a scalpel without being removed from the fruit. After treatment the fruit was closed, sealed with wax and kept in a humidified chamber to avoid dehydration.

To study the role of ethylene we used 100 μM ethephon, 50 μL L^{-1} 2,5-norbornadiene (NBD, competitive inhibitor of ethylene action) and 15 μM $AgNO_3$ (inhibitor of ethylene receptors); we injected 25 μM $CoCl_2$ (inhibitor of ethylene biosynthesis) directly into the endosperm. To study the role of nitric oxide we used 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxide (c-PTIO, nitric oxide scavenger), 200 μM sodium nitroprusside (SNP, nitric oxide donor) and 1 mM N-nitro-L-arginine methyl ester (L-NAME, nitric oxide synthase inhibitor) (all reagents are from Sigma, St Louis, MO, USA). For the study on indoleacetic acid translocation we injected 50 ng $[^{13}C]_6$ IAA (Cambridge Isotope Laboratories, Inc., Andover, MA) into the endosperms. To study the effect of auxin on nitric oxide production we treated the tissues with 10 μM 1-naphthaleneacetic acid (NAA). For each of the above treatments 30 fruits were used.