PLANTS RELATED TO EARLY EVOLUTIONARY EVENTS (BRYOPHYTES) CONTAIN LACANDONIA GRANULES PREVIOUSLY DISCOVERED IN FLOWERING PLANTS

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ABSTRACT
The early evolution of the plant kingdom is controversial, and bryophytes are argued to be the earliest divergent plants. Indeed, the origin and diversification of land plants marks an interval of unparalleled innovation in the history of plant life. Previous ultrastructural analysis of the nucleus revealed the presence of Lacandonia granules. Cytochemical, immunocytochemical and *in situ* hybridization studies suggested that Lacandonia granules are involved in mRNA storage. In addition, Lacandonia granules have been located in other flowering plants of the order Triuridales (such as *Triuris alata*) and also in non-flowering plants. Therefore, in order to understand the evolution of RNA processing, we demonstrated the presence of Lacandonia granules in three species of non-vascular plants (Bryophytes). In this study, we analyzed three species of Bryophytes using transmission electron microscopy and found granules in sporophyte cell nuclei of all species. Moreover, the presence of few granules in the interchromatin and perichromatin space was a constant feature of all nuclei of Bryophytes. Thus, non-abundant particles of around 32 nm in diameter were observed in the perichromatin and interchromatin space. Perichromatin fibers are usually present in continuity with granules, forming a fibrogranular environment. Finally, we demonstrated that these particles are positive after the EDTA regressive technique preferential for ribonucleoproteins. In summary, this study suggests that Lacandonia granules contribute to the understanding of the spatiotemporal organization of several mRNA processing factors in the nuclear subcompartments and verifies the conservation of the event throughout the evolutionary process in the Plant Kingdom.

Keywords: Lacandonia granules, non-vascular plants, nucleus, ribonucleoproteins.

RESUMEN
La evolución del reino vegetal es controversial y las briofitas han sido propuestas como uno de los grupos que divergió tempranamente dentro del proceso evolutivo. El origen y diversificación de las plantas terrestres marca un intervalo de innovación sin precedentes, un ejemplo es el descubrimiento *Lacandonia schismatica* por Martínez y Ramos, cuya característica principal es la inversión de sus órganos sexuales, la cual ha sido relacionada con eventos evolutivos. Estudios ultraestructurales de los núcleos de *Lacandonia schismatica* revelaron la presencia de una novedosa partícula llamada gránulo de Lacandonia. Análisis citoquímicos, inmunocitoquímicos e hibridación *in situ* sugirieron que los gránulos de Lacandonia están involucrados con el almacén y procesamiento del RNA mensajero. Los gránulos de Lacandonia se han localizado en otras angiospermas del orden triuridales (*Triuris alata*) y también dentro del grupo de las gimnospermas (*Ginkgo biloba*). Con el fin de comprender la evolución del procesamiento del RNA mensajero, hemos demostrado la presencia de los gránulos de Lacandonia en las plantas no vasculares (Briofitas). Se analizaron tres especies de Briofitas por microscopia electrónica de transmisión y encontramos gránulos en los núcleos de los esporofitos de todas las especies estudiadas. Se observaron partículas no abundantes de 32 nm de diámetro y fibras pericromatinianas en continuidad con dichos gránulos. Este estudio sugiere que los gránulos de Lacandonia contribuyen a la comprensión de la organización espacio-temporal de algunos factores que intervienen en el procesamiento del ARN mensajero y evidencia la conservación del evento a lo largo del proceso evolutivo en el reino vegetal.

Palabras clave: Gránulos de Lacandonia, plantas no vasculares, núcleo, ribonucleoproteínas.

INTRODUCTION

Phylogenetic analyses have demonstrated that embryophytes are monophyletic, and that charophycean green algae (possibly *Chara* or *Nitella*) are likely their closest sister taxa [1]. Although the early evolution of the Plant Kingdom is controversial, liverworts are argued to be the earliest divergent plants [2]. The relationships between the remaining bryophyte clades remain unsettled. Recently [3], multigene analyses suggest hornworts as sisters to vascular plants. These phylogenies suggest that bryophytes existed earlier than vascular plants [4].

The origin and early diversification of land plants marks an interval of unparalleled innovation in the history of plant life. From a simple plant body consisting of only a few cells, land plants (liverworts, hornworts, mosses and vascular plants) evolved an elaborate two-phase life cycle and an extraordinary array of complex organs and tissue systems. Specialized sexual organs, stems with an intricate fluid transport mechanism, structural tissues, epidermal structures for respiratory gas exchange, leaves and roots of various kinds, diverse spore-bearing organs, seeds, and the tree habit had all evolved by the end of the Devonian period. These and other innovations led to the initial assembly of plant-dominated terrestrial ecosystems, and had a great effect on the global environment [5].

In fact, the evolution of plant development can be studied in many different ways, each of which provides new insights into how plants have been modified over evolutionary time [6]. In this regard, the discovery of *Lacandonia schismatica* by Martínez and Ramos [7] from the Selva Lacandona, in Chiapas, México, whose most prominent characteristic is the spatial inversion of its sexual organs, may be related to evolutionary changes [8]. *L. schismatica* was once considered an endemic plant [9]; however, it is now under special protection [10].

In particular, Jiménez-García [11] discovered that the cell nucleus of *L. schismatica* is reticulated and is associated with a novel 32 nm-diameter nuclear ribonucleoprotein (RNP) particle, which they called the Lacandonia granule. Interestingly, this nuclear arrangement is also displayed by many related plants such as *Triuris brevistylis*.

Further studies found that Lacandonia granules are distributed widely in the Plant Kingdom. In angiosperms, the granules are present in *L. schismatica* and in other species of Triuridales such as *Triuris alata and Sciaphyla picta;* they also are present in gymnosperms, such as *Ginkgo biloba (Ginkgoaceae)*.

Developmental, immunocytochemical and high resolution *in situ* hybridization results suggest that Lacandonia granules are nuclear particles similar to the perichromatin and Balbiani ring granules present in mammalian and insect cells, respectively [12].

In fact, Vázquez-Nin and Echeverría [13] observed that Balbiani ring and perichromatin granules are nuclear RNP particles thought to be involved in intranuclear mRNA metabolism for storage and/or transport and also in the last steps of pre-mRNA processing.

Additionally, Jimenez-Ramirez [14] used electron and atomic force microscopy to show that the nuclei of *G. biloba* are reticulated. Furthermore, based on ultrastructural evidence such as size, distribution, cytochemical results and in situ hybridization to detect total RNA, they concluded that Lacandonia granules are present in the nuclei of non-flowering plant *G. biloba*.

Further atomic force microscopy (AFM) analysis in the contact mode provided sufficient resolution to visualize and analyze the nanometric structure and organization of granules [15]. Moreover, Fragoso-Soriano [16] showed

that visualization of the structural details of cellular organelles of *L. schismatica* can be enhanced using an atomic force microscope in the feedback loop error signal mode, compared to the constant force mode, which is more widely used. This marks a very important step toward an ever better understanding of the structure and role of the main nuclear sub-compartments.

Therefore, in order to understand the evolution of RNA processing, our research objective for the present study was to demonstrate the presence of Lacandonia granules in three species of non-vascular plants (Bryophytes). The species tested were *Marchantia polymorpha* (Liverworts), *Polytrichum juniperinum* (Mosses) and *Anthoceros punctatus* (Hornworts).

MATERIALS AND METHODS

Samples

Three species were collected in Los Dinamos National park, Magdalena Contreras, México. Small fragments of young sporophytes from *M. polymorpha, P. juniperinum* and *A. punctatus* were used for the present study.

Transmission electron microscopy

Sample preparation was conducted according to standard protocols [14]. Briefly, small fragments of sporophytes were fixed for 1 h at room temperature in a mixture of 6% glutaraldehyde and 4% paraformaldehyde, buffered in PBS (pH 7.2). Post-fixation was performed in 1% osmium tetroxide overnight. Samples were subsequently dehydrated in a graded series of ethanol and embedded in an epoxy resin. Thin sections of 60–90 nm thickness were contrasted with uranyl acetate and lead citrate.

We also used the EDTA regressive method for RNP originally described by Bernhard [17], with modifications for plants [18]. Again, small fragments of sporophytes were fixed for 1 h at room temperature in a mixture of 6% glutaraldehyde and 4% paraformaldehyde, buffered in PBS (pH 7.2), however, without osmium tetroxide postfixation. Then samples were dehydrated in a graded series of ethanol and embedded in an epoxy resin. Thin sections were contrasted using uranyl acetate for 3 minutes, followed by EDTA treatment for 18 minutes and finally lead citrate for 3 min.

All the preparations were observed in a Jeol JEM-1010 electron Microscope operating at 80kV.

RESULTS

Granules are present in Bryophyte cell nuclei of sporophytes by standard transmission electron microscopy. The non-abundant particles of around 32 nm in diameter were observed in the perichromatin and interchromatin space. As internal comparative structures, we showed ribosomes in the cytoplasm of cells of all three studied species. The granules are about twice the size of ribosomes (see Figs. 1-3, 6).

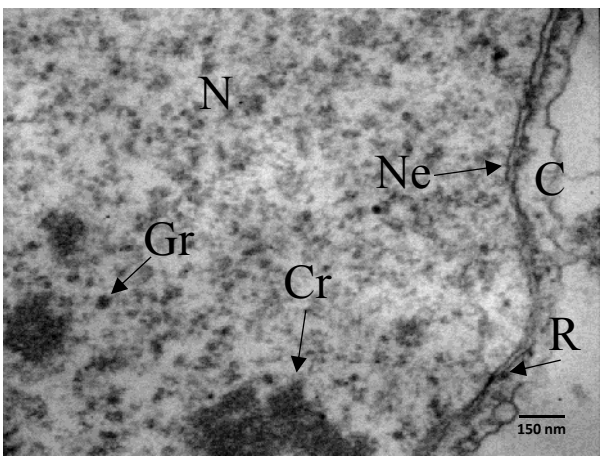

Fig. 1. Electron micrograph, *M. polymorpha* nucleus after uranyl acetate-lead citrate contrast, shows fibro-granular environment with Lacandonia granules twice the size of ribosomes. Nucleus (N), nuclear envelope (Ne), cytoplasm (C), chromatin (Cr), granule (Gr) and ribosome (R).

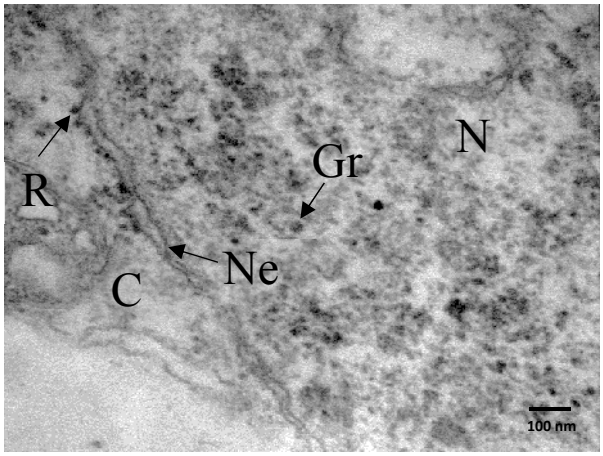

Fig. 2. Electron micrograph *P. juniperinum* nucleus after uranyl acetate-lead citrate contrast. Ribonucleoprotein particles (Ribosomes) in the cytoplasm were used to compare size versus granules. Nucleus (N), nuclear envelope (Ne), C (cytoplasm) granule (Gr) and ribosome (R).

The presence of few granules in the interchromatin and perichromatin space was a constant feature of all nuclei of Bryophytes, which differs from granules previously found in *L. schismatica* and *T. brevistylis,* which were abundant. Other RNP components were also observed as fibers intermingled with granules (as shown in Figs. 4 and 5).

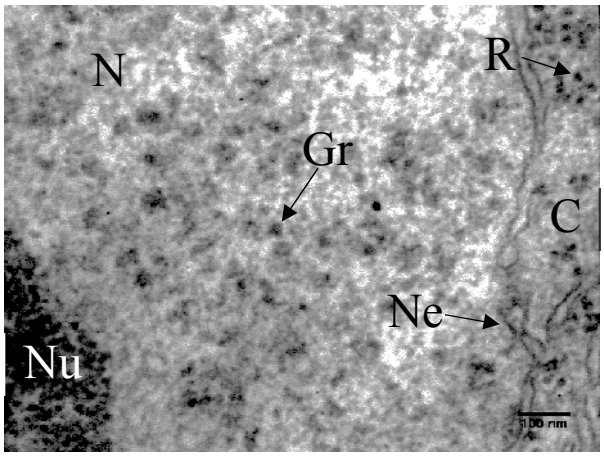

Fig. 3. Electron micrograph provides relation between granules and ribosomes; as a rule, granule is twice the size of a ribosome. A. punctatus nucleus after uranyl acetate-lead citrate contrast. Nucleus (N), nucleolus (Nu), nuclear envelope (Ne), cytoplasm (C), granule (Gr) and ribosome (R).

Ultrastructural analysis showed non-abundant and dispersed intranuclear particles (32 nm in diameter) in the nucleoplasm. These particles are present in Liverworts, Mosses and Hornworts; in addition, EDTA regressive staining suggested that the granules contain RNA (evidence for this is provided in Figs. 4-6).

Perichromatin fibers are usually present in a compact chromatin border in continuity with granules, forming a fibrogranular environment (displayed in Figs. 4 and 5).

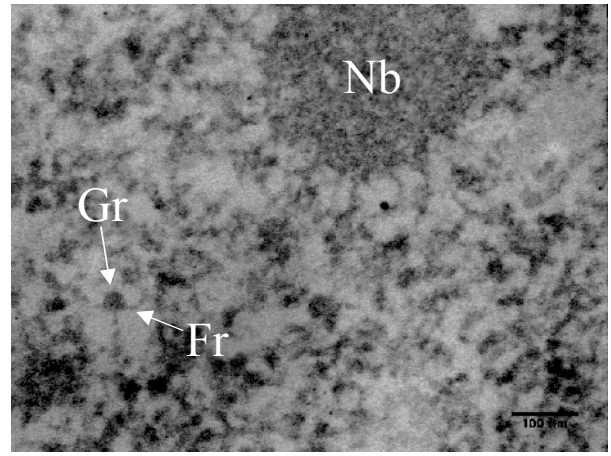

Fig. 4. Electron micrograph, *M. polymorpha* nucleus after EDTA regressive method for RNPs shows a Lacandonia granule associated with fibers. Granule Gr), nuclear body (Nb), fibers (Fr).

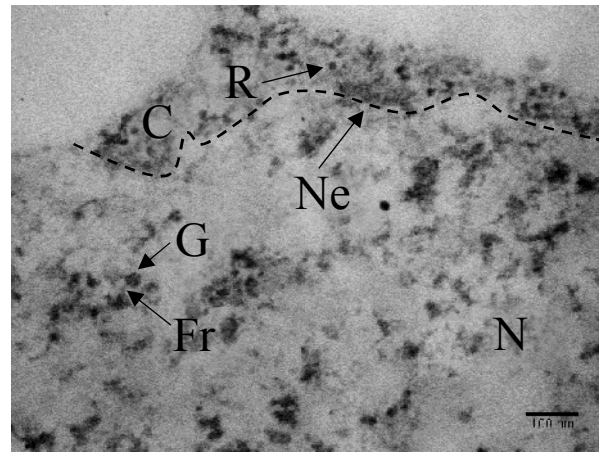

Fig. 5. Electron micrograph, *P. juniperinum* nucleus after EDTA regressive method for RNPs reveals fibrogranular environment of Lacandonia granules. Nucleus (N), nuclear envelope (Ne), cytoplasm (C), Granule (Gr), fibers (Fr), ribosome (R).

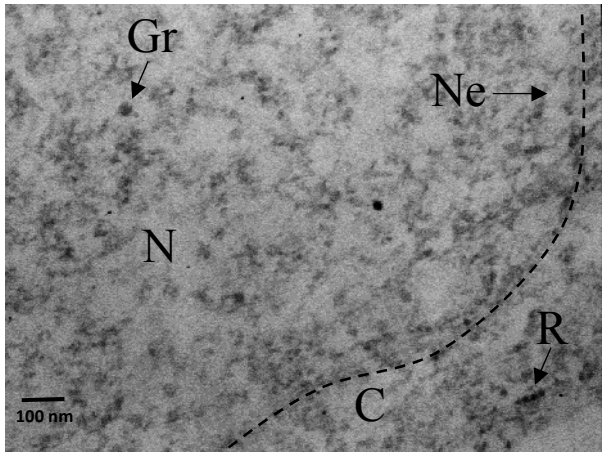

Fig. 6. Electron micrograph provides evidence of solitary particles (32 nm in diameter) in Hornworts. *A. punctatus* nucleus after EDTA regressive method for RNPs. Nucleus (N), nuclear envelope (Ne), cytoplasm (C), granule (Gr) and ribosomes (R).

DISCUSSION

We have found ultrastructural data for the presence of Lacandonia granules within cell nuclei of different species of Bryophytes. First, 32-nm diameter intranuclear granules are present in the nucleoplasm of different cells; in addition, Lacandonia granules are intermixed with RNP fibers; this suggests similitude to perichromatin and interchromatin granules where a structural continuity with RNP fibers is observed [19].

Second, in Bryophytes, Lacandonia granules are distributed in the perichromatin and interchromatin spaces. These characteristics have been previously reported [11]. However, the distribution of Lacandonia granules in both the perichromatin and interchromatin spaces, as well as the presence of SR proteins and RNA poly(A)+, suggest a similarity to intranuclear RNPs (Perichromatin and Balbiani ring granules) [12] .

Third, cytochemical results indicate that these particles are positive after the EDTA regressive technique that is preferential for RNPs; it is based on the proposition that after staining ultrathin sections with uranyl the stain is preferentially removed from DNA rather than RNA by the action of the chelating agent EDTA [17].

Although Lacandonia granules are present in the nuclei of Bryophytes, they are generally few in number and solitary. Specifically, they do not present the high numbers of granules in typically clumped distribution observed in *T. brevistylis*, *L. schismatica* [11] and *G. biloba* [14]. By contrast, characteristics such as form, size and general composition of RNPs are common to the Lacandonia granules seen in Bryophytes.

Lacandonia granules are present in the vascular plant species studied, and they have been located in other flowering plants of the Triuridales order (such as *T. alata*) [11] and in non-flowering plants (such as the gymnosperm *G. biloba*) [14]. The present results extend the presence of Lacandonia granules to members of Bryophytes; this information demonstrates that these granules are ubiquitous structures in the Plant Kingdom, which suggests that their ancestry predates the origin of the group.

CONCLUSIONS

We have confirmed the following ultrastructural and cytochemical evidence; first, by transmission electron microscopy we found intranuclear granules measuring on average, 32 nm in diameter; second, they are mixed with fibers; and third, these granules are distributed in the perichromatin and interchromatin spaces. These three characteristics have been previously reported [11, 14]. Fourth and finally, we have demonstrated that these particles are positive after the EDTA regressive technique preferential for RNPs; therefore, we conclude that Lacandonia granules are present in the nuclei of Bryophytes.

In brief, this study suggests that Lacandonia granules contribute to the understanding of the spatiotemporal

organization of several mRNA processing factors in the nuclear subcompartments and verifies the conservation of the event throughout the evolutionary process in the Plant Kingdom.

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