MARIA CAMILA MEDINA MONTES

DESENVOLVIMENTO DE LATICÍFEROS: ULTRAESTRUTURA, CITOQUÍMICA E EXPRESSÃO GÊNICA

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Maria Camila Medina Montes

Desenvolvimento de laticíferos: ultraestrutura, citoquímica e expressão gênica

Development of laticifers: ultrastructure, cytochemistry and gene expression

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ABSTRACT:

Laticifers are internal secretory structures, which produce and store latex; a complex secretion in plants made up of a wide mixture of compounds synthesized from both primary and secondary metabolism. The study of laticifers development necessarily includes observing the cell wall and the latex, because the definition of laticiferous is based on its structure and the nature of its secretion. Recent works still refers to anastomosed laticifers as growing intrusively, and the mechanisms involved in anastomosis described until now in some cases overlap with the type of growth. On the other hand, gene expression studies are mainly focused on latex composition and constituents related to rubber production, however, studies focused on the development of laticifers with a spatiotemporal approach remain poorly understood. In this work, we perform a comparative immunocytochemical study to evaluate the type of growth in articulated laticifers, with a representative for nonanastomosing, anastomosing unbranched, and anastomosing branched laticifers based on the pattern of microtubules. We observed that laticifers of the three different types presented microtubules with a pattern of organization typical of cells that grow diffusely. We also analyzed the structure, ultrastructure, and transcriptome of unbranched anastomosed laticifers of *Ipomoea nil*, which have not been reported as growing intrusively. Additionally, we analyzed the chemical composition of the chloroform fraction of latex of stems and petioles. We observed that anastomosing unbranched laticifers of *I. nil* have an early differentiation as is characteristic of laticifers as a whole, but in this species anastomosis occurs late. This allowed dividing the study of the transcriptome into two phases: 1) before and during the anastomosis and 2) after the anastomosis. This process recruits a diversity of enzymes that digest components of the wall, and proteolytic enzymes that participate in the autophagy process, responsible for the transformation of the protoplast. The wall of the laticifer has a suberin layer that, together with the cell wall, are dissolved. The latex components are produced at different times of development, but in a narrow time frame, since the development of laticifers occurs rapidly.

Keywords: laticifers, development, anastomosis, cell wall, type of growth.

RESUMO:

Laticíferos são estruturas secretoras internas, que produzem e armazenam látex, uma secreção complexa composta por uma ampla variedade de compostos sintetizados tanto no metabolismo primário quanto no secundário. O estudo do seu desenvolvimento deve passar necessariamente pela observação da parede celular e do látex, pois a definição de laticífero é baseada em sua estrutura e na natureza de sua secreção. Trabalhos recentes ainda se referem a laticíferos anastomosados como crescendo de forma intrusiva, e os mecanismos envolvidos na anastomose descritos até o momento, em alguns casos, se sobrepõem aos mecanismos envolvidos no seu tipo de crescimento. Por outro lado, os estudos de expressão gênica são focados principalmente no látex e componentes relacionados à produção de borracha, entretanto, são poucos os estudos focados no desenvolvimento de laticíferos com uma abordagem espaço-temporal. Neste trabalho, realizamos um estudo imunocitoquímico comparativo para avaliar o tipo de crescimento em laticíferos articulados, com um representante para não anastomosados, anastomosados não ramificados e anastomosados ramificados baseado no padrão de organização dos microtúbulos. Observamos que os laticíferos dos três diferentes tipos apresentaram microtúbulos com padrão de organização típico de células que crescem difusamente. Também analisamos a estrutura, ultraestrutura e transcriptoma de laticíferos anastomosados não ramificados de *Ipomoea nil*, que não foram relatados como crescendo intrusivamente. Adicionalmente analisamos a composição química da fração clorofórmica do látex dos caules e pecíolos. Notamos que laticíferos não ramificados anastomosados de *I. nil* diferenciam-se rapidamente como é característico de laticíferos como um todo, mas a anastomose ocorre tardiamente. Isso permitiu dividir o estudo do transcriptoma em duas fases: 1) antes e durante a anastomose e 2) após a anastomose. Esse processo recruta uma diversidade de enzimas que digerem componentes da parede e enzimas proteolíticas que participam do processo de autofagia, responsáveis pela transformação do protoplasto. A parede do laticífero possui uma camada de suberina que, juntamente com a parede celular, é anastomosada. Os componentes do látex são produzidos em diferentes momentos de desenvolvimento, mas em um intervalo de tempo estreito, uma vez que o desenvolvimento dos laticíferos ocorre rapidamente.

Palavras-chave: laticíferos, desenvolvimento, anastomose, parede celular, modo de crescimento.

GENERAL INTRODUCTION:

Plants are continuously exposed to diverse of biotic and abiotic damages, and as sessile organisms, they have developed innovative defense mechanisms against herbivorous and pathogenic microorganisms (Wink, 2008a; Parrotta et al., 2016). One of these innovations is the synthesis of an enormous and diverse secondary metabolites which can deter, poison, or repel herbivores and even inhibit the growth and development of other organisms (Wink, 2008a, 2010; Berni et al., 2019). Even some insects sequester the secondary metabolites produced by plants to protect themselves against predators (Wink, 2008b; Petschenka and Agrawal, 2016; Ramos et al., 2020).

It is common secondary metabolites be accumulated in entire organs or diverse tissues where they are stored in intracellular or extracellular spaces (Guern et al., 1987; Wink, 2010) and this storage can also be tissue or cell specific (Guern et al., 1987). Thus, plants have evolved specialized structures that can produce and store a large quantity of compounds. Moreover, the compounds are secreted through specialized mechanisms and can be released crossing the cell wall and cuticle or by injury. The secretions are not reused by the plant (Fahn, 1988). These various structures are called secretory structures and can be external, or internal (Crang et al., 2018), some of them having a defense function.

In relation to secretory structures with defense function, we can highlight trichomes, as external secretory structures located in the epidermis, which have diverse sizes and shapes and the capacity of producing diverse compounds secreted to the surface, establishing a protective barrier against herbivory (Levin, 1973; Tissier, 2018; Lu et al., 2021; Medina et al., 2021a). Inside the organs, secretory structures can be multicellular, formed by an epithelium which secretes many compounds into intercellular spaces, such as ducts and cavities, or single cells, such as idioblasts

and laticifers (Fahn, 1979, 1988; Tissier, 2018). The latter, despite being a single cell, can produce and accumulate a large quantity of both hydrophilic and lipophilic compounds (Wink, 2010).

Latescent plants have their laticifers distributed in all organs (Metcalfe and Chalk, 1950; Demarco et al., 2006; Demarco and Castro, 2008). They may be originated from the ground meristem and/or procambium during primary growth and from vascular cambium in the secondary growth (Pace et al., 2019; Medina et al., 2021b; Salomé et al., 2022).

Historically, laticifers have been classified in two types. Non-articulated laticifers have a unicellular origin, consisting in a coenocytic structure that can be branched or unbranched (Mahlberg, 1959, 1963; Mahlberg and Sabharwal, 1968; Wilson and Mahlberg, 1980a; Inamdar et al., 1988; Murugan and Inamdar, 1989; Serpe et al., 2001; Castelblanque et al., 2016) and articulated laticifers that have a multicellular origin, formed by a row of cells (Hagel et al., 2008). Anatomical studies of vegetative and floral apices and embryos showed that laticifers misclassified as non-articulated were actually articulated anastomosing laticifers with a multicellular origin in the ground meristem and procambium, becoming a tube-like structure since their terminal walls are early degraded (Milanez, 1978; Demarco et al., 2006, 2013; Demarco and Castro, 2008; Lopes et al., 2009; Demarco, 2015; Canaveze and Machado, 2016; Gama et al., 2017). Therefore, the distinction between articulated and non-articulated laticifers has no basis to support it. Here, we focused in anastomosing laticifers.

The identification of modifications in cell walls is important to differentiate different types of articulated laticifers. This is a relevant taxonomic character because the type of laticifers is constant in each taxon (Simpson, 2010; Demarco et al., 2013). Thereby, when terminal walls remain intact they are called non-anastomosing but if their terminal walls are degraded, partially or completely, they are called anastomosing (Mahlberg, 1961; Fahn, 1979). Anastomosing

laticifers can also be branched through lateral anastomosis between two laticifers, forming a system of interconnected tubes through the entire plant (Ramos et al., 2019). Laticifer ramifications can also been formed through the addition of adjacent meristematic cells into laticifer system, where cell walls of contact are degraded and then the protoplast are joined (Milanez, 1977, 1978). It seems that the incorporation of new meristematic cells to laticifer system is posterior to the differentiation of an established initial row (Canaveze et al., 2019) but the mechanisms by which the newly added cell is induced to differentiate into laticifer remains unclear.

One of the most discussed questions about laticifers is how they grow. The hypothesis that laticifers grow within the plant in an intrusive way has been applied initially to non-articulated ones. For a long time, it was believed that a single cell has the ability to grow through the entire plant, being the longest plant cell type (Mahlberg, 1963; Wilson and Mahlberg, 1980b; Murugan and Inamdar, 1989; Castelblanque et al., 2016, 2017). The intrusive growth of laticifers would occur through the expansion of their apices in intercellular spaces associated with the dissolution of the middle lamella between the laticifer and the adjacent cells. As mentioned, description of non-articulated laticifers is the result of a misinterpretation, nevertheless the hypothesis of intrusive growth has been maintained as an explanation for the type of growth of some articulated anastomosing branched laticifers (Lopes et al., 2009; Canaveze and Machado, 2016; Canaveze et al., 2019). However, anatomical analyses have revealed that the laticifer apices are observed close to the promeristem, where there are no intercellular spaces (Demarco et al., 2006). Conversely, approaches that specifically reveal the type of growth of the laticifers are lacking.

Enzymatic digestion of middle lamella is an important process that have been related to both anastomosis and intrusive growth (Marinho and Teixeira, 2019) and ultrastructural analyses have labeled cellulases and pectinases in the cell walls and vacuoles of laticifers, as well as in

adjacent cells (Allen and Nessler, 1984; Marinho and Teixeira, 2019). Latex serves as a strong defense for plants, and the high amount of diverse enzymes is common in latex (Konno et al., 2004; Agrawal and Konno, 2009; Wasano et al., 2009; Konno, 2011; de Freitas et al., 2016; Ramos et al., 2019, 2020; Cruz et al., 2020). However, it is not clear if the digestive enzymes detected in anastomosing laticifers are the product of the digestion of their terminal walls or a latex component with defensive function. The identification of enzymes related to laticifer development are needed.

During their development, laticifers produce a large quantity of compounds that form the latex. Latex production is phenotypically plastic (Agrawal and Van Zandt, 2003; Agrawal and Konno, 2009) because the environment influences the production of its components, including the herbivores. Depending on the taxa, each component can be present in more or less quantity, be absent, or exclusive for some species (Hagel et al., 2008; Rasmann et al., 2009; Patten et al., 2010; Mithöfer and Boland, 2012; Ramos et al., 2020). Important findings have also been obtained about bearing-latex plants in relation to responses to abiotic stress (Tan et al., 2017) and plant defense against pathogens (Fang et al., 2016; Havanapan et al., 2016; Montoro et al., 2018). Nevertheless, it is unknown if all components of the latex are produced at the same moment or if there are components produced later.

In this work, we studied the development of anastomosing laticifers using an integrative approach in order to understand how laticifers grow within the plant and to identify the expression profile in different moments of development. In the first chapter, we studied the type of growth of laticifers, in order to observe if they grow diffusely of polarly. For this, we performed a comparative immunodetection experiment, in three species with different types of articulated laticifers: nonanastomosing in *Urvillea ulmacea* Kunth (Sapindaceae), anastomosing unbranched in *Ipomoea nil* (L.) Roth (Convolvulaceae), and anastomosing branched in *Asclepias curassavica*

L. (Apocynaceae) to analyze the microtubule arrangement. It is known that the cytoskeleton of plant cells is assembled and disassembled during the cell cycle, forming different arrangements of microtubules (Cai, 2010) which has an essential role in determining the growth polarity (Gu and Nielsen, 2013). It has been observed that cells with apical growth have a characteristic microtubule pattern, being longitudinal or slightly helical organized (Anderhag et al., 2000). In contrast, cells with diffuse growth have microtubules transversely oriented (Wasteneys, 2002). We observed that laticifers of the three species exhibited the last type of pattern, indicating a diffuse type of growth.

In the second chapter, we performed anatomical, ultrastructural, chemical and transcriptome analyses to study the development of anastomosing unbranched laticifers of *Ipomoea nil* L. (Roth), in order to identify the expression profiles of different moments of laticifer development and stablish a relation between shape and gene expression. There are currently several ways to perform transcriptome analysis. When there is a reference genome, the transcriptome assembly can be built on it. When this reference genome does not exist, the strategy is to assemble *de novo* transcriptome, where overlaps are made between the reads and assemble them into transcripts. There is also a third strategy that consists of combining the two previous ones, aligning the reads to the reference genome and de novo assembling the reads (Martin and Wang, 2011). In this study we use the first strategy because *Ipomoea*, have a draft genome with a scaffold N50 of 2.88 Mb (contig N50 of 1.87 Mb), covering 98% of the 750 Mb genome. Scaffolds covering 91.42% of the assembly are anchored to 15 pseudo-chromosomes (Hoshino et al., 2016). This represents an advantage for the transcriptome analysis related to the development of the laticifers of *Ipomoea nil.* We observed that laticifers of *I. nil* have early differentiation but their terminal walls are degraded lately. The anastomosis is a complex mechanism that involves autophagy and diverse enzymes to form a tube. The laticifer walls have a suberin layer which is

degraded together with polysaccharide portion of the terminal walls. Enzymes participating of anastomosis can be distinguished from enzymes that are involved in defense and metabolism. It is important to highlight that enzymes related to intrusive growth were identified in *I. nil,* although these laticifers do not grow intrusively.

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GENERAL CONCLUSIONS:

In our study, we detected that laticifers grow diffusely in the same way as neighboring cells and their walls remain attached to adjacent cells during the whole development. The comparative study of microtubule organization in three types of anastomosing laticifers has also revealed that laticifers present the same type of growth. The complexity of the laticifer formation resides in the simultaneous differentiation of the latex containing tube-like structure and latex secretion accumulation. The work presented here reveals that laticifers development is restricted to the meristematic regions, independently of the age of the plant. It remains to be identified the developmental/molecular signal that initiates the laticifer formation and secretion as well as its cell circumscription. Autophagy is clearly a determinant as are members of aquaporin family and cell-wall degrading enzymes.

The effort to identify transcripts involved along the apical axis is relevant to understand the developmental steps towards a mature laticifer. We identified possible candidates of genes encoding enzymes which participates of anastomosis, and this represents an important advance to the understand of the rapid differentiation of this structure along the apical axis and establishes bases for future investigations in more complex forms, such as anastomosing branched laticifers. In addition, we identified transcripts related to other components that participates in the defensive function of the latex. Although diverse subcellular structures and genes have been studied in relation to rubber production, our results stand out since we observed that the machinery used to latex production and laticifer development is complex and participates in the identity of this secretory structure, not to be limited to production of latex.

Further studies are needed using most up-to-date approaches, in order to identify the laticifer development in a dynamic way, since all works made used fixed material. Live-cell observations would help to observe the formation of branches, which is an issue that remains currently poorly understood.