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MARCADORES BIOQUÍMICOS ASSOCIADOS AO METABOLISMO DE CARBOIDRATOS DURANTE A EMBRIOGÊNESE ZIGÓTICA E SOMÁTICA DE *ARAUCARIA ANGUSTIFOLIA* (BERTOL.) KUNTZE

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RESUMO

NAVARRO, B.V. **Marcadores bioquímicos associados ao metabolismo de carboidratos durante a embriogênese zigótica e somática de A*raucaria angustifolia* (Bertol.) Kuntze.** 2018. 241 f. Tese (Doutorado em Biotecnologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2018.

Araucaria angustifolia é uma espécie de conífera native do Brasil, com importância econômica, social e ecológica. Devido a sua intensa exploração ao longo dos anos, atualmente a espécie cobre apenas 2% do sua área florestal original. Neste sistema, a embriogênese somática pode ser integrada em programas de melhoramento e conservação. Além disso, a similaridade entre a embriogênese zigótica e somática tem sido usada para desenvolver estudos baseados em biologia de sistemas, a fim de otimizar o desenvolvimento do embrião somático *in vitro*, bem como para gerar uma melhor compreensão dos eventos moleculares, bioquímicos e fisiológicos que modulam a formação do embrião. O metabolismo dos carboidratos é uma rota central que desempenha um papel importante durante o crescimento e desenvolvimento das plantas. Além de seu papel essencial como substrato no metabolismo de carbono e energia, os açúcares também desempenham papéis importantes como moléculas sinalizadoras. Para *A. angustifolia*, os bancos de dados de transcriptoma e proteoma identificaram o metabolismo de carboidratos como uma via importante na modulação do processo embriogênico. Assim, o objetivo principal deste trabalho foi estudar o metabolismo de carboidratos durante três estádios da embriogênese zigótica (globular, cotiledonar e maduro) e nas fases de proliferação e maturação de linhagens celulares embriogênicas com potencial embriogênico contrastante (responsiva e bloqueada). Para tanto, foram analisados os perfis de carboidratos não estruturais e monossacarídeos de parede celular, bem como a identificação e caracterização dos principais genes e proteínas envolvidos nas respostas mediadas por carboidratos, na homeostase de comunicação célula-a-célula e na modulação do metabolismo de sacarose, amido, rafinose e parede celular. Adicionalmente, um banco de dados de metaboloma foi gerado e integrado ao transcriptoma e proteoma de

A. angustifolia através de redes de co-expressão, em uma abordagem de biologia de sistema. As respostas mediadas por carboidratos que ocorrem durante a embriogênese somática de *A. angustifolia* se assemelham às que ocorrem nos estádios iniciais da embriogênese zigótica. Além disso, o acúmulo de sacarose e amido durante o desenvolvimento embrionário foi modulado pelas respostas de detecção de açúcar e sinalização, destacando este processo como uma característica importante que direciona a responsividade das linhas celulares embriogênicas. Associado a isso, a seletividade mediada pela comunicação de plasmodesmas e transporte vesicular na linhagem celular responsiva, apareceu como importante controle de diferenciação e desenvolvimento de tipos celulares. Embora o mecanismo completo da embriogênese somática de *A. angustifolia* não tenha sido completamente elucidado, nossas análises sobre as alterações metabólicas (metaboloma) durante a embriogênese zigótica e somática indicam que as redes regulatórias envolvidas no crescimento e desenvolvimento estão altamente interconectadas aos níveis de metabólito, proteína e transcrito, mostrando altas correlações entre alvos envolvidos no metabolismo de carboidratos. Os resultados obtidos fornecem informações relevantes e inéditas sobre o metabolismo dos carboidratos na embriogênese zigótica e somática de *A. angustifolia*, bem como fornecem subsídios para a otimização das condições *in vitro* para o desenvolvimento de embriões somáticos.

Palavras-chave: Carboidratos, pinheiro brasileiro, sensoriamento de açúcar, embriogênese, biologia do sistema.

ABSTRACT

NAVARRO, B.V. **Biochemical markers associated with carbohydrate metabolism during the zygotic and somatic embryogenesis of *Araucaria angustifolia* (Bertol.) Kuntze.** 2018. 241 f. Ph. D. these (Biotechnology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2018.

Araucaria angustifolia is a native conifer species of Brazil, that with economic, social and ecological importance. Due to its intense exploitation, the species cover only 2% of its original forest area. In this system, somatic embryogenesis may be integrated into breeding and conservation programs. Beside this, the similarity between zygotic and somatic embryogenesis have been used to develop studies based on system biology, in order to optimize the *in vitro* somatic embryo development, as well as to generate a better understanding of molecular, biochemical and physiological events that modulate the embryogenesis. Carbohydrates metabolism is a central route that plays an important role during plant growth and development. In addition to its essential role as a substrate in carbon and energy metabolism, sugars also play important roles as signal molecules. For *A. angustifolia,* transcriptome and proteome databases identified carbohydrates metabolism as an important pathway in the modulation of embryogenic process. Thus, the main objective of this work was to study the carbohydrates metabolism during three zygotic embryogenesis stages (globular, cotyledonal and mature) and in proliferation and maturation phases of embryogenic cell lines with contrasting embryogenic potential (responsive and blocked). To achieve this purpose, the profiles of non-structural carbohydrates and cell wall monosaccharides were generated, as well as the identification and characterization of the main genes and proteins involved in carbohydrate-mediated responses, cell-to-cell communication homeostasis and modulation of sucrose, starch, raffinose and cell wall metabolism. Additionally, a metabolome database was generated and integrated with *A. angustifolia* transcriptome and proteome through co-expression networks in a system biology approach. The carbohydrate-mediated responses that occur during *A. angustifolia* somatic embryogenesis resembled those occurring in the early stages of zygotic embryogenesis, where the main

responses that affect the targeting of the tissue differentiation of the seed occur. Beside this, sucrose and starch accumulation during embryo development were modulated by sugar sensing and signaling responses, highlighting this process as an important trait that directs the responsiveness of embryogenic cell lines. Associated to this, the selectivity mediated by plasmodesmata communication and vesicular transport in the responsive cell line, appeared as an important control of cell types differentiation and development. Even though the complete mechanism of *A. angustifolia* somatic embryogenesis has not been completely elucidated, our analyses about the metabolic changes (metabolome) during zygotic and somatic embryogenesis indicate that the regulatory networks involved in growth and development are highly inter-connected at the metabolite, protein and transcript levels, showing high correlations between targets involved in carbohydrate metabolism. The results obtained provide relevant and inedited information about the carbohydrates metabolism in *A. angustifolia* zygotic and somatic embryogenesis, as well as provide news subsidies for optimization of *in vitro* conditions for somatic embryos development.

Key words: Carbohydrates, Brazilian pine, sugar sensing, embryogenesis, system biology.

GENERAL INTRODUCTION

Embryogenesis: building a plant from the zygote

The colonization of the terrestrial landscape by plants is one of the most important biological events in the history of life. The first successful land plants comprise a monophyletic group, the Embryophyta (Niklas and Kuttschera, 2009), that are able to retain the diploid zygote, generating a multicellular embryo. This achievement may be related to three important and interrelated reproductive attributes of embryophytes: (1) they possess a sexual life cycle that alternate between gametophyte (haploid generation) and sporophyte (diploid generation); (2) they develop parenchymatous structures that produce eggs (archegonia) and sperm (antheridia); (3) they retain the fertilized egg (i.e. the zygote) within the archegonium, for nutrition and protection purposes (Niklas and Kuttschera, 2009). Thus, embryogenesis figures as a highly controlled and complex biological process, which represents for higher plants a determining step in the life cycle (Santa-Catarina et al. 2006, von Arnold et al. 2002).

In plants, embryogenesis corresponds the developmental stages from fertilization to the seed maturation (Harada et al 2010). Unlike animals, it is a continuous process, which begins after fertilization, establishing the basic structure of the plant body, with the presence of meristems, which generate additional organs in the adult. The zygotic embryogenesis can be separated into two main phases: (1) the morphogenetic phase, in which occurs establishment of the embryogenic axis; (2) and the maturation phase, which occur metabolic modifications to prepare the embryo for des iccation, dormancy and/or to obtain the nutrients required for germination and initial growth.

For Angiosperms, the morphogenetic phase begins with the asymmetric division of the zygote, producing a smaller apical and a larger basal cell (Fig. 1). Except for its very basal region, the apical cell generates the entire embryo, whereas the basal cell generates the extra-embryogenic suspensor through a series of transverse divisions (ten Hove et al. 2015). The first division of the zygote is accompanied by the formation of the cell wall, by the determination of the three embryogenic axes (longitudinal, lateral and radial), and by a sequence of changes in the embryo morphology passing through globular, cordiform, torpedo and cotyledon phases, in the case of dicotyledons (Floh et al. 2015). In maturation phase, metabolic changes occur in the formed embryo and in the endosperm to allow the future germination process.

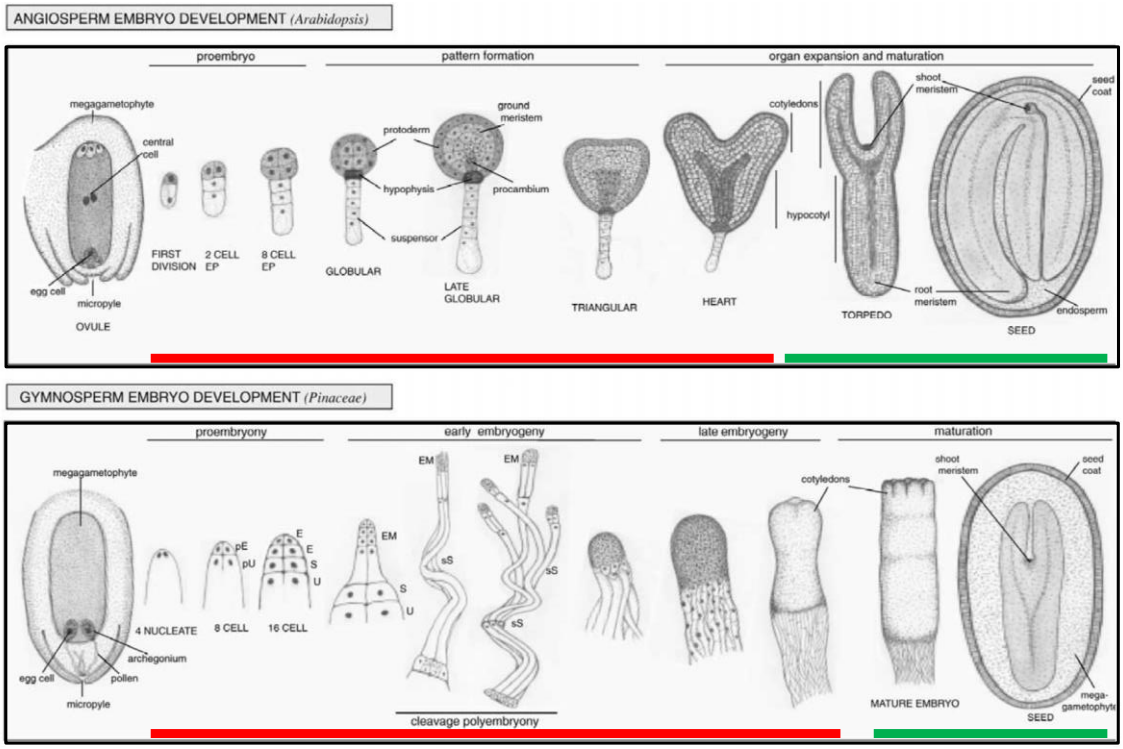

Figure 1: Angiosperm (Brassicaceae) and Gymnosperm (Pinaceae) embryo development steps, highlighting the morphogenetic and maturation phases. Drawings were prepared based on von Arnold et al. (2002), Goldberg et al. (1994) and Gifford and Foster (1989). Abbreviations: E – embryonal tier, EM – embryonal mass, EP – embryo proper, pE – primary embryonal tier, pU – primary upper tier, S – suspensor tier, U – upper tier, sS – secondary suspensor.
Source: Adapted from von Arnold et al. (2002).

For Gymnosperms, the embryogenesis differs in several aspects from Angiosperms (Fig. 1). First, the embryo development begins with a free nuclei stage, in which it is initially not possible to observe the cell wall formation (Floh et al. 2015, Hakman and Oliviusson 2002). The polarity of the proembryo occurs by the organization of free nuclei, constituting the cell wall and two well-defined cell types: the cells of the suspensor and the embryogenic group (Floh et al. 2015). In Gymnosperms, three distinct phases are recognized during embryogenic development: (1) proembryogenic phase from fertilization to archegonium rupture by proembryo (stages prior to elongation of the primary suspensor); (2) initial embryogenic phase, comprising the stages after the elongation of the secondary suspensor, and before the establishment of

meristems; (3) and late embryogenic phase, in which the protoderm and procambium are differentiated and the apical and radicular meristems are established (von Arnold et al. 2002, Haines e Prakash 1980, Singh 1978).

The formation of multiple embryos by the occurrence of simple polyembryony or by cleavage is common during the early embryogenic phase in Gymnosperms (Floh et al. 2015). In simple polyembryony occurs the formation of more than one embryo from the fertilization of more than one archegonium (Singh 1978). In polyembryony by cleavage, polyembryos can be formed from bipartition by cleavage of the proembryogenic cells, resulting in the formation of up to 24 proembryos. Regardless of the polyembryogenesis process (simple or by cleavage), only the embryo that reaches the cavity of corrosion is kept in the seed, and the other polyembryos (subordinate embryos) are eliminated by programmed cell death (Floh et al. 2015, Sharma e Thorpe 1995, Gifford e Foster 1989).

In the late stages of development, the embryo reduces the cell division process, starting the maturation phase (Ikeda et al. 2006). For both Angiosperms and Gymnosperms, maturation is considered the final and fundamental stage of embryo development (Perán-Quesada et al. 2004). During maturation occur the development of cotyledons and the accumulation of reserve substances (lipids, carbohydrates and proteins), which are considered fundamental for the post-germination period (initial seedling development) to autotrophy (Merkle et al. 1995, Bewley and Black 1994). These compounds can be mobilized during the initial germination and development of seedlings to provide amino acids, monosaccharides and free fatty acids for the formation of physical structures (i.e. cell wall) or for respiration, synthesizing metabolic intermediates or macromolecules with different purposes (Corte et al 2006, Buckeridge et al. 2004).

Depending on the type of seed (orthodox or recalcitrant) variations may occur during the maturation phase. These changes are related the decrease in metabolic activity and acquisition of desiccation tolerance, mediated or not by abscisic acid (ABA) and other regulators of stem growth and development (Floh et al. 2015, Walters et al. 2008, dos Santos et al. 2006, von Arnold et al. 2002, Bewley e Black 1994).

In addition to its physiological importance, zygotic embryogenesis is considered as a model for development and differentiation studies, where strict controls can be identified in the expression of hundreds of genes that trigger transcription, biosynthesis and transport of plant hormones, like auxins, along the embryogenic axis. Studies related to the establishment of apical and basal meristem in Gymnosperms and Angiosperms demonstrate a great similarity in the gene sequences (Cairney and Pullman 2007) suggesting that, despite the morphological, cytological and temporal differences observed during embryogenic development in both groups, the main genes related to embryogenesis were conserved throughout the evolution process (Uddenberg et al. 2011). Most of the embryogenesis studies are focus on model species, as *Arabidopsis thaliana, Medicago sativa* and *Pinus taeda*. However, recent advances in the use of state-of-the-art sequencers and the rapid expansion of the genetic sequence database have allowed molecular studies to be deepened in non-model systems such as long-lived trees (Ballester et al. 2016, Elbl et al. 2015a).

Somatic embryogenesis and biotechnology

The technique of plant tissue culture opened the possibility of the development of *in vitro* embryos from somatic cells, enabling the study of the stages of zygotic embryogenic development, mainly pro-embryogenic and embryogenic, characterized as difficult to manipulate in *in vivo* conditions. Somatic embryogenesis, considered as a biotechnological technique, is defined as a process, analogous to zygotic embryogenesis, where a single cell or group of somatic cells give rise to somatic embryos (Tautorus et al. 1991). For crops and others plant species of commercial interest, this technique allows the mass cloning of selected genotypes in breeding programs, as well as germplasm conservation programs by association with cryopreservation techniques (Santa-Catarina et al. 2013).

Due to the molecular, biochemical, physiological and morphological similarities between zygotic and somatic embryogenesis, the latter has been used as a model of experimental system for plant development in the physiological, biochemical and molecular aspects (Navarro et al. 2017, Elbl et al. 2015a, Pullman and Bucalo 2014, Schlögl et al. 2012a, 2012b, Steiner et al.

2012, Vieira et al. 2012, Durzan et al. 2012, Lara-Chavez et al. 2012, Balbuena et al. 2009, 2011, Silveira et al. 2008, Steiner et al. 2008, Steiner et al. 2007, Silveira et al. 2006, von Arnold et al. 2002). Integrative approaches to zygotic and somatic embryogenesis are fundamental for understanding these processes, establishing efficient protocols, and mimicking the conditions necessary for correct embryogenic development (Navarro et al. 2017, Elbl et al. 2015a, Garcia-Mendiguren et al. 2015).

In vitro embryo formation was first described, independently, by Steward and Reinert in carrot (Steward et al. 1958, Reinert 1958). Since then, several regenerative protocols, based on somatic embryogenesis, have been established in both Angiosperms and Gymnosperms (Floh et al. 2015). It is well accepted that the developmental switch resulting in somatic embryogenesis is triggered in cells transiently exposed to strong stress and/or high non-physiological concentration of growth regulators (Fehér 2015). Beside this, the somatic embryogenesis process is highly dependent on the developmental stage of the explant used for induction, the genotype of the mother plant and the culture conditions (dos Santos et al. 2002).

The somatic embryogenesis process can be divided into four distinct stages: (1) induction, which begins after inoculation of the explants in culture medium supplemented or not with growth regulators (especially auxins and cytokinins); (2) multiplication or proliferation, which is characterized by subculture cycles of the embryogenic cultures; (3) maturation, where the formation of somatic embryos occurs in the presence of ABA and/or osmotic agents; (4) and development and establishment of the seedling, which after the complete formation of somatic embryos, they are transferred to a medium free of growth regulators, allowing the seedling development (Floh et al. 2015, Fehér 2015, Stasolla and Yeung 2003, von Arnold et al. 2002, Stasolla et al. 2002, Attre e Fowke, 1993).

The first report of somatic embryos formatio in Gimnosperms was performed by Norstog and Rhamstine (1967) using zygotic embryos of *Zamia* spp. For conifers the process was initially described for *Picea abies* (Chalupa 1985, Hakman et al. 1985) and *Larix decidua* (Nagmani and Bonga 1985), and is currently described for 30 species of the Pinaceae and for species of Cupressaceae (four), Taxaceae (one), Podocarpaceae (one), Cephalotaxaceae

(one) and Araucariaceae (one) (Guerra et al. 2016, Klimaszewska et al. 2016, Fraga et al. 2016). The main use of somatic embryogenresis is related to clone propagation and conservation of forest species (Klimaszewska et al. 2016). It also provides the production of large-scale embryos for commercial multiplication of elite genotypes, and for conservation purposes, the generation of germplasm banks associated with cryopreservation (Klimaszewska et al. 2016).

During the somatic embryogenesis process, the formation of pro-embryogenic masses (PEM) can be classified as: PEM I, which consists of undeveloped embryogenic cells with only one suspensor cell; PEM II, which are composed of cell aggregates similar to PEM I, however with more than one suspensor cell; and PEM III characterized by an aggregate with a larger number of cells, consisting of suspensor and embryogenic cells, which form aggregates without any pattern of organization (Stasolla e Yeung 2003, von Arnold et al. 2002).

Some limitations are found in conifer embryogenesis systems, such as genotype-dependence, the rapid loss of embryogenic potential after a few months of subculture and the low rate of somatic embryos formed during maturation due to the diverse conditions imposed in this stage (Santa-Catarina et al. 2013). Much of these difficulties are associated with the culture conditions employed (physical and chemical stimuli) still ineffective for correct *in vitro* embryo development. Comparative studies involving molecular approaches and the identification of biochemical and molecular markers are being directed towards for an early selection of suitable and competent genotypes (Navarro et al. 2017, Jo et al. 2014, Dowlatabadi et al. 2009, Silveira et al. 2008, Lippert et al. 2005, Klimaszewska et al. 2004). Thus, studies of the different physiological, biochemical and molecular aspects during embryo development are fundamental for the understanding of the basic processes of cell differentiation, as well as for the establishment of more efficient protocols of propagation through somatic embryogenesis.

Araucaria angustifolia: a model for embryogenesis studies

The Araucariaceae family includes about 40 species, divided into three genera: *Agathis*, *Wollemia* and *Araucaria* (Codrington et al. 2005). The latter

genus is the most abundant, diversified and with a greater geographical amplitude, being found in part of Oceania, Southeast Asia and South America, where two species are found, *A. angustifolia* (Brazil and Argentina) and *A. araucana* (Argentina and Chile) (Mattos 2012). *A. Angustifolia* (Bert.) O. Kuntze, known as Araucaria, Paraná pine or Brazilian pine is found predominantly in the southern states of Brazil and some discontinuous points in the southeast, as well as small spots in Argentina (Fig. 2) (northeast) (Mattos 2012).

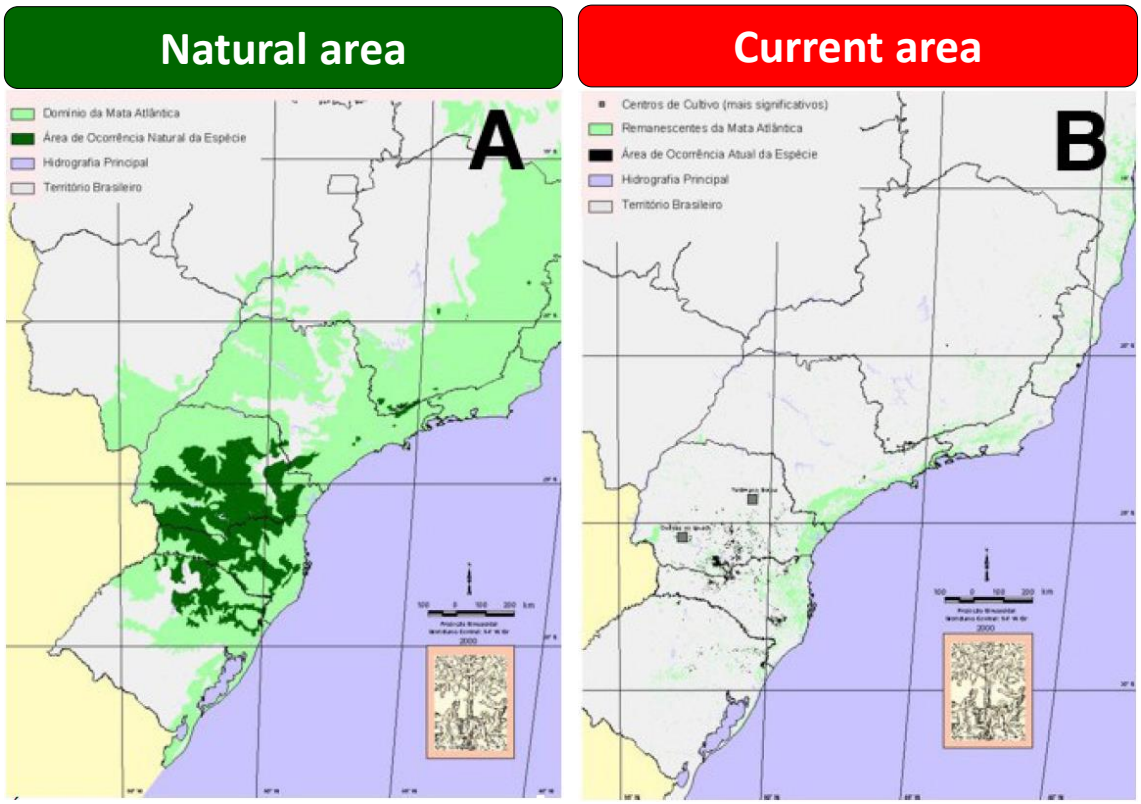

Figure 2: Geographic distribution of *Araucaria angustifolia* in Brazil. Occurrence area highlighted in dark green. Distribution in the early twentieth century (A), and the current coverage area of the species (B). Source: Conselho Nacional da Reserva da Biosfera da Mata Atlântica (2012).

This species constitutes the most important native Gymnosperm of Brazil. The high quality of the wood promoted an intense exploitation (Guerra et al. 2002), which was carried out in a predatory manner, either from a social, economic or ecological point of view (Guerra et al. 2008). Thus, natural populations were limited to values around 2% of their original extent (Fig. 2) (Guerra et al. 2008), which led them to be classified as endangered species in the Brazilian endangered species list (BRASIL 2008) and in critical danger of

extinction according to the listing of the International Union for Conservation of Nature (IUCN 2015). In this context, studies that support the genetic conservation of this species through the establishment of *in situ* and *ex situ* germplasm collections are of extreme importance (Guerra et al. 2008). Somatic embryogenesis has been considered one of the most promising techniques for mass cloning, *ex situ* propagation and conservation of germplasm for *A. angustifolia* (Steiner et al. 2008).

As for other species of conifers, *A. angustifolia* zygotic embryogenesis is characterized by the presence of a free nuclei stage, when occur successive nuclear divisions without cell wall biosynthesis (Cairney and Pullman 2007). In Araucariaceae species, 32 to 64 free nuclei are formed before the formation of cell walls (Guerra et al. 2008). The polarity of the pro-embryos occurs through the organization of the free nuclei, constituting three well-defined cell types: the cells of the suspensor, the cover and the embryogenic group (Guerra et al. 2008). In species of the genus Araucaria, only the occurrence of polyembryony is observed (Gifford and Foster 1989). The pro-embryogenic stage involves the post-fertilization stages, until the pro-embryo ceases the elongation of the suspensor (Haines and Prakash 1980). This phenomenon is of fundamental importance, since it allows the alignment of the embryo towards the megagametophyte (Dogra 1978).

During the *A. angustifolia* zygotic embryogenesis process, biochemical changes are identified in the various stages of embryo development. The stage of the initial embryogenesis, until the cotyledonal phase, is characterized by high levels of indoleacetic acid (AIA) and abscisic acid (ABA), accumulated mainly in the embryogenic axis and megagametophyte, respectively. As the embryos advance their development (complete formation of cotyledons), concentrations of AIA and gibberellic acid (GA) tend to decrease and ABA concentration increases, characterizing in this stage the maturation phase of the seed (Silveira et al. 2008, Astarita et al. 2003a, Astarita et al. 2003b). The polyamines (PAs) profile is also altered with a high concentrations of putrescine (Put) and spermidine (Spd) in the early stages of development (de Oliveira et al. 2017, Astarita et al. 2003b), with an increase in spermine (Spm) of the cotyledons and development of the mature seeds (de Oliveira et al. 2017, Pieruzzi et al. 2011, Astarita et al. 2003b). The existence of stage-specific

proteins, that could be used as biochemical markers, were identified. Thus, during the initial phase of the zygote embryo of *A. angustifolia*, the most expressed proteins are those of oxidative stress metabolism in contrast to the late stages of embryogenesis where the accumulation of reserve substances occurs (proteins, carbohydrates and lipids) (Balbuena et al. 2011, Silveira et al. 2006).

In 1992, with the first report of the induction of embryogenic cultures in *A. angustifolia* by Guerra and Kemper (1992), was opened the possibility of using the route of somatic embryogenesis and other associated techniques (synthetic seeds and cryopreservation) for the establishment of breeding and conservation programs based on the use of biotechnological tools. Since then, several studies have been developed, aiming the improvement and development of protocols for induction, proliferation and maturation of somatic embryos in this species (Santa-Catarina et al. 2013, Steiner et al. 2008, dos Santos et al. 2002).

For *A. angustifolia*, somatic embryogenesis is initiated by the induction of embryogenic cultures, originating from the apex of the immature zygotic embryo (Fig. 3) (i.e. globular stage) (dos Santos et al. 2002). The explants are inoculated *in vitro*, without the presence of auxins and cytokinins, resulting in the formation of pro-embryos that characterize the early stages of embryogenesis. The evolution of somatic embryos *in vitro* is stimulated when chemical signals of osmotic adjustment (polyethylene glycol and maltose) and hormonal (ABA) are supplied to the pro-embryos during the maturation stage (dos Santos et al. 2002). The main role of ABA during this stage of development is inhibition of the proliferation of embryogenic cultures, followed by induction of the various stages of development and maturation of somatic embryos (von Arnold et al. 2002). During this stage, somatic embryos present morphological changes (histodifferentiation of the protoderm, axial and radial growth, degradation of the suspensor and development of meristems) and biochemical (accumulation of reserve substances, reduction of metabolic activity and acquisition of tolerance to dehydration) (Stasolla and Yeung 2003, von Arnold et al. 2002).

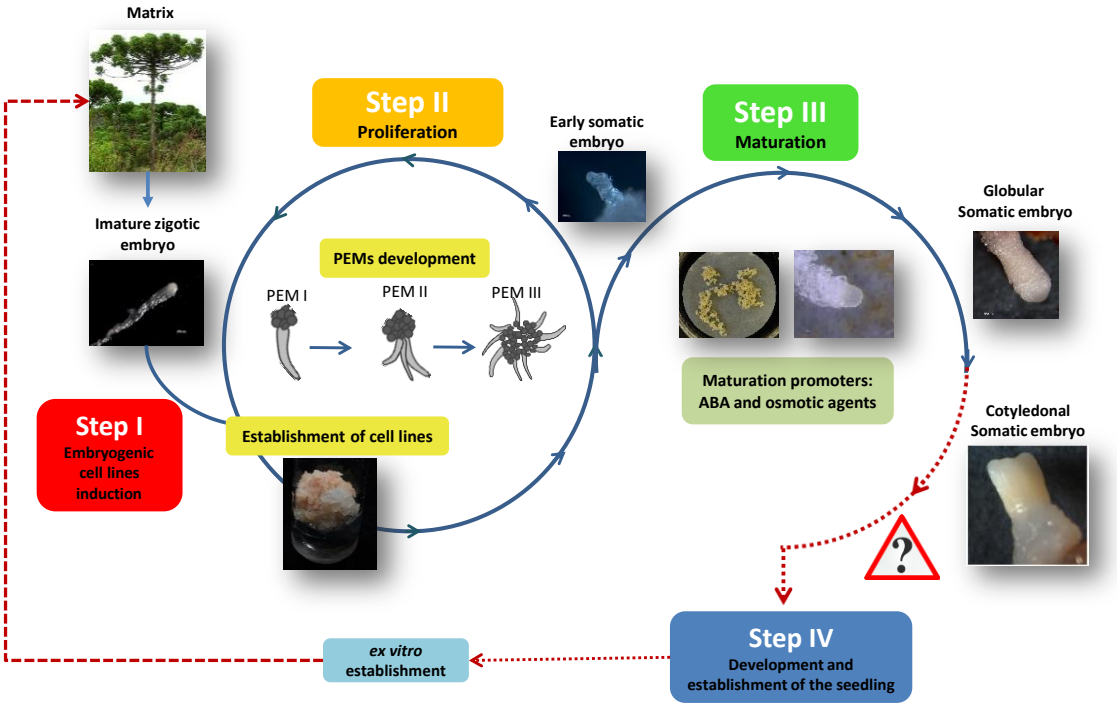

Figure 3: Schematic overview of the modulation of somatic embryogenesis in *Araucaria angustifolia*. PEM, proembriogenic masses; ABA, abscisic acid; Dashed red lines indicate steps in which the protocol is not yet fully established. Source: Adapted from de Oliveira (2017), Jo et al. (2014) and Steiner et al. (2008).

There are several difficulties for the use of somatic embryogenesis in the *A. angustifolia* system. Frequently, different cell lines develop a reduced number of globular embryos, which rarely progress into cotyledonal stage. When there is the formation of a large number of early embryos, the process of embryo maturation constitutes one of the limiting factors of the technique (Jo et al. 2014, Santos et al. 2008). Many efforts are being made to identify biochemical and molecular markers for an early selection of suitable and competent genotypes for the formation and evolution of viable somatic embryos (Navarro et al. 2017, dos Santos et al. 2016, Elbl et al. 2015a, Jo et al. 2014, Silveira et al. 2008). However, the main reason for limited production of *A. angustifolia* somatic embryos is the insufficient knowledge of the underlying regulatory process, especially in maturation phase during embryogenesis process (dos Santos et al. 2016, Elbl et al. 2015, Jo et al. 2014, Silveira et al. 2008).

Recently, Elbl et al. (2015a) and dos Santos et al. (2016) developed an extensive sequence datasets by de novo sequencing. These databases helped to understand the genetic factors that control embryogenesis process. Thus, a

comparative transcriptome analysis was performed to elucidate differences among seed development stage-specific tissues, and two cell lines with different embryogenic potential (Elbl et al. 2015a). Likewise, from *A. angustifolia* embryogenic cell lines that showed different propensities to form somatic embryos, a GeLC–MS/MS large scale based label free proteomic profiling was generated for the identification and quantification of proteins (dos Santos et al. 2016). These analyses provided insights into putative genetic factors that contribute to the zygotic embryogenesis, as well as to determine the potential of cell lines to develop somatic embryos, and the differences in gene and protein expression during the initial stages of somatic embryogenesis.

A total of 64 GB of sequence was used for *de novo* transcriptome assembly, derived from high-throughput Illumina RNA-seq profiling (Elbl et al. 2015a). In this study, a total of 149,510 transcripts were identified, representing 112,772 expressed *loci*, which were used for further annotation and differential expression analyses. Applying statistical analysis of differential gene expression, the genes were predicted to be involved in cell line embryogenic potential, early somatic embryo formation and recalcitrant seed development. The results expand our understanding of the complex molecular events that control embryogenesis, and suggest that the regeneration impairment of *A. angustifolia* cultures is consequence of the auxin signaling failure (Elbl et al. 2015a). The generated data allowed the understanding for future functional genomic and evolutionary studies that will contribute to conifer biology and unorthodox seed physiology studies.

Furthermore, using a predicted protein sequence database derived from *A. angustifolia* RNA-Seq data, 2,398 non-redundant proteins were identified (dos Santos et al. 2016). Among them, 106 proteins were significantly differentially abundant according to the cell lines potential to somatic embryogenesis. In the responsive cell line 35 proteins, related to storage reserve deposition, cell defense and anti-oxidative stress responses were more abundant, and for blocked cell line 71 proteins were highlighted (dos Santos et al. 2016). Moreover, in blocked cell line, an increased abundance of two proteins associated with seed development during the embryogenic cell proliferation stage was observed, suggesting an association with genotypes that show a low responsiveness to embryo formation. The proteomic analyses

confirm previous results of *A. angustifolia* embryogenic cell lines development, highlighting important proteins related to defense responses (dos Santos et al. 2008), anti-oxidative stress responses (Elbl et al. 2015a, Jo et al. 2014, Vieira et al. 2012), and gene expression (Steiner et al. 2012) in cell lines with high embryogenic potential. Besides being a valuable sequence and protein data resource for conifer proteomes studies, this database have valuable informations for the identification of proteins with potential roles in early somatic embryo formation, and also for the early detection of high embryogenic potential cell lines.

Both databases can allow us to provide insights into putative genetic determinants that contribute to the embryogenesis process. In addition to the potential for improving somatic embryogenesis protocols by revealing molecular markers for early detection of cell lines with high embryogenic capacity, these studies further advances, by correlation between both datasets, in the context of current understanding of molecular regulation during conifer embryogenesis.

Carbohydrates metabolism in plants

Several important characteristics differentiate plants from other organisms, and the metabolism of carbohydrates is an important property that assists in understanding the unique nature of an autotrophic living being (Dennis and Blakeley 2000). Carbohydrate metabolism is a central route in the global metabolism, which plays an important role during plant growth and development. Many environmental stresses like drought, cold and salinity lead to major alterations in carbohydrate metabolism, and the sugar signaling pathways interact with stress pathways to modulate the adaptive changes (Gupta and Kaur 2005).

From the photosynthetic process, plants are able to use light energy in the conversion of carbon dioxide, water and inorganic ions into organic compounds compatible with the need of the cell. The stored energy is then used in the cellular processes of plants, for example in supplying energy demand and numerous anabolic pathways (nucleic acids, proteins, lipids and polysaccharides) and as a source of energy for all other forms of life (Taiz and Zeiger 2010). The first stage of the photosynthetic process begins when sunlight is captured by the light collectors that direct the light energy to the

photochemical reaction centers in photosystems I and II, located in the thylakoid membranes within the chloroplasts (Cruz et al. 2004). The chemical energy released in the oxidation reaction of the water is processed by the electron transport chain by a series of reducing power carriers (NADPH) and high energy molecules (ATP), which will be used in the reduction reactions of carbon dioxide (Melis 1999). The CO_2 is diffused from the atmosphere into the intercellular spaces of the leaf mesophyll through the stomata. After diffusion into the chloroplasts, the C_3 pathway begins with carbonic gas being enzymatically combined with ribulose-1,5-biphosphate (RuBP) through the ribulose-1,5-biphosphate carboxylase/oxygenase (RUBISCO) enzyme and forming an unstable 6-carbon intermediate. The 6-carbon intermediate dissociates into two molecules of 3-phosphoglycerate (3-PGA) that are converted to triose-phosphate (triose-P) in reactions requiring ATP and NADPH (Sharkey 1985).

Together with the trioses-P, the pentoses phosphate and the hexoses phosphate feed the carbohydrate metabolism of plants. These metabolites are located in both the cytosol and the plastids, communicating through highly specific membrane-carrying proteins that export trioses-P in exchange for inorganic phosphate (Pi). The flow between these metabolites is determined by cellular demand (Dennis and Blakeley, 2000).

Hexoses phosphate (glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate) are in equilibrium due to the action of two enzymes: phosphoglucomutase and glucose 6-phosphate isomerase, responsible for the interconversion of these three metabolites. The biosynthesis can be done through metabolites derived from gluconeogenesis and phosphorylation of free hexoses, which are used in the synthesis of starch from glucose 1-phosphate; sucrose synthesis using glucose 1-phosphate and fructose 6-phosphate; cell wall formation from glucose 1-phosphate; and through oxidative reactions of the pentoses phosphate (glucose 6-phosphate) pathway (Dennis and Blakeley, 2000).

Source-sink dynamics

One of the key points in plant growth control is the coordination between availability and resource use (Foyer and Paul 2001). The organs that make

available and export the resources are denominated source (i.e. mature leaves), that produce photoassimilates. On the other hand, the organs that import these resources are defined as sink, which are non-photosynthetic tissues or do not produce sufficient photoassimilates to supply their demand (Taiz and Zeiger 2010).

In the source organs the availability of resources comes directly or indirectly from photosynthesis and the production of carbohydrates. The main carbohydrate from the photosynthetic process is sucrose (Dennis and Blakeley 2000), which is exported and used for growth and development of the sink organs. If the photosynthetic capacity exceeds the demand, the excess photoassimilates remain in the chloroplast and are stored as starch (Santos et al. 2004, Stitt 1991). Thus, it is believed that the sink organs can control the activity of the source (Foyer and Paul 2001). In addition, there is an intricate relationship between source and sink, since both activities are controlled by environmental factors and exchange internal signals between them (Santos et al. 2004). One of the control points of this process is the transport of photoassimilates via phloem (van Bel 2003), which directs the partitioning of sugars between tissues. The loading and discharging of phloem can occur in two ways: (1) symplastic way, where the sucrose flows passively through the plasmodesmatas without energy expenditure; (2) and the apoplastic pathway, in which the sucrose is carried through specific transporters of the plasma membrane (Lemoine et al. 2013, Lambers et al. 2008).

The dynamics between source and sink is also regulated by sensors and sugar signals, which are involved in the control of growth and development (Tiessen and Padilla-Chacon 2013, Rolland et al. 2002). Several genes related to photosynthesis, accumulation and mobilization of reserves, cell cycle and growth have been sensitive to changes in sugar concentrations (Rolland et al. 2006), which mainly involve sucrose and its degradation products, glucose and fructose (Tiessen and Padilla-Chacon 2013, Hanson and Smeekens 2009). Beside these, key players in sugar sensing and signaling have been highlighted as the major coordinators of sugar availability, allocation and cell energy status.

Sugar sensing and signaling in plants

All living organisms perceive and respond to environmental conditions and resources availability as a survival strategy to sustain cell metabolism and energy production. The responses that determine when to grow, to assimilate, store nutrients and to recycle reserves are the central coordinators of plant development. In eukaryotes, several interconnected signaling networks, involved in sugar sensing and signaling, perceive nutrient availability and direct growth and metabolic patterns (Dobrenel et al. 2016). In the literature, different types of sugars sensors and signals are described (Smith and Stitt 2007). Among them, trehalose-6-phosphate (T6P), SnRK1 (Sucrose Non- Fermenting-related protein kinase 1) and the TOR complex (Target of Rapamycin) connect sugar levels to expression of genes related to primary metabolism (Smeekens et al. 2010).

T6P has emerged as a key player metabolite in plants, which influences in growth and development. Trehalose is a non-reducing disaccharide that has various functions, such as storage reserve, osmolyte, transport sugar and stress protectant (Figueroa and Lunn 2016). In plants, there is a known trehalose synthesis pathway. In this two-step pathway a phosphorylated intermediate (T6P) is first synthesized from UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate synthase (TPS), and then dephosphorylated to trehalose by trehalose-6-phosphate phosphatase (TPP) (Cabib and Leloir 1958). Thus, T6P, as well as TPS and TPP, have emerged as essential for plant growth and development, acting as a signal of sugar availability (Tomé et al. 2014).

Similarly, the SnRK1 and TOR complex kinases found in plants are involved in the gene expression and enzymatic activity of the primary metabolism, as part of the signaling pathway of sugars, establishing a relationship in the regulation of cellular metabolic status. While the TOR complex acts on high metabolic status, triggering signaling cascades for growth and development, the SnRK1 protein responds to a low metabolic status, suppressing growth (Dobrenel et al. 2016, Lastdrager et al. 2014, Xiong and Sheen 2014).

These mechanisms of sugar sensing and signaling regulate many important cellular processes in plants, playing a role in embryogenesis, seedling

establishment, growth, metabolism, juvenile-adult transition, flowering and senescence (Navarro et al. 2017, Li and Sheen 2016).

Carbohydrates during embryogenesis

Carbohydrate metabolism is a very dynamic process, which its metabolic flux and concentration drastically altered during growth and development (Businge et al. 2012, Lipavská and Konrádová 2004, Iraqi and Tremblay 2001), or in response to environmental signals such as diurnal variations and biotic and abiotic stress (Blasing et al. 2005). In addition to its essential role as a substrate in carbon and energy metabolism and polymer biosynthesis (Pullman and Bucalo 2014), sugars play important roles as first messengers in signal transduction (Rolland et al. 2006).

The involvement of plant hormones at different embryogenesis process stages has been widely explored, however, carbohydrate signaling and its integration with other metabolic pathways has not been sufficiently studied (Eveland and Jackson 2012, Mehouachi et al. 1996). Not surprisingly, intricate regulatory interactions with plant hormones are an essential part of the carbohydrate signaling network (Tonini et al. 2010, Brandão et al. 2009, Rolland et al. 2006, Santos et al. 2004). Although the regulatory effect of carbohydrates on photosynthetic activity and plant metabolism has long been recognized, the concept of carbohydrates as central signaling molecules is relatively recent (Rolland et al. 2006).

When a material is cultivated and maintained *in vitro*, sugars produce direct metabolites participating in innumerable interconnected metabolic pathways, and also influence the osmotic condition of the medium and generate intermediates related to carbohydrate signaling (Smeekens et al. 2010, Rolland et al. 2006, Lipavská and Konrádová 2004). As mentioned earlier, sugar sensors respond to a complex and interconnected network of metabolic and molecular pathways (Smith and Stitt 2007), revealing strong interactions between sugar levels and the processes of growth and development (Eveland and Jackson 2012, Hey et al. 2010, Smeekens et al. 2010, Hartig e Beck 2006, Rolland et al. 2006).

Studies with conifers somatic embryogenesis pinpoint for the need to take into account the signaling caused by carbohydrates during proliferation and

maturation stages (Lipavská and Konrádová, 2004, Iraqi and Tremblay 2001a, 2001b). In somatic conifer embryos, carbohydrates play an essential role, with glucose being reported as the preferred sugar to supply metabolic demands (Lipavská and Konrádová, 2004). Different studies have shown that glucose and sucrose act as mediators of embryogenesis processes (Eveland and Jackson 2012). Probably sucrose is responsible for the protective action against desiccation and that it may affect cell differentiation and storage of reserve substances (Eveland and Jackson 2012). Thus, the combination and interaction of these factors *in vitro* can lead to regulation of the genetic and physiological processes, consequently in the control of induction, and finally the establishment of embryogenic cell cultures from somatic cells (Pullman and Buchanan 2008, Lipavská and Konrádová 2004).

System Biology: integrating data for understanding plant metabolism

In science, it is common to perform studies at different levels of organization for an individual separately, generating concepts and principles that explain biological phenomena on a particular scale (De Souza 2011). Although these studies have a great relevance to all the knowledge generated so far, the individualization of the phenomena can reduce the organizational complexity that exists between the different scales (Moore 2005). In this context, system biology has emerged as a strategy to integrate large numbers of complex data.

System biology is an interdisciplinary biology-based field of study that focuses on complex interactions within biological systems, using a holistic approach to biological research (Lucas et al. 2011). This approach considers the fact that all systems within the individual are interconnected through networks that have different hierarchies (Albert and Barabasi 2002). The progress in genome sequencing and high-throughput measurements enables us to collect and process comprehensive datasets of organisms and gain information on the underlying molecules (Kopka and Fernie 2018, Kitano 2017, Kitano 2002).

For *A. angustifolia* embryogenesis, recent studies focus on system biology approach, through the exploitation of transcriptome, and proteome databases (dos Santos et al. 2016, Elbl et al. 2015a). These approaches allow

deeper understanding of complex biological regulatory systems and provide major new opportunities for applied and theoretical studies.

Thesis contextualization

In the past twenty five years, the Laboratory of Plant Cell Biology (BIOCEL) at IB-USP and associated groups have been developing studies on plant embryogenesis and biotechnology in its basic and applied aspects, with native trees, including *A. angustifolia*. These studies present an integrative approach to the *A. angustifolia* zygotic and somatic embryogenesis, which has allowed the elucidation of control points of the embryogenesis process, such as the identification of different signaling agents, biochemical and molecular markers during differentiation and embryogenic development. Possible applications in propagation programs, including genetic improvement and/or conservation of germplasm, have been addressed. From the biotechnological point of view, the studies aim the improvement of the artificial conditions for *in vitro* culture of somatic embryos.

In this perspective, the research group has been using innovative methodologies that allow a deeper understanding of molecular aspects, including the study of high throughtput (RNAseq) and quantitative proteomics (GeLC-MS / MS) (dos Santos et al. 2016, Elbl et al. 2015a, 2015b), as well as on biochemical and physiological aspects (de Oliveira et al. 2018, Navarro et al. 2017, de Oliveira et al. 2017, Jo et al. 2014). Through the transcriptome, 112,772 unigenes were obtained from the *de novo* database assembly (Elbl et al. 2015a). In addition to the data generated by transcript and protein databases, the metabolism of carbohydrates was highlighted as an important pathway in the participation of the modulation of the embryogenic process.

In this work, three stages of zygotic development and two cell lines with different embryogenic potentials were compared, allowing an inference about genes or candidate processes to explain the differences found in these stages and the embryogenic potentials of the cultures. These transcripts were annotated automatically and categorized into functional groups related to biological processes, molecular functions and cellular components, obtaining 19,947 unigenes categorized. Among these, processes involved in the metabolism of carbohydrates were identified and subsequently used to select

and identify sequences of interest. In addition, the assembly of this database and the previously obtained results expanded the understanding of the molecular events that control embryogenesis, especially for *A. angustifolia*, generating perspectives for future studies in functional genomics that allow the advancement in understanding of the conifers and recalcitrant seeds biology and physiology.

In addition, a protein database was generated for *A. angustifolia*. The analysis of comparative proteomics between two cell lines with different embryogenic potentials allowed the identification of differentially expressed proteins, which were categorized in groups according to their performance in the metabolism. Among these, carbohydrate metabolism was once again an important factor in the difference for embryogenic potential acquisition of cell lines.

In this way, studies related to carbohydrate metabolism were started in a partnership established with the Laboratory of Ecological Plant Physiology (LAFIECO) at IB-USP. Beside this, collaboration was carried out with Center for Applied Plant Sciences of The Ohio State University. This collaboration comprised the development of a metabolomics database, and subsequent correlation with previously *A. angustifolia* transcriptome and proteome, providing the identification of conserved changes in the three regulation levels (transcript, protein and metabolite) during zygotic and somatic embryogenesis.The results obtained in the present thesis, besides unpublished for this species, allowed a greater understanding of the physiological, biochemical and molecular aspects of carbohydrate metabolism and carbon partitioning, during the process of plant cellular differentiation.

The workflow for the development of this work is presented in figure 4.

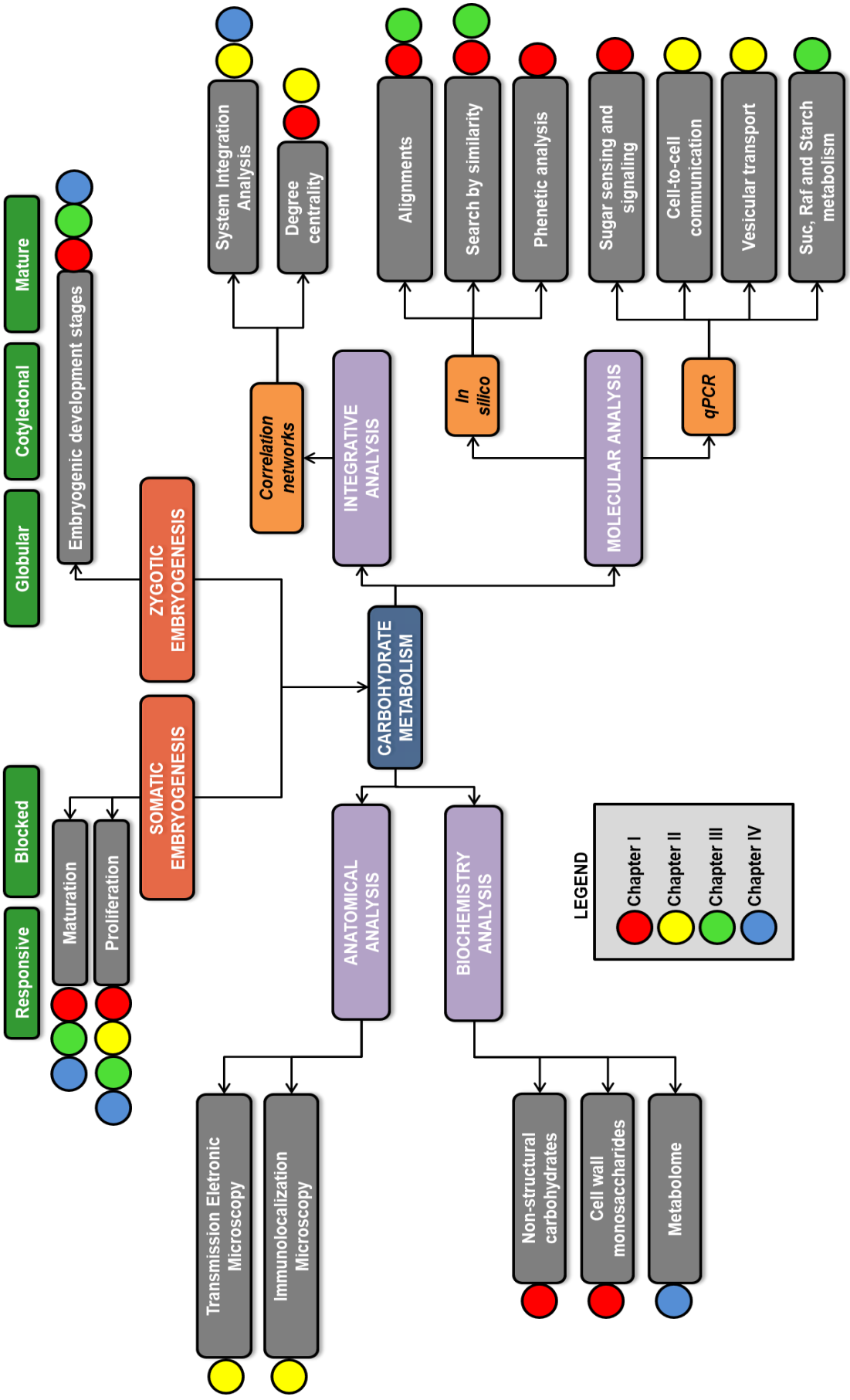

Figure 4: Thesis development workflow.

In this context, the present thesis was structured in the following chapters:

- **Chapter I:** Carbohydrate-mediated responses during zygotic and early somatic embryogenesis in *Araucaria angustifolia*
- **Chapter II:** Cell-To-Cell Trafficking Patterns in Brazilian Pine (*Araucaria angustifolia*) Cell Lines with Contrasting Embryogenic Potential
- **Chapter III:** Modulation of sucrose, raffinose and starch metabolism during *Araucaria angustifolia* zygotic and somatic embryogenesis
- **Chapter IV:** Changes in the metabolism during zygotic and somatic embryogenesis of *Araucaria angustifolia*

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OBJECTIVES

OBJECTIVE

The present work has as objective the study of carbohydrates metabolism during zygotic embryogenesis and in embryogenic cultures of *A. angustifolia*.

SPECIFIC OBJECTIVES

In order to achieve the main objective, were used different studies strategies including:

(1) Analysis of non-structural carbohydrates and cell wall monosaccharides profiles in different stages of development of zygotic embryos and in embryogenic cultures with different embryogenic potentials;

(2) Determination and characterization of cellular communication mediated by plasmodesmata and vesicular transport in embryogenic cultures with different embryogenic capacities;

(3) Identification of the main genes involved in carbohydrate metabolism through the Araucaria transcripts and proteins database, during zygotic embryo development and somatic embryogenesis cell lines.

(4) Characterization of the expression pattern of genes involved in carbohydrate metabolism and sugar sensing and signaling at different stages of zygotic embryos development and in embryogenic cultures;

(5) Development of a metabolite database (metabolome) and its integration with the transcript and protein databases during zygotic embryogenesis and in cell lines with different embryogenic potentials.

FINAL CONSIDERATIONS AND PERSPECTIVES

Final considerations and perspectives

The present work comprises the theme that involves signaling molecules analysis, aiming the study of carbohydrates metabolism, throughout *A. angustifolia* zygotic and somatic embryogenesis. For a long time, several researches have been carried out aiming the approach of basic aspects related to the competence, determination and cellular differentiation during zygotic and somatic embryogenesis. In particular for somatic embryogenesis, the main objective has been the improvement and the optimization of a protocol for the application of mass clonal propagation. This approach has been useful for many plant systems, especially for perennial species that demand conservation strategies, such as *A. angustifolia* an endangered native conifer species from Brazil, with recalcitrant seeds. In this context, studies employing this plant system reported the use of morphological, physiological, biochemical and molecular parameters during zygotic and somatic embryogenesis processes. Among different cell signaling pathways evaluated, we can highlight the metabolism of polyamine (PA), nitric oxide (NO), amino acids, and plant hormones, such as abscisic acid (ABA), indole-3-acetic acid (IAA) and ethylene. The participation of carbohydrates during the embryogenic process had not been explored until the present work. In this context, the present work aims to deepen the knowledge of these groups of substances and potential signaling in the modulation of the *A. angustifolia* zygotic and somatic embryogenesis processes.

The recent advances with the high-throughput techniques such as RNAseq, allowed the refinement of analyzes, making possible the data of the different physiological and biochemical processes during the cellular development. Therefore, an extensive database using this methodology was obtained for zygotic and somatic embryogenesis in *A. angustifolia*, allowing a better knowledge of the molecular regulation of *in vivo* and *in vitro* embryo development (Elbl et al. 2015). In addition, integrated transcriptome and proteome studies (dos Santos et al. 2016) were able to predict gene function, characterizing the biological processes involved, and the study of regulator-related metabolic networks, such as those selected for the present study. Associated with these studies, in the present work, the profiles of non-structural

carbohydrates and cell wall monosaccharides, as well as the identification and characterization of the main genes and proteins involved in carbohydrate-mediated responses, were fundamental to elucidate the involvement of carbohydrates metabolism during embryogenesis. Furthermore, it was possible to confirm the participation of carbohydrates as biochemical markers to identify the embryogenic potential of different cell lines.

The results described in chapter 1 and published at *PloS one* (Navarro et al. 2017), elucidated through biochemical and molecular parameters, the regulation of carbohydrates metabolism variation during zygotic and somatic embryogenesis. In this study, it was verified that: a) the TOR/SnRK1 system apparently operates in the modulation of the embryogenic responses; b) the sugar sensing and signaling-mediated responses during somatic embryo development resemble to zygotic embryogenesis, which occur mainly during the early stages; c) the accumulation of sucrose and starch during somatic embryo development were important and this pattern can be associated with the responsiveness for *A. angustifolia.*

At the morphological level, in chapter 2 we identified patterns that may be associated with the difference in embryogenic competence between two different cell lines related to embryogenic potential: responsive and blocked cell lines. In this way, the intense vesicular transport highlighted in the responsive cell line can be associated to a signal of division and differentiation. The high callose rates in the responsive cell line, the increased and differential expression of callose hydrolysis-related proteins and the higher number of PDs found in the blocked cell line could be a sign that the responsive cell line isolates its cells, unlike the blocked one. This isolation would be important for the selectivity of information passing through the cells, which would trigger a differentiation signal.

Chapter 3 indicates that sucrose and starch metabolism is as efficient markers for the identification of cell lines with high embryogenic potential. Key genes of sucrose and starch metabolism have identified similarities between the responsive cell line and the globular stage of zygotic embryogenesis. In this way, sucrose and starch can be used as markers of embryogenic competence during the somatic embryogenesis of *A. angustifolia*.

On behalf of the chapter 4, at the carbon metabolism, the differences between zygotic and somatic embryogenesis were highlighted. The main differences were observed in targets involved in post-translational changes, either by amino acids modifications or by protein biogenesis/degradation. In addition, metabolites related to sugars/starch and sugar alcohols, were the only ones that did not have a centrality variation between zygotic and somatic embryogenesis, highlighting that carbohydrate metabolism is important for the embryogenesis process.

Somatic embryogenesis is a complex process where the success of each step depends on the adequate performance in the previous step. The establishment of embryogenic cultures in this species is genotype-dependent, however, the embryogenic potential is only observed during the maturation stage. Thus, the results generated in this work pinpoint the carbohydrates metabolism as good targets for the determination of biochemical markers associated with somatic embryogenesis of *A. angustifolia*. In addition, the data generated reveal new perspectives on the study of carbon metabolism in embryogenesis, highlighting sugar sensing as an important signaling pathway during the embryo development.

As future perspectives, the data generated in this work will be valuable for the initiation of integrative approaches with other known embryogenesis signals for *A. angustifolia*, which several factors act at the same time modulating this process. In this way, integrative studies that take into account the regulation at different levels (transcript, protein and metabolite) should be performed in the same comparative approach between zygotic and somatic embryogenesis. In addition, more in-depth studies of sugar-mediated responses via sugar sensing and signaling process can be explored, based on the results presented previously. These studies can guide new insights in the manipulation of factors that improve the somatic embryo development, which may allow the modulation of sugar sensing responses for improved embryo development and optimization of *A. angustifolia* somatic embryogenesis protocols. Thus, in conjunction with the results generated, it is expected that our findings highlight that the modulation of embryogenesis is systemically controlled by concerted action of multiple factors, which carbohydrates metabolism is a high centrality during zygotic and somatic embryogenesis in *A. angustifolia.*