EXOGENOUS AUXIN IMPAIRS STORAGE RESERVE ACCUMULATION AND SOMATIC EMBRYO INDUCTION IN SUGARCANE EMBRYOGENIC CALLUS

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"Thesis submitted to the Centro de Ciências e Tecnologias Agropecuárias at the Universidade Estadual do Norte Fluminense Darcy Ribeiro in partial fulfillment of the requirements for the degree of Doctor in Genetics and Plant Breeding"

Adviser: Prof. Vanildo Silveira

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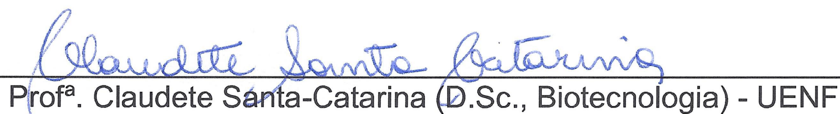

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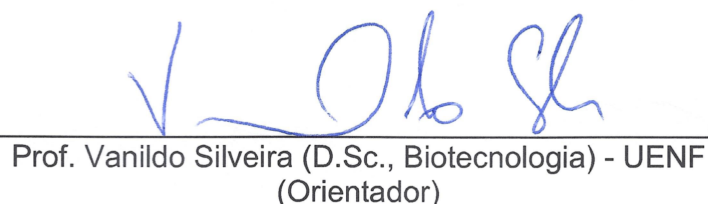

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic Acid
ABA	Abscisic Acid
ABI3	ABA INSENSITIVE3
ABI4	ABA INSENSITIVE4
ACC	1-Aminocyclopropane-1-Carboxylate
AGL15	AGAMOUS-LIKE15
ARF	Auxin Response Factor
BBM	BABY BOOM
CTC	Centro de Tecnologia Canavieira
CTNBio	National Technical Commission on Biosefety
DTT	Dithiothreitol
ER	Endoplasmic Reticulum
FASP	Filter-Aided Sample Preparation
FDR	False Discovery Rates
FM	Fresh Matter
FUS3	FUSCA3
FWHM	Full Width at Half Maximum
GA	Gibberellin
HDMSE	High Definition MSE
IAA	Indole-3-Acetic Acid
JA	Jasmonic Acid
LAFL	LEC1, ABI3, FUS3, LEC2

LEA	Late Embryogenesis Abundant
LEC1	LEAFY COTYLEDON1
LEC2	LEAFY COTYLEDON2
miRNA	microRNA
MS	Murashige and Skoog
PAS	Periodic Acid–Schiff
PB	Protein Body
PGR	Plant Growth Regulator
PHB	Polyhydroxybutyrate
PLGS	ProteinLynx Global SERVER
PM	Prematuration
PMSF	Phenylmethanesulfonyl Fluoride
PSV	Protein Storage Vacuoles
ROS	Reactive Oxygen Species
SERK	SOMATIC EMBRYOGENESIS RECEPTOR KINASE
TFA	Trifluoroacetic Acid
TOF	Time of Flight
UPLC	Ultra Performance Liquid Chromatography

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ABSTRACT

REIS, Ricardo de Souza dos; D.Sc.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; February 2019; EXOGENOUS AUXIN IMPAIRS STORAGE RESERVE ACCUMULATION AND SOMATIC EMBRYO INDUCTION IN SUGARCANE EMBRYOGENIC CALLUS; Adviser: Prof. Vanildo Silveira; Counselors: Profª. Virginia Silva Carvalho e Prof. Gonçalo Apolinário de Souza Filho

Several factors can influence the culture conditions and then the somatic embryogenesis responses during plant tissue culture, and plant growth regulators play important roles during this developmental process. Auxin is required for callus induction and embryogenic capacity acquisition; however, the removal of auxin is needed for further differentiation of somatic embryos. Thus, this study aimed to evaluate the effects of 2,4-dichlorophenoxyacetic acid (2,4-D) on differentiation of sugarcane somatic embryos. Embryogenic callus were submitted to a prematuration treatment (without 2,4-D; PM) or maintained in a multiplication culture medium (control). After 21 days, PM and control treatments were transferred to maturation into a plant growth regulator (PGR)-free culture medium. We observed that the presence of 2,4-D in the control treatment had a negative effect on differentiation of somatic embryos. Histochemical analysis demonstrated a higher accumulation of protein and starch reserves in PM treatment. Proteomic data showed a decrease in the abundance of abscisic acid (ABA)-induced proteins in the control treatment. Proteins of the ethylene biosynthetic pathway were also negatively affected, which could suggest a decrease in intracellular levels of both hormones in the control treatment. A disruption in ABA and ethylene levels may be responsible for the delay

in the accumulation of storage reserves, which may have impaired the onset of somatic embryogenesis in the control treatment. The identification of antioxidant enzymes (Peroxidase and Glutathione s-transferase) as more abundant in the control treatment may indicate a low level of reactive oxygen species (ROS). Other studies have demonstrated that a relatively high ROS level is important to promote somatic embryogenesis and the lower abundance of ROS-related enzymes in PM treatment may suggest a mild-to-high oxidative environment. Finally, our results also demonstrated that the efficient development of sugarcane somatic embryos seems to be preceded by a better accumulation of storage reserves during embryogenic callus differentiation, which in turn may be related to an optimal hormone homeostasis. Finally, our results also demonstrated that the efficient development of sugarcane somatic embryos seems to be preceded by a better accumulation of storage reserves during embryogenic callus differentiation, which in turn may be related to hormone homeostasis. In addition, several differentially regulated proteins, such as ABA and auxin-induced proteins, ethylene biosynthetic pathway proteins, as well as antioxidant and storage reserve-related proteins suggest a hormone balance is important for somatic embryo differentiation in sugarcane.

Key words: Hormones; Label-free Proteomics; Somatic Embryos; Histochemistry; Storage Reserves.

RESUMO

REIS, Ricardo de Souza dos; D.Sc.; Universidade Estadual do Norte Fluminense Darcy Ribeiro; Fevereiro de 2019; AUXINA EXÓGENA PREJUDICA O ACÚMULO DE RESERVAS E A INDUÇÃO DE EMBRIÕES SOMÁTICOS EM CALOS EMBRIOGÊNICOS DE CANA-DE-AÇÚCAR; Orientador: Prof. Vanildo Silveira; Conselheiros: Profª. Virginia Silva Carvalho e Prof. Gonçalo Apolinário de Souza Filho.

Vários fatores podem influenciar as condições da cultura e as respostas da embriogênese somática e os reguladores de crescimento vegetal desempenham papéis importantes durante esse processo de desenvolvimento. A auxina é necessária para indução de calo e aquisição de capacidade embriogênica; no entanto, a remoção da auxina é necessária para o progresso da diferenciação dos embriões somáticos. Assim, este trabalho teve como objetivo avaliar os efeitos residuais do ácido 2,4-diclorofenoxiacético (2,4-D) na diferenciação de embriões somáticos da cana-de-açúcar. Os calos embriogênicos foram submetidos a um tratamento de pré-maturação (sem 2,4-D; PM) ou mantidos em meio de multiplicação (controle). Após 21 dias, os tratamentos PM e controle foram transferidos para a maturação em meio de cultura livre de regulador de crescimento de plantas (PGR). Observamos que a presença de 2,4-D no tratamento controle teve um efeito negativo na diferenciação dos embriões somáticos. Análises histoquímicas demonstraram maior acúmulo de reservas proteicas e de amido no tratamento PM. Os dados proteômicos mostraram uma diminuição na abundância de proteínas induzidas pelo ácido abscísico (ABA) no tratamento controle.

Proteínas da via biossintética do etileno também foram afetadas negativamente, o que poderia sugerir uma diminuição no nível intracelular de ambos os hormônios no tratamento controle. Uma perturbação nos níveis de ABA e etileno pode ser a responsável pelo atraso no acúmulo de reservas de armazenamento, o que pode ter prejudicado o início do desenvolvimento da embriogênese somática no tratamento controle. A identificação de enzimas antioxidantes (Peroxidase e Glutationa s-transferase) como mais abundantes no tratamento controle possivelmente indica um baixo nível de espécies reativas de oxigênio (ROS). Tem sido demonstrado que um nível relativamente alto de ROS é importante para promover a embriogênese somática e a menor abundância de enzimas relacionadas à ROS no tratamento PM pode sugerir um ambiente oxidativo de leve a alto. Em conclusão, nossos resultados também demonstraram que o desenvolvimento eficiente do embrião somático de cana-de-açúcar parece ser precedido por um melhor acúmulo de reservas durante a diferenciação de calos embriogênicos, o que pode estar relacionado a um balanço hormonal. Isto foi indicado pela identificação de várias proteínas induzidas por ABA e auxina, proteínas da via biossintética do etileno, bem como proteínas antioxidantes e àquelas relacionadas à reserva de armazenamento que foram reguladas diferencialmente no tratamento PM quando comparado ao controle.

Palavras-chave: Hormônios; Proteômica Label-free; Embriões Somáticos; Histoquímica; Reservas de Armazenamento.

1. INTRODUCTION

The first observations of somatic embryogenesis occurred in the late 1950s (Krikorian and Simola, 1999; Vasil, 2008). The researchers observed that plant cells (and tissues) can be stimulated to reprogram their genetic information, acquiring the capacity to develop into somatic embryos and then a whole plant, thus expressing plant totipotency. Since then, much knowledge regarding this plant developmental process have been discovered. For sugarcane, the first studies reporting the development of somatic embryos were carried out in the 1980s through callus differentiation (Ahloowalia and Maretzki, 1983; Ho and Vasil, 1983b, a).

Most of these findings occurred in genomic studies, in which genes that control many aspects of plant cell dedifferentiation and differentiation are related to somatic embryogenesis (Horstman et al., 2017a; Lepiniec et al., 2018). This intricate signaling demonstrates the complexity of the molecular mechanisms that control the developmental plasticity of plant cells to generate a whole new organism. Proteomic studies have also been applied as a valuable tool to understand plant biological processes (Takac et al., 2011) and the use of such a tool has shed some light on the discovery of differentially abundant proteins during the development of somatic embryogenesis (Heringer et al., 2018).

Meanwhile, other studies focusing on the development of suitable conditions for somatic embryos induction became as much important as the genetic factors dictating the progress of this developmental program. Somatic embryogenesis often is species or genotype-dependent, requiring specific culture conditions to be used

for induction, maturation, and regeneration of the somatic embryos. Thus, the establishment of adequate protocols is important to applying this biotechnological technique in large scale production and genetic engineering programs.

Several factors can influence the culture conditions and the somatic embryogenesis responses, such as different light spectra (Heringer et al., 2017) and carbon source (Reyes-Díaz et al., 2017). These factors also include the plant growth regulators (PGR), which perhaps play the most important roles in plant tissue culture, and auxin is considered a crucial inducer of the embryogenic capacity, wherein the synthetic molecule 2,4-dichlorophenoxyacetic acid (2,4-D) is the most widely used (Jiménez, 2005; Fehér, 2015). Indeed, callus formation is dependent on a complex of auxin-induced transcription factors that is downstream of AUXIN RESPONSE FACTORs (ARFs), thus regulating cell fate (Xu et al., 2018). The diverse transcription factors related to somatic embryogenesis not only are induced by PGRs, but also activate the expression of hormone biosynthesis and signaling pathways (Horstman et al., 2017a). Depending on the plant tissue, hormones interaction can be synergistic, as demonstrated by the auxin-induced ethylene triggering abscisic acid (ABA) biosynthesis (Hansen and Grossmann, 2000), or antagonistic, as during seed dormancy (Beaudoin et al., 2000).

Depending on its concentration, auxin can also induce the generation of reactive oxygen species (ROS), mainly hydrogen peroxide and superoxide anions, as well as induce an increase in ROS-scavenging enzymes; however, at herbicide concentration, the production of ROS is too much elevated to be counteracted by the ROS-scavenging system (Romero-Puertas et al., 2004; Shi et al., 2014). The molecules of ROS are produced in every cellular compartment during metabolic reactions or by any stress perceived by plants and can be toxic, when in elevated levels, or function as signaling molecules when the homeostasis is maintained (Choudhury et al., 2017). At toxic level, ROS can oxidize proteins, lipids, RNA, and DNA, which could lead cells to an oxidative destruction (Choudhury et al., 2017). The ROS can also regulate the biosynthesis and signaling of several hormones, such as auxin, brassinosteroids, ABA, and gibberellin (Choudhury et al., 2017). In addition, ROS can also participate of regulation and signaling of cell differentiation processes during proliferation, elongation, expansion, and dedifferentiation (Schmidt and Schippers, 2015). It has also been demonstrated that the redox

signaling plays important roles in the induction of somatic embryogenesis (Becker et al., 2014; Zhou et al., 2016).

Different aspects of tissue culture play important roles during the development of somatic embryogenesis, especially, the type and concentration of PGR. However, establishing a universal protocol for somatic embryo induction is practically impossible because of the intrinsic nature of each species or genotype as a source of explant. Thus, different species, genotypes, and tissues may show different responses to the same PGR, sometimes even with opposite responses. In addition, not only the endogenous hormone level will influence on somatic embryos induction rates, but also the sensitivity of each plant material to the different stimulus perceived (Jiménez, 2005). Therefore, the present study aimed to investigate the effects of 2,4-D on the progress of embryogenic callus differentiation into somatic embryos. In our study, we used histochemical, morphological, and proteomic analyses to understand the relations between auxin and somatic embryo production. We found histochemical differences between the treatments regarding the accumulation of protein and starch storage reserves. In addition, comparative proteomic analysis also demonstrated that the 2,4-D induced a differential accumulation in ABA-induced proteins as well as those proteins related to ethylene biosynthetic pathway and ROS detoxification.

2. OBJECTIVES

Investigate the effects of 2,4-D on the progress of embryogenic callus differentiation into somatic embryos during sugarcane callus development.

2.1. Specific objectives

- Analyze the morphology of embryogenic callus to evaluate the effect of a prematuration treatment (PM) without 2,4-D on the development of somatic embryos during callus differentiation.
- Analyze the histomorphology of embryogenic callus submitted to PM and control treatments during callus differentiation to observe the accumulation of proteins (Coomassie staining) and starch (PAS staining).
- Analyze the proteomics of embryogenic callus submitted to PM and control treatments during callus differentiation.

3. REVIEW

3.1. Sugarcane

The modern sugarcane is the combination of a high-sugar content species, *Saccharum officinarum,* with the hardiness and disease resistance of the wild species *Saccharum spontaneum*. Because of that crossing, the cultivated varieties are highly complex interspecific hybrids, featuring a genome constitution of 2n=100-130 (Arruda, 2012). Sugarcane has been grown in more than 100 countries and is one of the world's most economically important crops (FAO, 2017). According to FAO (2017), Brazil, India, China, Thailand, and Pakistan are the world's five major sugarcane producers.

The sugarcane crop has had an economical importance to Brazil since the establishment of the first mill in the 16th century. Today, this importance is demonstrated by the contribution of approximately 43 billion USD of the sugar-energy sector to the gross domestic product (GDP) in the 2013-2014 harvest (Neves and Trombin, 2014). In addition to use in sugar and ethanol production, sugarcane has also been bred for biomass production as an alternative and renewable energy source to fossil resources (Kandel et al., 2018). This energy cane is a cane selected to have more fiber than sucrose in its composition, resulting in a significant increase in biomass yield that can be used for energy and second-generation ethanol production (Matsuoka et al., 2014). Considering the use of bagasse and straw for second-generation ethanol, Brazilian sugarcane industry is estimated to produce an

increase of up to 40% in the total ethanol production (De Souza et al., 2013). Regarding bioelectricity, Wilkinson (2015) estimates that by 2021 the potential for electricity generation will be 13.1 GW, which is equal to three Belo Monte hydroelectric plants.

3.2. Sugarcane and biotechnology

The bottlenecks of the conventional breeding and propagation of sugarcane are the long-term process, the complex genome, and the low multiplication rates. Thus, biotechnology studies and tools have a great potential to be applied in the sugar-energy industry by seeking to improve the agronomic performance and increase sugar and ethanol production of sugarcane crops. Therefore, micropropagation and genetic transformation could be helpful in addressing these problems through the large-scale multiplication of selected plants originated from protocols of *in vitro* embryogenesis and organogenesis and the development of new cultivated varieties with favorable agronomic characteristics. Biotechnological studies in sugarcane have faced challenges because of the genome size and complexity; however, this limitation has been recently addressed with sugarcane genome sequencing (Garsmeur et al., 2018; Zhang et al., 2018), which will allow the application of “omics” technologies to study and improve sugarcane characteristics and performance.

The first studies in the genetic transformation of sugarcane began in the 1980s (Chen et al., 1987), and several others are currently focused on the development of plants resistant to biotic (Schneider et al., 2017; Cristofoletti et al., 2018) and abiotic stresses (Ramiro et al., 2016), energy production (Zale et al., 2016; Kim et al., 2017a), biopolymers such as polyhydroxybutyrate (PHB) (McQualter et al., 2015), among others. However, in 2017, the first commercial use of a transgenic sugarcane plant was approved by the National Biosafety Technical Commission (CTNBio) in Brazil. The transgenic plant (CTC20BT™) confers resistance to the sugarcane borer and has been developed by the Centro de Tecnologia Canavieira (CTC) company (CTNBio, 2019). Although not yet approved for commercial use, Indonesia has also approved three transgenic varieties of a drought tolerant sugarcane (ISAAA, 2019). However, all this genetic transformation

technology depends on the application of another important biotechnological technique, the plant tissue culture.

3.2. Tissue culture and somatic embryogenesis

The tissue culture technique consists of the aseptic culture of cells, tissues, or organs in an *in vitro* environment under physical and chemical controlled conditions (Thorpe, 2007). The technique is based on the principle of totipotency, which states that plant cells can divide, differentiate, and then originate a whole new organism. This makes tissue culture an essential tool for genetic transformation and allows the production of disease-free plants, germplasm conservation, and mass propagation of plants. Since the first studies with tissue culture more than 100 years ago, several advancements have been achieved. These advancements include the development of new suitable culture media and the discovery of plant PGRs, which allow for the long-term growth of cultures (Vasil, 2008).

In sugarcane, the tissue culture technique was first applied in the 1960s through the induction of callus, which were used later to induce roots (Nickell, 1964). Sugarcane plants were later regenerated from callus-induced shoots (Barba and Nickell, 1969; Heinz and Mee, 1969), and somatic embryos were first observed (Ahloowalia and Maretzki, 1983; Ho and Vasil, 1983a, b). Since then, several other studies were performed aiming at different applications, such as micropropagation and disease-free plants, breeding, germplasm conservation, and genetic modification (Lakshmanan et al., 2005).

Micropropagation is the main application of tissue culture, and in comparison with the conventional propagation system, it allows for the large-scale and disease-free production of plantlets in a short time by using controlled environmental conditions during the culture development. Briefly, micropropagation is the clonal propagation of quality plant material, bypassing the conventional vegetative and sexual propagation. Commercial sugarcane is propagated by planting internodes, which is a slow process of multiplication because of the single bud per internode (Snyman et al., 2011). Additionally, the conventional method can spread pathogens in the crops, since the renewal of the crop is not annually (Snyman et al., 2011). Micropropagation could overcome all these drawbacks by ensuring the mass propagation of healthy plants. This clonal multiplication can be made by

organogenesis and somatic embryogenesis (Chavan et al., 2014; Ferreira et al., 2017; Mazri et al., 2017; Bayarmaa et al., 2018; Pérez-Alonso et al., 2018; Wang et al., 2018).

Somatic embryogenesis is the formation of embryos from somatic cells, a developmental process controlled by several endogenous and exogenous factors (Fehér et al., 2003; Gaj, 2004; Fehér, 2015; Horstman et al., 2017a). The embryos are formed after somatic (differentiated) cells perceive some stimulus that is usually provided by PGRs or some type of stress, reprogramming their genetic information and thus acquiring the status of totipotent cells. After that, other stimulus, usually the removal of auxin or occasionally the addition of maturation agents, may induce the redifferentiation of cells into bipolar structures (somatic embryos), which may develop into a whole new plant if placed in an appropriate condition (Gaj, 2004; Jiménez, 2005; Fehér, 2015; Sugimoto et al., 2019).

Somatic embryogenesis was first described independently by three researchers in the 1950s (Krikorian and Simola, 1999; Vasil, 2008). Since then, this developmental process has been reported for many other species with the development of species-specific protocols. These studies have aimed to identify and explain the molecular mechanisms that control somatic embryogenesis. Somatic embryos may be originated either from a callus phase, which is termed as indirect somatic embryogenesis, or from the surface of explants by a process called direct embryogenesis. The capacity of an explant to undergo the direct or indirect pathways seems to depend more on the combination of the culture conditions and developmental context of the explant than solely on the age of the tissue, although it is easier to induce somatic embryos from young tissues (Horstman et al., 2017a). When cells grow indefinitely, forming callus, two types of masses may usually be distinguished by the morphology: an embryogenic, which has the capacity to differentiate into embryos, and a non-embryogenic. Although the type and characteristics of cells that form somatic embryos are well known, their molecular mechanisms remain largely undescribed (Horstman et al., 2017a). However, genes expressing for transcription factors have already been described as relating to the development of somatic embryogenesis (Fehér, 2015; Horstman et al., 2017a; Horstman et al., 2017b).

Horstman et al. (2017b) demonstrated that the BABY BOOM (BBM) transcription factor activates the signaling network composed by *LEAFY*

COTYLEDON1 (*LEC1*), *LEC2*, *FUSCA3* (*FUS3*), and *ABA INSESITIVE3* (*ABI3*), termed *LAFL* genes, as well as the *AGAMOUS-LIKE15* (*AGL15*) to further induce somatic embryogenesis development. The overexpression of *BBM*, *LEC1*, and *LEC2* may induce the formation of somatic embryos in *Arabidopsis* in PGR-free culture medium (Horstman et al., 2017b). Meanwhile, mutations in *LEC/FUS* genes have been shown to be detrimental to embryo development, including completely repressing embryogenic capacity when there were multiple mutations in *LEC/FUS* loci (Gaj et al., 2005). However, organogenesis was not affected by these mutations, which points to a difference in the genetic determination of *in vitro* embryogenesis and organogenesis pathways with independent mechanisms that trigger these two developmental pathways (Gaj et al., 2005). This complex signaling network regulates plant cell totipotency by using an intricate feedback loop and involving the activation of many other gene pathways (Horstman et al., 2017a; Horstman et al., 2017b; Lepiniec et al., 2018; Méndez-Hernández et al., 2019).

The regulation of the *LAFL* genes during embryo or seed development can regulate, for instance, the accumulation of storage reserves, such as, oil, starch, and proteins (Lepiniec et al., 2018). However, the repression of the *LAFL* genes is essential for the transition from embryonic to vegetative growth (Jia et al., 2013; Jia et al., 2014). The *LAFL* genes can also regulate the biosynthesis of hormones (e.g., IAA, ethylene, ABA), which by feedback, can positively or negatively regulate *LAFL* genes expression in turn (Horstman et al., 2017a; Lepiniec et al., 2018; Méndez-Hernández et al., 2019).

Regarding hormone regulation, the overexpression of the *LEC2* gene promotes an increase in the auxin levels by activating the auxin biosynthetic pathway genes, which can induce the embryogenic capacity of the plant tissue (Stone et al., 2008; Wójcikowska et al., 2013; Wójcikowska and Gaj, 2015). LEC2 could also activate the expression of seed protein transcripts in vegetative tissues prior to the accumulation of *LEC1* and *FUS3* transcripts, which are both related to the maturation phase during seed development (Braybrook et al., 2006; Stone et al., 2008). Both LEC2 and FUS3 negatively regulate the expression of a key gene responsible for bioactive gibberellin (GA) biosynthesis, and FUS3 positively regulates ABA biosynthesis, while auxin induces *FUS3* expression (Curaba et al., 2004; Gazzarrini et al., 2004). Depending on their balance, ABA and GA also affect the stability of FUS3 protein in a positive and negative manner, respectively,

interfering in its accumulation inside cells (Gazzarrini et al., 2004). Wang and Perry (2013) have reported that FUS3 regulates *AGL15*, *ABI3*, *BBM*, as well as miRNA-encoding genes that participate in the phase of transition during seed development. Although auxin has been pointed to as important to embryogenic competence and AGL15 has been identified as an inducer of somatic embryogenesis, this transcription factor is responsible for repressing the auxin signaling pathway and GA accumulation, while inducing ethylene biosynthesis and response (Zheng et al., 2013; Zheng et al., 2016). Thus, all these interactions demonstrate the complexity among the regulation of embryo transcription factors, their target genes, and the somatic embryogenesis development. These interactions shed further light upon the molecular mechanisms controlling this developmental process.

3.3. Hormones and PGRs

Among the multiple factors influencing the plant tissue culture, the PGRs and hormones may play the most important roles in inducing organogenesis and somatic embryogenesis. Auxin, mainly 2,4-D, is a crucial inducer of embryogenic competence; however, its removal from or reduction in the culture medium is necessary for further morphogenetic development. Mutants, which contain several defective hormone-responsive genes, demonstrate the importance of hormones to somatic embryogenesis.

Gaj et al. (2006) showed that several *Arabidopsis thaliana* mutants have impaired formation of direct somatic embryos. These mutants are either insensitive or hypersensitive to auxin, ABA, and GA. The authors highlighted the fact that GA is usually considered a negative modulator of somatic embryo induction; however, both the elevated and deficient GA mutants showed low somatic embryo production (Gaj et al., 2006). This result seems to point to an optimal balance of plant hormones required for correct somatic embryogenesis development, including hormones that usually have negative effects.

Abscisic acid, the so-called stress hormone, also plays an important role in somatic embryo induction, since it can positively regulate auxin biosynthesis, polar transport, and distribution patterns; thus, ABA is required for somatic embryogenesis by controlling spatial auxin response (Su et al., 2013). In soybean seeds, exogenous auxin treatment represses germination by enhancing ABA

biosynthesis and signaling genes, as well as decreasing GA biosynthesis (Shuai et al., 2017). The levels of both ABA and GA followed the same pattern of their biosynthesis genes (Shuai et al., 2017). The antagonistic interaction between ABA and GA seems to be regular, because it occurs in a great diversity of tissues during plant growth and development (Liu and Hou, 2018). However, the same is not observed for the complex interaction between ABA and ethylene. This interaction is not entirely clear, because it can differ depending on the tissue in which it occurs (Beaudoin et al., 2000; Hansen and Grossmann, 2000).

Auxin and ABA appear to have a synergistic interaction in different tissues, presenting a positive feedback loop (Jiménez and Bangerth, 2001; Su et al., 2013; Shuai et al., 2017). Despite this synergism between natural auxin (IAA) and ABA, the synthetic auxin, 2,4-D, seems to have a direct or indirect negative effect on IAA and ABA levels in carrot callus cultures (Jiménez and Bangerth, 2001). Moreover, the overexpression of *LEC2* genes in immature zygotic embryo cultures of *Arabidopsis* resulted in low embryogenic potential, high IAA levels, and reduced ABA levels when cultured in the presence of 2,4-D, suggesting an over-optimal (disrupted) auxin level (Ledwoń and Gaj, 2009; Wójcikowska and Gaj, 2015).

In non-overexpressing cultures, the perception of 2,4-D may cause an increase in the ethylene levels, which down-regulates IAA biosynthesis genes, thus decreasing IAA levels. Conversely, removal of 2,4-D causes the opposite effect (Bai et al., 2013; Fehér, 2015; Nowak et al., 2015), which may take both hormones into homeostasis after some time. Now, in *LEC2*-overexpressing cultures, would 2,4-D indirectly (through ethylene) or directly inhibit ABA biosynthesis, even with the overproduction of IAA, a positive regulator of ABA? These interactions in callus cultures among 2,4-D, ABA, and ethylene are still not entirely clear, needing to be better elucidated.

3.4. Proteomics applied to somatic embryogenesis

Proteins are polypeptide chains of amino acids covalently bonded by the carboxyl groups of amino acids and the amino groups of others forming peptide bonds. Proteins are the functional molecules of living organisms. However, to perform their molecular functions properly, proteins must be correctly folded and frequently combined with other molecules, such as carbohydrates, lipids, and

phosphate groups, among others. These post-translational modifications may cause physical and chemical changes in the proteins, which then cause conformational alterations that may drive proteins to interact differently with their target. Moreover, proteins are dynamic molecules, and their accumulations can be influenced by different environmental factors perceived by cells, which usually do not occur in genomes. Thus, proteomic studies complement genomics by allowing a comprehensive monitoring of the metabolism and biochemistry of plants in response to external or internal stimuli and observing how plant development is affected (Takac et al., 2011; Heringer et al., 2018; Kosová et al., 2018).

Regarding plant tissue culture, the use of a proteomic approach to study somatic embryogenesis has been extensively explored in several works, and many proteins have been ascribed to be potentially related to somatic embryogenesis (Chin and Tan, 2018; Heringer et al., 2018; Loyola-Vargas and Aguilar-Hernández, 2018). In sugarcane, for instance, the embryogenic competence may be related to a differential abundance and activity of proton pumps (Passamani et al., 2018). While in responsive and non-responsive hybrids of *Elaeis oleifera* × *Elaeis guineensis*, embryogenic competence has been associated with a better adaptation of the responsive genotype to oxidative stresses, thus allowing the use of energy for cellular proliferation (Almeida et al., 2019). In *Gossypium hirsutum*, SERK protein and the LEC transcription factor have been found to be up-regulated in embryogenic callus cultures when compared to non-embryogenic (Zhu et al., 2018). In another study, Reis et al. (2016) described the effects of putrescine on somatic embryo induction in sugarcane and several proteins such as peroxidases, 14-3-3, heat shock proteins, and others as differentially abundant between treated and control cultures. Heringer et al. (2017) applied proteomic analysis to study the effects of different light spectra on the development of sugarcane somatic embryogenesis. Therefore, despite most proteomic studies are still descriptive, proteomics could be combined with metabolomic, genomic, and transgenic experiments to better explore the still-elusive molecular mechanisms of dedifferentiation and redifferentiation of somatic cells into embryos. Recently, two independent works reported the sequencing genome of sugarcane (Garsmeur et al., 2018; Zhang et al., 2018), which will be helpful in integrating the “omics” studies in the exploration of somatic embryogenesis.

4. MATERIALS AND METHODS

4.1. Plant material and callus induction

Callus induction was performed on sugarcane plants cv. SP80-3280 according to Silveira et al. (2013). Two-month-old plants were used as source of explants. The outer mature leaves were removed, and the resulting leaf rolls were surface sterilized in 70% ethanol for 1 min, then in 30% commercial bleach (2-2.5% sodium hypochlorite) for 15 min, and subsequently washed three times in autoclaved distilled water. The explants were obtained by transversely sectioning the leaf rolls into 3 mm-thick slices and culturing them in test tubes (150 x 25 mm) containing 10 mL of MS culture medium (Murashige and Skoog, 1962) (Phytotechnology Lab, Overland Park, KS, USA), supplemented with 20 g/L sucrose, 2 g/L Phytagel® (Sigma-Aldrich, St. Louis, MO, USA), 10 μM 2,4-D (Sigma-Aldrich), pH 5.8, and sterilized. pH was adjusted before adding phytagel. The cultures were kept in the dark at 25 °C ± 1 for approximately 45 days. Induced callus was transferred to Petri dishes (90 x 15 mm) containing 20 mL of the same culture medium (proliferation culture medium) and kept in the dark at 25 °C ± 1, with subcultures only of embryogenic callus every 21 days.

4.2. Prematuration treatment (PM) without 2,4-D

After three subcultures, part of the embryogenic callus was inoculated onto prematuration treatment (PM) consisting of PGR-free MS culture medium supplemented with 20 g/L sucrose and 2 g/L Phytagel, pH 5.8, and sterilized. Embryogenic cultures maintained onto proliferation culture medium were referred to as control treatment. The cultures were kept in the dark at 25 °C ± 1 for one more subculture (21 days) and then used for differentiation.

4.3. Differentiation and maturation

For somatic embryo differentiation, embryogenic callus from PM and control treatments were inoculated onto MS culture medium supplemented with 30 g/L sucrose, 2 g/L Phytagel, pH 5.8, and sterilized. After inoculation, the cultures were maintained in a growth chamber at 25 ± 1 ºC and grown in the dark for the first 7 days. Thereafter, the cultures were transferred to a 16h photoperiod, using GreenPower TLED 20W WmB (Koninklijke Philips Electronics NV, Netherlands) at 55 µmol/m^2/s and wavelength of 450/530 nm, at 25 ± 1 °C for a total of 42 days. Biological replicate consisted of one Petri dish containing four callus of 300 mg of fresh matter (FM) each. All experiments used five biological replicates per treatment. After 42 days, cultures were evaluated regarding the number of somatic embryos, by using a stereomicroscope, and FM increase. This experiment was repeated once more for validation of the results. Another short experiment for sample collection was performed and the samples were collected on days 0, 14, and 28 for further proteomic and histochemical analyses. Figure 1 shows the workflow of the experiment.

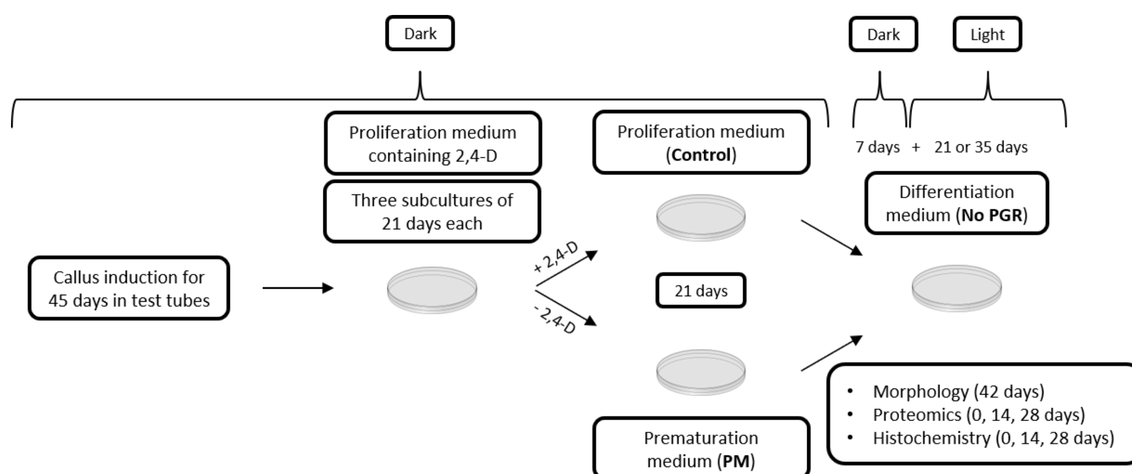

Figure 1. Complete workflow of the experiments.

4.4. Histochemical analysis

Samples of embryogenic callus from PM and control treatments were fixed for 24h at room temperature in aqueous solution of 2.5% glutaraldehyde, 4% formaldehyde, and 0.1 M sodium cacodylate, pH 7.3. After fixation, samples were dehydrated in increasing series of ethanol (30, 50, 70, 90, and 100%) for 3h each. Then, samples were infiltrated with Historesin (Leica, Wetzlar, Germany) and absolute ethanol at the same proportion for 12h, followed by pure Historesin for 24h. Infiltrated samples were embedded in Historesin and sectioned in 4 µm-thick slices, which were stained with Coomassie Brilliant Blue (CBB) for proteins (Gahan, 1984) and Periodic acid-Schiff (PAS) for starch (O'Brien and McCully, 1981). The samples were observed under an Axioplan light microscope (Carl Zeiss, Jena, Germany) using an Axiocam MRC5 digital camera (Carl Zeiss), and images analyzed by AxioVisionLE v4.8 software (Carl Zeiss).

4.5. Total protein extraction

The extraction buffer consisted of 7 M urea, 2 M thiourea, 2% triton X-100, 1% dithiothreitol (DTT, GE Healtcare), 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich), and cOmplete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Five biological samples (300 mg FM each sample) were pulverized using a mortar and pestle in liquid nitrogen. Then, in

microtubes, 1 mL of extraction buffer was added to the sample powder. Samples were vortexed for 30 min at 8 °C and centrifuged at 16,000 g for 20 min at 4 °C. The supernatants were collected, and protein concentration was measured using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA).

4.6. Protein digestion

Before the trypsin digestion step, protein samples were precipitated using the methanol/chloroform methodology to remove any interferent from samples (Nanjo et al., 2012). After protein precipitation, samples were resuspended in urea 7 M/thiourea 2 M solution for proper resuspension. Protein digestion was performed using the filter-aided sample preparation (FASP) methodology as described by Wiśniewski et al. (2009), with modifications. Before starting the digestion procedure, an integrity test was made to check for damaged filter units (Hernandez-Valladares et al., 2016), thus only the working units were used. After that, protein aliquots were added to the Microcon-30 kDa filter units (Millipore) (Lipecka et al., 2016), washed with 200 µL of 50 mM ammonium bicarbonate (solution A), and centrifuged at 10,000 g for 15 min at 25 °C (unless otherwise stated, all centrifugation steps were performed at this condition). This step was repeated once for complete removal of urea before reduction of proteins. Next, 100 µL of 50 mM DTT (GE Healthcare) freshly made in solution A were added, gently vortexed, and incubated for 20 min at 60 °C (1 min agitation and 4 min resting, at 650 rpm). Then, 200 µL of 8 M urea in 50 mM ammonium bicarbonate (solution B) were added, and centrifuged for 15 min. For protein alkylation, 100 µL of 50 mM iodoacetamide (GE Healthcare) freshly prepared in solution B were added, gently vortexed, and incubated for 20 min at 25 °C in the dark (1 min agitation and 19 min resting, at 650 rpm). Next, 200 µL of solution B were added and centrifuged for 15 min. This step was repeated once. Then, 200 µL of solution A were added and centrifuged for 15 min. This step was repeated twice. In the last washing, it should remain approximately 50 µL of sample. For protein digestion, 25 µL of 0.2% (v/v) RapiGest (Waters, Milford, CT, USA) and 25 µL of trypsin solution (1:100 enzyme:protein, V5111, Promega, Madison, WI, USA) were added, gently vortexed, and incubated for 18 h at 37 °C (1 min agitation and 4 min resting, at 650 rpm). For peptide elution, the filter units were transferred for new microtubes and centrifuged for 10 min. Then, 50 µL of solution A were added

and centrifuged for 15 min. This step was repeated once. For RapiGest precipitation and trypsin inhibition, 5 µL of 15% trifluoroacetic acid (TFA, Sigma-Aldrich) were added, gently vortexed, and incubated for 30 min at 37 °C. Then, samples were centrifuged for 15 min, the supernatants collected, and vacuum dried. Peptides were resuspended in 100 µL solution of 95% 50 mM ammonium bicarbonate, 5% acetonitrile and 0.1% formic acid. The resulting peptides were quantified by the A205 nm protein and peptide methodology using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific).

4.7. Mass spectrometry

Mass spectrometry was performed using a nanoAcquity UPLC connected to a Q-TOF SYNAPT G2-Si instrument (Waters, Manchester, UK). Runs consisted of five biological replicates of 1 µg of digested proteins. The injections were performed in a randomly fashion to avoid bias. During separation, samples were loaded onto the nanoAcquity UPLC M-Class Symmetry C18 5 µm trap column (180 µm × 20 mm) at 5 µL/min during 3 min and then onto the nanoAcquity M-Class HSS T3 1.8 µm analytical reversed phase column (75 µm × 150 mm) at 400 nL/min, with a column temperature of 45 °C. For peptide elution, a binary gradient was used, with mobile phase A consisting of water (Tedia, Fairfield, Ohio, USA) and 0.1% formic acid (Sigma-Aldrich), and mobile phase B consisting of acetonitrile (Sigma-Aldrich) and 0.1% formic acid. Gradient elution started at 5% B, then ramped from 5% B to 40% B up to 91.12 min, and from 40% B to 99% B until 95.12 min, being maintained at 99% until 99.12 min, then decreasing to 5% B until 101.12 min, and kept 5% B until the end of run at 117.00 min. Mass spectrometry was performed in positive and resolution mode (V mode), 35,000 FWHM, with ion mobility (HDMSE), and in DIA mode; ion mobility separation used an IMS wave velocity ramp starting with 800 m/s and ending with 500 m/s; the transfer collision energy ramped from 25 V to 55 V in high-energy mode; cone and capillary voltages of 30 V and 3000 V, respectively; nano flow gas of 0.5 Bar and purge gas of 150 L/h; and a source temperature of 100 °C. In TOF parameters, the scan time was set to 0.6 s in continuum mode with a mass range of 50 to 2000 Da. The human [Glu1]-fibrinopeptide B (Sigma-Aldrich) at 100 fmol/µL was used as an external calibrant and lock mass acquisition was

performed every 30 s. Mass spectra acquisition was performed by MassLynx v4.0 software.

4.8. Proteomic data analysis

Spectra processing and database search conditions were performed using ProteinLynx Global SERVER (PLGS) software v.3.02 (Waters). After optimization using the free software PLGS Threshold Inspector v2.4, the analysis utilized the following parameters: Apex3D of 150 counts for low-energy threshold, 25 counts for elevated-energy threshold, and 750 counts for intensity threshold; one missed cleavage; minimum fragment ions per peptide equal to three; minimum fragment ions per protein equal to seven; minimum peptides per protein equal to two; fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M) and phosphoryl (STY); default false discovery rate (FDR) of 1%; automatic peptide and fragment tolerance. For protein identification, we used the sugarcane EST protein databank from SUCEST project (Vettore et al., 2003), using a concatenated target-decoy approach for FDR estimation by reversing the database. Label-free quantification analyses were performed using ISOQuant software v.1.7 (Distler et al., 2014). Briefly, we used the following parameters: peptide and protein FDR 1%, sequence length of at least six amino acid residues, and minimum peptide score equal to six. Samples were normalized by a multidimensional normalization process, which corrects peak intensities based on the intensity and retention time domains. The software performed the relative protein quantification according to the TOP3 method. Based on relative abundances of uniquely assigned peptides, the abundances of shared peptides were redistributed to the respective source proteins, followed by the TOP3-based quantification (Distler et al., 2014). The output table contains the detailed settings used by ISOQuant for data analysis (Table S1). To ensure the quality of the results after data processing, only proteins present in at least three runs were accepted for differential abundance analysis. Proteins were considered as differentially abundant if fold change value was greater than 2 according to Student's *t*-test (Two-tailed, $P < 0.05$). Functional annotations were performed using Blast2Go software v.5.0 (Conesa et al., 2005) and UniprotKB (www.uniprot.org).

4.9. Statistics

All experiments were performed using completely randomized design. Data were analyzed using analysis of variance (ANOVA) ($P < 0.01$) followed by the Tukey test using the R statistical software (R Core Team, 2014) and the easyanova package (Arnhold, 2013). All analyses were performed with five biological replicates.

5. RESULTS

5.1. Sugarcane tissue culture

After 42 days of culture on differentiation medium, our results demonstrated that embryogenic callus from prematuration (PM) treatment were remarkably superior in the formation of somatic embryos than embryogenic callus from control treatment (Table 1 and Figure 2). The FM from PM treatment also increased slightly in comparison to the control (Table 1). Proteomic and histochemistry analyses used samples collected from another experiment of 28 days of culture.

Table 1. Number of somatic embryos (per gram of FM) and increase of FM in embryogenic callus from PM and control treatments after 42 days of incubation on differentiation medium.

Variables	Control	PM
Somatic embryos	43 b	243 a
FM*	4.91 b	5.54g a

Data are the average of two independent experiments. Values in the same line followed by different letters are significantly different according to the Tukey test ($P < 0.01$). Coefficient of variation (CV): somatic embryos (17%) and FM (5%). *Mean value of FM for each Petri dish containing four callus. n=5.

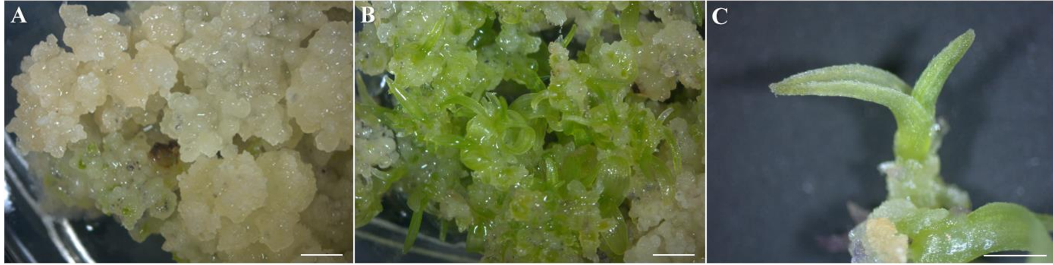

Figure 2. Morphological aspects of sugarcane embryogenic callus after 42 days of incubation on differentiation medium. Green points represent somatic embryo differentiation on callus surface. Control (A) and PM treatments (B). Sugarcane plantlet of PM treatment (C). Bars = 1 mm (A and B) and 0.5 mm (C).

5.2. Histomorphology

Histochemical analysis shows the differences between treatments in both CBB and PAS staining during the 28 days of culture (Figure 3). In CBB staining, the positive reaction showed conspicuous densely stained structures inside the cells from PM treatment on days 0 and 14, whereas on day 28 they decreased their abundance, demonstrating which seems to be a protein catabolic process (Figure 3A-C). These structures resemble either protein bodies (PBs) or protein storage vacuoles (PSVs). In clear contrast, the cells from control treatment did not form this storage protein structures (Figure 3D-F). In PAS staining, the positive reaction showed large starch grains on days 0 and 14 in the cells from PM treatment, while they are absent on day 28 (Figure 3G-I). In control treatment, starch grains did not form on day 0, but increased from day 14 to 28 (Figure 3J-L). However, these starch grains were smaller than those from PM treatment, which might be a slowly onset of energy reserve.

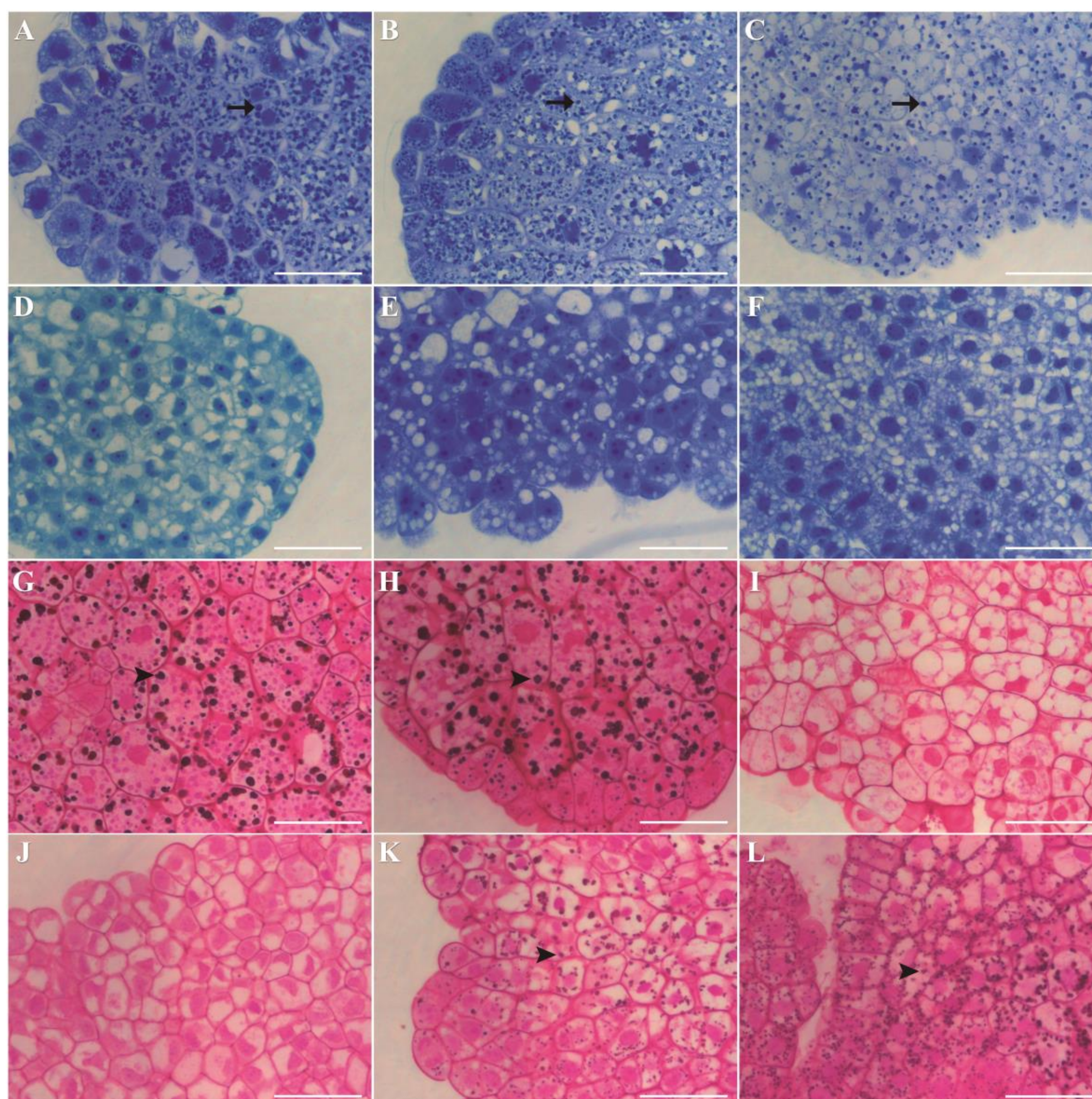

Figure 3. Histochemical analysis of sugarcane embryogenic callus from PM and control treatments over the 28 days of culture. CBB staining of PM (A-C) and control treatments (D-F) on days 0, 14, and 28, respectively. PAS staining of PM (G-I) and control treatment (J-L) on days 0, 14, and 28, respectively. Protein storage vacuoles (*arrow*) and starch grains (*arrowhead*). Bars = 50 µm.

5.3. Proteomic analysis

Embryogenic callus from PM treatment presented higher contents of total proteins in the three evaluated periods when compared to the control (Figure 4). Both conditions showed a peak on day 14, with a slight decrease up to day 28. These results, at least in part, are consistent with those observed in CBB staining (Figure 3A-F).

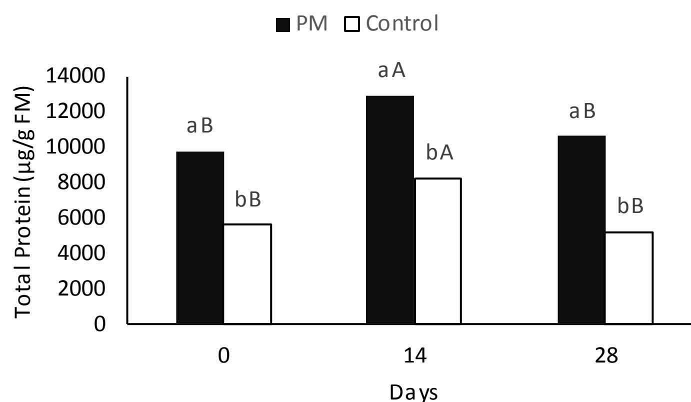

Figure 4. Total protein contents of embryogenic callus from PM and control treatments in 0, 14, and 28 days of incubation on differentiation medium. Lowercase letters denote significant differences between treatments within days of incubation. Capital letters denote significant differences among days of culture within same treatment. Means followed by different letters are significantly different ($P < 0.01$) according to the Tukey test. n = 5. CV = 12%.

Proteomic analysis was performed for samples collected on days 0 and 14, to evaluate the onset of somatic embryogenesis developmental process. The comparative proteomic analysis between embryogenic callus from PM and control treatments allowed the identification of 1986 and 1995 proteins for the days 0 and 14, respectively (Table S1). The ratio between PM and control treatments was used to evaluate the differential abundance of proteins tagged either as up- or down-regulated when presenting a fold-change of at least 2 ($P < 0.05$). Unique protein not necessarily means a completely absence of a given protein in one or another treatment, but also an abundance below the limit of detection of the mass spectrometer. Thus, 250 proteins were differentially abundant on day 0, with 84 proteins down-regulated, 109 up-regulated, 32 unique to control, and 25 unique to PM treatment. On day 14, 304 proteins were differentially abundant, in which 92 were down-regulated, 161 up-regulated, 29 unique to control, and 22 unique to PM treatment (Table S1).

Among the differentially regulated proteins, we found those related to response to auxin (IAA amido synthetase), ROS scavenging (peroxidases), chaperones, lipid metabolism (oleosins), storage proteins (globulins), response to ABA (LEA proteins), ethylene pathway (ACC oxidase), among others (Table S1). Based on selected regulated proteins and their functional classifications (Table 2 and S1), as well as the results obtained in the histochemical analysis, protein

quantitation, and somatic embryo differentiation, we formulated a molecular connection that could explain our results regarding the somatic embryos produced. These connections were plotted in a mind map (https://coggle.it) for the sake of clarity (Figure 5). In this figure, we represented the events occurring in the control that could justify a low production of somatic embryos. These events were possibly triggered by the presence of 2,4-D inside the cells causing a disruption in auxin homeostasis and interfering with the somatic embryogenesis developmental process. Thus, we highlighted the possible relation between ROS, ABA, ethylene, and auxin responsive proteins that could indicate the cellular metabolic state during the differentiation signaling to form somatic embryos in sugarcane.

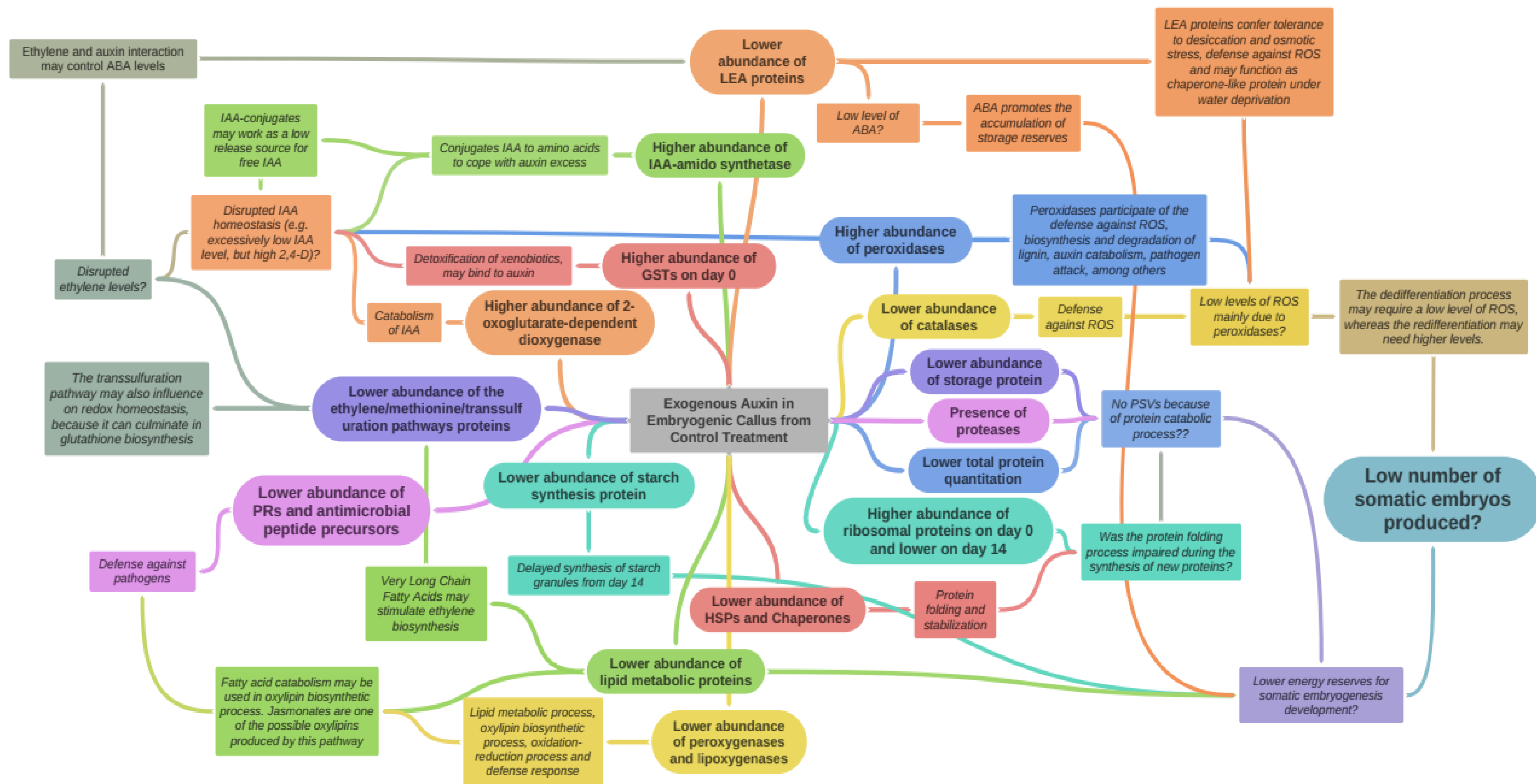

Figure 5. Schematic overview of events occurring in the cells from control treatment that might have impaired the development of sugarcane somatic embryogenesis when compared to the embryogenic callus from PM treatment.

6. DISCUSSION

6.1. PM treatment improves the protocol for sugarcane somatic embryogenesis

In previous studies with sugarcane, we established improvements in the protocol for somatic embryogenesis induction by investigating the effects of different compounds supplemented into the culture medium, such as activated charcoal (Silveira et al., 2013; Heringer et al., 2015) and polyamines (Reis et al., 2016), and using more efficient light sources, such as the Light-Emitting Diode (LED) technology (Heringer et al., 2017). However, in the present work, the simple removal of 2,4-D (PM treatment) from the culture medium just one subculture (21 days) in the dark prior to the maturation step proved to be the best protocol for somatic embryos production in the cultivated variety SP80-3280 (Table 1 and Figure 2). The protocol did not require the use of PGRs in the differentiation medium to promote the somatic embryo induction and could be improved by using temporary immersion systems for large scale production of sugarcane plants. Other studies demonstrated the feasibility of temporary immersion bioreactors for sugarcane mass micropropagation (Silva et al., 2015; Heringer et al., 2017).

In Scots pine (*Pinus sylvestris*), the prematuration treatment with auxin activity inhibitor or polar transport inhibitor increased somatic embryo yields in two different embryogenic lines (Abrahamsson et al., 2012). The removal of 2,4-D from the culture medium of *Arabidopsis* embryogenic cultures induced the expression of

WUS gene, the polar distribution of PIN1, and the establishment of auxin gradient in embryogenic callus prior to somatic embryo development (Su et al., 2009).

In plant tissue culture, the establishment of efficient protocols for somatic embryos induction and regeneration is important not only for simple micropropagation purposes, but also for breeding programs that use genetic engineering to improve agronomical traits in plants. Indeed, in previous works, we showed that the regeneration of sugarcane somatic embryos-derived plantlets was simple and efficient (Reis et al., 2016; Heringer et al., 2017) and did not require the regenerated plants to be in controlled environmental conditions during the acclimatization period right after the transferring from *in vitro* to *ex* vitro condition (data not shown). Moreover, the addition of either PGRs or mutagens, as well as the prolonged subcultures could inclusive be used to induce somaclonal variations as a source of plant variability to be explored by breeding programs (Krishna et al., 2016; Tawar et al., 2016; Manchanda et al., 2018). Altogether, based on our results, the PM treatment improved the somatic embryo differentiation in embryogenic callus of sugarcane. This demonstrates the suitability of this protocol to both mass micropropagation and genetic engineering-based breeding programs of sugarcane.

6.2. PM treatment promotes storage reserve accumulation

The histochemical analysis of embryogenic callus from PM and control treatments showed several differences between them over the 28 days of culture (Figure 3). In CBB staining, the presence of either PBs or PSVs in PM treatment (Figure 3A-C) is consistent with the higher content of total proteins (Figure 4) and the identification of storage proteins, such as vicilin-like and globulins, which were all strongly accumulated in the embryogenic callus from PM treatment (Table S1). However, the absence of these structures in the control might be related not only to a lower abundance of storage proteins, but also to a higher abundance of proteases (Table S1 and Figure 5). The lower abundance of lipid metabolic proteins (Table 2) could also indicate this absence, since PBs and PSVs are structures surrounded by membranes.

PBs and PSVs are distinctive structures and they differ from each other according to their biogenesis. PBs are formed mainly from the aggregation of

prolamins within endoplasmic reticulum (ER) and either are attached to its endomembrane system or bud off as a separate organelle, whereas PSVs are formed because of the accumulation of storage proteins synthesized in the ER (i.e., mainly globulins and other auxiliary proteins) and progressed through the secretory system toward the vacuole (Herman and Larkins, 1999; Ibl and Stoger, 2012). Although some of these storage proteins can also be present in both PBs and PSVs (Ibl and Stoger, 2012).

We also identified lectin, one of the auxiliary storage proteins (Etzler, 1985; Herman and Larkins, 1999), as more abundant in embryogenic callus from PM treatment (Table S1). It also seems that the PSVs are remodeled subdivisions of preexisting vacuoles and are coordinately formed with the onset of storage protein synthesis and accumulation (Feeney et al., 2018). Altogether, despite the need of a future confirmation using specific antibodies to PB and PSV coupled to fluorescence microscopy analysis, our results seem to show the accumulation of PSVs inside the cells of embryogenic callus from PM treatment. Therefore, PSVs work as a source of storage reserves to be further used in somatic embryo development. These reserves started to be mobilized between the 14 and 28 days of culture, as demonstrated by the histochemical (Figure 3A-F) and proteomic analyses (Figure 4 and Table S1). Besides, the absence of PSVs in the control may indicate a delayed development caused by the 2,4-D.

In PAS staining, we observed a high accumulation of conspicuous starch grains in the embryogenic callus from PM treatment on days 0 and 14, then a completely vanishing on day 28 (Figure 3G-I). While in the control, the onset of starch accumulation occurred from day 14, presenting small grains on day 28 (Figure 3J-L). Starch accumulation and mobilization occurred at same time of the storage protein reserves in PM treatment, pointing to a future period of extreme energy requirement, such that of somatic embryo development. These results are consistent with the higher abundance of starch synthesis proteins (e.g., ADP-glucose pyrophosphorylase and granule-bound starch synthase) on day 14 of the embryogenic cultures from PM treatment when compared to the control (Table S1). Likewise, there were a higher abundance of amylases, which suggests an energy mobilization from 14 days of culture (Table S1).

In *Nicotiana tabacum* cell cultures, Miyazawa et al. (1999) showed the negative effect of 2,4-D on both starch accumulation and biosynthesis transcripts

(i.e., ADP-Glucose pyrophosphorylase, granule-bound starch synthase, and starch branching enzyme), and a positive effect on cell proliferation. Conversely, when tobacco cells were cultured in auxin-free medium, the authors observed a decrease in cell proliferation and an increase in both starch granules and expression of biosynthesis genes, as well as an enhancement in this response caused by cytokinin addition. Altogether, this is consistent with our results, because embryogenic cultures from control seem to have been negatively affected by the 2,4-D, thus showing a low and delayed accumulation of starch from the 14 days of culture. We suggested possible explanations to these events in Figure 5.

6.3. ABA-induced proteins are affected by 2,4-D

Several studies have also demonstrated the synergistically positive influence of ABA and sugar on both starch and biosynthesis transcripts accumulation (Rook et al., 2001; Akihiro et al., 2005; Bossi et al., 2009; Hu et al., 2012; Huang et al., 2016). This inducibility caused by ABA occurs because it exists an ABA-inducible motif in the promoter regions of these genes, the ABA-responsive element (Bossi et al., 2009; Hu et al., 2012; Nakashima and Yamaguchi-Shinozaki, 2013). This region is also present in a great diversity of stress-induced gene families, including those that code for low-temperature-induced 65 kDa (Nakashima et al., 2006) and late embryogenesis abundant (LEA) proteins (Banerjee and Roychoudhury, 2016), both strongly accumulated in embryogenic callus from PM treatment on days 0 and 14 (Table S1). It has also been showed that ABA inhibits seed germination by avoiding storage reserve mobilization (Pritchard et al., 2002; Tonini et al., 2010). In *Brassica napus* microspore embryos, both ABA and osmotic stress stimulated the accumulation of storage proteins during embryo development (DeLisle and Crouch, 1989; Wilen et al., 1990).

Besides that, other two ABA-signaling proteins were identified in our study. Protein phosphatase 2A (PP2A) was more abundant in the control on days 0 (unique) and 14 (up-regulated). Serine/threonine-protein kinase SAPK3 (SnK2.3) was unique on day 0 of PM treatment (Table S1). PP2A has a negative regulatory activity in ABA signaling by inhibiting SnK2 activity, and the SnK2.3 has a positive effect by activating downstream genes in ABA signaling pathway (Waadt et al.,

2015; Yang et al., 2017). These evidences raise the hypothesis of a high level of ABA in embryogenic callus from PM treatment on days 0 and 14, thus allowing the accumulation of starch and storage proteins, followed by a decline in ABA level after 14 days of culture, which stimulated the mobilization of the storage reserves, as demonstrated by Figure 3 and 4. Conversely, in the control, the ABA level might be lower on day 0 and would have begun to slowly increase over the days of culture, thus allowing for a delayed starch accumulation; however, protein accumulation in PSVs would possibly be a slower process when compared to starch, since it was not observed over the 28 days of control treatment.

Abscisic acid has also been demonstrated to have influence on lipids and lipid-related proteins. In our study, we identified a strong regulation of lipid-related proteins, especially, the 18 kDa oleosins, which showed a very high abundance in PM treatment on days 0 and 14 when compared to the control (Table 2). The ABI4 transcription factor was shown to be responsible for the regulation of lipid mobilization in *Arabidopsis* embryos, thus avoiding excessive lipid breakdown (Penfield et al., 2006). In *B. napus*, Zou et al. (1995) and Jadhav et al. (2008) demonstrated that microspore-derived embryo cultures treated either with ABA or its metabolites have an increase in lipid and oleosin biosynthesis when compared to the control. The same could be observed for *Lesquerella fendleri* cell cultures (Kharenko et al., 2011). Kim et al. (2017b) showed that both rice seeds and cell culture increased oleosins expression upon ABA treatment and that oleosin genes contain the ABA-responsive element. Oleosins are integral components of oil bodies-monolayers, conferring stability and preventing oil bodies fusion, as well as protection against phospholipase action, and disappearing rapidly together with triacylglycerol mobilization, which is a storage lipid form that is used as energy source in plants (Frandsen et al., 2001; Shimada et al., 2018).

Peroxygenase (caleosin) is another ABA-responsive protein that were strongly accumulated in PM treatments on days 0 and 14 (Table 2) and, similarly to the oleosins, could also be found in oil bodies (Frandsen et al., 2001). This protein plays roles in oil body and lipid degradation, interaction of oil bodies and vacuoles, and defense response via oxylipin biosynthetic pathway (Poxleitner et al., 2006; Shimada et al., 2018). Besides peroxygenases, lipoxygenases, which were more abundant in embryogenic callus from PM treatment (Table 2), also play role in

oxylipin pathway by catalyzing the initial steps in oxylipin synthesis (Wasternack and Feussner, 2018).

Table 2. Proteins identified on days 0 e 14 in embryogenic callus from PM treatment compared to the control.

ACCESSION	DESCRIPTION	FUNCTIONAL CLASSIFICATION	MAX SCORE	REPORTED PEPTIDES	PM x Control DAY 0	PM x Control DAY 14
SCCCCL4005F01	oleosin 18 kDa	seed oilbody biogenesis; lipid storage; response to freezing	8921	4	UP	UP
SCSBSD2032F10	oil body-associated protein 2B	stability of oil bodies; embryo development	1534	2	UP	ND
SCEQLR1092H02	oleosin 18 kDa	seed oilbody biogenesis; lipid storage; response to freezing	6403	4	UP	UP
SCSBSD1031A09	oleosin 18 kDa	seed oilbody biogenesis; lipid storage; response to freezing	4665	5	UP	UP
SCCCCL7C05F08	peroxidase 2	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	10344	5	DOWN	DOWN
SCCCCL4008H04	peroxidase 56	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	5920	20	DOWN	DOWN
SCBGHR1058E08	peroxidase 4	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	2064	3	DOWN	UNCHANGED
SCSGRT2063H01	cationic peroxidase SPC4-like	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	36513	29	DOWN	UNCHANGED
SCSFCL6068C11	anionic peroxidase	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	29871	10	DOWN	UNCHANGED
SCCCCL3003B02.b	cationic peroxidase SPC4	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	26490	16	DOWN	DOWN
SCCCLB1002D05	peroxidase 16	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	2729	15	UNCHANGED	DOWN
SCCCCL4012A01	peroxidase 2	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	3021	7	UNCHANGED	DOWN
SCUTHR1063E12	peroxidase 4	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	2144	6	UNCHANGED	DOWN
SCVPRZ2035F03	peroxidase 2	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	9339	22	UNCHANGED	DOWN
SCRLAD1099B04	peroxidase 2	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	7107	12	UNCHANGED	DOWN
SCEQRT1026F09	peroxidase 1	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	6804	27	UNCHANGED	DOWN
SCEZRZ1014F10	peroxiredoxin-2E-1, chloroplastic	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	2385	7	UNCHANGED	UP
SCMCRT2087F01	peroxidase 16	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	1463	2	UNIQUE_CON_0	UNIQUE_CON_14
SCCCRT2002H08	probable L-ascorbate peroxidase 3	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	1965	4	UNIQUE_CON_0	UNIQUE_CON_14
SCEZRT2020H09	peroxidase 5	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	1455	2	UNIQUE_CON_0	UNIQUE_CON_14
SCEPCL6023G11	cationic peroxidase SPC4	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	3324	8	UP	UP
SCJLAM1062A05	probable phospholipid hydroperoxide glutathione peroxidase	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	8771	17	UP	UP
SCSBSD1033E10	cationic peroxidase SPC4	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	7666	10	UP	UP
SCJFRZ2013F04	peroxidase 24	response to oxidative stress; hydrogen peroxide catabolic process; auxin catabolism	2534	14	UP	UNCHANGED

Table 2. Continued

ACCESSION	DESCRIPTION	FUNCTIONAL CLASSIFICATION	MAX SCORE	REPORTED PEPTIDES	PM x Control DAY 0	PM x Control DAY 14
SCCCCL3005C05.b	antimicrobial peptide precursor	defense response	8135	7	UP	UP
SCUTCL6034D10	antimicrobial peptide precursor	killing of cells of other organism; defense response	20944	9	UP	UP
SCMCCL6059B02	probable indole-3-acetic acid-amido synthetase GH3.8	auxin homeostasis; response to auxin	1275	5	DOWN	ND
SCMCFL5008G05	probable indole-3-acetic acid-amido synthetase GH3.1	auxin homeostasis; response to auxin	2611	9	DOWN	DOWN
SCEPRZ3129G07	probable indole-3-acetic acid-amido synthetase GH3.1	auxin homeostasis; response to auxin	929	4	DOWN	UNCHANGED
SCJFRT2060F11	2-oxoglutarate-dependent dioxygenase (DAO)	auxin catabolic process; oxidation-reduction process	600	2	DOWN	DOWN
SCRLCL6031E11	probable indole-3-acetic acid-amido synthetase GH3.8	auxin homeostasis; response to auxin	1360	4	DOWN	ND
SCCCCL3002B05.b	probable indole-3-acetic acid-amido synthetase GH3.8	auxin homeostasis; response to auxin	5167	5	DOWN	UNCHANGED
SCCCCL6002A08	probable indole-3-acetic acid-amido synthetase GH3.2	auxin homeostasis; response to auxin	2567	5	UNCHANGED	DOWN
SCCCLR1072D06	cysteine synthase	cysteine biosynthetic process from serine	8892	30	UNCHANGED	UP
SCEZRZ1012F05	probable methylenetetrahydrofolate reductase	methionine metabolic process; tetrahydrofolate interconversion; oxidation-reduction process	1429	22	UNCHANGED	UP
SCBGLR1120D04	thioredoxin reductase NTRA	removal of superoxide radicals; oxidation-reduction process	2040	8	UNCHANGED	UP
SCMCST1054G01	thiosulfate/3-mercaptopyruvate sulfurtransferase 2 isoform X2	embryo and seed development	1328	11	UNCHANGED	UP
SCCCLR1065D04	beta-cyanoalanine synthase	cysteine biosynthetic process from serine; cyanide metabolic process	1068	5	UNIQUE_CON_0	DOWN
SCUTLR1058B02	S-adenosylmethionine synthase 1	one-carbon metabolic process; drug metabolic process; cofactor metabolic process	4730	12	UP	UNCHANGED
SCCCCL4015F02	Methionine synthase	methionine biosynthetic process; methylation; homocysteine metabolic process	22309	70	UP	UP
SCSBAD1050C06	1-aminocyclopropane-1-carboxylate oxidase 1	oxidation-reduction process; ethylene biosynthesis	3043	14	UP	UP
SCEQLB2018G06	probable inactive methyltransferase	aromatic compound biosynthetic process	823	2	ND	DOWN

Table 2. Continued

ACCESSION	DESCRIPTION	FUNCTIONAL CLASSIFICATION	MAX SCORE	REPORTED PEPTIDES	PM x Control DAY 0	PM x Control DAY 14
SCJFRT1007H07	Linoleate 9S-lipoxygenase 1	response to wounding; response to jasmonic acid; oxylipin biosynthetic process	1492	19	UNCHANGED	UP
SCSBFL5014H12	Linoleate 9S-lipoxygenase 1	response to wounding; response to jasmonic acid; oxylipin biosynthetic process	2593	4	UNCHANGED	UP
SCJLRZ1024A04	putative linoleate 9S-lipoxygenase 3	organic acid metabolic process; developmental process; cellular lipid metabolic process	2806	35	UP	UNCHANGED
SCCCCL3120D08.b	Peroxygenase 2 (Caleosin)	defense response; oxylipin biosynthetic process; oxidation-reduction process	16778	24	UP	UP
SCCCCL3120D08	Peroxygenase 2 (Caleosin)	defense response; oxylipin biosynthetic process; oxidation-reduction process	16034	16	UP	UP

UNIQUE_CON_0 and UNIQUE_CON_14 mean, respectively, proteins unique to the control on days 0 and 14. UNIQUE_PM_0 and UNIQUE_PM_14 mean, respectively, proteins unique to PM treatment on days 0 and 14. ND means proteins not detected in a given treatment. Proteins were considered up- or down-regulated when presenting a fold change higher than 2, according to *t*-test ($P < 0.05$).

Oxylipins are oxygenated fatty acids with a great diversity in biological functions (e.g., biotic and abiotic stresses, growth, and development), wherein jasmonic acid (JA) is one of the most studied molecules (Wasternack and Feussner, 2018). Anderson et al. (2004) demonstrated and proposed that depending on the stimulus perceived by the plant (i.e., pathogen, drought or wounding), different hormonal interactions may occur among JA, ABA, and ethylene. In our study, two antimicrobial peptide precursors (defense-related protein) were found in very high abundance in PM treatment over the culture period, especially on day 14 (Table 2). However, we hypothesize that these proteins are being used as storage proteins in the PSVs because of their higher accumulation.

6.4. Ethylene-induced proteins are affected by 2,4-D

Our results suggest a possible interaction between ABA and ethylene signaling in embryogenic callus from PM treatment in comparison to the control. In addition to the identification of several ABA-responsive proteins (Table S1), we also identified proteins related to the ethylene biosynthetic pathway, such as 1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase), which participates of the last step of ethylene synthesis, converting ACC into ethylene (Table 2). Does the higher abundance of ACC oxidase in the PM treatment suggest a higher level of ethylene than in the control? Considering the diverse lipid-related proteins and ACC oxidase abundance in our study, Qin et al. (2007) found that the very-long-chain fatty acids promote ethylene biosynthesis by increasing the transcripts for ACC oxidase.

Other ethylene-closely related proteins were also more abundant in the embryogenic callus from PM treatment, such as methionine synthase and S-adenosylmethionine synthase 1, which generate precursors to the ethylene pathway (Table 2). Furthermore, we also identified proteins of the folate and transsulfuration pathways, which work together the methionine synthesis pathway (Table 2). Despite that, future analysis will be important to confirm hormone levels in the cultures of both treatments. In *Arabidopsis* somatic embryo-induction experiments, Nowak et al. (2015) ascertained that a balanced ethylene production, perception, and signaling seem to be required for the efficient embryogenic capacity of explants. The authors observed that both higher and lower levels of ethylene production, as well

as mutations in receptors promoted a reduced somatic embryo efficiency and productivity (Nowak et al., 2015).

6.5. ABA, ethylene, and 2,4-D interactions

Taken together, these results show that the 2,4-D in the embryogenic callus from control might have disrupted the induction of both ABA and ethylene, thus precluding the downstream responses promoted by these two hormones during the differentiation and development of the somatic embryos (Figure 5). The crosstalk between ABA and ethylene can be either synergistic or antagonistic depending on the tissue that is occurring (Beaudoin et al., 2000). In *Arabidopsis* seeds, ethylene counteracts ABA signaling during seed dormancy, whereas it complements ABA effects on root growth inhibition (Beaudoin et al., 2000). ABA can negatively control the ACC oxidase transcript accumulation, while ethylene can affect ABA metabolism and signaling (Arc et al., 2013), and ethylene receptors can even affect ABA responses by an ethylene-independent pathway (Wilson et al., 2014; Bakshi et al., 2018), thus demonstrating the complexity of hormone signaling interactions.

In our study, we found ABA-induced proteins (LEAs, Oleosins, and ADP-glucose pyrophosphorylase) and ethylene biosynthesis-related proteins that were more abundant in the PM treatment when compared to the control, which could suggest the action of both hormones. Although additional confirmations are needed, we hypothesize that the ethylene signaling controls the ABA-responsive gene expression to further allow the progress of somatic embryo development by means of storage reserve mobilization, which is negatively controlled by ABA. However, as stated above, this ABA-ethylene crosstalking could also be synergistic, which means that ethylene could be positively inducing ABA production as well. Hansen and Grossmann (2000) demonstrated that shoots of *Galium aparine* plants root-treated with IAA or other synthetic auxins presented elevated production of ethylene, which, in turn, triggered the production of ABA. Consistently, the authors also observed that the inhibition of ethylene production inhibited ABA accumulation (Hansen and Grossmann, 2000).

6.6. Auxin-reponsive proteins are up-regulated in response to 2,4-D

The 2,4-D present in the control treatment induced the accumulation of several auxin-responsive proteins on days 0 and 14 (Table 2 and S1). Hence, the involvement of auxin in the hormone interaction has also to be considered as an important factor in our study (Figure 5). We identified the indole-3-acetic acid (IAA)-amido synthetase and 2-oxoglutarate-dependent dioxygenase (DAO) as more abundant in the control treatment (Table 2). IAA-amido synthetase is a member of the GH3 protein family that is induced by auxin and catalyzes the conjugation of IAA to amino acids, thus decreasing the excess of endogenous content of free IAA and helping to keep auxin homeostasis (Hagen et al., 1984; Hagen and Guilfoyle, 1985; Staswick et al., 2005).

Different types of auxin conjugates (e.g., ester-linked to carbohydrates and amide-linked to amino acids, peptides, and proteins) are used either as storage or inactivation forms of auxin (Ludwig-Müller, 2011; Korasick et al., 2013; Ma et al., 2018). IAA-amido synthetase has been described as not acting on 2,4-D (Staswick et al., 2005); however, a recent study demonstrated that this protein can also conjugate 2,4-D to amino acids, although with a lower activity, and that 2,4-D-conjugates can be hydrolyzed back to free-2,4-D (Chiu et al., 2018). The 2,4-D can promote the expression of early auxin-induced genes, including IAA-amido synthetase (Hagen and Guilfoyle, 1985; Hagen and Guilfoyle, 2002), which is consistent with the higher abundance of this protein in the control. Furthermore, 2,4-D has a low metabolism rate (Staswick et al., 2005; Hošek et al., 2012; Ma et al., 2018), suggesting the possible presence of free-2,4-D in the control, thus increasing the auxin pool and then disrupting auxin homeostasis and preventing the progress of the embryogenic differentiation (Figure 5).

DAO and several peroxidases were more abundant in the control on days 0 and 14 (Table 2). The former is also induced by auxin and participates of auxin catabolism to keep the homeostasis (Zhao et al., 2013; Stepanova and Alonso, 2016; Zhang and Peer, 2017). In tissue culture, it is well known that auxin (i.e., exogenous and endogenous) plays important role during callus induction and embryogenic capacity acquisition, and that either an auxin decreased level or its completely removal is required for further culture development into somatic embryos (Jiménez, 2005).

The local expression of *YUCCA* genes, which is responsible for IAA biosynthesis, is required for somatic embryogenesis induction in *Arabidopsis* (Bai et

al., 2013). High production of ethylene down-regulates the expression of *YUCCA* genes, thus impairing somatic embryo induction (Bai et al., 2013). *Arabidopsis* embryogenic cultures with 2,4-D showed a higher level of ethylene than cultures without 2,4-D, demonstrating that auxin stimulates ethylene production (Bai et al., 2013). Ethylene also down-regulates the expression of *LEC2* (Nowak et al., 2015). *LEC2* overexpression promotes embryogenic induction by stimulating *YUCCA*-auxin biosynthesis (Wójcikowska et al., 2013). This overexpression increases auxin levels and decreases ABA levels (Wójcikowska and Gaj, 2015). These evidences show the existence of a tight regulation of the auxin biosynthetic pathway, which could negatively affect ABA biosynthesis.

Although our study demonstrated that the callus from PM treatment presented a higher abundance of ACC oxidase, this may not necessarily mean a higher production of ethylene. Besides that, embryogenic callus from PM treatment would eventually contain a lower level of 2,4-D, which consequently could present a lower level of ethylene as well. This result may suggest that the 21 days of PM treatment took embryogenic callus to an optimal endogenous auxin level that could stimulate an optimal production of ethylene. The ethylene production in turn could be regulating auxin homeostasis and, perhaps, also ABA levels. However, future hormonal analyses are still needed for confirmation or refutation of this possibility. Other studies also show that the AGL15 gene promotes somatic embryogenesis by controlling ethylene biosynthesis and signaling (up-regulation), as well as auxin signaling (down-regulation), thus demonstrating the complexity of somatic embryogenesis gene regulation (Zheng et al., 2013; Zheng et al., 2016).

6.7. ROS-related proteins are differentially regulated

Differential accumulation of peroxidases during somatic embryogenesis have been observed in several works (Heringer et al., 2018). Peroxidases belong to a multigenic family that can be found in most living organisms and are divided into three classes: I, the intracellular, II encoded exclusively by fungi, and III, secreted by plants (Passardi et al., 2005; Cosio and Dunand, 2009). This enzyme plays a great diversity of roles in plants, such as hydrogen peroxide detoxification, ROS production, auxin catabolism, lignification, defense, among others (Hiraga et al., 2001; Passardi et al., 2005; Cosio and Dunand, 2009). AGL15 was identified

negatively controlling a peroxidase isoform in *Arabidopsis thaliana* plants (Cosio et al., 2017). In addition, higher levels of exogenous and endogenous auxins promoted the activity of antioxidant enzymes, such as peroxidases, and a decrease of ROS in *Arabidopsis* plants under drought stress (Shi et al., 2014).

However, 2,4-D at a herbicide dose caused a negative effect in growth and other physiological parameters, presenting an increase in both ROS and antioxidant enzymes (Romero-Puertas et al., 2004). In our study, we identified a great number of peroxidases and most of them were more abundant in the control on days 0 and 14 (Table 2). This suggests that the presence of 2,4-D inside the cells promoted an increase in both the ROS levels and the antioxidant system. This accumulation of antioxidant enzymes possibly caused a decrease in ROS levels in the control, keeping it under homeostasis. Besides that, peroxidases might be acting on auxin catabolism (Figure 5).

Reactive oxygen species are molecules generated in great number of reactions inside the cell and may present antagonistic responses (i.e., toxic or signaling molecules) depending on the level. Plants use different tools to cope with ROS excess and keep homeostasis, such as antioxidant enzymes and compounds. In somatic embryogenesis of cotton, ROS homeostasis is important during dedifferentiation of explants (callus induction), whereas a mild oxidative environment is required in differentiation of embryogenic cultures into somatic embryos (Zhou et al., 2016). The cotton suppression lines for ascorbate peroxidase and GST increased the hydrogen peroxide content and therefore induced somatic embryo formation (Zhou et al., 2016).

In our study, GSTs were also more abundant in the control treatment (Table S1). Fraga et al. (2016) also found GSTs as more abundant in *Araucaria angustifolia* embryogenic culture supplemented with 2,4-D than in the PGR-free culture. This result seems to be consistent with our hypothesis that embryogenic cultures from PM treatment might also be in a mild and still safe oxidative state, while the cultures from control treatment are possibly keeping ROS homeostasis or even with a level below homeostasis (Figure 5).

6.8. Concluding remarks

We observed that a simple prematuration treatment, prior the transfer to differentiation medium, is efficient and the best procedure for somatic embryo production in sugarcane cv.SP80-3280. The direct transfer of sugarcane callus cultures from a proliferation medium, which has 2,4-D (control), to a PGR-free medium during the differentiation is not suitable for sugarcane somatic embryo production. We congregated the results of this study and proposed a possible scenario to explain why 2,4-D interfered with somatic embryogenesis induction in the control treatment (Figure 5). Finally, our results also demonstrated that the efficient development of sugarcane somatic embryos seems to be preceded by a better accumulation of storage reserves during embryogenic callus differentiation, which in turn may be related to hormone homeostasis. In addition, several differentially regulated proteins, such as ABA and auxin-induced proteins, ethylene biosynthetic pathway proteins, as well as antioxidant and storage reserve-related proteins suggest a hormone balance is important for somatic embryo differentiation in sugarcane.

7. FUTURE PESPERCTIVES

The present study demonstrated the possible relation between storage reserve accumulation in sugarcane embryogenic callus and somatic embryogenesis development. We also pointed to several hormone-induced proteins that could suggest a disruption in auxin, ABA, and ethylene homeostasis. We identified several proteins of the antioxidant system that may also indicate a difference in ROS balance between embryogenic callus from PM and control treatments. However, to confirm the raised hypotheses, we propose some future analyses to be performed over the incubation period of 28 days in embryogenic callus from PM and control treatments:

- Analyze the levels of auxin, ABA, and ethylene
- Analyze starch content
- Analyze total lipid content
- Evaluate the effects of ethylene modulators (ethephon, cobalt chloride, and silver nitrate) during the incubation of embryogenic callus from PM treatment in differentiation medium
- Validate the profile of genes for proteins identified in our study
- Analyze the profile of genes related to somatic embryo development

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