Scientific Bulletin. Series F. Biotechnologies, Vol. XXVII, No. 1, 2023
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

PLANT SOMATIC EMBRYOGENESIS - A FOCUS ON THE ROLE OF CALCIUM IN EMBRYO INDUCTION AND DEVELOPMENT

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Abstract

Upon fertilization, plants produce zygote-derived embryos that will develop into new individuals. The study of its regulation is important to overcome the post-zygotic barriers that may prevent hybridization, requiered for the development of new cultivars. The study of zygotic embryo development is challenging, since they are surrounded by a multilayer maternal tissue, and are difficult to reach. Alternatively, plants produce embryos from specific somatic cells under certain in vitro conditions. This process, referred to as somatic embryogenesis, has been established as a model to study plant embryogenesis, due to its similitudes with zygotic embryogenesis and its technical feasibility. Moreover, the production of somatic embryos allows to efficiently propagate plant material. Somatic embryogenesis is regulated by stimuli of different nature. Among others, calcium gradients within cells and tissues are important to achieve a proper consecution of embryogenesis from somatic cells. In this review, we summarize the most relevant advances in different plant species by using multiple approaches that shows the role of calcium in the regulation of somatic embryogenesis.

Key words*: somatic embryogenesis, calcium signalling, ionophore, FRET.*

SOMATIC EMBRYOGENESIS AS A MODEL SYSTEM

Embryos are structures with the capacity of generating a new organism. Plant zygotic embryos are formed upon fertilization of a female egg cell by a male sperm cell, and its development is finely controlled by a specific embryogenic program (Wendrich and Weijers, 2013). Zygotic embryos develop within the embryo sac, surrounded by the nucellus and the ovular teguments. Thus, their study is somehow challenging. Alternatively, under certain in vitro conditions, differentiated cells, both somatic and gametic, can develop embryos with a high morphological similarity to these of the zygotic embryos. Specifically, somatic embryogenesis (SE) results in the production of embryos derived from somatic cells. Somatic embryos are bipolar structures that undergo through the same globular, heart, torpedo and cotyledonary stages as zygotic embryos, although several differences related to the overall morphology, cell and organelle sizes, water status, and biochemical composition, have been detected when comparing both types of embryos in different species (Winkelmann, 2016; Zimmerman, 1993). Although SE can occur in nature, being the case of several species of the *Kalanchoe* genus one of the most fascinating (Garcês and Sinha, 2009), this phenomenon is rare. However, SE can be induced under certain *in vitro* culture conditions. A special case of non-sexual embryogenesis is the formation of seeds without fertilization or apomixis. In this process, which can occur naturally or be induced by different techniques, apomictic seeds are produced either from sporophyte cells of the ovule, or from the megaspore mother cell or nucellar somatic cell contained in unreduced embryo sac cells, and are therefore, genetically identical to the mother plant (Yin et al., 2022).
The first evidence of *in vitro* induced somatic embryogenesis was reported on *Oenanthe aquatic* (Waris, 1957), and carrot (Steward et al., 1958; Reinert, 1958). Steward and co-workers described the formation of roots, shoots, and eventually entire living plants from cell aggregates obtained by liquid culture of carrot root phloem somatic cells (Steward et al., 1958). A closer view to this process showed that initiation of SE is limited to a single or few somatic cells (Backs-Hüsemann and Reinert,

1970), although embryos develop from multicellular proembryogenic cell masses (Goldberg et al., 1989). Due to its valuable biotechnological application (see below), many research groups focused in the study of SE in different species, and currently efficient protocols are available for more than one hundred species, including tobacco (Takebe et al., 1971), woody species (reviewed in Wann, 1988), several palm trees species (reviewed in Ree and Guerra, 2015), maize (Armstrong and Green, 1985), grapevine (Gambino et al., 2011), and the model plant *Arabidopsis thaliana* (Gaj, 2001), among others.

APPLICATIONS OF SOMATIC EMBRYOGENESIS

In vitro culture techniques have helped to increase agronomic features of current crops, including productivity, pathogen and herbicide resistance, and food quality. Such techniques, together with molecular approaches including GMO generation and gene editing, are the basis to further improve plant performance under the uncertain growing conditions imposed by climate change, and to develop new commercial products to satisfy new consumer demands. In this context, SE can be useful at different levels. On the one hand, SE allows for large scale propagation of plant material, which is especially useful for woody species (Guan et al., 2016). Contrary to organogenesis and conventional micropropagation protocols, SE allows for the propagation of green material in a single step, rather than sequential root and shoot regeneration steps. Moreover, SE has a higher multiplication rate than alternative micropropagation techniques, although it depends on the genetic background, and the possibility of scaling up using bioreactors (Egertsdotter et al., 2019). Additionally, the encapsulation of somatic embryos enables their direct delivery as synthetic seeds, thereby facilitating the maintenance of elite cultivars (Onishi et al., 1994). On the other hand, SE is a valuable technique for the regeneration of plants upon genetic transformation. Efficient protocols to produce somatic embryos greatly facilitate the production of high numbers of independent transgenic lines, in order to select the most appropriate in terms of transgene expression, stability and phenotype (Deo et al., 2010). Furthermore, somatic embryogenesis has been used for the elimination of viruses from plant material of different species, including grapevine (Olah et al., 2022; Gambino and Perrone, 2022). Finally, due to the developmental similarities among zygotic and somatic embryogenesis and the absence of maternal tissues surrounding *in vitro* embryos, SE is a very appropriated model to study basic features and regulation of plant embryogenesis.
Altogether, the advantages for plant improvement of SE versus other techniques are evident. However, SE has also certain limitations. For example, the non-synchronous formation of somatic embryos within the same explant, the recalcitrance of some species or cultivars to SE induction, the accumulation of undesired mutations due to somaclonal variation along in vitro-cultured generations, and the loss of embryogenic capacity of calli over time (Deo et al., 2010). The study of the basic mechanisms that regulate SE may help us to reduce the negative impact of these constraints, and to establish improved protocols for recalcitrant species.

FACTORS AFFECTING SOMATIC EMBRYOGENESIS

SE is a complex process whose efficiency, generally measured as number of embryos per explant, is affected by several factors of different nature. On the one hand, somatic embryogenesis is highly dependent on the explant type, as many other *in vitro* culture approaches. Somatic embryos have been obtained using different explant types such as leaves (Martins et al., 2022), leaf main veins (Hanh et al., 2022), spikelets (Ornellas et al., 2022), anthers and ovaries (Gray and Mortensen, 1987; Gambino et al., 2007; Perera et al., 2007), flower tepals (de Almeida et al., 2022), roots (Chen et al., 1987), stems (Cuenca et al., 1999), petioles (Rugini and Caricato, 1995), mature and immature embryos (Chan et al., 1998; Fernando and Gamage, 2000; Calabuig-Serna et al., 2023a), seedling hypocotyls (Calabuig-Serna et al., 2023b), and cotyledons (Leva et al., 1995). Not only the explant type, but the explant age determines to a great extent the efficiency of SE, as shown

for coffee (Molina et al., 2002), eucalyptus (Prakash and Gurumurthi, 2010) and soybean (Yang et al., 2009), among others. As for any other in vitro culture-based morphogenic process, SE efficiency strongly depends on the genetic background, not only at the species level, but also within cultivars. For instance, the SE efficiency and the regeneration capability of 25 inbred maize lines were analyzed, observing that some lines were highly efficient on SE production, whereas other were completely recalcitrant (Hodges et al., 1986). Further genetic analysis of F1 crosses with the highly embryogenic line A188, and corresponding F2 plants, demonstrate the genetic dependence of SE induction and plantlet regeneration (Hodges et al., 1986). The genotypic effect has also been studied for other species, such as cotton (Trolinder and Xhixian, 1989), coffee (Molina et al., 2002), soybean (Parrot et al., 1989), and cocoa (Florez et al., 2015), among others. Finally, composition of the *in vitro* culture medium, as well as application of certain abiotic stresses, determines the SE efficiency in different species and genotypes. The nature of the nitrogen source (NO3 versus $NH4^{+}$) in Medicago sativa (Meijer and Brown, 1987), feijoa (dal Vesco and Guerra, 2001) and coffee (Samson et al., 2006), influence both the velocity of the SE process and the efficiency of the process. Specific culture media composition, and incubation conditions for different species has also been described (Fernández-Guijarro et al., 1995; Chen and Chang, 2002). More importantly, the type and concentration of growth factors on growing media are crucial for SE, being auxins a major regulator. Generally, auxin favors the initial formation of callus, whereas SE initiates under auxin-free media supplemented or not with low concentrations of other growth regulators (Chambhare and Nikam, 2022; Su et al., 2009; Filippov et al., 2006). Auxin is considered, indeed, one of the main SE regulators (reviewed in Wójcik et al., 2020; Winnicki, 2020). In summary, SE protocols use to be slightly different among species and even cultivars, including variations in the type of explant, medium composition, and in vitro culture conditions. Therefore, specific protocols have been established for different genetic backgrounds.

These factors affecting SE not only determines the efficiency of the process, but also the degree of de-differentiation of somatic cells prior to the embryogenic process. In this sense, formation of somatic embryos can be either direct from somatic cells or indirect, through an intermediate callus phase, where embryos are formed from cells of the surface of the callus (Sharp et al., 1980). Generally, indirect SE produces more embryos, although the incubation time is longer and the effect of somaclonal variation is higher (Miguel and Marum, 2011). Why a concrete explant under certain conditions produces somatic embryos through a direct or indirect way is not clearly understood, although it may be related to the age of the explant (Horstman et al., 2017b; Merkle et al., 1995), the type of explant and/or the *in vitro* culture conditions. For instance, both direct and indirect SE protocols are established for *Camellia oleifera*, and the difference among them relies in different combination of different growth regulators (Zhang et al., 2021). Similarly, protocols for direct and indirect SE have been established for *Coffea arabica* (Quiroz-Figueroa et al., 2002), carrot (Mizukami et al., 2008; Steward et al., 1958), and maize (Lowe et al., 2018), among many others. Indeed, in some cases, both direct and indirect SE are induced in the same explant (Turgut et al., 1998; Gaj, 2004). An example of the occurrence of both, direct and indirect SE, is arabidopsis, a model species where many molecular tools are developed, and an ideal system to study the molecular regulation of SE (Gaj, 2004). Different protocols and explants are used to induce SE in arabidopsis (Horstman et al., 2017b), although the most extended protocol is based on the application of 2,4-D to immature zygote embryos (IZEs) at the late cotyledonary stage (Figure 1; Wu et al., 1992; Gaj, 2001). IZEs are isolated from green siliques of arabidopsis adult plants, sterilized, and placed on in vitro plates (Figure 1A). Upon 5 days on auxin inductive media a lump, referred as protrusion, is formed on the adaxial side of the cotyledon of responding explants (Figure 1B,C). The embryogenic nature of the protrusion is demonstrated by the expression of embryo identity genes, such as *WOX2* (Godel-

Jedrychowska et al., 2020) and *WUS* (Calabuig-Serna et al., 2023a), and histological studies showed that embryos emerged from protodermal and subprotodermal layer (Kurczyńska et al., 2007). The appearance of the protrusion is the result of an inner proliferating cell mass that, upon 7 days of culture, usually emerges and breaks the explant epidermis (Figure 1D). Finally, upon 14 days of culture, visible embryos arise from the embryogenic cell mass formed (Figure 1E; Calabuig-Serna et al., 2023a).

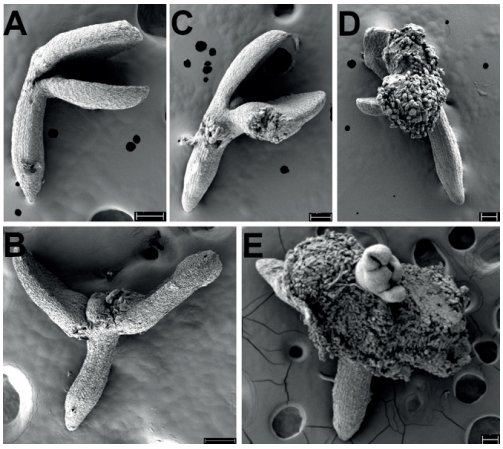

Figure 1. Somatic embryogenesis process from immature zygote embryos of *Arabidopsis thaliana*. A. Freshly isolated immature zygote embryos (IZEs) at the late cotyledonary stage. B, C. IZEs upon 5 days of culture, showing (B) discrete or (C) strong growth of protrusion. D. IZE upon 7 days of culture showing drastic protrusion growth. E. Somatic Embryo growing on the callus-like structure formed on the IZE. Scale bar: 100 μm

GENETIC REGULATION OF SOMATIC EMBRYOGENESIS

During somatic embryogenesis, cells undergo a process of "dedifferentiation" to become embryogenic and, therefore, acquire the capability to develop into a new whole organism. These complex processes require a fine-tuned genetic regulatory network, that has mostly been studied in arabidopsis. The genetic regulatory network controlling SE has been recently reviewed by Horstman et al. (2017b) and Elhiti and Stasolla (2022). Different approaches have been followed to decipher such genetic network. On the one hand, the effect of the ectopic expression of embryo and meristem identity genes on SE activation has revealed their role during SE. For instance, overexpression of *LEAFY COTYLEDON 1* (*LEC1*) induces the formation of embryos in arabidopsis cotyledons (Lotan et al., 1998). Other genes within the same family, such as *LEC2* and *LEAFY COTYLEDON1-LIKE* (*L1L*), are also able to induce SE when overexpressed (Horstman et al., 2017b). Accordingly, *lec1* and *lec2* mutants exhibit low SE response (Lotan et al., 1998; Stone et al., 2008; Gaj et al., 2005). These genes are known to regulate auxin metabolism and signaling, as well as gibberellin/ABA ratios, thus their function during SE can be explained, at least in part, by their role In the hormonal response within the explants (Elhiti and Stasolla, 2022). Transcriptional activation of LEC1 and LEC2 is controlled by *BABY BOOM* (*BBM*; Horstman et al., 2017a). Accordingly, ectopic expression of the transcription factor *BBM*, induces the formation of somatic embryos in leaves and cotyledons, even in the absence of growth regulators (Boutilier et al., 2002). Similar observations were made upon *WOUND INDUCED DEDIFFERENTIATION 1* (*WIND1*; Ikeuchi et al., 2015) and *WUSCHEL* (*WUS*; Chatfield et al., 2013). As a step forward, microarray analysis and chromatin immunoprecipitation experiments helped to the identification of downstream target genes. For instance, microarray analysis of *LEC2* overexpression identified genes related to auxin metabolisms that were important for SE (Stone et al., 2008). On the other hand, the expression of master regulators of SE is controlled by epigenetic signals, such as DNA methylation, chromatin remodeling and, micro-RNA mediated regulation (reviewed in Kumar and van Staden, 2017). First, different methylation patterns during SE have been observed in different species. Whereas high methylation levels were detected during *Daucus carota* (Yamamoto et al., 2005), *Castanea sativa* (Viejo et al., 2010) and *Picea omorika* (Levanic et al., 2009), in some other species, such as *Eleutherococcus senticosus* (Chakrabarty et al., 2003) and *Coffea canephora* (Nic-Can et al., 2013), DNA methylation increased throughout SE development. No matter how, the application of the DNA methylation inhibitor 5-Azacytidine reduces SE in *Daucus carota*

(Yamamoto et al., 2005) and *Coffea canephora* (Nic-Can et al., 2013), which points to the need of controlled DNA methylation stage during SE. Moreover, a specific methylation signature, i.e. H3K27me3, has been described to activate *LEC1* and *BBM1* (Nic-Can et al., 2013). Finally, the pattern of specific non-coding microRNAs changed throughout the embryogenic induction, compared to non-induced calli (Kumar and van Staden, 2017). For instance, arabidopsis microRNA167 controls somatic embryogenesis through regulating its target genes *ARF6* and *ARF8* (Su et al., 2015). In conclusion, SE genetic regulation is a highly complex process that requires both genetic and epigenetic regulation. Some specific genes and gene targets, as well as epigenetic marks that control their expression, have been described. The identification of specific genes involved in SE can help us to obtain genetic backgrounds that produce somatic embryos at a higher efficiency.

CALCIUM SIGNALLING IN PLANT CELLS

Calcium is a microelement needed for proper plant growth and development and is present within cells in its free cationic form (Ca^{2+}), stored or loosely-bound, and covalently bound to macromolecules (Ge et al., 2007). Whereas covalently bound calcium has mainly a structural function, the equilibrium among free Ca^{2+} and stored (loosely-bound) Ca^{2+} plays an important signaling role within cells, and regulates precise cellular responses by its interaction with specific Ca^{2+} sensors or Ca^{2+}-binding proteins such as calmodulin, calmodulin-like proteins, Ca^{2+} dependent protein kinases, and Calcineurin B-like proteins (Tuteja and Mahajan, 2007). Calmodulin (CaM) is a small protein that is present in almost all eukaryotic organisms and, upon Ca^{2+} binding, it is activated and changes its conformation favoring the interaction with diverse targets including specific kinases, which triggers specific signaling cascades (Bredow and Monaghan, 2022). Free intracellular Ca^{2+} is present in the cytoplasm at very low concentrations (50-100 nM) due to its cytotoxicity, although it can reach up to 5 mM when confined in organelles such as the endoplasmic reticulum and vacuoles, or in the cell wall, acting as cellular Ca^{2+} reservoirs. When required, Ca^{2+} is released to the cytosol by selective channels and, upon signaling, it is translocated back to the reservoirs by specific pumps (Pirayesh et al., 2021). These mechanisms allow cells to trigger transient Ca^{2+} signatures (peaks) that act as a second messenger for signal transduction (Ge et al., 2007), avoiding the toxic effects of continuous high Ca^{2+} levels. Detection of cellular Ca^{2+} accumulation during biological processes can shed some light about the role exerted by this substance, and the mechanisms underneath.

Ca^{2+} within cells can be detected by several biotechnological tools (Kanchiswamy et al., 2014). Traditionally, cytosolic Ca^{2+} has been detected using specific, Ca^{2+}-sensitive fluorescent probes, such as Indo-1 and Fura-2 (Bush and Jones, 1990), FluoForte (Rivas-Sendra et al., 2017; Rivas-Sendra et al., 2019), fluo-4/AM (Gee et al., 2000), chlorotetracycline (Tirlapur et al., 1995), and fluo-3/AM (Qiu et al., 2020), among others. However, these techniques, although very informative, require the incubation of the probe with the sample, which allows solely for the detection of Ca^{2+} accumulation at specific time points, precluding the continuous observation of Ca^{2+} changes in living cells and the identification of fast, Ca^{2+} transient peaks. Instead, the use of confocal microscopy to register specific signal of Ca^{2+} binding proteins coupled to a fluorescence resonance energy transfer (FRET) system has been a valuable tool to study in vivo Ca^{2+} dynamics. Briefly, a donor (CFP) and acceptor (YFP) fluorescent proteins are linked by calmodulin bridge in the so-called cameleon constructs (Figure 2A). As the Ca^{2+} concentration increases, calmodulin incorporates Ca^{2+} and changes its conformation, thereby approaching both fluoroproteins and allowing for the FRET emission (Figure 2A; Miyawaki et al., 1997). The availability of these tools allows for the detection for Ca^{2+} signatures throughout biological processes and, therefore, the potential implication of Ca^{2+} on the signaling and regulation of these processes.

Ca^{2+} SIGNATURES DURING SOMATIC EMBRYOGENESIS

Ca^{2+} is an important cation for sexual reproduction in angiosperms, and Ca^{2+} peaks have been observed during pollen germination and pollen tube elongation, pollen-pistil interaction, and gametic interaction and fertilization (reviewed in Ge et al., 2007). At the very initial phase of zygote embryogenesis, it was observed transient elevation of cytosolic Ca^{2+} concentration upon maize *in vitro* egg cell fertilization, that is triggered by gamete fusion (Digonnet et al., 1997). Later, it was demonstrated that Ca^{2+} cellular uptake starts in the fusion site, and then is spread throughout the whole egg cell plasma membrane, resulting in a transient increase of cytosolic Ca^{2+} (Antoine et al., 2000). Moreover, addition of the Ca^{2+}-channel inhibitor gadolinium blocked gamete fusion, what indicates that Ca^{2+} influx is required for sperm incorporation (Antoine et al., 2001). Further *in vivo* experiments showed that different female ovule cell types (i.e. egg cell, synergid cells and central cells) showed specific Ca^{2+} signatures upon fertilization, pointing to a complex regulation of plant fertilization mediated by Ca^{2+} (Denninger et al., 2014). These works demonstrate a prominent role of Ca^{2+} signatures to fine-tune the initial steps of plant zygote embryogenesis, as it has been seen for animal systems (Miao et al., 2012). However, to the best of our knowledge, Ca^{2+} accumulation has not been reported in later plant zygotic embryogenic stages, probably due to the relative inaccessibility of the embryos within the developing seeds.

In alternative systems, such as rapeseed microspore embryogenesis, Ca^{2+} overaccumulation was observed by FluoForte staining in the cytosol, nucleus, and vacuoles of embryogenic microspores, but not in non-embryogenic forms, such as callus-like and pollen-like structures. Mature microspore-derived embryos showed decreased Ca^{2+} levels (Rivas-Sendra et al., 2017; Rivas-Sendra et al., 2019). However, most of the knowledge regarding Ca^{2+} accumulation and signatures during embryogenesis has been obtained in different somatic embryogenesis systems. For instance, measurement of free intracellular Ca^{2+} by different approaches, including incubation with fluo-3, showed a clear increase of Ca^{2+} specific signal, compared to the low signal in proembryogenic masses, in induced embryogenic explants, mostly in the nuclei of cells belonging to the protoderm of late globular to torpedo stages (Timmers et al., 1996). Similarly, high fluorescent signal derived from Fura-2AM was detected in embryogenic *Coffea canephora* embryogenic calli treated with $CaCl_2$, whereas treatment with specific Ca^{2+} channel blockers resulted in a low cytosolic Ca^{2+} accumulation (Ramakrishna et al., 2011). Accordingly, a high expression of calmodulin was observed in sugarcane somatic embryos, compared to non-responsive cells (Suprasanna et al., 2004; Overvoorde and Grimes, 1994). Ca^{2+} measurement by biochemical approaches demonstrates that proembryogenic sandalwood cell clumps transferred to cell differentiation media accumulate more Ca^{2+} than the ones kept on callus proliferation media (Anil and Rao, 2000). Recently, the use of cameleon carrot transgenic lines and confocal FRET signal detection, revealed that Ca^{2+} accumulation was highly dynamic along the different stages of the SE process, being higher in proliferating cells and embryogenic structures, what supports the signaling role of free Ca^{2+} in SE regulation (Calabuig-Serna et al., 2023b). Similarly, FRET analysis of arabidopsis cameleon transgenic lines (Krebs et al., 2012), revealed that Ca^{2+} accumulates in the adaxial side of the cotyledons of the induced IZEs, coincident with the formation of the protrusion (Figure 1B, C; Figure 2B, C), and with the embryo-identity gene *WUS* (Calabuig-Serna et al., 2023a). Ca^{2+} accumulation increased with incubation time in inner cell layers of the cotyledon and was especially high during the protrusion emergence (Calabuig-Serna et al., 2023a).

All these data are coincident on the increase of Ca^{2+} accumulation in induced embryogenic plant somatic and gametophytic cells, what is indicative of a relevant role of Ca^{2+} in the induction of embryogenesis. Whereas Ca^{2+} has been showed to be transiently accumulated at the initiation of zygotic embryogenesis, it seems likely that Ca^{2+} signatures are also important during the following stages, as shown for somatic and microspore embryogenesis.

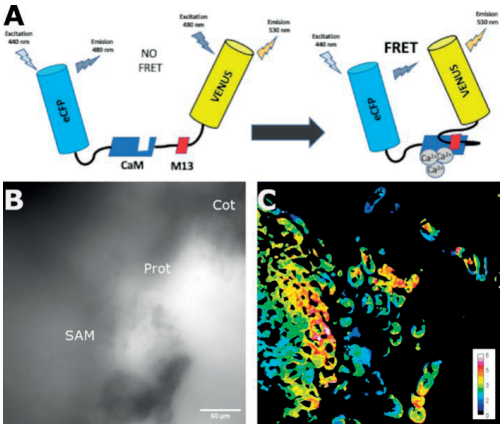

Figure 2. (A) Schematic representation of cameleon system to detect Ca^{2+} accumulation by FRET. Under low Ca^{2+} concentration (left) no FRET signal is detected. Upon Ca^{2+} increase (right), CaM and M13 form a complex that brings closer donor (i.e. eCFP) and acceptor (i.e. VENUS), what results in a FRET signal registration by confocal microscopy. (B) Bright field and (C) FRET assay, of cameleon transgenic line of arabidopsis IZE upon 5 days on embryogenic induction media

Ca^{2+} MODULATION AND EFFICIENCY OF SOMATIC EMBRYOGENESIS

The different works presented in the previous section demonstrate the occurrence of transient Ca^{2+} increases of different duration during specific stages of somatic embryogenesis. This observation, however, does not demonstrate the functional role of Ca^{2+} in this process, as Ca^{2+} increases could alternatively be due to the use of growth regulators (i.e. auxin) in the culture medium, as described for different plant organs and cell types (Vanneste and Friml, 2013). To shed light on this, pharmacological approaches consisting on the application of chemical compounds that induce free Ca^{2+} release within the cytoplasm or avoids Ca^{2+} transport and/or signaling within cells, have been used. The enrichment with $CaCl_2$ of in vitro culture media results in an increase of the SE efficiency and embryo quality in *Hevea brasiliensis* (Etienne et al., 1997), *Manihot esculenta* (Li et al., 2009), carrot (Calabuig-Serna et al., 2023b), and sandalwood (Anil and Rao, 2000). However, in other species, such as *Pinus patula* (Malabadi and van Staden, 2006) and arabidopsis (Calabuig-Serna et al., 2023a), $CaCl_2$ application showed no positive effect on SE. Even a negative effect was observed in *Cocos nucifera* (Rivera-Solís et al., 2018). Moreover, different species within *Musa* genera have different SE responses to increasing $CaCl_2$ concentrations on SE induction media (Marimuthu et al., 2019). Thus, there is not a clear pattern for the effect of Ca^{2+} enrichment in the efficiency of SE, and the reason why SE of different genotypes respond differently to specific Ca^{2+} concentrations on induction *in vitro* medium is not clearly understood. A possible explanation is that, although extracellular Ca^{2+} is higher on $CaCl_2$-supplemented media, Ca^{2+} influx is finely regulated at the plasma membrane level in a genotype-dependent manner. This could result in different species presenting different capabilities to regulate Ca^{2+} cellular homeostasis and, therefore, to accumulate Ca^{2+} within cells. In this scenario, highly responding species would accumulate high Ca^{2+} peaks, that would activate SE, whereas low or non-responsive species, would be more sensitive to intracellular Ca^{2+} toxicity, thus being Ca^{2+} peaks and SE activation low or absent.

Similarly, application of ionophore A23187, a chemical compound that favors the membrane permeability and, therefore, facilitates the cellular Ca^{2+} uptake, results on a higher SE response in carrot (Takeda et al., 2003; Calabuig-Serna et al., 2023b), *Coffea canephora* (Ramakrishna et al., 2011), and *Cocos nucifera* (Rivera-Solís et al., 2018), which points to a positive role of intracellular free Ca^{2+} on SE of different species. However, in other species such as sandalwood (Anil and Rao, 2000) and arabidopsis (Calabuig-Serna et al., 2023a), application of ionophore do not have any effect, or even results on a decrease of SE efficiency at high concentrations. Decreased SE efficiency provoked by the addition of ionophore could be explained by a possible toxic effect of high intracellular Ca^{2+} concentration, a deleterious effect of high ionophore concentration, or both. Moreover, deregulation of Ca^{2+} influx and thus, intracellular Ca^{2+} gradients, may provoke negative effects on SE. As mentioned before for Ca^{2+}-supplemented experiments, different species may have different capabilities for ionophore uptake within cells, or a more fine-tuned mechanism to tolerate high intracellular Ca^{2+} peaks. In any case, all these results

reinforce the notion that specific intracellular Ca^{2+} concentration or the maintenance of Ca^{2+} gradients are required for proper embryo development.

On the other hand, different chemical compounds that block Ca^{2+} signaling, either chelating Ca^{2+} or inhibiting CaM, as well as Ca^{2+} channel blockers, have been used to demonstrate the effect of low intracellular Ca^{2+} and Ca^{2+}-signaling during SE. In this sense, Ca^{2+} chelation by the application EGTA and blocking Ca^{2+}-mediated signaling by the addition of W-7, result on a reduction of SE in sandalwood, but does not reduces calli viability, what points to a specific role of Ca^{2+} on the SE process (Anil and Rao, 2000). Similarly, a reduction of SE efficiency was observed in *C. canephora* (Ramakrishna et al., 2011), arabidopsis (Calabuig-Serna et al., 2023a) and carrot (Calabuig-Serna et al., 2023b) upon media supplementation with Ca^{2+} chelators (i.e. EGTA), Ca^{2+} channel blockers (i.e. verapamil and chlorpromazine), or Ca^{2+} signaling inhibitors (i.e. W-7). Accordingly, reduction of intracellular Ca^{2+} by the application of the Ca^{2+} channel blockers La^{3+} ($LaCl_3$) and EGTA completely eliminated the cold-induced enhancement of SE in *P. patula* (Malabadi and van Staden, 2006). Therefore, inhibition of Ca^{2+} intracellular accumulation and signaling by different chemical approaches results in a reduction of SE in different species, which, again, demonstrates the importance of intracellular Ca^{2+} accumulation and signalling for a proper somatic embryogenic process.

Altogether, the functional role of Ca^{2+} during SE has been demonstrated in different plant species. Increasing Ca^{2+} intracellular concentrations to levels that positively regulates SE might be challenging, since different results have been obtained for different species. Indeed, specific Ca^{2+} signatures (peaks) are needed to regulate embryogenesis (Ge et al., 2007), thus exogenous ionophore application might deregulate Ca^{2+} cellular homeostasis and, therefore, its proper embryogenic activator capability. On the other hand, different studies coincide on the need of a correct Ca^{2+} accumulation and signalling during SE, since Ca^{2+} chelation and inhibition of Ca^{2+} transport and signaling results on a drastic reduction of SE efficiency of different species.

Whether the described effect of Ca^{2+} on SE is specific for this process, or can be extrapolated to other embryogenic processes, either zygotic or microspore embryogenesis, is not fully elucidated. Microspores isolated at the inducible stages from highly embryogenesis-responsive genotypes (i.e. the *B. napus* DH4079 line) accumulate more Ca^{2+} than those from genotypes with lower response (i.e the *B. napus* DH12075 line and the DH36 *S. melongena* line; Rivas-Sendra et al., 2017; Rivas-Sendra et al., 2019), which may indicate a positive effect of Ca^{2+} in the sensitivity to microspore embryogenesis induction. Moreover, pharmacological approaches aimed to study the effect of cytosolic Ca^{2+} in microspore embryogenesis revealed a positive effect of increased Ca^{2+} levels in *Triticum aestivum* (Reynolds, 2000) and Brassica napus (Calabuig-Serna et al., our unpublished data). These observations point to a general effect of Ca^{2+} during embryogenesis. The exact mechanism by which Ca^{2+} regulates SE is unknown. A possible scenario would be a direct or indirect genetic regulation of master embryogenic genes in response to Ca^{2+} signatures, which would be crucial for cells to activate an embryogenic developmental pathway (Sharma et al., 2022). Moreover, callose symplastically isolate somatic arabidopsis cells, prior to the adquisition of totipotency during SE, through its deposition on plasmodesmata (Godel-Jedrychowska et al., 2020), and it has been recently shown that application of 2-desoxy-D-glucose, a compound that inhibits callose synthesis, results in a lower SE efficiency in both carrot (Calabuig-Serna et al., 2023b) ad arabidopsis (Godel-Jedrychowska et al., 2020). Thus, the mechanisms through which Ca^{2+} regulates SE could be related to callose synthesis and deposition. Accordingly, a functional relationship was demonstrated between the embryogenic response of *B. napus* isolated microspores and Ca^{2+} influx and callose deposition at their subintinal layer (Rivas-Sendra et al., 2019), which points to a key role of Ca^{2+} in the establishment of the proper chemical environment for a successful

reprogramming of somatic cells towards embryogenesis.

CONCLUSIONS

Somatic embryogenesis is regulated by a complex genetic and epigenetic network, as well as different growth regulators. Pharmacological approaches to modify Ca^{2+} cellular homeostasis result in altered SE responses, demonstrating the need for a proper cellular Ca^{2+} homeostasis. SE protocols have been developed for many different species due to the biotechnological applications of the process. Specific Ca^{2+} signatures are detected during zygote fertilization and initial zygotic embryogenesis, microspore embryogenesis, and somatic embryogenesis. Due to these and other similarities between in vivo and in vitro embryogenesis, SE is a valuable system to study plant embryogenesis, but there are still many questions to be elucidated. Further research directed to understand the basic cellular and molecular mechanisms that control SE would help us to (1) increase its efficiency and establish new protocols for recalcitrant species, and (2) study factors that determine post-zygotic barriers during intra- or interspecific hybridizations, useful to obtain new cultivars.

ACKNOWLEDGEMENTS

This work was supported by grant PID2020-115763RB-I00 funded by MCIN/AEI/10.13039/501100011033 to JMSS and by Generalitat Valenciana, grant number CDEIGENT 2018/023 to RM. ACS is recipient of a predoctoral contract from the “FPU” program of the Spanish Government.

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