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REVIEW

Abnormalities in somatic embryogenesis caused by 2,4-D: an overview

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Abstract

Somatic embryogenesis is a morphogenetic event where somatic cells have the ability to produce embryos without gamete fusion. It is used as a technique for plant mass propagation. It is a process that has six well defined steps such as induction, expression, development, maturation, germination and plant conversion. These steps are characterized by distinct physiological, morphological and molecular events. Although somatic embryogenesis has been established in several plant species, there remains many problems to be solved. The main problem in somatic embryogenesis is the large number of abnormal embryos produced which cannot germinate nor convert into normal plants. Abnormalities in somatic embryos (SE) can be generated by genetic or epigenetic changes in the DNA. These changes in the DNA can be influenced by external factors such as the use of plant growth regulators and mutagenic substances or stress factors applied to the plant tissue such as high and low temperatures, drought, salinity, and heavy metals. Abnormalities generated by genetic changes in the DNA are hardly reversible; however, abnormalities generated by epigenetic changes may be reversible and the abnormal embryos are able to produce normal plants in most cases. This review focuses on the identification of the main factors that can cause abnormal SE development in different plant species, suggest how SE abnormalities are related to somaclonal variations and identify which genes may be involved with embryo abnormalities. Zygotic embryo abnormalities in *Arabidopsis thaliana* mutants are listed with the aim to understand the main genetic mechanisms involved in embryo aberrations.

Key message

The abnormalities in somatic embryos are related to the use of 2,4-D in most of the published protocols, this sintetic auxin disrupts the endogenous auxin balance and the auxin polar transportation interfering with the embryo apical-basal polarity.

Keywords Physiological disorders · Embryo-to-plant conversion · Somaclonal variation · Epigenetic · Mutation

Introduction

Plants propagation by in vitro culture is usually achieved through organogenesis and somatic embryogenesis. Somatic embryogenesis is a morphogenetic event where any somatic cell has the capacity to produce a whole plant without gamete fusion (Fehér et al. 2003). SE can have either multicellular or unicellular origin, emerging from a group of cells or a unique cell, respectively (Maximova et al. 2002). Somatic embryogenesis is produced by in vitro conditions using various plant tissue explant sources and different treatments almost exclusively consisting in supplementing the medium with plant growth regulators (PGR). Other stress treatments such as heavy metal ions, ultraviolet radiation, antibiotic applications, osmotic shocks, dehydration, thermal or cold

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shocks, as well as mechanical or chemical stimuli (Zavattieri et al. 2010) have also been used but remain anecdotal.

The capacity of the plant cell to produce embryos depends on its totipotency, whereby plant cells have the ability to generate new plants (Germana and Lambardi 2016). Somatic embryogenesis can follow two different pathways called direct and indirect somatic embryogenesis (Horstman et al. 2017). The direct pathway occurs when a plant cell produces embryos without callus formation. The indirect pathway requires one additional step for callus formation prior to embryo development (Horstman et al. 2017). Somatic embryogenesis is a tissue culture technique with six steps enumerated as induction, expression, multiplication, development, maturation, germination and plant conversion. These steps are characterized by distinct physiological, morphological, and molecular events (Zavattieri et al. 2010). It has been known that auxins mainly 2,4-dichlorophenoxyacetic acid (2,4-D) is required for somatic embryogenesis induction and embryo multiplication to scale-up the number of embryos which can be potentially produced by indirect somatic embryogenesis (Lloyd et al. 1980; Pasternak 2002; Raghavan 2004; Vondráková et al. 2011) but it is necessary to remove this PGR at the subsequent expression, development and maturation steps because its effect hampers the embryo development and their subsequent conversion into plant (Pasternak 2002; Zavattieri et al. 2010).

The shape of SE can be compared with their equivalent zygotic embryos (ZE) that display the same developmental stages, such as globular, heart, torpedo and cotyledon in dicotyledonous species; globular, scutellar and coleoptilar stages in monocotyledonous species; and early and late embryogenesis in gymnosperm species. However, they are different in the maturation and germination steps because ZE grow and become mature inside the seed surrounded by endosperm, while SE develop from somatic cells without endosperm (Dodeman et al. 1997; Kumar and Van Staden 2017).

There are problems still to be solved in successfully carrying out the somatic embryogenesis process in many plant species, including the processes of maturation, germination and plant conversion. High production of abnormal embryos has been reported in different plant species (Tremblay et al. 1999; Chanatásig 2004; Hashemloian et al. 2008; Vila et al. 2010). The main abnormalities exhibited in SE in different plant species are: fusion of two or more embryos, lack of apical and radical meristems, translucent embryos, multiple cotyledons, and loss of bipolarity (Chanatásig 2004; Hashemloian et al. 2008; El Dawayati et al. 2012; Ruffoni and Savona 2013). Abnormalities in SE may be associated with physiological disorders and/or somaclonal variations (SV) where mutations or epigenetic changes can influence the embryo development and, consequently, the morphology of the resulting plants (Tremblay et al. 1999; Finnegan et al. 2000; Xiao 2006; Bobadilla Landey et al. 2015). The main objectives of this review are to discuss the principal factors that can cause abnormal SE development in different plant species, elucidate how SE abnormalities are related to SV, and identify which genes are potentially involved with embryo abnormalities. ZE abnormalities in some *Arabidopsis* mutants will be listed in this work with the aim to understand the main genetic mechanisms involved in embryo aberrations.

Abnormal somatic embryo production associated with cell culture conditions and PGA

Abnormalities in somatic embryogenesis have been reported in different studies during embryo development that are influenced by components in the growth media and environmental conditions. Medium composition and in vitro environmental conditions such as osmotic components, PGR, amino acid sources, culture medium pH, and light intensity and quality (Table 1) can induce changes in the SE phenotype (Zavattieri et al. 2010). Any cell culture condition that stimulates stress in the plant cells can induce different physiological responses and, as a consequence, phenotypic abnormalities (Krishna et al. 2016). Inadequate in vitro conditions in somatic embryogenesis can cause big losses such as low rate of germination and plant conversion of the embryos in a plant production process, which can make these methods inefficient (Garcia et al. 2016). It is also important to highlight that depending of the genotype, variation in culture conditions can improve SE development, germination and plant conversion, so it is often necessary to devise specific protocols.

Auxins are important in many plant species to induce somatic embryogenesis. 2,4-D is the PGR more used for this purpose. However, this auxin can produce epigenetic and genetic changes in the cells, such as methylation and mutations in the DNA (LoSchiavo et al. 1989; Leljak-Levanić et al. 2004; Fraga et al. 2012; Fehér 2015). PGR (especially 2,4-D) in high concentration or high exposure of the explant to auxins can block normal embryo development (Cruz et al. 1990; Gaj 2004; Vondráková et al. 2011). Thus, 2,4-D has been related to abnormal SE formation, because in high concentration it disrupts normal genetic and physiological processes in cells treated with this PGR (Stuart and McCall 1992; Tokuji and Masuda 1996; Gaj 2004; Pescador et al. 2008). Normal SE development can be arrested because of long exposition or accumulation of exogenous auxins inside the tissue. As it is in the case of somatic embryogenesis in carrot where exogenous application of 2,4-D was used to induce globular SE. Although

Table 1 Examples of effects of culture conditions and PGR on the quality of somatic embryos of different species

Crop specie	Cell culture condition	Effects	References
Culture conditions that decrease abnormalities in somatic embryos			
Juglans regia L	Carbohydrate source and polyethylene glycol	Polyethylene glycol 7.5% and sucrose 3.0% enhances morphogenesis stimulating embryo maturation and reducing embryo abnormalities	Jalali et al. (2017)
Picea glauca	Polyethylene glycol	Polyethylene glycol 7.5% improves the number and quality of SE by promoting normal differentiation of the embryonic shoot and root	Stasolla (2003)
Larix x lepto-europaea	Abscisic acid	The embryos present dense cytoplasmic cells and high rate of cell division, after 60 µM ABA treatment the embryos show high storage proteins content and improve their germination	Gutmann et al. (1996)
Carum carvi L	Abscisic acid	The embryos develop normally, healthy in appearance with well-defined cotyledons, unexpanded radicles and axis free of any proliferations using 10^{-7} M ABA	Ammirato (1974)
Carica papaya L	Abscisic acid	ABA 0.5 µM allows embryogenesis induction, improves embryos maturation, and prevents early germination	Cipriano et al. (2018)
Crocus sativus L	Abscisic acid and polyethylene glycol	Embryo maturation using polyethylene glycol 5% and ABA 3.8 mM increases embryo germination and plant conversion	Vahedi et al. (2015)
Glycine max	Carbohydrate source	Sucrose 6% enhances the conversion of abnormal embryos into plants	Körbes and Droste (2005)
Cucumis melo	Carbohydrate source	Sucrose 3–6% allows germination of abnormal embryos into plants	Gray et al. (1993)
Pseudotsuga menziesii	Gelling agents (Gellan gum)	Gellan gum 1% stimulated embryo production, and abnormal embryo development is reduced	Lelu-Walter et al. (2018)
Acacia Senegal L	L-glutamine	L-glutamine 15 mM stimulates the maturation of SE and reduces embryo abnormalities (cup-shaped structures)	Rathore et al. (2012)
Abelmoschus esculentus L. Monech	L-glutamine	L-glutamine 2 mM increases plant conversion reducing embryo abnormalities	Daniel et al. (2018)
Carica papaya L	White light	White light with intensity of 32 µmol m^{-2} s^{-1} improves SE germination and normal root development	Ascencio-Cabral et al. (2008)
Ostericum korean	Low pH	Medium with pH 4.3 improves embryo morphology reducing the cup-shaped embryo production	Cho et al. (2003)
Glycine max	Low pH	Medium with pH 5.5 increases normal embryo production	Lazzeri et al. (1987)
Theobroma cacao L	Myo-inositol	0.27 M myo-inositol increased the embryo-to-plantlet conversion rate from 13 to 16% to 40–48%	Guillou et al. (2018)

Table 1 (continued)

Crop specie	Cell culture condition	Effects	References
Culture conditions that increase abnormalities in somatic embryos			
Pseudotsuga menziesii	Abscisic acid	ABA 60 µM blocks cotyledon development inducing embryo abnormalities	Lelu-Walter et al. (2018)
Phoenix dactylifera L	Abscisic acid	Concentration of ABA in a range of 10–50 µM hampers embryo development and induces embryo abnormalities	Mazri et al. 2018)
Agave angustifolia	Carbohydrate source	High sucrose concentrations (8–10%) induce embryo abnormalities and blocks embryo development	Reyes-Díaz et al. (2017)
Eucalyptus globules Labill	Casein hydrolysate and L-glutamine	Addition of 0.05% casein hydrolysate and 3.4 mM glutamine increase abnormal embryo formation	Pinto et al. (2002)
Vitis vinifera L	Benzyladenine	BAP in a range of 0.8–3.5 mM increase abnormal embryos production with fascicular shape	Ji et al. (2017)
Hovenia dulcis	2,4-dichlorophenoxyacetic acid	2,4-D 4.5 µM induces embryo abnormalities that cannot germinate in normal plants	Yang et al. (2013)
Theobroma cacao L	2,4-dichlorophenoxyacetic acid	Depending of the genotype, induces embryo abnormalities in a concentration of 9.05 µM	Garcia et al. (2016)
Glycine max L	2,4-dichlorophenoxyacetic acid	2,4-D 22.5 µM induces embryo abnormalities	Shoemaker et al. (1991)
Capsicum chinense Jacq	2,4-dichlorophenoxyacetic acid	2,4-D 9.05 µM stimulated fused SE formation due to the prolonged exposure to the hormone	Lopez-Puc et al. (2006)
Rosa chinensis Jacq	White light or dark conditions	White light with an intensity of 27 µmol m^{-2} s^{-1} causes embryo formation without cotyledons	Chen et al. (2014)
Daucus carota	White light	White light with an intensity of 1.4 µmol m^{-2} s^{-1} induces embryo abnormalities such as multiples cotyledons, branched radicles and hypocotyl elongation	Michler and Lineberger (1987)
Arachis hypogaea	Thidiazuron	Embryogenic masses cannot develop into SE when TDZ is used in a range of 13.62–45.41 µM	Joshi et al. (2008)
Oncidium sp.	Thidiazuron	TDZ in a range of 0.4–13 µM stimulated globular embryos abnormalities inhibiting their development and induce necrosis and dead of the embryogenic masses	Chen and Chang (2000)
Rosa chinensis Jacq	Thidiazuron	TDZ applied in a concentration of 11.25 µM to the induction medium furthered embryogenic masses formation but the embryos cannot develop normally becoming necrotic and finally die	Chen et al. (2014)

embryogenic masses were rinsed with auxin-free medium before to be transferred into the expression medium, 2,4-D accumulation occur inside the globular embryos interfering with their ability to set up the internal auxin gradients that allows cells polarization (Nissen and Minocha 1993). In consequence, these PGR disrupt the normal endogenous auxin balance and the auxin polar transportation, which is important for bilateral symmetry during early plant embryogenesis (Chée and Cantliffe 1989; Venkatesh et al. 2009). Auxin polar transportation in SE when disrupted induces embryo abnormalities (Liu et al. 1993; Abrahamsson et al. 2012; Verma et al. 2018). Research developed with *Picea abies* identified the influence of polar transportation of indol-3-acetic acid (IAA). A polar transportation inhibitor (1-naphthylphthalamic acid, NPA) was applied at different stages of SE development of *P. abies* (Larsson et al. 2007). As a result, abnormalities in SE were shown when NPA was used in the initiation and maturation medium. The main abnormalities found were cotyledon fused and irregular cell division in shoot and root meristems (Larsson et al. 2007). Other studies in *Arabidopsis thaliana* were made inducing a mutation in the auxin transporter. The experiments showed that *A. thaliana* embryos developed the same morphologies as in *P. abies*, which suggests that the role of polar auxin transport is important for the normal embryo development into plants (Laux 2004).

Abnormalities resulting from the cellular origin

Somatic embryos arise from a single cell (unicellular origin) or from a group of cells (multicellular origin). Genetic uniformity has been associated with cellular origin, where multicellular origin promotes genetically variable chimeric plants from group of cells transformed genetically. Thus, unicellular origin produces plants that are genetically uniform (Puigderrajols 2001). As mentioned before, SE are produced by direct or by indirect somatic embryogenesis pathways. Direct somatic embryogenesis has a unicellular origin, while indirect somatic embryogenesis has a multicellular origin (Fernando et al. 2001). It is believed that normal SE are developed through direct somatic embryogenesis and abnormal embryos are derived from a group of cells by indirect somatic embryogenesis (Gaj 2004). SE arise from individual epidermic cells directly without callus formation, while indirect somatic embryogenesis requires callus formation before pro-embryonic complexes are formed and subsequent embryo development (Fernando et al. 2001). Somatic embryogenesis can occur either directly or indirectly in the same tissue simultaneously (Puigderrajols 2001; Corredoira et al. 2006). This occurs in *Quercus suber* L. (cork oak) where SE originated both directly from the individual epidermal cells in the superficial layers without callus formation, and from multiples cells in a compact mass originated from the epidermal cells of the ZE hypocotyls used as explants.

Leaf cultures of *Quercus robur* tree used to induce somatic embryogenesis also showed the origin of the SE. SE emerged directly from the superficial layers of the epidermis in expanded leaves of *Q. robur* (normal embryos) or from embryonic nodular structures (by indirect somatic embryogenesis) giving rise to abnormal embryos due to the loss of bipolarity (Corredoira et al. 2006). The same pattern was reported in *Acca sellowiana* somatic embryogenesis where abnormalities developed when SE arose from a group of cells (Pescador et al. 2008). Abnormal embryos might have formed through alteration in cell division patterns of the pro-embryogenic masses induced by disturbances in auxin transport brought about by exogenous PGR (Gaj 2004). It was reported that abnormalities have been associated with multicellular origin in species such as *T. cacao* (Maximova et al. 2002), *Q. robur* (Corredoira et al. 2006), *Quercus suber* L (Puigderrajols 2001) and *Carica papaya* L. (Fernando et al. 2001) generally by primary somatic embryogenesis but some SE obtained by secondary somatic embryogenesis have normal development probably due to their unicellular origin (Maximova et al. 2002; Yang et al. 2013). The theory is that SE produced through secondary somatic embryogenesis arises from cells without mutations. Cells with mutation are eliminated by programmed cell death, which is a cellular mechanism that removes DNA damage (Rodríguez López et al. 2010b).

Abnormalities related to SV in somatic embryogenesis

SV are known as epigenetic and genetic changes produced in an in vitro propagation program where the resulting plantlets show phenotype and/or genotype variability with regard to the donor plant (Larkin and Scowcroft 1981; Jain et al. 1998; Tremblay et al. 1999; Krishna et al. 2016). Frequency of SV in tissue culture derived plantlets depends on genotype, explant type, culture environment, exogenous PGR, mode of regeneration and culture age (Table 2) (Hitomi et al. 1998; Etienne and Bertrand 2003; Rodríguez López et al. 2010b; Krishna et al. 2016). The main SV shown in tissue culture are variation in the number and structure of the chromosomes, DNA sequence variation, mobile element activation, chromatin remodeling and DNA methylation (Ruiz et al. 1992; Viehmannova et al. 2014).The first report of SV was made by Heinz and Mee (1971) in sugarcane (Heinz and Mee 1971), and after that many reports followed in different species (reviewed in (Krishna et al. 2016)). SV has also been associated with oxidative stress where reactive oxygen species may be involved in DNA alterations of epigenetic or genetic nature (Krishna et al. 2016).

Table 2 SV type in somatic embryogenesis of important crops

Crop specie	Somaclonal variation type	Causes of variation/analysis type	References
Glycine max L	Genetic	Teratogenic effect on embryo morphology and development was generated at 22.5 µM 2,4-D. The progeny of all regenerated plants was screened for variants (R3 generation) and found phenotypic abnormalities associated to the use of this auxin suggesting mutations	Shoemaker et al. (1991)
Picea glauca Moench	Genetic	Long period of in vitro cultivation. *Variegata* phenotype was identified in somatic embryo-derived plantlets. Random amplified polymorphic DNA (RAPD) analysis was carried out and identified variation in a sequence of 700 pb suggesting mutation	Isabel et al. (1996)
Theobroma cacao L	Genetic	Mutations associated with tissue culture protocol. In the protocol a high concentration of 2,4-D (9.08 µM) was used. Simple sequence repeat (SSR) analysis was carried out which identified putative chimeric mutants for slippage mutation or allele loss across two loci	Rodríguez Lopez et al. (2004)
Saccharum officinarum L	Genetic	Causes unknown. Genotypic stability in direct SE was evaluated using RAPD molecular markers. The analysis revealed polymorphisms with 0.89% of variation in regenerated plants derived through direct somatic embryogenesis suggesting mutation	Suprasanna et al. (2007)
Bactris gasipaes	Genetic	High concentration of Picloram. Amplified fragment length polymorphism (AFLP) analysis was carried out to identify mutations. 25% of variation was detected in regenerants cultured on medium with 600 µM Picloram	Steinmacher et al. (2007)
Carica papaya	Genetic	High concentration of 2,4-D (68 µM). RAPD analysis was carried out and the DNA fingerprints revealed both monomorphic and polymorphic bands which were associated with morphological abnormalities in the resulted plants	Homhuan et al. (2008)
Theobroma cacao L	Genetic	Mutations associated with tissue culture protocol. In the protocol was used high concentration of 2,4-D (9.08 µM). Cleaved amplified polymorphic sequence (CAPS) analysis was used to detect mutation amongst a clonal population of in vitro cocoa plants. Polymorphisms were detected comprising 26% of the samples screened	Rodríguez López et al. (2010a)

Table 2 (continued)

Crop specie	Somaclonal variation type	Causes of variation/analysis type	References
Olea europaea L cv. Kroneiki	Genetic	Tissue culture method. High concentration of auxins are used to induce somatic embryogenesis (24,6 µM IBA and 2,4 µM 2-ip). To identify mutations a RAPD analysis was carried out in somatic embryogenesis plantlets. Regenerated plantlets were less than 75% similar to the mother plants suggesting mutations	Peyvandi (2010)
Smallanthus sonchifolius	Genetic	Tissue culture method. Indirect somatic embryogenesis using 4.5 µM 2,4-D in combination with 0.05–0.5 µM zeatin or 0.04–0.4 µM N6-benzyladenine (BA) generated genetic variation. Simple sequence repeat (ISSR) analysis was carried out in 60 regenerated plantlets. 6% of the somatic embryogenesis plants showed genetic variability compared to the donor plant	Viehmannova et al. (2014)
Cymbopogon winterianus Jowitt	Genetic	Method of regeneration. Indirect somatic embryogenesis using 2,4-D in a range of 6.8–13.6 µM with long exposition of the explants to this PGR. Abnormalities were identified in regenerated plants established in the field. RAPD analysis was carried out in these plants and genetic variability was identified	Dey et al. (2015)
Cuminum cyminum L	Genetic	Method of regeneration through indirect somatic embryogenesis using 2,4-D as source of auxin in a range of 2.3–9.08 µM for callus formation. RAPD analysis was carried out and presence of polymorphic bands was detected between regenerated plantlets and mother plants	Bahmankar et al. (2017)
Picea sp.	Genetic	Long culture periods and cryopreservation method. Culture medium for somatic embryogenesis initiation was supplemented with 9 µM Picloram as source of auxin and BA as source of cytoquinin in a range of 2.2–8.8 µM. Cryopreservation was carried out using pregrowth-dehydration method and freezing with liquid nitrogen. Somaclonal variation was analyzed using SSR markers. 52 different mutations were identified in both long culture period (42–44 months) and cryopreservation with liquid nitrogen (2 weeks) of somatic embryogenic lines	Hazubska-Przybył and Dering (2017)

Table 2 (continued)

Crop specie	Somaclonal variation type	Causes of variation/analysis type	References
Theobroma cacao L	Genetic and epigenetic	Mutation and epigenetic changes associated with culture age. SSR markers were used to detect genetic changes. 35% of regenerated plants exhibited at least one mutation with polymorphisms in two categories (Allele loss and formation of new alleles). For epigenetic changes, methylation-sensitive amplified polymorphism (MSAP) markers were used. The analysis revealed epigenetic divergence between regenerated plants and explant source. Epigenetic and mutation divergences between source of explant and regenerated plants decreased after 10 weeks in culture. The hypothesis is that secondary somatic embryogenesis in cacao generated SE from cells without mutation or epigenetic changes	Rodríguez López et al. (2010b)
Coffea arabica	Genetic and epigenetic	Tissue culture method. Cellular suspensions using 2.3 µM 2,4-D as source of auxin induce genome and epigenome instability. The use of low concentration of 1.36 µM 2,4-D in suspension cultures reduces somaclonal variations in the resulted somatic embryo-derived plantlets. Molecular AFLP and MSAP analysis in regenerated plantlets showed that polymorphism between mother plants and regenerated plantlets by somatic embryogenesis was extremely low (Less than 0.18%)	Landey et al. (2013)
Coffea arabica and Coffea canephora	Genetic and epigenetic	Culture age and method of culture. Multiplication of cell suspension is carried out in liquid medium supplemented with 2.26 µM 2,4-D, 4.92 µM indole-3-butyric acid (IBA) and 9.84 µM isopentenyladenine (iP). Age of embryogenic cells suspension induces somaclonal variation. Morphological analysis was carried out in somatic embryo-derived plants established in the field. Somaclonal variation increase from 6 to 25% in plantlets produced from cell suspension aged 6 to 12 months	Etienne and Bertrand (2003)
Hordeum vulgare	Epigenetic	Long exposition to 2,4-D in a concentration of 9.0 µM. Phenotypic variants were detected in the regenerated plantlets, but in the cellular analysis mitotic alterations were not detected. This suggests epigenetic variation	Ruiz et al. (1992)

Table 2 (continued)

Crop specie	Somaclonal variation type	Causes of variation/analysis type	References
Cucurbita pepo L	Epigenetic	Long exposition of 2,4-D 4.5 µM and NH4Cl in a range of 1 − 0 mM blocks embryos development. Genomic DNA digestion and random PCR amplification was carried out to analyze DNA methylation. High levels of DNA methylation were observed in cell lines cultured on medium supplemented with 2,4-D and NH4Cl	Leljak-Levanić et al. (2004)

Phenotypic abnormalities related to genetic nature were identified in somatic embryo-derived plantlets (Table 2). Variations in the plant architecture and leaf morphology are some of the most important features identified in coffee somatic embryo-derived nursery plants after 12 months of development. The predominant abnormalities were angustifolia, variegata and dwarf found in these somatic embryogenic plants. Chromosome number were evaluated in the abnormal plants and it was reduced compared with the number of chromosomes of the mother plants (Bobadilla Landey et al. 2015). AFLP markers were also used to analyze DNA variations in coffee somatic embryos-derived plants. The molecular analysis revealed differences in polymorphisms profiles between plantlets and the donor plants (Landey et al. 2013). Similar morphological variations were reported in papaya plants in the field where production was compromised. Somatic embryogenesis plants obtained by the indirect pathway showed abnormalities in number of flowers, floral length, number of fruits, fruit length and fruit morphology. Papaya embryogenic callus was grown in induction medium supplemented with 15 mg L^{-1} of 2,4-D. Probably the high concentration of 2,4-D used to induce embryogenic callus had a strong influence in genetic variations and in consequence morphological abnormalities. Therefore, molecular analysis was done using RAPD. The results revealed differences in band patterns between somatic embryo-derived plantlets (emerged from callus) and the donor plant due to mutations in the DNA (Homhuan et al. 2008; Aydin et al. 2016).

Abnormalities were also identified in somatic embryo-derived plants associated with epigenetic changes (Table 2). Epigenetic is any process that changes gene activity due to alterations in the chemistry of the DNA without sequence variations (Zhang 2013; Kumar and Van Staden 2017). During the various plant development phases, the gene expression is programmed to allow organ formation. This program must be erased and replaced with a new program within hours, days or weeks following their exposure to the in vitro culture conditions that induce somatic embryogenesis. These events are mediated by the interaction among endogenous and exogenous hormone levels, other components in the culture medium and the environment conditions (De-la-Peña et al. 2015). PGA and stressful environmental conditions applied to cells in in vitro culture affect the processes of histone modification, chromatin structural reorganization and DNA methylation, which in turn can change the overall gene expression patterns (De-la-Peña et al. 2012; Ruffoni and Savona 2013, Us-Camas et al. 2014; Fehér 2015). These epigenetic mechanisms are important factors that have strong influence in organism phenotypes and consequently in SE abnormalities (Zhang 2013).

DNA methylation is an epigenetic event that consists of the addition of a methyl group at the 5′-position of the cytosine in the contexts of CpG (5′—C—phosphate—G—3′), CHG or CHH (with H being A/T/G) repetitive sequences in the plant DNA (Finnegan et al. 2000; Zhang et al. 2010). In tissue culture, mainly in somatic embryogenesis there is a growing appreciation that DNA methylation is an important mechanism influencing SV in the resultant plantlets altering their physiology and phenotype and it can be inherited by generations (Chakrabarty et al. 2003; Tchorbadjieva and Pantchev 2004; Francischini et al. 2017). Chromatin remodeling is also related to SV when sudden variation in the gene expression can cause phenotypic and physiologic alterations in the plantlets generated by in vitro culture. Chromatin adopts two major stages, heterochromatin (chromatin more compacted) and euchromatin (chromatin more relaxed) when the histones are methylated or acetylated, respectively. Heterochromatin, different to euchromatin, blocks DNA transcription (Kim and Kaang 2017). DNA methylation and chromatin remodeling interact mutually in regulating gene expression.

Abnormalities in plantlets induced by epigenetic factors have been reported in different crops (Kitimu et al. 2015; Ghosh et al. 2017; Goyali et al. 2018). In *Agave angustifolia* Haw., albinism was related to the tissue culture environment where the missing of color in leaves was correlated with high levels of DNA methylation (Duarte-Aké et al. 2016). In *Elaeis guineensis* Jacq. (oil palm) the *mantled* phenotype was identified in plants propagated by somatic

embryogenesis. This phenotype is a homeotic flowering abnormality that results in plant sterility and in consequence lower oil yields (Morcillo et al. 2006). In that study the expression of two genes (*EgM39A* and *EgIAA1*) displayed increased transcription accumulation in auxin-treated callus as a consequence of DNA methylation. The auxins used in this experiment were 2,4-D and naphthalene acetic acid. It was demonstrated that exogenous auxin induced changes in DNA methylation and this change generated abnormalities in plants.

Some research has been developed to evaluate DNA methylation in somatic embryogenesis using methylation inhibitors, such as 5-azacytidine (5-azaC). 5-azaC is a potent drug that has the ability to incorporate itself into DNA, trap DNA methyltransferases (DMNTs) and lead them to their degradation, promoting DNA hypomethylation (Issa and Kantarjian 2009). It has been shown that plants submitted to treatments with 5-azaC produce abnormalities such as dwarfism and it can also induce early flowering and vegetative growth inhibition (Kondo et al. 2006). Embryogenic callus of *Coffea canephora* treated with 5-azaC in different points during the whole process showed some interesting results. 5-azaC added every 7 days in the somatic embryogenesis cultures showed embryo formation inhibition, but when the drug was added at the 21 day of culture it increased pro-embryogenic masses proliferation preventing SE development (Nic-Can et al. 2013). In this research, it was noted that the methylation level decreased when the drug was added to the culture medium independently of the phase of the somatic embryogenic process where it was applied. Methylation levels in SE decreased during embryo development and maturation but, when somatic embryogenesis is starting methylation levels are higher (Nic-Can et al. 2013). Similar results were obtained in *Medicago truncatula* where it was observed that the use of 5-azaC in somatic embryogenesis cultures decreased methylation levels and this interrupted SE development (Santos and Fevereiro 2002). Differently, the use of 5-azaC in somatic embryogenesis in other crops such as *Acca sellowiana* maintained high methylation levels, but these levels of methylation did not allow embryo development and plant conversion (Fraga et al. 2012). Those results suggest the important dynamic role of DNA methylation in SE induction, development and conversion to true-to-type plants.

Somatic and ZE morphology has also been associated with PIN-like proteins regulated by DNA methylation (Xiao 2006; Fraga et al. 2016). In zygotic embryogenesis DNA methylation was related with *PIN1* expression, which in turn, are necessary to establish auxin transport in the embryo (Xiao 2006). By contrast, for induction of somatic embryogenesis, the DNA methylation levels are affected by PGR addition to the culture medium and, in consequence, the transcription of PIN-like proteins can be down-regulated and auxin polar transportation affected, blocking the right SE morphogenesis (Friml et al. 2003). In other studies, DNA methylation in the mother plant, and normal and abnormal in vitro plantlets of sugarcane was measured using the technique MSAP. Sixteen selective primers for *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II restriction enzymes were used. Abnormal plantlets were identified as somaclonal variants. Genetic similarity between mother plant and abnormal plantlets was compared and the range of similarity was 87.7–91.1% for *Eco*RI/*Msp*I and 92–95% for *Eco*RI/*Hpa*II. These results confirm that there is a relationship between abnormalities in in vitro plantlets and DNA methylation (Francischini et al. 2017).

Molecular view of abnormalities in plant embryogenesis

Plant embryogenesis depends on a sophisticated morphogenetic program where several genes, mainly those that encode transcription factors (TF), regulate all the process from zygote formation until embryo maturation. Zygotic and SE show several similarities in the morphogenetic program that control them, for example the HAEM ACTIVATOR PROTEINS 3 (HAP3) a multimeric transcriptional activator complex, where the TF *LEAFY COTYLEDON1* (*LEC1*) and *LEAFY COTYLEDON LIKE* (*LIL*) are members (Smertenko and Bozhkov 2014). *LEC1* and *LIL* are important regulators of early zygotic embryogenesis in the cotyledon cell identity and suspensor cell maintenance, and in later stages these TF control maturation initiation and maintenance, as well as precocious germination (West et al. 1994). Beside the role of *LEC1* and *LIL* in zygotic embryogenesis, they are essential for somatic embryogenesis induction and embryo maturation. Along with *LEC1* and *LIL, LEC2* and *FUSCA3* also members of the *LEC* genes family are important TF that regulate embryogenesis development (Harada 2001; Guo et al. 2013). *Lec* mutants in *A. thaliana* zygotic embryos exhibit defects in cotyledons morphology and problems in maturation and desiccation tolerance. Mutations in *LEC1* and *FUSCA3* (*FUS3*) genes cause embryo lethality due to loss of desiccation tolerance during late seed development, while mutations in the *LEC2* gene block embryo development (Harada 2001; Stone et al. 2001). In somatic embryogenesis *lec1, lec2* and *fus3* mutants submitted to in vitro culture formed SE in low frequency compared with wild-type cultures, and double (*lec1 lec2, lec1 fus3,lec2 fus3*) and triple (*fus3 lec1 lec2*) mutants show total somatic embryogenesis repression (Gaj et al. 2005). *LEC 2* gene has also an important role in zygotic and somatic embryogenesis in *T. cacao*. Young cacao leaves and ZE cotyledons were transformed using *Agrobacterium tumefaciens* to overexpress the

TcLEC2 gene. After that, somatic embryogenesis induction was carried out in the transformed tissues. Surprisingly, high frequency of SE were induced, but they expressed developmental and morphological abnormalities keeping the embryos at the globular and heart stage, therefore, cotyledon development was compromised, and new secondary SE came out of the malformed cotyledons (Zhang et al. 2014). *LEC2* gene has the capacity to cause changes in auxin activity (Ledwoń and Gaj 2009) through activation of the *YUCCA2* (*YUC2*) and *YUC4* genes involved in auxin biosynthesis via the tryptophan-dependent pathway (Yamamoto et al. 2007; Stone et al. 2008).

After embryogenesis induction, zygotic and somatic cells divide asymmetrically producing an apical and basal cell. This spatial cell distribution results in the apical-basal axis formation where the PGR auxin plays an important role together with proteins such as WOX and PINFORMED. The *WUSCHEL (WUS)*—related homeobox *(WOX)* homeo domain gene family codified an important TF that is involved in the earlier phases of ZE development and also regulates the lateral organ development in plants (Haecker 2004). *WOX* genes are expressed in the single-cell zygote when the embryo apical-basal axis is forming in angiosperm plants, such as *A. thaliana* (origin of early pattern divisions). The apical daughter cell of the zygote gives origin to the apical meristem, cotyledons, hypocotyl, embryonic root and root meristem. The basal daughter cell gives origin to the suspensor. Both cell fates occur through asymmetric divisions (Haecker 2004). Three important *WOX* genes are activated in the apical and basal cell division patterns, i.e., the *WOX2* and *WOX8/9* respectively (Breuninger et al. 2008).The *WOX2, WOX8* and *WOX9* genes were also expressed in early SE development, but their expression decreased in SE maturation (Palovaara and Hakman 2009, Su et al. 2009; Bouchabké-Coussa et al. 2013; Zhu et al. 2014; Kumar and Van Staden 2017). Abnormalities in the suspensor formation and lack of protoderm were the main features in SE produced from *WOX2* transgenic cell lines *(35S:WOX2i)* in the gymnosperm plant Norway spruce (*Picea abies* L. Karst) (Zhu et al.

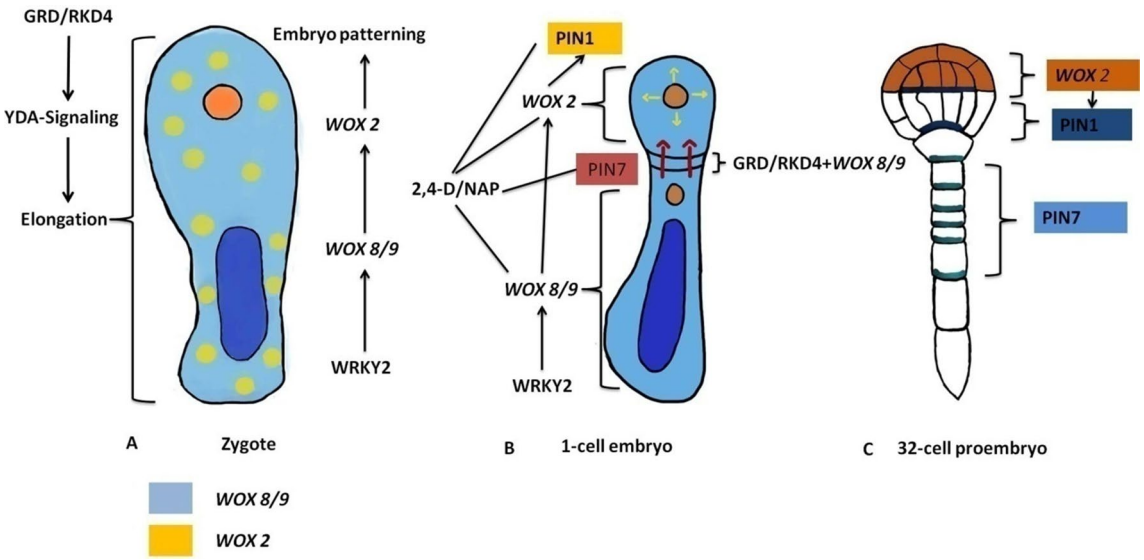

Fig. 1 Model of the zygotic elongation and asymmetric division in *Arabidopsis thaliana*. **a** YDA-signaling activation by GRD/RKD4 protein through unknown mechanism to zygote elongation (Jeong et al. 2011; Waki et al. 2011; Mursyanti et al. 2016), and the embryo patterning activation by WRKY2 protein, which in turn activates the *WOX 8/9* gene expression (Ueda et al. 2011). Subsequently, *WOX 2* is activated by WOX 8/9 proteins to start the embryo polarization (Breuninger et al. 2008). **b** The WRKY/WOX pathway promotes polar organelle localization where the small apical daughter cell is formed by dense cytoplasm and the bigger basal daughter cell is formed by a large vacuole (Ueda et al. 2011; Zhang and Laux 2011). Exogenous application of 2,4-D/NAP blocks the *WOX* genes and the auxin polar transportation via PIN proteins, it results in the disruption of embryos asymmetric division, and in the production of several embryo abnormalities (Friml et al. 2003; Palovaara and Hakman 2009). *WOX 2* genes are expressed in the apical daughter cell of the 1-cell embryo, while the *WOX 8/9* genes are expressed in the basal-cell (Breuninger et al. 2008). The GRD/RKD4 works in cooperation with the *WOX 8/9* genes in the establishment of embryo polarity (Jeong et al. 2011; Waki et al. 2011; Mursyanti et al. 2016). Loss-of-function of the *GRD* gene induces the reduction of the elongation of the zygote and abnormal early cell division (Jeong et al. 2011). **c** *WOX 2* affects auxin polar transport and distribution in the 32-cell pro-embryo via activation of PIN1 protein (Friml et al. 2003).. PIN7 proteins are localized in the apical side of the suspensor cells facing the pro-embryo (Friml et al. 2003). These proteins lead the auxin transport from the suspensor to the pro-embryo since 1-cell embryo onwards (Friml et al. 2003). The solid lines represent inhibition and solid arrows indicated activation. Vacuoles (dark blue ovals), nuclei (brown circles) are indicate in **a, b**. The *WOX 2* and *WOX 8/9* expression are indicate in color-code as shown in **a, c**. PIN1 and PIN7 distribution in the 1-cell and 32-cell embryos are indicated in color-code shown in **b, c**

2016). Aberrant cell division in basal cells of the zygote, and apical-basal polarity were completely lost in SE from *WOX8/9* transgenic cell lines of *P. abies* (Zhu et al. 2014). Embryos development in *wox8wox9* double mutants of *A. thaliana* is arrested, resulting in abnormal development in the suspensor and the pro-embryo, suggesting that *WOX8* and *WOX9* gene expression influence the expression of the *WOX2* gene (Fig. 1a) (Breuninger et al. 2008). *WOX* genes have an evolutionary conserved function in apical-basal axis formation in angiosperms and gymnosperms. Loss-of-function of these TF in zygotic and SE generates serious problems in embryo development.

The WRKY proteins are a superfamily of zinc-finger domain TF specific to plants, which are involved in environmental and developmental interactions (Eulgem et al. 2000). These proteins are important in cell fate and embryonic patterning because they regulate *WOX8* and *WOX9* expression in the basal embryo lineage (Fig. 1a) (Ueda et al. 2011). The *WOX8* and *WOX9* carry an intron fragment *cisB* with a canonical W-box (TTGACC/T) where the WRKY proteins bind with high affinity (Eulgem et al. 2000; Ueda et al. 2011). *WOX8/9* expression in the *wrky2-1* mutants is completely lacking, and the uppermost cells of the basal lineage of these mutants and secondary pro-embryo-like structures arisen from them divided abnormally (Ueda et al. 2011). The zygote polarity is established by the WRKY2-WOX8/9 transcription cascade through the organelle organization (vacuolization of the basal daughter cell in the zygote); thus, it establishes the asymmetric cell division in the zygote with the subsequent *WOX2* gene activation in the embryo proper by WOX8/9 proteins, which in turn activates the *PIN1* gene and the correct auxin transport and distribution in the 1-cell embryo stage (Fig. 1b, c) (Ueda et al. 2011; Zhang and Laux 2011).

Along with the *WOX* TF the *PINFORMED* (*PIN)* genes that codified PIN1, PIN4 and PIN7 proteins are involved in the formation of the apical-basal axis of the embryo providing the intercellular transport of the auxin (Tvorogova et al. 2015). PIN1 and PIN7 proteins act directly in the apical-basal auxin transport in early embryogenesis. At the 32-cell pro-embryo stage PIN1 protein is localized in the provascular cells facing the basal embryo pole (hypophysis), while PIN7 is localized in the apical side of the suspensor cells facing the pro-embryo (Fig. 1b, c). PIN1 and PIN7 localization reflect early apical-basal polarization and presumably, auxin is transported through the auxin efflux factor (PIN7) to the apical daughter cell of the zygote where it accumulates, and after that the PIN1 proteins play an important role in auxin flux from the globular embryo stage and during the lifespan of the plant (Friml et al. 2003). Abnormalities in the apical daughter cell of the zygote were shown in *A. thaliana pin7* mutants where specification was compromised due to its horizontal division instead of vertical cell division. As a consequence, *pin7* mutants failed to establish the pro-embryo properly. On the other hand, *pin1* mutants showed defects at the basal embryo pole compromising the normal development of the hypophysis (Friml et al. 2003). Despite the abnormalities in pro-embryos, *pin1* and *pin7* mutants recovered and produced fertile plants. Probably, the recovery of these mutants is because of functional redundancy among *PIN* genes. Interestingly, abnormalities are developed in double, triple and quadruple *pin* mutants to avoid *PIN* genes functional redundancy, *pin4 pin7* double mutants showed abnormal cotyledon number, *pin1 pin3 pin4* triple mutants showed fused cotyledons and the root was very short, and *pin1 pin3 pin4 pin7* quadruple mutants displayed misplaced or fused cotyledons, apical structures absent, non-functional root formation, and loss of apical-basal polarity generating ball-shaped embryos. Similar defects were shown in embryos after interfering with auxin homeostasis when they are cultured in artificial conditions using 2,4-D (as an influx substrate) or auxin-efflux inhibitors as NAP (Fig. 1b) (Friml et al. 2003; Palovaara and Hakman 2009).

Another important player involved in the apical-basal polarity in the early embryo is the MAPK (mitogen activated protein kinase) kinase kinase gene *YODA* (*YDA)* that leads the (YDA)-signaling pathway (Lukowitz et al. 2004). The *yda* mutants show a dwarf phenotype because the loss of function of the *YDA* gene affects zygote elongation and the normal development of the suspensor (the suspensor cells generally have morphology similar to the pro-embryos cells and the large vacuole characteristic of these cells is lacking). As a result, the apical-basal polarity of the future embryo is disrupted (Lukowitz et al. 2004; Musielak and Bayer 2014). Conversely, *YDA* gain-of-function alleles promote exaggerated suspensor growth and suppress embryo development (Lukowitz et al. 2004). The *MPK3* and *MPK6* are other MAPK kinase kinase genes that act redundantly downstream of the *YDA* gene in the embryogenic pathway.

The *mpk3 mpk6* double mutants displayed the same phenotype of *yda* mutants (Musielak and Bayer 2014). It was identified in zygotic embryogenesis that the *short suspensor (SSP)* and *grounded (GRD)* genes also act by controlling the YDA-signaling pathway especially the *SSP* gene that is a member of the interleukin-1 receptor-associated kinase (IRAK)/Pelle-like kinase family of receptors like-kinases (Wendrich and Weijers 2013). The *SSP* protein is a membrane-bound protein that is under the control of pollen cells, which is translated specifically after fertilization and after that, it activates the YDA-signaling pathway in zygotic embryogenesis (Wendrich and Weijers 2013). The protein RKD4 encoded by the *GRD* gene was identified in somatic embryogenic cultures inducing the embryogenic fate in somatic cells when overexpressed, and it is also expressed in ZE for pattern formation from the first division onward (Fig. 1b) (Waki et al. 2011; Mursyanti et al. 2016).

Loss-of-function of the *GRD* gene induces the reduction of the elongation of the zygote and abnormal early cell division. Phenotypically, the *grd*/*rkd4* mutant looks like the *yda* and *spp* mutants probably because of the interaction of the GRD/RKD4 with the YDA-signaling pathway (Jeong et al. 2011).It was also found that GRD/RKD4 acts in cooperation with *WOX8 and WOX9* for establishing embryo polarity (Fig. 1b) (Jeong et al. 2011). In this way, GRD/RKD4 is related to the signaling cascade (mechanism unknown) where *YDA, WOX2, WOX8, WOX9* genes and theWRYK2 and PIN proteins are interconnected in synergy for the normal zygotic and SE development (Fig. 1a, b).

Abnormalities in ZE can be used as a model of somatic embryogenesis development

The use of mutants in zygotic embryogenesis has been an important tool to understand embryo development in their different stages. Investigations in *A. thaliana* and other species such as *Zea mays* have increased our understanding of abnormalities in ZE (Clark and Sheridan 1988; Meinke 1995; Vernon and Meinke 1995; Bastida et al. 2006). Abnormalities have been induced by mutation in the DNA sequence of the ZE, generally by chemical and physical mutagenesis or by loss-of-function phenotypes using gene knockout techniques (mainly T-DNA insertion mutagenesis) resulting in embryo-defective (*emb*) mutants (Meinke 1985; Devic 2008). As a result, embryogenic processes from the *emb* mutants are disrupted. The mutants do not develop beyond the cotyledonary stage and show morphogenic alterations, anomalous differentiation and problems in the maturation stage. The principal abnormalities found in these *emb* mutants are lack of pigmentation, disorganized vegetative growth and dwarfism (Clark and Sheridan 1988; Vernon and Meinke 1995). Several *emb* mutants with interesting defects in embryo development have been described by a wide number of publications (Meinke and Sussex 1979a, b; Meinke 1991, 1995; McElver et al. 2001; Tzafrir 2004; Muralla et al. 2011). In order to collect all the information about *A. thaliana emb* mutants available, a database at http://www.seedgenes.org is accessible. This database includes more than 400 *EMBRYO-DEFECTIVE* (*EMB*) genes and 888 mutant alleles, where 1% is related to defective seeds, 15% to abnormalities in seed pigmentation and 84% is related to *emb* (747 out of 888 mutant alleles). With this complete information, a classic genetic map has been saturated in order to facilitate the analysis of important genes in *A. thaliana* embryo development (McElver et al. 2001; Meinke 2003; Meinke et al. 2009).

Plant embryogenesis is a process generally divided in four phases such as embryo development, maturation, desiccation and germination (Apuya et al. 2001). Embryo development and conversion into plant are processes controlled by molecular mechanisms as gene expression, where *emb* mutants can help in understanding the correlation between phenotypes and gene functions. Vernon and Meinke (1995) reported several representative *emb* mutants called late embryo defectives in *A. thaliana*. These mutants develop beyond the globular stage, but cannot become normal plants (Vernon and Meinke 1995; Patton et al. 1998). Representative late embryo mutants were chosen for further morphological analyses (Table 3). Comparing wild-type embryos with mutant embryos it is easier to identify differences in the size and morphologies. Mutant embryos are small and white, but some can develop green cotyledons such as *emb266* and *emb270*. The embryo mutants of *emb224, emb232* and *emb256-1* developed until the heart stage, but the young cotyledons were malformed, and the hypocotyl was reduced. While *emb152, emb163* and *emb201* develop until the cotyledonal stage, but the cotyledons were excessively reduced. Conversely, *emb163* and *emb266* showed multiple cotyledons in rosette shape, which were occasionally observed in *emb223* and *emb209* (Vernon and Meinke 1995).

Several embryo-lethal mutants were also evaluated to understand the molecular function of some genes in developmental processes from *A. thaliana*. The *emb49* is an embryo mutant in biotin precursors (dethiobiotin) that shows multiple cotyledons deformation and its development is slow compared with wild-type embryos (Patton et al. 1998). Mutants with low expression of the gene *Alanyl-tRNA synthetase* induce weak pro-embryos and its expression stops along with globular embryo development. Although embryos at the globular stage are aborted, some of them can be germinated by embryo rescue techniques. The resulting seedlings showed short hypocotyl and the shoot meristem twisted (Sun et al. 1998).The *schlepperless* is a mutant in the *chaperonin-60*α gene. Embryo mutants for the *chaperonin-60*α gene show normal development but their growth is slow and the development is arrested at the heart stage (Apuya et al. 2001). The T-DNA insertion mutant *slow embryo development1* that affects a protein targeted to mitochondria showed a slower development compared to wild-type embryos. These mutants at the early globular stage are white in appearance. In the maturation stage, production and accumulation of proteins and lipids in their body is poor (Ju et al. 2016). The enzyme serine decarboxylase (SDC1), which directly converts serine to ethanolamine, is essential in plant embryogenesis. Mutations in the gene coding for this enzyme can arrest embryo development in *A. thaliana*. In this study, it was shown that embryo lethal defects are produced with the knockout of the *SDC1* gene (Yunus et al. 2016). Another important enzyme for ZE development is the putative glutamate carboxypeptidase encoded by *Altered Meristem Program 1* (*AMP1*) gene.

Table 3 Several characteristics observed in lethal embryos in the model plant *A. thaliana*

Mutant/mutation	Characteristic	References
emb22	Arrested embryo at globular stage that failed to produce hypocotyls and cotyledons	Patton and Meinke (1990)
emb30	Aborted seeds with green embryos with hypocotyls reduced and distorted cotyledons fused forming a conical structure	Patton and Meinke (1990)
emb31	Normal hypocotyls with pale cotyledons that varied in size and morphology	Patton and Meinke (1990)
fusca3	Dormancy and desiccation tolerance is altered, storage proteins levels reduced and cotyledons with trichomes	Keith et al. (1994)
sus	Enlarged suspensor phenotype at maturity stage	Schwartz et al. (1994)
fusca	High levels of anthocyanin accumulation, inhibition of hypocotyls elongation, apical hook opening, unfolding of cotyledons	Mis et al. (1994)
emb266	Dwarfism	Vernon and Meinke (1995)
emb270	Dwarfism	Vernon and Meinke (1995)
emb224, emb232, emb256-1	Large heart shape with abnormal cotyledons and reduced hypocotyl	Vernon and Meinke (1995)
emb163	Cotyledons reduced	Vernon and Meinke (1995)
emb201	Cotyledons reduced	Vernon and Meinke (1995)
emb223	Rosette cotyledons malformation	Vernon and Meinke (1995)
emb209	Rosette cotyledons malformation	Vernon and Meinke (1995)
Shoot meristemless	Shoot meristem reduced or completely absent in mature embryos	Endrizzi et al. (1996)
wus	Fail to properly organize a shoot meristem in the embryo	Laux et al. (1996)
emb49	Cotyledons deformed as rosettes	Patton et al. (1998)
Monopteros	Vascular strands formation interference and body axis alteration	Hardtke and Berleth (1998)
Alanyl-tRNASynthetase	Weak embryos in globular stage	Sun et al. (1998)
emb30	Abnormal cell wall and deficient polar cell growth control	Shevell et al. (2000)
Schlepperless	Retardation of embryo development before the heart stage	Apuya et al. (2001)
Topless-1	Transformation of the shoot pole of the embryo into a root	Long et al. (2002)
hoc	Over production of cytokinin, bushy phenotype with supernumerary rosettes	Catterou et al. (2002)
Pex10 null mutant	Retarded development and morphological abnormalities	Sparkes (2003)
Embryo ball	Cotyledons are absent, the apical region produced leaf-like structures with trichomes	Kristof et al. (2008)
bobber1	Arrest at the globular stage of development	Jurkuta et al. (2009)
gnom	Root meristem deficient and cotyledon primordial formation fails	Wolters et al. (2011)
Dicer-like1	Early embryo maturation	Willmann et al. (2011)
lec1	Cotyledon identity altered and ectopic trichomes formation	Huang et al. (2015)
Slow embryo development1	Slower development in globular stage and white appearance	Ju et al. (2016)
Serine decarboxylase	Arrest embryo development	Yunus et al. (2016)
Altered Meristem Program 1	Seeds develop deficient coat	López-García et al. (2016)

When this enzyme is defective, an increased frequency of embryo abortion occurs because the seeds develop deficiency in coat formation (López-García et al. 2016). Complementary information about other important mutants with gene dysfunction is listed in the Table 3. The abnormalities presented in embryos-defective mutants have similarities with some SE abnormalities such as cotyledons lacking or malformed, lacking apical and radical meristems, translucent color and loss of bipolarity, among others (Chanatásig 2004; Hashemloian et al. 2008; El Dawayati et al. 2012; Ruffoni and Savona 2013). Reverse genetics can be an interesting methodology to evaluate SE abnormalities using the information about abnormalities in the counterpart ZE mutants with the aim to identify the phenotypes generated by a specific mutation; thus, in this way implement strategies to improve somatic embryogenesis protocols.

Conclusion

Morphological deformation occurs commonly in SE. These morphological abnormalities are a result of physiological disorders or somaclonal variation. Depending on the genotype, some in vitro culture conditions can improve embryo quality by reducing abnormalities (Table 1). 2,4-D is one of the most important PGR used to induce somatic embryogenesis; high concentration and long exposition to this PGR generates embryo abnormalities (Cruz et al. 1990; Gaj 2004; Vondráková et al. 2011). It is well known that 2,4-D disrupts auxin

homeostasis in embryos when applied in vitro (Friml et al. 2003; Palovaara and Hakman 2009). Epigenetic and genetic changes are also attributed to the use of 2,4-D because this PGR can induce methylation or mutations in the DNA (LoSchiavo et al. 1989; Leljak-Levanić et al. 2004; Fraga et al. 2012; Fehér 2015). Auxin polar transportation can be compromised with the use of 2,4-D in high concentrations. This PGR affects the right expression of *PIN* genes (especially *PIN1*) by inducing methylation of the DNA (Xiao 2006; Fraga et al. 2016). Two important PIN proteins are expressed in the embryo patterning (PIN1 and PIN7). Mutation or inactivation of genes that codify these proteins affect the establishment of apical-basal polarity in the early embryo formation (Tvorogova et al. 2015). Another important protein that is involved in the cell fate and embryo patterning is the WRKY protein. This protein takes part in regulating *WOX8* and *WOX9* expression in the basal embryo lineage (Ueda et al. 2011; Zhang and Laux 2011). *WOX8/9* genes are important players in establishing cell bipolarity because they are activators of *WOX2* genes, which in turn, activate *PIN1* expression (Breuninger et al. 2008). *WOX2, WOX8* and *WOX9* genes are the main actors in embryo polarization. WOX8/9 induce the basal cell division forming the suspensor, while *WOX2* induces the apical cell division developing the embryo proper (Breuninger et al. 2008). Along with *WOX* and *PIN* genes, *LEC, YDA* and *GRD* are related to the morphogenetic network in ZE, and they also play an important role in somatic embryogenesis (Lukowitz et al. 2004; Gaj et al. 2005; Waki et al. 2011; Mursyanti et al. 2016). Abnormal embryo development can be caused by mutation or inactivation of the above-mentioned genes by epigenetic repression when 2,4-D is applied in artificial culture conditions. Several studies in somatic embryogenesis use 2,4-D as the main inducer. It is known that somatic embryogenesis can also be induced by stress treatments with other chemical or physical agents (Fehér 2015). This can be an alternative to reduce the use of 2,4-D to stimulate embryo generation. This potent auxin remains in the tissue for a long period and is difficult to remove. Abnormal embryo development can occur because of this accumulation.

Mutations in ZE can help us to understand which genes are involved in the embryo morphogenesis (Table 3). Several abnormalities displayed in *emb* mutants are similar to those produced in SE. The application of reverse genetics in somatic embryogenesis using the broad information available about *emb* mutants allows us to identify the cause of the abnormalities. Some abnormalities in *emb* mutants are produced by disruption of metabolic pathways where a protein or precursor is defective. The comprehension of this biochemical information enables us to create strategies to manipulate culture conditions for improving specific somatic embryogenesis protocols.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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