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REVIEW ARTICLE

# Mechanisms of plant somatic embryogenesis *

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**Abstract** Based on our experimental results and the related research reports, this review addresses some important issues about plant somatic embryogenesis. Once plant somatic cells develop into embryonic cells, the organelles of the cells increase, and their function is active. In the early stage of embryogenic cells, the reaction products of ATPase are mainly deposited on the plasma and vacuole's membranes, then the activity of ATPase can be found in the cytoplasm, vacuole and nucleus in the late stage of embryogenic cells. ATPase-catalyzed reaction occurs actively in the thickened wall of embryogenic cells. Endogenous hormone is a critical factor affecting somatic embryogenesis. Superoxide dismutase (SOD) activity, which is closely related to embryogenic cell differentiation, is much higher in differentiated embryogenic cells than in undifferentiated cells, indicating that embryogenic cells have stronger function of antioxidation. In addition, the lower level $H_2O_2$ can promote embryogenic cell differentiation. The programmed cell death (PCD) exists in the process of embryogenic cell differentiation and development, and the active oxygen species plays an important role in inducing of plant PCD.

**Keywords: plant somatic embryogenesis, ultrastructure, ATPase, endogenous hormone, antioxidation system, programmed cell death.**

## 1 Characteristics and significance of plant somatic embryogenesis

Sexual reproduction of plant begins with the forming of a zygote through fertilization, and then it forms an embryo called zygotic embryo through predetermination. In the late 1950s, Steward[1] discovered *in vitro* carrot root cell culture that some somatic cells were similar to the zygotic embryo in morphology and development, so he called those embryos that originate from somatic cells somatic embryos or embryoids. It is obvious that the somatic embryo formed in the cell culture differs from apomixia that is not from azygote. The formation of a somatic embryo goes through the process of embryogenesis, which is different from the differentiation of root and bud in organogenesis in tissue culture.

The regenerated plant can be formed either through the somatic embryogenesis or through organogenesis in plant cell culture. These two processes are different as shown by the following characteristics. (i) Somatic embryogenesis produces double polarity and intact structure at the early stage of the

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cells, like a seed. Its germination and conversion rates are higher than organogenesis. (ii) When a somatic embryo is formed, it is less associated with the maternal tissue or the explant's vascular bundle, a phenomenon called the physiological isolation. The somatic embryo is like a zygotic embryo and can grow into an intact plant. The cell's differentiation and totipontency expression might be easier when a somatic embryo is in a relatively independent stage. (iii) The inherited characteristics of the regenerated plant through somatic embryogenesis remain relatively stable. The variation in the genome is less than those occurring in organogenesis. This suggests that only the cells without genetic variations or the cells with less variation can form a somatic embryo and fulfill their totipotency expressions.

The plant somatic cells have a potential, like the production of a zygotic embryo in sexual reproduction. This potential can be induced under suitable cultural condition. It is a common knowledge that there exist mutual effect, mutual influence and mutual restriction among plant tissues or cells. Especially, when an angiosperm embryo is deeply rooted in the embryonary sac, collecting the materials for study becomes very difficult. Some investigators have been thinking of finding a feasible technique and setting up a suitable experimental system to overcome the difficulty in the study. The plant somatic embryogenesis system was developed for the purpose of reducing the effects of mutual restriction, and simulating the characteristics of the zygotic embryo morphogenesis. It is a useful tool to study the cell's totipotency expression, its differentiation, developing mechanism, morphogenesis and the zygotic embryo development. In addition, it is useful in making artificial seeds, improving crops, cloning elite germplasma and selecting transgenic acceptor and mutant.

Based on our research results and the published reports[2—4], we summarize in this review the developments in some very important aspects in plant somatic embryogenesis such as the ultrastructural changes, cytochemical localization of ATPase, the effects of endogenous hormone and antioxidant system, the programmed cell death (PCD) in the process of somatic embryogenesis.

## 2 Ultrastructural changes and localization of ATPase in somatic embryogenesis

In recent years, we have made a systematic study on the ultrastructure and the localization of ATPase in the processes of somatic embryogenesis and the development of various plants[5—8] by the observation of cellular embryology.

### 2.1 Ultrastructural changes

*Lycium barbarum* L. and wheat form callus through dedifferentiation. The thin-walled cells from the surface of callus change into embryogenic cells which are characterized by larger nucleus, disappearance of vacuoles, and regular spherical shape. This kind of cells has abundant organelles including plasmid, ribosome, and particularly mitochondria in division with plentiful cristae. The degree of development of cristae is an index of respiratory capacity. The more cristae, the stronger the respiratory rate, and the more energy is released. Once somatic cells become embryonic cells, a great amount of starch particles are accumulated in plasmid, providing the necessary energy for embryogenic cell differentiation and development. All these changes indicate that once the somatic cells turn into embryogenic cells, they are in active metabolism. A lot of ribosomes as well as polyribosome are also

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presented, the latter is the site of mRNA translation, so the increased polyribosome is not only a marker of protein synthesis, but also a marker of cell differentiation.

There are a lot of plasmodesmata between the early embryogenic cells and the surrounding cells. During embryonic cellular development, the cell's shape becomes longer, the nucleus inclines to one side of the cell, the cell walls are thickened, and plasmodesmata disappears. However, mitochondria increase in number, golgi body and microtubules are present in cytoplasm. It implies that (i) in the early stage of embryogenic cells, the materials and the signals have to be transferred through plasmodesmata, by which embryonic cells acquire the energy and the information for their differentiation and division, the formation of physiological isolation between embryonic cells and the other surrounding cells is a step-by-step process; (ii) the structural changes of thickened cell walls and disappearance of plasodesmada might be a prerequisite for differentiation and development of the somatic embryo, making the cell get rid of surrounding cells' control and have totipotency expression; (iii) the appearance of dictyosome is obviously associated with the cell wall's thickening, because some polysaccharide such as cellulose and pectin are synthesized in and secreted from dictyosomes; (iv) the formation of microtubules in embryogenic cells plays an important role in substance transportation.

Embryonic cells are not only with large nuclei, but also abundant in organelle such as a body of ribosome. There are a number of nucleoli in the nucleus and the nucleolus vacuoles are contained in the nucleoli. The division of the cells forms bi-cellular, then multi-cellular proembryo and a globular embryo. There are many small vacuoles in the embryo-forming cells in which the phagocytic residual bodies and protein bodies can be seen. In addition, there are plasmodesmatas between the embryo-forming cells. The embryo is wrapt by a thick wall, and a boundary is formed between the embryo and surrounding tissues or cells. It can be inferred that the cells of forming multi-cellular proembryo or globular embryo have active rRNA synthesis and the synthesized rRNA is transferred quickly into cytoplasm where the ribosome synthesis takes place. Ribosome is a place of mRNA translation, a process involved in embryogenic cell development. In cytoplasm, the vacuoles play an important role in metabolism, because the permeability and physical property of the vacuole membrane are different from plasmalemma's. The vacuoles contain many kinds of substances and the amount of those substances are several times or even tens of times more than those in cytoplasm. There are residual bodies, protein bodies and various kinds of hydrolytic enzymes in the vacuoles, the latter ones have important functions in making use of material, replacing organelle and maintaining the stability of cell's metabolism.

### 2.2 Cytochemical locatlization of ATPase in somatic embryogenesis

ATPase is a kind of membrane-bond protein widely connected with all kinds of cellular membrane systems and organelles, and maintains the concentration of all kinds of ions both inside and outside the cells. For these reason, some researchers have studied ATPase activity changes in organs or cells of different plants[9—11]. But there is no report on ATPase activity changes in the process of somatic embryogenesis. In recent years, we have studied ATPase activity and its distributive dynamics in the formation of embryogenic cells in *Triticum aestivum* L and *Lycium barbarum* L.[6]. All the evidence indicates that in the early stage of embryogenic cells, ATPase products mainly deposit on plas-

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molemma and vacuole membrane, and especially on the former. It implies that the formation of embryogenic cells in wheat and *Lycium barbarum* L. comes from the hormone induction. The exogenous hormones act on cell membrane and induce membrane protein synthesis, so ATPase activity is increased on cell membrane. It also indicates that the embryonic cells are in active metabolism. These results are in consistence with the observations of the ultrastructural studies[4—5].

In the late stage of embryogenic cells, the transfer of ATPase activity from plasmolemma to inside of the cells is observed. ATPase reaction is active in cytoplasm, vacuoles and even nuclei. With embryogenic cell wall thickening and embryogenic cells dividing, ATPase reaction products appear on the thickened cell wall, suggesting that ATPase reaction is activated in the process of embryogenesis. ATPase in nuclei takes part in chromatical assembly, which promotes the formation of nucleosome and stabilizes the space configuration of nucleosome[12].

## 3 Endogenous hormone in somatic embryogenesis

It is a prerequisite for transforming plant somatic cells into embryogenic cells that these cells must be isolated from the whole body and cultured *in vitro*. However, it is not sufficient for embryogenic cell differentiation, and some inductive factors, in particular, hormones have to be involved.

### 3.1 Metabolic kinetics of endogenous auxin and kinin

At the beginning of the culture, the cells are always lacking in auxin and kinin synthesis abilities. However, the differentiation, division and morphogenesis of these cells require a mutual response between auxin and kinin. Therefore, adding different kinds of exogenous hormones with different concentrations becomes a feasible way to induce morphogenesis for the cultured cells. But few research papers about the changes of endogenous hormone in cell differentiation and somatic embryogenesis have been published. Some experiments have proved that the metabolism and kinetic equilibrium of endogenous hormones is a crux to cell differentiation. In the cultured *Freesia refracta telatt* the somatic embryo was induced only at the basilar end of rhachis but was not induced at the upper end of rhachis. Before the culturing, there was no obvious difference of endogenous indole-3-acetic (IAA) level between basilar end and upper end of the explant. However, IAA content was much higher at the end of embryogenesis than that at the end of non-embryogenesis after 6 days of culturing, suggesting that endogenous IAA has a key function in inducing of somatic embryogenesis[13]. Same as in *Freesia refractu tellatt*, the formation of embryogenic cells in rice cell culture was caused by 2,4-D induced increased endogenous IAA content[14]. It can be inferred that the increasing of endogenous IAA content and the maintaining of it at a higher level are crucial for embryogenesis induction. It is well known that 2,4-D is necessary for inducing the formation of early embryogenic cells in carrot cell suspension culture. The mechanism might be that 2,4-D promotes the production of IAA binding protein and increases the sensitivity of the cells to IAA[15]. The contents of endogenous hormones in embryogenic callus of wheat are found to be much higher than that in non-embryogenic callus during embryogenic callus induction and differentiation, and especially for IAA content. In addition, endogenous auxin content is closely associated with the frequency of embryogenic cell differentiation[16].

It is considered that 2,4-D affects the regulating and balancing of endogenous hormone. Some

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scholars studied the absorption and metabolic dynamics of 2,4-D using $^{14}C$ labeled 2,4-D and the explant of immature embryo of the self-bred corn lines A188 and A623 to determine the effect of 2,4-D on the induced somatic embryogenesis. Their results showed that both corn lines began to absorb 2,4-D after being cultured for 24 h, but the levels of absorption were different between the two, 70% of radioactive 2,4-D was accumulated in A188, which was mainly in free form, while for A632 it was 37%, with mostly combined form of 2,4-D (with sugar and amino acid). And the ability of inducing the differentiation of embryogenic cells was also different between the two self-bred lines, A188 was more capable than A623[17]. The increasing of endogenous auxin is very crucial in establishing the rice embryogenic cell line and the high level of kinin is good for cell division and proliferation, and even for somatic embryo development[18].

### 3.2 Endogenous abscisci acid content and its metabolism

Some research results showed that abscisci acid (ABA) has an important function in plant somatic embryogenesis and development[19,20]. At the early stage of carrot somatic embryogenesis, endogenous ABA maintained at a low level in the embryo-cells forming and embryo-cells un-forming. After 10 days of culturing, ABA content reached the maximum, then dropped down[21]. In *Lycium bazbarum* L. we studied endogenous ABA content and the effect of exogenous ABA[22]. The results showed that endogenous ABA increased considerably after one day of culturing, and the first peak of ABA content appeared. At this point, a large number of embryogenic cells were formed in callus, and a 35 kD protein was synthesized which is an ABA inducer. After 15 days of the differentiation culture, endogenous ABA content reached the second peak value. At this time the globular embryo was formed and the 35 kD protein increased to the maximum as well. The results also showed that both exogenous and endogenous ABA had regulatory and promotive effects on somatic embryogenesis. The results from other studies[23] support our observation.

In plant somatic embryogenesis, ABA induces the expressions of some genes that encode storage-protein, Lea (late-embryogenesis-abound protein) and the embryogenesis-specific protein. In chickpea somatic embryogenesis, ABA treatment produced three gene products which were associated with embyo maturation[24—29]. In somatic embryogenesis of *Picea glauca*, the callus treated with ABA showed not only a higher frequency of somatic embryogenesis but also an increased protein synthesis[30]. Exogenous ABA is also a necessary substance for barley embryo germination[31]. ABA could increase DC8 mRNA level in carrot somatic embryogenesis, and fluridione, an inhibitor of ABA synthesis, could decrease ABA synthesis, which in turn influenced DC8 mRNA transcription level[32].

The ABA-regulated gene promoter sequences have several *cis*-acting elements called ABA responsive element (ABRE), and combined with some specific proteins. The study of rab16A gene promoter induced transitory gene expression using Cat as a report-gene in rice pratoplant proved that the region of nt ~290— +27 upstream of rab16A gene was a necessary sequence for ABA-induced expression[33]. They combined the sequence of nt −290— −52 upstream of rad16A gene with the promoter of CaMV35s and found that the chimeric promoter kept the ability of ABA-induced expression. In further analysis of the different gene promoter sequences, it was found that several regions of them are quite conservative. The sequence of TACGTGGY (Y is pyridine nucleotide) is the core sequence

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of ABA-induced enhancing element and it is also the binding site of *trans*-acting factors Taf-1. This sequence is homologous to 5 Lea genes in cotton, Em gene in wheat and the conserved sequence in promoter region of Win gene in rice as well as light regulation gene GT-box. In barley ABA response complex (ABRC) produced from the ABA-induced Lea gene expression also contains this conserved sequence. ABRC is composed of a 10 bp core-box containing ACGT and a 11 bp upstream sequence, and only one copy of ABRC is required for the initiation of ABA-induced effect[34]. All the above results prove that *cis*-acting element of TACGTGGC plays a critical role in the ABA-induced gene expression, and this sequence is similar to the binding sequence of TGACGTCA of cAMP response factor in mammals for it can bind to several *trans*-acting factors[35,36]. The experimental evidences showed that ABA combines firstly with the receptor at the binding site of the target cell, then the signal is transferred, the *trans*-acting factors are activated and bound to *cis*-acting factors through cellular second-messenger molecule, which regulates gene expression at transcription level[19,20]. The study of barley showed that the concentration of $Ca^{2+}$ affected ABA-induced RAB gene expression[37]. If exogenous $Ca^{2+}$ was added, the expression level of RAB's mRNA increased while the ABA content remained the same. In protoplasm of barley eleurone layer, RAB gene expression was inhibited by the $Ca^{2+}$ channel blocker. $Ca^{2+}$ inhibitors could inhibit RAB expression. It suggests that the $Ca^{2+}$/CaM system functions actively in the ABA-induced gene expression, which is indicated by the transfer of the information into the specific location, the activation of a series of proteins for signaling and the phosphorylation of these proteins, and the modification of GIP and GDP[37].

## 4 Antioxidant system and PCD in somatic embryogenesis

### 4.1 Metabolic dynamics of antioxidant system in somatic embryogenesis

In cultured plant cells, the hormone or annexation has to be added to induce the dedifferentiation and redifferentiation. However, these substances are mostly prooxidant compounds which further increase the oxidative stress to the cultured cells. As a result, more active oxygen species will be produced and the callus will turn brown and lose the differentiation ability, or even die. To solve this problem vitamin C (AsA), glutathion (GSH reduced form) and active carbon are usually added into the culture for removing of excess oxidative radicals. We found an interesting phenomenon that the embryonic callus of several plants were protected by these antioxidants from the damage of the radicals, suggesting a highly efficient antioxidant system is necessary for maintaining the normal function of the cells. We studied the metabolic dynamics of several protective enzymes in somatic embryogenesis of *Lycium barbarum* L.[38,39]. The results showed that the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) presented the inter-undulant changes in the process of somatic embryogenesis[38]. The SOD activity presented an ascending trend along with the formation of embryogenic cells. It reached to the maximum level in the multi-cellular embryos. The increased SOD activity could promote the differentiation and development of embryogenic cells, and the interreaction of SOD, $O_2^-$ and $H_2O$ determined cell differentiation[40]. Diethyldithiocarbonate (DDC) not only inhibited SOD activity, but also reduced the frequency of somatic embryogenesis. Our recent results on embryogenic cell differentiation further confirm that SOD is closely associated with the embryogenic cell differentiation and the early development processes, including the morphological changes[4,8], the accumulated polyhexose[4,41], the increase of endogenous ABA content[22], the ac-

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tivated nucleic acid and protein syntheses[42], the differential expression of the genes[43] in embryogenic cells, and the increased ATPase activity on cell membrane[6], etc. We suppose that all these changes are associated with embryogenic cell's antioxidant ability.

We found in *Lycium barbarum* L. that a certain concentration of $H_2O_2$ could promotes embryogenic cell differentiation. When the callus was cultured in the differentiation medium containing different concentrations of exogenous $H_2O_2$ the highest frequency of somatic embryogenesis was obsereved at the concentration of 200 μmmol/L for 15 days. If the concentration of $H_2O_2$ was below 200 μmol/L, its effect on the frequency of somatic embryogenesis could not be observed. A higher concentration of $H_2O_2$(300 μmmol/L) inhibited the formation of somatic embryos due to its harmful effect on the cells[39]. In addition, the content of endogenous $H_2O_2$ was much higher in the process of somatic embryogenesis than that in the callus. So we considere that (i) $H_2O_2$ increases the synthesis of lignin, which leads to the thickening of cell wall, the latter is an important indicator of the cell differentiation and development; (ii) $H_2O_2$ might be a substance for cell signaling and regulating the expression of embryogenesis-related gene through signal transduction; (iii) $H_2O_2$ probably promotes embryogenic cell differentiation through increasing $Ca^{2+}$ concentration in cytoplasm or changing $Ca^{2+}$ distribution in the cells.

### 4.2 Programmed cell death in somatic embryogenesis

In recent years, people discovered that PCD exists in plant cell development and in response to the environmental factors. However, it is not known what signals induce PCD in plant. Some researchers found that there was the accumulation of $O_2^-$ and $H_2O_2$ in the aging leaf that had PCD, indicating the active oxygen species are the triggers inducing plant PCD[44]. In fern macrospore, a single gene was expressed and the product of the gene expression caused $H_2O_2$ accumulation and induced PCD[45,46]. In hypersensitive response (HR) of tabocco, a speed-up oxidation stimulated the production of $O_2^-$ and $H_2O_2$. The NADPH oxidoreductase induced PCD through producing $O_2^-$ and $H_2O_2$, but it prevented the cells from death under the anoxic condition[47]. The expressions of CAT and POD genes can decrease intracellular content of $H_2O_2$ and prevents or reduces PCD[48,49]. PCD can be induced in plant cell culture at low cell density, while it can be reduced or inhibited by a nursing culture. This implies that plant cells can get death inhibitory molecules from the other cells[50].

We have known that the first asymmetric division of the embryogenic cells forms a small apical cell that will generate an embryo and a large basal cell that will not divide until dead. The electron microscopic images showed that the cytoplasm in dead cells was condensed and shrunk, with this morphological change, the 3'-OH group in the DNA of these cells was also accumulated, moreover, a DNA fragment of about 0.14 kb was found, indicating the cells underwent PCD[50,51].

It is possible that other cells involved in the formation and development of plant embryogenic cells undergo PCD as well. We observed in several plants[4,38] that when embryogenic cells developed to a multi-cellular embryo, the cells around embryogenic cells began to die. However, cell death is a physilogical feature of the tissues from which an apomictic embryo originates, therefore, it is also possible that PCD process is activated in plant embryogenesis.

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