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SOMATIC EMBRYO GENESIS: A MODEL OF DEVELOPMENT IN PLANT

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ABSTRACT

Somatic embryogenesis (SE) is a powerful tool for plant genetic improvement when used in combination with traditional agricultural techniques, and it is also an important technique to understand the different processes that occur during the development of plant embryogenesis. SE onset depends on a complex network of interactions among plant growth regulators, mainly auxins and cytokinins, during the proembryogenic early stages, and ethylene and gibberellic and abscisic acids later in the development of the somatic embryos. In recent years, epigenetic mechanisms have emerged as critical factors during SE. Some early reports indicate that auxins and in vitro conditions modify the levels of DNA methylation in embryogenic cells. The changes in DNA methylation patterns are associated with the regulation of several genes involved in SE, such as WUS, BBM1, LEC, and several others. In this review, we highlight the types, procedure, factors, role of epigenetic regulation and application of SE.

Keywords: Somatic embryogenesis, Epigenesis, Developmental pattern

Introduction

Somatic embryogenesis is a process whereby somatic cells (non zygotic cells) differentiate into embryos and ultimately into fertile plants (Zimmerman, 1993). Somatic embryogenesis is a multi-step regeneration process which involves transition of plant cells from differentiated state to tot potent state and is different from organogenesis. Growth regulators control the spatial and temporal expression of multiple genes in order to initiate change in the genetic program of the somatic cells, as well as the transition between embryo developmental stages (Fehér, 2015). In certain cases like *Citrus* and *Mangifera a*ny cell of the female gametophyte (embryo sac) or sporophytic tissue around the embryo sac may give rise to an embryo. Somatic embryogenesis is not reported in monocots like Gramineae but in case of dicots is very common.

Reports of somatic embryogenesis in vitro

The first observations of in vitro somatic embryogenesis were made about 50 years ago in *Daucus carota* (Reinert, 1958, 1959; Steward *et al.*, 1958). Other plants in which this phenomenon has been studied in some detail are *Citrus* sp. (Rangaswamy, 1961; Sabharwal, 1963; Rangan *et al.*, 1968; Kochba and Spiegel-Roy, 1977; Tisserat and Murashige, 1977;

Gavish *et al*., 1991, 1992).Coffee sp. (Monaco *et al*., 1977; Sondahl *et al*., 1979; Sharp *et al*., 1980; Nakamura *et al*., 1992).Guava (Madhu *et al*., 2011).*Medicago* sp. (Redenbaugh and Walker, 1990; McKersie *et al*., 1993; Rose and Nolan, 2006) *Zea mays* (Emons and Kieft, 1991; Songstad *et al*., 1992; Emons, 1994).Rice *(*Sah *et al*, 2014).*Carica papaya* (Anandan *et al*, 2012).

Table 1. SOMATIC EMBRYOGENESIS (SE) VS ZYGOTIC EMBRYOGENESIS (ZE)

SOMATIC EMBRYOGENESIS	ZYGOTIC EMBRYOGENESIS
Originates from **superficial cells of calli** or PEMs.	Developed from **germ cells.**
Non-sexual propagation.	**Sexual propagation.**
No Stimulant required for **fertilization.**	**Stimulant** required for **fertilization.**
Does not follow a fixed pattern of early segment (McWilliams *et al*., 1974).	**Early segmentation pattern of zygote is fi** (Bhojwani and Bhatnagar, 1990).
Accumulate seed-specific storage reserves and pr in **less amounts** than the zygotic embryos (Kim Janick, 1990; Stuart *et al*., 1988).	**Accumulate seed-specific storage reserves** proteins in **sufficient amounts** than the zyg embryos (Kim and Janick, 1990; Stuart 1988).
Absence endosperm or seed coat	**Presence endosperm or seed coat**
Suspensor development is variable in nature sometir found **absent** (Williams and Maheswaran, 1986).	**Suspensor development** is fixed in r sometimes it found **absent** (Williams and Maheswaran, 1
Show **double or triple vascular system** develop caused by polar transport of auxins (Chee and Cantliffe, 1989).	**Doesnot** show **double or triple vascular sy** development caused by polar transport of auxins (Che Cantliffe, 1989).
Lack a dormant phase and often show **pluricotyled**	**Presence of dormant phase** and does not **pluricotyledony.**

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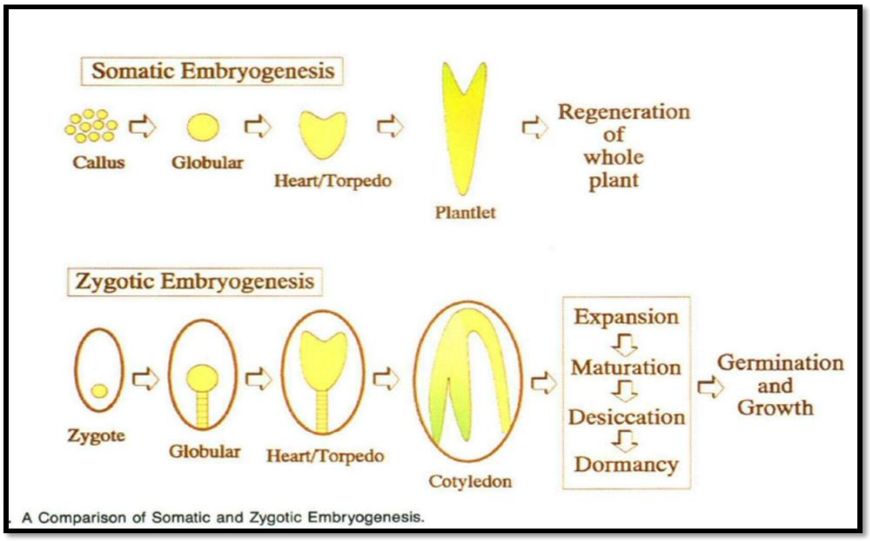

Figure 1

Table 2. DIFFERENCE BETWEEN SOMATIC AND ZYGOTIC EMBRYC	
Somatic embryo	**Sexual embryo**
Embryo arises from sinle cell	Arises from multi cell
Embryo have bipolar structure	It is monopolar sructure
Embryo has no vascular connection with cultured explant	Embryo has vascular connection with culture plant
Induction of somatic embryogenesis requires single hormonal signal	Requires two hormonal signals

Hypothesis related to somatic embryogenesis

Physiological Hypothesis

Loss of embryogenic potential can be restored by adding 1-4% activated charcoal in auxin free medium.Eg: Carrot culture

Competitive Hypothesis

Few embryogenic cells are tot potent and some are unable to express due to inhibitory effect of non embryogenic cells.

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Two types of Somatic embryogenesis (Sharp *et al.*, 1980)

Direct Somatic embryogenesis

Direct Somatic embryogenesis Cells of cultured tissues directly develop into embryos.Explants contain Pre-embryogenic determined cells(PEDC) which develop directly into embryos (Komamine *et al* ,1990).Such cells are present in embryonic tissues(Scutellum of cereals, nucellus, embryo sac etc.Eg: *Daucus carota, Brassica napus*

Indirect Somatic embryogenesis

Cells of cultured tissues first form callus then some of these cells develop into embryo.Cells of callus contain **Induced embryogenic determined cells(IEDC)** which develop into embryos.Such cells are present in secondary phloem, leaf tissues(coffee, *Petunia, Asparagus* etc.).In majority of cases embryogenesis is through indirect method. Eg: Leaf tissues of coffee.

Explants used during somatic embryogenesis.

CROP	EXPLANT	REFERENCES
Rice	Imature embryo	William and Maheshawan,1986
Wheat	Imature embryo	William and Maheshawan,1986
Conifers	Imature embryo	Bornman,1993
Alfalfa	2-3 Young fully expanded leaf	Mckersie *et al*, 1989
Soyabean	Cotyledons	Lui *et al* ,1992
Orchard grass	Two innermost leaves	Conger *et al* ,1983
Maize	Hypocotyl	Kamada and Hazada ,1979
Banana	Secondary suspensor	Kosky *et al*, 2002

Surfacesterilization

70% ethyl alcohol for 30-60s and 20%-40% Sodium hypochlorite for 15-20 min

Condition required for incubation of culture

25˚C 16hrs 1000 lux for 1-2 weeks (Immature embryo) and 25˚C 16hrs for 4-8 weeks (seed).

Induction medium

- Auxin- 2,4 D (0.5 -27.6 mM) , NAA (0.5 – 5.0 mM) (Halperin and Wetherell,1964)
- 2,4 D(50%),NAA(28%),IAA(6%) and IBA(6%)

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- Cytokinin – Kinetin (0.5 – 5.0 mM)
- BAP (57%), Kinetin (37%), ZEATIN (3%) and TZ (3%)

Embryo development medium

- Removal of Auxin (Halperin and Wetherell,1964)
- Use Gibberlic Acid if chilling treatment is not given (Takeno *et al* , 1983) required for germination of somatic embryo.
- ABA regulates desiccation and maturation phase.
- Antiauxin (7-azaindole, 2,4 ,6 - tri chlorophenoxy acetic acid and 5- hydroxy nitro benzyl bromide) promotes maturation of embryo.

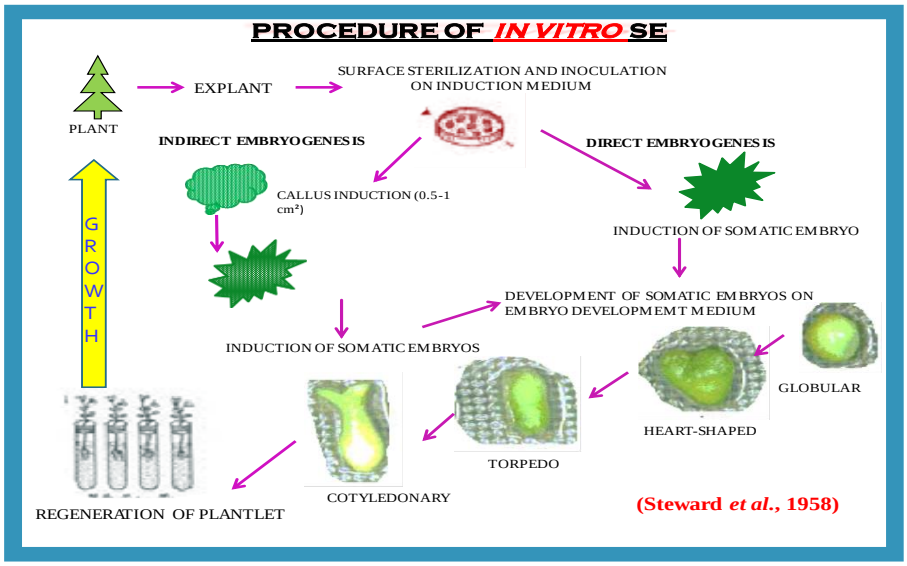

Figure 2

Hardening of regenerated plantlets

Removal of rooted plants and wash with water. Transfer of plants to small pots enclosed inside plastic container in sterile soil.Do not waters the plant and allow under sunlight for 1 hr per day. Then finally expose to natural environments and remove all the plastic containers.

Stages of somatic embryogenesis

Somatic embryogenesis is a multistep process which involves transition of plant cells from **differentiated** or **pluripotent state** to **totipotent state** and then again to differentiated state.

1. **Induction of callus-** This stage is encountered during indirect embryogenesis, involves transition of cells from differentiated state to totipotent state.

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2. **Induction of somatic embryo**-This stage involves formation of **Pre-embryogenic mass(PEM)** from division of PEDC(Direct SE) (de vries *et al* ,1988) or IEDC(Indirect SE).
3. **Embryo development**- The PEM divides to form somatic embryo.
4. **Embryo maturation**- The SE passes through various stages of development like globular, heart -shaped, torpedo- shaped and prepares for germination.
5. **Regeneration of plantlet**- The SE is germinated in vitro to give plantlet.
6. **Growth of plant**-The acclimatized plantlet is grown if natural conditions to give complete plant

Induction

Induction requires coordinated role of stress and hormones. Auxins required for induction mostly 2,4-D which established bilateral symmetry(Lui *et al* , 1993).Auxin affects electrical patterns, membrane permeability and IAA binding protein(ABP1) (Deshpande and Hall , 2000).Proembryogenic masses form from PEDCs or IEDCs.PEMs comprise embryogenic cells, which are small (400-800μm).

Development

Auxin must be removed for embryo development. Continued use of auxin inhibits embryogenesis.Stages are similar to those of zygotic embryogenesis viz globular, heart, torpedo, cotyledonary.

Developmental stage in SEs

Globular stage

It requires auxin free medium (Cooke,1987).In Indian mustard transition takes place even in presence of auxin (Lui *et al* , 1993).It takes 5-7 days to develop suspensor like structures (Halperin and Wetherell, 1964). After 2-3 days iso diametric growth is followed by oblong stage (Schiavane and Cooke, 1985).Exhibit electric polarity (Brawley *et al*, 1989).

Heart stage

Signal shifts from iso diametric to bilateral growth beginning of heart stage.

Torpedo stage

Transition is marked by outgrowth of two cotyledons

a) Elongation of hypocotyl.
b) Development of radicle.

Cotyledonary stage

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Green cotyledons can be identified after 2.5 – 3 weeks with clearly distinguished root hairs. This stage is continued in liquid medium.

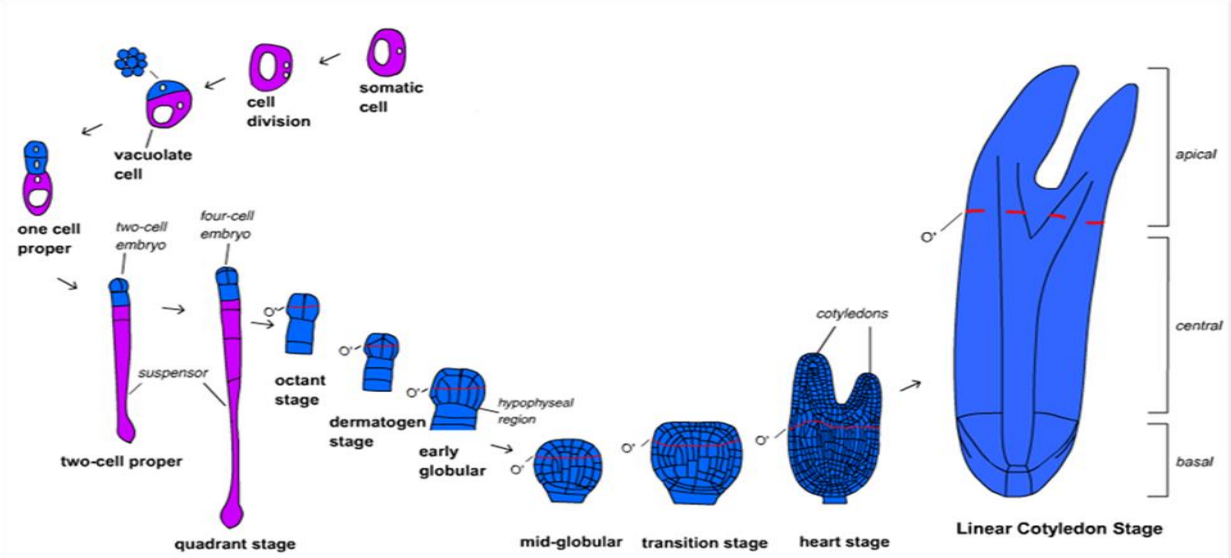
Regenerated plantlets

This stage is continued in solid medium. Transfer plantlets into sterile soil for hardening process and then grown in field under natural environments.

Maturation

The SEs normally do not go through the final phase of embryogenesis, called 'embryo maturation'.ABA, which prevents precocious germination and promotes normal development of embryos by suppression of secondary embryogenesis to promote embryo maturation in several species (Ammirato, 1983). Temperature shock, nutrient deprivation and high density inoculum as stimulate endogenous stimulate endogenous synthesis of ABA (Mc Kersie *et al.* 1990).Embryo size also remains constant

Germination



Only about 3-5% SE germinate. Sucrose (10%), mannitol (4%) may be required.

Figure 3 .Cellular pathway of somatic embryogenesis

Factors affecting somatic embryogenesis

Explant

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Immature zygotic embryo is best to raise embryogenic cultures**.** SEs increases from mesophyll cells as the distant of explant from base of leaf increase then formation of callus reduces and promotes direct SE (Conger *et al*. 1983).

Genotype of explant

Genotypic variation could be due to endogenous level of hormones (Carman,1990).Out of 500 varieties of rice tested 19 showed 65-100 % SEs , 41 showed 35-64% SEs, remaining 440 cultivars were less efficient.

ph of culture medium

At pH 4 embryogenic clumps continued to proliferate without the appearance of embryo. Embryo developed when the pH increased to 5.6.The pH of **4.86-6.86** is favourable for the development and maturation of SE while acidic **(pH 3.86)** or alkaline **(pH 7.86)** is favourable for differentiation of SEs.

Nutrient medium

Liquid medium is most frequently used as compare to solid medium. Carbohydrate used is **Sucrose** in case of monocots but Glucose, Fructose and Sorbitol also used in culture media.

Nitrogen source

Mixture of amino acids in combination of proline and serine or threonine each at 12.5mM enhances in production and quality of SE.The form of nitrogen in the medium significantly affects in vitro embryogenesis. Presence of reduced nitrogen was critical in the induction medium for carrot (Halperin and Wetherell, 1965).Meijer and Brown (1987) found an absolute requirement for ammonium during induction and differentiation of SEs in alfalfa.

Growth regulators

Hormones and stress-induce cell dedifferentiation and initiate an embryogenic program in plants with a responsive genotype (Rose and Nolan, 2006). Auxin- Auxin is a stress inducing hormone and play key role in the induction of SE and the formation of somatic embryos. Synthetic auxin necessary for the induction of somatic embryogenesis (PEMs formed on induction or proliferation medium) followed by transfer to an auxin-free(embryo development) medium for embryo differentiation. **Cytokinin**s have a mixed effect, **important for embryo maturation**. Eg.BAP and kinetin were inhibitory for embryogenesis in carrot; zeatin at a concentration of 0.1µM promoted the process (Fujimura and Komamine 1975).The relative concentrations of the two growth regulators in the induction medium determines the type of morphogenic

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differentiation after transfer to hormone-free medium**. High 2, 4-D to kinetin favours embryo/shoot differentiation the reverse ratio favours rooting. ABA and ethylene interfere with auxin. Gibberellin inhibits somatic embryogenesis** (Halperin, 1975).

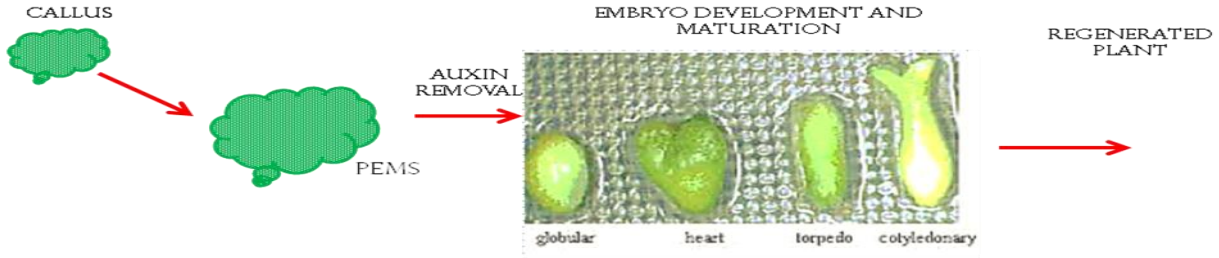

Figure 4.Diagrammatic representation of in vitro embryogenesis and role of auxin in SE

Polyamines

Putrescine, Spermidine and Spermine. Putrescine increases SE by 6 fold as compare to other polyamines.It required for embryo development in vivo and in vitro (Altman *et al,* 1990).

Oxygen concentration

Oxygen tension results in larger number of SEs were converted into a unipolar root structures (Carman, 1990). Higher oxygen concentration induces more rooting .Low oxygen level results in abnormal scutellar enlargement and supplemented by addition of ATP.

Electrical stimulation

It promotes the differentiation of organized structure by affecting cell polarity by organization of microtubules (Dijak and Simonds, 1988).Induction of asymmetric first division coupled with a short period of cell expansion and results in spherical structures.

Inoculum cell density

0.4 g /40ml of culture medium produced highest average number of SEs (Rizvi *et al.*2010) .3g/20ml of culture medium in *Lilium sps* (Ho *et al.*2006). 0.6/25ml of culture medium in Banana (Kosky *et al.*2002).

Subculture

White or pale yellow, compact and often nodular exhibits embryogenic differentiation. In cotton, yellow callus yielded embryogenic culture.

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Stress

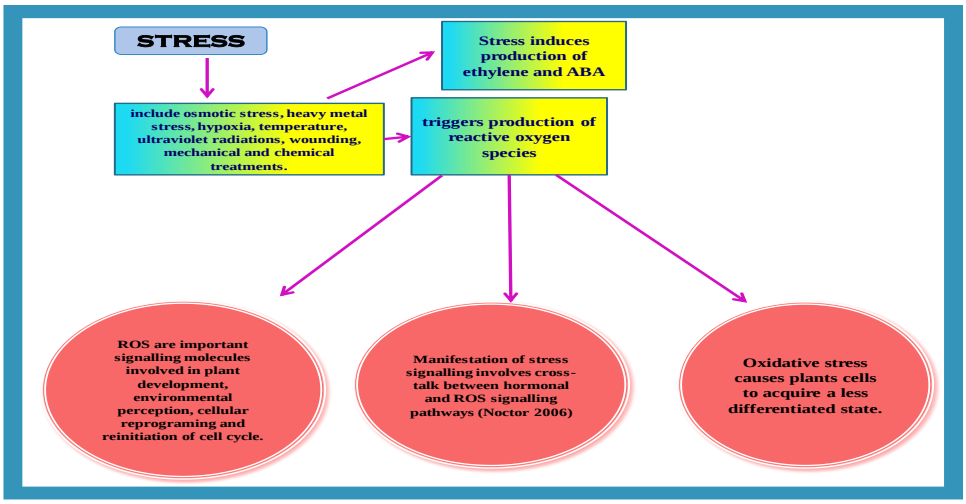

Figure 5.Effect of stress on SE

Molecular aspects (genes inducing somatic embryogenesis)

Wuschel(wus):

It is Homeotic transcription factor. These genes encode proteins called transcription factors that direct cells to form various parts of the body. Homeotic genes contain a sequence of DNA known as a homeobox, which encodes a segment of 60 amino acids within the homeotic transcription factor protein It regulates the stem cell population in the shoot meristem. It acts as both a meristem and embryo organizer (Zuo *et al.* 2002).It can be use as an early marker for totipotency/embryogenic cell fate.

Leafy cotyledon (lec):

LEC1 and LEC2 is regulator of plant stem cell fate in shoot meristem.It is expressed before maturation phase and necessary for cotyledon formation.LEC1 and LEC2 in presence of ABA induce auxin(Ogas *et al.* 1999; Rider *et al.* 2003).

Agamous-like15 (agl15):

Transcription factor associated with seed development and maturation.AGL15 promotes SE in Arabidopsis (Harding *et al.* 2003).

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Somatic embryogenesis receptor kinase(serk):

The SERK promoter contains ethylene and auxin response elements and binding site for WUS.The expression of SERK is dependent on not only ethylene but also auxin and cytokinin. SERK has been shown to express in single cells that developed into somatic embryos and, in carrot, it acted as a marker of cells competent for SE (Schmidt *et al.* 1997).

Baby boom gene (bbm)

It is isolated from *Brassica napus.*promotes organogenesis and embryogenesis in absence of exogenous auxin. It stimulates the production of growth regulators for ED medium.

Epigenetic influence on somatic embryogenesis

Epigenesis is stable heritable changes in gene function that do not involve changes in the DNA sequence. Examples of mechanisms that produce such changes are DNA methylation and histone modification, each of which alters how genes are expressed without altering the underlying DNA sequence.

DNA methylation:

DNA methylation is carried out by the addition of a methyl group attached to 5^{th} position of the pyrimidine ring of cytosine in the DNA (5mC). In animals, this methylation occurs in a cytosine that is adjacent to aguanine (CpG) (Vanyushin,1984).However, methylation in plants is not always in the CpG islands (Gruenbaum etal.,1981; Belanger and Hepburn,1990); it can also be done in CpHpG and CpHpHp (where H is any nucleotide except G; Finnegan etal.,1998; Feng etal.,2010).High methylation levels are associated with high cellular proliferation(Wang *et al*., 2012).Loss of methylation pattern of WUS leads to enhanced shoot regeneration in *mrt*1 mutant.DNA methylation decreased on removal of auxin followed by over expression of WUS suggesting that methylation modulates auxin signalling within callus. DNA methylation decreases to 53.4% after 8 weeks of maturation (Teyssier et al., 2014). The treatment of embryogenic lines with a variety of auxin/cytokinin ratios before placement onto a maturation medium containing 40 µM ABA changes the methylation of DNA in the original embryogenic line.The decrease of 2,4- D concentration or its exclusion causes a reduction in the methylation and improves the maturation of somatic embryos in the presence of ABA (Levanicetal.,2009).

Histone acetylation:

A histone modification is a covalent post-translational modification (PTM) to histone proteins which includes methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation. The

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PTMs made to histones can impact gene expression by altering chromatin structure or recruiting histone modifiers Acetylation associated with release of HETEROCHROMATIN PROTEIN1 (HP1) from chromatin during dedifferentiation of tobacco leaf derived protoplast. Transcription ally active euchromatic regions are often associated with hyper acetylated histones, while silent heterochromatic regions associate with hypo acetylated forms (Grunstein 1997). Besides acetylation, the histone H3 N terminus is also phosphorylated during different cellular processes. Phosphorylation of serine 10 in histone H3 (H3-S10) is required for proper chromosome condensation and segregation (Wei et al. 1999). The phosphorylation of H3-S10 also correlates with transcriptional activation of immediate-early genes upon mitogen stimulation (Mahadevan et al. 1991). In addition to acetylation and phosphorylation, methylation of histone tails at different residues has been implicated in transcriptional regulation (Jenuwein and Allis 2001; Zhang and Reinberg 2001). It has been shown that the histone methyltransferase (HMTase) responsible for methylation of Arg 2, Arg 17, and Arg 26 in H3 (Ma et al. 2001; Xu et al. 2001) and Arg 3 in H4 (Strahl et al. 2001; Wang et al. 2001b) plays an important role in the transcriptional activation of certain genes. Methylation of Lys 4 in H3 (H3-K4) localizes to heterochromatin boundaries and transcriptionally active loci (Litt et al. 2001; Noma et al. 2001). Pericentric heterochromatin contains enriched HP1 proteins from specific interaction between methylated H3-K9 and the chromodomain of HP1 (Bannister et al. 2001; Lachner et al. 2001).

Applications of somatic embryogenesis

i. It is a means of micro propagation to produce large no of plants (Zimmerman, 1993).

ii. Direct somatic embryogenesis offers several advantages in crop improvement; cost-effectiveness and large-scale clonal propagation is possible using bioreactors (Vasil, 1987; Philips and Gamborg, 2005).

iii. It is used for development of synthetic seeds. It is also a method commonly used in large scale production of plants and synthetic seeds (Philips and Gamborg, 2005).

iv. It is used to study developmental pattern of embryo and study the cellular and molecular events occurring during embryogenesis.

v. Embryo production from non-zygotic cells in anther and isolated microspore culture in the production of doubled haploids is important in breeding programs (Hosp et al., 2007).

vi. It produces virus free plants. Eg. Citrus trees propagated from nucellar embryos are free of viruses.

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Conclusion

Somatic embryogenesis is a highly regulated process involving many genes that determine embryo identity. It is a very efficient technique of micro propagation in which somatic cells undergo a very complex series of events to form embryos resembling those formed from zygote. Stress and hormones are the two most important factors which help in cellular (metabolic and genetic) reprogramming to form somatic embryos. It occurs via two pathways: direct and indirect which occur in various stages of development. Auxins play a very critical role in induction of embryogenesis while other hormones are particularly required for embryo maturation. It is a promising field of study from academic view point (to know similarity in various developing stages of immature zygotic embryo) as well as commercial aspect (i.e.; production of synthetic seed).

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