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Chapter 1

Exploring the molecular mechanism behind somatic embryogenesis-a multistep event of cellular reprogramming

Kumari Anjani[1*], Harshit Soni[1] and Harsh Kumar[1]

[1]*Department of Agricultural Biotechnology and Molecular Biology Dr. Rajendra Prasad Central Agricultural University, Pusa-848125, Bihar, India.*

Abstract

Somatic embryogenesis is an important technique of plant tissue culture through which somatic cells deviate from normal developmental pathway and undergo an embryogenic pathway to differentiate into embryo and ultimately fertile plant. It is a process involving complete cellular reprogramming and is an excellent means of understanding the morpho-molecular regulation of plant embryogenesis. SE involves progression of somatic cells from differentiated / pluripotent state to totipotent state and then back to differentiated state. An insight into this process suggests that it is a very complex process involving many stages of development which are controlled by different factors. Most of these factors help somatic cells to gain embryogenic competence and follow embryogenic developmental pathway. A great number of genes and transcription factors (TF) plays part in this event of cellular re-programming, most of which are involved in development pathways. Some of these are MADS box gene, WUSCHEL, LEC, LEA and others. The vital role of these factors in somatic embryogenesis have been described in this article.

Keywords: Somatic embryogenesis, WUSHEL, LEC, AGL15, Transcription factors.

1. Introduction

Somatic embryogenesis (SE), an event of cellular reprogramming, is one of the most studied processes of development in plants. SE is a series of events of differentiation of somatic cells into embryos that may develop into fertile plants, via a series of stages, which resemble developmental stages of zygotic embryogenesis [1, 2]. It is a process where either a cell or a group of cells obtained from somatic tissue forms an embryo without fertilization. Somatic embryos have closed radicular ends with shoot and apical meristem and their development is similar to zygotic embryo. That is why somatic embryogenesis has been used as a paradigm to study morpho-molecular and regulatory mechanism of embryo development in many plants. [3] SE, as an important technique of biotechnology has many applications like micropropagation, to produce large number of plants; main method of automated production of plants in bioreactors on commercial scale and major method of artificial seeds production [4]. It is an essential component of majority of plant transformation protocols as well as has potential to improve plants by producing somaclonal variants. In future, cloning of desirable/ideal genotypes using SE can be advantageous in profitable plants of importance for example in forestry [5], horticulture [6], and other important vegetatively propagated plants.

SE is regarded as an in vitro phenomenon, but it is not confined to laboratory. The non-zygotic embryos have been found to develop from cells of female gametophyte (i.e. embryo sac) or sporophytic tissue surrounding the embryo sac in nature. As like, somatic embryos present on succulent leaves of Kalanchô have been discovered, as well as adventive embryos formed from nucellar cells in numerous Citrus and Mangifera species. Many plant species that are evolutionarily distinct exhibit apomixis, the process by which embryos form from unfertilised ovule walls [7]. Thus, depending on the source, non -zygotic embryos have been termed as adventive embryos for embryos being formed directly from other organs or embryos, parthogenetic embryo for embryo formed from unfertilized egg and androgenic embryo for the embryo formed from male gametophyte. However, the term somatic embryo is generally used for the embryo developed from somatic tissue in culture.

Somatic embryos are bipolar structures possessing many similarities with zygotic embryos [8] but there are many differences between the two. SE is a process of non-sexual propagation where somatic cells develop into embryos under environments which are too different from those which are experienced by zygotic embryos [9]. A fixed pattern of early segmentation similar to zygotic embryogenesis is not observed

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in SE. The stimulus for zygotic embryogenesis is fertilization, while SE is stimulated by hormones, stress and many other factors. The accumulation of less amount of storage proteins and other reserves is observed in somatic embryo [10, 11]. The suspensor may or may not develop in SE thereby showing different hypophysis – root pole than zygotic embryogenesis. Since somatic embryos develop independent of the influence of maternal tissue, they are less organized and comparatively larger containing a greater number of cells than zygotic embryos [12]. Somatic embryos show multi-vascular system development due to polar transport of auxins. The failure of the development of root or shoot apical meristem is observed in SE [13] occurs in SE. Somatic embryos directly develop into plants encompassing the stages of dormancy and show secondary embryogenesis and pluricotyledony.

Somatic embryogenesis can be recorded back to about 58 years ago when Steward et al. obtained somatic embryos from carrot's storage root phloem in 1958. Because of this many research articles have been published covering various aspects of somatic embryogenesis in many varieties of plant species such as *Zea mays* (Emons, 1994); *Macleaya cordata* [14]; *Podophyllum hexandrum*; *Medicago* sp. [15]; *Citrus* sp.; *Carica papaya*; *Cyclamen* [16]; Coffee sp, [17] and many others. These works have helped in furthermore understanding of the process.

Somatic embryogenesis can occur through either a direct or an indirect pathway. In case of the direct SE process, somatic embryos originate from explants containing pre-embryogenic determined cells (PEDC) without undergoing an intermediate callus formation stage. In indirect SE, the explant forms a callus in which some cells form induced embryogenic determined cells (IEDC) which ultimately form somatic embryo. Indirect SE is more common than direct SE [18]. Whatever be the pathway, the SE is a very complex process involving complete cellular reprogramming. It is a complex process in which cell transforms from differentiated to totipotent state and later again gains specialization. It occurs in four stages: induction stage in which PEDC or IEDC form pre-embryogenic mass (PEM); development in which PEM forms somatic embryo; maturation in which the embryogenic development pathway is followed and finally germination in which mature somatic embryo germinates to form plantlet Figure 1.

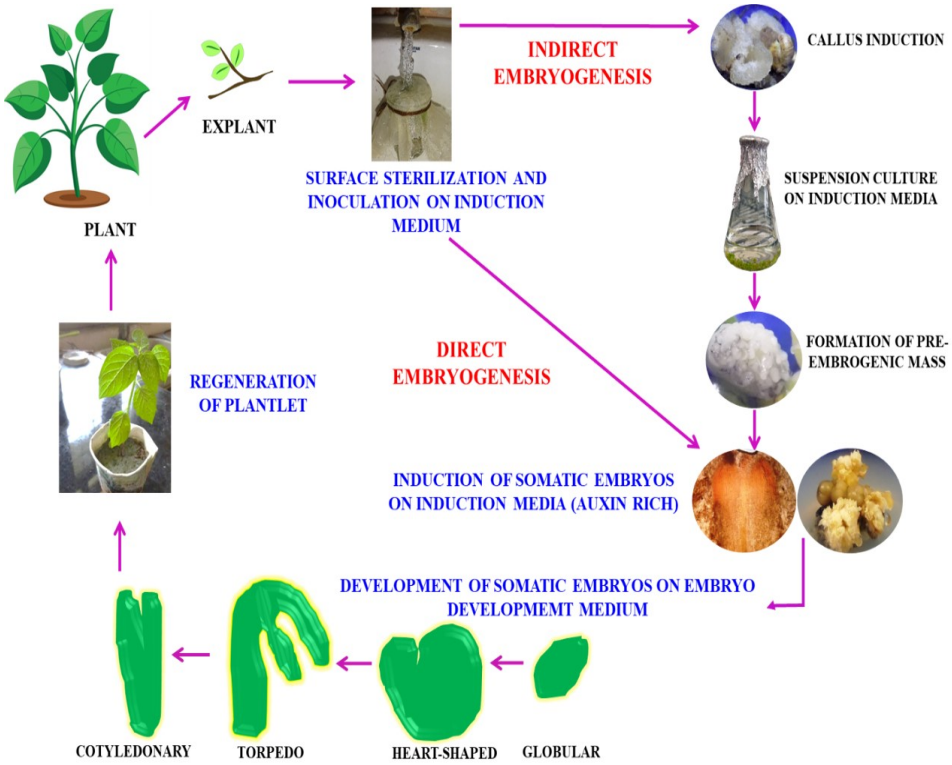

Figure 1: Stages of Direct and Indirect Somatic Embryogenesis

Induction

Somatic embryogenesis refers to the expression of plant cell totipotency which requires a change in gene expression and complete remodeling of cellular functions. To induce SE, gene expression pattern existing for vegetative growth in the explant should be diverted to embryogenic pathway to form competent cells [19]. Such competent cells which are destined to make somatic embryos are termed as embryogenic determined cells (i.e. PEDC in direct SE and IEDC in indirect SE). These cells through the combined role of a large number of factors are induced to divide and form pre-embryogenic masses (PEM). PEM comprises of small (400-800 μm) embryogenic cells which further develop to form somatic embryos. To induce SE the developmental pattern of the cells have to be changed from differentiated/pluripotent state to totipotent state. This transition is brought about mainly by stress and hormones and a combined role of a large no. of various different factors. The genotype, donor plant's physiological state and choice of the explant are major factors in SE Figure 2.

Development

The embryogenic pathway's initiation leads to formation of PEM that develops to form globular somatic embryo, the first stage of embryo development. The somatic embryo then develops in a manner which is very similar in nature to zygotic embryo passing through stages - globular, heart, torpedo and cotyledonary stages. Development of somatic embryo shows more or less the same pattern, but some variations

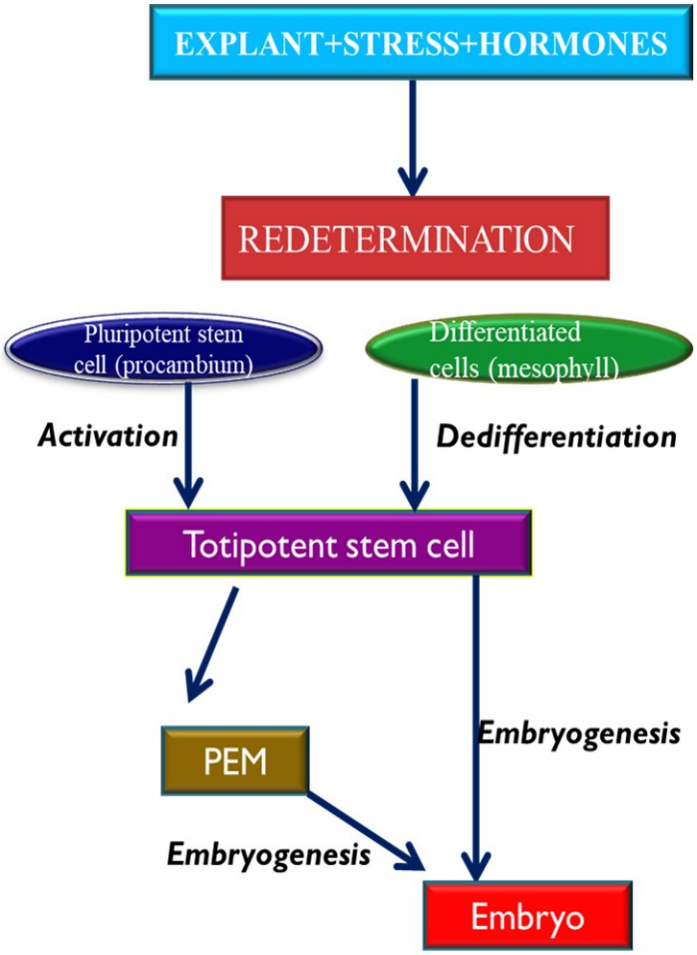

Figure 2: Steps of cellular reprogramming for induction of somatic embryogenesis

are also seen. These variations arise mainly due to difference in constitution of the medium used in the development of somatic embryos, which result in morphological changes. Based on the size and shape, embryos have been described. In *Quercus suber* L. for instance, five types of somatic embryos were identified as E1, E2, E3, E4 and E5. Immature cotyledonary translucent embryos, 3–4 mm in size were described as E1, E2 type were 6–7 mm log with white opaque appearance without secondary embryogenesis and E3 had a size of 1–2 cm with bigger cotyledons and white appearance with yellow embryonic axis. E4 type had were embryos with big cotyledons, while E5 type were embryos with roots and seed [20].

Maturation

The maturation of developed zygotic embryo is an essential prerequisite for its conversion into plant. However, for both somatic and zygotic embryos, maturation is a stage where embryo prepares for germination by accumulating proteins, carbohydrates and other reserves. Simultaneously, embryos show dehydration and reduced respiration [21]. Etienne [22] stated, "maturation is a transitory, frequently indispensable stage between embryo development and embryo germination phases". Failure of proper maturation phase leads to premature germination and reduced number of viable plants. The SEs normally do not go through 'embryo maturation' but directly germinate to form complete plants [23]. Whenever somatic embryos need maturation, they require certain optimal conditions controlled by a variety of factors major being ABA and the culture medium. It is observed that growth inhibitors promote maturation by countering the effects of growth promoters [11].

Germination

Unlike zygotic embryos which are found to desiccate after maturation phase and normally exhibit a dormant stage before germination, somatic embryo neither desiccate nor become dormant but directly develop into plantlets. Like other phases of SE, this stage also depends on many factors. The composition of medium and hormone present play the most crucial role but no generalization can be drawn since a large number of combinations have been found to be useful. Sucrose (10%), mannitol (4%) and activated charcoal inside the medium promotes somatic embryo development. Among hormones ABA is found to promote germination of somatic embryo as in case of *P. hexandrum*. Conversely, the MS medium having IAA produced the best result for plantlet regeneration and BAP in sugarcane [24]. Similarly, the mature somatic embryos of Indian chicory were converted into plantlets in medium supplemented with IBA (indole butyric acid), IAA, CH and kinetin [25]. However, growth regulators may not be required for maturation as in *C. orchiodies* [26]. To optimize maturation of the somatic embryo and counter the adverse effect of hyperhydricity, the embryo may be alternatively cultured on liquid and solid media. The incorporation of osmotic agents like polyethylene glycol in the medium (Al-Khateeb; 2006).

Genes and Transcription Factors Playing Key Role In Gaining Embryogenic Potential

Somatic embryogenesis is an event of complete cellular reprogramming involving simultaneous activation and suppression of many genes leading to transition of cells or tissues from vegetative to embryogenic pathway. Various studies involving identification of genes with altered function during SE have revealed that this process is under the control of many genes involved in gain of embryogenic competence by the non-zygotic cells [27]. The genetic transformation experiments have also helped in identifying the function and mechanism of the genes involved. For most of these studies carrot and *Arabidopsis* have served as a model but analogous genes have also been identified in cotton, Vitis vinifera and other plants. Most of these genes are transcription factors (TF) and signaling molecules involved in crucial developmental pathways promoting SE when ectopically expressed [28]. The genes involved in SE can be grouped as embryo-specific genes, embryo development genes and cell division genes. The hormones play a key role in activation and regulation of these genes Figure 3. Some of them like SERK, WUSCHEL, LEC and AGL15 are described here.

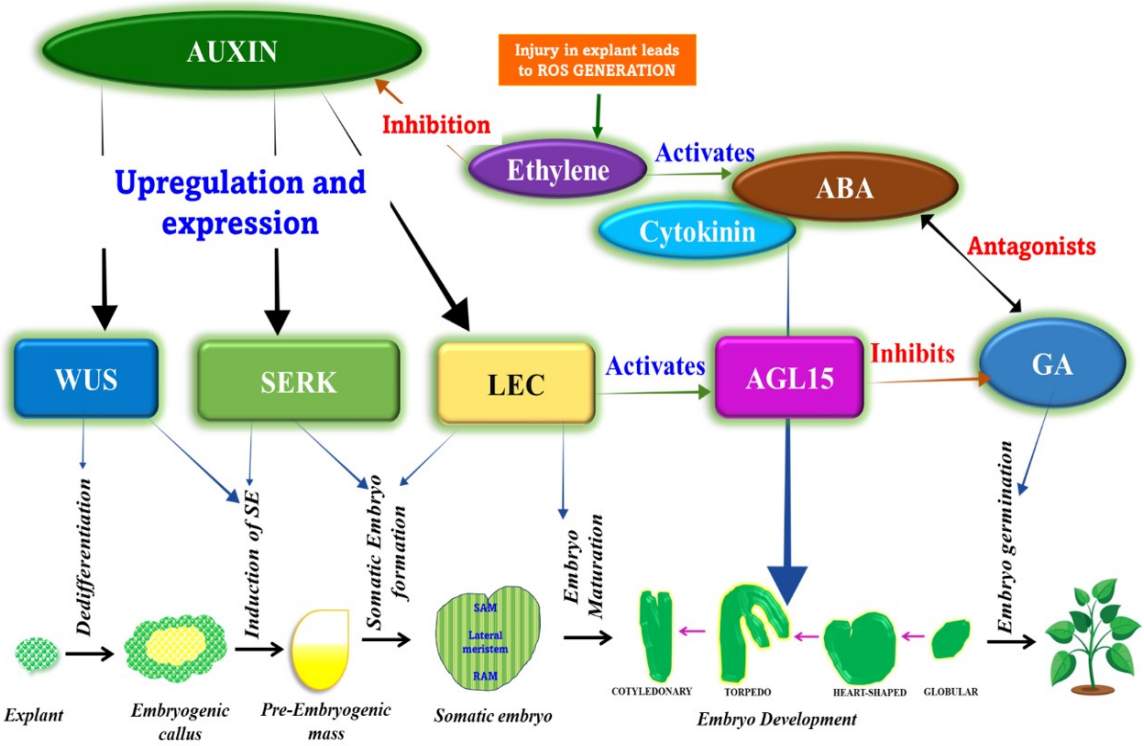

Figure 3: Hormone–Gene Interaction Network in Somatic Embryogenesis

SERK genes

Somatic embryogenesis receptor kinases (SERK) are transmembrane receptor kinases having leucine rich repeats, which were first identified for carrot [1, 29]. They play vital role for cell communication to other cells and environment while plant development [29]. It has been found to express during initial stages of zygotic and somatic embryogenesis, revealing the similarity in these two processes. SERK was identified as a marker for determining cell competency for SE in carrot [1]. Homologs of carrot *SERK* genes have been identified in various plant species, including Potato [30], *Japanese mandarin*, *Cacao* [31], *Rice* [32], *Sunflower* [33], *Barrel medic* (MtSERK1) [34] and *Arabidopsis* (SERK1). Five homologs, namely SERK1 to SERK5, were described in *Arabidopsis*. SERK1 was found to express in gametophyte and embryo upto heart stage [35]. It has been also found to express in vascular bundles [36]. Over expression of SERK1 was associated for promoting gradual change of somatic cells into embryogenic state and initiation of SE in *Arabidopsis*. Further, it has been found to enhance somatic embryo development from the shoot apical region of *Arabidopsis* seedlings germinated in liquid culture medium. Auxin treatment was found to induce this gene's expression Figure 3. Thus, auxin as inducer of SE seems to act through SERK1 gene in *Arabidopsis*. MtSERK1 was also found to be induced by auxin and determines embryogenic competence [34]. In maize and *Medicago truncatula* expression of SERK gene is found to be tightly correlated with SE [37]. Besides SE, SERKs are also found to be associated with brassinosteroid signaling pathways and have an important role in various pathways in plants such as controlling cell death, development of anther and modulation of multiple plant innate immunity responses [29].

Wuschel

The homeodomain transcription factor (in the shoot meristem) encoded by WUSCHEL (WUS) regulates the number of stem cells [38, 39]. It was initially discovered to be a function-losing mutant in *Arabidopsis* which resulted in zygotic embryos and adult plants developing shoot and floral meristems less effectively [40]. The abnormal expression of WUS during embryogenesis leads to formation of defective shoot apical meristem, central zone is occupied by large, vacuolated cells [38, 41]. WUS at first expressed during the heart stage of zygotic embryo in organizing center of shoot meristem and helps in formation of organs for the entire plant life cycle by regulating the stem cell pool.

The spatial presence of WUS gene is confirmed by the work of [42], who observed that the WUS reporter expression was rarely found in the outermost layer of cells. WUS produces TF which act on numerous targets to determine stem cell identity. WUS act as transcriptional activator of CLAVATA (CLV) genes, AGAMOUS (AGA) gene, TOPLESS (TPL) gene and transcriptional repressor of cytokinin-signaling genes [43, 44]. While the CLV gene is involved in feedback loop with WUS, the transcription factor TPL inhibits root forming genes by suppressing auxin signaling [44, 45] Figure 4. The AGA gene is involved in formation of floral meristem.

The protein produced by WUS migrates to the overlying cells of the central zone of shoot meristem and is involved in determination of stem cell fate during the entire life cycle of the plant [46, 47] Figure 4. According to Bhalla and Singh [48], a self-regulatory feedback loop involving WUS and CLV genes sustains the stem cell population in this zone. WUS overexpression in shoot meristem leads to increase in number of stem cells, that activates CLV3, which negatively regulates WUS expression. CLV3 through CLV1/CLV2 receptors reduces WUS expression and decrease the number of stem cells [39–41]. The suppression of SAM production in roots is also mediated by CLV3, as the expression of CLV3 in neighboring cells is stimulated by the misexpression of WUS in roots [38, 46]. Thus, WUS appears in promoting the formation of shoot apical meristem (SAM) but repress the root meristem in stems [42] Figure 4.

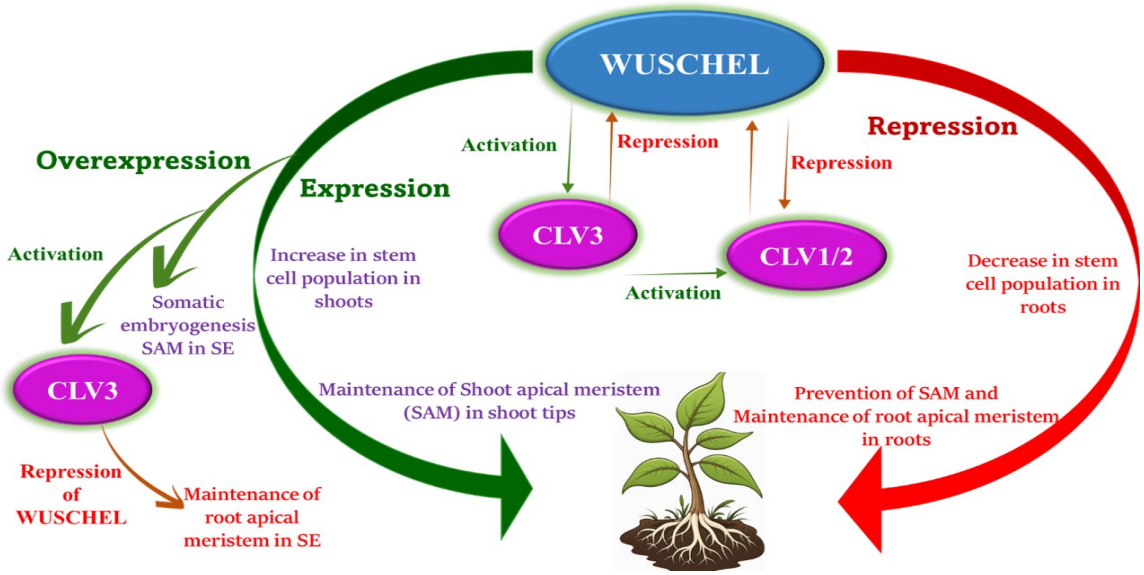

Figure 4: Role of WUSCHEL in maintaining stem cell identity through WUS–CLAVATA (CLV) Feedback Loop during somatic embryogenesis

WUS is mainly associated for maintenance of stem cell identity in the SAM but its ectopic expression regulates various developmental pathways in many plants Figure 4. WUS has the ability to act as transcriptional activator (AGAMOUS) and repressor (cytokinin response gene), suggesting that its role is modified based on process of development [43, 49, 50]. In somatic cells, it can cause dedifferentiation and formation of new stem cells leading to organogenesis or somatic embryogenesis [51] Figure 3. In *Arabidopsis* it is found to cause caulogenesis from callus [46]. Over-expression of WUS in *Arabidopsis* formed embryo-like structures and embryogenic callus without any external hormones. This homeobox gene shows critical role during transition from vegetative cells to embryonic cells which leads to form the somatic embryos [39, 40]. Other homeobox genes which are found to be associated with somatic embryogenesis include plant Growth Activator 6 (PGA6) and WUSCHEL homeobox 2 (WOX2) in *Arabidopsis* and pine [40, 52]. While PGA6 can induce SE without external hormones, WOX2 shows high expression in cold induced embryogenic tissues in pine. It is used as a marker to determine embryogenic potential of pine, spruce and Brassica cultivars [51, 53].

WUS is the only regulatory protein that controls both meristematic and embryogenic stem cell [39]. The ectopic expression of WUS caused shoot formation in roots at low auxin and SE in presence of high auxin in *Arabidopsis* [38]. The function of WUS was also dependent on tissue and level of expression. WUS over-expression in highly differentiated cells caused callogenesis while low expression lead to SE in a few cells. The out-of-place expression of WUS causes abnormal growth for most of less differentiated cells in somatic embryos. This may be because of reorganization in heterochromatin and autonomous replicating factors (ARFs) by WUS which leads to abnormal growth [40, 54]. WUS thus, seem to be involved in SE by inducing embryogenic transition and maintenance of embryogenic identity [40]. WUS seems auxin responsive and promotes SE in presence of auxin in *Arabidopsis* and *Antirrhinum majus* [38, 40]. However, AtWUS gene was capable of producing embryogenic lines in cotton without any external hormones. These lines however differed in development from *Arabidopsis* somatic embryos. The constitutive expression of AtWUS resulted in abnormal embryo-like structure while over expression resulted in leaf-like structures [55]. In *Medicago truncatula*, MtWUS caused both callogenesis and somatic embryo induction. However, unlike AtWUS, the expression of MtWUS gene showed dependance on the hormone present in the medium. Auxin alone was not enough to induce expression of MtWUS, but did require the presence of cytokinin inside medium. It was also observed that cytokinin induced MtWUS expression was not itself capable of inducing SE in certain wild type species such as Jemalong. Also, MtWUS expressed in the entire somatic embryo instead of localized expression as observed in *Arabidopsis* [56]. WUS was also involved in SE of *Coffea canephora* through the same regulatory pathway as *Arabidopsis*. But unlike *Arabidopsis*, WUS was not necessary for induction of SE and only affected the number of somatic embryo [43].

Leafy cotyledon

LEAFY COTYLEDON (LEC) genes are a group of embryo specific genes which play a key role in determining embryo identity. These genes are TF which belong to a regulatory network that control smooth initiation and progress of zygotic embryogenesis by expressing in early

stages. They control events like establishing embryogenic competence in zygote, seed maturation, maintenance of cotyledon and suspensor identity, inhibition of germination and others [39, 57, 58] Figure 4. LEC genes were originally identified in *Arabidopsis* (AsLEC) where their loss of expression led to loss of embryo identity and maturation defects. The expression of these genes resulted in somatic embryogenesis in many plants such as *Arabidopsis*, *Medicago truncatula*, maize, carrot [59–61]. Ectopic LEC gene expression alters cellular hormone balance that favours SE from meristematic type cells or tissues. In *Arabidopsis* ectopic overexpression of LEC genes in seedlings formed embryogenic calli and somatic embryos on surface of cotyledons [58]. The LEC genes were found to be essential for SE in *Arabidopsis*. It was observed that the LEC mutants showed a substantial loss in embryogenic competence and inability for direct SE [37]. Though LEC genes played a vital role for both in vivo and in vitro embryogenesis, there is a contradiction in their role in the two events. While AsLEC genes are essential for initiation of SE, they aren't required for zygotic embryogenesis till heart stage. This difference may be due to different hormonal environment during the two processes [39]. LEC genes are categorized into two distinct groups. The first group consists of genes that encode Heme-Activated Protein 3 (HAP3)-related TF, with LEC1 in *Arabidopsis* being a representative member [62]. The second group includes genes that encode B3 domain TF, such as LEC2, FUS3 and ABI3 in *Arabidopsis* and Viviparous1 (VP1) in maize. It has been demonstrated that both gene classes are critical for somatic embryogenesis in addition to being necessary for zygotic embryogenesis [8, 63].

LEC1 gene of *Arabidopsis thaliana*codes for a protein associated to HAP3 subunit of CCAAT box-Binding LRR-RLKs factor (CBF). It is known to be vital in both early and late zygotic embryogenesis, also performs all the function of LEC genes. The out-of-place expression of LEC1 helps in the development of embryo [64]. Mutation in LEC1 results in defective embryo in which cotyledons are converted to leaf-like structures, hence the name of the gene [65]. Its constitutive overexpression forms somatic embryo-like structures. However, LEC1 overexpression is lethal and occurs prior to germination, after which it is controlled by XVE-inducible system. Also, the expression of LEC1 was localized to embryos and seeds and expressed in presence of embryo or seed specific co-factors [66]. The overexpression of WUS represses LEC1 [40]. LEC1 is also responses to auxin concentration [67] Figure 3. This gene controls the protein accumulation in the seeds through FUS3 and ABI3 genes along with LEC2 [68]. Through the ABA-response element (ABRE) binding factor, LEC1 may also activate the enzyme SUCROSE SYNTHASE 2 (SUS2) and the seed storage protein CRUCIFERIN C (CRC) [69]. It is found to be essential for induction of SE. Its ectopic post-embryonic expression in vegetative cells of *Arabidopsis* causes SE induction. Its overexpression has been observed while somatic embryogenesis in various plant species, like Grapes [70], Alfalfa [71], Sunflower [72], Cacao [57], Daucus carota [73] and *Zea mays* [59]. Additionally, it was reported that, induction of somatic embryogenesis is hampered by loss of function mutation in this gene [63]. LEC1 is needed during induction of somatic embryogenic pathway and maturation but not for zygotic embryogenesis Figure 3. LEC1 is also not involved in the post embryogenic regulation. This is evident by the presence of repressive H3K27me3 mark in LEC1 which is enriched during early stages of zygotic embryogenesis but decrease during transition from heart to torpedo stages of development [64]. The LEC genes link maturation of zygotic embryo and initiation of somatic embryo by inducing favorable conditions for cellular differentiation [74]. However, the insight into the expression pattern of LEC1 gene in embryogenic culture of *Arabidopsis* suggested the role of this gene in differentiation and development instead of induction pathway of SE (Ledwon and Gaj, 2011). The homologue of LEC1 gene and other genes with HAP3 subunit has been found in many other plants including *Medicago truncatula*, carrot, *Isoetes sinensis*, *Brassica napus*, *Pistacia chinensis*, maize, rice, *Phaseolus coccineus* (LEC1-LIKE), *Helianthus annuus* (lec1L) and *Bixa orellana* (LEC1) [37, 62]. LEC1-LIKE (LIL) genes were also found in *Arabidopsis*. They are required for normal embryo development along with LEC1 and can complement LEC1 functions on ectopic expression [62]. However, LIL and LEC1 mutants have different phenotypes indicating different role of these genes in embryogenesis.

LEC2, belonging to the 2nd class of LEC genes, codes for a DNA binding B3 domain TF. These TF are necessary for normal seed development [60]. In *Arabidopsis*, LEC2 assumes central role for developing embryo and seed via inducing expression of seed-specific genes, controlling oil and protein metabolism and regulating carbon partitioning to storage compounds in growing seeds [74, 75]. The loss of function mutations in this gene during initial stages of zygote development leads to tragic results like impairment in embryonic cell fate, formation of leafy cotyledons and defective suspensor formation. The mutations in later stages results in cotyledon tips devoid in storage reserves, desiccation tolerance leading to the formation of desiccation intolerant seeds [37, 76]. The ectopic overexpression of this gene causes SE, callogenesis, formation of leaf like cotyledons in *Arabidopsis*. This phenotype is similar to WUS mutants suggesting functional similarity between the two [40, 60]. During SE, LEC2 is believed to cause reprogramming of somatic cells to divert them to embryogenic pathway since it triggers spontaneous somatic embryo formation Figure 5. While if we see another side, a mutation in this gene limits in vitro embryogenic response of explants. This is due to the fact that LEC2 controls the response of the explants to different plant hormones. For instance, it controls both endogeneous level of auxin and auxin response by controlling YUCCA4 (YUC4), IAA INDUCIBLE30 (IAA30) and YUCCA2 (YUC2) genes [76]. LEC2 controls GA biosynthesis through regulation of AtGA3ox2 [61] Figure 5. Also, LEC2 directly induce expression of MADS transcription factor AGAMOUS LIKE 15 (AGL15), which promotes SE by regulating levels of ABA and GA [61, 77] Figure 3. LEC2 is expressed mainly during early stages of embryogenesis. In post-embryogenic tissue, its expression is controlled by PICKLE (PKL), a chromatin remodeler [78, 79]. PKL mutants can form somatic embryos due to their ability to over express LEC2 gene [78].

Some other LEC genes also play important roles in SE apart from LEC1 and LEC2. For instance, FUS3, a AFLB3 subfamily gene, controls transition of vegetative to embryogenic phase during zygotic embryogenesis and has function similar to other LEC genes in controlling embryo maturation [68]. It also shows expression similar to LEC1 and it's expression is during early stages of embryo development also it decreases during germination [80]. However, its expression is constant and not auxin dependent. But, since LEC1 and LEC2 control the level of FUS3, upregulation of FUS3 level is observed in some cultured zygotic embryos in response to auxin particularly due to increase [60, 68]. FUS3 commands SE in decreasing GA accumulation by down-regulating GA-biosynthesis genes, GA3ox1 and GA3ox2 and up- regulating ABA accumulation [60, 81] Figure 5. The activity of FUS3 is not limited to a particular stage of SE (Ledwon and Gaj, 2011). The mutual action of LEC1 and LEC2 to control zygotic and somatic embryogenesis was also found to partially involve FUS3 and ABI3 suggesting minor involvement of these two genes in both the processes [31, 57, 68](Ledwon and Gaj, 2011). Action of LEC genes in SE is either by increasing hormone production or the sensitivity of cells towards hormones [37] Figure 4.

The LEC genes form regulatory loops and modulates cellular hormonal balance enabling induction of embryogenic pathway in meristematic cells [39]. Firstly, LEC1 (in the presence of ABA) and LEC2 initiates auxin synthesis, by activating the tryptophan-dependent IPA-YUCCA pathway of auxin biosynthesis through activation of YUCCA genes YUC1, YUC2, YUC4 and YUC10 [82] Figure 5. This is evident by the fact that higher LEC2 and auxin accumulation were found to be associated with SE induction [36, 83]. Moreover,

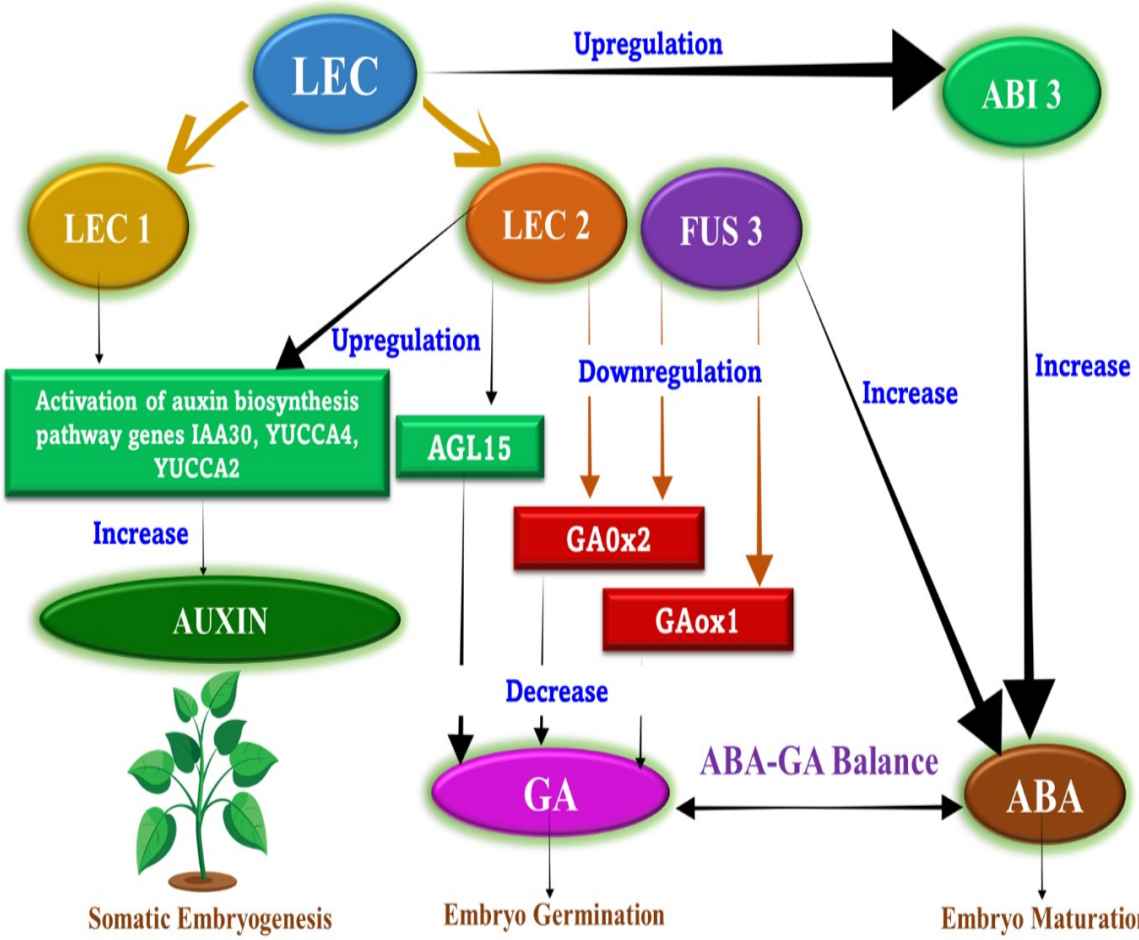

Figure 5: LEC Regulatory Network Governing Somatic Embryo Identity and Maturation by regulating endogenous Auxin, Abscisic Acid and Gibberellic Acid Levels

YUC4 showed elevated expression in regions having higher auxin concentrations like cotyledon tips also shoot apex of immature zygote. Upon upregulating these genes was also observed in cultured explants of *Arabidopsis*, where regions with LEC2 overexpression showed accumulation of auxin [58, 76, 77]. Stone et al [58] found that induction of LEC2 activates enzymes associated in auxin biosynthesis during SE of *Arabidopsis* within 1 hours. Since SE was observed in auxin free medium, overexpression of LEC2 seems to counterbalance auxin treatment. It was also found that the exogenous auxin inhibited embryogenesis induction ability of LEC2 and thus this ability of LEC2 was limited to auxin free conditions. LEC2 was also found to increase endogenous auxin level since shoot regeneration was substituted by callogenesis in explants (cultured on standard hormone medium) overexpressing LEC2 (Ledwon and Gaj, 2009). LEC2 is also found to influence INDOLE-3-ACETIC ACID INDUCIBLE30 (IAA30) (associated in auxin signaling) gene, which results in increased sensitivity of the explants to auxin in the medium. The expression of 35S:LEC2:GR transgene was found to activate IAA30 gene expression and increase in indole compounds such as IAA, in tissues showing overexpression of LEC2 [77] The exogenous auxin on the other hand affects the LEC2 expression. The up regulation of LEC2 was observed in auxin induced embryogenic cultures [76]. The MsLEC1 was also found to be upregulated in alfalfa embryogenic culture, after auxin treatment [71]. Thus, it can be hypothesized that LEC genes mediate SE by modifying the auxin response of the explants by affecting both auxin biosynthesis and sensitivity. Besides auxin, LEC genes also influence levels of gibberellins and ABA in the medium. During *Arabidopsis* embryogenesis, LEC2 and FUS3 genes reduce the level of GA by controlling its biosynthesis through repression of AtGA3ox2. FUS3 is also known to repress GA3ox1, that is also involved in GA biosynthesis. The low level of GA results in SE [8, 61]. A decrease in GA results in increased levels of ABA due to antagonistic activity of both the hormones. The ratio of these two hormones ultimately decides whether embryonic or adult leaf develops. High ABA to GA ratio favors formation of somatic embryos [60, 61]. Additionally, ABA-responsive gene ABI3 expression is regulated by LEC1, LEC2 and FUS3 [84] Figure 5.

Agamous-Like15

AGAMOUS-Like15 (AGL15) is a MADS box gene of *Arabidopsis thaliana*that controls seed development [85]. The proteins of this family are involved in spatial and temporal regulation of development of flowers, fruits and seed [61]. This gene was originally reported in *Arabidopsis* as a MADS-box gene, showing differential mRNA in embryo. AGL15 gene expression is limited largely for young embryos where its accumulation is the highest [37, 86]. Thus, it seems to be involved mainly in embryonic development in plants, although its role in commanding flowering time has been described [87]. Ectopic overexpression of AGL15 can enhance embryogenic response in *Arabidopsis*, soybean and other plants (Zheng and Perry, 2014). The ectopic expression of AGL15 increased the number of somatic embryos in zygotic embryos and SAMs of seeds cultured on liquid medium with 2,4-D [88]. SE in *Arabidopsis* is impeded by the mutation in AGL15 either by itself or in conjunction with AGL18, a gene belonging to the same family [61].

AGL15 affects SE by altering the levels of hormones in the cultured tissues. It promotes auxin, ABA and ethylene pathways but negatively regulates GA pathway (Fig. 3). AGL15 was found to upregulate At5g61590 gene in *Arabidopsis* [67]. This gene is involved in interconnection of hormone pathways and is essential for SE [89]. AGL15 controls the expression of FUS3 and ABI3 and is involved in regulatory loop of ABA and GA. By stimulating the GA2ox6 enzyme and suppressing the production of GA3ox2, AGL15 blocks the GA pathway [39]. Expression of these enzymes decrease the level of GA in the cultured tissues thereby affecting somatic embryo development [37]. AGL15 expression is induced by LEC2. Together these two TF upregulate the auxin signaling inhibitor IAA30 which results in increased sensitivity of explants to auxin in the medium, thus promoting formation of somatic embryos [61, 77]. AGL15 expression is found to be self -regulated (Zhu and Perry, 2005). Without exogenous auxin, zygotic embryos from plants with the 35S:AGL15 transgene continue to develop in embryonic mode for prolonged periods of time [61]. This transcription factor is also a component of SERK1 protein complex [90]. Auxin treatment promotes SE through SERK1, AGL15 and FUS3 [61]. AGL15 function in a similar way to auxins particularly 2,4-D. Both AGL15 and 2,4-D act by affecting ethylene production and response [3, 39]. In soybean, GmAGL15, an ortholog of AGL15 gene, has been identified to regulate the expression of key enzymes associated in ethylene biosynthesis, including 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), ACC oxidase (ACO) and ACC itself. ACS is responsible for converting S-adenosyl-Met into ACC, while ACO subsequently transforms ACC into ethylene. Furthermore, genes associated with ethylene signaling and response, particularly ERF1, exhibited differential regulation [67]. In *Arabidopsis*, ERF1 was also identified as being regulated by AGL15. However, unlike in soybean, the accumulation of AGL15 in *Arabidopsis* did not lead to the upregulation of ACS or ACO orthologs.

Other genes

Somatic embryogenesis is a highly intricate process that involves significant alterations in gene expression patterns. Apart from the previously mentioned genes, several other genes and TF contribute critically to this process. Among the key players are BABY BOOM (BBM) [91], *MtSERF1* [89], Late-Embryogenesis Abundant (LEA), PICKEL [78] and PGA6 [52]. BBM and *MtSERF1* encode TF belonging to the AP2/ERF superfamily. This transcription factor family is notably one of the biggest in *Arabidopsis*, encompassing around 150 genes categorized into five distinct phylogenetic subfamilies. These subfamilies vary in the number of AP2/ERF domains [37]. Certain genes from two of these subfamilies have been linked to improve in vitro regeneration, while others regulate meristem cell fate and organogenesis [37, 92]. LEA genes encode hydrophilic proteins that play a crucial role in the late stages of zygotic embryogenesis across multiple plant species, including cotton, barley, rice and wheat. These proteins function primarily in safeguarding the embryo from desiccation. The majority of LEA genes possess abscisic acid response elements (ABRE) in their promoter regions, making them responsive to ABA [8]. They are notably abundant in the heart stage of both zygotic and somatic embryos, localizing within the procambium, as well as shoot and root apical meristems. Additionally, these proteins accumulate in meristematic tissues of somatic embryos at the plantlet stage, though they are absent in young zygotic plantlets. The Plant Growth Activator 6 (PGA6) gene, which is homologous for WUSCHEL (WUS), functions as a homeobox gene. Its overexpression is observed to induce somatic embryogenesis from many vegetative tissues and zygotic embryos without requiring external plant hormones [52].

BBM gene was initially described in *Brassica napus* microspore embryo cultures, where it is used like a marker for microspore-derived embryogenesis [91]. Research has made it evident that in *Arabidopsis*, BBM exhibits preferential expression in basal region of the embryo [93]. In *Medicago truncatula*, it has been detected in root tissues and is identified to be driven by auxin [94]. BBM plays a central role in regulating pathways of development, which are linked to proliferation and growth [64]. It is essential for the zygotic and pollen-derived embryogenesis also it contributes to the regulation of organ primordia development. BBM1, a key variant, is known to activate genes responsible for cell wall modifications in actively dividing and growing cells [64, 95]. The ectopic expression of BBM in transgenic plants has been involved with spontaneous somatic embryo formation in seedlings and the emergence of embryo-like structures along the cotyledon and leaf margins [37, 91]. Additionally, abnormal cell division leading to defective floral and leaf morphology, uncontrolled division and hormone independent growth have been reported. These findings suggest that BBM shows a significant role during embryogenesis [91]. In *Arabidopsis*, overexpression of BBM is involved for a minor role in shoot and callus formation. In *Nicotiana tabacum*(tobacco), heterologous expression of BBM has been shown to induce spontaneous shoot and callus formation, though somatic embryo formation requires an initial cytokinin pulse (Souter and Lindsey, 2000). The capacity of BBM to trigger somatic embryogenesis and organogenesis in the absence of externally supplied plant hormones suggests that it enhances endogenous hormone production while also increasing cellular sensitivity to these regulatory compounds [37, 63].

MtSERF1 gene codes for transcription factor included in B-3 subfamily of AP2/ERF transcription factor family. It was initially identified in *Medicago truncatula*, where it was found to be associated with somatic embryogenesis (SE) induced by auxin and cytokinin in leaf explants [89]. Research has demonstrated that *MtSERF1* is essential for SE in M. truncatula, as RNA interference (RNAi)-mediated suppression of *MtSERF1* expression resulted in a nearly complete inhibition of somatic embryo formation [89]. The SE-inducing activity of *MtSERF1* is regulated by ethylene and also relies on auxin and cytokinin signaling [37, 39, 85, 89]. The *MtSERF1* promoter contains specific response elements for ethylene and auxin, as well as cytokinin-responsive ARR motifs. Additionally, it features binding sites for the WUSCHEL (WUS) transcription factor [39, 89]. Studies in *Arabidopsis* and soybean have shown that orthologs of *MtSERF1* are direct targets of the AGL15 transcription factor and contribute to the derepression of SE [39, 96, 97]. Furthermore, *MtSERF1* expression is initiated following the expression of SERK, showing its crucial role for hormone signal transduction also acting as key regulator linking stress responses with hormone signaling pathways.

The PICKEL (PKL) gene codes a CHD3 protein associated with chromatin remodeling and functions as a repressor of embryogenic development in *Arabidopsis* [37]. Its primary role is to prevent the expression of embryonic and seed-specific traits following germination [78]. Mutations in PKL results in the post-germination expression of embryo-specific characteristics, like the accumulation for seed storage reserves and also it enhances the potential for somatic embryogenesis [37, 78]. The repression of embryonic traits by PKL is thought to occur through the suppression of embryo-specific LEC genes and seed storage protein-coding genes [15, 37, 78]. Additionally, PKL is involved in regulating transcription during stress-induced developmental responses. Given that the mutant phenotype of PKL can be suppressed by gibberellin (GA), it is believed that PKL operates within the GA signaling pathway. This suggests that PKL helps create a cellular environment conducive for somatic embryogenesis, even in absence of exogenous hormone treatments.

2. Conclusion

Somatic embryogenesis is a well-regulated process during which somatic cells undergo complex series of events in order to form embryos resembling those which are formed from zygote. It involves a complete cellular reprogramming and redetermination of the fate of the cells in culture to initiate embryogenic program in non-embryogenic cells. SE involves many phases of development, each of which are commanded by a huge number of factors. Each step of this important process differs in its requirement and is highly regulated. The most significant factor controlling SE is media composition particularly the type of hormone. Auxin is essential for the initiation step and is deciding factor to initiate SE while other hormones like GA, ABA, ethylene play important role in subsequent steps of development. Stress plays an equally important role in vegetative to embryogenic transition. SE can be rightly be regarded as a process involving interplay of stress and hormones, which helps in cellular dedifferentiation and initiation of embryogenic program in plants with a responsive genotype (Rose and Nolan, 2006). A huge number of genes and TF controls this complex process. Most of the genes associated in SE controls many aspects of plant development simultaneously and thus act as master regulators. These genes act in coordination to initiate and maintain the embryogenic program. The most important role is played by genes such as WUS, LEC, AGL15, SERK, BBM, *MtSERF1* and others. Some of these are embryo-specific genes while others are ectopically expressed to form somatic embryo. They control SE by controlling the level of hormones in the cultured cells and coordinating different signaling pathways. A significant amount of cross talk is present between hormone and stress signalling pathways which help in SE. The combined role of all the factors produces a cellular environment that forces the somatic, non-embryogenic cells to undergo embryogenic pathway and form somatic embryo.

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