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Secretome-Derived Cultured Cell System: Overview Towards Extracellular Protein Characterization and Biotechnological Applications

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Abstract:

Secretome released by plant cells into the extracellular space, play crucial roles during development, embryonic potential acquisition, nutrient backing and stress acclimation. The dynamic nature of the extracellular proteome presents the challenge of identifying an array of extracellular proteins involved in the regulation of somatic embryogenesis in embryogenic suspension cultures. Extracellular proteins produced by cell cultures are perceived here as a central node of overlapping regulator factor network of totipotent somatic embryo developmental process. This paper reviews in a morphogenetic aspect the biological processes associated with extracellular protein-derived plant cultured cells and explores their prospective biotechnological applications in laboratories and biofactories retated to cell signaling and metabolism, developmental process, and biotic / abiotic stress tolerance. The role of extracellular proteins in acquisition and maintenance of embryonic potential and their relevance are especially emphasized.

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INTRODUCTION

The broad scientific and technical progress in the field of biotechnology, plant genetic engineering and molecular farming owes its success to the development of tissue culture mainly based on plant regeneration capacity. Besides culture on solid gelified media, culture in shacked liquid system provides a more suitable environment for better control of cell multiplication, interaction, synchronized cell-cluster development, increased relevant cell-cell signaling, and enhanced uptake of nutrients. When friable callus is placed into the appropriate liquid medium and agitated, single cells or small clumps of cells are released into the medium and continue to grow and divide, producing an established cell suspension. The inoculum used to initiate these suspension cultures should neither be too small to affect critical cell number no too large to generate toxic products or led stressed cells to lethal levels. Cell suspension is a very useful system for studying differentiation process in plants as it is suitable to build up an understanding of de novo organ or embryo formation . Two types of cell clusters can be found, embryogenic and non-embryogenic, showing distinct morpho-histological features and differential gene expression pattern explaining the disparity in embryogenic competence of clusters [1,2]. During developmental reprogramming, somatic cells de-differentiate to generate non-embryogenic cells which can further differentiate to produce somatic embryos through pro-embryogenic cells [3].

CELL TOTIPOTENCE AND EMBRYOGENIC COMPETENCE: MORPHOGENETIC VIEW

Plant cells display an exceptional feature of indefinite growth and developmental plasticity, as they have conserved during evolution besides meristematic zones acting as stem cell generator, a multi-level cell dedifferentiation capacity producing undifferentiated competent somatic cells. Moreover, embryogenesis takes places in the ovule after fusion of the female and male gametes with the formation of the unicellular zygote. Zygotic embryo resulting from sexual fertilization appears as an extremely totipotent bipolar structure to be capable to give an entire individual. Although due to high level of totipotency differentiated plant cells are able to induce dedifferentiation and bud neoformation through organogenesis, but it is too difficult to process as a zygotic embryo. Conversely, cells derived from somatic macrospore mother cell layers and flower tissues (i.e. ovaries and anthers) are more disposed to go back to an ultimate juvenile phase (embryonic stage) and undergo a somatic embryogenesis (SE) process. These both regeneration pathways, namely organogenesis and SE, differ in terms of at which level the cell could be able to return in the differentiation way to be so close to the starting embryonic stage that it can trigger an embryogenic program. During this somatic-to-embryogenic transition, cells have to dedifferentiate, reset their biological clock by remodeling their nuclear chromatin and reorganizing their gene expression patterns [4]. The fully totipotence that a somatic plant cell can acquire, outside the case of zygotic embryo, when cultured in an artificial medium under specific hormonal balance still that of SE, which provides an unique model to understand the molecular and cellular bases of developmental plasticity in plants. Application of stress treatment or exogenous auxin can induce SE, an intriguing process that illustrates plant cell totipotency expression [5]. Embryo induced via SE goes through specific-cell division and histo-differentiation programs under the same morphologically adopted developmental stage sequence (globular, heart, torpedo) of zygotic embryos [6] as illustrated in Figure **1**.

EMBRYOGENIC CELL SUSPENSION PLATFORM : IMPLEMENTATION AND RELEVANCE

SE is already known as the most appropriate regeneration system based on the exceptional totipotency of pro-embryonic masses (PEMs) giving rise to hundred of somatic embryos able to regenerate into plants. Several explants have been used to induce PEMs including immature flower tissues [7], anthers [7,8], ovaries [7,9], microspores [10], mature and immature zygotic embryos [9,11], cotyledons [12,13], hypocotyls [13], petioles [14], root tip [15,16], shoot apex [17] and leaf explants [16,18]. Somatic embryos developed over the surface of embryogenic callus (indirect SE) or occasionally, directly from explants without intervening calls phase (direct SE).

Cell suspension cultures are able of regenerating plants via SE and were first reported in carrot liquid culture more than 25 years ago [19]. So far, anther-derived friable embryogenic callus has been commonly used to initiate embryogenic cell suspension (ECS) cultures in liquid medium supplemented with growth regulators, essentially auxins and cytokinins.

Crop improvement through genetic engineering depends mostly on the availability of embryogenic material, the development of ECS and the proficiency

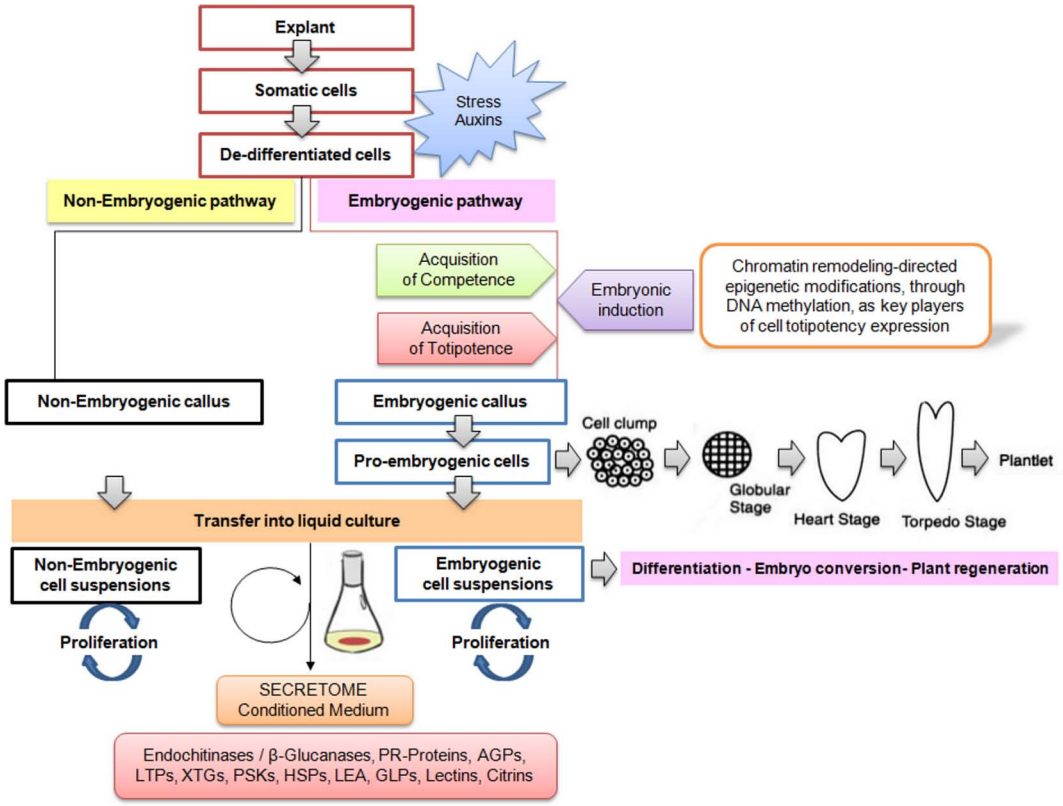

Figure 1: Flow-chart illustrating the main developmental pathways of somatic cells and their transfer in liquid system. Cell suspension establishment is mostly relied on several extracellular proteins released into the culture medium and act as plant growth factors.

of reliable in vitro regeneration process. A highly efficient and well-synchronized regeneration system is greatly requested for investigation in tissue culture, developmental biology and cell manipulation including protoplast isolation, gene transfer, somaclonal variation, clonal selection, directed mutagenesis, cryopreservation, and metabolite production [20-23].

IMPORTANCE OF EXTRACELLULAR PROTEIN PATTERN FOR ESTABLISHMENT OF EMBRYOGENIC SUSPENSION CULTURES

The transfer of embryogenic cultures into liquid medium represents a tricky step for initiation of a new starting ECS before being established. Basically, PEMs were initiated in erlenmeyer flask containing 4 to 6-week old yellow well-growing PEMs at high cell density in liquid medium supplemented with specific growth regulators and grown under continuous agitation on a rotary shaker, at 22-26°C in darkness. Numerous attempts to initiate ECS were unsuccessful due to the lack of cell signaling factors modulating cell interactions and developmental morphogenesis. Proliferation of dispersed plant cells in culture is strictly dependent on cell density, and cells in a low-density culture can only grow in the presence of conditioned medium (CM), in which cells have been grown previously. No known plant hormones have been able to substitute for CM [24]. It was already shown that the cellular environment, determined by the dynamics of the cell wall matrix, as a source of extracellular proteins (EPs) play a crucial role in cell nursing, signaling, and supporting young PEMs transferred into liquid system. Besides hormones known to stimulate embryo formation, other classes of molecules have been identified as embryo-stimulating factors, especially those secreted into the CM [25]. Thus, over the last three decades, several research groups investigated in optimizing the initiation and maintenance of these ECS by studying the effect of donor material, cell density, subculture period, phytohormones or growth factors, and substances involved in ECS establishment. Embryogenic potential in carrot (*Daucus carota* L.), revealed by the number of PEMs present, slowly increased in starting cultures over a period of six weeks. Addition of excreted, high-molecular-weight, heat-labile cell factors from an established embryogenic culture considerably accelerated the acquisition of embryogenic potential in starting cultures [26]. A battery of EPs produced by embryogenic material and released into the medium could constitute

an inducing environment which maintain the potential of PEMs and boost newly initiated ECS (Figure **1**), thus making the big difference with non-embryogenic cultures [27,28]. The pattern of EPs was reported to reflect the embryo morphology and to control the morphogenetic pathway that will be taken by somatic cell lines in *Picea abies* [29]. The CM contains a mixture of EPs with embryonic signature involved in the attenuation of stress response and maintenance of highly proliferating embryogenic cell lines in grapevine cell cultures [28,30]. In zucchini (*Cucurbita pepo*), EP-derived highly embryogenic cell lines have been proven to enhance a lower-performing line, suggesting their effect as PEM-stimulating factors [31]. In fact, these EPs serve for the conditioning of the medium, in which fresh cells will grow efficiently and faster in liquid culture system (Figure **1**).

In contrast, PEMs from which somatic embryos develop going through consecutive developmental stages could be feasible under conditions of low cell density in the absence of phytohormones. EP expression patterns have been associated with induction of SE in grapevine cultured cells that allowed somatic embryo emergence under particular subculture frequency [28,32].

MAJOR EXTRACELLULAR PROTEINS AND THEIR BIOTECHNOLOGICAL APPLICATIONS

EPs released into the medium of ECS cultures are regarded as one of the key morphogenesis-oriented factors that control cell division and differentiation as well as further development of PEMs in cell culture. The pioneer group of plant EP of Sacco C. de Vries and coll. (Wageningen-Netherlands) firstly described an EP1 only secreted by non-embryogenic cells [33], then they reported an EP2, identified as a lipid transfer protein produced by carrot embryogenic cells [34], and later an endochitinase EP3 that rescue proliferation of somatic embryos of mutant carrot cell line [35,36]. Studies have revealed that suspensions of somatic embryo masses secrete a vast array of proteins that could be involved in SE [30,37]. Some of these molecules have been suggested to work as inductors and others as inhibitors of this process. In this context, profiling of this extracellular proteome has been described and to date the widely documented EPs include: Arabinogalactan proteins (AGPs), Endochitinases/ Glucanases/ Pathogen-related (PR) proteins, Lipid transfer protein (LTP), Xyloglucan transglycosylases (XTGs), Heat shock proteins (HSPs), Late embryogenesis abundant proteins (LEA), Phytosulfokines (PSKs), Germin-like proteins (GLPs), Citrins, and Lectins, (Table **1**) among others [38]. Investigations focused on functional characterization studies of these EPs have opened impressive field of their applications starting from cell growth and proliferation, embryo differentiation and maturation, to defense-related mechanisms during biotic and abiotic stresses with secondary metabolite production (Figure **2**). The putative functions and potential biotechnological applications of these EPs are listed in Table **1**.

1. Endo-Chitinases, Endo-Glucanases, and Pathogen-Related Proteins

Endo-chitinases (32-kDa) and β-1,3-D-glucanases (38-kDa) are well-known pathogenesis-related (PR) proteins that are constitutively expressed at low levels in plants, but are dramatically expressed upon pathogen infection. Both of these EPs have a plant hydrolase enzymatic activity catalyzing respectively the degradation of N- acetylated-glucosamine residues (chitin unit) and β -1,3/1,4-glucans. Enhanced accumulation of β-1,4-glucanase mRNA transcript level in suspension-cultured poplar (*Populus alba*) cells was reported by addition of 2,4-dichlorophenoxyacetic acid (2,4-D) in the presence of sucrose and was correlated with solubilization of cello-oligosaccharides and xyloglucan [39]. In ECS cultures, endochitinases are differentially expressed and accumulated only in PEMs and not in the developing somatic embryos. The endochitinase secreted in the CM has been well documented in carrot cell suspensions. Extra-cellular glycosylated acidic class IV endochitinase secreted into culture medium of ECS exclusively and known as extracellular protein 3 (EP3) was shown to be responsible for the transition from globular to heart-shaped stage in carrot embryogenic cultures [35]. This endochitinase is also able to rescue proliferation and to lift the arrest of somatic embryos of mutant carrot cell line ts11 at non-permissive temperatures [35]. Glucanase transcripts were induced, from very low basal levels, with similar kinetics to chitinase transcripts in elicitor-treated cell suspension cultures [40]. Chitinases and glucanases are shown to generate signal oligosaccharide molecules acting as messengers regulating developmental programs and likely involved in cell proliferation [30]. Zhong *et al*. [41] reported that mutation of chitinase-like gene causes an alteration of developmental process and cellular senescence by ectopic deposition of lignin, aberrant cell shapes and overproduction of ethylene.

Table 1: Putative Functions and Potential Applications of Extracellular Protein from the Secretome

Extracellular protein from the Secretome	Putative functions	References	Other potential applications	References
Endo-Chitinase Endo-Glucanase	Somatic embryogenesis Osmotic stress tolerance Proliferation of Cell Suspensions Inhibition of spore germination Antimicrobial activity	[92] [93] [30] [94] [95]	Antifungal role in plant disease resistance against pathogens	[96, 97]
Arabinogalactan proteins (AGPs)	Somatic embryogenesis Cellular signaling Cell elongation / extension Cell division / proliferation Plant growth / development Cell cryopreservation Biotic / abiotic stress tolerance	[46] [99] [100] [28,30,31] [44] [101] [102,103]	Food applications-Dietary fiber / hydrocolloids Immune-stimulating phytochemical (Allergy prevention) Fruit ripening	[104] [105,106] [107]
Lipid transfer protein (LTP)	Somatic embryogenesis / conversion Biotic / abiotic stress tolerance	[27,34,52,53] [55,58]	Stress tolerance and pathogen resistance Anti-microbial function Anti-proliferative	[57] [56] [59]
Xyloglucan transglucosylase (XTGs)	Cell wall reorganisation and cell competence	[60,61]		
Heat shock proteins (HSPs)	Somatic embryogenesis Cell signaling / immunity Stress tolerance	[61] [80] [70]	Stress tolerance	[70]
Late embryogenesis abundant proteins (LEA)	Somatic embryogenesis/stress tolerance Cell membrane protection	[71] [73]	Abiotic tolerance	
Phtosulfokines (PSKs)	Cell signaling / communication Stress mitigation/ Mitogenic activity	[74] [77,78]	Osmotic stress tolerance	[79]
Germin like proteins (GLPs)	Somatic embryogenesis In vitro stress tolerance/ Desiccation/ Maturation	[81,82]		
Citrins	Somatic embryogenesis	[84]	Human health (mitochonrial carrier regulating cell energy) oncogenesis	[108]
Lectins	Cellular signaling /communication Cell recognition system Pathogen detection / Defense system	[86] [80]	Immuni-modulating effects Selective cytotoxicity against cancer cells Antimicrobial and insecticidal activities	[89] [90] [91]

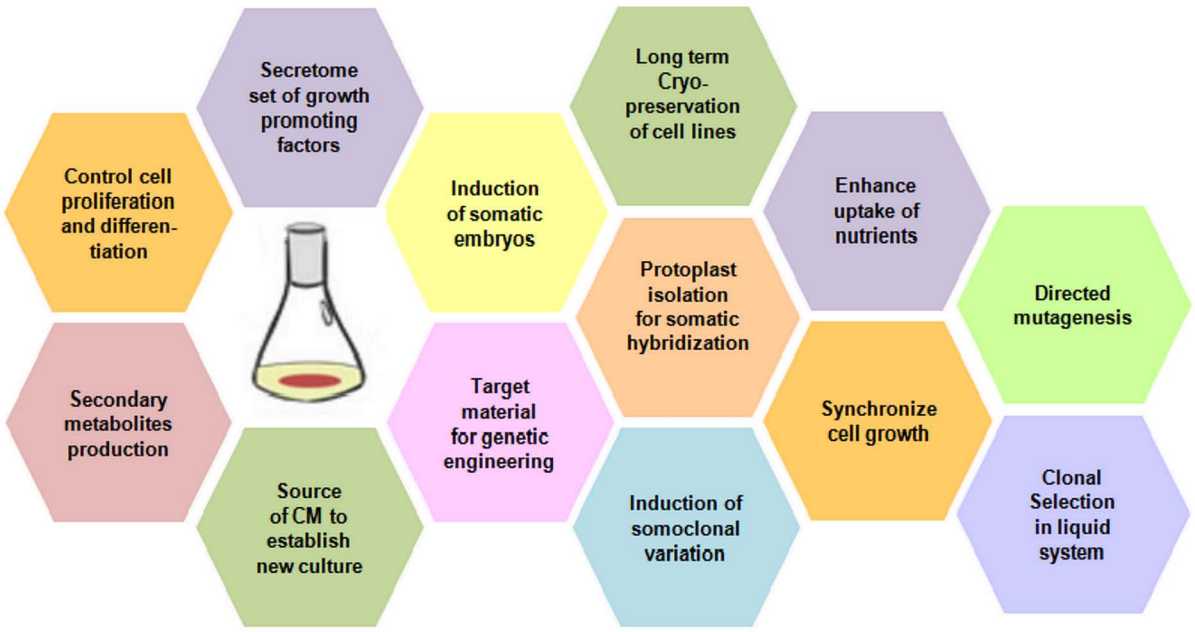

Figure 2: Biotechnological applications of secretome-derived suspension-cultured cells.

Conversely, exogenous addition of purified chitinase or its substrate resulted in an increase of cell population and restored multiplication rate in ECS cultures through promotion of highly dividing cells of PEMs [30,42]. Cell death, induced by withdrawal of plant growth regulators, can be suppressed by extra supply of lipo-chito-oligosaccharides (LCOs), which are most likely produced by extracellular chitinases [43].

2. Arabino-Galactan Proteins (AGPs)

AGPs represent the most highly glycosylated hydroxy-prolin-rich proteoglycans (>90% of carbohydrates) found in cell walls, plasma membranes, and extracellular secretions [44]. Recent research has led to the identification of the glycosyl-transferases responsible for the biosynthesis of two of the most functionally abundant families of plant cell wall proteins, extensins and AGPs [45]. AGPs have been suggested to play a key role in plant development since AGP epitopes are known to display developmentally regulated patterns of expression in several plant tissues [46]. Their complexity arises from the diversity of their carbohydrate extensions. SE is regulated by AGPs as well as by EP3-endochitinases. AGPs are detected by specific antibodies [47] or by precipitation with Yariv-reagent [48] which bind to AGPs producing a red complex, resulting in an inhibition of their function. AGPs have been identified in cell culture medium of carrot, grapevine and squash [28,31,49], microspores haploid cell cultures of maize and barley [50,51]. Therefore, AGPs are supposed to have a central role in embryo formation [25] and mainly involved in cell proliferation and attenuation of stress response during cell culture and differentiation [28,30,31]. Addition of AGPs to culture medium, even at nanomolar concentrations, significantly improves the efficiency of cellular proliferation of PEMs, even when the initial cell population is far below the critical density [28].

3. Lipid Transfer Proteins (LTPs)

LTPs are a group of highly-conserved proteins found in higher plant tissues, and are responsible for shutting of phospholipids and other fatty acid groups between cell membranes. An EP of 10-kDa identified as lipid transfer protein (LTP) has been reported as produced by embryogenic cells in carrot cell lines [34], and grapevine ECS [32]. The level of LTP expression in cotton cell lines is high before induction of embryogenesis and during the globular stage, whereas it diminishes during post-globular stages [52]. Recently, using global scale transcriptome analysis, these LTPs are identified as differentially expressed genes involved in early SE and thus revealed to be greatly related to the SE process [53]. The finding that these proteins are located in the cell wall and can be secreted outside the cell has led to the suggestion that they are not required for intracellular lipid transport. Instead, they may be involved in cutin biosynthesis, pathogen-defense reactions, signaling or adaptation of plants to environmental changes [54]. Transcript accumulation of LTP genes occurs as a strategy to induce a tolerance mechanism under both biotic and abiotic stress

conditions in plants [55]. Studies on purified LTPs have confirmed their extracellular location as well as their anti-microbial functions [56] and roles in abiotic stress tolerance [57]. LTPs are accumulated upon water deficiency, most likely via up-regulation of genes encoding LTPs providing an adaptive mechanism through which cuticle thickness can be increased to minimize water loss [58]. In fact, LTPs are known to be pathogenesis-related proteins and may contribute in plant innate immune system as antibacterial, antifungal, antiviral and/or in vitro anti-proliferative [59]. The enzyme inhibitor members are thought to regulation plant development and seed germination, also to be involved in lipid metabolism and fruit ripening [59].

4. Xyloglucan Transglucosylases (XTGs)

Huge structural changes were achieved in pro-embryogenic cells to alter the mechanical properties of their extra-cellular matrix and change their cell-wall plasticity in order to prepare themselves for acquisition of competence. Thus, several cell wall-modifying enzymes, particularly those modifying the structure of xyloglucan chains, are differentially expressed and induction of SE is commonly accompanied by up-regulation of xyloglucan endo-transglycosylases [60,61].

5. Heat Shock Proteins (HSPs)

Heat shock proteins (HSPs) have been identified for a long time in cellular biology as proteins, which concentration dramatically increases when cells are grown at higher temperatures. They play important role in cell signaling and immunity mediated by HSP specific-cell wall receptors [62]. While the identity and functions of intracellular heat stress-responsive proteins have been extensively studied, HSPs secreted to the extracellular matrix is unknown. Under heat stress, plants alter their gene expression reducing the synthesis of regular proteins, while promoting that of HSPs induced by diverse stress factors, including heat and other stresses. These stress-responsive bio-molecules act as molecular chaperones most likely for thermoprotection of cellular structures enhancing membrane stability and detoxifying the reactive oxygen species (ROS), and thus play a pivotal role in conferring biotic and abiotic stress tolerance [63,64]. These responses require expression of stress-responsive genes, which are regulated by a network of transcription factors, including heat stress transcription factors (HSFs) modulating the expression of HSPs [65]. The involvement of Ca^{2+}-calmodulin in the expression of HSPs by activating of heat shock factor and signal transduction in wheat, maize and Arabidopsis has been documented [66-68]. Upon stress conditions, Mita *et al*. [69] revealed specific heat stress-induced protein accumulation in sunflower suspension cell cultures. Correspondingly, when exposed to 40 ◦C for 72 h, heat-sensitive Arabidopsis cell suspension cultures died, while sorghum cell cultures survived by activation of a transcriptional response characterized by the induction of HSP70 and HSP90 genes providing evidence that heat stress triggers differential protein accumulation in the extracellular matrix within the sorghum secretome [70].

6. Late Embryogenesis Abundant Proteins (LEA)

Late embryogenesis abundant (LEA) proteins constitute a large family of proteins that typically accumulate to high levels during seed dehydration, at the later stages of embryogenesis, and involved in development and abiotic stress responses [71]. Although firstly described as abundant in seeds and pollens, LEA proteins have been later found to protect against protein aggregation due to desiccation or osmotic stresses associated with freezing temperatures or high salinity which cause cellular water deficit [72]. LEA proteins are particularly protective of membranes against dehydration damage [73].

7. Phytosulfokine Proteins (PSKs)

Phytosulfokine (PSK), a 5-amino-acid sulfated peptide, acts as an extracellular ligand involved in the initial step of cellular dedifferentiation, proliferation and re-differentiation. PSK was first purified from the CM of Asparagus cell culture based on its ability to promote cell division of Asparagus mesophyll cells incubated at low cell density [24]. PSKs play a role in intercellular signaling process and cell-cell comunications in meristematic cell lines [74]. Hanai *et al*. [75] demonstrated the presence of PSKs in CM of embryonic cell culture in carrot. Although PSK does not induce embryogenic competence but its stimulating action on embryo formation was proven. In fact, PSK which has been identified as peptide growth factor, had a dramatic stimulatory effect on the formation of somatic embryos in Cryptomeria japonica [76]. It was also reported to attenuate stress response during trans-differentiation of zinnia mesophyll cells [77] and to stimulate mitogenic activity in protoplast-derived cell culture [78]. PSKs contribute to drought stress tolerance and are found to be up-regulated in response

to osmotic stress in Arabidopsis with elevated expression of PSK as peptide signal for stress mitigation [79].

8. Germin-Like Proteins (GLPs)

Germin-like proteins (GLPs) were first discovered in germinating wheat grains, but later found in different plant species, organs, tissues and cell lines as glycoproteins somehow associated to the extra-cellular matrix and involved in developmental modulation and response to diverse stress conditions [80]. GPLs belong to one of the most abundant groups of EPs found in embryogenic lines of *Pinus caribaea* [81]. Studies showed transcript up-regulation of GLP-encoding genes in embryogenic lines of wheat [82] and increase of GLP level in the secretome of suspension-cultured cells of sorghum [83]. GLP expression was strictly limited to embryogenic cells [61].

9. Citrins

Citrins are citrus seed storage proteins that show differential expression during embryogenesis. Transcripts of citrin-coding genes were found to accumulate during the later stages of somatic embryogenesis and released in the extracellular compartment [84].

10. Lectins

Lectins are fundamental to plant life and have important roles in cell-to-cell communication, development and defense strategies. Specific glycoproteins, proteoglycans that bind these endogenous lectins, appear to play a role in shaping extracellular environment [85]. At the cell surface, lectins form the extracellular domains of receptor-like kinases (LecRLKs) and receptor-like proteins (LecRLPs) which constitute together a versatile recognition system at the cell wall surface [86]. Lectines are non-catalytic sugar-binding proteins, such as legume lectins contributing to the detection of symbionts and pathogens, and are relatively stable against heat denaturation and proteolytic digestion. Lectin-like protein was identified among released proteins in sunflower and immuno-localization assays confirmed its extracellular location [87]. Meanwhile, many interesting biological functions have been discovered and reviewed in lectins [88] originating from foods or foodstuffs, including immuno-modulating effects [89], selective cytotoxicity against cancer cells [90], antimicrobial and insecticidal activities [91].

CONCLUSION AND OUTLOOK

Although research investigation in plant cell suspensions have been started since several decades, suspension cultures still a mystery to be decoded and deciphered. One of the most outstanding piece of evidence is the Secretome-derived cell suspensions with a wide diversity of extracellular proteins that could modulate development, signaling and defense system. These proteins released into the medium have numerous advantages and potential applications (Figure **2**) in diverse sectors including fundamental studies on cell differentiation and totipotence, applied research for bioreactor cell culture, cold storage cryopreservation, production of secondary metabolites, regulation of immune system and production of bioactive anti-cancer/anti-pathogen substances, enhanced environmental stress tolerance and pathogen-acquired resistance, and potentially used in pharmaceutical biofactories.

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