PROTOPLAST EVOLUTION: FROM EARLY DISCOVERIES TO CONTEMPORARY ADVANCEMENTS IN PLANT BIOTECHNOLOGY

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Malaysian Cocoa J. 15 (1): 81-89 (2023)

ABSTRACT - *The study of plant protoplasts, isolated plant cells devoid of cell walls, has evolved remarkably, shaping our understanding of plant biology and revolutionising biotechnology. This evolution has contributed to our understanding of plant biology and revolutionised biotechnology. This review article traces the trajectory of protoplast research from its origins in the 1960s to the cutting-edge advancements in the current era. Early breakthroughs allowed for the isolation and culture of protoplasts, paving the way for further investigations into regeneration capacity and cellular behaviour. CRISPR-Cas9 technology enabled precise genome editing in protoplasts in the 21st century, marking a turning point in cell biology. Molecular protoplast responses have been better understood through integrative systems biology approaches and the "omics" technologies. In addition, protoplasts have been reprogrammed for the production of valuable compounds, biofuels, and pharmaceuticals through the convergence of protoplast research and synthetic biology. This review presents a comprehensive narrative of plant protoplast research, demonstrating its relevance and potential for crop improvement, genetic engineering, and sustainable biotechnology.*

Key words: Plant protoplasts, Biotechnology, CRISPR-Cas9, Systems biology, Omics technologies, Genetic engineering

INTRODUCTION

Protoplasts, the cellular components of plants lacking cell walls, present a distinctive and captivating facet of plant biology. In plant cells, protoplasts, also known as spherical naked cells with totipotency are isolated from their cell walls, leaving the plasma membrane behind (Torrey & Landgreen, 1977; Navrátilová 2004; Eeckhaut *et al*., 2013). Plant cells are liberated from rigid cell walls through enzymatic digestion or mechanical disruption (Anand *et al*., 2020). This procedure results in the development of discrete cellular units that maintain their plasma membrane, cytoplasm, organelles, and genetic material enclosed within the nucleus (Ziv & Altman, 2003). In contrast to conventional plant cells, protoplasts exhibit a deficiency in the structural reinforcement offered by the cell wall, rendering them more vulnerable to alterations in their surroundings and cellular interventions.

The plasma membrane of protoplasts is paramount in upholding cellular integrity, regulating the transport of substances, and in serving as a dynamic interface for cellular communication and signalling (https://opentextbc.ca). The cytoplasm is the intracellular compartment where the cell's metabolic machinery is located, encompassing enzymes and a diverse array of molecular constituents participating in various cellular processes (Luby-Phelps, 2013). Organelles, such as chloroplasts and mitochondria, persist in their functionality within protoplasts, playing a crucial role in energy generation and diverse cellular processes (Luby-Phelps, 2013).

Protoplasts exhibit a high degree of receptiveness to experimental manipulations, including genetic engineering techniques, which enable researchers to introduce exogenous DNA and investigate gene expression patterns under controlled and precise conditions (Reed *et al*., 2021; Lin *et al*., 2018). These organisms' regenerative abilities render them as highly valuable instruments for propagating genetically identical plants via cellular division and subsequent tissue culture, which significantly contributed to the progress of biotechnology.

HISTORICAL BACKGROUND AND EARLY DISCOVERIES IN PROTOPLAST RESEARCH

The study of protoplasts dates back to the 1960s when researchers first initiated research on the

feasibility of extracting plant cell walls to access the enclosed protoplasts (Navrátilová 2004). The initial trailblazers in the field of protoplast research, includes Ian Cocking, who effectively employed enzymatic techniques that involved the use of cellulase preparations to isolate protoplasts from plant tissues (Navrátilová 2004). These groundbreaking findings established a basis for investigating plant cells in their indispensable state, devoid of the structural limitations imposed by the cell wall.

As protoplast research advanced, the studies done in the 1970s was primarily directed towards comprehending protoplast regenerative potential and behavioural characteristics. Protoplasts' ability to undergo cellular proliferation and form cell colonies was observed under suitable culture conditions. Thus, this significant discovery has presented promising opportunities for clonal propagation, offering a method to efficiently and expeditiously produce genetically identical plants (Takebe *et al*. 1971).

Furthermore, the technique of isolating and culturing protoplasts has been successfully applied to a wide variety of cultivar such as potato, tobacco, petunia, carrot (Naing *et al*., 2021, Soriano *et al*., 2012), and many more, thereby broadening the potential applications of this method in crop enhancement. Significant progress was made in the subsequent decades in the protoplast culture techniques, focusing on optimising media formulations and developing genetic transformation methods.

PROTOPLAST ISOLATION AND CULTURE TECHNIQUES

The techniques employed for the isolation and culture of protoplasts have progressed throughout the years. Multiple techniques have been devised to isolate protoplasts, with each method being customised to suit the specific plant tissue and species. The efficacy of protoplast isolation is contingent upon various factors, including the developmental stages of plant tissue and the selection of appropriate enzymes or mechanical methods (Navrátilová, 2004).

The use of enzymatic methods has been extensively used in isolating protoplasts. Enzymatic treatment is employed on plant tissues by treating with enzymes such as cellulases, pectinases, and hemicellulases to facilitate cell wall degradation (Reed *et al*., 2021). The selection of enzymes and their concentrations can be modified according to specific plant species and cell types under consideration (Navrátilová 2004; Blackhall *et al*., 1994a).

In contrast, mechanical techniques encompass the physical disruption of the cell wall. Various techniques such as chopping, grinding, or using a mortar and pestle can be employed, albeit with potentially diminished yields compared to enzymatic methodologies (Bauer, 1990; Navrátilová 2004; Davey *et al*., 2005). Other studied methods include osmotic shock, a technique involved in alterations of osmotic pressure to plant tissues. This process induces the rupture of the cell wall, thereby facilitating the release of protoplasts.

CULTURE CONDITIONS AND MEDIA FORMULATIONS FOR PROTOPLAST GROWTH AND REGENERATION

Following isolation, protoplasts necessitate specific culture conditions and suitable media to sustain their survival, growth, and regeneration into viable plant cells. Providing a properly formulated culture medium, which encompasses a harmonious combination of vital nutrients, including sugars, minerals, vitamins, and plant growth regulators, is pivotal in facilitating the prosperous cultivation of protoplasts (Reed *et al*., 2021). The composition of the medium may exhibit variations contingent upon the particular objectives of the culture, encompassing investigations related to cell division, regeneration, or gene expression (Davey *et al*., 2005).

Osmolarity and pH are other critical factors that necessitate precise regulation in the culture medium to mitigate cellular stress and uphold the essential physiological equilibrium necessary for protoplast viability (Ruesink, 1978, Pearce & Cocking, 1973). In addition to the osmolarity and pH, temperature and light must be carefully considered to ensure the successful survival of protoplasts during the regeneration process, as these two factors are essential in optimising cell growth and regeneration (Sinha *et al*., 2003b). Extreme temperatures, whether too hot or too cold, can adversely affect protoplasts' metabolic processes and membrane integrity. Optimal temperature ranges are species-specific and must be meticulously determined for each plant type under investigation. On the other hand, light is an energy source for photosynthetic organisms, and certain plant protoplasts may require specific light regimes to support their metabolic activities. Adequate light exposure or light deprivation can be essential to induce desired physiological responses in regenerating protoplasts.

Furthermore, adding plant growth regulators, such as auxins and cytokinins, to the medium can stimulate cell division and facilitate the development of shoots or roots during regeneration (Reed *et al*., 2021).

ADVANCEMENTS IN PROTOPLAST CULTURE TECHNIQUES OVER THE YEARS

With time, notable progress has been achieved in protoplast culture techniques, resulting in improved suitability and efficacy for a wide range of research and biotechnological endeavours. The enzymatic procedures employed for isolating protoplasts have been refined, incorporating enzyme combinations and optimising the concentrations to enhance cellular yield and mitigate cellular harm.

Suspension culture methodologies also have been devised to facilitate the cultivation of protoplasts in liquid media, thereby creating a uniform culture milieu and facilitating large-scale studies (Tang *et al*., 2001). The protoplast fusion method has also been utilised to generate hybrid cells by the fusion of protoplasts derived from distinct plant species (Davey *et al*., 2004). This technique enables the development of novel plant varieties with desirable traits. Furthermore, various innovative methodologies have been developed to augment the efficiency of protoplast regeneration. These include the utilisation of bioreactors, delivery systems based on nanotechnology, and plant growth regulators manipulation.

REGENERATION AND CLONAL PROPAGATION

Various research has been conducted on the regenerative capabilities of protoplasts. However, several obstacles can be seen in generating complete plants from protoplasts. Identifying the ability to regenerate protoplasts started in the 1970s and represented a significant milestone in scientific advancement, presenting a promising opportunity to produce complete plants from individual cells (Kao *et al*., 1971; Nagata & Takebe, 1971). The primary emphasis in research on protoplast regeneration has been placed on comprehending the various factors that influence the process of regenerative callus formation and the subsequent growth of shoots and roots (Darvey *et al*., 2005). Most of the studies also indicated that protoplasts frequently exhibit distinct developmental pathways in their regenerative capacity, typically involving the formation of callus (Zhao *et al*., 2001; Williams *et al*., 2003; Grafi, 2004; Chupeau *et al*., 2013) as an intermediate stage before the shoot and root development.

Researchers have conducted numerous investigations to examine a range of factors that influence the process of protoplast regeneration. These factors include the age and physiological condition of the plant tissue utilised for protoplast isolation, the composition of the culture medium employed, and the plant growth regulators used (Yue *et al*., 2021). Young and healthy plant tissues have been found to exhibit higher regeneration potential compared to older or stressed tissues. This is attributed to the presence of more active cells and a greater capacity for cell division and tissue regeneration.

However, the capacity for regeneration is not solely determined by tissue age and physiological condition. Variability in regeneration potential is also observed across different plant species and genotypes. Some plant species have demonstrated a remarkable ability to regenerate from protoplasts, making them valuable candidates for genetic transformation and other biotechnological applications. On the other hand, certain plant species may exhibit limited or even no potential for regeneration, posing challenges for researchers attempting protoplast-based techniques.

This variability in regeneration capacity highlights the importance of carefully selecting plant materials and considering the specific requirements of each species or genotype when attempting protoplast regeneration. Researchers must tailor their protocols, including the composition of the culture medium and the use of plant growth regulators, to optimize the chances of successful regeneration. Such considerations are essential for advancing the field of plant biotechnology and harnessing the full potential of protoplast-based techniques in crop improvement and genetic engineering (Yue *et al*., 2021).

APPLICATIONS OF PROTOPLAST REGENERATION FOR CLONAL PROPAGATION AND CROP IMPROVEMENT

The protoplast regeneration process is a highly effective method for achieving clonal propagation, thereby facilitating the generation of genetically identical plants from a protoplast. This methodology is notably advantageous in conserving and disseminating superior cultivars possessing desirable characteristics, facilitating the swift proliferation of valuable crops. Other than that, protoplast fusion techniques have been employed to generate somatic hybrids, thereby facilitating the incorporation of desirable traits from diverse plant species. Genetic transformation, characterised by the capacity to regenerate plants from genetically

modified protoplasts, has brought about a significant revolution in genetic engineering. As somatic hybrids possess significant potential as genetic reservoirs, the protoplast regeneration process also facilitates the incorporation of exogenous DNA and the subsequent development of transgenic plants exhibiting enhanced characteristics (Zhao *et al*., 2015).

CHALLENGES AND ACHIEVEMENTS IN PRODUCING WHOLE PLANTS FROM PROTOPLASTS

Although protoplast regeneration has enormous potential, it has several difficulties and barriers to overcome. Low regeneration efficiency for some plant species through somatic embryogenesis is the most difficult barrier to overcome (Reed *et al*., 2021). Somatic embryogenesis is a process by which embryonic structures emerge from somatic cells (Tamchek *et al*., 2020). The induction of somatic embryogenesis and the subsequent growth of robust plantlets remains an area of intensive research for many plants. Other than that, protoplast regeneration faces an ongoing challenge in ensuring the stability and uniformity of regenerated plants, as it can induce epigenetic changes in regenerated plants, which may influence their phenotypic characteristics. The possibility of genetic diversity also persists due to somaclonal variation and genetic instability during the regeneration process (Ahuja, 1987; Saieed *et al*., 1994a).

Even though protocols for protoplast isolation, regeneration, transfection, and transformation have been established for a long time, the need for protoplast regeneration systems for specific crops continues to hinder protoplast transfection as a DNA-free genome method. Furthermore, the study more focused on dicotyledonous species (Woo *et al*., 2015; Andersson *et al*., 2017; Lin *et al*., 2018; Hsu *et al*., 2019; Yu *et al*, 2019; De Bruyn *et al*., 2020; Hsu *et al*., 2021).

CHARACTERISATION AND APPLICATIONS OF *THEOBROMA CACAO* PROTOPLASTS

Theobroma cacao L., commonly referred to as cocoa, is a cash crop commodity in cocoa-producing countries, including Ghana, Ivory Coast, Indonesia, and Malaysia. Cocoa production faces numerous challenges, such as the adverse effects caused by pests, diseases, and environmental stresses. Investigating protoplasts in *Theobroma cacao* presents a fruitful avenue for enhancing crop quality and advancing biotechnological applications.

The successful process of isolating protoplasts from *Theobroma cacao* entails the enzymatic degradation of the cell walls (deMelo & Brar, 1998). Characterising cocoa protoplasts entails morphological and physiological evaluations, cell viability tests, and protoplast yield estimation. In the cocoa industry, preventing the spread of debilitating diseases like witches' broom (*Moniliophthora perniciosa*) and frosty pod (*Moniliophthora roreri*) is one of the most significant issues (Sousa Filho *et al*., 2021; Philips-Mora & Wilkinson, 2007). Furthermore, the application of genetic engineering techniques on cocoa protoplasts presents a promising avenue for addressing the vulnerability of cocoa plants to various abiotic stresses, including drought, heat, and waterlogged conditions, all of which significantly impact crop productivity and quality. Through the introduction of specific genes into cocoa protoplasts, the potential exists to confer resistance against these stressors and enhance the plant's resilience in adverse environmental conditions. By doing so, the cocoa industry can actively mitigate yield losses and reduce its reliance on chemical pesticides, ultimately facilitating the development of disease or stress-resistant cocoa varieties that contribute to sustainable and improved cocoa production.

Theobroma cacao is renowned for its intricate genetic variability. The study of protoplasts presents a valuable method for conserving and exploiting scarce and distinctive genetic materials through cryopreservation and the subsequent regeneration of plants derived from protoplasts (Engelmann, 2000). This action facilitates the preservation and responsible utilisation of cocoa genetic diversity.

PROTOPLASTS AND GENETIC ENGINEERING

Integrating protoplasts and genetic engineering has significantly transformed plant biotechnology, providing a robust framework for accurate and focused alterations of plant genomes (Yue *et al*., 2021). The onset of the 21st century marked a significant transformation in the field of genetic engineering, as the CRISPR-Cas9 technology emerged as a groundbreaking tool for editing genes (Zhang *et al*., 2019). Using protoplasts in early genetic transformation techniques yielded significant knowledge regarding the mechanisms underlying DNA uptake and integration into the plant genome, laying the foundation for developing more advanced genetic engineering methodologies.

The framework for genetic modification of protoplasts was set by the groundbreaking work done in the 1980s, which introduced foreign DNA

into these isolated plant cells (Ranaware *et al*., 2023). In the early studies, protoplasts were exposed to DNA fragments that the cells could ingest directly. The technique referred to as direct DNA uptake has established the preliminary research for subsequent advancements in the field of genetic transformation (Kozlowski & Pallardy, 1997). Other than that, Polyethylene Glycol (PEG) was used as a fusogen during PEG-mediated transformation to enable protoplasts DNA uptake (Reed *et al*., 2021). The technique dramatically increased the rate of genetic transformation in protoplasts.

The CRISPR-Cas9 system is an innovative gene-editing tool that has attracted considerable interest within the scientific community. The CRISPR-Cas9 system is derived from a bacterial immune system. This system uses a guide RNA to direct the Cas9 enzyme towards a specific DNA target sequence. This ultimately results in the precise cleavage of the DNA at the intended location (Wu *et al*., 2014). This procedure facilitates precise and efficient gene editing with unparalleled accuracy. Protoplasts present various benefits in the context of CRISPR-Cas9-mediated gene editing, such as their convenient DNA delivery mechanism and capacity to assess gene modifications efficiently.

OMICS TECHNOLOGIES AND PROTOPLASTS

In order to fully understand the complexity of protoplast biology, omics technology could be used. Omics technology combines genomics, transcriptomics, proteomics, and metabolomics, thus revealing the protoplast's gene expression, protein profiles, and metabolite dynamics.

Analysing the entire set of RNA transcripts in a cell, also known as transcriptomic, provides important knowledge about gene expression patterns and regulatory networks (Morinaka *et al*., 2023). RNA-Seq is the field of transcriptomics by facilitating the high-throughput sequencing of RNA molecules within protoplasts. This methodology facilitates quantifying gene expression levels, characterising alternative splicing events, and identifying non-coding RNAs. In addition, transcriptomics allows for comparing gene expression across various protoplast circumstances, including developmental stages and genetic backgrounds. Differential gene expression analysis reveals crucial genes implicated in distinct cellular processes and responses (Sugimoto *et al*., 2010; Shang *et al*., 2016; Fan *et al*. 2012; Iwase *et al*., 2011). Functional Annotation and Pathway Analysis involve assigning biological functions to genes by leveraging existing databases of known information. Pathway analysis is a valuable tool for understanding the intricate networks of genes and regulatory pathways within protoplasts (Shang *et al*., 2016; Fan *et al*., 2012)

Proteomics is mainly involved in thoroughly examining the proteins found within protoplasts. This analysis aims to capture a comprehensive view of the cell's proteome, including any post-translational modifications that may have occurred (Wang *et al*., 2017). Mass spectrometry in proteomics enables the discernment and measurement of proteins within protoplasts. This method makes identifying proteins that display variable expression, post-translational changes, and interactions with other proteins easier. The protein localisation and dynamics approach make detecting the subcellular localisation of proteins within protoplasts easier (Wang *et al*., 2017).

Additionally, proteomics makes it possible to identify changes in protein abundance in response to various circumstances. Other than that, the information about the dynamic nature of biological activities can be gathered. Proteomics can also identify and characterise post-translational changes, such as ubiquitination, glycosylation, and phosphorylation. This method enables the elucidation of the regulatory mechanisms that govern protein function.

A complete picture of the metabolite composition in protoplasts is provided by metabolomics, which entails a thorough examination of small molecules. Mass Spectrometry and Nuclear Magnetic Resonance (NMR) can be used to detect and quantify metabolites (Nagana Gowda & Raftery 2021) within protoplasts. The provided information elucidates alterations in metabolic pathways and cellular reactions. Combining metabolomics with other omics data enables the construction of metabolic pathways and facilitates comprehending the metabolic network within protoplasts. This process facilitates the identification of crucial metabolic nodes and potential targets for metabolic engineering.

In order to create a comprehensive understanding of biological processes and their dynamic interconnections, systems biology incorporates data from many omics technologies. Network analysis is a technique used in systems biology to comprehend the complex interactions between protoplasts' genes, proteins, and metabolites (Brady & Benfey 2006). Network models play a crucial role in enabling the detection of central hubs and emergent properties within cellular processes. Furthermore, computational modelling techniques, such as kinetic modelling and metabolic flux analysis, also facilitate the simulation of cellular behaviour by leveraging omics data (Locke *et al*., 2005a). This approach offers a

predictive framework for understanding how cells respond to various stimuli.

Implementing omics technologies in protoplast research encompasses a wide range of applications, which contribute to a deeper understanding of fundamental biological processes and facilitate advancements in the biotechnology field. For example, functional genomics utilises omics data to investigate the functionality of genes and regulatory elements to the behaviour of protoplasts and their responses to environmental stimuli. The application of omics technologies has also proven as instrumental in elucidating the intricate molecular mechanisms that govern stress responses in protoplasts, offering valuable insights into stress tolerance and adaptation mechanisms (Hennig *et al*., 2015). These approaches facilitate the identification of potential targets, thereby facilitating the development of enhanced crop varieties.

SYNTHETIC BIOLOGY AND PROTOPLASTS

Synthetic biology techniques, exemplified by CRISPR-Cas9, offer a precise means of modifying plant genomes to incorporate advantageous traits, including diseases resistance, enhanced nutritional composition, and improved tolerance to abiotic stresses. Together with that, protoplast-based synthetic biology techniques are employed to enhance the domestication process of wild plant species by introducing desirable traits that increase their suitability for agricultural cultivation. Furthermore, the technique of gene stacking and trait pyramiding, facilitated by synthetic biology, allows for simultaneously incorporating multiple genes into protoplasts, combining desirable traits within a single plant variety. The experimental validation of this method has been conducted in multiple plant species, including the protoplast of potatoes (Andersson *et al*., 2017; Tuncel *et al*., 2019; González *et al*., 2020; Zhao *et al*., 2021), *N. tabacum* (Lin *et al*., 2018), *Brassica oleracea* (Park *et al*., 2020), lettuce (Woo *et al*., 2015), and petunia (Yu *et al*., 2020).

The advent of protoplast-based synthetic biology presents a range of prospects and concerns regarding its environmental and agricultural ramifications. Using protoplast-based synthetic biology, a viable and environmentally conscious substitute for conventional industrial methodologies in generating valuable substances can be achieved. The utilisation of this approach decreases the dependence on resource-intensive chemical synthesis methods and mitigates the environmental consequences associated with pharmaceutical and chemical manufacturing processes. The utilisation of protoplast-based synthetic biology has the potential to enhance agricultural sustainability through the development of crop varieties with improved traits. The enhancement of disease resistance and stress tolerance in crops reduces reliance on chemical inputs, thereby fostering the adoption of environmentally sustainable farming methods.

PROSPECTS FOR THE FUTURE AND EMERGING TRENDS

Promising potential uses and improvements and the advent of cutting-edge technologies and instruments make the future of protoplast research extremely promising. Research on protoplasts is still developing, bringing with it new applications and developments that have the potential to revolutionise many industries and branches of science. As protoplasts offer a flexible framework for synthetic biology and metabolic engineering, enabling the reprogramming of cellular metabolism to generate valuable crops using protoplasts is also affordable. With the evolution of new genetic engineering methods, such as CRISPR-Cas9, plant genome is possible to manipulate precisely. This will speed up efforts to improve crops and solve some of the biggest problems in agriculture around the world. Protoplasts also contribute to the advancement of our fundamental understanding of plant biology by helping to decipher the complex processes of plant development, from cell differentiation through organogenesis.

Technological developments are changing the field of protoplast study and giving scientists new instruments to investigate the molecular complexity of plant cells. Through profiling individual protoplasts, single-cell omics technologies, such as single-cell RNA sequencing and single-cell proteomics, can reveal cellular heterogeneity and regulatory dynamics. Platforms for high-throughput screening make it possible to quickly evaluate genetic alterations and functional assays in protoplasts, hastening the identification of genes and pathways important for particular features.

CONCLUSION

Protoplast research has led to revolutionary plant biotechnology discoveries. This comprehensive overview covered protoplast research, from early discoveries to cutting-edge advances. Protoplast has transformed biology, biotechnology, and agricultural development. Protoplast research has bright prospects. In order to address global concerns in food security, agriculture, and sustainable biotechnological solutions, protoplast research holds

transformative promise. Protoplast-based biotechnology can aid in climate-resilient agriculture, assuring steady yields in climate change by precisely modifying crop genomes for greater stress tolerance. In addition, protoplasts provide a green and sustainable alternative to resource-intensive chemical procedures for synthesising important chemicals, resulting in environmentally friendly biofactories. Furthermore, protoplast technology can aid in conserving endangered plant species, preserving their genetic diversity and contributing to biodiversity conservation efforts. Finally, protoplast-based techniques for micropropagation and plant breeding can hasten the creation of new crop types and increase agricultural production.

ACKOWLEDGEMENT

The study was financially supported under Temporary Research Fund (02-03-TRF0008) and 12th Malaysian Plan Development Fund (P20001001210005 – Development of Cocoa Agri-Biotechnology Innovation Programme). Special thanks to the Director General of Malaysian Cocoa Board, Deputy Director General Research and Development and Director of Biotechnology Division for their continuous support and kind approval to publish this research.

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