Plant protoplasts in the age of synthetic biology

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Highlight

Protoplasts have long contributed to plant biology. This review discusses how protoplasts aid in the "design-build-test-learn" cycle in synthetic biology and how this approach can help other areas of research.

Abstract

Protoplasts, which are plant cells with their cell walls removed, have been used for decades in plant research and have been instrumental in genetic transformation and the study of various aspects of plant physiology and genetics. With the advent of synthetic biology, these individualized plant cells are fundamental to accelerate the "design-build-test-learn" cycle, which is relatively slow in plant research. Despite their potential, challenges remain to expanding the use of protoplasts in synthetic biology. The capacity of individual protoplasts to hybridize to form new varieties, and to regenerate from single cells, creating individuals with new features is underexplored. The main objective of this review is to discuss the use of protoplasts in plant synthetic biology and to highlight the challenges to exploiting protoplast technologies in this new "age of synthetic biology".

Keywords

Protoplasts, Synthetic biology, Regeneration, Genetic transformation, Miniaturization, Genetic circuits, Cell wall digestion.

Introduction

The concept of protoplasts, which are individualized plant cells that have had their cell walls removed, was first introduced by Hanstein in 1880 as a way to describe the "living matter" enclosed by cell walls (Hanstein, 1880). Then in 1892, the isolation of protoplasts was achieved through the use of microsurgery on plasmolyzed cells, as described by Klercker (Klercker, 1892). In 1960, Cocking published the first method for isolating protoplasts using cell wall digesting enzymes, a technique that is still widely used today (Cocking, 1960). During the 1970s, protoplasts were extensively used to study various aspects of plant cell wall regeneration, cell division, and differentiation (as discussed in Sheen, 2001). In the 1980s and 1990s, protoplasts became a popular tool for genetic transformation due to the development of efficient methods such as electroporation (as described by Fromm et al., 1985; Hauptmann et al., 1987; H. Jones et al., 1989; Negrutiu et al., 1987; Nishiguchi et al., 1987; Ou-Lee et al., 1986 and polyethylene glycol (PEG)-based transfection (as described by Krens et al., 1982; Potrykus et al., 1985). Since then, protoplasts have been widely used to study various aspects of plant physiology, cell ultrastructure, and genetics (as discussed in Davey et al., 2005).

In plants, the time required for generation of transgenic organisms ranges from months to years. Additionally, once a transgenic locus has been incorporated into the host, the number of inserted copies or the site of insertion might have a significant effect on the overall activity of the transgene, resulting in the need to generate multiple independent lines to reduce variability. In this regard, researchers have adopted the use of transient transformation methods where DNA is not integrated into the host's genome and phenotypes can be observed within hours after transformation. The most widely used system for rapid gene testing is agroinfiltration, most commonly in *Nicothiana benthamiana* leaves (Yang et al., 2000), a wild relative of tobacco. While *N. bentamiana* agroinfiltration is useful to rapidly assess the heterologous expression of genetic circuits, not all species are amenable to agroinfiltration limiting the application of this approach.

Synthetic biology involves the design and construction of biological systems following an iterative process that combines engineering principles and molecular biology. By assembling biological components such as DNA, proteins, and cells in a modular and predictable way, synthetic biology has the potential to systematically study biological function or to engineer new features that do not exist in nature. With the interest in using synthetic biology for the generation of high yielding, resilient crops, or the production of industrial and therapeutic compounds, the adoption of protoplasts has become a powerful alternative to accelerate the “design-build-test-learn” cycle that is the basis of synthetic biology because efficient transient transformation methods can be applied to a wide variety

of species. Indeed, the high yields of protoplast isolation and transfection has allowed the qualitative and quantitative characterization of genetic parts in a high-throughput manner. This is demonstrated in recent papers aiming at defining the regulatory logic of genetic parts (Cai et al., 2020; Pfotenhauer et al., 2022; Schaumberg et al., 2015). For instance, Cai and collaborators used protoplasts derived from *A. thaliana, N. benthamiana, B. rapa* and *H. vulgare* for the rapid characterization of minimal synthetic plant promoters. In this work, the authors were able to design a suite of promoters with varied strengths and with the option of being activated by either endogenous or exogenous transcription factors. Many of these synthetic promoters were then validated in whole plants offering scientists a toolset for the control of gene expression of synthetic genetic circuits. A similar approach was followed by Pfotenhauer who characterized 91 plant expression cassettes in protoplasts obtained from transfected *N. benthamiana* leaves. In this case, the characterization was extended to other regulatory regions including 3´and 5´untranslated regions (UTRs) highlighting the importance of pairing promoters with appropriate UTRs to achieve desired levels of gene expression.

The need to expand the current *cis*-regulatory repertoire in plants for synthetic biology has motivated the application of techniques for the parallel characterization of native promoters like self-transcribing active regulatory region sequencing (STARR-seq) (Jores et al., 2020, 2021; Ricci et al., 2019; Sun et al., 2019) In STARR-seq, arrays of putative promoters or regulatory elements are synthesized and fused to specific barcode sequences and a reporter gene. The resulting reporter library is transfected into a desired organism followed by mRNA extraction and barcode sequencing. In this case, the use of protoplasts derived from species like *Z. mays* where methods of agroinfiltration are less suitable has been useful. As the number of sequenced genomes expands and both gene synthesis and sequencing costs are reduced, protoplasts will play a pivotal role in the characterization of regulatory elements across the plant kingdom.

Protoplasts have been extensively used for validating the mutagenesis efficiency of DNA-free gene editing protocols in which the CRISPR/Cas machinery is delivered directly to the cells without DNA insertions (see (Yue et al., 2021)). As the community engages in the application of these techniques to non-model species, protoplasts will play a pivotal role especially in plants less suitable to stable transformation methods. At the same time, many challenges remain in the use of protoplasts for plant synthetic biology. One of the biggest challenges is the regeneration of whole plants from single protoplasts by exploiting the inherent capacity of protoplasts to retain their regenerative potential. Indeed, some plant species are more amenable for regeneration and so, understanding the mechanisms involved in this process might help to overcome an important bottleneck for plant cell reprogramming.

Limitations and challenges

A successful protoplast isolation assay will depend on both intrinsic (plant species, age and source of the plant tissue) and extrinsic factors (conditions of plant growth, protoplast culture and enzyme combinations). Indeed, protoplast isolation is possible in most of the current models for plant synthetic biology such as *Marchantia polymorpha*, *Physcomitrella patens*, *Camelina sativa*, *Arabidopsis thaliana*, *Nicotiana sp.*, *Lycopersicum esculentum* or the monocots *Brachypodium distachyon*, *Zea mays* and *Setaria italica*. Protoplasts can be extracted from a variety of source tissues. There are examples of protoplasts isolated from most plant tissues and organs, including leaves, roots, stems, flowers, pollen, embryos, aleurone layers, spores, callus or cell suspension cultures (Eriksson, 1985). Previous reviews offer further detail in terms of the main variables to consider for a successful protoplast isolation (Eriksson, 1985; Jen Sheen, 2001; Yue et al., 2021). While most methods for protoplast isolation are based on the initial work by Cocking, as the number of plant chassis for synthetic biology expands, the experimenter is required to calibrate the protoplast isolation protocol according to his/her own needs.

An important factor to consider is the environmental conditions in which the plants are grown and harvested. This has a significant influence in the yield and viability of isolated protoplasts (Yoo et al., 2007). For instance, some of the most well-established methods for protoplast isolation incorporate the use of etiolated or greening tissue for both monocots and dicots (J. Sheen et al., 1991; Jen Sheen, 2001). This might not be compatible with some of the applications intended for the protoplasts in some species. In fact, the plant species or the specific tissue for protoplast isolation will also dictate the combination of cell wall digesting enzymes, incubation times or even the need to mechanically assist in the separation of tissue. Some improvements have been done in this regard. For instance, the "Tape-Arabidopsis Sandwich", whereby epidermis is physically removed with the use of tape, leaving the leaf mesophyll more exposed to digestion (F. H. Wu et al., 2009).

Perhaps the biggest concern of employing protoplasts is not related with their isolation efficiency but with the actual conclusions that could be drawn from their use. The process of cell separation and removal of the cell wall could have an impact in the genetic program of these cells. While protoplasts could remain viable and retain many of their biochemical and cellular activities (Figure 1A), the prolonged exposure of plant tissue to cell wall degrading enzymes can influence the transcriptional programme of the cell to different degrees thus affecting the physiology of the plant (Covshoff et al., 2013; Sawers et al., 2007). This is particularly relevant for the isolation of protoplasts derived from tissues requiring longer incubation periods. To account for the potential noise caused by protoplast

isolation in itself, researchers have implemented a series of stress controls. First, protoplast viability can be tested using different vital staining protocols (Huang et al., 1986; K. H. Jones & Senft, 1985; Widholm, 1972). On the other hand, protoplast stress levels can be quantified by monitoring the activity of stress responsive genes or stress responsive promoters fused to reporter genes (Yoo et al., 2007). Alternatively, genes of interest can be compared against values obtained from whole tissue, and against values obtained from whole tissue that has undergone a mock protoplast digestion without cell-wall digesting enzymes (Covshoff et al., 2008; Sawers et al., 2007). Although some applications might not allow for such controls, these are important considerations especially when the conclusions generated from protoplasts are used to infer the behavior of whole plants. One potential solution to validate inferences from experiments with protoplasts could be to incorporate a regeneration step, in which single cells with the desired behavior are selected to regenerate into whole individuals.

Box 1.- Integration of protoplasts into "Lab-on-a-Chip" technologies

A "Lab-on-a-chip" takes advantage of miniaturization for the creation of closed environments in which single cells can be manipulated, grown and phenotyped under different conditions (Linshiz et al., 2016). In conjunction with protoplasts, this approach has shown great versatility for the study of plant growth and development (Sanati Nezhad, 2014). For example, microfluidic platforms have been generated for studying the developmental progression of regenerating protoplasts obtained from mosses (Bascom et al., 2016; Sakai et al., 2019) and tobacco (Zaban et al., 2014). Microfluidics platforms have also been used to generate suitable microenvironments for protoplast fusion. Wu et al., created a platform for protoplast fusion that reached similar fusion rates to those obtained in macro-scale environments (Wu et al., 2011). Indeed, the capacity of microfluidics to handle low volumes provide an ideal platform for plant synthetic biology. There are several examples where microfluidics has facilitated the collection (Hung & Chang, 2012), encapsulation (Grasso & Lintilhac, 2016; Yu et al., 2018), culture (Ko et al., 2006; Sakai et al., 2019; Wu et al., 2011) and screening of protoplasts at a high-throughput scale (Yu et al., 2018). Indeed, microfluidic platforms could be seen as miniaturized experimental modules that could be integrated together. A full integration of chips developed independently would require the establishment of a "common syntax" for microfluidics similar to that used for the generation of genetic circuits (Patron et al., 2015). For example, one could assemble different chips to create a system where protoplasts are incubated and transformed into the same chip. Then, a particular genetic circuit or novel metabolic route could be phenotyped in a different chip module. Finally, those cells showing the desired phenotypes could be selected and regenerated into full plants (**Figure 2**). This would certainly help overcome some of the "design-build-test" bottlenecks currently present in plant synthetic biology.

Regeneration of protoplast as the next frontier?

Reliable control of protoplast regeneration remains challenging, and the genetic cause of why some tissues, species or cultivars, can more easily be regenerated than others is an active area of research. Protoplast regeneration requires cell wall regeneration followed by the establishment of cell wall identity through the expression of key transcriptional regulators (Sugimoto et al., 2019). In somatic cells, developmental regulators are typically under epigenetic silencing, but stress can induce changes in chromatin structure, altering gene expression (Probst & Mittelsten Scheid, 2015) and is proposed to be responsible for stochastic reactivation of stem cell regulators (Xu et al., 2021). These findings explain why various additives and stress treatments can stimulate protoplast regeneration (Reed & Bargmann, 2021). Removal of the cell wall results in a change in mechanical properties, triggering stress induced alterations in gene expression. Genes involved in sensing and signaling changes in mechanical force are poorly understood, but stretch sensitive ion channels, polysaccharide signaling molecules, proteins that bridge the cell-wall and plasma membrane could act as mechanosensors that activate the regeneration process. Further research could provide insight into their potential role in the regeneration of the cell wall prior to the reestablishment of cell division (Fruleux et al., 2019). In addition, global increases in chromatin accessibility during protoplast regeneration have been observed by ATAC-SEQ, and treatment with trichostatin A (TSA), an inhibitor of histone deacetylases, increased chromatin accessibility and promoted regeneration efficiency (Xu et al., 2021). Moving forward, combining insights into the interplay between stress signaling, chromatin accessibility and stem cell regulators in individual cultivars may provide the basis for regenerating recalcitrant genotypes.

Plants can be regenerated from both embryonic material or somatic cells (Reed & Bargmann, 2021), but the ability to regenerate plants from protoplasts is highly dependent on the species, genotype and tissue involved (Roest & Gilissen, 1989). Due to species and cultivar specific variation, the process of regeneration from protoplasts continues to be refined on a genotype specific basis, with further reports of field cress (*Lepidium campestre*) (Li et al., 2021), forage and turf grass (Wang & Spangenberg, 2022), chicory (*Cichorium intybus var. foliosum*)(De Bruyn et al., 2020), and citrus (Mahmoud et al., 2022) recently reported.

However, significant advances have been made over the past decade in understanding the molecular basis of somatic embryogenesis, the process by which embryos are formed from mature cells. Evidence suggests the first stage of regeneration of protoplasts derived from somatic cells is de-differentiation, this includes loss of expression of cell-fate determinants, de-condensing of chromatin,

epigenetic reprogramming and resumption of mitotic cell division (as reviewed by Ikeuchi et al., 2019). These processes are triggered by digestion of the cell wall, and transiently incubating cells in medium containing high concentrations of auxin (Rather et al., 2022). The addition of further stresses, such as heat shock, can be further used to increase the efficiency of regeneration (Reed & Bargmann, 2021). Several key transcription factors have been identified and overexpression of master regulators have been shown to aid plant regeneration and protoplasts as reviewed (Ikeuchi et al., 2019; Rather et al., 2022). In particular, although a variety of transcription factors have been identified (e.g. GRFs), research suggests expression of the master regulator WUSCHEL (WUS) is required for activation of a transcription factor network involving LEAFY COTELYLEDON 1 (LEC1) and LEAFY COTELYLEDON 2, (LEC2) which work together with BABYBOOM (BBM) and AGL15 to promote somatic embryogenesis (Ikeuchi et al., 2019). Overexpression of BBM has proven able to promote embryogenesis in a range of species, and importantly the combination of BBM and WUS expression was able to facilitate regeneration of recalcitrant monocot species (Figure 3)(Lowe et al., 2016).

A combination of single cell imaging, transcriptomics and chromatin accessibility assays demonstrated protoplasting of mesophyll cells from Arabidopsis induced widespread stochastic changes in gene expression due to altered chromatin accessibility (Xu et al., 2021). These findings indicated that cells competent for regeneration are produced by stochastic activation of key regulators, providing an explanation for the low efficiency of regeneration. Consistent with these suggestions, the transcription factors DRN and WUS were necessary for protoplast regeneration, and efficiency of regeneration could be increased by overexpression. Moving forward it will be important to test whether a similar strategy can be adopted to increase the rate of protoplast regeneration in a wider variety of species along with greater control over stem cell identity, and whether using RNP-based CRISPR activation systems to activate BBM and WUS expression can stimulate transgene free regeneration.

Genome instability following protoplast regeneration is a significant concern for realizing the potential of protoplast regeneration (Fossi et al., 2019). A comparison of potato lines derived from either protoplasts or explants revealed increases in aneuploidy and genome rearrangements (Fossi et al., 2019). However, the precise cause, and the prevalence of abnormalities caused by protoplasting in different species is currently unclear. In contrast to Fossi et al. (2019), no evidence of chromosome instability was found in plants regenerated from protoplasts of wild tomato species *Solanum peruvianum* (Lin et al., 2022). The proposed reason for this difference in outcomes lies in the process of chromosome pairing during cell division: in contrast to the diploid *S. peruvianum*, potato is an autotetraploid consisting of two pairs of non-homologous chromosomes which may lead to a higher

rate mis-segregation during mitosis (Lin et al., 2022). Given these findings, further research is required to better understand the potential for and causes of genomic instability in target species.

Gene editing using preassembled CRISPR-Cas9 ribonuclease protein (RNP) complexes affords the opportunity of creating transgene-free edited cells (Woo et al., 2015); because the Cas9 is not integrated into the host genome only the target sequence is modified and a selectable marker is not required (reviewed by Zhang et al., 2021). Transgene-free edited calli or protoplasts can be regenerated into plants, potentially alleviating some concerns associated with transgenic crops (Lin et al., 2018; Reed & Bargmann, 2021). RNP editing can be particularly beneficial for sterile, slow growing, or clonally propagated crops, such as sugarcane, potato and especially woody trees such as Prunus, Malus or Vitis, where CRISPR-Cas9 transgenes cannot be removed from germplasm by backcrossing. Editing using biolistic transformation of calli frequently results in the production of chimeras (Morales & Thomson, 2022), which can prevent transgenerational inheritance of mutations, when somatic rather than germline cells are modified (Zheng et al., 2020). The chance of chimera production is significantly reduced when regenerating from a single cell. In addition, for many species both viral and agrobacterium mediated transformation protocols suffer from a lack of host sensitivity. Transformation and regeneration from protoplasts may therefore be preferable if a suitable regeneration procedure exists.

There has been significant interest in developing procedures for RNP-based gene editing and regeneration of protoplasts from a variety of species over recent years (Table I). While initially focusing on gene editing, these approaches have the potential to assist in the creation of improved varieties by targeting candidate genes, such as those known to be involved in susceptibility to pathogens. This approach could be adapted for *de novo* domestication of crops, using wild varieties with greater stress resilience and better resource use efficiency as starting material (Fernie & Yan, 2019). Such efforts are aided by a growing list of over >25 genes commonly implicated in domestication that could be potential targets (Fernie & Yan, 2019), and was recently demonstrated by RNP-based editing and regeneration of wild tomato (*Solanum peruvianum*) protoplasts, in which genes responsible for pathogen susceptibility were edited, with modified plants obtained within six months (Lin et al., 2022).

A remaining challenge involves the possibility to control of genetic modification. When looking to create targeted deletions, Andersson et al. 2018 found a high percentage of lines (~80%) derived from CRISPR RNP mutagenesis included fragments of integrated DNA, from either in vitro transcribed donor RNA or chromosomal DNA, at cut sites, reducing the frequency of lines of commercial importance (as defined as lines without DNA integration) (Andersson et al., 2018). However, this phenomenon was turned into an advantage by Jiang et al. 2021, who demonstrated the full potential of CRISPR-RNP based editing in protoplasts through targeted insertion by homology directed repair co-

transfecting the Cas9-RNP with a single-stranded oligodeoxynucleotide containing 70 nt homology flanking region, as well as in the case of prime-editing, achieving efficiencies of up to 7% and 4.6% respectively (Jiang et al., 2021). These findings were further validated by (Hsu et al., 2021), who also demonstrated targeted replacement using RNP and a donor DNA molecule, followed by regeneration in *Brassica oleracea* and *N. benthamiana* (Hsu et al., 2021). In summary, these studies are an encouraging demonstration and provide a future platform for further development towards the ideal of routine targeted replacements in plant cells.

Designing, building and testing plant biology

Moving forward, there is scope to combine conventional protoplast assays with synthetic biology techniques to create high-throughput discovery platforms for the study of gene function, to exploit plant metabolic diversity or to develop novel breeding and synthetic biology tools (Figure 4). Next, we highlight some potential applications of this approach in plant sciences.

High-throughput screening of genetic parts

Modular plasmid construction, coupled to bulk transformation and screening can be used to study and optimize the function of regulatory elements, such as promoters, enhancers and terminators. These approaches have been used in microbial systems for comprehensive analysis of part libraries, such as screening >200,000 synthetic sequences (Cambray et al., 2018) or the optimization of metabolic pathways while reducing toxicity (Jones et al., 2015). However, characterization on a similar scale in plants remains challenging. One of the largest studies in plants to date, involved screening >100 genetic parts using protoplasts from Arabidopsis and sorghum and individually synthesized constructs (Schaumberg et al., 2015). While this work opened the possibility to build and test tunable gene circuits in plants, the authors identified batch effects between protoplasts. To solve this, they developed a mathematical models to account for such effects on reporter expression (Schaumberg et al., 2015). An additional factor limiting the use of protoplasts in high-throughput pooled screens is the requirement for large amounts of DNA for efficient transformation via PEG, electroporation, or microinjection (Yoo et al., 2007). One potential solution could be to leverage advancements in DNA delivery systems used in mammalian, microbial and algal systems which are largely unexplored in the context of protoplasts, including cell-penetrating peptides, nanomaterials and membrane disruption techniques (as reviewed by Lv et al., 2020). While there have been a few reports of using different species of nanoparticles to transform protoplasts, the efficiency was still relatively low (as reviewed by Burlaka et al., 2015; Lv et al., 2020). As mentioned before, other approaches using protoplasts for high-throughput characterization of regulatory elements like STARR-seq have contributed to the optimization of synthetic promoters and to our understanding of transcription in plants. For instance, Ricci et al., used this technique to test the activity of distal *cis*-regulatory elements obtained from accessible chromatin regions defined by ATAC-seq (Ricci et al., 2019). This work supported the

functional relevance of distal loci acting as *cis*-regulatory elements. Similar works also found that enhancers positioned immediately upstream the promoter had the highest activity and that localization of such elements towards the 3´-UTR position reduced gene expression considerably (Jores et al., 2020). If established routinely, techniques like STARR-seq coupled with protoplast technologies could be applied to test the activity of regulatory element in different cellular and environmental contexts. For instance, a library of regulatory elements could be assayed on protoplasts derived from different species, isolated from distinct tissues or exposed to particular stresses.

Metabolic diversity

Plants possess a remarkable ability to synthesize a wide range of molecules which contribute to their metabolic diversity. The study of secondary metabolites is very important for the industrial sector including chemical, food and pharmaceutical industries. However, one important bottleneck for the study of metabolic diversity is the lack of understanding of how these metabolites are produced or how their synthesis is regulated *in planta* (Oksman-Caldentey & Inzé, 2004). Cell heterogeneity can obscure the discovery of new enzymes and metabolites since the production of such compounds is limited to specific cells or tissues (de Souza et al., 2020; Misra et al., 2014; Schenck & Last, 2020). Protoplasts are particularly useful for providing sufficient tissue-specific material to overcome the detection limits for certain metabolites. Despite the challenges associated in obtaining metabolites from uncommon cell types, protoplasts derived from cell-specific tissue has been used for the study of plant metabolism. For instance, Jin et al., used protoplasts derived from guard cells of Arabidopsis to profile several metabolites related to Abscisic Acid (ABA) signaling (X. Jin et al., 2013). Also, Tohge et al., employed mesophyll derived protoplasts to profile the metabolic content of vacuoles in Barley (Tohge et al., 2011). Secondary metabolites can be elicited under specific environmental conditions adding an extra layer of complexity in metabolic profiling (Dixon, 2001). In this context, protoplasts could be very useful for screening the metabolomic profiles of cultures exposed to different environmental conditions. At the same time, this approach could be complemented with the use of single cell transcriptomics technologies (Giacomello, 2021) to identify putative genes whose expression is correlated with the accumulation of metabolites at a cell-specific scale. Once enzymes and genes involved in secondary metabolite biosynthesis have been identified, protoplasts can also be used for optimizing the engineering of specific metabolic pathways. For instance, genetic circuits containing different combinations of enzymes could be transformed and screened for specific metabolites using a high-throughput screening system like the one mentioned in **Box 1**.

Development of breeding and synthetic biology tools

Protoplasts can be transfected with CRISPR-Cas9 constructs or RNP to perform gene editing. However, a major challenge is identifying regenerants which possess the desired edits. Common approaches involve destructive techniques which limit throughput, including T7 endonuclease I assay,

PCR coupled to restriction digest, and sequencing. To address this issue, Petersen et al. developed a protocol which transformed protoplasts with Cas9 fused to GFP with a 2A self-cleaving peptide, followed by FACS to enrich for edited lines (Petersen et al., 2019). While not a direct test of editing efficiency, this allowed for enrichment of edited cells from ~20 to 80% (Petersen et al., 2019) with the potential to significantly reduce the number of protoplasts that need to be regenerated to full plants to identify positive lines.

In addition, protoplasts can be used to accelerate the process of plant breeding. Somatic hybridization, or the process by which protoplasts from different species or varieties are fused together to create hybrids, was first established in the early 1900s and developed as a method for introgression of novel traits (Sivanesan et al., 2023), such as abiotic and biotic stress resilience (Jia et al., 2022; Tu et al., 2021), to increase the uniformity of open pollinated crops (Bruznican et al., 2021), and establish triploid varieties for the production of seedless fruit (Ćalović et al., 2019). When coupled to plant regeneration, protoplast fusion can overcome reproductive barriers, such as sexual incompatibility between species, and bypass long juvenility (Ćalović et al., 2019). However, somatic hybridization is limited by low efficiency and difficulty in identifying of successful events. Tagging cell lines with different fluorescent proteins allows for facile identification of hybrid progeny (Gieniec et al., 2020; Olivares-Fuster et al., 2002); if coupled to fluorescence activated cell sorting (FACS), or sorting in a microfluidic device, it may be possible to enrich for successful events at an early stage in the regeneration process.

Coupling protoplast assays to methods for sorting and regeneration has the potential to speed up development of new improved varieties. For example, several assays have been developed for screening pathogen resistance using protoplasts, including screening for cell surface receptors involved in immunity using fluorescent tagged quantum dots (MgCina et al., 2015), and cell death assays when investigating receptor effector interactions (Saur et al., 2019). It would be intriguing to see whether these methods could be developed to create a selection system for development of pest resistance. In addition, further adoption of RNA aptamers (Mou et al., 2022), or biosensors (Beltrán et al., 2022), as reporters for small molecule production could provide opportunities for high-throughput screening and optimization of metabolite production. An approach for which protoplasts have been under-used is conventional mutagenesis screens for gene discovery. As highlighted previously, genome wide CRISPR screens, which have been widely applied in microbial and mammalian systems have not been adapted for plant protoplasts (Gaillochet et al., 2021). It was speculated this may be a result of multiple guides entering a cell simultaneously, if this is true, coupling protoplast transformation to droplet microfluidics may represent an opportunity for increasing control over DNA:cell ratios (Kim et al., 2019). However, conventional mutagenesis has been used to generate

diversity in protoplast physiology which can be screened: Jin et al. reported a protoplast screen using Gamma irradiation, followed by culturing in presence of salt and tissue culture to identify plantlets of *Nicotiana benthamiana* that were able to grow in NaCl up to 200 mM (D. M. Jin et al., 2020). A similar approach, without application of a mutagen, was pursued with three carrot accessions, using spontaneous mutation in the presence of salt to identify resistant lines (Kiełkowska et al., 2019). These studies suggest that coupling selective screens to plantlet regeneration may be more broadly effective as a means of developing stress resilient cultivars.

Conclusions

For many decades, protoplasts have been an enabling tool to overcome challenges including increasing genotypic diversity, through creating interspecies and intraspecies hybrids, and performing cell biology and transcriptional assays, by providing a rapid system for transient transformation that avoids issues relating to relatively long life cycles and difficulties transforming or regenerating recalcitrant genotypes. With the advent of plant synthetic biology, protoplasts have the potential to accelerate engineering efforts, but current techniques are still far from reaching this goal. As the field of plant synthetic biology moves forward, protoplast technologies need to advance at the same pace. There is a need for more standardized methods for the generation and maintenance of good quality protoplasts that are less dependent on the proficiency of the experimentalist. In addition, normalization strategies for different batch effects together with controls related to the effects of cell-wall digesting enzymes on cellular activity need to be considered. Finally, significant steps are still required to fully understand the process of protoplast regeneration to fully capitalize on advantages in gene editing, and leverage the potential for applied research. This is perhaps one of the areas with more potential for plant synthetic biology. To reach this goal a better understanding of the underlying biology of protoplasts is fundamental.

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Figure Legends

Figure 1. Protoplast isolation and transfection. Both intrinsic and extrinsic factors influence protoplasts separation in plants. Viable protoplasts will retain an intact plasma membrane. This can be observed with the fluorescent dye fluorescein diacetate (FDA) (A). Genetic circuits can be rapidly tested using protoplasts. (B) PEG-transfected *Marchantia polymorpha* protoplast expressing a nuclear GFP marker under a constitutive promoter (35S). Bright field image of a protoplast (left), GFP fluorescence visible in the nucleus and cytoplasm (middle) and chlorophyll autofluorescence observed in the chloroplasts (right). White bar indicates 30 µm.

Figure 2.- Integration of protoplast with microfluidics can help overcome many of the bottlenecks of plant synthetic biology. For instance, microfluidic platforms could be implemented for protoplast transformation. Transformed protoplasts could then be encapsulated and incubated under different environmental conditions. Then, single cells producing a particular compound or carrying the desired levels of expression of a genetic circuit could be sorted and selected for the regeneration phase. As a result, whole plants could then be regenerated from this process.

Figure 3.- Simplified transcription network depicting master regulators of somatic embryogenesis and their interactions, adapted from (Ikeuchi et al. 2019). Auxin stimulates the production of WUS which induces embryonic regulators LEC1 and LEC2. These factors, along with AGL15 and BBM, form a network of interactions that activate expression of downstream regulators to control auxin production.

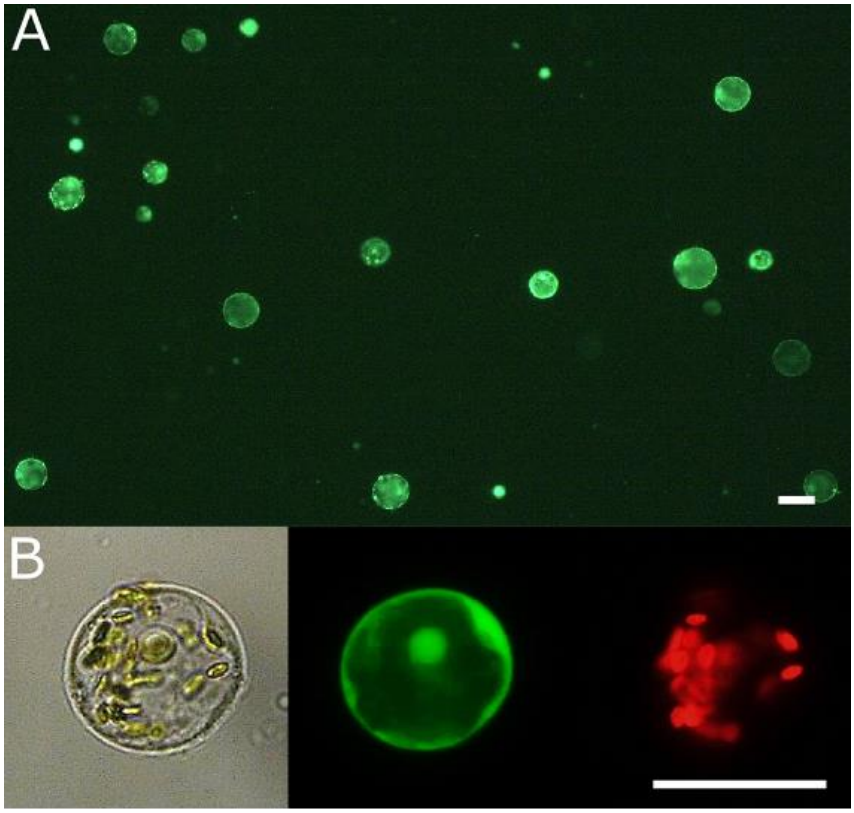
Figure 4.- The utilization of protoplast technologies and synthetic biology in a design, build, test and learn approach provides an opportunity to explore various aspects of plant biology. The screening of gene parts that modulate gene expression, the exploration of metabolic diversity at single cell resolution or the generation of novel breeding and synthetic biology tools for crop improvement are some of the areas with more potential for this approach.

Tables

Species	Source of protoplasts	Gene editing tool	Reference
Brassica napus L.	Hypocotyls	RNP CRISPR-Cas9 gene editing (LbCas12a)	(Sidorov et al., 2022)
Brassica napus L.	Leaf tissue	RNP CRISPR-Cas9 gene editing (SpCas9)	(X. Li et al., 2021)
Daucus carota subs. sativus	Leaf tissue	Cytidine base editor (A3A-PBE)	(Meyer et al., 2022)
Nicotiana benthamiana	Leaf tissue from in vitro plantlets	RNP CRISPR-Cas9 gene editing, cytidine base-editing	(Hsu, Lee, et al., 2021)
Nicotiana benthamiana	Leaf tissue	RNP CRISPR-Cas9 gene editing (SpCas9)	(Banakar et al., 2022)
Nicotiana benthamiana *Brassica oleracea*	Leaf tissue from in vitro plantlets	Targeted insertion using ssDNA and RNP CRISPR-Cas9 gene editing (SpCas9)	(Hsu, Yuan, et al., 2021)
Nicotiana tabacum	Leaf tissue from in vitro plantlets	RNP CRISPR-Cas9 gene editing. (Cas12a, nCas9-activation-induced cytidine	(Hsu et al., 2019) and (Wu et al., 2022)

		deaminase)	
Petunia cv. 'Madness Midnight'	Leaf tissue from in vitro plantlets	RNP CRISPR-Cas9 gene editing (SpCas9)	(Yu et al., 2021)
Solanum peruvianum	In vitro shoots (stem)	RNP CRISPR-Cas9 gene editing (SpCas9)	(Lin et al., 2022)
Solanum lycopersicum	Leaf tissue (cotyledons and first true leaves	RNP CRISPR-Cas9 gene editing (TrueCut™ Cas9 v.2)	(Liu et al., 2022)
Vitis vinifera	Embryogenic callus	RNP CRISPR-Cas9 gene editing (eSpCas9)	(Najafi et al., 2022)

Table I: Studies since 2019 investigating the combination of protoplasting, CRISPR RNP editing and plant regeneration. Species name, the source of protoplasts, and the CRISPR system used is provided.



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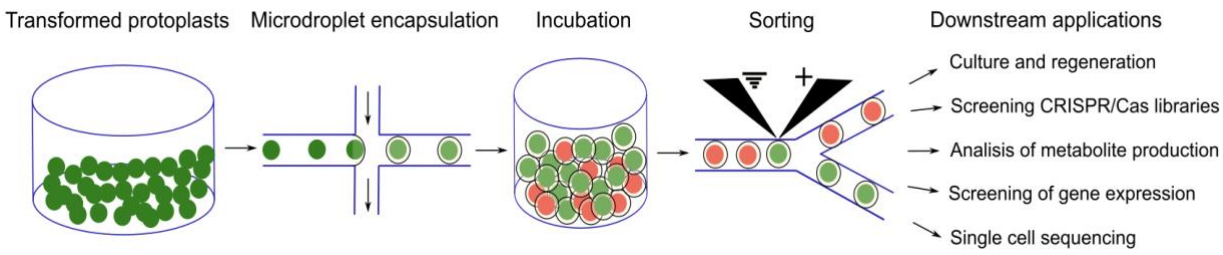

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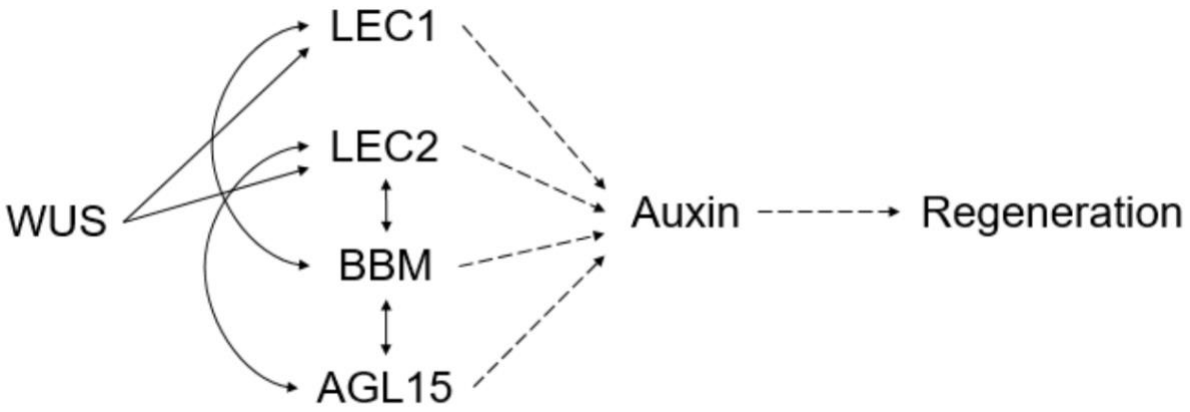

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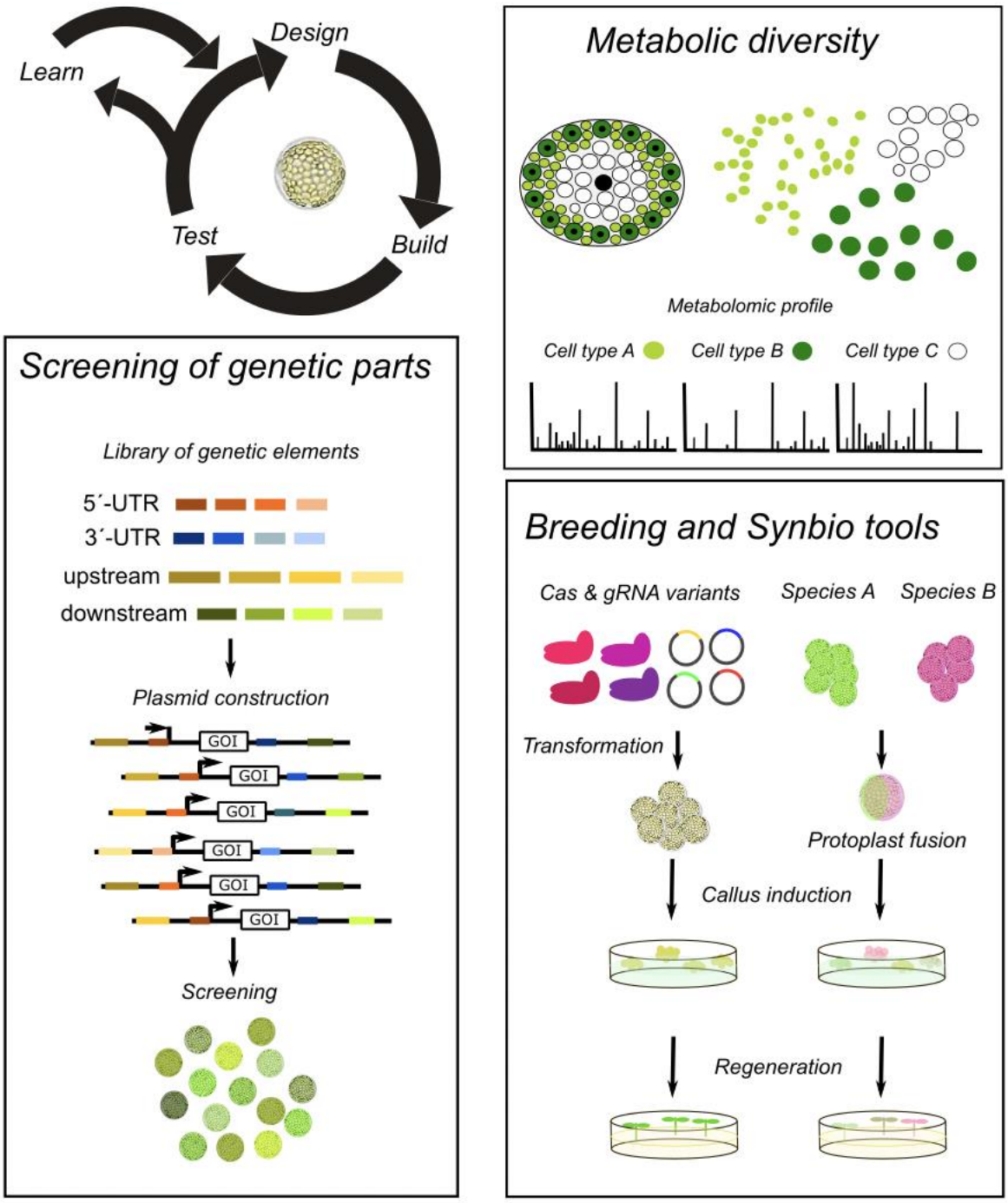

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