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PROTOPLAST AT THE TIME OF GENOME EDITING

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Abstract: Many uses of protoplasts, plant cells with the cell wall removed, have been explored. Many advantages of the system have been realized and proven in recent years in various physiological, biochemical, genetic, and molecular biological studies. Reliable methods to isolate viable protoplasts from a broad variety of plant species have been established. Regeneration of plants from protoplasts has become one of the options involved in crop gene manipulation and crop improvement. Here, we present how protoplast system may help crop gene editing and novel trait development, and discuss the potentials and challenges of this approach.

Keywords: CRISPR, crop improvement, gene editing, protoplast, regeneration, transient expression.

Introduction

Crop improvement through genetic manipulation has been in practice for decades. T-DNA-based gene overexpression, RNAi, or transposon insertional mutagenesis play a significant role in the manipulation of gene expression levels or changes of phenotypes. However, there is an increasing demand for simultaneous multi-gene manipulations for two main reasons: (1) The current wealth of different data types annotating the genome and how the many molecules in the parts interact with each other poses a demand to develop methods of integration that seamlessly connect genome-wide data; (2) Multi-traits development such as value added food, enhanced stress and pathogen-resistant crops, energy efficient architecture and increased yield also requires tools for multi-targeting and multi-manipulation. In the past a few years, multiplex genome editing strategies have been developed and become available for such a need. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are proteins that can produce double-strand DNA breaks that when repaired introduce site-specific mutations or insertions [JAGANATHAN & al. 2018]. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system uses RNAs to target nucleases to specific sites; when repaired, site-specific mutations or insertions are introduced [JAGANATHAN & al. 2018]. Multiple single guide RNAs (sgRNAs) with various target sequences can also direct Cas9 to multiple sites [CONG & al. 2013]. This feature of Cas9 allows simultaneous editing of multiple loci in the same individual.

Transgene-based delivery systems and non-transgene delivery systems are both applied to gene-editing. In the latter, additional advantages exist in protoplast system. Protoplasts have been successfully, and in some cases, routinely applied to complex signaling analysis in many plant species including *Arabidopsis*, tomato, tobacco, broad bean, maize, rice, wheat, barley, poplar, petunia, and moss [XING & WANG, 2015]. Various plant tissues can provide the cells for protoplast production. Protoplasts have been isolated from suspension cultures, callus cultures, embryos, shoots, and seedlings [XING & WANG, 2015]. The versatile cell-based assays have significantly facilitated an integrated

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understanding of some complex mechanisms such as plant signaling network [SHEEN, 2001; LI & al. 2015; XING & WANG, 2015; XING & al. 2017]. In addition to the above two main demands, protoplast system is a critical alternative in crop engineering in that they can be regenerated into plants [WOO & al. 2015; LIN & al. 2018]. Here, we will highlight the significant opportunities and challenges for plant protoplast system in crop genome editing.

Protoplast and large-scale screening

Genomic data of model systems and crop species have provided us with overwhelming amount of resources and discovery of the function of any genes in the genome (e.g. 25,000 in *Arabidopsis* and 41,000 in rice) is now within reach. While stable transformation takes considerable amount of time, initial screening can be achieved in a cost-effective manner in protoplast system [JUNG & al. 2008; XING & WANG, 2015]. The system is applicable for the analysis of gene expression effect, knock-out or knock-in gene editing effect and protein-protein interactions [EHLERT & al. 2006; LI & al. 2011, 2015; XING & WANG, 2015; SAKAMOTO & al. 2020]. Further developed robotic systems for protoplast isolation and transformation facilitated automated high throughput screening [XING & al. 2014; LOWDER & al. 2015; QUÉTIER, 2016; ČERMÁK & al. 2017].

As tissue culture and regeneration procedures to generate gene-edited events are time consuming, large-scale screening will facilitate rapid validation of genome-editing reagents and screening for resulting targeted mutagenesis [DLUGOSZ & al. 2016; NADAKUDUTI & al. 2019]. In the past a few years, protoplasts were successfully applied to gene editing analysis in *Arabidopsis* [LI & al. 2013, 2015], tobacco [LI & al. 2013], maize [LIANG & al. 2014], brassica [MUROVEC & al. 2018], rice [SHAN & al. 2014], wheat [WANG & al. 2014b; ZHANG & al. 2016; LUO & al. 2019], soybean [SUN & al. 2015], tomato [ČERMÁK & al. 2015], potato [ANDERSSON & al. 2017], strawberry [GOU & al. 2020], grapevine and apple [MALNOY & al. 2016]. Transient protoplast transfection is also an alternative strategy to test multiple mutagenesis parameters rapidly [LIN & al. 2018]. Hence, transient assays using protoplasts from various plant species hold great promise for increasing the speed at which genes can be studied, bridging the gap between the large data sets coming from high-throughput assays and the time consuming and laborious *in planta* investigations. Protoplast is also one of the main plant materials for Cas9 system delivery in various studies of crop species [MANGHWAR & al. 2019].

Selection of plant materials

Protoplast generation involves removal of tissue surface and enzyme treatment. The protocols for *Arabidopsis* mesophyll or maize mesophyll protoplast systems and *Arabidopsis* or tobacco BY-2 suspension cultured cells could serve as guidelines [SHEEN, 2001; YOO & al. 2007]. A simpler protoplast isolation method involving the use of two different adhesive tapes to sandwich *Arabidopsis* leaves was also developed [WU & al. 2009]. One should evaluate the isolation success before moving to any analysis. Intact viable protoplasts could be identified by a few common methods including (1) viable protoplasts exclude Evan's blue appearing clear or yellowish against a blue background; (2) viable protoplasts can accumulate neutral red and turn red; (3) fluorescent dyes are also used to stain viable protoplasts [XING & WANG, 2015].

Use of healthy leaves at the proper developmental stage is a very important factor in the production of viable protoplasts from *Arabidopsis* while stressed leaves (e.g. those

under drought, flooding, extreme temperature and constant mechanical perturbation) may seemingly give protoplasts but they only lead to low transfection efficiency when used in gene expression analysis [YOO & al. 2007]. In a sense, protoplast isolation remains a bottleneck to testing genome-editing reagents in many crop species. It is critical in the study of gene-editing effect to give considerable time to set up a reproducible protoplast system.

Transfection efficiency

The plant species, source materials, isolation methods and transfection methods all play a role in determining the transfection efficiencies. From one case to another, this can vary dramatically, e.g. around 50 to 70% in tomato leaf mesophyll system [XING & al. 2001, 2008] down to only 5% to 20% in *Arabidopsis* root protoplasts [BARGMANN & BIRNBAUM, 2009]. The best so far probably is the *Arabidopsis* mesophyll protoplast system, which may reach 90% transfection efficiency [SHEEN, 2001; YOO & al. 2007]. However, further improvement in transfection efficiency is always possible in any systems. Protoplasts from six species in *brassicaceas* also gave 43-83% transfection efficiency [WU & al. 2009]. Transfection efficiency was also tested and improved in multiple species from a single study, where protoplast transfection efficiency was shown to be 44-63% for rice, maize, wheat, millet, bamboo, and tomato [LIN & al. 2018]. In this study, the efficiency of CRISPR/Cas9-mediated mutagenesis (insertions, deletions) in the isolated protoplasts from different species varied dramatically ranging from 0.2% and 1.1% for *Zea mays* to 75.2% in *Brassica oleracea* [LIN & al. 2018]. Co-expressing GFP along with site-specific nuclease (SSN)-reagents in protoplasts may help the detection of the delivery and expression of genome-editing reagents and the co-expression approach facilitated direct comparison of the transformation efficiencies of CRISPR/Cas9 and TALEN reagents [NADAKUDUTI & al. 2019]. Although protoplast transfection efficiency is not correlated to the efficiency of CRISPR/Cas9-mediated mutagenesis, a healthy population of protoplasts with a high transfection efficiency is critical for CRISPR/Cas9-mediated mutagenesis. It should be noted that there is considerable variability in gRNA efficiency, and this does not seem to change with expression system or Cas9 delivery method. The feasibility of improving CRISPR/Cas9 editing efficiency by Fluorescence Activated Cell Sorting (FACS) of protoplasts was examined and protoplasts expressing GFP tagged CRISPR/Cas9, delivered through *A. tumefaciens* leaf infiltration, could be enriched by FACS [PETERSEN & al. 2019].

Regeneration from protoplast

Started with gene editing in protoplast, whole plants were generated with targeted modifications for various plant species [LI & al. 2013; SHAN & al. 2014; WANG & al. 2014b; SUN & al. 2015; WOO & al. 2015; CLASEN & al. 2016; MALNOY & al. 2016; KIM & al. 2017; LIANG & al. 2017; MANGHWAR & al. 2019]. CRISPR/Cas12a and base editing systems along with DNA-free CRISPR delivery methods were also implemented in protoplasts, targeted mutagenesis achieved, and plants regenerated with desired edited mutations [WOO & al. 2015; ANDERSSON & al. 2017; KIM & al. 2017]. For plant species that can be regenerated from protoplasts, the phenotypic changes are assessed at the whole plant level. With high delivery efficiencies and effective nucleases, a significant number of the plants regenerated from transformed protoplast populations harbor mutations at the target locus [CLASEN & al. 2016; LI & al. 2016]. A majority of them were shown not to have foreign DNA, which indicate that the nuclease was expressed only transiently,

and the construct was degraded before integration. This certainly is an additional advantage as the lack of foreign DNA is often desirable due to biotechnology regulation.

Difficulties do exist, together with concerns. Protoplast regeneration is difficult in most plant species [LIN & al. 2018], particularly monocots [BERNARD & al. 2019]. The regeneration process is time-consuming and is often preferred for quick efficiency test of gene editing systems or the mutagenesis effect [SOYARS & al. 2018]. Biolistic or *A. rhizogenes*-mediated transformation could be a common alternative. *A. rhizogenes* has been widely used to study rhizosphere, metabolic or hormone pathways [XING & al. 1996; GOMES & al. 2019]. Knocking out the CiPDS gene (phytoene desaturase) in chicory plants regenerated from both hairy roots and protoplasts was successfully shown [BERNARD & al. 2019]. We could expect that the list of protoplast-generated crops to expand because of the merit of ribonucleoprotein (RNP)-based genome editing technology. The significant potential is that while transient expression screening in protoplasts provides information for short listing of genes, further functional analysis with gene editing approaches can be followed by regeneration of plants so that gene editing effect will be analyzed at organismal level (Figure 1).

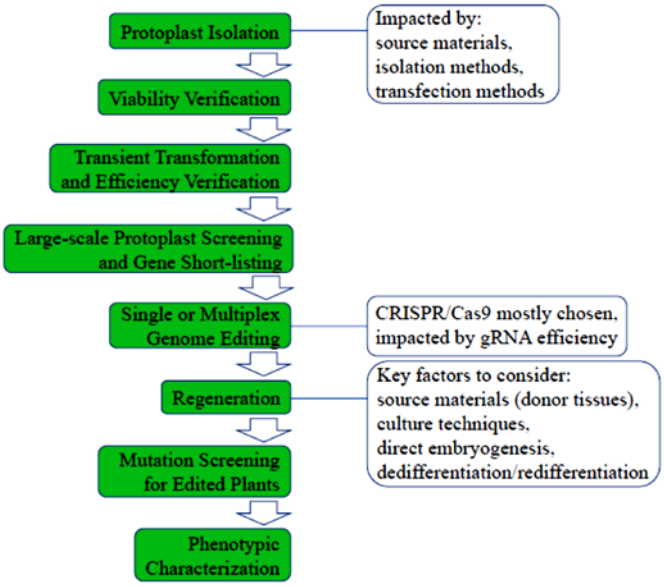

Figure 1. Schematic diagram of protoplast application for single or multiplex gene editing and plant regeneration. Some technique notes are indicated by boxes on right.

Conclusions

It is important that findings from large-scale omics analysis are confirmed by functional analysis. With the gene editing development such cell-based assays and functional screening will continue to facilitate the comprehensive understanding of the complexity of many processes in plants. Studies in the past several decades have indicated the usefulness of protoplasts and defined protoplast expression systems [XING & WANG,

2015]. Well established *Arabidopsis*, maize, tobacco, and tomato protoplast systems were applied to analysis of responses to oxidative, heat and osmotic stress signals, and pathogen elicitors [SHEEN, 1996; KOVTUN & al. 2000; TENA & al. 2001; XING & al. 2001, 2008; XING & WANG, 2015]. Protoplast system has also been applied to the analysis of developmental reprogramming [WANG & al. 2014a] and detailed metabolite investigation in specific cell types such as guard cells [JIN & al. 2013; RUBAKHIN & al. 2013]. As indicated in our previous work, a major application is protoplast transient transfection assay for the analysis of (1) gene expression in response to various signals and treatments; (2) promoter elements involved in regulating expression of genes; (3) roles played by signaling proteins such as protein kinases and transcription factors in regulating gene expression; (4) subcellular localization of proteins; (5) genetic interactions of genes; (6) protein-protein interactions; (7) gene interference effect; (8) proteomic and metabolomic profiles; and (9) functions of large number of genes derived from large-scale studies as an initial screening process [XING & WANG, 2015]. With such a broad application to mechanism analysis, we could be confident that the protoplast system will play an increasingly significant role in the coming years when high-throughput approaches and gene editing approaches meet.

Notes on contributor

Tim XING is an associate professor and a plant molecular biologist with a special interest in cell signaling and plant-microbe interactions. He teaches plant physiology, molecular plant development, and cell signaling.

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