View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Edinburgh Research Explorer

THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

CRISPR/Cas in Arabidopsis

Citation for published version:
Khumsupan, P, Donovan, S & McCormick, A 2019, 'CRISPR/Cas in Arabidopsis: overcoming challenges to accelerate improvements in crop photosynthetic efficiencies', *Physiologia plantarum*.
https://doi.org/10.1111/ppl.12937

Digital Object Identifier (DOI):
10.1111/ppl.12937

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Physiologia plantarum

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 17. Aug. 2021

Accepted Article

CRISPR/Cas in Arabidopsis: overcoming challenges to accelerate improvements in crop photosynthetic efficiencies

Panupon Khumsupan†, Sophie Donovan† and Alistair McCormick*
SynthSys and Institute of Molecular Plant Sciences, School of Biological Sciences, University of Edinburgh, Edinburgh, EH9 3BF, UK

† these authors contributed equally to the work

Correspondence
* Corresponding author,
e-mail: alistair.mccormick@ed.ac.uk

The rapid and widespread adoption of CRISPR/Cas technologies has allowed genetic editing in plants to enter a revolutionary new era. In this mini-review we highlight the current CRISPR/Cas tools available in plants and the use of *Arabidopsis thaliana* as a model to guide future improvements in crop yields, such as enhancing photosynthetic potential. We also outline the current socio-political landscape for CRISPR/Cas research and highlight the growing need for governments to better facilitate research into plant genetic editing technologies.

Introduction

Genetic editing via CRISPR/Cas has been used by plant biologists for a range of purposes, from generating novel mutants for fundamental biological studies to improving crop plant performance and enhancing crop yields (recently reviewed in Scheben and Edwards 2018). Although CRISPR/Cas is now a well-known tool, its first use in plants and other eukaryotes was reported only five years ago (Nekrasov et al. 2013). Thus, CRISPR/Cas is still a relatively immature technology and new findings and applications continue to emerge, promising to further enhance our capabilities for precise genetic

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ppl.12937

This article is protected by copyright. All rights reserved.

Accepted Article

editing in plants. The purpose of this short review is to provide an update on the current successes and challenges of CRISPR/Cas in plant research with a key focus on *Arabidopsis thaliana* (Arabidopsis), the most well studied model plant species. We will discuss the continued usefulness of Arabidopsis as a model for guiding genetic editing strategies, in particular, for improving photosynthetic efficiencies and crop yields. This review is also particularly timely, given the recent opposing rulings in the US and EU on the status of genetically edited plants. Therefore, we will also briefly consider the political, social and commercial aspects of the CRISPR/Cas research landscape.

CRISPR/Cas gene editing in action

The CRISPR/Cas gene editing system is a repurposed domestication of the class II CRISPR (clustered regularly interspaced short palindromic repeats) interference mechanism from the adaptive immune response of prokaryotes (Cong et al. 2013, Nekrasov et al. 2013). CRISPR/Cas relies on the interaction of a CRISPR-associated endonuclease (Cas) enzyme with a synthetic guide RNA (gRNA) designed to target and induce cleavage at specific DNA or RNA sites (for a detailed mechanistic review see Jiang and Doudna 2017). In brief, sequence-specificity is achieved by a short region (19-22 nt) in the gRNA that is complementary to the host target sequence and next to a 3-6 nt protospacer adjacent motif (PAM) sequence (Table 1; Jinek et al. 2012). Off-target mutations can occur at undesired sites that have mismatches distal to the PAM (Zhang et al. 2018). However, several bioinformatic tools are now available to predict off-target activity based on the gRNA(s) and Cas used, which can subsequently be screened for during analysis (reviewed in Zischewski et al. 2017). As such, the majority of studies in plants report a low frequency of mutation at off-target sites (Xie et al. 2014, Jacobs et al. 2015). Class II Cas (comprising types II, V and VI Cas) are currently the most attractive targets to domesticate for genetic editing as they can perform several tasks in one, including formation of a ribonucleoprotein complex with gRNAs and the processing of those gRNAs, as well as

This article is protected by copyright. All rights reserved.

Accepted Article

recognition of the PAM site within the host target sequence (Shmakov et al. 2016). Host sequence disruption is achieved via the error-prone repair of double-stranded breaks (DSBs) by the native eukaryotic non-homologous end joining pathway (NHEJ), which typically induces small insertions or deletions (indels) at the DSB site. More precise genomic deletions or insertions can be generated through homology-directed repair (HDR) of DSBs with a template or 'donor' sequence (Li et al. 2013, Knoll et al. 2014).

The type II Cas9 from *Streptococcus pyogenes* (SpCas9) was the first reported Cas isoform to be domesticated (Jinek et al. 2012). Although several others Cas9 variants have since been studied (Table 1), SpCas9 is still the most commonly used to generate loss of function mutations in plants (Nekrasov et al. 2013, Sánchez-León et al. 2018). The two nuclease domains of Cas9, RuvC and HnH, induce a blunt-ended DSB, three base pairs upstream of the PAM sequence (Jiang and Doudna 2017). More recently, additional structurally and functionally distinct class II Cas homologs have been identified with the capacity to cleave DNA or RNA (Shmakov et al. 2016). For example, the type V Cas12a (previously known as Cpf1) is functionally similar to Cas9, but generates a staggered DSB (i.e. a four-nucleotide overhang) upstream of the PAM and outside of the gRNA sequence using a single RuvC nuclease domain (Zetsche et al. 2015). The PAM sequences of Cas12a are well-suited for targeting AT-rich genomic regions, such as promoters. Cas12a also has RNase activity and can process a sequential string of gRNAs from a single promoter to more easily facilitate multiple gene targeting (known as multiplexing; Wang et al. 2017a). The type VI Cas13a (previously known as C2c2) targets and cleaves RNA using the ribonuclease domain HEPN (Abudayyeh et al. 2018). CRISPR/Cas-based RNA targeting could have important applications in functional RNA studies and generating resistance to common single-stranded RNA plant viruses (e.g. Cauliflower mosaic virus; Aman et al. 2018). Modified variants of Cas have also expanded the capabilities of CRISPR/Cas. For example, catalytically inactivated Cas (dCas) retains the capacity for target binding and can be used to

This article is protected by copyright. All rights reserved.

Accepted Article

regulate gene expression through transcriptional interference (a process called CRISPRi by Qi et al. 2013). dCas fused to transcriptional repression or activation domains can be used in plants for modulation of gene expression (Tang et al. 2017, Lowder et al. 2018) and epigenetic modification (Gallego-Bartolomé et al. 2018). More recently, the potential applications for targeted mutagenesis have been further developed by fusing dCas9 with a cysteine or adenine deaminase domain for precise base editing of C/G to T/A or T/A to C/G, respectively (Eid et al. 2018, Li et al. 2018). Base editing could be applied to engineer alternative variants of enzymes and/or regulatory sequences in cases requiring a single nucleotide change, thus removing the need to supply a donor template for HDR-based approaches.

Arabidopsis – leading the way or playing catch up?

CRISPR/Cas has been central to a recent surge in genetic editing studies in a variety of crop species. A key aim is to engineer desirable agronomic traits, such as abiotic stress resilience and pathogen resistance, and develop transgene-free edited plant lines (Scheben and Edwards 2018). Two outstanding examples are the generation of low-gluten wheat lines (Sánchez-León et al. 2018) and the domestication of wild tomato (Zsögön et al. 2018). Improvements in Cas and gRNA(s) delivery methods, high frequencies of editing in transformants and better tissue culture regeneration methods have accelerated the development of non-model, polyploid plants for functional and applied reverse genetic research (e.g. Li et al. 2017, Lin et al. 2018). As such, CRISPR/Cas will likely continue to drive efforts to expand the availability of reference genomes in different plant species.

In contrast, CRISPR/Cas work in the model species Arabidopsis has been highlighted by several challenges associated with localisation of Cas expression, relatively low transformation efficiencies and issues with heritability (Feng et al. 2014, Mao et al. 2016). One drawback has arisen from a key advantage: Arabidopsis can be transformed at high efficiencies by the floral dip method, which is

This article is protected by copyright. All rights reserved.

Accepted Article

more rapid than tissue culture approaches required for most other plant species (Fig. 1). Although floral dipping has significant benefits over tissue culture approaches, the frequency of heterozygous, homozygous and bi-allelic CRISPR/Cas induced mutations initially reported in T1 Arabidopsis lines has been low (Table 2), with 1-bp indels and chimeric mutations in somatic cells accounting for the majority of mutation types (Feng et al. 2014). This issue has been linked to the use of common, strong promoters to drive Cas expression [e.g. the Cauliflower mosaic virus promoter (CaMV35S)], which have been shown to have a low activity in germ-line cells or at the one-cell stage of embryogenesis (Hyun et al. 2015, Wang et al. 2015). In contrast, plants regenerated through tissue culture can arise through embryogenesis from a single somatic cell where such promoters are highly active. Tissue culture-based studies using CaMV35S to drive Cas expression have reported homozygous mutations associated with NHEJ in T0 lines for a variety of species, including the woody plant *Populus tomentosa* (Fan et al. 2015).

Several crop species have also made significant progress in HDR-based editing (Butler et al. 2016, Sun et al. 2016). However, the frequency of HDR events varies considerably between plant species, with low efficiencies initially being reported in Arabidopsis. For example, Li et al. (2013) compared the delivery of a double stranded DNA (dsDNA) donor template (via PEG-mediated transformation) to *Nicotiana tabacum* (tobacco) and Arabidopsis protoplasts. HDR-mediated integration of the donor template was unsuccessful in Arabidopsis, whereas an integration frequency of 9% was reported for tobacco. Similarly, Schiml et al. (2014) reported only a low frequency of donor template integration (ca. 0.1%) in Arabidopsis. *Agrobacterium*-mediated transformation of geminivirus-based vectors can help to increase the abundance of the donor template, and has yielded improved donor integration frequencies in tomato and rice (6 and 8.5%, respectively; ermák et al. 2015, Wang et al. 2017b). Viral-mediated genome editing was recently reported in Arabidopsis (Ali et al. 2018), although it has not yet been applied for HDR-mediated strategies.

This article is protected by copyright. All rights reserved.

Return of the King: increasing the efficiencies of CRISPR/Cas editing in Arabidopsis

Numerous studies have now shown that using germline-specific promoters to express Cas can significantly improve the frequency and heritability of mutations in Arabidopsis (Table 2; Hyun et al. 2015, Wang et al. 2015, Yan et al. 2015, Mao et al. 2016). Germline-specific promoters can increase the frequencies of homozygous mutations in T1 plants and lower the rate of chimerism compared to non-germline specific promoters such as CaMV35S (Wang et al. 2015, Yan et al. 2015, Mao et al. 2016). This has reduced the sample size needed for screening and the requirement for multi-generational analyses of mutations. To date, 13 germline-specific promoters have been reported, with heritable mutation rates of up to 17% in the T1 generation with EC1.1/EC1.2 (Hyun et al. 2015, Wang et al. 2015, Yan et al. 2015, Eid et al. 2016, Mao et al. 2016, Osakabe et al. 2016). Recently, a robust protocol for HDR-based editing using a germ-line specific promoter (DD45) to drive Cas9 expression was reported in Arabidopsis with a knock-in efficiency of 16-55% observed in the T2 generation (Miki et al. 2018). Additionally, replacing the constitutive promoter PcUbi4-2 with EC1.1 improved the rate of HDR from 1 to 6% (Wolter et al. 2018). Together, these results indicate that the timely expression of Cas in germ cells or during early embryogenesis is a critical factor for HDR-directed editing in Arabidopsis.

Based on recent work, there are many other opportunities to enhance gene editing efficiencies in Arabidopsis. Ordon et al. (2018) reported that improvements in vector design coupled with a paired gRNAs approach resulted in high frequencies (1.6%) for a 70 kb deletion using a constitutive ubiquitin promoter (Ordon et al. 2018). Better transformant screening strategies, improvements in gRNA expression and a more detailed understanding of the variability in gRNA efficiencies should help to further increase the detection and frequency of heritable mutations (Ordon et al. 2018, Wu et al. 2018). Increases in mutation frequencies can also be achieved by subjecting plants to periodic heat

This article is protected by copyright. All rights reserved.

stress that favours the activity of currently used Cas enzymes (LeBlanc et al. 2017). Future work could focus on identifying Cas variants that have maximal activity at temperatures used for plant growth.

From labs to fields: how Arabidopsis can guide improvements in crop photosynthesis

CRISPR/Cas has successfully improved agronomic traits in a variety of crops. Although the transformation and selection of some crops is now routinely achieved (e.g. in rice and wheat), generating transgenic lines remains a labour-intensive process for many species. Arabidopsis has historically been a powerful model species to study gene function and regulation. Due to recent advances in CRISPR/Cas editing, Arabidopsis remains well-positioned as a rapid and convenient tool to screen crop improvement strategies for complex traits that involve multiple genes and/or gene families. Increasing the efficiency of photosynthesis to improve productivity is one key example. Many approaches have been suggested, which include (and sometimes combine) enhancing the capacity for light capture, reducing photorespiration, and increasing flux through the Calvin cycle (Zhu et al. 2007, Ort et al. 2015, Rae et al. 2017 South et al. 2018). Recent lab- and field-based studies using transgenic plants have now shown that enhancing photosynthesis is a transformative strategy that can increase yields (Simkin et al. 2015, Kromdijk et al. 2016, Driever et al. 2017, Lopez-Calcagno et al. 2018). The multiplexing approaches achievable with CRISPR/Cas can be screened more rapidly in Arabidopsis to enable the progression of more complex strategies, as typically numerous genes are involved that require appropriate regulation. For example, there are 38 enzymes directly involved in photosynthetic carbon assimilation in C3 plants [e.g. the Calvin-Benson cycle (CB) and photorespiratory pathway] and multiple chaperones and regulatory components encoded by several gene families (Zhu et al. 2007).

Accepted Article

This article is protected by copyright. All rights reserved.

Models to optimise photosynthetic carbon metabolism indicate that modifying the activity of photorespiratory enzymes, Rubisco, and increasing the activity of other CB enzymes can increase photosynthesis (Zhu et al. 2007). For the latter, overexpression of a single enzyme, Sedoheptulose-1, 5 bisphosphatase (SBPase), can increase photosynthetic rates in model species and crops, including wheat and tomato (Lefebvre et al. 2005, Ding et al. 2016, Driever et al. 2017). This approach has been developed further by overexpressing SBPase with an additional CB enzyme (Fructose 1,6-bisphosphate aldolase) in Arabidopsis and tobacco (Simkin et al. 2017, Simkin et al. 2015, respectively). These examples represent significant progression towards increasing photosynthesis by manipulating the activity of multiple enzymes simultaneously. CRISPR/Cas-mediated HDR or NHEJ could allow similar strategies to be commercially applied to crops, for example, by modulating the activity of native enzymes and/or promoter-driven transcription. However, progress in understanding the regulation and diversity of other pathways related to photosynthesis (e.g. photorespiration, C4 and crassulacean acid metabolism (CAM) pathways, and pyrenoid-based CO_2-concentrating mechanisms) would also be accelerated by the approaches discussed. Therefore, in the immediate future, CRISPR/Cas will likely be applied to functional studies in model species to elucidate the regulation and activity of new targets for manipulation.

A key challenge in genetic engineering has been the manipulation of enzymes that are represented by, or regulated by gene families. For example, the small subunit of Rubisco (rbcS) has multiple isoforms (four in Arabidopsis and up to 22 in other species), while assembly with the plastid encoded Rubisco large subunit (rbcL) requires at least five species-specific chaperones in plants (Spreitzer 2003, Aigner et al. 2017). CRISPR/Cas-based approaches could overcome challenges associated with engineering gene families, including Rubisco. Engineering the catalytic site, which is located on the rbcL, is mostly limited to species amenable to routine plastid transformation, such as tobacco. However, the nuclear-encoded rbcS is also known to influence Rubisco catalysis (Sprietzer 2003,

Accepted Article

This article is protected by copyright. All rights reserved.

Atkinson et al. 2017). Recently, a new group of specialised rbcS, called rbcS-T, have been identified that are expressed exclusively in plant organs with specialised metabolism (e.g. trichomes; Laterre et al. 2017, Pottier et al. 2018). Endogenous rbcS-T isoforms likely maintain chaperone specificity but can alter the catalytic properties of Rubisco (Morita et al. 2014, Laterre et al. 2017). Thus, replacing rbcS expressed in mesophyll cells with an rbcS-T isoform could improve the efficiency of leaf CO_2 assimilation. Alternatively, as rbcS-T isoforms are not found in all species (Pottier et al. 2018) it may be desirable to express isoforms that are significantly divergent from the native family. Although the chaperones involved in Rubisco assembly appear to be highly species-specific (Aigner et al. 2018), little is known of the mechanisms underlying chaperone specificity in planta. Replacing and/or modifying endogenous chaperones via CRISPR/Cas-mediated HDR or base-editing could accelerate fundamental studies underpinning chaperone involvement in rbcS and rbcL assembly.

CRISPR/Cas based approaches offer the potential to improve existing strategies to increase photosynthesis, including enzyme overexpression (as HDR-mediated strategies improve), and to overcome key challenges, such as manipulating Rubisco. With improved plastid transformation protocols in different species (Yu et al. 2017), it may soon be possible to reliably engineer both plastid and nuclear expressed photosynthetic enzymes and/or associated regulatory proteins (Avila et al. 2016). Thus, Arabidopsis remains a critically important platform to rapidly test novel strategies in planta and examine the impact of photosynthetic efficiency and productivity before undertaking time-consuming translational studies.

When politics triumphs over science: the possibility of gene-edited food on your plate

Although CRISPR/Cas has resulted in a significant increase in agri-tech investment, social acceptance and discrepancies surrounding the regulation of gene editing technologies still hinders basic research and commercialisation in most countries (Smart et al. 2016, Brinegar et al. 2017). The adoption of

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

genetically modified (GM) crops by farmers is increasing globally (Parisi et al. 2016), but opposition by consumers to GM food is still highly prevalent (Blancke et al. 2015). Whilst the public perception of products engineered by gene editing is unclear, the regulatory status of organisms produced by these methods will play a central role in social acceptance. The US Department of Agriculture (USDA) have recently announced that plants produced by gene editing, which could be made by traditional breeding techniques, will not be subject to genetically modified organism (GMO) regulations (USDA 2018). This ruling includes plants with gene deletions of any size, single base pair substitutions and *cis*-genic plants. The announcement is a boon for the US agri-tech industry and will encourage biotech companies to invest in plant genetic editing research without the risk of facing costly regulatory processes (Smart et al. 2017). Products already planned for the market include sweeter strawberries with a longer shelf life (Monsanto) and drought-resistant maize lines (DuPont Pioneer).

In contrast, a recent landmark ruling by the EU Court of Justice has applied the same stringent regulations for conventional GMOs to genetically-edited plants (for an excellent review of the regulatory framework see Agapito-Tenfen et al. 2018). In the EU, a GMO is defined as “an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.” This definition covers both the process of genetic modification and the final product, so plants produced through lab-based technologies, including *cis*-genic plants that contain genes from sexually compatible species must be labelled as GMO (Agapito-Tenfen et al. 2018). The ruling came as a disappointment to EU-based scientists, as the negative effect of GMO legislation, that has hindered research for the past 15 years, will continue to impact new gene editing technologies and commercial uptake in the EU (Callaway 2018). In the future, the European commission may seek to overturn the court’s ruling. Resolving the current international discrepancies between gene editing regulations will help global efforts to ameliorate impending food security concerns.

This article is protected by copyright. All rights reserved.

Accepted Article

Author contributions

P.K. and S.D. had a primary role in writing the manuscript, including drawing figures and tables. A.J.M. read and corrected the manuscript.

Acknowledgment

S.D. was funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC) East of Scotland Bioscience Doctoral Training Partnership (EASTBIO) program. P.K. was funded by a postgraduate research scholarship from the Darwin Trust of Edinburgh.

References

Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, David BT, Kellner MJ, Regev A, Lander ES, Voytas DF, Alice Y (2018) RNA targeting with CRISPR-Cas13. Nature 550: 280–284

Agapito-Tenfen SZ, Okoli AS, Bernstein MJ, Wikmark OG, Myhr AI (2018) Revisiting risk governance of GM plants: the need to consider new and emerging gene-editing techniques. Front Plant Sci 9: 1874

Aigner H, Wilson RH, Bracher A, Calisse L, Bhat JY, Hartl FU, Hayer-Hartl M (2017) Plant RuBisCo assembly in *E. coli* with five chloroplast chaperones including BSD2. Science 358: 1272–1278

Ali Z, Eid A, Ali S, Mahfouz MM (2018) Pea early-browning virus-mediated genome editing via the CRISPR/Cas9 system in *Nicotiana benthamiana* and Arabidopsis. Virus Res 244: 333–337

Aman R, Ali Z, Butt H, Mahas A, Aljedaani F, Khan MZ, Ding S, Mahfouz M (2018) RNA virus interference via CRISPR/Cas13a system in plants. Genome Biol 19: 1–9

This article is protected by copyright. All rights reserved.

Accepted Article

Atkinson N, Leitão N, Orr DJ, Meyer MT, Carmo-Silva E, Griffiths H, Smith AM, McCormick AJ (2017) Rubisco small subunits from the unicellular green alga *Chlamydomonas* complement Rubisco-deficient mutants of Arabidopsis. New Phytol 214: 655–667

Avila EM, Gisby MF, Day A (2016) Seamless editing of the chloroplast genome in plants. BMC Plant Biol 16: 1–13

Blancke S, Van Breusegem F, De Jaeger G, Braeckman J, Van Montagu M (2015) Fatal attraction: the intuitive appeal of GMO opposition. Trends in Plant Sci 20: 414-418

Brinegar K, K. Yetisen A, Choi S, Vallillo E, Ruiz-Esparza GU, Prabhakar AM, Khademhosseini A, Yun SH (2017) The commercialization of genome-editing technologies. Crit Rev Biotechnol 37: 924–932

Butler NM, Baltes NJ, Voytas DF, Douches DS (2016) Geminivirus-mediated genome editing in potato (*Solanum tuberosum* L.) using sequence-specific nucleases. Front Plant Sci 7: 1045

Callaway E (25 July 2018) CRISPR plants now subject to tough GM laws in European Union. Nature. Available at https://www.nature.com/articles/d41586-018-05814-6.

ermák T, Baltes NJ, egan R, Zhang Y, Voytas DF (2015) High-frequency, precise modification of the tomato genome. Genome Biol 6: 232–246

Cong L, Ran F, Cox D, Lin, Shuailiang L, Barretto R, Habib N, Hsu P, Wu X, Jiang W, Marraffini L, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 340: 216–219

Ding F, Wang M, Zhang S, Ai X (2016) Changes in SBPase activity influence photosynthetic capacity, growth, and tolerance to chilling stress in transgenic tomato plants. Sci Rep 6: 1–14

Driever SM, Simkin AJ, Alotaibi S, Fisk SJ, Madgwick PJ, Sparks CA, Jones HD, Lawson T, Parry MAJ, Raines CA (2017) Increased SBPase activity improves photosynthesis and grain yield in wheat grown in greenhouse conditions. Philos Trans R Soc B Biol Sci 372: 1730–1740

This article is protected by copyright. All rights reserved.

Accepted Article

Eid A, Ali Z, Mahfouz MM (2016) High efficiency of targeted mutagenesis in Arabidopsis via meiotic promoter-driven expression of Cas9 endonuclease. Plant Cell Rep 35: 1555–1558

Eid A, Alshareef S, Mahfouz MM (2018) CRISPR base editors: genome editing without double-stranded breaks. Biochem J 475: 1955–1964

Endo A, Masafumi M, Kaya H, Toki S (2016) Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from *Francisella novicida*. Sci Rep 6: 1–9

Fan D, Liu T, Li C, Jiao B, Li S, Hou Y, Luo K (2015) Efficient CRISPR/Cas9-mediated Targeted Mutagenesis in Populus in the First Generation. Sci Rep 5: 1–7

Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang D-L, Wang Z, Zhang Z, Zheng R, Yang L, Zeng L, Liu X, Zhu J-K (2014) Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. Proc Natl Acad Sci USA 111: 4632–4637

Gallego-Bartolomé J, Gardiner J, Liu W, Papikian A, Ghoshal B, Kuo HY, Zhao JM-C, Segal DJ, Jacobsen SE (2018) Targeted DNA demethylation of the Arabidopsis genome using the human TET1 catalytic domain. Proc Natl Acad Sci USA 115: 2125–2134

Hyun Y, Kim J, Cho SW, Choi Y, Kim JS, Coupland G (2015) Site-directed mutagenesis in *Arabidopsis thaliana* using dividing tissue-targeted RGEN of the CRISPR/Cas system to generate heritable null alleles. Planta 241: 271–284

Jacobs TB, LaFayette PR, Schmitz RJ, Parrott WA (2015) Targeted genome modifications in soybean with CRISPR/Cas9. BMC Biotechnol 15: 16-25

Jiang F, Doudna JA (2017) CRISPR-Cas9 Structures and Mechanisms. Annu Rev Biophys 46: 505–529

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A Programmable dual-RNA-guided endonuclease in adaptive bacterial immunity. Science 337: 816–822

This article is protected by copyright. All rights reserved.

Accepted Article

Kim H, Kim ST, Ryu J, Kang BC, Kim JS, Kim SG (2017) CRISPR/Cpf1-mediated DNA-free plant genome editing. Nat Commun 8: 1–7

Knoll A, Fauser F, Puchta H (2014) DNA recombination in somatic plant cells: Mechanisms and evolutionary consequences. Chromosom Res 22: 191–201

Kromdijk J, Glowacka K, Leonelli L, Gabilly S, Iwai M, Niyogi K, Long SP (2016) Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. Plant Sci 354: 857–862

Laterre R, Pottier M, Remacle C, Boutry M (2017) Photosynthetic Trichomes Contain a Specific Rubisco with a Modified pH-Dependent Activity. Plant Physiol 173: 2110–2120

Lawrenson T, Shorinola O, Stacey N, Li C, Østergaard L, Patron N, Uauy C, Harwood W (2015) Induction of targeted, heritable mutations in barley and *Brassica oleracea* using RNA-guided Cas9 nuclease. Genome Biol 16: 1-13

LeBlanc C, Zhang F, Mendez J, Lozano Y, Chatpar K, Irish V, Jacob Y (2017) Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. Plant J 9: 377–386

Lefebvre S, Lawson T, Zakhleniuk O V, Lloyd JC, Raines CA (2005) Increased Sedoheptulose-1,7-Bisphosphatase Activity in Transgenic Tobacco Plants Stimulates Photosynthesis and Growth from an Early Stage in Development. Plant Physiol 138: 451–460

Li C, Zong Y, Wang Y, Jin S, Zhang D, Song Q, Zhang R, Gao C (2018) Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. Genome Biol 19: 59-67

Li J, Aach J, Norville JE, Mccormack M, Bush J, Church GM, Sheen J (2013) Multiplex and homologous recombination-mediated plant genome editing via guide RNA/Cas9. Nat Biotechnol 31: 688–691

Lin CS, Hsu CT, Yang LH, Lee LY, Fu JY, Cheng QW, Wu FH, Hsiao HCW, Zhang Y, Zhang R, Chang WJ, Yu CT, Wang W, Liao LJ, Gelvin SB, Shih MC (2018) Application of protoplast

This article is protected by copyright. All rights reserved.

Accepted Article

technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration. Plant Biotechnol J 16: 1295–1310

Lopez-Calcagno PE, Fisk S, Brown KL, Bull SE, South PF, Raines CA (2018) Overexpressing the H-protein of the glycine cleavage system increases biomass yield in glasshouse and field grown transgenic tobacco plants. Plant Biotechnol J: doi 10.1111/pbi.12953

Lowder LG, Zhou J, Zhang Y, Malzahn A, Zhong Z, Hsieh TF, Voytas DF, Zhang Y, Qi Y (2018) Robust Transcriptional Activation in Plants Using Multiplexed CRISPR-Act2.0 and mTALE-Act Systems. Mol Plant 11: 245–256

Mao Y, Zhang Z, Feng Z, Wei P, Zhang H, Zhu J, Sciences F, Lafayette W (2016) Development of germ-line-specific CRISPR-Cas9 systems to improve the production of heritable gene modifications in Arabidopsis. Plant Biotechnol J 14: 519–532

Miki D, Zhang W, Zeng W, Feng Z, Zhu JK (2018) CRISPR/Cas9-mediated gene targeting in Arabidopsis using sequential transformation. Nat Commun 9: 1–9

Morita K, Hatanaka T, Misoo S, Fukayama H (2014) Unusual small subunit that is not expressed in photosynthetic cells alters the catalytic properties of Rubisco in rice. Plant Physiol 164: 69–79

Nekrasov V, Staskawicz B, Weigel D, Jones JDG, Kamoun S (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. Nat Biotechnol 31: 691–693

Ordon J, Bressan M, Kretschmer C, Dall'Osto L, Marillonnet S, Bassi R, Struttmann J (posted August 2018) Optimized Cas9 expression systems for highly efficient Arabidopsis genome editing facilitate isolation of complex alleles in a single generation. BioRxiv 2322: doi 10.1038/nbt.4272

Ordon J, Gantner J, Kemna J, Schwalgun L, Reschke M, Streubel J, Boch J, Stuttmann J (2017) Generation of chromosomal deletions in dicotyledonous plants employing a user-friendly

This article is protected by copyright. All rights reserved.

Accepted Article

genome editing toolkit. Plant J 89: 155–168

Ort DR, Merchange SS, Alric J, Barkan A, Blankenship RE, Bock R, Croce R, Hanson M, Hibberd JM, Long SP, Moore T, Moroney J, Niyogi KK, Parry MA, Peralta-Yahya P, Prince RC, Redding KE, Spalding MH, van Wijk K, Vermaas WFJ, von Caemmerer S, Webber APM, Yeates TO, Yuan JS, Zhu XG (2015) Redesigning photosynthesis to sustainably meet global food and bioenergy demand. Proc Natl Acad Sci USA 112: 8529–8536

Osakabe Y, Watanabe T, Sugano SS, Ueta R, Ishihara R, Shinozaki K, Osakabe K (2016) Optimization of CRISPR/Cas9 genome editing to modify abiotic stress responses in plants. Sci Rep 6: 1–10

Parisi C, Tillie P, Rodriguez-Cerezo E (2016) The global pipeline of GM crops out to 2020. Nat Biotechnol 34: 31-36

Pottier M, Gilis D, Boutry M (2018) The Hidden Face of Rubisco. Trends Plant Sci 23: 382–392

Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA (2013) Repurposing CRISPR as an RNA-Guided Platform for Sequence- Specific Control of Gene Expression. Cell 152: 1173–1183

Rae BD, Long BM, Förster B, Nguyen ND, Velanis CN, Atkinson N, Hee WY, Mukherjee B, Price GD, McCormick AJ (2017) Progress and challenges of engineering a biophysical CO_2-concentrating mechanism into higher plants. J Exp Bot 68: 3717–3737

Sánchez-León S, Gil-Humanes J, Ozuna C V., Giménez MJ, Sousa C, Voytas DF, Barro F (2018) Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. Plant Biotechnol J 16: 902–910

Scheben A, Edwards D (2018) Towards a more predictable plant breeding pipeline with CRISPR/Cas-induced allelic series to optimize quantitative and qualitative traits. Curr Opin Plant Biol 45: 1–8

Schiml S, Fauser F, Puchta H (2014) The CRISPR/Cas system can be used as nuclease for in planta

This article is protected by copyright. All rights reserved.

Accepted Article

gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. Plant J 80: 1139–1150

Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Abudayyeh OO, Gootenberg JS, Makarova KS, Wolf YI, Severinov K, Zhang F, Koonin E V. (2016) Diversity and evolution of class 2 CRISPR–Cas systems. Nat Rev Microbiol 15: 1922–2013

Simkin AJ, Lopez-Calcagno PE, Davey PA, Headland LR, Lawson T, Timm S, Bauwe H, Raines CA (2017) Simultaneous stimulation of sedoheptulose 1,7-bisphosphatase, fructose 1,6-bisphophate aldolase and the photorespiratory glycine decarboxylase-H protein increases CO_2 assimilation, vegetative biomass and seed yield in Arabidopsis. Plant Biotechnol J 15: 805–816

Simkin AJ, McAusland L, Headland LR, Lawson T, Raines CA (2015) Multigene manipulation of photosynthetic carbon assimilation increases CO_2 fixation and biomass yield in tobacco. J Exp Bot 66: 4075–4090

Smart RD, Blum M, Wesseler J (2017) Trends in Approval Times for Genetically Engineered Crops in the United States and the European Union. J Agric Econ 68: 182–19

South PF, Cavanagh AP, Lopez-Calcagno PE, Raines CA, Ort DR (2018) Optimizing photorespiration for improved crop productivity. J Integr Plant Biol: doi 10.1111/jipb.12709

Spreitzer RJ (2003) Role of the small subunit in ribulose-1,5-bisphosphate carboxylase/oxygenase. Arch Biochem Biophys 414: 141–149

Steinert J, Schiml S, Fauser F, Puchta H (2015) Highly efficient heritable plant genome engineering using Cas9 orthologues from *Streptococcus thermophilus* and *Staphylococcus aureus*. Plant J 84: 1295–1305

Sun Y, Xin Z, Wu C, He Y, Ma Y, Hou H, Guo X, Du W, Zhao Y, Xia L (2016) Engineering herbicide-resistant rice plants through CRISPR/Cas9-mediated homologous recombination of acetolactate synthase. Mol Plant 9: 628-631

This article is protected by copyright. All rights reserved.

Accepted Article

Tang X, Lowder LG, Zhang T, Malzahn AA, Zheng X, Voytas DF, Zhong Z, Chen Y, Ren Q, Li Q, Kirkland ER, Zhang Y, Qi Y (2017) A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. Nat Plants 3: 1–5

Tsutsui H, Higashiyama T (2017) PKAMA-ITACHI vectors for highly efficient CRISPR/Cas9-mediated gene knockout in *Arabidopsis thaliana*. Plant Cell Physiol 58: 46–56

USDA (28 March 2018) Secretary Perdue Issues USDA Statement on Plant Breeding Innovation. U.S. Department of Agriculture. Available at https://www.usda.gov/media/press-releases/2018/03/28/secretary-perdue-issues-usda-statement-plant-breeding-innovation

Wang M, Mao Y, Lu Y, Tao X, Zhu J kang (2017a) Multiplex Gene Editing in Rice Using the CRISPR-Cpf1 System. Mol Plant 10: 1011–1013

Wang M, Lu Y, Botella JR, Mao Y, Hua K, Zhu J kang (2017b) Gene Targeting by Homology-Directed Repair in Rice Using a Geminivirus-Based CRISPR/Cas9 System. Mol Plant 10: 1007–1010

Wang Z, Xing H, Dong L, Zhang H, Han C, Wang X, Chen Q (2015) Egg cell-specific promoter-controlled CRISPR / Cas9 efficiently generates homozygous mutants for multiple target genes in Arabidopsis in a single generation. Genome Biol 16: 114–126

Wolter F, Klemm J, Puchta H (2018) Efficient in planta gene targeting in Arabidopsis using egg cell-specific expression of the Cas9 nuclease of *Staphylococcus aureus*. Plant J 94: 735–746

Wu R, Lucke M, Jang Y ting, Zhu W, Symeonidi E, Wang C, Fitz J, Xi W, Schwab R, Weigel D (2018) An efficient CRISPR vector toolbox for engineering large deletions in *Arabidopsis thaliana*. Plant Methods 14: 1–9

Xie K, Zhang J, Yang Y (2014) Genome-wide prediction of highly specific guide RNA spacers for CRISPR-Cas9-mediated genome editing in model plants and major crops. Mol Plant 7: 923-926

Xing H-L, Dong L, Wang Z-P, Zhang H-Y, Hang C-Y, Liu B, Wang X-C, Qi-Jun C (2014) A

This article is protected by copyright. All rights reserved.

CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol 14: 327–337

Yan L, Wei S, Wu Y, Hu R, Li H, Yang W, Xie Q (2015) High-Efficiency Genome Editing in Arabidopsis Using YAO Promoter-Driven CRISPR/Cas9 System. Mol Plant 8: 1820–1823

Yu Q, Lutz KA, Maliga P (2017) Efficient Plastid Transformation in Arabidopsis. Plant Physiol 175: 186–193

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV, Zhang F (2015) Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. Cell 163: 759–771

Zhang, Q, Xing HL, Wang ZP, Zhang HY, Yang F, Wang XC, Chen, QJ (2018) Potential high-frequency off-target mutagenesis induced by CRISPR/Cas9 in Arabidopsis and its prevention. Plant Mol Biol 96: 445-456

Zischewski J, Fischer R, Bortesi L (2017) Detection of on-target and off-target mutations generated by CRISPR/Cas9 and other sequence-specific nucleases. Biotechnol Adv 35: 95-104

Zhu X-G, de Sturler E, Long SP (2007) Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: A numerical simulation using an evolutionary algorithm. Plant Physiol 145: 513–526

Zsögön A, ermák T, Naves ER, Notini MM, Edel KH, Weinl S, Freschi L, Voytas DF, Kudla J, Peres LEP (2018) De novo domestication of wild tomato using genome editing. Nat Biotechnol: doi 10.1038/nbt.4272

Figure legend

Fig. 1. A comparison of workflows and timescales to obtain genetically-edited transgene free plants using CRISPR/Cas. (A) Floral dipping of Arabidopsis. (B) Agrobacterium-mediated transformation of other dicot species (e.g. tobacco) and regeneration of explants or calli by tissue culture. (C)

Accepted Article

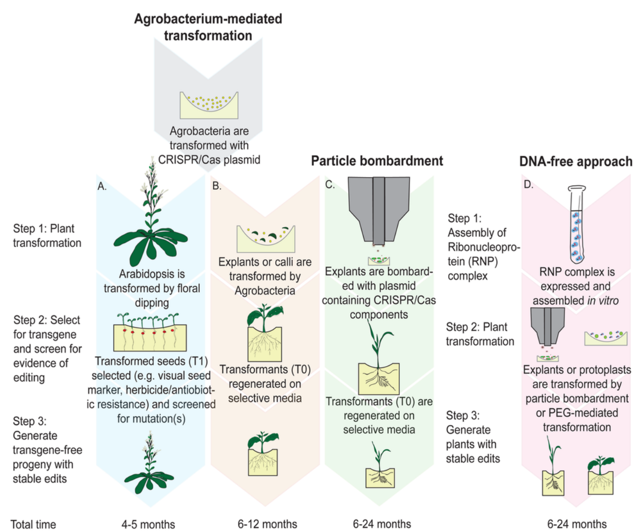
This article is protected by copyright. All rights reserved.

Transformation of monocot species (e.g. wheat) by particle bombardment and regeneration by tissue culture. (D) Intracellular delivery of a ribonucleoprotein (RNP) gRNA-Cas complex and regeneration by tissue culture. Transformants are screened for editing events by PCR and sequencing or commonly used mutation assays (e.g. Surveyor assay, T7 endonuclease assay). The period of time until transgene-free progeny are identified is species-dependent.

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article



PPL_12937_190125_Figure_1.tif

This article is protected by copyright. All rights reserved.

Accepted Article

Table 1. List of Cas variants used in some of the plant studies referenced in this review. Cas targets different PAM sequences, thus expanding the available genomic sites for gene editing. The RNA-targeting Cas13a variants used in plants so far have been PAM-independent. PAM sequence abbreviations: N: any nucleotide, V: A, C or G; R: A or G; Y: C or T; M: A or C

Cas variant	Source species	Size (a.a.)	PAM sequence(s)	Plant species used	References
SpCas9	*Streptococcus pyogenes*	1368	NGG	Species referenced in this review include Arabidopsis, *Nicotiana benthamiana, Zea mays* (maize), *Hordeum vulgare* (barley), *Brassica oleracea (*broccoli*)*, *Bambusa oldhamii* (bamboo), *Setaria italic* (millet), *Brassica napus* (rapeseed) *Oryza sativa* (rice), *Solanum pimpinellifolium* (wild tomato)	Nekrasov et al. 2013, Xing et al. 2014, Lawrenson et al. 2015, Lin et al. 2018, Zsögön et al. 2018
StCas9	*Streptococcus thermophilus*	1122	NNAGAA, NNGGAA	Arabidopsis	Steinert et al. 2015
SaCas9	*Staphylococcus aureus*	1053	NNGGGT, NNGAA	Arabidopsis	Steinert et al. 2015, Wolter et al. 2018
LbCas12a	*Lachnospiraceae bacterium* ND2006	1228	TTTV	*O. sativa*, Arabidopsis, *Glycine max* (soybean)*, Nicotiana attenuata* (wild tobacco)	Kim et al. 2017, Tang et al. 2017
AsCas12a	*Acidaminococcus* sp. BV3L6	1307	TTTV	*O. sativa*, Arabidopsis, *G. max*, *N. attenuata*	Kim et al. 2017, Tang et al. 2017
FnCas12a	*Francisella novicida*	1300	TTV	*O. sativa*, *N. benthamiana*	Endo et al. 2016
LwaCas13a	*Leptotrichia wadei*	1212	PAM-independent	*O. sativa*	Abudayyeh et al. 2018
LshCas13a	*Leptotrichia shahii*	1389	PAM-independent	*N. benthamiana*	Aman et al. 2018

This article is protected by copyright. All rights reserved.

Accepted Article

Table 2. Promoters used to drive Cas expression in Arabidopsis. The frequencies of heritable mutations in T1 and T2 generations are indicated.

Promoter	Terminator	Target gene(s)	Targeted organ expression	Heritable mutation in T1 (%)	Heritable mutation in T2 (%)	Reference
AtEC1.1; AtEC1.2; EC1.1/1.2	PsRbcSE9	*ETC2, TRY, CPC, CHLI1/2*	Egg cells, embryo	1.8; 8.3; 17.0	N/A	Wang et al. 2015
AtYAO	Nopaline synthase (Nos) from *A. tumefacians*	*BRI1*	Embryo sac, embryo, endosperm and pollen	6.7	66	Yan et al. 2015
AtINCURVATA2	Nos	*FT, SPLA4*	Endosperm and embryo	13.0	N/A	Hyun et al. 2015
AtSPOROCYTELESS (SPL)	AtSPL	*AP1, TT4*	Early microsporocytes and megasporocytes	0	70	Mao et al. 2016
AtDD45	Nos	*GL2*	Zygotes and early embryo	5.6	38	Mao et al. 2016
SlLAT52	Nos	*GL2*	Pollen	0	39	Mao et al. 2016
AtRPS5A	AtHSP	*Adh1*	Constitutive	81	N/A	Tsutsui and Higashiyama 2017
AtUBQ10	PsRbcSE9	*At3g04220*	Constitutive	74	N/A	Wu et al. 2018
PcUBQ4-2	Nos	*DM2C*	Constitutive	10	N/A	Ordon et al. 2017
CaMV35S	Nos	*BRI1, JAZ1, GAI, CHLI, TT4*	Constitutive	0	22	Feng et al. 2014

This article is protected by copyright. All rights reserved.