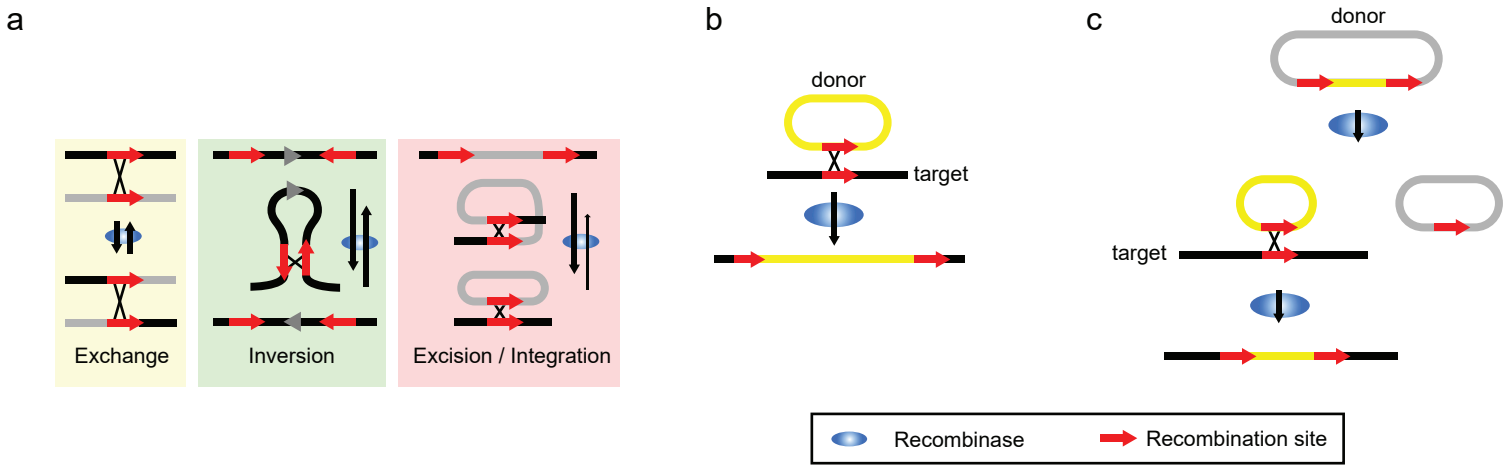
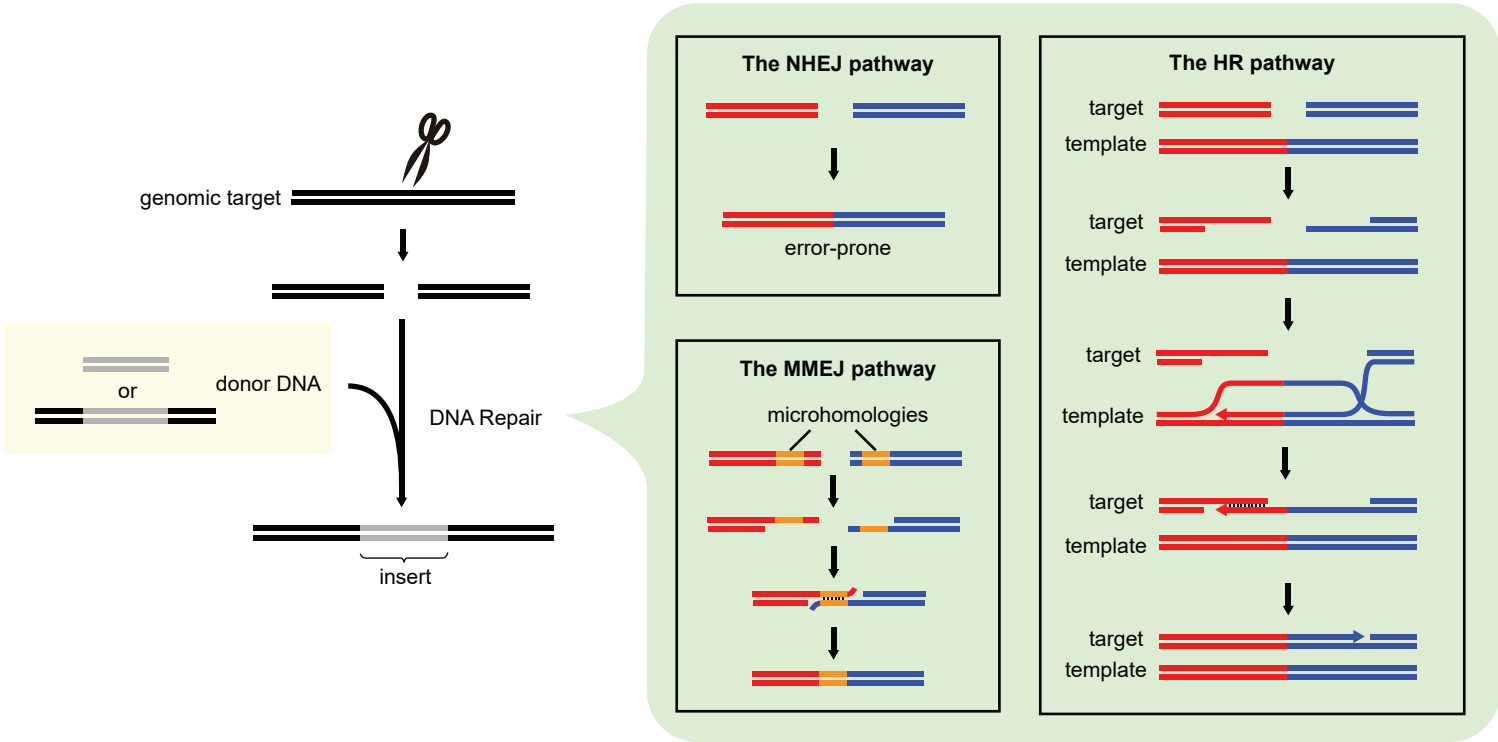


Supplementary Figure 1. Using a recombinase system for targeted gene insertion. (a) A recombinase (oval) catalyzes the exchange between two recombination sites (arrows) placed in proximity. The positioning of the recombination sites dictates the outcome of the recombination. This process is reversible. **(b-c)** Targeted insertion of DNA at a designated genomic target may occur via the genetic recombination between recombination sites. The two panels illustrate two examples of insertion strategies. In (b), a single recombination results in the integration of the entire donor DNA at the genomic target. In (c), two subsequent recombination reactions result in the targeted insertion of a defined section of the donor DNA.



Supplementary Figure 2. Applying various DNA repair pathways in targeted DNA insertion. Various DNA repair mechanism have been employed to insert donor DNA at designated genomic targets. In these methods, a site-directed nuclease creates a double-stranded break at the designated insertion target. The repair of the break in the presence of a properly designed donor DNA can lead to the insertion of a specific DNA sequence. In NHEJ, the broken ends of the DNA are re-joined directly. Repair through NHEJ is error-prone, often accompanied by the insertion or deletion of bases at the junction. In MMEJ, the Watson-Crick base pairing of microhomology sequences present on both DNA ends before end joining results in the deletion of the nucleotide sequence between the two microhomologies. During HR-mediated repair, a homologous repair template is employed to guide the repair of the double-stranded break. The diagram for the HR pathway illustrates synthesis-dependent strand annealing, the prevailing mechanism of HR-mediated repair in somatic plant cells. DSB, double-stranded break; NHEJ, non-homologous end joining; MMEJ, microhomology-mediated end joining; HR, homologous recombination.

Supplementary Table 1. Examples of targeted DNA insertion in plants

Molecular Tool	Species	Delivery	Outcome	Reported efficiency* of targeted insertion	Whole plant obtained?	Reference
Homologous Recombination	Tobacco	Direct gene transfer	Marker restored	0.5 - 4.2 x 10^{-4} (targeted insertion / random insertion)	Y	[1]
	Tobacco	Agrobacterium	Marker restored	3 x 10^{-5} (targeted insertion / random insertion)	Y	[2]
	Arabidopsis	Agrobacterium	Marker restored	10^{-4} (targeted insertion / random insertion)	Y	[3]
	Tobacco	Direct gene transfer	Marker restored	up to 10^{-4} (targeted insertion / random insertion)	Y	[4]
	Arabidopsis	Agrobacterium	Gene disrupted by marker insertion	7.7 x 10^{-4} (targeted insertion / random insertion)	N	[5]
	Tobacco	Agrobacterium	Marker restored	3.2 x 10^{-6} (targeted insertion / random insertion)	Y	[6]
	Lotus japonicus	Agrobacterium	Gene disrupted by marker insertion	Below 5.3 x 10^{-5} (targeted insertion / random insertion)	N	[7]
	Arabidopsis	Agrobacterium	Gene disrupted by marker insertion	1.3 x 10^{-3} (targeted insertion / random insertion)	Y	[8]
	Tobacco	Agrobacterium	Gene disrupted by marker insertion	5.7 x 10^{-3} (targeted insertion / dual-selected events)	Y	[9]
	Physcomytrella patens	Direct gene transfer	Marker inserted	93% of the transformation events	Y	[10]
	Rice	Agrobacterium	Gene disrupted by marker insertion	1% of the dual-selected events	Y	[11]
	Arabidopsis	Agrobacterium	Reporter gene inserted in frame	3% to 17% (targeted insertion / random insertion)	Y	[12]
	Rice	Agrobacterium	Gene disrupted by marker insertion	2% of the dual-selected events	Y	[13]
	Arabidopsis	Agrobacterium	Reporter gene inserted in frame	4.17 x 10^{-5} (targeted insertion / random insertion)	Y	[14]
Recombinase	Tobacco	Direct gene transfer	Marker restored	Up to 10^{-4} (targeted insertion / total treated) Comparable to random insertion frequency	Y	[15]
	Arabidopsis	Agrobacterium	Marker restored	About 2 x 10^{-2} (targeted events / explants used)	Y	[16]
	Arabidopsis	Agrobacterium	Marker restored	About 10^{-3} (targeted events / explants used)	Y	[17]
	Rice	Bombardment	Marker restored	Not specified	Y	[18]
	Tobacco	Bombardment	BAC inserted	Not specified	Y	[19]
	Tobacco	Direct gene transfer	Marker restored	Not specified	Y	[20]
	Tobacco	Agrobacterium	Marker inserted	1% - 3%	Y	[21]

				(targeted events / explants used)		
	Arabidopsis	Agrobacterium	Marker restored	Up to 3.5 x 10^{-3} (targeted events / explants used)	Y	[22]
	Soybean	Bombardment	Marker restored	Not specified	Y	[23]
	Rice	Bombardment	Marker restored	0.2 - 0.3 targeted events per plate bombarded	Y	[24]
	Maize	Agrobacterium	Marker restored	6.7% - 6.9% (targeted events / explants used)	Y	[25]
	Maize	Agrobacterium/ Bombardment	Trait genes inserted	4% for bombardment 5.9% - 9.3% for Agrobacterium (targeted events / explants used)	Y	[26]
Meganuclease	Tobacco	Agrobacterium	Marker restored	Up to 1.88 x 10^{-3} (targeted insertion / random insertion)	Y	[27]
	Tobacco	Agrobacterium	Marker restored	2.58% of the herbicide-tolerant events	Y	[28]
	Tobacco	Agrobacterium	Marker restored	1% - 2% (targeted insertion / explants used)	Y	[29]
	Maize	Agrobacterium/ Bombardment	Marker restored	Up to 0.3 (targeted insertion / random insertion)	Y	[30]
	Maize	Agrobacterium + Cross	Marker restored	0.085% (targeted insertion / embryos screened)	Y	[31]
	Cotton	Bombardment	Marker inserted	1.8% of the herbicide-tolerant events	Y	[32]
	Barley	Agrobacterium	Marker restored	About 1% (targeted insertion / explants used)	Y	[33]
ZFN	Tobacco	Direct gene transfer	Marker restored	About 0.17 (targeted insertion / random insertion)	Y	[34]
	Maize	Silicon carbide whiskers	Gene disrupted by marker insertion	18.7% - 40% of the herbicide-tolerant events	Y	[35]
	Tobacco	Agrobacterium	Gene disrupted by marker insertion	Up to 10% of the herbicide-tolerant events	Y	[36]
	Arabidopsis	Agrobacterium	Marker inserted	0.1% of the herbicide-tolerant events	Y	[37]
	Maize	Bombardment	Marker inserted	Up to 5% of the herbicide-tolerant events	Y	[38]
	Arabidopsis Tobacco	Agrobacterium	Marker inserted	4.8% of herbicide-tolerant Arabidopsis events 6.7% of herbicide-tolerant tobacco events	Y	[39]
	Arabidopsis	Direct gene transfer	DNA fragment inserted	Up to 5.32% of all cells used based on the sequencing reads	N	[40]
	Tobacco	Agrobacterium + Replicon	Marker restored	Not specified	Y	[41]
	Maize	Bombardment	Marker inserted	Up to 30% of the herbicide-tolerant events	Y	[42]
	Potato	Agrobacterium + Replicon	Marker restored	Not specified	N	[43]

	Soybean	Bombardment	DNA fragment inserted	2.8% of the herbicide-tolerant events (7.1kb) 0.23% of the herbicide-tolerant events (16.2kb)	Y	[44]
TALEN	Tobacco	Direct gene transfer	Reporter gene inserted in frame	Up to 14% of all cells used	N	[45]
	Tomato	Agrobacterium + Replicon	Gene activated and marker inserted	7.28% of the herbicide-tolerant events	Y	[46]
	Potato	Agrobacterium + Replicon	Gene modified and marker inserted	Up to 41.7% of the herbicide-tolerant events	Y	[43]
	Potato	Agrobacterium	Marker restored	Up to 96% of the herbicide-tolerant events	Y	[47]
CRISPR-Cas	Rice	Direct gene transfer	DNA fragment inserted	Not specified	N	[48]
	Arabidopsis	Agrobacterium	Marker inserted	0.14% of the seeds screened	Y	[49]
	Tomato	Agrobacterium + Replicon	Gene activated and marker inserted	2.75% - 8.8% (targeted events / explants used)	Y	[46]
	Maize	Bombardment	Marker inserted	2.5% - 4.1% of the herbicide-tolerant events	Y	[50]
	Soybean	Bombardment	Marker inserted	3.8% - 4.6% of the herbicide-tolerant events	Y	[51]
	Rice	Bombardment	DNA fragment inserted	2.2% of the CRISPR-Cas-expressing events	Y	[52]
	Arabidopsis	Agrobacterium	Reporter gene inserted	0.2% of the CRISPR-Cas-expressing events	Y	[53]
	Potato	Agrobacterium + Replicon	Gene modified and marker inserted	Up to 12.5% of the herbicide-tolerant events	Y	[43]
	Tobacco	Agrobacterium + Replicon	Marker restored	Not specified	N	[54]
	Wheat	Bombardment + Replicon	Reporter gene inserted in frame	Not specified		
	Wheat	Bombardment + Replicon	Reporter gene inserted	5.74% of the transformed cells	N	[55]
	Rice	Ribonucleoprotein transfection	Epitope tag inserted in frame	2.13% - 4.69% of the target DNA	N	[56]
	Rice	Agrobacterium + Replicon	Reporter gene inserted in frame	4.7% - 8.5% of the herbicide-tolerant events	Y	[57]
	Maize	Bombardment	DNA fragment inserted	1% of the CRISPR-Cas-expressing events	Y	[58]
	Physcomytrella patens	Direct gene transfer	Gene disrupted by marker insertion	Up to 100% of the herbicide-tolerant events	Y	[59]
	Rice	Bombardment	Gene disrupted by marker insertion	8% of the herbicide-tolerant events	Y	[60]
	Tomato	Agrobacterium + Replicon	Gene repaired	25 % of the CRISPR-Cas-expressing events	Y	[61]
	Arabidopsis	Agrobacterium	Gene repaired	0.12% of the examined plants	Y	[62]

	Arabidopsis	Agrobacterium	Reporter gene inserted in frame	6.3% - 9.1% of the examined plants	Y	[63]
	Tomato	Agrobacterium	Gene repaired and marker inserted	1.29% of the herbicide-tolerant events	Y	[64]
	Rice	Agrobacterium	Marker inserted	3.8% - 5.3% of the herbicide-tolerant events	Y	[65]
	Rice	Bombardment	Epitope tag inserted in frame	Not specified	N	[66]
	Tomato	Agrobacterium + Replicon	Gene activated and marker inserted	Up to 12.8% of the herbicide-tolerant events	Y	[67]
	Rice	Bombardment	DNA fragment inserted	6.25% of the CRISPR-Cas-expressing events	Y	[68]
	Maize	Bombardment	Landing pad with marker inserted	Up to 18% of the herbicide-tolerant events	Y	[26]
	Rice	Bombardment	DNA fragment inserted	An average of 25% of the CRISPR-Cas-expressing events	Y	[69]
	Maize	Agrobacterium	DNA fragment inserted	Up to 4.7% of the herbicide-tolerant events	Y	[70]

*Not all efficiencies are comparable due to the difference among calculation methods.

Supplementary Table References:

1. Paszkowski, J., et al., *Gene targeting in plants.* EMBO J, 1988. **7**(13): p. 4021-6.
2. Offringa, R., et al., *Extrachromosomal homologous recombination and gene targeting in plant cells after Agrobacterium mediated transformation.* EMBO J, 1990. **9**(10): p. 3077-84.
3. Halfter, U., P.C. Morris, and L. Willmitzer, *Gene targeting in Arabidopsis thaliana.* Mol Gen Genet, 1992. **231**(2): p. 186-93.
4. Hrouda, M. and J. Paszkowski, *High fidelity extrachromosomal recombination and gene targeting in plants.* Mol Gen Genet, 1994. **243**(1): p. 106-11.
5. Miao, Z.H. and E. Lam, *Targeted disruption of the TGA3 locus in Arabidopsis thaliana.* Plant J, 1995. **7**(2): p. 359-65.
6. Risseeuw, E., et al., *Targeted recombination in plants using Agrobacterium coincides with additional rearrangements at the target locus.* Plant J, 1995. **7**(1): p. 109-19.
7. Thykjaer, T., et al., *Gene targeting approaches using positive-negative selection and large flanking regions.* Plant Mol Biol, 1997. **35**(4): p. 523-30.
8. Kempin, S.A., et al., *Targeted disruption in Arabidopsis.* Nature, 1997. **389**(6653): p. 802-3.
9. Risseeuw, E., M.E. Franke-van Dijk, and P.J. Hooykaas, *Gene targeting and instability of Agrobacterium T-DNA loci in the plant genome.* Plant J, 1997. **11**(4): p. 717-28.
10. Schaefer, D.G. and J.P. Zryd, *Efficient gene targeting in the moss Physcomitrella patens.* Plant J, 1997. **11**(6): p. 1195-206.
11. Terada, R., et al., *Efficient gene targeting by homologous recombination in rice.* Nat Biotechnol, 2002. **20**(10): p. 1030-4.
12. Shaked, H., C. Melamed-Bessudo, and A.A. Levy, *High-frequency gene targeting in Arabidopsis plants expressing the yeast RAD54 gene.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(34): p. 12265-12269.

13. Terada, R., et al., *Gene targeting by homologous recombination as a biotechnological tool for rice functional genomics.* Plant Physiol, 2007. **144**(2): p. 846-56.
14. Even-Faitelson, L., et al., *Localized egg-cell expression of effector proteins for targeted modification of the Arabidopsis genome.* Plant J, 2011. **68**(5): p. 929-37.
15. Albert, H., et al., *Site-Specific Integration of DNA into Wild-Type and Mutant Lox Sites Placed in the Plant Genome.* Plant Journal, 1995. **7**(4): p. 649-659.
16. Vergunst, A.C., L.E.T. Jansen, and P.J.J. Hooykaas, *Site-specific integration of Agrobacterium T-DNA in Arabidopsis thaliana mediated by Cre recombinase.* Nucleic Acids Research, 1998. **26**(11): p. 2729-2734.
17. Vergunst, A.C. and P.J. Hooykaas, *Cre/lox-mediated site-specific integration of Agrobacterium T-DNA in Arabidopsis thaliana by transient expression of cre.* Plant Mol Biol, 1998. **38**(3): p. 393-406.
18. Srivastava, V. and D.W. Ow, *Biolistic mediated site-specific integration in rice.* Molecular Breeding, 2002. **8**(4): p. 345-350.
19. Choi, S., et al., *A new approach for the identification and cloning of genes: the pBACwich system using Cre/lox site-specific recombination.* Nucleic Acids Res, 2000. **28**(7): p. E19.
20. Day, C.D., et al., *Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced.* Genes & Development, 2000. **14**(22): p. 2869-2880.
21. Nanto, K., K. Yamada-Watanabe, and H. Ebinuma, *Agrobacterium-mediated RMCE approach for gene replacement.* Plant Biotechnology Journal, 2005. **3**(2): p. 203-214.
22. Louwerse, J.D., et al., *Stable recombinase-mediated cassette exchange in Arabidopsis using Agrobacterium tumefaciens.* Plant Physiology, 2007. **145**(4): p. 1282-1293.
23. Li, Z.S., et al., *Site-Specific Integration of Transgenes in Soybean via Recombinase-Mediated DNA Cassette Exchange.* Plant Physiology, 2009. **151**(3): p. 1087-1095.
24. Nandy, S. and V. Srivastava, *Site-specific gene integration in rice genome mediated by the FLP-FRT recombination system.* Plant Biotechnol J, 2011. **9**(6): p. 713-21.
25. Anand, A., et al., *High efficiency Agrobacterium-mediated site-specific gene integration in maize utilizing the FLP-FRT recombination system.* Plant Biotechnol J, 2019. **17**(8): p. 1636-1645.
26. Gao, H., et al., *Complex Trait Loci in Maize Enabled by CRISPR-Cas9 Mediated Gene Insertion.* Front Plant Sci, 2020. **11**: p. 535.
27. Puchta, H., B. Dujon, and B. Hohn, *Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination.* Proc Natl Acad Sci U S A, 1996. **93**(10): p. 5055-60.
28. Tzfira, T., et al., *Site-specific integration of Agrobacterium tumefaciens T-DNA via double-stranded intermediates.* Plant Physiol, 2003. **133**(3): p. 1011-23.
29. Chilton, M.D. and Q. Que, *Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration.* Plant Physiol, 2003. **133**(3): p. 956-65.
30. D'Halluin, K., et al., *Homologous recombination: a basis for targeted genome optimization in crop species such as maize.* Plant Biotechnol J, 2008. **6**(1): p. 93-102.
31. Ayar, A., et al., *Gene targeting in maize by somatic ectopic recombination.* Plant Biotechnol J, 2013. **11**(3): p. 305-14.
32. D'Halluin, K., et al., *Targeted molecular trait stacking in cotton through targeted double-strand break induction.* Plant Biotechnology Journal, 2013. **11**(8): p. 933-941.
33. Watanabe, K., et al., *Stable gene replacement in barley by targeted double-strand break induction.* J Exp Bot, 2016. **67**(5): p. 1433-45.

34. Wright, D.A., et al., *High-frequency homologous recombination in plants mediated by zinc-finger nucleases.* Plant J, 2005. **44**(4): p. 693-705.
35. Shukla, V.K., et al., *Precise genome modification in the crop species Zea mays using zinc-finger nucleases.* Nature, 2009. **459**(7245): p. 437-41.
36. Cai, C.Q., et al., *Targeted transgene integration in plant cells using designed zinc finger nucleases.* Plant Mol Biol, 2009. **69**(6): p. 699-709.
37. de Pater, S., et al., *ZFN-induced mutagenesis and gene-targeting in Arabidopsis through Agrobacterium-mediated floral dip transformation.* Plant Biotechnol J, 2009. **7**(8): p. 821-35.
38. Ainley, W.M., et al., *Trait stacking via targeted genome editing.* Plant Biotechnol J, 2013. **11**(9): p. 1126-34.
39. Weinthal, D.M., R.A. Taylor, and T. Tzfira, *Nonhomologous end joining-mediated gene replacement in plant cells.* Plant Physiol, 2013. **162**(1): p. 390-400.
40. Qi, Y., et al., *Increasing frequencies of site-specific mutagenesis and gene targeting in Arabidopsis by manipulating DNA repair pathways.* Genome Res, 2013. **23**(3): p. 547-54.
41. Baltes, N.J., et al., *DNA replicons for plant genome engineering.* Plant Cell, 2014. **26**(1): p. 151-63.
42. Kumar, S., et al., *A modular gene targeting system for sequential transgene stacking in plants.* J Biotechnol, 2015. **207**: p. 12-20.
43. Butler, N.M., et al., *Geminivirus-Mediated Genome Editing in Potato (Solanum tuberosum L.) Using Sequence-Specific Nucleases.* Front Plant Sci, 2016. **7**: p. 1045.
44. Bonawitz, N.D., et al., *Zinc finger nuclease-mediated targeting of multiple transgenes to an endogenous soybean genomic locus via non-homologous end joining.* Plant Biotechnol J, 2019. **17**(4): p. 750-761.
45. Zhang, Y., et al., *Transcription activator-like effector nucleases enable efficient plant genome engineering.* Plant Physiol, 2013. **161**(1): p. 20-7.
46. Cermak, T., et al., *High-frequency, precise modification of the tomato genome.* Genome Biol, 2015. **16**: p. 232.
47. Forsyth, A., et al., *Transcription Activator-Like Effector Nucleases (TALEN)-Mediated Targeted DNA Insertion in Potato Plants.* Front Plant Sci, 2016. **7**: p. 1572.
48. Shan, Q., et al., *Targeted genome modification of crop plants using a CRISPR-Cas system.* Nat Biotechnol, 2013. **31**(8): p. 686-8.
49. Schiml, S., F. Fauser, and H. Puchta, *The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny.* Plant J, 2014. **80**(6): p. 1139-50.
50. Svitashev, S., et al., *Targeted Mutagenesis, Precise Gene Editing, and Site-Specific Gene Insertion in Maize Using Cas9 and Guide RNA.* Plant Physiol, 2015. **169**(2): p. 931-45.
51. Li, Z., et al., *Cas9-Guide RNA Directed Genome Editing in Soybean.* Plant Physiol, 2015. **169**(2): p. 960-70.
52. Li, J., et al., *Gene replacements and insertions in rice by intron targeting using CRISPR-Cas9.* Nat Plants, 2016. **2**: p. 16139.
53. Zhao, Y., et al., *An alternative strategy for targeted gene replacement in plants using a dual-sgRNA/Cas9 design.* Sci Rep, 2016. **6**: p. 23890.
54. Cermak, T., et al., *A Multipurpose Toolkit to Enable Advanced Genome Engineering in Plants.* Plant Cell, 2017. **29**(6): p. 1196-1217.
55. Gil-Humanes, J., et al., *High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9.* Plant J, 2017. **89**(6): p. 1251-1262.
56. Butt, H., et al., *Efficient CRISPR/Cas9-Mediated Genome Editing Using a Chimeric Single-Guide RNA Molecule.* Frontiers in Plant Science, 2017. **8**.
57. Wang, M., et al., *Gene Targeting by Homology-Directed Repair in Rice Using a Geminivirus-Based CRISPR/Cas9 System.* Mol Plant, 2017. **10**(7): p. 1007-1010.

58. Shi, J., et al., *ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions.* Plant Biotechnol J, 2017. **15**(2): p. 207-216.
59. Collonnier, C., et al., *CRISPR-Cas9-mediated efficient directed mutagenesis and RAD51-dependent and RAD51-independent gene targeting in the moss Physcomitrella patens.* Plant Biotechnol J, 2017. **15**(1): p. 122-131.
60. Begemann, M.B., et al., *Precise insertion and guided editing of higher plant genomes using Cpf1 CRISPR nucleases.* Sci Rep, 2017. **7**(1): p. 11606.
61. Dahan-Meir, T., et al., *Efficient in planta gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system.* Plant J, 2018.
62. Hahn, F., et al., *Homology-Directed Repair of a Defective Glabrous Gene in Arabidopsis With Cas9-Based Gene Targeting.* Front Plant Sci, 2018. **9**: p. 424.
63. Miki, D., et al., *CRISPR/Cas9-mediated gene targeting in Arabidopsis using sequential transformation.* Nat Commun, 2018. **9**(1): p. 1967.
64. Danilo, B., et al., *The DFR locus: A smart landing pad for targeted transgene insertion in tomato.* PLoS One, 2018. **13**(12): p. e0208395.
65. Lee, K., et al., *CRISPR/Cas9-mediated targeted T-DNA integration in rice.* Plant Mol Biol, 2019. **99**(4-5): p. 317-328.
66. Ali, Z., et al., *Fusion of the Cas9 endonuclease and the VirD2 relaxase facilitates homology-directed repair for precise genome engineering in rice.* Communications Biology, 2020. **3**(1).
67. Vu, T.V., et al., *Highly efficient homology-directed repair using CRISPR/Cpf1-geminiviral replicon in tomato.* Plant Biotechnol J, 2020. doi.org/10.1111/pbi.13373
68. Dong, O.X., et al., *Marker-free carotenoid-enriched rice generated through targeted gene insertion using CRISPR-Cas9.* Nat Commun, 2020. **11**(1): p. 1178.
69. Lu, Y., et al., *Targeted, efficient sequence insertion and replacement in rice.* Nat Biotechnol, 2020. doi.org/10.1038/s41587-020-0581-5
70. Barone, P., et al., *Efficient Gene Targeting in Maize Using Inducible CRISPR-Cas9 and Marker-free Donor Template.* Mol Plant, 2020. doi.org/10.1016/j.molp.2020.06.008