Advances in Agrobacterium transformation and vector design result in high-frequency targeted gene insertion in maize

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Introduction

Demonstration that the presence of a DNA double-strand break (DSB) increases frequency of homologous recombination at the target site by more than 1000-fold (Puchta et al., 1993; Smith et al., 1995) has led to development of several classes of site-directed nucleases (SDNs) – homing endonucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR associated) nucleases – capable of cutting genomic DNA and generating DSBs at predetermined locations (Chandrasegaran and Carroll, 2016; Voytas, 2013). Although all SDNs have been demonstrated to be useful DSB reagents, CRISPR-Cas has quickly become the technology of choice for most laboratories due to its high activity, versatility and low cost (Barrangou and Doudna, 2016; Voytas, 2013). In eukaryotic cells, DSBs can be repaired via two highly conserved pathways – non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Puchta, 2005; Puchta et al., 1996). NHEJ is prone to imperfect repair and may lead to variable size insertions and/or deletions (INDELS) resulting in frame-shifts and consequent gene knockouts. If two guide RNAs (gRNAs), with homology to sites flanking a gene or a DNA fragment of interest, are used, it can result in a deletion of the entire sequence between the two sites (Gao et al., 2020a; Srivastava et al., 2017). HDR enables precise insertion of a donor sequence to a specific target site and relies on the presence of homology regions to sequences upstream and downstream of the DSB. By using a repair template that contains nucleotide alterations, specific changes to the coding sequence of an endogenous gene can be introduced. Alternatively, if the repair template includes a new sequence (e.g. gene of interest), the DSB repair can result in the sequence insertion into a specific chromosomal site avoiding endogenous gene disruption often observed during random integration. This approach allows for consecutive insertions of several genes into the same chromosomal region (gene stacking), which can significantly simplify breeding programmes (Ainley et al., 2013; Gao et al., 2020b; Kumar et al., 2016a; Que et al., 2010).

Recently, two new CRISPR-Cas-based technologies, base editing and prime editing, have been reported (Anzalone et al., 2020). These technologies use either dead Cas9 or Cas9 nickase fusions with DNA deaminases (base editing), or Cas9 nickase fusion with reverse transcriptase (prime editing) and enable targeted nucleotide modifications without generating DSBs. Although these approaches open new exciting opportunities in gene editing, they do not allow site-specific insertion of large DNA sequences.

HDR is the preferred mechanism for targeted gene insertion in comparison to the NHEJ pathway, but its application remains limited due to low efficiency and a high attrition rate of recovered events. Additionally, in most successful gene insertion experiments reported in plants, the repair template contains a
selectable marker as the gene of interest (Begemann et al., 2017; Čermák et al., 2015; Gao et al., 2020b; Lee et al., 2019; Li et al., 2015; Svitashev et al., 2015; Wang et al., 2017). With some rare exceptions (Gao, 2020b), the presence of a selectable marker in the repair template, in addition to the gene of interest, is highly undesirable and needs to be removed in the consecutive generation(s). Such design increases the size of the repair template and may further reduce the frequency of insertion events (Vu et al., 2019). A targeted insertion with a selectable marker outside the repair template is more challenging as it requires two independent integration events in a single cell. HDR-mediated targeted gene insertion and NHEJ-mediated random integration of a selectable marker gene. Due to the DSBR repair mechanisms, the selectable marker gene frequently co-integrates with the repair template into the same locus, further lowering the frequency of usable events (Svitashev et al., 2015). As a result, very few examples of HDR-mediated selectable marker-free DNA insertions have been reported and usually relate to rather short DNA fragments (Li et al., 2016; Miki et al., 2018; Peng et al., 2020; Shi et al., 2017; Zhao et al., 2016).

During the past decade, several approaches to improve frequency of targeted insertions in plants have been explored. One strategy is associated with modulating HDR and NHEJ repair pathways. For example, mutations in key NHEJ pathway genes, ku70 and lig4, in Arabidopsis and rice have been reported to increase HDR frequency (Endo et al., 2016; Qi et al., 2013). Alternatively, overexpression of RAD54 gene in egg cells increased gene insertion frequency in Arabidopsis (Even-Faitelson et al., 2011). However, permanent knock-out of genes involved in the NHEJ repair pathway may lead to serious abnormalities, genome instability, chromosomal rearrangements and even plant death. Although transient suppression of the NHEJ pathway is theoretically possible, this approach could significantly complicate the experimental design and may still lead to genome instability.

Another important factor influencing the efficiency of targeted insertions is the presence and amount of repair template in the nucleus. For this reason, most successful gene insertion experiments have used particle bombardment, which allows delivery of a higher copy number of DNA molecules in comparison to Agrobacterium-mediated transformation. Nevertheless, Agrobacterium infection remains the preferred method of plant transformation due to its less invasive nature, simplicity and reproducibility. To increase repair template copy number using Agrobacterium-mediated delivery, several groups have used a geminival replication system (Baltes et al., 2014; Čermák et al., 2015; Dahan-Meir et al., 2018; Gil-Humanes et al., 2017; Vu et al., 2020). Although it shows positive results, this approach requires complex vector designs, may lead to uncontrolled replication of the repair template and has problems with plant regeneration (Afzal et al., 2020).

In planta (Ayar et al., 2013; Fauser et al., 2012; Fauser et al., 2014; Schimi et al., 2014) and infra-genomic (Kumar et al., 2016a; Kumar et al., 2016b; Wolter et al., 2018) homologous recombination approaches have been used to address plant transformation inefficiency and quality of targeted insertion events. These approaches rely on stable random integration of the repair template, flanked by SDN target sites, into the genome. Introduction of SDN (usually by crossing) releases the repair template and simultaneously generates DSB at the intended target site. Long generation time and low efficiency have been two major limitations of this method in plants. However, a promising new approach with high efficiency intra-genomic targeted gene insertion has recently been reported (Barone et al., 2020).

It has been suggested that linear DNA can be a better substrate for HDR-mediated DSB repair and increase frequency of targeted gene insertions (Beumer et al., 2006; Song and Stieger, 2017). Flanking the repair template with target sites and the release of linear repair template has been shown to provide a 2- to 5-fold improvement of targeted gene insertion in human culture cells in comparison to circular plasmid DNA (Zhang et al., 2017). However, in several experiments conducted in plant species using particle bombardment (Dong et al., 2020; Sun et al., 2016; Zhao et al., 2016) and Agrobacterium-mediated (Peng et al., 2020) delivery of editing components, no such effect was demonstrated.

Here, we report on significant increase of HDR-based targeted gene insertion frequency in maize using Agrobacterium-mediated transformation. Advancements in transformation approaches, combined with improvements in vector design, allowed us to increase the frequency (by two orders of magnitude) and improve the quality of HDR-mediated gene insertion events. Moreover, our data demonstrate that targeted insertion of a repair template with or without selectable marker gene occurs with comparable frequencies. These results further enable the application of genome editing for trait product development in a wide variety of crop species amenable to Agrobacterium-mediated transformation.

**Results**

**Target site selection**

Frequency of targeted gene insertion may vary for different sites and likely relates to chromatin structure, target site accessibility, cleavage efficiency and the length of homology regions. In our experience, frequency of gene insertion can vary 4- to 5-fold or more, for different target sites. TS45 was identified in previous particle bombardment experiments as a site with medium-to-high HDR frequency (Gao et al., 2020b). Selection of this target site was essential for this study as it allowed us to generate a substantial number of HDR-based gene insertion events, compare results of different experiments and draw reliable conclusions.

**Vector design**

Three different T-DNA vectors were used in this study (Figure 1). Two nearly identical vectors (Figure 1a and b) included morphogenic genes, Wus and Bbm, regulated by the Axig1 and PLTP promoters, respectively (Lowe et al., 2018), maize Ubiquitin promoter regulated Streptococcus pyogenes Cas9 (Svitashev et al., 2015), Polymerase III U6 promoter regulated gRNA for previously described genomic target site TS45 (Gao et al., 2020b), the first selectable marker gene – acetalactate synthase (Als) promoter regulating highly herbicide-resistant Als (Hra) gene (Green et al., 2009), and the repair template comprised of the NptII (neomycin phosphotransferase II) gene as the second selectable marker gene under the maize Ubiquitin promoter (Anand et al., 2019) flanked with regions of homology (HR1 and HR2). The only difference is the presence of TS45 sequences with protospacer adjacent motif (PAM) flanking HR1 and HR2 fragments in the second vector (Figure 1b) that results in release of the repair template from the T-DNA upon target sites cleavage by Cas9 nuclease. This vector design allowed for evaluation of the repair template release effect on the frequency of HDR-mediated
targeted gene insertion. In addition, the presence of two selectable marker genes, one inside (\textit{NptII}) and one outside (\textit{Hra}) the repair template, allowed us to evaluate the impact of selectable marker gene position on the frequency and quality of insertion events. It is important to emphasize that the two selectable marker genes used in this vector have different modes of action and require different selective agents, \textit{Geneticin} (G418) and \textit{Imazapyr}, respectively. Based on our experience, both agents provide very tight selection with approximately 4\%–5\% escape rate for G418 and 2\%–3\% for \textit{Imazapyr}. Therefore, transformation and regeneration frequencies using both genes and the corresponding selective agents are practically indistinguishable and have no impact on the reported HDR frequency.

The third vector (Figure 1c) contained two morphogenic genes, \textit{Wus2} and \textit{Bbm}, regulated by \textit{Axig1} and PLTP promoters, respectively, maize \textit{Ubiquitin} promoter regulated \textit{Streptococcus pyogenes Cas9}, Polymerase III U6 promoter regulated gRNA for genomic target site TS45, the repair template comprised of the trait gene, \textit{Arabidopsis carboxylesterase 20} (\textit{CXE-20}) (Allen et al., 2015) under rice \textit{Actin} promoter (McElroy et al., 1990) flanked with homology regions (HR1 and HR2) and TS45 target sequences with PAM, and selectable marker gene \textit{NptII} under maize \textit{Ubiquitin} promoter. In this vector, a selectable marker gene is placed downstream from the repair template. With this configuration, cleavage of the target sites flanking the repair template at the transient stage results not only in the release of the repair template, but also in separation of the selectable marker gene from the rest of the T-DNA (Figure 1c). This design allowed us to evaluate the effect of selectable marker gene position in T-DNA on both transformation and targeted gene insertion frequencies.

The described vectors were transformed into \textit{Agrobacterium LBA4404} cells containing the pVIR9 accessory plasmid (Anand et al., 2018), which provides increased virulence and T-DNA transmission to maize embryo cells.

**Targeted gene insertion**

In multiple experiments conducted in our group using conventional \textit{Agrobacterium}-mediated transformation with different SDNs (meganucleases, ZFNs and gRNA/Cas9), the rates of targeted gene insertions were less than 0.1\% (Barone et al. and Svitashev et al., unpublished data). This study is the first attempt to evaluate the effect of our advanced transformation system (morphogenic genes and a new accessory plasmid) on the frequency of HDR-based genome editing. Although we did not include the conventional transformation system (no morphogenic genes and a new accessory plasmid) as control into our experimental design, the 0.1\% frequency of gene insertion observed in our previous experiments is used as a baseline to evaluate the new advanced transformation approach.

The insertion of a gene of interest flanked by homology regions into a DSB site can occur through HDR, NHEJ, or a combination of both pathways. As a result, a broad spectrum of different outcomes is possible. Single copy gene insertions can occur via perfect HDR-based repair, combination of HDR and NHEJ, and NHEJ-mediated repair only. More complex integration events that involve multiple copies of the donor template and fragments of other transgenic and/or genomic sequences are also very

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic description of T-DNA constructs. (a) T-DNA vector containing morphogenic genes \textit{Wus2} and \textit{Bbm}, \textit{Cas9}, gRNA, selectable marker gene 1 (\textit{Hra}) and repair template, comprising selectable marker gene 2 (\textit{NptII}) flanked with homology arms (HR1 and HR2). (b) T-DNA vector as on figure (a) supplemented with Cas9/gRNA TS45 target sites (TS) flanking homology arms. (c) T-DNA vector containing morphogenic genes \textit{Wus2} and \textit{Bbm}, \textit{Cas9}, gRNA, repair template (\textit{CXE-20}, homology arms, HR1 and HR2) flanked with TS45 target sequences, and selectable marker gene (\textit{NptII}).}
\end{figure}
| Experiment (Genotype) | Selectable marker gene (position in T-DNA) | Selective agent | Embryos infected (%) of embryos infected | T0 plants recovered (% of T0 plants (%) of T0s analysed) | HR1-positive T0 plants (% of T0s analysed) | HR2-positive T0 plants (% of T0s analysed) | HR1HR2-positive T0 plants (% of T0s analysed) | Long PCR-positive T0 plants (% of T0s analysed) | % of HR1HR2-positive T0 plants that are long PCR-positive |
|----------------------|-------------------------------------------|----------------|------------------------------------------|------------------------------------------------|-------------------------------------------|-------------------------------------------|-----------------------------------------------|-----------------------------------------------|--------------------------------------------------|
| EXPERIMENT 1 (PH184C) | NPTII (in repair template) | G418 | 889 | 343 (39%) | 21 (6.1%) | 16 (4.4%) | 12 (3.5%) | 6 (1.7%) | 50% |
| EXPERIMENT 1 (PH184C) | NPTII (in repair template) | G418 | 865 | 425 (49%) | 50 (11.8%) | 57 (13.4%) | 39 (9.2%) | 26 (6.1%) | 67% |
| EXPERIMENT 2 (PH184C) | NPTII (in repair template) | G418 | 1155 | 591 (51%) | 25 (4.2%) | 17 (2.9%) | 14 (2.4%) | 7 (1.2%) | 50% |
| EXPERIMENT 2 (PH184C) | NPTII (in repair template) | G418 | 1185 | 365 (31%) | 52 (14.2%) | 38 (10.4%) | 33 (9.0%) | 21 (5.7%) | 64% |
| EXPERIMENT 3 (PH184C) | NPTII (in repair template) | G418 | 909 | 320 (35%) | 43 (13.4%) | 37 (11.5%) | 33 (10.3%) | 20 (5.8%) | 60% |
| EXPERIMENT 3 (PH184C) | HRA (upstream from repair template) | Imazapyr | 1975 | 766 (39%) | 90 (11.7%) | 83 (10.8%) | 64 (8.4%) | 34 (4.6%) | 53% |
| EXPERIMENT 4 (PH1V5T) | NPTII (downstream from repair template) | G418 | 1000 | 1403 (140%) | 165 (11.8%) | 163 (11.6%) | 116 (8.3%) | 55 (3.9%) | 47% |

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For new product development, perfect, single copy HDR-based insertion events are highly preferred and, therefore, our analytical approach was designed to identify only the desired insertion outcomes.

The results of T0 plant analysis from the four experiments conducted using the constructs described above are summarized in Table 1. In Experiment 1, 889 and 865 maize immature embryos (PH184C genotype) were transformed using Agrobacterium carrying T-DNA constructs shown in Figures 1a and b, respectively. Embryos were then subjected to tissue culture and regeneration procedures described in the Methods section. A total of 343 and 425 T0 plants, respectively, were regenerated on G418 containing media using selectable marker gene (NptII) within the repair template and analysed as illustrated in Figure 2. Due to the high number of samples, instead of a gel-based analysis of PCR amplification products, HDR-mediated insertion events were first detected by diagnostic HR1- and HR2-junction qPCR (Figure 2a and b) as described in Methods using primers and probes listed in Table S1. For the T-DNA vector without target sites flanking the repair template (Figure 1a), 21 out of 343 T0 plants (6.1%) were HR1-junction qPCR-positive, while HR2-positive junctions were detected in 16 (4.4%) plants. For the construct with flanking target sites allowing donor DNA release (Figure 1b), the numbers were significantly higher with 50 (11.8%) and 57 (13.4%) HR1- and HR2-junction qPCR-positive T0 plants, respectively. Twelve (3.5%) and 39 (9.2%) plants were positive for both HR1 and HR2 junctions using vectors without and with target sites flanking donor template, respectively (Table 1, Experiment 1).

Southern blot hybridization has been the main analytical tool for both random and targeted gene insertion events (Svitashev et al., 2015). In the past decade, however, Southern hybridization has been successfully replaced by rapidly developing PCR-based (qPCR, improved long PCR using ultrahigh fidelity DNA polymerase) and sequencing (NextGen sequencing) technologies due to their sensitivity, precision, reproducibility, high throughput capability and low cost. Therefore, HR1/HR2-positive events were further analysed by long PCR spanning the entire insertion using genomic primers located outside the HR1 and the HR2 homology regions (Figure 2a and c).

Long PCR products were analysed by agarose gel electrophoresis (Figure 2d shows results for a group of 15 T0 plants with representative patterns as an example). The presence of an approximately 4.6 kb band indicates events with putative perfect HDR-mediated gene insertions. A lower, approximately 1.0 kb band, represents PCR product of the allele without insertion. Size variation of this band indicates various size deletions and/or insertions. Plasmid 4 has 3 bands indicating that the plant is chimeric. Plant 15 has only one band, which is slightly smaller than 4.6 kb indicating incomplete gene sequence insertion in one allele and a large insertion and/or deletion in the second allele resulting in no lower band amplification.

**Figure 2** Plant analysis for gene insertion events. (a) Schematic depiction of HDR-mediated targeted gene insertion locus. (b) Diagnostic qPCR primers and expected products from 5’ and 3’ PCR indicating gene insertion. (c) PCR primers and expected product of long PCR used for further characterization of HR1/HR2 junction qPCR-positive gene insertion events. (d) Example of long PCR diagnostic agarose gel. Upper, approximately 4.6 kb band, corresponds to a putative perfect single copy gene insertion. Lower, approximately 1 kb band, corresponds to amplification of the allele without insertion. Size variation of the shorter fragment reflects the presence of various size deletions and/or insertions. Plant 4 has 3 bands indicating that the plant is chimeric. Plant 15 has only one band, which is slightly smaller than 4.6 kb indicating incomplete gene sequence insertion in one allele and a large insertion and/or deletion in the second allele resulting in no lower band amplification.
The exact same constructs and experimental design were used in Experiment 2 to validate the high frequency and quality of Agrobacterium-mediated gene insertion observed in Experiment 1. A total of 1155 and 1185 embryos were transformed and produced 591 and 365 T0 plants using constructs without and with target sites flanking the repair template, respectively. For T-DNA vector without flanking target sites, 25 (4.2%) and 17 (2.9%) T0 plants were HR1- and HR2-junction qPCR-positive, respectively. For the construct with flanking target sites, the numbers were 52 (14.2%) for HR1 junction and 38 (10.4%) for HR2 junction. Overall, for T-DNA vector without target sites, 14 T0 plants (2.4%) were HR1/HR2-junction qPCR-positive, while for the construct with flanking target sites, 33 T0 plants (9.0%) were HR1/HR2-positive when analysed by diagnostic junction qPCR (Table 1, Experiment 2). Long PCR analysis demonstrated that 7 (1.2%) and 21 (5.7%) T0 plants had putative perfect gene insertions for the two constructs, respectively. In this experiment, only two HR1/HR2-junction positive T0 plants did not show the presence of randomly inserted T-DNA.

Experiments 1 and 2 showed very close results demonstrating highly efficient targeted gene insertion using our advanced transformation technology – a 25-to-35-fold improvement over conventional Agrobacterium-mediated delivery. In addition, release of the repair template from the T-DNA increased HDR-based insertion frequency by approximately 3-fold bringing the total frequency improvement to approximately 100-fold.

To compare insertion frequencies of repair templates with and without selectable marker gene, we used the T-DNA construct shown in Figure 1b. In this experiment (Experiment 3), after Agrobacterium infection, a total of 2884 transformed embryos were divided in two groups. For the group of 909 embryos, tissue culture steps and T0 plant regeneration were performed on selective media containing G418 using NptII as selectable marker gene (inside the repair template), while for 1975 embryos, these steps were conducted using Imazapyr as the selective agent and Hra as the selectable marker gene (outside of the repair template). The number of embryos used in the treatment with Imazapyr selection was intentionally doubled to compensate for potentially lower frequency of HDR-mediated insertion events with selectable marker gene outside of the repair template. A total of 320 and 766 plants were regenerated using G418 and Imazapyr selection, respectively.

In the case of G418 selection, 43 (13.4%) and 37 (11.5%) T0 plants were HR1- and HR2-junction qPCR-positive, respectively. When Imazapyr was used as the selective agent, the numbers were 90 (11.7%) and 83 (10.8%), respectively. With the selectable marker gene (NptII) inside the repair template, 33 (10.3%) regenerants were both HR1- and HR2-positive by junction qPCR, while 64 (8.4%) plants were HR1/HR2-positive when selectable marker gene (Hra) outside the repair template was used (Table 1, Experiment 3). All but two HR1/HR2-junction positive T0 plants (both regenerated on media with G418) were also positive for random T-DNA insertions.

Experiment 4 was designed to test the frequency of targeted insertion using a drought tolerance-related gene (CXE-20), and the effect of selectable marker gene position (downstream of repair template, Figure 1c) on transformation and trait gene insertion frequencies. To test potential differences in gene insertion frequency between genotypes, this experiment was conducted using a different Corteva Agriscience inbred line, PH1VST, which has higher regenerability than PH184C. One thousand immature embryos were transformed, and 1403 T0 plants regenerated in this experiment. One hundred and sixty-five (11.8%) and 163 (11.6%) T0 plants were either HR1- or HR2-junction qPCR-positive, respectively. A total of 116 (8.3%) T0 plants were positive for both HR1 and HR2 junctions, and 55 (3.9%) plants were identified to have putative perfect integration events by long genomic PCR (Table 1, Experiment 4). As expected, all HR1/HR2 junction positive T0 plants were also positive for randomly inserted T-DNA.

It is important to emphasize that, in the four experiments conducted, the proportion of long PCR-positive events varied and possibly reflects differences in vector design and position of selectable marker genes (Table 1). For the construct without flanking target sites, this number was 50% in both Experiments 1 and 2. In Experiments 1, 2 and 3 when the repair template was flanked with TS45 target sites and selectable marker was inside the repair template, 60 to 67% of all HR1/HR2-positive events were also long PCR-positive. When selectable marker gene (Hra) upstream of the repair template was used, this number was 53% (Experiment 3). In Experiment 4 when selectable marker gene (NptII) was placed downstream from the repair template, the proportion of long PCR-positive events dropped to 47%. Although these percentages likely reflect the differences in experimental and vector designs, they cannot be statistically validated due to the number of events analysed.

Edit transmission to next generation and segregation analysis

To evaluate transmission of targeted gene insertions to the next generation, segregation pattern and attrition rates, a group of 68 out of 86 long PCR-positive T0 plants from Experiments 1 and 3, were sent to the greenhouse. Nineteen out of 68 T0 plants either died or were sterile. A total of 49 fertile T0 plants were used as pollen donors and crossed with wild-type PH184C plants to generate T1 progeny (Table 2).

Fifteen to 40 T1 plants (based on seed availability) for each of the 49 T0 events were analysed by qPCR for the presence of targeted gene insertion and T-DNA components (Cas9, gRNA, Bbm, Wus2, Hra and NptII). Results of this analysis are summarized in Table 2, with more detailed information provided in Tables S3 and S4. Five T0 plants (Plants 2, 4, 38, 46 and 48, Tables S3 and S4) did not show transmission of the insertion to the T1 generation likely due to the chimeric nature of the original events. Of the remaining 44 events, 33 showed approximately 1:1 segregation for the targeted gene insertion, consistent with stable Mendelian inheritance of a mono-allelic locus. One T0 plant in Experiment 3 with Imazapyr selection (Plant 45, Table S4) transmitted the gene insertion to the entire T1 progeny likely indicating a perfect bi-allelic insertion event. A T0 plant from Experiment 1 with donor template release (Plant 8, Table S3) also had insertions in both alleles: one allele containing a putative perfect insertion, while the second one was only HR1-junction qPCR-positive. For 9 T0 plants, gene insertion alleles were transmitted to T1 progeny with frequencies lower than 30% likely due to the chimeric nature of the T0 plants. In addition, 5 out of these 9 T0 plants showed two types of insertions: HR1/ HR2-positive and either HR1- or HR2-positive only, as indicated by the T1 segregation analysis (Plants 20, 29, 31, 32 and 33, Table S4).

Progenies positive for gene insertion and T-DNA-free were obtained from 39 T0 plants. In the remaining 5 events, random T-DNA integration likely occurred either into the same chromosome as the targeted gene insertion, or into multiple genomic sites. In
these cases, additional rounds of backcrosses are needed to complete segregation of T-DNA components.

**Gene insertion verification by sequencing**

T1 progeny plants of 13 insertion-positive and properly segregated T0 events from Experiment 1 were further analysed by sequencing to verify quality and integrity of the insertions. Long PCR products from 13 selected T1 plants (one plant representing each of the 13 T0 events) were sequenced by NGS in both forward and reverse directions using primers listed in Table S3 and assembled into contigs. Comparison of all 13 contigs showed no sequence variation; confirming every plant had a precise, HDR-mediated gene insertion.

**Discussion**

Targeted gene insertion is the most challenging type of genome editing in plants. Its success depends on multiple factors, including transformation and regeneration processes of the insertions, efficient delivery of genome editing components, copy number of the repair template and its availability at the DSB site, size of the DNA sequence to be inserted, presence or absence of a selectable marker gene in the repair template, and HDR efficiency at a given target site. In addition, gene insertion experiments usually have a high attrition rate of generated events due to the complexity of the insertion, plant chimerism and inability to segregate intended edits from helper genes (e.g. Cas9, gRNA and selectable marker). Different approaches have been suggested and evaluated to overcome some of these issues and increase the overall success rate of targeted gene insertion: particle bombardment and Agrobacterium transformation, delivery of editing components as DNA, RNA and ribonucleoprotein (RNP), suppression of NHEJ and activation of HDR pathways, viral replication to increase repair template copy number, linear and circular repair template molecules, tethering repair template to Cas9-gRNA RNP complexes, inducible, cell cycle-specific and developmental promoters, *in planta* and intra-genomic approaches, and exploiting different insertion mechanisms – HDR, NHEJ, homology-mediated end joining (HMEJ), and microhomology-mediated end joining (MMEJ) (Atkins and Voytas, 2020; Huang and Puchta, 2019; Mao et al., 2019). Despite new genome-editing approaches and a better understanding of underlying mechanisms of DSB repair, the progress in developing a simple, robust and reliable technology for targeted gene insertion in plants at a higher efficiency has remained limited.

Because the high copy number of donor template is required for efficient targeted gene insertion, most reported experiments have been conducted using particle bombardment as the delivery method. However, this high copy number of DNA molecules, which are not always intact, often results in a very complex integration pattern making the generated events unusable. This method is also invasive and may lead to chromosomal rearrangements (Liu et al., 2019; Svitashev et al., 2000), further contributing to an already high attrition rate.

**Agrobacterium-mediated transformation** is the preferred method for delivery of editing components into the plant cell (Gordon-Kamm et al., 2021). This system is reproducible, amenable to a broad variety of crop species, and cost effective since it does not require special equipment and associated consumables. In addition, Agrobacterium-mediated delivery is less invasive and has a lower attrition rate during the regeneration stage (Jackson et al., 2013; Shou et al., 2004). The major limitation of this transformation method is the low number of T-DNA molecules transmitted into the plant cell during infection, resulting in low copy number of repair template and, therefore, very low frequency of targeted gene insertion. Based on our experience, these limitations make conventional Agrobacterium-mediated delivery not feasible for targeted gene insertion and deployment in commercial trait product development programmes. In multiple experiments conducted by our group (Barone et al. and Svitashev et al., unpublished data), the rates of successful targeted gene insertions using Agrobacterium-

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**Table 2** Summary of edits transmission from T0 plants to T1 generation and event attrition rate

| Experiment (Genotype) | Selectable marker gene (position in T-DNA) | HR1/HR2-positive T0 plants | Long PCR-positive T0 plants sent to greenhouse | Fertile T0 plants used as pollen donors and set seeds | T0 plants transmitted insertion to T1 progeny | T0 plants transmitted insertion and segregated T-DNA in T1 progeny | Attrition rate based on number of HR1/HR2-positive T0 plants |
|-----------------------|-------------------------------------------|---------------------------|-----------------------------------------------|-------------------------------------------------|------------------------------------------|-------------------------------------------------|--------------------------------------------------|
| **EXPERIMENT 1 (PH184C)** |                                          |                           |                                               |                                                 |                                          |                                                 |                                                   |
| No repair template release | NPTII (in repair template) | G418 | 12 | 6 | 5 | 5 | 3 | 3 | 75% |
| Repair template release | NPTII (in repair template) | G418 | 39 | 26 | 14 | 12 | 12 | 12 | 69% |
| **EXPERIMENT 3 (PH184C)** |                                          |                           |                                               |                                                 |                                          |                                                 |                                                   |
| Repair template release | NPTII (in repair template) | G418 | 33 | 20 | 18 | 11 | 11 | 11 | 67% |
| Repair template release | HRA (upstream from repair template) | Imazapyr | 64 | 34 | 31 | 21 | 18 | 15 | 77% |

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mediated delivery were less than 0.1%. Similar frequencies were demonstrated in several in planta experiments. For example, 0.15% frequency of gene insertion was demonstrated in Arabidopsis (Schmi et al., 2014), while in maize, this number was even lower (0.085%) despite targeting the restoration of a selectable marker gene (Ayar et al., 2013).

To overcome the disadvantages of Agrobacterium-mediated delivery associated with low T-DNA copy number transmission to plant cells, several groups have used a geminivirus replication system to amplify the repair template sequence (Baltes et al., 2014; Dahan-Meir et al., 2018; Gil-Humanes et al., 2017; Vu et al., 2020). Although increased frequencies of targeted gene insertions using this approach have been demonstrated, in most studies, selectable marker genes were part of the insertion and the efficiency of the donor template amplification has been shown to be limited by its size (Baltes et al., 2014). Insertion of a very short (281 bp) DNA fragment was demonstrated in tomato with no selection for HDR events during the tissue culture stage (Dahan-Meir et al., 2018). In wheat, although this approach has been reported to increase targeted gene insertion by approximately 12-fold (Gil-Humanes et al., 2017), experiments were conducted on protoplasts and ballistically transformed scutellum cells with no plant regeneration. Furthermore, viral replication approach requires a more complicated vector design, results in uncontrolled DNA replication, may lead to unintended random integration of DNA into the genome and can result in lower regeneration frequency (Afza et al., 2020).

Another serious impediment to genome editing, also associated with low frequencies of HDR-based modifications, is the necessity to regenerate hundreds or even thousands of events to recover plants with the desired outcome. However, many important crop species, especially their elite genotypes, have low regenerability and are extremely challenging for complex HDR-based edits. A rapid maize transformation system developed at Corteva Agriscience (Lowe et al., 2018) that relies on expression of two morphogenic genes (Bbm and Wus2) allows recovery of transgenic plants from transformed immature embryos at high frequencies and significantly improves transformation efficiency of elite maize genotypes (Lowe et al., 2016), resulting in a faster trait product development.

Furthermore, it has been suggested that DSB repair via homologous recombination occurs predominantly during the late S, G2 and early M phases of the cell cycle, while being actively suppressed at the G1 stage (Baltes et al., 2014; Heyer et al., 2010; Mao et al., 2008; Saleh-Gohari and Helledge, 2004; Yang et al., 2016). Consequently, efficient HDR-based genome editing is largely restricted to actively proliferating cells. Therefore, an additional, and very important, advantage associated with the use of morphogenic genes in genome editing experiments is the stimulation of cell division, providing an HDR-friendly cellular environment critical for efficient genome editing.

Another improvement of maize Agrobacterium-mediated transformation has been accomplished by developing a new ternary vector system that utilizes an optimized accessory plasmid, pVIR9 (Anand et al., 2018). This system increased transient T-DNA delivery and recovery of stable callus events, resulting in 6- to sevenfold improvement over conventional random transformation using the plasmid pSB1 in elite maize inbreds. The combination of morphogenic genes and new ternary vector design resulted in high transformation efficiency, increased number of T-DNA molecules transmitted and high frequency of plant regeneration. Implementation of this enhanced transformation protocol allowed us to increase HDR-based insertion event frequencies at target site TS45 from less than 0.1% (Barone et al., Svitashev et al., unpublished data) to approximately 3% (a 30-fold improvement), making this type of genome editing application not only feasible, but also practical for crop species amenable to Agrobacterium-mediated transformation.

Target sites flanking the repair template were described in in planta and intra-genomic targeted gene insertion experiments (Fauser et al., 2012; Fauser et al., 2014; Schmi et al., 2014), where they were used to release the repair template from random integration loci. Introduction of the flanking target sites in our T-DNA vectors benefits the targeted gene insertion process in several ways. First, a linear repair template potentially increases frequency of HDR-based gene insertion (Beumer et al., 2006; Song and Steiger, 2017). Second, transient release of donor template from the T-DNA makes it readily available for homology-based repair of the DSB at the intended target site. Third, it allows single-strand invasion in both directions – from genomic DNA and from donor template (Huang and Puchta, 2019). Fourth, it probably simplifies the second end resolution through homologous recombination, thus increasing proportion of perfect HDR-based insertion events (Verma et al., 2020). Finally, it might be even more advantageous when considering recent results indicating that damaged DNA might be transported to specific loci in the nucleus for further repair (Caridi et al., 2018).

Target sites flanking the repair template have been used in several particle bombardment experiments in Arabidopsis and rice (Dong et al., 2020; Sun et al., 2016; Zhao et al., 2016). However, the positive effect of this vector design on frequency of gene insertion could not be validated as no controls were provided in these experiments. Recently, this vector design has also been tested in gene targeting experiments in Arabidopsis using Agrobacterium-mediated delivery (Peng et al., 2020). However, based on a very limited set of data, the authors concluded that excising the donor template from the T-DNA does not increase but rather reduces the efficiency of the insertion. Contrary, the introduction of flanking target sites has resulted in 2-to-5-fold increase of integration frequencies relative to circular plasmids in human culture cells (Zhang et al., 2017). In our experiments, direct comparison of T-DNA vectors with and without target sites flanking repair templates, consistently, demonstrated an approximately threefold increase of targeted gene insertion frequency.

Besides high frequency of HDR-based gene insertion, our approach also resulted in a significant improvement in the quality of edited events. This improvement is demonstrated by the results of long PCR analysis, which shows high proportion of putative perfect insertion T0 events, similar insertion frequencies of donor templates with and without selectable marker, and by the Mendelian transmission of the edit to T1 progeny, indicating their non-chimeric nature.

Attrition of HDR-based insertion events is an important factor that can significantly affect the success rate of gene targeting experiments but is rarely discussed in the literature. In this report, the frequency of HR1/HR2-junction qPCR-positive events (8-10%) drops by approximately 40-50% after long PCR analysis. This is likely related to complex insertions with multiple copies of transgene components and/or genomic DNA fragments due to the concurrent action of HDR and NHEJ pathways, which results in co-integration of various DNA molecules (vector DNA and/or genomic DNA sequences) into the target site (Svitashev et al., 2002; Svitashev et al., 2015). Other attrition factors are associated...
with position of selectable marker gene, regenerants survival rate, fertility of T0 plants and chimerism. The combined attrition rate, reported here, ranges from 64% to 77%, with the highest rate observed in the experiment with selectable marker gene outside the repair template (Table 2).

In summary, the advancements in Agrobacterium-mediated maize transformation (Anand et al., 2018; Lowe et al., 2018; Lowe et al., 2016), combined with optimized vector design, enabled an approximately 100-fold improvement in the efficiency of targeted gene insertion. Our experiments showed reliable and reproducible results for two maize genotypes with different transformability, two different genes, and for constructs with selectable marker genes both inside and outside repair templates. These results open new opportunities for accelerated precision breeding in a wide range of crop species amenable to Agrobacterium-mediated transformation.

Methods

Plant material

Maize (Zea mays L.) inbred lines PH184C and PH1V5T were obtained from internal Corteva Agriscience sources.

Plasmids and reagents used for plant transformation

Cas9 expression cassette (Svitashev et al., 2015), target site TS45 and guide RNA cassette (Gao et al., 2020b), morphogenic transcription factors, PLTP promoter regulated Babyboom (Bbm), and Axig1 promoter regulated Wuschel2 (Wus2) (Lowe et al., 2016), acetolactate synthase (Als) promoter regulated highly herbicide-resistant Als (Hra) gene (Green et al., 2009), and new ternary vector and pVIR9 accessory plasmid (Anand et al., 2018) have been previously described. Repair template consisted of either Ubiquitin promoter-driven NptII (neomycin phosphotransferase II) gene (Anand et al., 2019), or Arabidopsis carboxylesterase 20 (Cxe-20) gene under rice Actin promoter (Allen et al., 2015) flanked with HR1 and HR2 homology regions (411 bp and 417 bp, respectively). For vectors depicted on Figure 1(b and c), HR1 and HR2 fragments were flanked with target site TS45 sequences with PAM.

Maize transformation

Two Corteva Agriscience inbred lines used in this study, PH184C and PH1V5T, are proprietary. All plants used as a source of immature embryos were grown in greenhouse conditions. Ear harvest, immature embryo isolation, Agrobacterium-mediated transformation and plant regeneration were performed as previously described (Jones et al., 2019).

T0 and T1 plant analysis

Genomic DNA was extracted from leaf tissue as previously described (Gao et al., 2010). Quantitative PCR (qPCR) was used to detect T-DNA components (Cas9, gRNA, Bbm, Wus2, Hra and NptII) and mutation frequency at endogenous target site TS45 using Qiagen QuantiTect Multiplex PCR Master Mix (Qiagen, Germany) with the primers and probes listed in Table S1. Junction PCR assays were used to detect gene insertion at the target site TS45. In this assay, PCR amplification of the target region was coupled with a nested qPCR using primers and probes listed in Table S2. PCR was performed using 2x Extract-N-amp PCR Ready Mix (MilliporeSigma, St. Louis, MO, USA) or 2x Phusion Flash High-fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s recommendations. For nested PCR used in screening of the insertion events, the first PCR was carried out in 5 μL of reaction mixtures for 20 cycles. Fifteen μL of the reaction mixture containing 2xTaqMan Master Mix (LGc Limited, Teddington, UK) and primers then were added, and the second PCR was performed using LightCycler 480 (Roche Life Science, Germany) for 30 cycles. Data were analysed using the Endpoint Genotyping Software (Roche Life Science, Germany). Long PCR was performed using Extensor Master Mix (Thermo Fisher Scientific) or LongAmp™ Hot Start Taq 2X Master Mix (New England Biolabs, Ipswich, MA, USA) with primers and probes listed in Table S2. All qPCR copy number assays were calibrated using Corteva Agriscience proprietary housekeeping gene assay (VIC).

Integrity of the gene insertion was confirmed in T1 plants by NGS sequencing of long genomic PCR amplicons. Two sets of sequencing primers (22 forward and 22 reverse) were used in the analysis (Table S3). Approximately 250 bp-long reads were then assembled into contigs and compared using Sequencher v. 4.8.

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Conflicts of interest

The authors declare that they have no conflict of interests.

Author contributions

S.S. conceived the project, D.P., P.B. and S.S. designed experiments; D.P., P.B., L.F., S.J. and G.S.C. conducted plant transformation and regeneration; B.L. and C.S. analysed plant materials; D.P., P.B. and S.S. analysed the data; S.S. and P.B. wrote the manuscript.

Data availability statement

The authors declare that the data supporting the findings are available within the paper or are available from the corresponding author upon reasonable request. Corteva Agriscience will provide plasmids to academic investigators for non-commercial research under an applicable material transfer agreement subject to proof of permission from any third-party owners of all or parts of the material and to governmental regulation considerations. Completion of a stewardship plan is also required. The Corteva Agriscience inbred lines PH184C and PH1V5T described in this research are proprietary.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 PCR and qPCR primers and probes used to identify HR1 and HR2 positive T0 and T1 plants.

Table S2 QPCR primers and probes used to identify T-DNA vector components and their copy number in T0 and T1 plants.

Table S3 Results of segregation analysis of the T1 progeny from Experiment 1.

Table S4 Results of segregation analysis of the T1 progeny from Experiment 3.

Table S5 List of sequencing primers used to validate gene insertion in a group of 13 T1 plants.