Replacement of stacked transgenes \textit{in planta}

Weiqiang Chen$^{1,1}$, Gurminder Kaur$^{1,2,1}$, Lili Hou$^{1,2}$, Ruyu Li$^{1}$ and David W. Ow$^{1,*}$

$^1$Plant Gene Engineering Center, Chinese Academy of Sciences Key Laboratory of South China Agricultural Plant Molecular Analysis and Genetic Improvement, Guangdong Key Laboratory of Applied Botany, South China Botanical Garden, Guangzhou, China

$^2$University of Chinese Academy of Sciences, Beijing, China

Received 27 March 2019; revised 5 May 2019; accepted 10 May 2019.

Correspondence (Tel +86 20 3708 5863; fax +86 20 3708 5863; email: dow@scbg.ac.cn)

These authors contributed equally.

Keywords: gene stacking, transgene replacement, GMO, recombinase, Bxb1, Cre.

While GM crops of past years contain one to few traits, the continuous discovery of new trait genes would mean that over time, crops could end up with a large number of transgene insertions. If they were dispersed throughout the genome, extensive breeding would be needed to reassemble all of them into a single breeding line. Stacking new DNA to a preexisting transgenic locus insures that the package of transgenes can be transmitted through breeding programmes as a single locus rather than as segregating loci. Several versions of recombinase-mediated gene stacking have been described (Srivastava and Thomson, 2016), and we previously reported on an \textit{in planta} gene stacking method using the Bxb1 integrase for site-specific integration followed by the Cre recombinase for removal of unneeded DNA (Hou et al., 2014). The described method permits the sequential addition of transgenes as each integrating molecule brings a new recombination target for the next round of integration. However, should a need arises later on that requires removal of existing transgenic DNA, one possibility would be to use sequence-specific nucleases such as zinc-finger nucleases, TALEN or CRISPR/Cas9 (Murovec et al., 2017; Weeks et al., 2016) to cut at specific targets and induce host-mediated repair through non-homologous end joining or homologous recombination. Alternatively, as we show here, it is possible to delete or replace preexisting transgenes by the same Bxb1/Cre recombinase-mediated gene stacking system. Moreover, commercial crop improvement using this gene deletion/replacement strategy has freedom to operate, as opposed to patent licences needed for use of sequence-specific nuclease-based tools (Chen and Ow, 2017).

We had previously reported stacking two rounds of transgenes into a tobacco target line (Hou et al., 2014) that led to creation of line 23.C.4.9.d8.BC1 with the structure depicted in Figure 1a. To test a gene replacement strategy, microparticle bombardment (Li et al., 2016) was used to deliver pHL002 (Figure 1b) along with a Bxb1 integrase-expressing construct (not shown) into leaf explants of line 23.C.4.9.d8.BC1. This integrating molecule differs slightly from the vectors previously described in that there is an additional lox site situated between the npt-distal attB and the trait gene exemplified by OsO3L2-2B. OsO3L2-2B is a rice gene that has been shown to lower rice cadmium accumulation (Wang et al., 2016, 2019), and here, it serves as a DNA fragment to replace the previously stacked transgenes. Bxb1-mediated site-specific integration of pHL002 inserts OsO3L2-2B into the target locus placing directly oriented lox sites to flank the previously stacked transgenes gus, luc and gfp (Figure 1c). This should permit the subsequent deletion of the previously stacked transgenes to produce the configurations shown in Figure 1d or e depending on inversion of the DNA bound by the outermost lox sites.

Out of ~200 shoots (due to npt) regenerated on kanamycin-containing plates, a first PCR analysis detected 60 shoots positive for the npt DNA. A second PCR analysis for the expected integration junctions (Figure 1c) found only 11 plants with the correct junctions $a$ and $b$ (~1.7 and ~1.5 kb, respectively). The rest of the shoots could represent random or imperfect insertions of pHLO02. After cloning into a vector for sequencing, the PCR products from all 11 plants confirmed the expected left and right junction sequences.

Southern hybridization with an npt probe (p1) detected a 6.0 kb HindIII band and 0.9 kb Xhol band in the genomic DNA from each of the 11 integrants (Figure 1g), and sizes of these bands were consistent with site-specific integration. For the genomic DNA from WT (wild type) or the parental stacked line 23.C.4.9.d8.BC1, hybridization was not found. As there was no other Xhol or HindIII band detected in integrant lines #1, #2, #6, #8 and #11, these five plants most likely lack additional copies of pHLO02 elsewhere in the genome.

To remove the lox-flanked DNA, we took hemizygous lines #2, #8, #11 to pollinate a homoyzogous cre-expression line where cre is expressed from the CaMV 35S RNA promoter (Hou et al., 2014). A total of 78 F1 seedlings were genotyped by PCR to detect OsO3L2-2B and cre DNA. The cre gene was detected in all 78 F1 seedlings as expected for a homoyzogous recipient. However, OsO3L2-2B was detected in only 18 F1 individuals, which is lower than the 50% expected for Mendelian segregation. This may be caused by inefficient pollinisation or false negatives from difficulty in amplifying the OsO3L2-2B gene.

Due an incidence of contamination, only 12 of the 18 seedlings survived for further analysis. PCR was used to detect the expected deletion-specific recombination junctions $c$ and $d$ (Figure 1d), or if an inversion also took place, junctions $e$ and $f$ (Figure 1e). Recombination leading to inversions without deletions would yield only the parental $a$ and $b$ junctions. Of the 12 plants, junctions $c$, $d$, $e$ and $f$ were detected from #8.3, #8.14 and #11.14 (Figure 1f), while junctions $d$, $e$ and $f$ were found in #2.1. Junction $c$ was found in one plant, and junction $d$ in another, but for the remaining five plants, new junctions were not found.
However, for plants #8.3, #8.14 and #11.14, gus and gfp were also detected to indicate incomplete deletion (data not shown), which means that the F1 plants must be chimeric for the desired recombination. Along with the need to obtain germinal transmission of the deletion event, we took plants #8.3, #8.14 and #11.14 to the F2 generation. From the PCR analysis on 148 F2 seedlings (88 from #8.3, 48 from #8.14 and 12 from #11.14), only three seedlings from #8.3 (#8.3.1, #8.3.2 and #8.3.3) showed a PCR pattern lacking luc and gus, while having OsO3L2-2B, and junctions c and d. Additionally, plant #8.3.3 also showed PCR products e and f, which could indicate that #8.3.3 harbours both the inverted and the noninverted transgene structure. This might mean that cre is still active, and indeed, a PCR could amplify the cre coding region in #8.3.3 but not in #8.3.1, #8.3.2 or in parental lines. (Figure 1f).

**Figure 1** Bxb1-mediated gene replacement. Tobacco stacked line 23.C.4-9.d8.BC1 (Hou et al., 2014) with structure shown in (a) harbours an attP site for the integration by pHL002 (b) mediated by the co-introduction of Bxb1 recombinase-expressing pC35S-BNK (not shown, Yau et al., 2011). Recombination with the npt-distal attB yields the configuration shown in (c). Configuration from recombination with the npt-proximal attB not shown. Cre-lox reaction deletes DNA between lox sites to yield the structure shown in (d) or inverts DNA between oppositely oriented lox sites to yield the structure in (e). (f) Representative PCR detection of junctions a, b, c, d, e, f and cre gene. (g) Representative Southern blots of regenerated plants probed with npt DNA (p1) show a 6 kb HindIII band spanning from gfp to npt DNA and a 0.9 kb Xhol npt specific band, but only lines 1, 2, 6, 8 and 11 (blue lettering) show them as the only hybridizing band. Other lines with additional bands indicate additional copies integrated elsewhere in the genome. (h) Southern blot of F2 plants #8.3.1, #8.3.2 and #8.3.3 with O3L2-2B probe (p2) detects a 2.6 kb HindIII band and a 6.3 kb SpeI band, as well as a 1.8 kb SpeI band in #8.3.3. Symbols for recombination sites as indicated. gus: beta-glucuronidase gene, luc: firefly luciferase gene, gfp: green fluorescent protein gene, OsO3L2: rice O3L2 gene 2B fragment, npt: neomycin phosphotransferase gene. L and R: T-DNA left and right borders. HindIII (H), Xhol (X) and SpeI (S) sites and expected sizes (kb) of DNA fragments shown in blue. Red lines show PCR-detected recombination junctions. M is marker lane, fragment sizes in kb. Gene promoters and terminators not shown; all genes transcribe from left to right except for inverted O3L2-2B in (e). Protocol for biolistic site-specific integration described in Li et al. (2016).

© 2019 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 17, 2029–2031
Southern blotting of the F2 plants #8.3.1, #8.3.2 and #8.3.3 showed that the O3L2-2B probe (p2) hybridized a 2.6 kb HindIII band in all three F2 plants (Figure 1h). Since the HindIII sites are located outside of the inverted lox sites, the 2.6 kb HindIII band should be common for both transgene orientations. However, when cleaved with SpeI, which cuts within O3L2-2B, the p2 probe detected a 6.3 kb band in all three F2 plants indicating the orientation shown in Figure 1d, while it also detected a 1.8 kb band in plant #8.3.3 indicating the inverse orientation in Figure 1e. This confirms that plant #8.3.3 harbours both the inverted and the noninverted copy. Since cre is still present in #8.3.3, Cre-mediated inversion may be an ongoing reaction and is consistent with the relative abundance of the 6.3 kb and 1.8 kb bands. As for the control lanes, p2 hybridized to a 6.0 kb HindIII band and an 11.3 kb SpeI band in parental line #8, but to neither HindIII nor SpeI treated DNA from the WT or the parental stacked line 23.C.4-9.d8.BC1.

To conclude, we demonstrate how one can use the same gene stacking steps of DNA site-specific integration followed by removal of unneeded DNA to reconfigure a preexisting transgenic locus. Moreover, the new structure with a single attachment site (attB or attP) can continue to permit subsequent rounds of gene stacking or gene replacement. Whether this is used for deleting preexisting transgenes, replacing with new versions of the same gene or replacing with a completely new transgene, this transgene locus editing feature extends the flexibility of the Bxb1/Cre recombinase-mediated system for transgene stacking.

Acknowledgements
Support provided by the Ministry of Science and Technology of China (2016YFD0101904) and the Chinese Academy of Sciences (QYZDY-SSW-SMC010).

Conflict of interest
The authors declare no conflict of interests.

Author Contributions
D.W.O designed the project. G.K., W.C., L.H. and R.L conducted the experiments. D.W.O., G.K. and W.C prepared the manuscript. All authors approved the final version of the manuscript.

References
Chen, W. and Ow, D.W. (2017) Precise, flexible and affordable gene stacking for crop improvement. Bioengineered 8, 451–456.
Hou, L., Yau, Y.-Y., Wei, J., Han, Z. and Ow, D.W. (2014) An open-source system for in planta gene stacking by Bxb1 and Cre recombinases. Mol. Plant 7, 1756–1765.
Li, R., Han, Z., Hou, L., Kaur, G., Qian, Y. and Ow, D.W. (2016) Method for biolistic site-specific integration in plants catalyzed by Bxb1 integrase. Methods Mol. Biol. 1469, 15–30. (Chromosome and Genomic Engineering in Plants, Ed. M. Murata, New York: Humana Press).
Murovec, J., Pirc, Z. and Yang, B. (2017) New variants of CRISPR RNA-guided genome editing enzymes. Plant Biotechnol. J. 15, 917–926.
Srivastava, V. and Thomson, J. (2016) Gene stacking by recombinases. Plant Biotechnol. J. 14, 471–482.
Wang, C., Guo, W., Ye, S., Wei, P. and Ow, D.W. (2016) Reduction of Cd in rice through expression of OXS3 like gene fragments. Mol. Plant 9, 301–304.
Wang, C., Guo, W., Cai, X., Li, R. and Ow, D.W. (2019) Engineering low-cadmium rice through stress-inducible expression of OXS3-family member genes. New Biotechnol. 48, 29–34.
Weeks, D.P., Spalding, M.H. and Yang, B. (2016) Use of designer nucleases for targeted gene and genome editing in plants. Plant Biotechnol. J. 14, 483–495.
Yau, Y.-Y., Wang, Y., Thomson, J.G. and Ow, D.W. (2011) Method for Bxb1-mediated site-specific integration in planta. Methods Mol. Biol. 701, 147–166. (Plant Chromosome Engineering, Ed. J.A. Birchler, New York: Humana Press).