Stacking Multiple Transgenes at a Selected Genomic Site via Repeated Recombinase-Mediated DNA Cassette Exchanges [OA]

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Recombinase-mediated DNA cassette exchange (RMCE) has been successfully used to insert transgenes at previously characterized genomic sites in plants. Following the same strategy, groups of transgenes can be stacked to the same site through multiple rounds of RMCE. A gene-silencing cassette, designed to simultaneously silence soybean (Glycine max) genes fatty acid ω-6 desaturase 2 (FAD2) and acyl-acyl carrier protein thioesterase 2 (FATB) to improve oleic acid content, was first inserted by RMCE at a precharacterized genomic site in soybean. Selected transgenic events were subsequently retransformed with the second DNA construct containing a Yarrowia lipolytica diacylglycerol acyltransferase gene (DGAT1) to increase oil content by the enhancement of triacylglycerol biosynthesis and three other genes, a Corynebacterium glutamicum dihydrodipicolinate synthetase gene (DHPS), a barley (Hordeum vulgare) high-lysine protein gene (BHL8), and a truncated soybean cysteine synthase gene (CGS), to improve the contents of the essential amino acids lysine and methionine. Molecular characterization confirmed that the second RMCE successfully stacked the four overexpression cassettes to the previously integrated FAD2-FATB gene-silencing cassette. Phenotypic analyses indicated that all the transgenes expressed expected phenotypes.

Many vegetable oils for human consumption are rich in 18-carbon ω-6 fatty acids, which, if excessively consumed, can lead to the depletion of ω-3 fatty acids in human body tissues, with numerous negative health consequences. Edible vegetable oils are also often hydrogenated to improve oxidative stability, maintain flavor, and provide necessary solid fat functionality. But hydrogenation leads to the formation of trans-unsaturated fatty acids, which have been linked to cardiovascular diseases. Healthy alternatives are oils rich in oleic acid, 18:1, that can be obtained from mutant oilseed plants with defective ω-6 desaturase (FAD2) genes. FAD2 is directly responsible for the desaturation of 18:1 to linoleic acid, 18:2 (Fig. 1). Many plants have several FAD2 genes contributing to seed 18:2 content that need to be simultaneously mutated in order to get high enough levels of 18:1 (Heppard et al., 1996; Lightner et al., 2006). However, the fatty acid contents of nonseed organs can also be affected by the FAD2 mutations, causing agronomic problems (for review, see Damude and Kinney, 2007, 2008a, 2008b).

A transgenic approach that is able to simultaneously knock out several FAD2 genes in seeds only can overcome the problems associated with the FAD2 mutants mentioned above. Furthermore, additional genes involved in fatty acid biosynthesis can be simultaneously targeted (Fig. 1). The acyl-acyl carrier protein thioesterase 2 (FATB) gene, primarily responsible for the accumulation of the saturated fatty acids palmitic acid, 16:0, and stearic acid, 18:0, can be knocked out to reduce saturated fatty acids and increase 18:1 (Hitz, 2001). The diacylglycerol acyltransferase (DGAT) gene responsible for transferring a fatty acyl group from acyl-CoA to a diacylglycerol substrate to form triacylglycerol can be overexpressed to increase the overall oil content (Cahoon et al., 2007; Meyer et al., 2008). The advantage of a transgenic approach is that several genes in the fatty acid biosynthesis pathway can be simultaneously manipulated through gene silencing or overexpression using one or a few DNA constructs (Wu et al., 2005; Kinney, 2006).

Another nutritional trait of crops is the content of essential amino acids such as Lys and Trp, which are often low in legumes, and Met, Cys, and Thr, which are often low in legumes and cereals (Hesse et al., 2001; Sun and Liu, 2004; Galli et al., 2005). Both Lys and Met, including Cys and Thr intermediates, are synthesized through the Asp family biosynthesis pathway by two branches, the Lys branch and the Thr-Met branch, which compete for some common substrates. Complex feedback controls on key enzymes in the pathway, such as dihydrodipicolinate synthase (DHPS) and cystathionine γ-synthase (CGS), maintain a dynamic balance of...
the amino acids levels (Chiba et al., 1999; Falco et al., 1999; Falco, 2006). The overexpression of a feedback-insensitive \( \text{DHPS} \) gene alone, or combined with the knockout of Lys catabolism key enzymes Lys keta-
tarate reductase/saccharopine dehydrogenase and the overexpression of a feedback-insensitive \( \text{CGS} \) gene, can dramatically increase the levels of free Lys or both Lys and Met (Zhu and Galili, 2003, 2004; Hacham et al., 2007). But the increased levels of free amino acids may not necessarily be stored unless enough sink, such as in the barley (\( \text{Hordeum vulgare} \)), high Lys (BHL8), a small storage protein engineered to be silenced, leading to the increase of 18:1 and decrease of saturated fatty acids 16:0 and 18:0 (Fig. 2C). While \( \text{FAD2} \) and \( \text{FATB} \) were constructed in one cassette for gene silencing, the others were constructed as separate cassettes for overexpression with different promoters. Any transgene cassette that contains two incompatible \( \text{FRT} \) sites can be used as a target for FLP-mediated RMCE to have the intervening DNA replaced by the DNA between two corresponding \( \text{FRT} \) sites of a donor DNA construct. The \( \text{QC288A329A} \) and \( \text{QC288A436A} \) transgenes were used as targets for the two rounds of SSI transformation in which the selectable marker genes \( \text{ALS} \) and \( \text{HPT} \) were used alternately for transgenic event selection (Fig. 2, A and B). The first round of SSI transformation was done on embryogenic callus derived from the homozygous prog-
ey of reported RMCE plants B5-1 and B5-2 containing the \( \text{QC288A329A} \) transgenes (Li et al., 2009). Since \( \text{QC288A329A} \) contains the \( \text{ALS} \) gene, the first donor DNA construct, \( \text{QC436} \), consisting of all the components between the \( \text{FRT1} \) and \( \text{FRT87} \) sites of \( \text{QC288A436A} \), has to contain a different selectable marker gene, \( \text{HPT} \) (Fig. 2, A and B). The \( \text{HPT} \) gene and the \( \text{FAD2-FATB} \) gene-silencing cassette replaced the \( \text{ALS} \) and \( \text{CFP} \) genes of \( \text{QC288A329A} \) during the first round of RMCE to form \( \text{QC288A436A} \). The \( \text{FRT12} \) site was simultaneously introduced between the \( \text{FRT1} \) and \( \text{FRT87} \) sites. The bom-
bardment of 11 plates of B5 embryogenic tissues with the donor \( \text{QC436} \) and FLP \( \text{QC292} \) DNA produced 37 hygromycin-resistant events, and 23 of them were iden-
tified to be SSI positive. So the first-round SSI transformation frequency is approximately two SSI events per bombarded plate. Four selected RMCE events, B51, B52, B53, and B54, were proliferated and used directly as the targets for the second round of SSI transformation. B52 retransformation events were subsequently abandoned, since B52 somatic embryos did not display the expected fatty acid phenotype.

### RESULTS

**DNA Construction and SSI Transformation**

Two rounds of SSI transformation were done to stack seven transgenes, including the selection gene \( \text{ALS} \), the three genes \( \text{BHL8}, \text{DHPS}, \) and \( \text{CGS} \) for improving essential amino acids, and the three genes \( \text{DGAT1}, \text{FAD2}, \) and \( \text{FATB} \) for high oil, high 18:1, and low 16:0 and 18:0 (Fig. 2C). While \( \text{FAD2} \) and \( \text{FATB} \) were constructed in one cassette for gene silencing, the others were constructed as separate cassettes for overexpression with different promoters. Any transgene cassette that contains two incompatible \( \text{FRT} \) sites can be used as a target for FLP-mediated RMCE to have the intervening DNA replaced by the DNA between two corresponding \( \text{FRT} \) sites of a donor DNA construct. The \( \text{QC288A329A} \) and \( \text{QC288A436A} \) transgenes were used as targets for the two rounds of SSI transformation in which the selectable marker genes \( \text{ALS} \) and \( \text{HPT} \) were used alternately for transgenic event selection (Fig. 2, A and B).

![Figure 1. Alteration of fatty acid biosynthesis for high oleic acid and high oil production. Two genes, \( \text{FATB} \) and \( \text{FAD2} \), are silenced, leading to the increase of 18:1 and decrease of saturated fatty acids 16:0 and 18:0. The \( \text{DGAT1} \) gene encoding a key enzyme for fatty acid accumulation in oil bodies is overexpressed, leading to increased oil. \( \text{ACP} \), Acyl carrier protein; \( \text{KASII} \), \( \beta \)-ketocarboxy-
acyl-ACP synthase II; \( \Delta 9 \) DES, \( \Delta 9 \)-desaturase; \( \text{FATA} \), acyl-carrier protein thioesterase 1; \( \text{FATB} \), acyl-carrier protein thioesterase 2; \( \text{FAD2} \), \( \omega-6 \) desaturase; \( \text{FAD3} \), \( \omega-3 \) desaturase; \( \text{DGAT} \), diacylglycerol acyltransferase, \( \text{TAG} \), triacylglycerol; \( \text{ER} \), endoplasmic reticulum. \( \text{FATA} \) or \( \text{FATB} \) in smaller font indicates a minor role for the step.](image-url)
The second donor DNA construct QC438 contains all the components between the FRT1 and FRT12 sites of QC288A436A438A (Fig. 2C). The ALS selectable marker gene and the FRT1 and FRT12 sites are required for RMCE to stack all the genes onto the QC288A436A target. The second round of SSI transformation was expected to be challenging for several reasons: (1) it required the embryogenic callus of newly identified transgenic events to be directly retransformed; (2) the performance of the four first-round RMCE events as targets for another round of transformation was unknown; (3) the QC438 donor DNA is larger than any previously transformed DNA; and (4) the complication of three FRT sites on both the QC288A436A target and the QC438 donor DNA had never been evaluated. There were 32 chlorsulfuron-resistant events produced, but most of them were determined to be SSI negative. Only one event, B531, was confirmed to be a gene-stacking RMCE event containing the complete QC288A436A438A transgenes (Fig. 2C).

Characterization of the First-Round RMCE Events

Somatic embryo samples of all 37 hygromycin-resistant events produced by the first SSI transformation were analyzed by four quantitative (q)PCR assays. The target qPCR is specific to the FRT1 site of QC288A329A; it checks the SCP1 and ALS junction for the copy number change of the target (Fig. 2A). The SSI qPCR is specific to the FRT1 site of QC288A436A; it checks the new SCP1 and HPT junction that resulted from a recombination event at the FRT1 site (Fig. 2B). Since the integration of transgenes is site specific for the FRT1 site, any events positive for the SSI qPCR are considered SSI positive. Only those SSI-positive events that are shown, by subsequent border-specific PCR, to also have predicted FRT87-end DNA recombination are considered as RMCE events. The donor and FLP qPCR assays are each specific to a unique region of the donor QC436 or the FLP QC292 circular plasmid DNA and were used to check for their random integration. Since the B5 target cultures were initiated from homozygous transgenic plants, the target qPCR should identify two copies of the target QC288A329A if SSI recombination did not occur. If DNA recombination occurred on only one target chromosome, the SSI qPCR would detect one copy of SSI QC288A436A, while the target qPCR would detect a copy of the target contributed by the other target chromosome. Since the FRT1 and FRT87 sites are not completely incompatible, all components between them can possibly be excised, resulting in gene excision product QC288ME that cannot be detected by either SSI or target qPCR (Li et al., 2009). It was observed that events with one copy of SSI often no longer contained any target, probably due to gene excision. All of the 23 SSI-positive events contained a single-copy SSI; 10 of them retained one copy of the target while the remaining 13 had the other copy of the target excised. Many of the 23 SSI-positive events also contained randomly integrated donor or FLP DNA, or both. Four selected events, B51, B52, B53, and B54, are all
samples were analyzed by qPCR specific to the germinated to T1 plants that provided leaf DNA for the qPCR assay. Decreases in 16:0 to less than 10% are indicative of successful transformation and are reproducible to approximately 3% of total fatty acids. Untransformed control somatic embryos typically contain 12.6% to 20.8% 16:0, 4.2% to 6.6% 18:0, 12.3% to 22.9% 18:1, 39.3% to 46.9% 18:2, and 12.4% to 23.5% 18:3 (Meyer et al., 2008). Increases in 18:1 to greater than 30% and decreases in 16:0 to less than 10% are indicative of successful FAD2 and FATB gene down-regulation, respectively. The chipped B53 seeds later germinated from the same chipped seeds were analyzed by the SSI, target, donor, and FLP-specific qPCR assays to check for transgene segregation. Mendelian segregation was observed in nine of the 10 events, and the high 18:1 and low 16:0 and 18:0 phenotypes are linked to the transgenes. Although the SSI qPCR-negative plants (null) contain the wild-type levels of fatty acids in their seeds, homozygous and hemizygous T1 plants contain similar high levels of 18:1 (approximately 90%) and low levels of 16:0 and 18:0 (approximately 5%) in their seeds, indicating that one copy of the QC288AQC436A transgenes is sufficient to suppress endogenous FAD2 and FATB genes. The qPCR results and fatty acid profiles for B53 T1 progeny, including two homozygous B53-1 and B53-2, two hemizygous B53-3 and B53-4, and two null B53-5 and B53-6, are shown as examples (Table I).

Characterization of the Second-Round RMCE Events

Putative events selected from the direct retransformation of the four QC288A436 RMCE events B51, B52, B53, and B54 with the second donor QC438 DNA were analyzed by the same four qPCR assays; how-

<table>
<thead>
<tr>
<th>Event</th>
<th>SSI</th>
<th>Gene Copy No. by qPCR</th>
<th>Fatty Acid Content by GC</th>
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</thead>
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<tr>
<td></td>
<td>Donor</td>
<td>Target</td>
<td>FLP</td>
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<td>0.0</td>
</tr>
<tr>
<td>B52</td>
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</tr>
<tr>
<td>B53</td>
<td>0.8</td>
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<td>0.0</td>
</tr>
<tr>
<td>B54</td>
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<td>0.0</td>
</tr>
<tr>
<td>B53-1</td>
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<td>0.0</td>
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</tr>
<tr>
<td>B53-2</td>
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<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
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<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>B53-4</td>
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<td>0.0</td>
</tr>
<tr>
<td>B53-5</td>
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<tr>
<td>B53-6</td>
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*Events B51 to B54 are representative hygromycin-resistant events selected from the retransformation of homozygous target QC288A329A with the first donor QC436 and FLP QC292 DNA. B53-1 to B53-6 are segregating T1 plants derived from event B53. Embryogenic callus or T1 plant leaf samples were analyzed by qPCR specific to the SCP1 and HPT junction of QC288A436A (SSI), a QC436-specific region (Donor), the SCP1 and ALS junction of QC288A329A (Target), and a QC292-specific region (FLP). A heat shock protein gene, HSP, was used as an endogenous control in duplex qPCR. A genomic DNA sample containing one copy of the respective transgene was used as the calibrator to calculate relative transgene copies in other samples using the Applied Biosystems 7500 system software. A value of less than 0.3 or between 0.4 and 1.3 was considered as zero or one copy. A value between 1.4 and 2.3 was considered as two copies. Fatty acids were determined on the bulk of 10 mature somatic embryos for B51 to B54 or on T1 seed chips for B53 by GC and expressed as the percentage of total fatty acids. Fatty acid measurements by GC as described here are reproducible to approximately 3% of total fatty acids. Untransformed control somatic embryos typically contain 12.6% to 20.8% 16:0, 4.2% to 6.6% 18:0, 12.3% to 22.9% 18:1, 39.3% to 46.9% 18:2, and 12.4% to 23.5% 18:3 (Meyer et al., 2008). Increases in 18:1 to greater than 30% and decreases in 16:0 to less than 10% are indicative of successful FAD2 and FATB gene down-regulation, respectively. The chipped B53 seeds later germinated to T1 plants that provided leaf DNA for the qPCR assay.
ever, the SSI and target qPCR were switched because the HPT gene in QC288A436A was exchanged to the ALS gene in QC288A436A438A (Fig. 2). There were only four events identified as SSI qPCR positive, including B531 and B541 shown in Table II. The other two SSI-positive events failed to survive. Border-specific PCR assays indicated that only one event, B531, was an RMCE event positive for both the 5’ and 3’ end borders specific to QC288A436A438A (data not shown).

Event B531 and a few others were analyzed by GC and NMR to check for fatty acids and total oil in somatic embryos (Table II). Event B531 retained the high level of 18:1 and low levels of saturated fatty acids (16:0 and 18:0) conferred by the RMCE, QC288A436A, is too large to be amplified (right). B. Expected PCR fragments of the 5’ border (left) and 3’ border (center) of target QC288A329A and the FRT12 region of RMCE QC288A436A (right) are 967, 1,180, and 693 bp. Wild-type DNA (wt) was included as a negative control. The four events were all chimeras containing the RMCE, target, and excision transgenes at the embryogenic callus stage. The FlashGel DNA markers are 4, 2, 1.25, 0.8, 0.5, 0.3, 0.2, and 0.1 kb (Lonza Rockland).

Figure 3. Analysis of the first-round SSI events. PCR assays specific to the genomic borders of the B target site hosting different transgenes was done using combinations of the 5’ border, 3’ border, and transgene-specific primers (Fig. 2). A, Expected PCR fragments of the 5’ border (left), 3’ border (center), and excision QC288ME (right) of the first RMCE, QC288A436A, are 886, 1,116, and 986 bp. The expected 9,108-bp-long full-length QC288A436A is too large to be amplified (right). B, Expected PCR fragments of the 5’ border (left) and 3’ border (center) of target QC288A329A and the FRT12 region of RMCE QC288A436A (right) are 967, 1,180, and 693 bp. Wild-type DNA (wt) was included as a negative control. The four events were all chimeras containing the RMCE, target, and excision transgenes at the embryogenic callus stage. The FlashGel DNA markers are 4, 2, 1.25, 0.8, 0.5, 0.3, 0.2, and 0.1 kb (Lonza Rockland).

Molecular Characterization of the Gene-Stacking RMCE Plant

A T0 plant, B531-1, regenerated from event B531 was analyzed by the same qPCR assays previously done on embryogenic callus. Consistent with previous results (Table II), the qPCR assays confirmed that B531-1 contains one copy of the RMCE DNA QC288A436A438A and is free of any donor, target, or FLP DNA (data not shown).

The T0 plant B531-1 containing the stacked genes QC288A436A438A and its ancestors, including the original target B containing QC288A, the first-round SSI transformation target B5 containing QC288A329A, and the second-round SSI transformation target B53 containing QC288A436A, were analyzed by PCR using nine sets of primers (Fig. 2). The 70-1S/Hygro-A primer set specific to the SCP1 and HPT junction of QC288A and QC288A436A detected the expected band in B and B53 (Fig. 5A, left). The Yfp-3/70-1A primer set specific to the QC288A 3’ end detected the band expected only in B (Fig. 5A, center). The 70-1S/70-1A primer set specific to both the 5’ and 3’ end genomic borders of the B target site detected the full-length QC288A band in homozygous B, the full-length QC288A329A band in homozygous B5, and the exci-
tion band in hemizygous B53 and B53-1 (Fig. 5A, right). The full-length QC288A436A of B53 and QC288A436A438A of B53-1 are too large to be amplified by PCR. The 70-1S/Als-3 primer set specific to the SCP1 and ALS junction of QC288A436A438A (SSI), a QC438-specific region (Donor), the SCP1 and HPT junction of QC288A436A (Target), and a QC292-specific region (FLP). The qPCR assays were done as described in the Table I legend. 1Fatty acids in mature somatic embryos were determined by GC as described in the Table I legend and are expressed as the percentages of total fatty acids. Oil contents in mature somatic embryos determined by NMR are presented as the percentages of total sample dry weight. Oil measurements made by NMR as described here are reproducible to approximately 1% oil of sample dry weight. Untransformed control somatic embryos typically contain 2.2% to 6.2% oil (Meyer et al., 2008). Increases in oil contents to above 7% oil of sample dry weight are indicative of a functional DGAT1 gene. Oil content for event B532 was not determined (ND) due to low tissue amounts.

Table II. Transgene integration and fatty acid contents of the second-round SSI events

<table>
<thead>
<tr>
<th>Event</th>
<th>Gene Copy No. by qPCR</th>
<th>Fatty Acid Content by GC</th>
<th>Oil</th>
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<tbody>
<tr>
<td></td>
<td>SSI</td>
<td>Donor</td>
<td>Target</td>
</tr>
<tr>
<td>B531</td>
<td>1.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>B532</td>
<td>0.0</td>
<td>0.7</td>
<td>1.3</td>
</tr>
<tr>
<td>B541</td>
<td>1.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>B511</td>
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<td>0.7</td>
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</tr>
<tr>
<td>B512</td>
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</table>

Events B531 to B512 are representative chlorsulfuron-resistant events selected from the retransformation of the embryogenic callus of RMCE events B51, B53, and B54 containing QC288A436A with the second donor QC438 and FLP QC292 DNA. 1Embryogenic callus samples were analyzed by qPCR specific to the SCP1 and ALS junction of QC288A436A438A (SSI), a QC438-specific region (Donor), the SCP1 and HPT junction of QC288A436A (Target), and a QC292-specific region (FLP). The qPCR assays were done as described in the Table I legend. 1Fatty acids in mature somatic embryos were determined by GC as described in the Table I legend and are expressed as the percentages of total fatty acids. Oil contents in mature somatic embryos determined by NMR are presented as the percentages of total sample dry weight. Oil measurements made by NMR as described here are reproducible to approximately 1% oil of sample dry weight. Untransformed control somatic embryos typically contain 2.2% to 6.2% oil (Meyer et al., 2008). Increases in oil contents to above 7% oil of sample dry weight are indicative of a functional DGAT1 gene. Oil content for event B532 was not determined (ND) due to low tissue amounts.

Figure 4. Transgene expression in the second-round SSI events. The expression of genes DHPS, BHL8, and CGS in mature somatic embryos of selected second-round SSI events was checked by western blotting. Only the RMCE event B531 expressed all three proteins DHPS (A), BHL8 (B), and CGS (C). Event B532 expressed only BHL8. Event B541 expressed both DHPS and BHL8 but not CGS. Event B511 expressed both DHPS and CGS but not BHL8. Event B512 expressed only DHPS. Positive controls containing respective genes are transgenic events from unrelated projects. The protein markers are in kD. wt, Wild type.
RMCE QC288A436A438A. As expected, both the QC288A436A438A-specific 3,681-bp band and the QC288ME-specific 3,987-bp band were detected in B53-1. The same 3,681-bp band, also specific to QC288A329A of the B5 ancestor, was detected in B5. The QC288A-specific 7,839-bp band was detected in B (Fig. 6A). The HPT probe hybridized to the same QC288A436A-specific 12,205-bp band in B53-1, B53-2, B53-3, and B53-4 and the QC288A-specific 7,839-bp band in B. As expected, no HPT band was detected in B5-3, B5-6, B53-1, or B (Fig. 6B). The FATB-5 probe hybridized to two endogenous homolog gene bands in all the samples in addition to the same QC288A436A-specific 12,205-bp band in B53-1, B53-2, B53-3, and B53-4. But the expected QC288A436A438A-specific 12,931-bp FATB-5 band was not detected. Instead, a larger band seems overlapped with the large wild-type band (Fig. 6C). The discrepancy was caused by an internal deletion of QC288A436A438A, as discussed below.

The DGAT1 probe hybridized to only B53-1 as expected, but the band is much larger than the expected 6,352-bp QC288A436A438A-specific band (Fig. 6D). To resolve this discrepancy, the entire 21,277-bp QC288A436A438A transgenes plus some 5′ and 3′ end genomic borders were amplified by PCR as five overlapping approximately 5-kb fragments (data not shown). An approximately 3-kb-long deletion involving the DHPS gene and the UBIQ10 promoter was identified only in the B531 T0 plant but not in its embryogenic callus B531 (Fig. 5D). Sequencing the corresponding fragments revealed that a 2,946-bp segment including the entire DHPS gene and the UBIQ10 promoter was looped out between two 165-bp repeats in B531-1. The 165-bp sequence encoding a soybean ribulose-1,5-bisphosphate carboxylase small subunit transit peptide was engineered at the 5′ end of both DHPS and CGS to target the enzymes to chloroplast. The loss of two MfeI sites contained in the DHPS region (Fig. 2C) changed the predicted 12,931-bp internal deletion of QC288A436A, as discussed below.

DISCUSSION

RMCE using different recombinase systems has been achieved successfully in several plants (Nanto et al., 2005; Louwerse et al., 2007; Li et al., 2009). However, stacking multiple genes at a precharacterized genomic site using RMCE or any other technology has not been reported. Although the gene-stacking event B531 in this report has defects and cannot be used as a product, the integration of seven functional trait genes at one genomic site by two rounds of SSI technology has not been reported. Although the gene-stacking event B531 in this report has defects and cannot be used as a product, the integration of seven functional trait genes at one genomic site by two rounds of SSI technology demonstrates the usefulness of RMCE technology in agricultural biotechnology and gene expression research.
Taking advantage of reversible DNA cassette exchange in RMCE, an RMCE product can be used as a new target for subsequent SSI transformation. If additional incompatible recognition sites can be introduced, successive rounds of RMCE can stack genes at a precharacterized genomic target site repeatedly, in theory. The work described here required three rounds of RMCE. The first round of RMCE described previously by Li et al. (2009) was an SSI transformation that switched the HPT gene of QC288A in the original target event B to the ALS gene of QC288A329A in RMCE event B5. This is necessary to ensure that the final gene-stacking event B531 contains the regulatory-acceptable ALS gene. Thus, the first round of SSI transformation in this study was done on RMCE B5 as the target to integrate the first group of trait genes, FAD2 and FATB, and meanwhile switching the selectable marker back to HPT to create RMCE B53 containing QC288A436A. The second group of trait genes, DGAT1, DHPS, BHL8, and CGS, were subsequently integrated by the second SSI transformation on B53 to create RMCE B531 containing QC288A436A438A, which consisted of the above six and ALS genes. If desired, the process can continue to stack more genes as long as additional incompatible FRT recognition sites are available. Outcrossing to wild-type plants may be necessary to rejuvenate the target lines, as they may lose vigor by repeated transformation.

The proper expression of the stacked seven genes ALS, DGAT1, DHPS, BHL8, CGS, FAD2, and FATB demonstrated that gene overexpression and silencing can be simultaneously achieved at one genomic site. One copy of hairpin-structured FAD2 and FATB is sufficient to trigger the silencing of corresponding endogenous genes. Careful evaluation of the phenotypes conferred by all the genes will help to check if the seven genes placed in the linear configuration interfere with each other. Due to the 2,946-bp internal deletion of QC288A436A438A and other defects in the T0 plant B531-1, we will not be able to study this event further. New gene-stacking events produced from other experiments will need to be evaluated to address the question.

RMCE is a complex process especially when there are two targets, one on each homologous chromosome, and the two recombinase recognition sites involved are only partially incompatible (Li et al., 2009). The process is further complicated in gene stacking by using three recognition sites and large donor DNA such as QC438 containing multiple genes with some repeated sequences. Since it is known that the three FRT sites used in B53 and B531 are not completely incompatible (Tao et al., 2007), DNA recombination can happen between any pairs of them when exposed to FLP recombinase. As a result, genes already integrated in the first-round SSI (i.e. FAD2 and FATB) can be excised during the second round of SSI transformation, as indicated by the reduced levels of 18:1 in events B541, B511, and B512 (Table II).

Figure 6. Confirmation of gene stacking by Southern hybridization. Genomic DNA of B53 T1 plants, homozygous RMCE B53-1 and B53-2, hemizygous (RMCE/excision) B53-3 and B53-4, and excision B53-5 and B53-6, the T0 plant B531-1 (RMCE/excision), and homozygous B5 and B ancestor plants were digested with MfeI and hybridized sequentially with probes SCP1, HPT, FATB-5, and DGAT1. The expected sizes of Southern bands are specific to transgenes integrated at the B target site, where the transgenic QC288A lost 17-bp 5' end and 49-bp 3' end sequences. There is an MfeI site 2,131 bp upstream and 1,230 bp downstream, respectively, of the transgenes that contain MfeI sites (Fig. 2). A, The SCP1 probe hybridized to the expected 12,205-bp band of QC288A436A in B53-1 and B53-2, the 3,987-bp band of QC288ME in B53-5 and B53-6, and both bands in hemizygous plants B53-3 and B53-4. The same 3,987-bp QC288ME band and a 3,681-bp QC288A436A438A band were hybridized in B531-1. As expected, the same 3,681-bp band also specific to QC288A329A was detected in B5 and a 7,839-bp QC288A band was detected in B. B, The HPT probe hybridized to the same 12,205-bp QC288A436A band in B53-1, B53-2, B53-3, and B53-4 and to the same 7,839-bp QC288A band in B. C, The FATB-5 probe hybridized to two endogenous bands in all samples in addition to the same 12,205-bp QC288A436A band in B53-1, B53-2, B53-3, and B53-4. Instead of the expected 12,931-bp band of QC288A436A438A, a larger band, likely the 16,402-bp band expected from modified QC288A436A438A with its 2,946-bp DHPS-MYB2-UBIQ10 deleted, overlapped with the top wild-type band (wt) in B531-1. D, The DGAT1 probe detected in B531-1 the same 16,402-bp band of the modified QC288A436A438A instead of an expected 6,352-bp QC288A436A438A band. The DIGVII DNA markers are 8,576, 7,427, 6,106, 4,899, and 3,639 bp (Roche).
Furthermore, even the perfect gene-stacking RMCE event B531, confirmed at the embryogenic callus stage, lost an internal segment of the transgenes later during plant regeneration, possibly by DNA looping out between two 165-bp repeats. Although longer DNA fragment inverted repeats, such as the two FAD2-1 and FATB-4 fragments designed for gene silencing, were maintained in B531-1 T0 plants, direct repeats should be generally avoided in DNA constructs to prevent intervening DNA from being looped out via similar intrachromosomal homologous recombination that is involved in creating extrachromosomal circular DNA from tandem repeats in plant genomes (Cohen et al., 2008).

Recent developments in plant gene targeting demonstrate that endogenous genomic sites can be specifically targeted for modification through DNA double-strand break-induced homologous recombination. DNA double-strand breaks can be created with either designed zinc finger nucleases or modified homing endonucleases. Customized zinc finger nucleases have been employed to introduce successfully an herbicide resistance gene, PAT, to a tobacco (Nicotiana tabacum) endochitinase gene locus, a maize (Zea mays) inositol-1,3,4,5,6-petakishosphate 2-kinase gene locus, or to introduce specific mutations to a tobacco acetolactate synthase gene to gain resistance to sulfonyl urea (Cai et al., 2009; Shukla et al., 2009; Townsend et al., 2009). Similarly, an engineered I-CreI endonuclease derivative designed to recognize a selected sequence adjacent to the maize LIGULELESS1 gene has been used to produce mutations with small deletions or insertions specifically at expected cleavage sites (Gao et al., 2010). Thus, the random integration problem of creating initial SSI target sites can be resolved by using double-strand break-induced homologous recombination to introduce the FRT1-FRT87 recognition sequences at specifically selected genomic sites. Then, more transgenes can subsequently be added or exchanged through FLP recombinase-mediated RMCE, which may have the advantages of being reversible, more effective and flexible, and able to deliver large transgenes. Future challenge for the application of RMCE in agricultural biotechnology is to develop highly transformatible target lines that are able to accept transgenes with different preferences for optimal gene overexpression, gene silencing, tissue-specific expression, and agronomic performance.

MATERIALS AND METHODS

DNA Construction and Plant Transformation

Donor constructs QC436 containing FRT1-HPT:NOS-FRT12+KTI3:ALS:PINII+UBQ:CFP:NOS-FRT87 and QC438 containing FRT1-ALS:PINII+yGt1:DGAT1:LEG+CONG:BHL8:PH3+ALB:DHP5:MYB2+UBIQ10:CGS:PH3-FRT12 were made following standard molecular cloning procedures using components from existing DNA constructs (Falco et al., 1999; Roesler and Rao, 2000; Hitz, 2001; Kinney et al., 2004; Falco, 2006; Lightner et al., 2006; Meyer et al., 2008; Li et al., 2009; Xing et al., 2010). Soybean (Glycine max) embryogenic cultures were initiated from homozygous progeny of RMCE plants B5-1 and B5-2 containing the QC288A329A transgen SCP1:FRT1:ALS:PINII+UBQ:CFP:NOS-FRT87 as the target (Li et al., 2009). The first donor DNA QC436 and the FLP expression DNA QC292 SCP1:FLP:PINII were cotransformed at 9.3 ratios following the biolistic transformation protocol using 30 μg mL−1 hygromycin B for transgenic event selection (Li et al., 2009). The embryogenic cultures of selected QC436 RMCE events were directly retransformed with the second donor DNA QC438 and the same FLP expression DNA QC292 at 9.3 ratios using 90 mg mL−1 chlorosulfuron (DuPont) for transgenic event selection. All the donor and FLP DNAs used are circular plasmid DNA.

qPCR Analysis

Gene-specific qPCR assays were done on somatic embryo or leaf DNA samples using the same primer/probe sets specific to the original target QC288A, the first target QC288A329A (i.e. RMCE event B5), and the FLP DNA QC292 (Li et al., 2009). The first donor QC436-specific qPCR assay used a new primer, 409-1F (5′-CGAGCGTGATCGATAACCTTGTTAAC-3′), and the previous primer Hydro-116R and probe Hydro-79T. The second donor QC438-specific qPCR used primers 409-1F, Als-163R, and probe Als-110F. SSI-specific qPCR assays used the same QC288A-specific primer/probe set of QC288A1F, Hydro-116R, and probe Hydro-79T for the first-round RMCE QC288A436A and the same QC288A329A-specific primer/probe set of QC288A1-1F, Als-163R, and probe Als-110T for the second-round RMCE QC288A436A438A.

PCR Analysis

PCR assays were similarly done on somatic embryo or leaf samples using some primers previously described (Li et al., 2009). The 5′ border PCR assays used primers 70-1S and Als-3 for QC288A329A and QC288A436A438A or 70-1S and Hydro-A for QC288A436A. The 3′ border PCR assays used primers Cry-1 and 70-1A for QC288A329A or soyfad2-A (5′-GAAGGCTCAACCAACCAACA-ACATCT-3′) and 70-1A for both QC288A436A and QC288A436A438A. The FRT12 site of QC288A436A was checked with primers Hygro801 (5′-CCCTGCAGCAGAAAGGAATAGTGAGG-3′) and Ph3-A (5′-GGCCGG-TTGATATTATTTATACACACC-3′). The FRT12 site of QC288A436A438A was checked with primers 3′-CAATGCTTGGAAATGTTGACC-3′ and Kti3P-A. A QC288A436A438A middle region was checked with primers Cong-1 (5′-TCAAACCTGCAAACTCGATG-3′) and Ph3-A (5′-GCAATTC-CATAAACGCGCTACACACC-3′). A QC288A436A438A internal deletion was checked by two PCR assays with primers Ph3-3′-5′-GAATGCTGCACGGCT-TATGGAATG-3′ and CRRSII-1 (5′-GCCGAGGGCAAAGGAATAGTGAGG-3′) and primers Dgt-1R (5′-CTGTTGTCGTTGTTGATTGAGG-3′) and CRRSII. The expected sizes of all PCR bands are given in the figure legends.

Fatty Acid and Oil Analysis

Soybean seeds or somatic embryos were ground and their fatty acid compositions were determined by GC. Approximately 5 mg of embryo powder was incubated while shaking with 50 μL of 0.25 M trimethylsulfonium hydroxide in methanol and 0.5 mL of hexane for transesterification in a GC vial at room temperature for 30 min. Fatty acid methyl esters (5 μL injected from the hexane layer) were separated and quantified using a Hewlett-Packard 6890 gas chromatograph fitted with an Omegawax 320 fused silica capillary column (Supelco). The oven temperature was programmed to hold at 220°C for 2.6 min, increase to 240°C at 20°C min−1, and then hold for an additional 2.4 min. Carrier gas was supplied by a Whatman hydrogen generator. Retention times were compared with those for methyl esters of commercially available standards (Nu-Chek Prep), and the peaks corresponding to 16:0, 18:0, 18:1, 18:2, and 18:3 were analyzed using the Chemstation software (Agilent).

The oil content was analyzed on remaining embryo powder by NMR using a Maran Ultra NMR system. The samples were weighed and scanned in a commercial 40-mHz NMR tube (Adopt). The oil content was determined by comparing the NMR readings with standard curves and expressed as the percentage of the total sample weight.

Western Blotting

Total proteins were extracted from 5 mg of lyophilized somatic embryo powder in a buffer containing 50 mM Tris, pH 7.5, 10 mM β-mercaptoethanol,
and 0.1% SDS. The proteins were resolved on 12% NuPAGE Tris-Glycine protein gels and blotted to nitrocellulose membranes using the XCell SureLock Novex Mini-Cell system (Invitrogen). Protein loadings and MultiMark weight markers (Invitrogen) were checked by staining the blots with the pores of Pureteam S (Sigma). The blots were then incubated at 4°C overnight with DHPS, BHL8, or CGS antibodies (rabbit) diluted 1:1500 in 5% dry milk dissolved in TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.5) followed by four 5-min washes in TBS buffer (TBS with 0.05% Tween 20; Sigma). Specific proteins were detected using the horseradish peroxidase-labeled mouse antibody of the Lumi-Light western blotting (mouse/rabbit) kit (Roche). Signals were captured on BioMax films (Eastman Kodak).

**Southern Hybridization Analysis**

Soybean genomic DNA was prepared from leaf samples and analyzed by Southern hybridization with digoxigenin-labeled probes (Li et al., 2009). DNA was digested with EcoRI and hybridized with a 581-bp SCPI probe (Li et al., 2009), a 588-bp HPT probe made by PCR with primers Hpt-1 (5’-TTCGAATTCGAGAGGCCG-3’) and Hpt-2 (5’-CATGTTTCGCGACCCTGATTGGG-3’), a 459-bp FATB-5 probe made with primers TE2-F (5’-GGTGAATCTTGGCAAGCTCTCCACG-3’) and TE2-R (5’-CAACTCTAGGACCCATTITCCAG-3’), and a 616-bp DGAT probe made with primers Dgat-1-F (5’-CCTCTCTTCTTCTGATGTAGGAGGGCG-3’) and Dgat-1-R using the PCR digoxigenin probe synthesis kit (Roche).

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