**PureMlb® technology reduces vector backbone integration and increases efficiency of Agrobacterium-mediated transformation in cassava**

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**Introduction**
Agrobacterium is an indispensable tool for the genetic transformation of a large number of plant species. The basic component of the Agrobacterium gene transfer system is a vector that houses the T-DNA bordered by two 25bp imperfect repeats termed the left (LB) and right border (RB)(Fig.1).

Use of pureMlb® technology to reduce vector backbone integration

Multiple left border (PureMlb®) technology was adopted in order to reduce vector backbone integration in transgenic cassava plants. Binary vector pCambia2300 was modified by introducing two and three LB repeats in close proximity (19bp apart) to the existing LB. A 35S promoter-driven GFP cassette was also cloned 260bp downstream of the LB to allow visual screening for integration of backbone sequences (Fig. 3). These constructs were tested in transgenic BY2 (tobacco) cells and tobacco plants in addition to the production of transgenic cassava plants.

Ideally T-DNA integration into the host genome involves transfer of only the DNA sequences starting from the RB, progressing and terminating at the LB. However, a growing number of reports have documented that this process is prone to mistakes, whereby integration occurs of the vector plasmid backbone past the LB (Fig. 2) and less frequently past the RB. In extreme cases, the entire vector backbone sequence can be integrated into the plant genome.

Integration of vector DNA could be the result of read-through at the LB which prevents the normal termination of the T-DNA transfer. Alternatively, DNA transfer could start at the LB and proceeds towards the RB leading to read-through at the RB.

The ratio of transfectants that acquire backbone sequences differs between species but typically ranges between 20% and 50% and can be greater than 75%, as reported in Arabidopsis, tobacco, petunia, potato, rice, maize, tomato, grape, barley and strawberry. Initial studies identified that vector backbone integration ranged from 60-80% in transgenic cassava plants generated in our laboratory by transformation of friable embryogenic callus (FEC) via Agrobacterium strain LBA4404 (Fig. 4).

Regulatory requirements determine that transgenic plants possessing integrated vector backbone cannot be commercialized, most especially if such sequences code for bacterial genes or functional elements. The goal of ILTAB is to generate regulatory compliant, viral resistant and nutritionally enhanced cassava for release to farmers in Africa. To increase the proportion of plants devoid of backbone sequence we employed a technology reported by Kuraya et al. 2006.

This technology, patented by Japan Tobacco Inc., is known as PureMlb® (multiple left border) and employs the use of additional LB sequences cloned close to the original LB of the transformation vector.

**Table 1. Frequency of vector backbone integration in BY2 cells**

<table>
<thead>
<tr>
<th>CONSTRUCT</th>
<th>PRESENCE OF VECTOR BB</th>
<th>LB (NO RB)</th>
<th>RB (NO LB)</th>
<th>LB &amp; RB</th>
</tr>
</thead>
<tbody>
<tr>
<td>pILTAB 602 (+GFP)</td>
<td>before LB &amp; RB- presence of both beyond LB &amp; RB sequences (integration of whole vector plasmid)</td>
<td>61/95 (64%)</td>
<td>44/95 (47%)</td>
<td>12/50 (24%)</td>
</tr>
<tr>
<td>pILTAB 607 (+2LB+GFP)</td>
<td>before LB- presence of beyond LB sequence only; RB (NO LB)- presence of beyond RB sequence only</td>
<td>63/95 (66%)</td>
<td>26/95 (28%)</td>
<td>13/95 (14%)</td>
</tr>
<tr>
<td>pILTAB 607 (+3LB+GFP)</td>
<td>before LB- presence of beyond LB sequence only; RB- presence of both beyond RB (LB &amp; RB sequences) (integration of whole vector plasmid)</td>
<td>57/100 (57%)</td>
<td>23/100 (23%)</td>
<td>15/100 (15%)</td>
</tr>
</tbody>
</table>

**Results**

Transgenic tissues transformed with Agrobacterium carrying PureMlb® technology were analyzed for the presence of backbone sequences by PCR amplification of sequences immediately beyond the LB and RB (Tables 1-4). PCR analysis for presence of Agrobacterium confirmed absence of any such contamination.

**Table 2. Frequency of vector backbone integration in N. tabacum plants**

<table>
<thead>
<tr>
<th>CONSTRUCT</th>
<th>PRESENCE OF VECTOR BB</th>
<th>LB (NO RB)</th>
<th>RB (NO LB)</th>
<th>LB &amp; RB</th>
</tr>
</thead>
<tbody>
<tr>
<td>pILTAB 602 (+GFP)</td>
<td>before LB &amp; RB- presence of both beyond LB &amp; RB sequences (integration of whole vector plasmid)</td>
<td>55/84 (65%)</td>
<td>23/84 (28%)</td>
<td>7/84 (8%)</td>
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<tr>
<td>pILTAB 607 (+2LB+GFP)</td>
<td>before LB- presence of beyond LB sequence only; RB (NO LB)- presence of beyond RB sequence only</td>
<td>22/35 (63%)</td>
<td>8/35 (23%)</td>
<td>5/35 (14%)</td>
</tr>
<tr>
<td>pILTAB 608 (+3LB+GFP)</td>
<td>before LB- presence of beyond LB sequence only; RB- presence of both beyond RB (LB &amp; RB sequences) (integration of whole vector plasmid)</td>
<td>15/47 (32%)</td>
<td>6/47 (13%)</td>
<td>4/47 (9%)</td>
</tr>
</tbody>
</table>

**Table 3. Frequency of vector backbone integration in cassava**

<table>
<thead>
<tr>
<th>CONSTRUCT</th>
<th>PRESENCE OF VECTOR BB</th>
<th>LB (NO RB)</th>
<th>RB (NO LB)</th>
<th>LB &amp; RB</th>
</tr>
</thead>
<tbody>
<tr>
<td>pILTAB 602 (+GFP)</td>
<td>before LB &amp; RB- presence of both beyond LB &amp; RB sequences (integration of whole vector plasmid)</td>
<td>16/23 (70%)</td>
<td>5/23 (22%)</td>
<td>5/23 (22%)</td>
</tr>
<tr>
<td>pILTAB 607 (+2LB+GFP)</td>
<td>before LB- presence of beyond LB sequence only; RB (NO LB)- presence of beyond RB sequence only</td>
<td>25/41 (61%)</td>
<td>4/41 (10%)</td>
<td>13/41 (32%)</td>
</tr>
<tr>
<td>pILTAB 608 (+3LB+GFP)</td>
<td>before LB- presence of beyond LB sequence only; RB- presence of both beyond RB (LB &amp; RB sequences) (integration of whole vector plasmid)</td>
<td>25/46 (54%)</td>
<td>3/46 (6%)</td>
<td>19/46 (41%)</td>
</tr>
</tbody>
</table>

**Conclusions**

- Inclusion of PureMlb® technology was effective for reducing vector backbone integration in all three tissue systems tested. In each case the triple LB construct was more effective than the double LB version.
- Molecular analysis of transgenic BY2 cells and tobacco plants indicated that inclusion of PureMlb® reduced vector backbone integration by between 33% and 50% compared to the comparative, single LB construct. Analysis of transgenic cassava is still ongoing. To date, depending on the specific experiment, reductions in backbone integration is also between 35 and 50% compared to the single LB controls.
- Inclusion of GFP in the vector backbone resulted in identifiable GFP expression in only 50% of total lines in which vector backbone had been integrated. We conclude therefore that this is not an effective strategy for elimination of vector backbone integration events.
- PureMlb® reduced the frequency of integration past the LB but did not reduce frequency of integration past the RB. Transgenic events with integrations past the RB (but not past the LB), can account for 25-30% of the vector backbone integration events.
- PureMlb® technology was found to increase transformation efficiencies in cassava approximately two fold compared to the single LB control constructs.
- Japan Tobacco Inc. has kindly donated access to PureMlb® technology through a Humanitarian License for application in cassava transgenic technologies.

**References**

**Acknowledgements**
We thank Japan Tobacco Inc. for providing the PureMlb® technology
We thank Kevin Lutke (Plant Tissue Culture and Transformation Facility, Danforth Plant Science Center) for production of BY2 cells and transgenic tobacco plants.