Running head;

Gateway™ vectors for high-throughput functional analysis

Mark D. Curtis
Institute of Plant Biology and Zürich-Basel Plant Science Centre,
University of Zürich,
Zollikerstrasse 107,
CH-8008 Zürich,
Switzerland

Tel: +4116348241
Fax: +4116348204
email: mcurtis@botinst.unizh.ch

Breakthrough Technologies
A Gateway™ cloning vector set for high-throughput functional analysis of genes

in planta

Mark D. Curtis* and Ueli Grossniklaus

Institute of Plant Biology and Zürich-Basel Plant Science Centre, University of

Zürich, Zollikerstrasse 107, CH-8008 Zürich, Switzerland

* Corresponding Author
Institute of Plant Biology and Zürich-Basel Plant Science Centre,
University of Zürich,
Zollikerstrasse 107,
CH-8008 Zürich,
Switzerland

Tel: +4116348241
Fax: +4116348204
email: mcurtis@botinst.unizh.ch
Supplementary Materials and Methods

Plasmid construction

Each of the three att cassettes supplied by Invitrogen was cloned into the blunt-ended HindIII and EcoRI sites of the pUCAP vector, so that each cassette was obtained in both orientations with respect to the eight-nucleotide recognition sites of AscI and PacI that flank the blunted HindIII and EcoRI sites, respectively. This allows any DNA fragment that has recombined into the att sites to be identified easily using AscI and PacI restriction digestion. One of the new cassettes (cassetteB) was subcloned into the plasmid PER8 (Zuo et al., 2000) to make the plasmid pMDC7. The AscI and PacI sites in this vector are flanked by XhoI and SpeI sites, respectively. Using XhoI and SpeI the cassette was subcloned into the XhoI and SpeI sites of pBluescript SK- (Clontech). In this way the attR1 site was adjacent to the AscI, XhoI and KpnI sites and the attR2 site was adjacent to the PacI, SpeI and XbaI sites. This plasmid was digested with KpnI and XbaI, and the fragment containing the cassette was ligated between the heat-shock promoter and the nos terminator of pMDC23 (Mark Curtis, unpublished) using the KpnI and XbaI sites to orientate the cassette correctly, thus producing plasmid pMDC30. Similarly the same KpnI-XbaI fragment containing the cassette, was also ligated between the 2 X 35S promoter and the nos terminator of a second plasmid to produce pMDC32. The arrangement of the attR2 site, adjacent to the PacI, SpeI and XbaI sites, provides at least one stop codon in each reading frame so that cDNAs amplified without a stop codon in the Entry clone can utilize the stop codon adjacent to the recombination site in the destination construct.
**WUSCHEL cDNA amplification**

WUSCHEL cDNA was amplified from the plasmid A1-1 with primers \textit{WusF} (5’AAA AAG CAG GCT ATG GAG CCG CCA CAG CAT CAG CAT C 3’) and \textit{WusR} (5’AGA AAG CTG GGT GTT CAG ACG TAG CTC AAG AGA AGC 3’). The PCR conditions were 94°C for 1 min, then 10 cycles of 94°C for 15s, 55°C for 30s and 68°C for 60s, followed by further PCR of this product with \textit{att} recombination adapter primers and the manufacturers recommended conditions.

**C-terminal GFP6 constructs**

The GFP6 gene was amplified from plasmid pSKpmgfp6, a histidine tagged derivative of gfp6 (Schuldt et al., 1998) (a gift from David Jackson, Cold Spring Harbor Laboratory), with primers \textit{GFP6KpnI} (5’AAA AGG TAC CGG TAG AAA AAA TGA GTA AAG ACC TTT TC 3’) and \textit{GFP6AscI} (5’GCT TGG CGC GCC TTT GTA TAG TTC ATC CAT GCC 3’). The PCR conditions were 94 °C for 2 min, then 10 cycles of 94 °C for 30s, 53 °C for 30s and 72 °C for 60s, followed by 20 cycles of 94 °C for 30s, 55 °C for 30s and 72 °C for 60s, and finally 72 °C for 2 min. The PCR product was digested with \textit{AscI} and \textit{KpnI}, and subcloned into the \textit{AscI} and \textit{KpnI} sites of pMDC32, to produce the plasmid pMDC44. The other two reading frame cassettes in pUCAP, with the \textit{attR1} site adjacent to the \textit{AscI} site and the \textit{attB2} site adjacent to the \textit{PacI} site, were subcloned, using \textit{AscI} and \textit{PacI}, into pMDC44, replacing cassette B with cassette C1, to produce pMDC43. This cloning strategy was repeated for cassette A to produce pMDC45. The arrangement of the \textit{attR2} site adjacent to the \textit{PacI}, \textit{SpeI} and \textit{XbaI} sites again provides at least one stop codon for each reading frame. This means that the same Entry clone containing the gene of interest without a
stop, used for ectopic expression or heat-inducible expression, can be used to make N-terminal fusions with GFP6 or GUS.

**N-terminal GFP6 constructs**

The N-terminal GFP6 constructs, pMDC83, pMDC84, pMDC85 were derived from pMDC32. GFP6 was amplified from the plasmid pSKpmgfp6 with primers \textit{GFP6XbaI} (5’AAA ATC TAG ATG AGT AAA GGA GAA GAA CTT TTC3’) and \textit{GFP6AscI}. The PCR was carried out using the conditions described for primers \textit{GFP6KpnI} and \textit{GFP6AscI} (see C-terminal GFP6 constructs). The PCR product was digested with \textit{AscI} and \textit{XbaI}, and subcloned into the \textit{AscI} and \textit{XbaI} sites of pMDC32 to produce the plasmid pMDC84. The other two reading frame cassettes in pUCAP, with the \textit{attR2} site adjacent to the \textit{AscI} site and the \textit{attB1} site adjacent to the \textit{PacI} site, were subcloned using \textit{AscI} and \textit{PacI} into plasmid pMDC84, replacing cassette B with cassette A, to produce pMDC83. This cloning strategy was repeated with cassette C1 to produce pMDC85. The arrangement of the \textit{AscI} site, adjacent to the \textit{attR2} site provides no stop codons in any reading frame so that the same cloned gene in the Entry vector (without a stop codon) can be used to make in-frame N-terminal fusions with GFP6 in the vectors pMDC83, pMDC84, pMDC85.

**N-terminal GUS constructs**

To make C-terminal GUS constructs, the \textit{XbaI-EcoRI} fragment of pBI121 (Jefferson et al., 1987) was subcloned into the \textit{XbaI} and \textit{EcoRI} sites of pCambia 1300, to make pMDC91. The \textit{BamHI} site in pMDC91 was end-filled, using T4 polymerase, and religated to remove the site and shift the reading frame by two nucleotides, to produce plasmid pMDC127. The \textit{HindIII-XhoI} fragments from each plasmid pMDC83, pMDC84, and pMDC85, containing the 2 X 35S promoter and the \textit{att} cassettes A, B
and C1 respectively, were subcloned individually into plasmid pMDC127 using HindIII and SalI. This produced the plasmids pMDC139, pMDC140 and pMDC141, respectively.

**Promoter-reporter constructs**

Promoter-reporter constructs with GFP6 and with GUS were generated simply by removing the SpeI / HindIII fragment, containing the 2 x 35S promoter, blunt-ending and religating the vector fragment. In this way the GFP6 promoterless constructs pMDC107, pMDC110 and pMDC111, and the GUS promoterless constructs pMDC162, pMDC163 and pMDC164, were generated. These constructs have 3 reading frames so that gene fusions (genes without their native stop codon), linked to their native promoter, can be made to either GFP6his or GUS.

**Genomic complementation constructs**

Vectors for complementation using genomic fragments were generated in pCambia 1300 (hygromycin resistant) and pCambia 2300 (kanamycin resistant), by subcloning the KpnI-SacI fragment of pBluescript SK+, containing cassette C1 flanked by the AscI and PacI sites, to produce pMDC99 and pMDC100, respectively. The pCambia 3300 (Basta resistant) construct was produced by subcloning the HindIII-AscI fragment from pMDC100 into the HindIII-AscI sites of pCambia 3300. All vectors containing att cassettes were maintained in E.coli strain DB3.1 (Invitrogen) in which the ccdB gene has no lethal effect.
Plant materials, growth conditions and plant transformation

MS agar was supplemented with the appropriate antibiotics and or the inducer 17-β-estradiol. Induction experiments with 17-β-estradiol were carried out as previously described by Zuo et al., (2000), except that 2µM 17-β-estradiol was used in the media. Plants for heat shock induced gene expression were grown for 5 days under continuous white light at 22°C and then transferred to a 37°C incubator overnight and histochemically stained with GUS staining solution.

GUS assays

In-situ GUS staining was carried out by vacuum infiltrating GUS staining solution (50 mM sodium phosphate buffer, pH 7.0, 1mM EDTA, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-D glucuronic acid [X-Gluc, Biosynth AG.], 0.4% Triton X-100, 100 mg/ml chloramphenicol and 5 mM each of potassium ferri/ferrocyanide), and incubating at 37°C for 24 hours.

Transient expression assays

Transient expression assays in onion epidermal cells using Biolistic PDS-1000/He DNA delivery system (Bio-Rad), were carried out at 900psi, following the manufacturers instructions. Typically 5µg of plasmid was used per assay. Tissue bombarded with GFP constructs was examined on a Ziess Axioplan microscope (Carl Zeiss, Oberkochen, Germany) with a 100W mercury arc lamp and an excitation filter at 450-490nm, a dichroic mirror at 510nm, and a barrier filter at 515-565nm. Images were acquired using an Optronics digital camera and MagnaFire image analysis software.
Distribution of Materials

All the vectors described in this publication will be made freely available through the University of Zurich for non-commercial research purposes (http://www.unizh.ch/botinst/Devo_Website/curtisvector/).

If these vectors are to be used for a commercial purpose, permission must be obtained from: - Cornelia Boesch, Dr. sc. techn. ETH Unitectra Inc. Technology Transfer Universities of Bern and Zurich, Gesellschaftsstrasse 25; CH-3012 Bern, Tel. ++41-31-631 37 81; Fax ++41-31-631 37 89, e-mail: boesch@unitectra.ch; web: http://www.unitectra.ch

Additional permission must be obtained for use of the PER8 derived vectors from the Rockefeller University. Contact Kathleen A. Denis, Ph.D. Associate Vice President, Technology Transfer, The Rockefeller University, 1230 York Avenue - Box 81, New York, New York 10021. Tel. 212-327-8266, Fax: 212-327-8267.

The Centre for Application of Molecular Biology to International Agriculture (CAMBIA) supplied the backbone of the vectors. All vectors described in this set of vectors, except the PER8 derived vectors, contain pCambia sequences. For further information on the possible commercial use of these vectors contact Carol Nottenburg PhD JD, Chief Legal Counsel / Director of cambiaIP Resource, CAMBIA, GPO Box 3200, Canberra ACT 2601 Australia, Tel. +61 2 6246 4504, Fax. +61 2 6246 4533 www.cambiaIP.org or www.cambia.org

All of these vectors contain proprietary Gateway™ sequences. For-profit organisations must obtain a license from Life Technologies (See also limited label licenses in Gateway™ Cloning Technology Instruction Manual).
Supplementary Results

In planta expression of the uidA reporter gene using destination vectors for constitutive ectopic gene expression

An 8-day old seedling of a representative T1 generation transformant (dual 35S CaMV promoter driving the GUS reporter gene), showing constitutive ubiquitous expression throughout most tissues of the plant.
In planta expression of the uidA reporter gene using destination vectors for inducible gene expression

Heat-inducible vector

Heat shock induced (a, b and c) and non-induced (d) 5-day old seedlings showing GUS activity in induced dividing cells.

Estrogen-inducible vector
17-β-Estradiol induced (a and b) and non-induced (c and d) 8-day old seedlings of two independent transformants.

In planta expression of the uidA reporter gene using destination vectors for promoter-reporter (or native promoter-gene fusion) constructs

The expression pattern of the 35S CaMV promoter, recombined upstream of the gusA reporter gene, showing a representative example of constitutive expression in leaves of 28-day old transgenic plant and the flowers from the same plants. A and B are the T1 generation of one representative plant line.