The *ble* marker

The *ble* gene originates from the tallysomycin-producing actinomycetes species *Streptoalloteichus hindustanus* and encodes a small (13.7 kDa) protein conferring resistance to tallysomycin and related antibiotics including bleomycin, phleomycin and zeomycin. These glycopeptide antibiotics act by breaking down DNA: the BLE protein prevents this by binding the antibiotics with strong affinity.

The gene has been developed as a dominant selectable marker for both prokaryotes and lower and higher eukaryotes. The marker and the antibiotics are marketed by the French company CAYLA (http://www.cayla.com/). See CALYA's home page also for further details of the antibiotics and key references relating to the *ble* sequence, the protein's crystal structure and use of the marker in various organisms.

note: phleomycin is also available from Sigma, and zeomycin (trade name Zeocin^{TM}) is also available from Invitrogen (http://www.invitrogen.com/).

Development of a *ble* marker for *Chlamydomonas* nuclear transformation

The *ble* coding region was fused to the 5' and 3' regulatory regions of the *Chlamydomonas RBCS2* gene to create a marker (plasmid pSP108) that allows direct selection for phleomycin-resistant transformants.

This was reported in:

Stevens, D.R., Rochaix, J.-D. and Purton, S. (1996). The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*. Mol. Gen. Genet. 251, 23-30.

However, the transformation efficiency using this marker was low. We have therefore improved this by introducing *Chlamydomonas* intronic sequences into the *ble* gene and shortening the 5' *RBCS2* region. These modifications are described in:

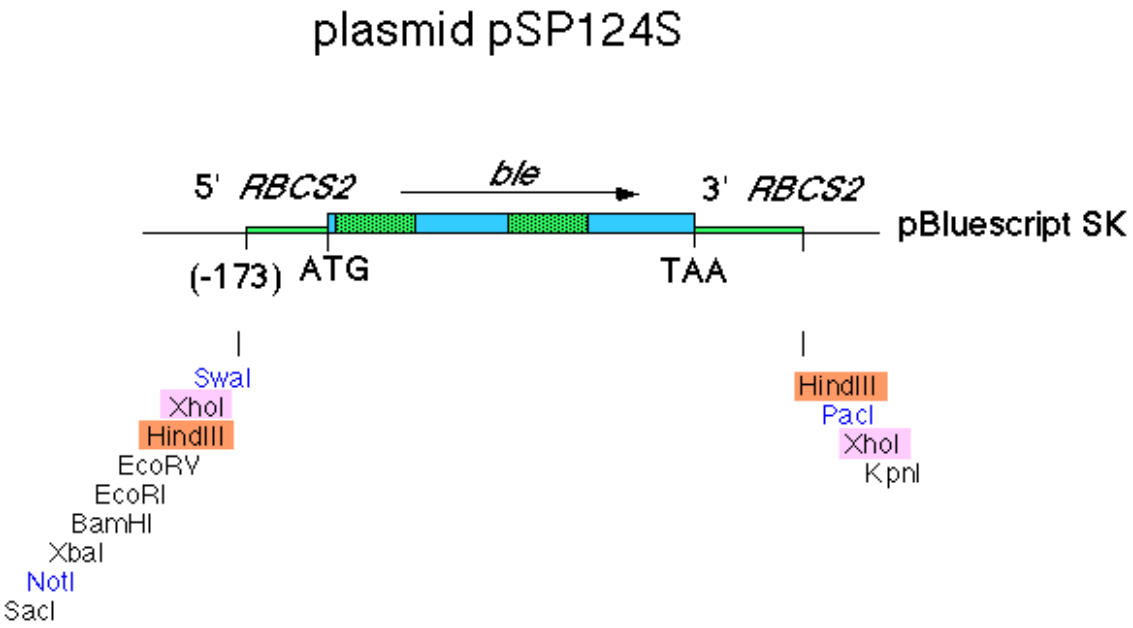
Lumbreras, V., Stevens, D.R. and Purton, S. (1998). Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. Plant J. 14, 441-448

The best version of the construct (pSP124) described in the paper then underwent some additional minor modifications to give pSP124S as described below.

pSP124S: the latest version of the *ble* marker

As shown in the diagram below, the marker comprises: i) the *RBCS2* promoter region extending from the -173 position (relative to the transcription start) to the translation start; ii) the *ble* coding region, into which has been inserted two copies of *RBCS2* intron 1 [one intron immediately downstream of the ATG, one in the middle of the coding

region]; iii) a 231 bp fragment containing the 3' untranslated region of *RBCS2*, including the putative polyadenylation signal. The cassette is 1.2 kb in size (can be excised using *Xho*I or HindIII) and is cloned into pBluescript SK-. Several rare RE sites (*Pac*I, *Swa*I, *etc.*) have been introduced at the ends of the marker to facilitate DNA analysis of transformants and the rescue of genomic flanking sequence.



The pSP124S plasmid is available from the Chlamydomonas Resource Center (https://www.chlamycollection.org/).

The DNA sequence of pSP124S

The construct has been completely sequenced. Presented below is the sequence of the coding strand (including the pBluescript polylinker - from *Sac*I to *Kpn*I). Coding sequence is shown in UPPERCASE.

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gagctccaccgcggtggcggccgctctagaactagtggatcccccgggctgcaggaattcgatatcaagcttatcgatac
cgtcgacctcgagatttaaatgccagaaggagcgcagccaaaccaggatgatgtttgatggggtatttgagcacttgcaa
cccttatccggaagccccctggcccacaaaggctaggcgccaatgcaagcagttcgcatgcagcccctggagcaggtgcc
ctcctgataaaccggccaggggggcctatgttctttacttttttacaagagaagtcactcaacatcttaaaATGGCCAGgt
gagtcgacgagcaagcccggcggatcaggcagcgtgcttgcagatttgacttgcaacgcccgcattgtgtcgacgaaggc
ttttggctcctctgtcgctgtctcaagcagcatctaaccctgcgtcgccgtttccatttgcagGATGGCCAAGCTGACCA
GCGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGAC
TTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATCAGCGCGGTCCAGGACCAGgtgagtcg
acgagcaagcccggcggatcaggcagcgtgcttgcagatttgacttgcaacgcccgcattgtgtcgacgaaggcttttgg
ctcctctgtcgctgtctcaagcagcatctaaccctgcgtcgccgtttccatttgcagGACCAGGTGGTGCCGGACAACAC
CCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACG
CCTCCGGGCCGGCCATGACCGAGATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGC
GTGCACTTCGTGGCCGAGGAGCAGGACTAAccgacgtcgacccactctagaggatcgatccccgctccgtgtaaatggag
gcgctcgttgatctgagccttgccccctgacgaacggcggtggatggaagatactgctctcaagtgctgaagcggtagct
tagctccccgtttcgtgctgatcagtctttttcaacacgtaaaaagcggaggagttttgcaattttgttggttgtaacga
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tcctccgttgattttggcctctttctccatgggcgggctgggcgtatttgaagcttaattaactcgaggggggggcccggt
acc

Transformation of *C. reinhardtii* with the *ble* marker

The method used in the Purton lab is as follows and is based on the original glass bead method of Karen Kindle:

• Grow the cells to mid-log phase (1 - 2 x 10^6 cells/ml) in TAP medium under continuous bright light (~90 µE/m2/s).

note: we have tested various cell-wall-deficient strains and have found that *cw10* mt– (obtained from the Chlamy stock center: CC-849) is a healthy strain that gives consistently good transformation rates and also mates reasonably well.

• Pellet the cells by spinning in a GSA rotor at 6,000 rpm, room temp, for 5 minutes.

• Discard the medium and resuspend the cells in fresh TAP by gentle pipetting, to a concentration of 2x10^8 cells/ml.

• Transfer 300 µl of this cell suspension to 5 ml test tubes containing ~0.3 g of 0.4mm diameter glass beads.

note: we buy our beads from B.D.H. We wash them several times in distilled water, and then in ethanol to remove any contaminants. The beads are then dried in an oven. They are dispensed into the tubes using a 'scoop' fashioned from the bottom of a microfuge tube heat-fused to a 1 ml pipette tip (I got frustrated trying to weigh the beads and sought a simpler solution!). The tubes are then loosely capped and autoclaved.

• Add 1 µg of linearised DNA (cut with *e.g. Kpn*I). Also set up a 'no DNA' control.

note: We have found that the merits of adding 5% PEG are dependent on the particular *cw* mutation - sometimes PEG improves transformant numbers, sometimes it has no effect. In the case of *cw*10, it appears toxic to the cells - so we don't add it.

• Vortex the cell/glass bead/DNA suspension for 15 seconds at top speed using a Vortex genie-2 (Scientific Industries).

• Transfer the cells to a 25 ml screw-top tube (we use sterile plastic tubes that fit into a bench-top centrifuge) containing 10 ml of TAP medium. Grow the cells overnight (~18 h) by shaking at 100 rpm to allow for recovery and expression of the *ble* gene.

• Pellet the cells by centrifugation for 5 minutes at 3,000 rpm.

• Gently resuspend the cells in 0.5ml TAP medium and then plate in 3.5 ml of TAP + 0.5% agar (the molten agar should be below 42°C and contain no antibiotic) onto TAP 2% agar supplemented with Zeocin @ 20μg/ml.

note: we always buy our antibiotic from CAYLA and have never had any problems in terms of selection (we obtain good numbers of transformants and never get any resistant colonies on our 'no DNA' plates). Furthermore, we have only ever used TAP as the growth medium. It may be that antibiotic levels may have to be altered when using preparations from other suppliers or in different media (pH and salt concentrations affect the potency of the drug).

• Allow the agar to set, invert the plates and leave in the light (~45 μE/m2/s) at ~22°C. Colonies are visible after 6-7 days.

We typically obtain ~500 colonies per plate using pSP124S.

FAQs

1. *Do you need to maintain transformants on the drug?*
No. Although we have not carried out a rigorous study, all the transformants we have tested show stable expression of *ble* in the absence of selective pressure and we have not observed the loss of the resistance phenotype in these strains.

2. *Can you use ble as an insertional mutagen?*
Yes. We have generated respiratory mutants and various pigment mutants, and others (*e.g.* Rachel Dent) have used it to generate photosynthetic mutants. We find that most of our transformants contain a single copy of *ble*, therefore genetic analysis is fairly straightforward - cross to a WT strain and test progeny for the co-segregation of Zm resistance and the mutant phenotype.

3. *Is the mutagenic nature of the drug a problem when generating insertional mutants?*
I don't think so. The cells are left to express the resistance gene prior to plating on the drug and the resistance levels obtained amongst the transformant populations are typically many times higher that the selection level (20 μg/ml). [See Lumbreras et al. paper]. Also, the affinity of the binding protein for the drug is remarkably strong so I doubt whether the drug ever sees the nuclear DNA in the transformants. So far, we have only carried out genetic analysis on three insertional mutants, but all three are tagged.

Saul Purton. June 2002.