Mike's Moss Transformation Protocol  
(Compiled from various sources, mostly Cove protocol)  Updated 8/18/2012

I. Preparation
A. 8.5% D-Mannitol Solution (8+ mL/ transformation + ~30 mL/ protoplast strain)
B. 10.28% D-Mannitol Solution (10 mL/ protoplast strain)
C. CaPW (8.5% D-Mannitol + 0.219% CaCl\textsubscript{2}•6H\textsubscript{2}O; autoclave) (10 mL/ protoplast strain)
D. 1% MES pH 5.6 (autoclave) (1 mL/ protoplast strain)
E. 1M MgCl\textsubscript{2} (autoclave) (<1 mL/ protoplast strain)
F. 1M Ca(NO\textsubscript{3})\textsubscript{2} (autoclave) (<1 mL/ protoplast strain)
G. 1M Tris•HCl pH 8 (autoclave) (50 µL/~20 transformations)
H. Autoclave 2.0 g PEG-6000 in a 15 mL tube (1 tube/~20 transformations)
I. PRMT medium BCD + 2.5 mM DAT, 8% D-mannitol, 0.4% Agar + 10 mM CaCl\textsubscript{2} (@50°C)
J. PRMB plates BCD + 2.5 mM DAT, 6% D-mannitol, 0.8% Agar, 10 mM CaCl\textsubscript{2} (@50°C)
K. Week-old moss chloronema grown on BCD+DAT + 0.6 mM CaCl\textsubscript{2} (± glucose)
L. 10 to 30 µg linearized plasmid DNA in <30 µL (Digest, extract, ethanol precipitate, dry)
M. BCD+DAT Medium (700 ml): 700 µl 500 mM MgSO\textsubscript{4}; 700 µl 616 mM KH\textsubscript{2}PO\textsubscript{4}/303 mM K\textsubscript{2}HPO\textsubscript{4} pH 6.5; 7 ml 1 M KNO\textsubscript{3}/4.5 mM FeSO\textsubscript{4}; 0.7 ml Trace element solution; 5.6 g Agar; 0.644 g diammonium tartrate; [±175 µg PABA ±350 µg thiamine] 1 mM CaCl\textsubscript{2}(@50°C)

II. Day 1
A. Prepare 2% Driselase Solution in 8.5% D-Mannitol 15 minutes at RT, Centrifuge 2500 g, Filter sterilize supernant.
B. Collect protonema to a sterile 50 ml tube, pipet away excess liquid, and add ~15 mL 8.5% D-Mannitol per plate of tissue.
C. Add 1/3 volumes of 2% Driselase Solution (0.5% final). Incubate 30 to 60 at RT with occasional gentle shaking (<2 hours). (Label and place cellophanes on plates during wait)
D. Start PEGT preparation: add 4.45 mL 8.5% D-mannitol 500 µL Ca(NO\textsubscript{3})\textsubscript{2} 50 µL Tris pH 8, to a sterile PEG tube. Dissolve at 65° then allow to cool completely before use.
E. Filter digested tissue through 70 µ mesh basket.
F. Pellet at 100-200 g for 4 minutes with no braking.
G. Resuspend in 10 mL CaPW. Repeat once but use 8.5% D-Mannitol.
H. Count protoplasts and prepare enough fresh MMM Medium
   Total # protoplasts = [# protoplasts in 1 mm\textsuperscript{2} (5x5) grid] x [# mL protoplasts] x 10\textsuperscript{4}
   MMM = 8.85 mL 10.28% D-Mannitol, 150 µL 1M MgCl\textsubscript{2}, 1 mL 1% MES pH 5.6.
I. Pellet as before and resuspend in MMM at 1.67x10\textsuperscript{6} cells/mL
J. Add <30 µL linearized plasmid DNA to 15 mL conical tubes (or round-bottom tubes)
K. Add 300 µL protoplasts
L. Add 300 µL PEGT (5 minutes)
M. 45°C for 5 minutes
N. 25°C for 10 minutes
O. Add 300 µL 8.5% D-Mannitol, repeat 4 additional times with ≥1 minute in between.
P. Add 1 mL 8.5% D-Mannitol, repeat 4 additional times with ≥1 minute in between.
Q. Pellet as before (if round-bottom tubes were used, transfer to conical tubes.)
R. Resuspend in 1 mL 8.5% D-mannitol.
S. Add 5 mL molten PRMT (45°C).
T. Dispense 2 mL per PRMB+cellophane plates.
U. Put in growth room

III. Day 5 or 6: Transfer cellophane to Selective BCD(±DAT) plates (grow 10-17 days)
20-50 µg/ml G418, 15-50 µg/ml Hygromycin, 50 µg/ml Zeocin (replace weekly), 100-150 µg/ml Gentamicin, 80 µg/ml Finale, 150 µg/ml sulfadiazine

IV. Day ~20: Move (or pick resistant protonemata) to non-selective media (grow 7-14 days)

V. Day ~34: Pick a few cells at 2-3 edges to selective media (grow 10+ days)

VI. Day ~48: Check insertion site PCR (5’ native/foreign, 3’ native/foreign, empty site)