Transformation Method for *Brassica napus* (and *B. oleracea*): A Cotyledonary Petiole Based System

This method is adapted from the *B. napus* system developed by M. M. Moloney, J.M Walker and K. K Sharma (1989) Plant Cell Reports 8: 238-242. Should you require any further information please contact us via e-mail at enquiries@bract.org

Seed sterilisation and germination

Surface sterilise seed in 100 % ethanol for 2 minutes, 15 % sodium hypochlorite (containing 1 drop of Tween20) for 15 minutes and rinse three times for 10 minutes in sterile distilled water.

Seed are sown on germination medium at a density of 20 seed per 90 mm petri dish, sealed with micropore tape and transferred to a 10^{o}C cold room overnight before being transferred to a 23^{o}C culture room under 16 hour day length of 70µmol m^{-2} sec^{-1} for 4 days.

***Agrobacterium* preparation**

Prior to inoculation *A. tumefaciens* strains are streaked out onto solid LB medium containing the appropriate level of selection. Plates are incubated at 28^{o}C for 48 hours, after which a single colony is transferred to 10 ml of Minimal A liquid medium, containing selection and transferred to a 28^{o}C shaker for 48 hours. A 50 µl aliquot of the resulting bacterial suspension is transferred to 10 ml of Minimal A liquid medium containing no selection and grown over night in a 28^{o}C shaker. Overnight suspensions of O.D$_{650}$ = 0.1 should be used for inoculations (dilutions made using Minimal A liquid medium).

Isolation and inoculation

After 4 days cotyledons are excised from the seedlings. This is carried out by gently holding the base of the cotyledon with forceps, and slicing through the petiole just

above the meristem region using a sharp scalpel blade. Care should be taken not to include any meristematic tissue, which does not transform easily and will regenerate "escape" shoots rapidly on selection medium. Once excised the cotyledons are immediately placed onto co-cultivation medium in 90mm petri single vent dishes ensuring that at least 1-2mm of the cut petiole is implanted in the agar.[1] Ten explants are established on each plate.

Once all explants have been isolated, they are inoculated by dipping briefly into an *Agrobacterium* solution (described above) ensuring that only the cut end of the petiole is immersed. The cotyledons are then returned to co-cultivation plates and sealed with micropore tape before being transferred to the culture room under scattered light ($40\mu molm^{-2}sec^{-2}$) for 3 days.

Selection

After co-cultivation, cotyledons are transferred to selection medium in 90 mm diameter by 20mm deep tissue culture dishes.[2] The plates are resealed with tape and returned to the culture room under scattered light.

N.B Always add controls: In each experiment it is recommended that you have 2 non-transformed controls, where you incorporate all constituents except selection (i.e. no kanamycin), containing

(i) Cotyledonary petioles with *Agrobacterium* inoculation and
(ii) Cotyledonary petioles without *Agrobacterium* inoculation

For effective transformation the cut ends should initiate callus after the first couple of weeks[3], and shoots develop from this callus after four to ten weeks. Green shoots are

[1] This is a good way to determine when cotyledons are the right size/age. If cotyledons have just turned green and can easily be excised (the two cotyledons come away freely without any meristematic tissue) then they are of the right size and age. The correct size will depend on your genotype, culture room conditions etc. If excised too late, cotyledons will just expand rapidly when on regeneration media and not form shoots.

[2] At this stage petioles will have extended and it should be possible to embed them into selection media so that the cotyledons themselves are clear of the media – therefore preventing dieback.

excised and transferred to 100 ml jars containing Gamborgs B5 medium (Gamborg et al. 1968), 1 % sucrose, 0.8% Phytagar, plus selection. Where dense multiple shoots are isolated further subculturing after shoot elongation should be made to ensure a main stem is isolated, thus reducing the likelihood of escapes and producing multi - stemmed plants when transferred to the glasshouse. Shoots are maintained on Gamborgs B5 medium until roots develop. Plantlets are then transferred to sterile peat pots (Jiffy No.7) to allow further root development, before being transferred to the glasshouse.

Media

Germination

Full strength Murashige and Skoog Plant Salt Base (xg/l depending on supplier's recommendation).
Phytagar (Gibco BRL) 8g/l
3 % Sucrose
pH 5.7

Add after autoclaving
Myo-inositol 100mg/l
Thiamine 10mg/l
Nicotinic acid 1mg/l
Pyridoxine 1mg/l

Co-cultivation

As germination media but incorporating:
Benzyl amino purine 2mg/l (this can be added before autoclaving)

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[3] Not all genotypes regenerate via a callus phase, but swelling of the cut base should be seen after a week. If extreme blackening to the petiole base occurs, it is likely the genotype you are working with will be difficult (if not impossible) to transform!

Selection

As co-cultivation but including

25mg/l kanamycin * (if using the nptII gene)

500mg/l carbenicillin **

* levels may vary depending on genotype

** check the strain of Agrobacterium you are using is not resistant to carbenicillin (e.g. when using AGL1, replace carbenicillin with Timentin)

Gambourgs B5 Medium

Full strength Gambourgs B5 (xg/l depending on supplier's recommendation)

1% Sucrose

Phytagar 8g/l

PH 5.7

Plus selection

LB medium

Yeast Extract	5 g/l
NaCl	10 g/l
Tryptone	10 g/l
Bactoagar (Difco)	15 g/l

Minimal A medium: To make 1L

50 ml Minimal A Salts

50 ml Minimal A Buffer

10 ml 20% Sucrose

1 ml 1M $MgSO_4$

890 ml SDW

All components should be autoclaved **separately** before combining, with the exception of $MgSO_4$, which is unstable at high temperatures and therefore should be filter sterilised.

20X Minimal A Salts

$(NH4)_2SO_4$	20 g/l
Sodium Citrate	10 g/l

20X Minimal A Buffer

K_2HPO_4	274 g/l
KH_2PO_4	90 g/l

Diary of Events

Day 1 (a.m.)

Surface sterilise seed and sow onto germination medium (2g of seed is typically used to produce 500 explants).

Transfer plates to the cold room over night.

Day 2 (a.m.)

Transfer plates to the culture room.

Day 2 (p.m.)

Single colony of *Agrobacterium* to 10ml Minimal A liquid medium plus selection, transfer to 28^{o}C shaker. [Alternatively if you have reliable glycerol stocks, add 50ul to 10ml Min A plus selection]

Day 4 (p.m.)

Transfer 50µl of *Agrobacterium* (from day 2) to 10ml Minimal A liquid medium with no selection.

Day 5 (a.m.)

Isolate explants and inoculate with *Agrobacterium* (diluted to $O.D_{650}$ = 0.1). If seedlings are too small leave for another 24hrs before isolating.

Day 8

Transfer explants to selection media.

Other dates

- Well defined shoots that develop within the first 2 weeks are likely to have resulted from residual meristematic tissue, not properly avoided during explant isolation. These shoots are unlikely to be transformed.

- Explants are transferred to fresh selection medium after 3 weeks. White escape shoots are removed during subculture and discarded.

- After 4 to 5 weeks and onward, green shoots should develop. Isolate and transfer to shooting medium. Following shoot elongation, a further subculture is recommended to ensure single main stem plantlets are produced.

- Shoots are left to develop roots prior to transferring to sterile peat pots. After 2 weeks in soil they can be transferred to the glasshouse, where they should be maintained under propagators for the first week.