Brassica Transformation

The following protocol outlines an *A. tumefaciens*-mediated transformation method for *B. oleracea* using 4-day-old cotyledonary explants and a model *B. oleracea* doubled haploid genotype, AG DH1012. After only 3 weeks on kanamycin selection the first transgenic shoots can be isolated. Transformation efficiencies in the region of 10-25% are typically achieved. For researchers wishing to use their own plant genotype, we highlight the tissue culture phenotypes that are conducive to efficient transformation.

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1. Introduction

The production of transgenic plants as a research tool for testing gene function is now a routine procedure for many model species, such as *Arabidopsis*, tobacco and rice. Plant transformation is enabling researchers to introduce genes, with known function from the model plants, into crop plants. It is also increasingly used to introduce homologous sequences from the crops, back into the crops, to either over express or silence native genes. A better understanding of gene function in crops will facilitate the transfer of knowledge of plant systems from model species and support targeted selection of key traits in plant breeding programmes.

Transformation of *Brassica oleracea* (e.g. cabbage, broccoli, kohl rabi, cauliflower, Brussels sprouts and kale) using *Agrobacterium tumefaciens* has been reported by a number of groups (***1-7***). Despite considerable advances in methodologies over recent years, the routine transformation of *B. oleracea* is still hindered by genotype restrictions, with some genotypes remaining recalcitrant to transformation. For gene testing to become a routine procedure it is important to identify easy to

transform genotypes, with reproducible and reliable transformation efficiencies, that respond well when handled by different users (***7-8***, *see also* **Note 1**).

We have identified a number of key factors affecting transformation that are highly genotype dependent; these include susceptibility to *Agrobacterium* and *in vitro* tissue culture response (***9-10***). These studies have shown a number of stages within the transformation process to be under strong genetic control, suggesting that altering the tissue culture conditions alone will have a limited effect on transformation and regeneration efficiencies of recalcitrant material. This has led to a better understanding of why some genotypes can be transformed, whilst others remain recalcitrant and has enabled the screening of germplasm to identify high throughput candidate genotypes for routine transformation.

In this document we describe a method for the transformation of *B. oleracea*; this protocol can also be applied for transformation of *B. napus* (e.g. oilseed rape/canola). The accompanying notes highlight tissue culture phenotypes that are conducive to efficient transformation, and those that are likely to remain recalcitrant (*see* **Note 1**).

2. Materials

2.1. Plant Culture Media and Components

1. MS Basal medium: 4.3 g/L Murashige and Skoog (***11***) (MS) salts (Duchefa-M0221 without vitamins), 30g/L Sucrose, pH 5.7, 8g/L phytagar (Duchefa-P1003). Autoclave at 120^{o}C for 20 minutes (*see* **Note 2**).
2. BAP (6-Benzylaminopurine (Sigma – B-9395)) stock solution: 4mg/mL. Prepare by dissolving the powder in a few drops of 1M NaOH. Make to final volume with sterile distilled water (SDW). Store at 4^{o}C.
3. Vitamin stocks: 10g/L *myo*-inositol (Sigma I-3011), 10mg/L Thiamine-HCL (Sigma T-3902), 1mg/L Pyridoxine (Sigma P-8666), 1mg/L Nicotinic acid (Sigma N-0765). All vitamins are

made up in SDW, filter sterilised and stored individually at 4°C, with the exception of *myo*-inositol which is stored at room temperature.

4. Kanamycin monosulphate (Sigma K1377) stock solution: 100mg/mL. Prepare by dissolving 1g of powder in 10mL of SDW. Filter-sterilise and store in 1mL aliquots in sterile 1.5mL tubes and store at -20°C. (*see* **Note 3**).
5. Timentin (Sold as Ticarcillin disodium/clavulanate potassium: Duchefa T0190) stock solution: 160mg/mL. Filter-sterilise and store at -20°C in 1mL aliquots.
6. Germination medium: 1L MS basal medium, plus 1mL of each of the 4 vitamin stocks added prior to pouring. One litre typically pours 30 petri-dishes (15 x 90mm).
7. Co-Cultivation medium: 1L MS basal medium plus 2mg/L BAP (0.5mL of 4mg/L BAP stock solution added prior to autoclaving); prior to pouring add 1mL each of the 4 vitamin stocks. One litre typically pours 30 petri-plates (15 x 90mm).
8. Selection medium: As Co-Cultivation medium, with the addition of 15mg/L kanamycin (150µL of Kanamycin stock) and 160mg/L Timentin (1mL of Timentin stock), added prior to pouring. One litre typically pours 20 petri-plates (20 x 90mm). Kanamycin is not included in the control plates (a single control plate can be poured/L before kanamycin is added to the medium for the other plates). *See also method* ***section*** **3.4** ***part*** **2**.
9. Gamborg's B5 medium (***12***): 3.1g/L Gamborg's B5 Salts (Duchefa-G0209), 10g/L Sucrose, pH5.7, 8g/L Phytagar (Duchefa-P1003). Autoclave at 120°C for 20 minutes. Prior to pouring add filter-sterilised kanamycin 25mg/L (50mg/L for later steps) and Timentin 160mg/L.
10. Sterile peat: Sterile peat pots (Jiffy No.7) are placed into Magenta pots (Sigma), and soaked in water until fully expanded. Excess water is poured off and Magentas autoclaved at 120°C for 20 minutes.

2.2 *Agrobacterium* culture media

1. LB Medium: 5 g/L Yeast Extract (Duchefa Y1333), 10g/L NaCl, 10g/L Tryptone and 15g/L Bactoagar (Difco).

2. MGL medium: 5g/L Tryptone, 2.5g/L Yeast, 100mg/L NaCl, 5g/L Mannitol, 1g/L glutamic acid, 250mg/L KH2PO4, 100mg/L MgSO4, 1ug/L Biotin. pH7. Dispense in 10ml aliquots, autoclave and store at RT
3. Liquid MS: as germination media but without the agar. Dispense into 10ml aliquots and store at RT.

Alternative media

4. Minimal A liquid Medium (for 1L): 50 ml 20X Minimal A Salts (20g/L $(NH4)_2SO_4$ and 10 g/L Sodium Citrate), 50 mL 20X Minimal A Buffer (274 g/L K_2HPO_4 and KH_2PO_4 90 g/L), 10 mL 20% Sucrose, and 1 mL 1M $MgSO_4$ made up to 1L with sterile SDW. All components should be autoclaved separately before combining, with the exception of $MgSO_4$, which should be filter sterilised as it is unstable at high temperatures. Store as 100ml aliquots in sterile Duran bottles at room temperature.

   [Liquid Minimal A media can be used for the overnight cultures (without selection); and at the inoculation stage. Some genotypes prefer this. Some *Agrobacterium* also grows better in this for ALL stages, especially fast growing cultures where bacteria may clump if a rich media like LB is used].

2.3 Seed source

AG DH1012 is the *B. oleracea* genotype described in this protocol. AG DH1012 is a doubled haploid genotype from the *Brassica oleracea* ssp *alboglabra* (A12DHd) and *B. oleracea* ssp *italica* (Green Duke GDDH33) mapping population (***13-14***). Seed can be obtained from Dr. Penny Sparrow,

BRACT group, John Innes Centre. A cost recovery charge will be issued per 1g of seed supplied, and seed supplied under an MTA.

3. Methods

The protocols described below are applicable for *B. oleracea* and *B. napus* transformation, and are based on a previously reported method for *B. napus* transformation (***15***).

3.1 Seed sterilisation and germination

1. Seeds are surface sterilised in 100% ethanol (BDH) for 2 minutes, 15% sodium hypochlorite (Fluka 71696) plus 0.1% Tween-20 (Sigma P-9416) for 15 minutes and rinsed three times for 10 minutes in sterile distilled water (*see* **Note 4**).

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

3.2 *Agrobacterium* preparation

1. Prior to inoculation, *A. tumefaciens* strains (*see* **Note 5**) are streaked out onto solid LB medium containing the appropriate level of selection. Plates are incubated at 28^{o}C for 48 hours.
2. A single colony (or alternatively 50 µL of a glycerol stock) is transferred to 10 mL of LB liquid medium, containing selection and transferred to a 28^{o}C shaker for 48 hours.
3. A 50 µL aliquot of the resulting bacterial suspension is transferred to 10 mL of MGL liquid medium containing selection and grown over night in a 28^{o}C shaker.

4. Over night cultures are spun down @ approximately 3,000 rpm for 5 minutes at R.T and resuspended in 10ml liquid MS media, and left on a shaker at RT to recover while explants are isolated.

$O.D_{650}$ = 0.1 to 0.3 are used for inoculations (dilutions made using liquid MS medium). [Alternatively Minimal A liquid media can be used for all/ part of the growth stages; particularly useful for fast growing bacterial cultures that are prone to clumping].

3.3 Explant isolation, inoculation and co-cultivation

1. Cotyledons are excised from 4-day-old seedlings (**see also pictorial protocol**, *see* **Note 6**). 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 (**pictorial protocol**). Care should be taken not to include any meristem tissue, which does not transform easily and will regenerate "escape" shoots rapidly on selection medium.
2. Once excised, cotyledons are immediately placed onto co-cultivation medium in petri dishes (20 x 90mm) ensuring that at least 1-2mm of the cut petiole is implanted into the agar (*see* **Note 7**). Ten explants are established on each plate.
3. Once all explants have been isolated they are inoculated, one at a time, by dipping briefly into an *Agrobacterium* suspension (Section 3.2) ensuring that only the cut end of the petiole is immersed.
4. The cotyledons are then returned to co-cultivation plates and sealed with micropore tape before being transferred to a $23^{o}C$ culture room under 16 hour day length of $40\mu mol\ m^{-2}sec^{-2}$ for 72 hours.

3.4 Selection

1. After co-cultivation, cotyledons are transferred to selection medium in deep petri dishes (20 x 90mm) (*see* **Note 8**). Plates are sealed with micropore tape and returned to the culture room under scattered light.

   In each experiment it is recommended that 3 control plates (without kanamycin) are also included. One plate containing explants inoculated with *Agrobacterium,* one with explants not inoculated, but dipped in the suspending media, and one plate where explants are transferred straight to co-cultivation media without dipping. Look at shoot regeneration from each of your controls, if there is a significant difference between them, you may need to optimise *Agrobacterium* concentration and/or kanamycin levels; in rare cases the suspension media itself (in the absence of *Agrobacterium*) may also have a negative effect on shoot regeneration – alternative media should then be investigated.

2. Explants are transferred to fresh selection medium after 3 weeks, with kanamycin levels increased to 25mg/l at this stage. During this subculture, any white escape shoots are removed. For effective transformation the cut ends should initiate callus after the first couple of weeks (*see* **Note 9**), and shoots develop from this callus after three to five weeks on selection - **Fig. 3.**

3.5 Shoot isolation

1. When using AG DH1012 the emergence of transgenic (green) shoots can be seen after just 2 weeks. After 3-5 weeks transgenic shoots can be isolated.

2. Green shoots are excised and transferred to 100 mL jars (75 x 50mm) containing 25mL of Gamborgs B5 medium, 25mg/L kanamycin and 160mg/L timentin. When shoots are initially isolated, it is often not possible to isolate a single meristem. Where multiple shoots subsequently develop, further subculturing should be carried out to ensure a single-

stemmed shoot is isolated. This will reduce the number of multi-stemmed plants transferred to the glasshouse and the likelihood of escapes/chimeras going through. Such plants will complicate the molecular analysis of the primary transgenics.

3. Shoots are maintained on Gamborgs B5 medium (at an increased kanamycin concentration of 50mg/l), and at 23^{o}C under 16 hour day length of 40μmol $m^{-2}sec^{-2}$, until roots develop (*see* **Note 10)**.

4. After root elongation (to approximately 20 mm in length) plantlets are transferred to sterile peat pots to allow further root growth before being transferred to the glasshouse.

3.6 Transfer of plants to greenhouse

1. Plants are transferred to soil (John Innes No.2 commercial compost) and maintained under shade within a propagator for the first week (a large plastic bag tied up at the top can be used). This ensures that plants gradually adjust to reduced humidity and increased light intensity. Glasshouse light conditions; day/ night temperatures of 18/ 12 ± 2ºC, 16 h day length, with supplementary lighting (5.35 x 102 μE m-2 s-1). Plants were fed weekly with a 2:1:1 NPK fertiliser

2. AG DH1012 is a rapid cycling genotype and should flower approximately 6-8 weeks after transfer. This line is also highly self compatible and readily sets seed (approx. 8-10 weeks after bud break) without the need for hand pollination.

3. When in bud, plants are covered with clear, perforated bags (Cryovac (UK) Ltd) to prevent cross-pollination and shaken daily once in flower to encourage seed set. Pods are allowed to dry on the plant, before being threshed. Seed yield is generally high (>2-4g/plant).

4. Notes

1. In *B. oleracea,* tissue culture phenotypes associated with transformability have been identified (***7***). One of the critical factors for transformation success is the absence of tissue culture blackening (**Fig. 1A, 1D**). Genotypes that regenerate shoots from more than 50% of cotyledonary petioles, through a distinct swelling or callus phase and in the absence of blackening were subsequently found to have higher transformation efficiencies. Transformation efficiency was further improved in genotypes with a high susceptibility to *Agrobacterium*. These phenotypic markers have successfully been applied to *B. napus* material in order to select genotypes amenable to transformation (***8***).
2. Medium can be allowed to set and microwave to re-melt if not poured immediately.
3. Alternative selective agents (e.g. hygromycin, phosphinothricin etc) have not been tested with this protocol, kanamycin is therefore recommended.
4. Typically, 2g of seed is enough for a 500 explant transformation (depending on seed quality and germination rate). Petri-dishes (15 x 50 mm) are ideal for sterilising seed in. Allowing the seed to air dry will make seed sowing easier. Seed should be placed onto the surface of the medium and not embedded.
5. AGL 1 is the *Agrobacterium tumefaciens* strain routinely used with the pBRACT vectors. LBA 4404, EHA 101 and EHA 105 have also been used successfully for Brassica transformation.
6. When setting up a large experiment, change the scalpel blades frequently. Flame sterilising will blunt the blade over time. The cut surface of the petiole base is the target tissue for transformation and regeneration. Petiole tissue that is torn or damaged tends not to respond well in culture.
7. 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 (see also **pictorial protocol**).

The correct size will depend on your genotype, culture room conditions etc. If excised too late, cotyledons will expand rapidly when on regeneration media and not produce shoots.

8. At this stage petioles will have extended and it should be possible to embed them into selection medium and ensure that the cotyledonary lamella is clear of the medium, therefore preventing dieback.
9. 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 that the genotype you are working with will be difficult (if not impossible) to transform (**Fig. 1A and 1C**).
10. Preliminary molecular analysis (i.e. PCR analysis) can be carried out while shoots are still *in vitro.*

Further illustrative photographs of the transformation of AG DH1012 can be seen on the Biotechnology Resources for Arable Crop Transformation (BRACT) website, www.bract.org .

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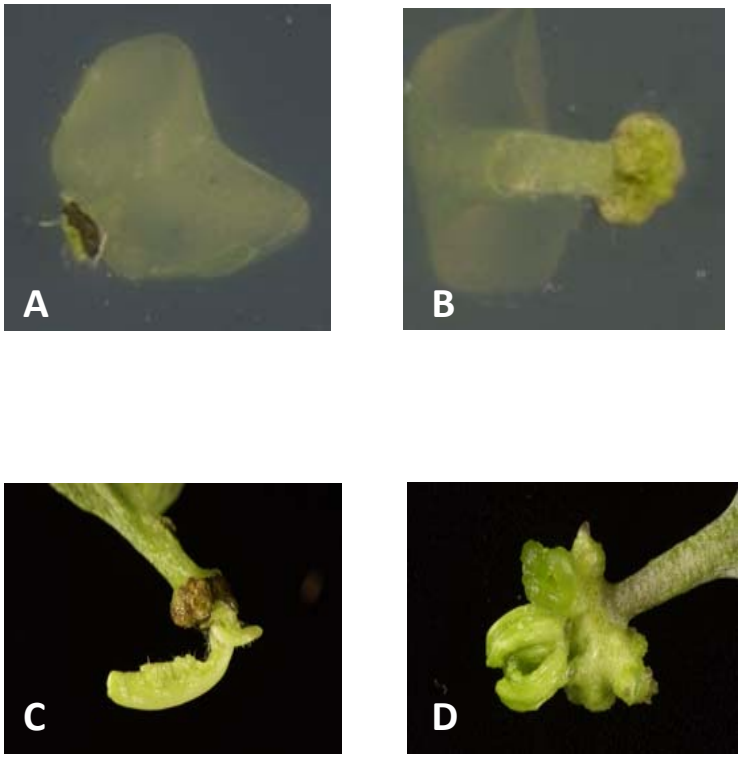

Figure 1:

Cotyledonary petioles from 4 separate genotypes of *B. napus* photographed *in vitro* after 16 days in culture: Explant A) shows slight swelling to petiole base and the presence of tissue culture blackening; in explant B) early callus formation to petiole base is observed and tissue culture blackening is absent. Shoot regeneration via C) a direct mode and associated with tissue culture blackening and D) via an indirect callus mode and in the absence of tissue culture blackening.