Chapter

Brassica oleracea Transformation

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Abstract

With easier access to genome sequences and genome editing technology over the last few years, transformation technologies are routinely being used now as a research tool for elucidating gene function. In this chapter, we outline a simple *A. tumefaciens*-mediated transformation method for the diploid brassica species *B. oleracea*, using a doubled haploid spring line called AG DH1012. This genotype has become our model genotype for training purposes and for most of our routine work, with a transformation efficiency of 25% (no. independent shoots/no. explants). This easy-to-follow protocol has made for a 100% technology transfer success rate to other researchers and laboratories around the globe. In this chapter, we detail our method using 4-day-old cotyledonary explants, with a step-by-step guide and supplemented with an online video to show the explant and subsequent shoot isolation stages. The video helps to remove any ambiguity that written protocols sometimes have. Primary transgenics ready for DNA extraction and early molecular analysis can be generated within 5–7 weeks from explant isolation.

Keywords: *Brassica oleracea*, *Agrobacterium tumefaciens*, cotyledon, transformation, CRISPR, gene function, AG DH 1012

1. Introduction

The genus *Brassica* is known not only for its agricultural oilseed crops but also for the diverse array of horticultural crops it has to offer. When GM plant technologies first emerged in the early 1980s, with the first publication being tobacco in 1983 [1], it was not long before the success followed in *Brassica* with reports by the late 1980s/early 1990s for all six of the major economically important species; *B. rapa* [2], *B. oleracea* [3], *B. nigra* [4], *B. napus* [5], *B. juncea* [6] and *B. carinata* [7]. Yet despite the considerable advances in methodologies that followed over the years [8–11], the routine transformation of *Brassica* remains highly genotype dependent, with some genotypes simply remaining recalcitrant to transformation. Several key factors affecting the *Brassica* transformation efficiency have been identified, including susceptibility to *Agrobacterium* [12] and *in vitro* tissue culture responses [13]. This has enabled us to screen large numbers of genotypes to identify the ones that fit our protocols, rather than tailoring methods for individual genotypes [14, 15]. For gene testing to become a routine procedure, it is important to identify the easy-to-transform genotypes, with reproducible transformation efficiencies, that respond well when handled by different users. This is especially important in the era of genome editing, which to date is still heavily reliant on the tissue culture transformation approaches in most cases. The frequency of genome edits is still relatively low in primary transgenics and

therefore requires the generation and screening of large numbers of primary transgenics, to maximise the potential of identifying edits within the primary generation. The transformation protocol described in the current chapter was used in the first published report of CRISPR genome editing in *Brassica* [16], and methods associated with screening for CRISPR/Cas9 edits in this genotype are detailed in [17]. Since 2015, there has been a steady flow of publications using CRISPR in *Brassica*. The use of transformation/CRISPR as a tool for genetic improvement in *Brassica* has recently been reviewed [18]. The AG DH1012 *B. oleracea* spring genotype, detailed in this chapter, has a broccoli type phenotype and was recently used in a study to generate MYB28 knock outs, which were subsequently grown under field conditions and were the first CRISPR/Cas9 edited plants to enter field trials in the UK, regulated under the EU GMO Directive [19].

The current era of genome editing, and other new plant breeding technologies, to help address issues of climate change, sustainability and food security is an exciting time. For some countries simple edits, which result in a product indistinguishable to conventional mutagenesis, will see them freed from the regulatory burden associated with GMOs, and this will help to rapidly advance plant breeding. However, for Europe and other regions, the technology remains predominantly a research tool for helping to increase our understanding of gene function, as such easily assessable transformation methods and resources provide a valuable resource to researchers.

While access to written protocols is essential for the successful transfer of technology to different users, seeing the protocol in real time is invaluable as it removes a lot of the subjective interpretation of a written protocol. Within the methods section we provide a link to our YouTube video which shows the explant isolation and later shoot isolation steps of the protocol.

2. Transformation method

The protocol described below has been optimised for *B. oleracea* genotype AG DH1012 but has successfully been applied to many other *B. oleracea* genotypes and *B. napus* (using a slight higher BAP concentration for *B. napus* of 3.75 mg/L). This protocol is based on a previously reported method for *B. napus* transformation [5], using 4-day-old cotyledonary petioles as the explant choice. A link to a video showing aspects of the transformation procedure can be found here (https://www.youtube.com/watch?v=cbtpCtc2lw0) or by Google search 'Brassica Transformation' YouTube John Innes Centre. Details of the stock solutions and media components used are detailed after the method, in Section 4.

2.1 Plant material

AG DH1012 is the *B. oleracea* genotype described in this chapter. AG DH1012 is a doubled haploid genotype from the *Brassica oleracea* ssp. *alboglabra* (A12DHd) crossed with *B. oleracea* ssp. *italica* (Green Duke GDDH33) mapping population [19]. AG DH1012 is a spring type and self-compatible. Seeds can be obtained from Dr Penny Hundleby from the Biotechnology Resources for Arable Crop Transformation group, at the John Innes Centre, UK. Cost recovery charges, for generating the seed, will apply.

2.2 Seed sterilisation and germination

In a flow bench, seeds are surface sterilised in 75% ethanol for 2 min followed by a 15% bleach sterilising solution (15 mL sodium hypochlorite [Sigma-Aldrich supplied at 10% RT] added to 85 mL distilled water with a drop (0.1%) of surfactant

Tween-20). Typically, 2 g of seed is used per transformation, and sterilised in a 5-cm deep petri dish, or similar, and shaken on an orbital shaker for 15 min. Seeds are rinsed three times with sterile distilled water to ensure no bleach remains and allowed to air dry before sowing. Seeds are placed (not embedded) onto germination medium at a density of 20–25 seeds per 90 mm petri dish and sealed with a micropore tape. Plates are incubated at 22°C culture room under high intensity (full light) with a 16-h photoperiod length of 70 μmol/m^2/s for 4 days (i.e. sow seed on a Monday to transform on a Friday).

2.3 *Agrobacterium* preparation and preparation of inoculum

- *A. tumefaciens* strains containing the appropriate plasmid(s) with the gene(s) of interest are streaked out onto the solid LB medium containing the appropriate level of selection. Plates are incubated at 28°C for 48 h. We routinely use *A. tumefaciens* strain AGL1 [20], see also Note 1.

- A single colony is transferred to 10 mL of a liquid LB medium containing selection and transferred to a 28°C shaker at 200 rpm for 48 h. After this stage, standard inoculums can be prepared for later use or proceed as follows.

- 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°C shaker.

- Pellet the over-night cultures by centrifugation at approximately 3000 rpm for 5 min and resuspend the pellet in a liquid MS medium, dilute to an OD_{600} = 0.1–0.3.

- Alternatively, we make up 'standard inoculums' from the resulting LB cultures; using 2 mL of culture to 2 mL of 50% glycerol. Gently mix by inverting the tube upside down, several times, aliquot 200 μl in each 0.5 μl tubes and store at −80°C until required. To use, 50 μl of a standard inoculum is added to 10 mL of MGL media without selection and grown overnight in a 28°C shaker at 200 rpm.

- Dilute the overnight suspension with liquid MS media to an OD_{600} = 0.1–0.3. Typically, 1 mL of the overnight culture with 5 mL of MS liquid will give an OD within this range.

2.4 Explant isolation, inoculation and co-cultivation

Cotyledons are excised from 4-day-old seedlings by gently holding the base of the cotyledon with forceps (Dumont No 24/45) and slicing across the petiole just above the meristematic region using a sharp scalpel blade (surgical blades No. 11) aiming for a petiole length of approximately 1–2 mm, see **Figure 1**. Avoid taking any meristematic tissue as it does not transform easily and will regenerate 'escape' shoots rapidly on the selection medium.

Explants are immediately placed onto co-cultivation medium in petri dishes (20 × 90 mm) ensuring that at least 1–2 mm of the cut petiole is implanted into the agar. Place at a density of approximately 10 explants/plate. Once all explants have been isolated, explants are then removed from the media individually and dipped briefly into an *Agrobacterium* suspension, ensuring only the cut end of the petiole is immersed (see video). Two control plates are set up per experiment, each with 10 explants. For one plate, explants are inoculated, and for the other plate, explants are not inoculated with *A. tumefaciens*.

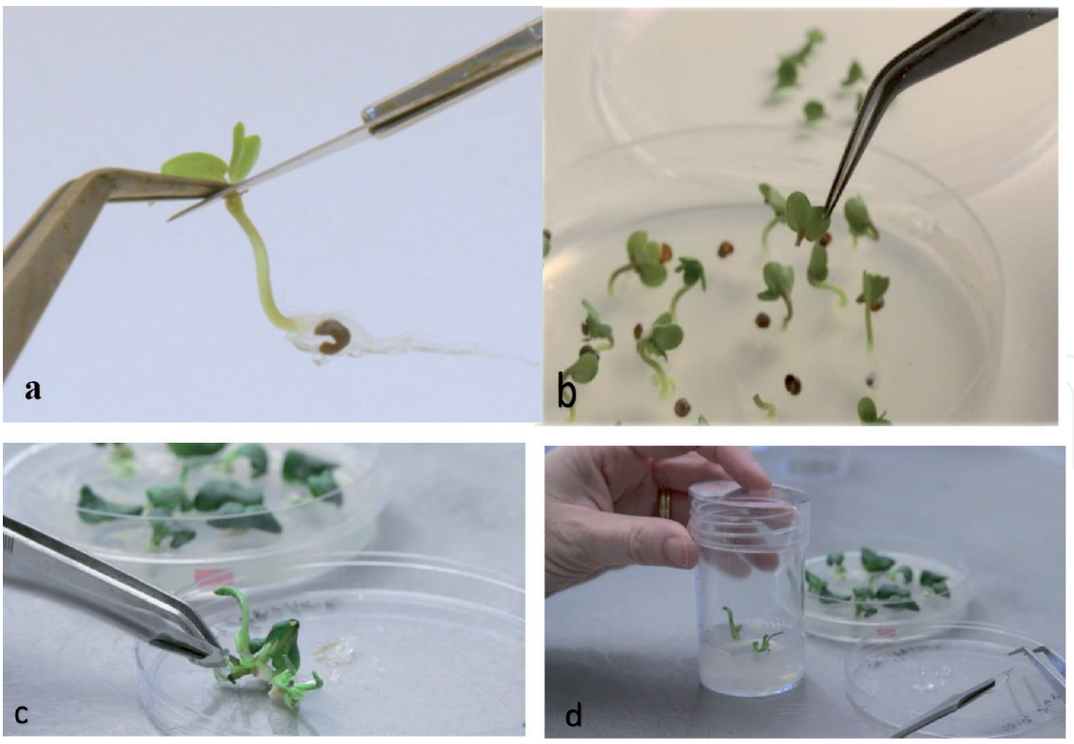

Figure 1.
(a) Explant isolation from 4-day-old seedlings. Forceps are used to hold the seedling without pressure and used as a guide to run the scalpel blade down. (b) Cotyledons with approximately 1–2 mm of petiole are used as explants. The petiole base is later inoculated by dipping briefly in Agrobacterium*. (c) Putative transgenic green shoot forming at approximately 4 weeks from a small callus at the petiole base; (d) shoots are isolated and transferred to rooting medium.*

All cotyledons are then returned to co-cultivation plates and sealed with micropore tape and transferred to a 22°C culture room with a 16-h photoperiod of 40 μmol/m^2/s for 72 h.

2.5 Selection

- After co-cultivation, cotyledons are transferred to selection medium in deep petri dishes (20 × 90 mm). Plates are sealed with a micropore tape and returned to the culture room under scattered light.
- For each experiment, two control plates (as selection plates but with no kanamycin) are established; one contains 10 explants that have not been inoculated with *Agrobacterium*, and the other 10 explants that have been inoculated.
- Explants are transferred to fresh selection medium after 3 weeks. During this subculture, any white shoots (escapes) are removed.

2.6 Shoot isolation

- When using AG DH1012, the emergence of transgenic (green) shoots can be seen after just 3 weeks. After 3–5 weeks, transgenic shoots can be excised and transferred to 100 mL jars (75 × 50 mm), containing 25 mL of Gamborgs B5 medium, containing 25 mg/L kanamycin and 160 mg/L Timentin, and maintained at 22°C under 16-h day length of 40 μmol/m^2/s. In some cases, further subculture maybe needed to 'thin' out multiple shoots to ensure a single-stemmed shoot is allowed to root. 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.

- After root elongation (to approximately 20 mm in length), plantlets are transferred to sterile peat pods (Jiffy No. 7) in magentas (Stratlab Laboratories Supplies) SPL Life Sciences, (Cat no 451-AA-002/004) to allow further root growth (approx. 2 weeks) before being transferred to the glasshouse.

3. Transfer of plants to the glasshouse and seed generation

- Plants are transferred to soil and maintained under shade within a propagator for the first week. This ensures that plants gradually adjust to reduced humidity and increased light intensity. Glasshouse light conditions; day/night temperatures of 18/12 ± 2°C, 16 h day length, with supplementary lighting (high-pressure sodium lamps with an average bench reading of 200 μmol/m^2/s). Plants are fed weekly with a 2:1:1 NPK fertiliser.

- 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 (approximately 8–10 weeks after bud break) without the need for hand pollination (see **Figure 2**).

- When in bud, plants are covered with clear, perforated 'bread' bags (Cryovac (UK) Ltd), to prevent cross-pollination, and shaken daily once in flower to encourage the seed set. Pods are allowed to dry on the plant before being threshed. Seed yield is generally high (>4 g/plant for wild type plants, or subsequent transgenic plants sown from seed; however, seed yield can often be reduced in the primary transgenics to in the region of 0.5–4 g/plant).

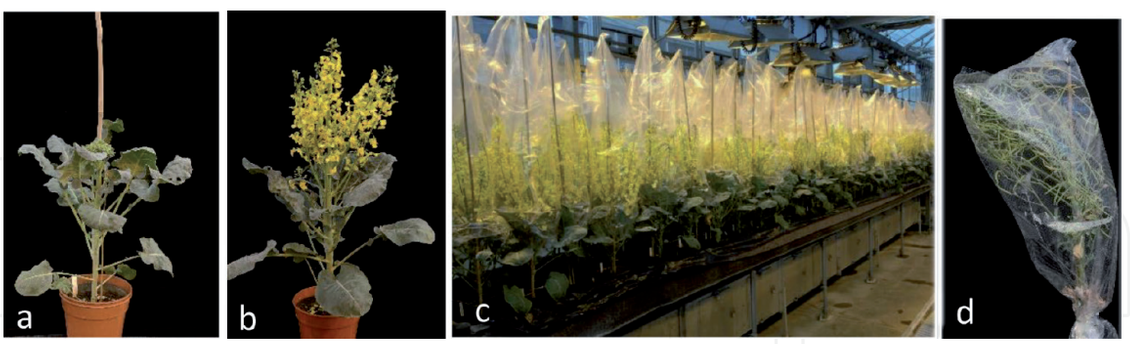

Figure 2.
Images show the phenotype of the Brassica oleracea *genotype AG DH1012; (a) bud formation (broccoli type phenotype); (b) flowering; (c) bagged transgenics flowering in the glasshouse; (d) seed set—in this image pods are still green, when approximately half the pods start to brown watering is stopped.*

4. Stock solutions and media

4.1 Antibiotic stock solutions

- **Kanamycin monosulphate stock solution** (Sigma K1377): 100 mg/mL (2.5 g dissolved in minimal 1 M NaOH and made up to 25 mL with sterile distilled water (SDW) filter-sterilise and store in 1 mL aliquots in sterile 1.5 mL tubes at −20°C).

- **Timentin stock solution** [sold as Ticarcillin disodium/clavulanate potassium (Duchefa T0190)]: 160 mg/mL (2 g dissolved in 12.5 mL SDW) filter-sterilise and store in 1 mL aliquots in sterile 1.5 mL tubes at –20°C.

- **Augmentin stock solution** [sold as Amoxicillin sodium/clavulanate potassium (Duchefa A0189)]: 600 mg/ml and 300 mg/ml, filter-sterilise, store in 1 mL aliquots in sterile 1.5 mL tubes, and prepare just before use (see Note 2).

4.2 Plant culture media and components

- **6-Benzylaminopurine (BAP) stock solution**: 4 mg/mL. We prepare by dissolving 0.1 g of powder in minimal drops of 1 M NaOH. Make to final volume of 25 mL with sterile distilled water (SDW). Store at 4°C.

- **MS Basal medium + 2 mg/L BAP**: 4.3 g/L Murashige and Skoog (MS) salts (Duchefa, M0221) supplied without vitamins, 30 g/L Sucrose, 2 mg/L BAP (add 500 µl of BAP 4 mg/L stock), 500 mg/L 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.7, and 8 g/L phytagar (Duchefa P1003). Autoclave at 120°C for 20 min (see Note 3).

- **Vitamin stocks**: prepare 10 mg/mL Thiamine HCL, 1 mg/mL Pyridoxine, 1 mg/mL Nicotinic acid and 100 mg/mL *myo*-inositol in SDW, and filter-sterilise. Store individually at 4°C except *myo*-inositol, which is stored at room temperature.

- **Germination medium:** MS including vitamins (M0222, Duchefa) 4.41 g/L, Glucose 1 g/L, 500 mg/L MES, 10 g/L Difco Bacto Agar (214,030, Becton Dickinson, Thermofischer), adjust pH to 5.7, autoclave (see Note 4).

- **Co-cultivation medium: To the MS basal medium + 2BAP detailed above:** add 1 mL each of the four vitamin stocks. One litre makes around 40 petri dishes (90 mm single vent, Slaughter Ltd, R&L).

- **Selection medium:** add 160 mg/L Timentin (1 mL Timentin stock) and 15 mg/L Kanamycin (150 µL of 100 mg/mL kanamycin stock) to 1 L of co-cultivation media and pour in petri dishes (100 × 20 mm STD, Sarstedt Limited). Alternatively replace Timentin with Augmentin at 600 mg/ml dropping to 300 mg/ml at later subcultures. One litre is enough for 20 petri dishes. The two control plates can be poured from a 1L flask of media before the kanamycin is added.

- **Rooting medium: (B5 + 1% Sucrose).** 3.05 g/L Gamborg B5 salts (Duchefa, G0209), 10 g/L Sucrose and 8 g/L Phyto agar (Duchefa P1003). Adjust pH to 5.8 with 1 M KOH and autoclave. Prior to pouring add filter-sterilised 160 mg/L Timentin and 25 mg/L Kanamycin. Raising the selection level from 15 to 25 mg/L reduces further the number of escapes that occur.

All media detailed above can be allowed to set and microwaved to re-melt if not poured immediately.

4.3 *Agrobacterium* culture media

- **LB solid medium:** 5 g/L Yeast Extract, 10 g/L NaCl, 10 g/L Tryptone and 15 g/L Bactoagar; autoclave at 120°C for 20 min.

- **LB liquid medium:** 5 g/L Yeast Extract, 10 g/L NaCl, 10 g/L Tryptone and dispense into 10 mL aliquots. Autoclave at 120°C for 20 min.

- **MGL liquid medium:** 5 g/L Tryptone, 2.5 g/L Yeast, 100 mg/L NaCl, 5 g/L Mannitol, 1 g Glutamic acid, 250 mg/L KH_2PO_4, 100 mg/L $MgSO_4$, 1 μg/L Biotin and pH 7.

- **MS Liquid medium:** 4.3 g/L Murashige and Skoog (MS) salts, 30 g/L Sucrose, and pH 5.7 dispense into 100 mL aliquots. Autoclave at 120°C for 20 min.

5. Notes

1. AGL1 is the *Agrobacterium tumefaciens* strain routinely used in our lab in conjunction with the pBRACT series of vectors. LBA 4404, EHA101 and EHA105 are also suitable *Agrobacterium* strains with other plasmids such as pCAM2200 (from the pCAMBIA series) and the SLJ vectors SLJ1714 and SLJ1711.

2. Augmentin should preferably be made up fresh before use. However, it can be stored frozen and defrosted once. Prolonged storage will see a discoloration from pale yellow to dark brown.

3. MS salts with vitamins are available commercially in combination or separate. We prefer to use MS Salts without vitamins from Duchefa, and make up fresh Gamborg's B5 vitamins to add prior to pouring. However, we have also used MS salts with MS vitamins (M0221) and understand that MS medium including Gamborg's B5 vitamins (M0231) is also available. We believe adding vitamins after autoclaving maybe preferable, but we have not carried out enough direct comparisons to see if it significantly makes a difference.

4. Our in-house Arabidopsis germination media is used, for convenience, to germinate Brassica seeds. We have used a range of different germination media in the past successfully and believe it is the size of the seedling on the day of explant isolation that is most critical.

6. Conclusion

Access to easy-to-follow and reproducible transformation protocols is highly desirable. The authors aim is that the protocol described in this chapter, when read in conjunction with watching the video, will help to demystify those specialist skills often associated with tissue culture success.

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crop-transformation/ together with a list of construct resources also available. These transformation resources were developed as part of the Biotechnology Resources for Arable Crop Transformation (BRACT) facility, initially funded by Defra (UK) and now operating on a cost-recovery basis as a transformation resource for the research community.

Conflict of interest

The authors declare there is no 'conflict of interest'.

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