Chapter 28

# *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF CANOLA

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## 1. INTRODUCTION

### *1.1. Importance of Canola*

Canola (*Brassica napus* L.) is an important oil crop, ranking third only to soybean and palm oil in global production. It is a member of the family *Brassicacea* (syn. *Cruciferae*). It is a winter or spring crop and is amenable to growth in cooler climates. Once considered a specialty crop for Canada, it is now a global crop. Many other countries including the USA, Australia and those in Europe also grow canola. However, Canada and the United States account for most of the canola crop. It is grown mostly in Western Canada and North Central United States. In the year 2002, in Canada alone, 9.6 million acres of canola was grown (*1*) and in the USA, 1.5 million acres was devoted to canola cultivation (*2*). The term 'canola' was adopted by Canada apparently as an acronym of the Canadian Oilseed Association in 1979. Although canola has been commonly also known as rapeseed or oilseed rape, in the strict sense canola oil is defined as an oil that must contain less than 2% erucic acid, and the solid component of the seed must contain less than 30 μM of any one or a mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of air-dry, oil-free solid.

Canola oil is widely used as cooking oil, salad oil, and making margarine. Of all edible vegetable oils widely available today, it has the lowest saturated fat content, making it appealing to health-conscious consumers. Canola oil is also used in lubricants and hydraulic fluids especially when there is a significant risk of oil leaking to waterways or into ground water (*3*). It is also used in the manufacture of inks, biodegradable grease, in pharmaceuticals and cosmetics.

Canola meal, which is the leftover product of the seeds after extracting oil, is used as a protein supplement in dairy, beef, swine and poultry rations and is recognized for its consistent high quality and value. The increased processing activity within Canada has led to rising consumption and exports of meal and product mixtures. Feed mixtures include such items as pea-can meal (combining dry peas and canola meal) as well as incorporating canola meal with dehydrated alfalfa products. The bulk of Canada's canola meal exports supply the USA feed ingredient market, which imports over a million tonnes annually and represents 80% or more of



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total meal exports. Feed deficit Asian countries such as Japan, Taiwan and South Korea are also consistent markets for Canadian canola meal.

### *1.2. Need for Genetic Improvement of Canola*

With the increasing demand for canola oil and the need to meet the demands of consumers, there is more research being pursued in improving canola breeding. Conventional breeding techniques are time consuming and laborious. It takes at least 8-10 generations to develop a new variety using conventional breeding. An alternative to trait improvement without conventional breeding techniques is by genetic engineering which reduces the time needed to develop a new variety. A considerable amount of research has already been undertaken in this direction and canola has been exploited for genetic engineering purposes. However of the total genetically modified (GM) crops grown in the world, GM canola represents only 7% of the total area and there is a need to increase the world cultivation of genetically modified canola in order to develop high yielding and pathogen-resistant canola plants. Genetic engineering can also be used to improve the oil quality in canola.

### *1.3. Genetic Improvement of Canola*

Genetic engineering approaches in canola have mainly focused on improving oil quality (*4-8*) or making it herbicide tolerant (*9, 10*) and these plants are now commercially available. In Canada in the year 2002, 900,000 hectares of transgenic canola were grown which corresponds to 20% of the total canola cultivation area in the country. In addition to these traits found in the commercial varieties, canola has also been engineered for insect (*11*) and fungal (*12*) resistance. *Brassica juncea,* which is closely related to *Brassica napus* is known for its hyperaccumulator properties and is a well known phytoremediator. The prospects of using *Brassica napus* in phytoremediation remains unexploited. Although there have been a few reports of transformation of *Brassica napus* for heavy metal tolerance (*13*), there is still much research to be explored in this area. By genetically engineering canola with genes that make it tolerant to heavy metals, canola could be a potential candidate for phytoremediation. In addition to phytoremediation, there are numerous non-food traits that might prove useful. Canola has been used in the production of biopolymers (*14*). There is also significant interest in increasing fuel-oil capabilities or the oil and using the whole plant for biofumigation as a substitute for noxious gases. Canola might also be especially useful as a vehicle to overproduce pharmaceutically active proteins and edible vaccines (*15*). One especially attractive feature is the ability to target proteins to oil bodies for easy downstream processing. Since canola is very closely related to *Arabidopsis thaliana,* which is the principle genomic dicot model, the crop should benefit widely from bioinformatics advances that can be parlayed into transgenic improvements. Canola has an increasing market share in food-stuffs that should continue to drive agronomic and quality trait improvement.

### *1.4. Tissue Culture and Transformation Techniques Applied to Canola*

Tissue culture techniques are quite advanced for canola, which has made it an attractive candidate for use in transformation studies. Various explants have been used for regeneration, however, hypocotyls remain the most attractive explants for transformation and have been used in most transformation experiments. Transformation has been performed using stem internodes (*16*), stem segments (*17*), cotyledonary petioles (*18*), hypocotyl segments (*11, 19-21*), microspores (*22-25*) and protoplasts (*26*).

Spring type canola varieties have proven to be most amenable for transformation. The model variety that has been transformed most often has been Westar, an old spring type cultivar from Canada. However, Westar is not black leg resistant and has other agronomic deficiencies. Technical solutions to address genotype dependency are still needed.

Various techniques such as microprojectile bombardment, electroporation, and *Agrobacterium tumefaciens*-mediated transformation have been used for canola transformation. A combined treatment of DNA-imbibition of desiccated embryos and microprojectile bombardment has been used to enhance transformation efficiency (*27*). However, most transformation procedures have been carried out using *Agrobacterium* because of its ease and cost effectiveness. The virulence of *Agrobacterium* can be increased by the use of acetosyringone, a phenolic compound and it is now being routinely used in canola transformation (*28, 21*). The efficiency of transformation using *Agrobacterium* is enhanced by preconditioning of the explant on callus inducing media before co-cultivation (*19, 29, 30, 21*). Co-cultivation period also plays a critical role in transformation with *Agrobacterium*. A Co-cultivation period of 2 days was found to be optimal (*21*).

The protocol we have described here (*21*) gives the highest transformation efficiency in our hands (25%) compared with other canola transformation protocols published. It is also consistent and reproducible. Increasing transformation efficieny is desirable to decrease the amount of resources to produce transgenic plants, and to also potentially provide a higher baseline for subsequent transformation of other canola varieties. This protocol describes an increase in efficiency by optimizing two important parameters; preconditioning and co-cultivation periods. *A. tumefaciens*-mediated transformation is described using hypocotyls as explant tissue. After obtaining the transgenic shoots, the two important prerequisites for efficient transgenic plant recovery are the promotion of healthy shoots by avoiding hyperhydration and the subsequent rooting of these shoots. This protocol describes methods to circumvent problems of hyperhydration of transgenic shoots and also a very efficient rooting system for transformed shoots, which gives 100% rooting.

## 2. MATERIALS

Seeds used: *B. napus* L. cv. Westar.
Surface-sterilization: 10 % commercial sodium hypochlorite bleach with 0.1% Tween 20 added as a surfactant followed by 95% ethanol with sterile distilled water rinses.
Germination: Seeds are germinated in Magenta GA-7 boxes containing Murashige and Skoog (MS) (*31*) basal medium comprising of MS macro and micro salts and vitamins (*see* Note 1) to which 20 g/l sucrose is added and semi-solidified with 2 g/l Gelrite (Sigma).
*A. tumefaciens* strain and plasmid: *A. tumefaciens* strain GV3850 strain harbouring pBinmGFP5-ER which contains the mGFP5-ER gene (reporter) and *npt*II gene (selectable marker) which confers resistance to kanamycin (*32*) were used. The mGFP5-ER gene is driven by the CaMV 35S promoter and *npt*II is driven by the NOS promoter (*see* Note 2).
Media: All media stock solutions were made with Type I water as solvent. Stock solutions have a refrigerated shelf-life of approx. 90 days, after which they are discarded.
Callus induction medium: MS medium with 1 mg/l 2,4-D (Sigma) and 30 g/l sucrose and semi-solidified with 2 g/l Gelrite.
Selection medium: Callus induction medium with 400 mg/l timentin (GlaxoSmithkline, USA) (to kill *Agrobacterium*) and 200 mg/l kanamycin (Sigma) to select for transformed cells (*see* Note 3). Stock solutions for the antibiotics were made by dissolving in water and filter-sterilization using a 0.2 μm membrane filter.
Organogenesis medium: MS medium with 4 mg/l BAP (Sigma), 2 mg/l zeatin and 5 mg/l silver nitrate (Sigma), antibiotics as mentioned above and 30 g/l sucrose and semi-solidified with 2 g/l Gelrite.
Shoot regeneration medium: MS medium containing 3 mg/l BAP, 2 mg/l zeatin, antibiotics and 30 g/l sucrose and 2 g/l Gelrite for shoot development.
Shoot elongation medium: MS medium with 0.05 mg/l BAP and 30 g/l sucrose and semi-solidified with 3 g/l Gelrite. Antibiotics are added as above.
Rooting medium: Half-strength MS salts, 10 mg/l sucrose, 3 g/l Gelrite and 0.5 mg/l IBA (Sigma). Antibiotics are added as above.

## 3. METHODS

### *3.1. Tissue Culture and Transformation*

1. Seeds are surface-sterilized for 5 min with 10 % sodium hypochlorite with 0.1 % Tween 20 added as a surfactant. The sterilization is followed by a 60 sec rinse with 95% ethanol. The seeds are washed thoroughly 3 times with sterile distilled water. Seeds are surface-sterilized by shaking with the sterilants, manually in 1.5 ml Eppendorf tubes.

2. Seeds are germinated in Magenta GA-7 boxes (10 seeds per box) with 50 ml MS medium for 8-10 days. The cultures are grown in a light intensity of 40-60 $\mu mol/m^2/sec$.
3. Hypocotyls from the 8-10 day-old seedlings are cut into 1 cm pieces and preconditioned for 3 days on callus induction medium (*see* Note 4) in 100 x 15 mm polysterene disposable dishes (Petri dishes). Make sure to excise the apical meristem completely. Ten to fifteen explants were placed in each Petri dish containing 25 ml of the medium.
4. *Agrobacterium* cells are grown overnight in flasks to an $OD_{600} = 0.8$ in 50 ml liquid LB medium, pelleted, and re-suspended in 15-20 ml liquid callus induction medium to which acetosyringone (Sigma) is added to a final concentration of 0.05 mM (*see* Note 5).
5. The preconditioned hypocotyls are inoculated with *Agrobacterium* for 30 min in a Petri dish, shaking the dish at intervals manually to make sure the explants are in constant contact with *Agrobacterium*. About 100-150 explants are used per 15-20 ml of the *Agrobacterium* solution.
6. The explants are picked carefully from the *Agrobacterium* solution (so that excess *Agrobacterium* is drained) and then co-cultivated with *Agrobacterium* for 48 h on callus induction medium in 100 x 15 mm Petri dishes (*see* Note 6). Twenty explants can be placed on each dish.
7. For selection of transformed cells, the explants are transferred to the callus induction medium with antibiotics (400 mg/l timentin and 200 mg/l kanamycin).
8. After 2 weeks, the transformed organogenetic calli are transferred to 25 ml of organogenesis medium in 100 x 15 mm Petri dishes.
9. After another 2 weeks, the calli are transferred to shoot induction medium in Petri dishes. Shoots are produced after 2-3 weeks on this medium.
10. Shoots (2-4 per Magenta box) are then transferred to the shoot elongation medium in Magenta boxes which contain 25 ml of the culture medium. The shoots elongate in approx. 3 weeks (*see* Note 7).
11. The elongated shoots are transferred to the root induction medium. Roots develop in 1-2 weeks (*see* Note 8). Only one shoot is rooted in each Magenta box which allows the formation of well developed roots and easy removal of the rooted shoot from the Magenta box.
12. The rooted plants are transferred to soil (from STA-GREEN, California, USA which is a mixture of composted forest products, sphagnum peat moss, perlite, ground dolomitic limestone, a wetting agent and water holding polymer) in 1 litre pots and grown in a plant growth chamber. They are covered with a plastic dome to retain humidity.
13. After 3 days, the dome is gradually removed and the plants are transferred to 3.5 litre pots and grown for seed set in the glasshouse.

### *3.2. Culture Conditions*

All the cultures should be maintained at 25 ± 2 °C under a 16 h photoperiod using cool white daylight fluorescent lights at 40-60 $\mu mol/m^2/sec$. The rooted shoots are

transferred to soil and grown at a photoperiod of 16 h at 20°C in a plant growth chamber at 300 μmol/$m^2$/sec.

### *3.3. Progeny Analysis*

The progeny of transformed plants are analyzed using PCR for transgene presence. Western blots or RT-PCR may be used for expression analysis. Transgene segregation of the T1 seedlings can be performed using kanamycin selection (200 mg/l kanamycin in MS media—seeds germinated in Petri dishes). A 3:1 Mendelian ratio is expected for single-locus insertion inheritance. Southern analysis and more recently RT-PCR, which is a fluorescence based kinetic RT-PCR, is used to confirm gene copy number.

## 4. NOTES

MS micro and macro salts and vitamins are used in the medium wherever we state MS medium.

The *gfp* gene allows real-time monitoring of the transformed callus and shoots. GFP positive callus and plants glow bright green when excited by UV light.

Appropriate antibiotics need to be used according to the selectable marker genes on the plasmid. We use kanamycin, since the selectable marker gene in this work is *npt*II which confers resistance to kanamycin.

4. A preconditioning time of 72 h was found to be optimal for high transformation efficiency.
5. Acetosyringone increases the virulence of *Agrobacterium* thus increasing the transformation efficiency.
6. A co-cultivation time of 48 h is critical to obtain good transformation efficiency. With a co-cultivation time of less or more than 48 h the transformation efficiency decreases significantly.

A major problem encountered during transformation of canola is the hyperhydration of transformed shoots. This problem is overcome by increasing the Gelrite concentration from 2 g/l to 3 g/l in the rooting and elongation medium. By increasing the Gelrite concentration, the water availability for the shoot is reduced which would otherwise make the plant hyperhydrate.

Rooting is very efficient using half-strength MS medium vs. full-strength MS medium and reducing the sucrose concentration from 30 g/l to 10 g/l. The rooting medium described gives 100% rooting in a very short duration of time (1-2 weeks). When full-strength medium is used the plants grow tall instead of producing roots, hence we use a low strength, low sugar medium which facilitates rooting.

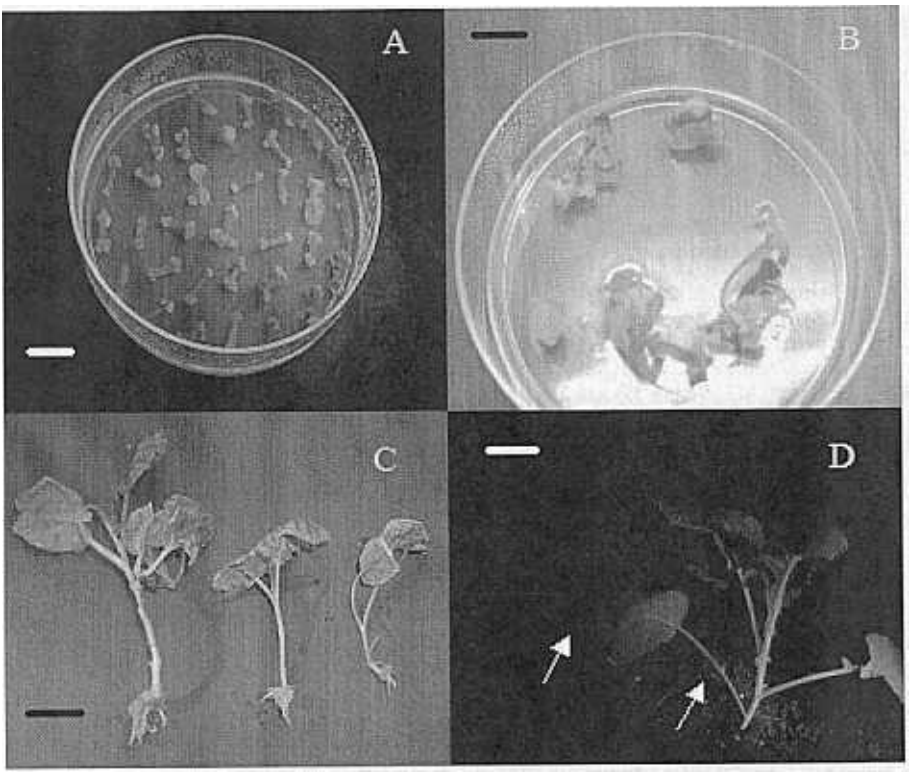


*Figure 1. Progression of genetic transformation of canola. A. Hypocotyl segments are inoculated with* A. tumefaciens *harbouring a* GFP *gene. B. Shoots regenerating from transgenic calli. C. Transgenic rooted shoots. D. Transgenic plant expressing* GFP *(right arrow). Panels A and D are under UV illumination. Horizontal scale bars in images A and B represent 2 mm; in C and D, 5 mm.*

## CONCLUSION

Using the transformation method we have described, it is possible to obtain a transformation efficiency of 25%. All the factors which include, preconditioning and co-cultivation time, overcoming hyperhydricity and improving rooting together has brought about this improvement in transformation efficiency. We have used only one variety of canola. We see no reason why these techniques cannot be extended to other varieties of canola. With the increase in demand for canola oil, there is still a need for variety improvement. Various genes that improve oil quality can be genetically engineered in canola using the transformation technique we have described.

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