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Brassica Transformation: Commercial Application and Powerful Research Tool

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ABSTRACT

Transformation systems are now available for all six of the main economic *Brassica* species; with a wealth of introduced traits reported (reviewed in Cardoza and Stewart 2004; Christey *et al.* in press). *Agrobacterium*-mediated transformation remains the method of choice for *Brassica*, yet despite the significant progress in enhancing efficiencies some genotypes remain recalcitrant to transformation. Recent advances in our understanding of the genetics behind transformation have enabled researchers to identify more readily transformable genotypes for use in routine high-throughput systems. These developments open up exciting new avenues to use model *Brassica* genotypes as powerful research tools for understanding gene function in complex genomes. In this paper advances in *Brassica* transformation methodologies and applications are reviewed.

Keywords: *Brassica*, genetic modification (GM), *Agrobacterium*, tissue culture, gene function, model-to-crop

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INTRODUCTION

The *Brassica* genus belongs to the family Brassicaceae and includes a group of six inter-related species of worldwide economic importance. *Brassica rapa* (genome AA, 2n = 20) is used both as a vegetable crop (turnip, Chinese cabbage) and as an oil crop (turnip rape). *B. nigra* (BB, 2n = 16) is grown as a condiment (black mustard) and *B. oleracea* (CC, 2n = 18) contains numerous vegetable crops with a wide range of different morphologies including cabbage, cauliflower, kale, broccoli and Brussels sprouts. The hybrids *B. juncea* (AABB, 2n = 36, brown mustard) and *B. napus* (AACC, 2n = 38, oilseed rape) are important oilseed crops and *B. carinata* (BBCC, 2n = 34) is grown in Ethiopia as both a vegetable and oil crop (Ethiopian/Abyssinian mustard).

The diverse array of vegetable and oilseed crops outlined above has been the result of years of hybridization between and within the *Brassica* species, accompanied by intense selection for different morphologies. The application of conventional breeding has led to the development of many superior cultivars within this genus. With increasing knowledge of the function of genes, and the development of techniques for plant transformation, the potential for further improvement of these species is considerable. To date, genetically modified (GM) *B. napus* is the only *Brassica* species to gain commercial regulatory approval; with herbicide tolerant *B. napus* being the fourth most planted GM crop in 2006 (James 2006). Another 'first generation' trait to be introduced into vegetable *Brassicas* is *Bacillus thuringiensis*

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Invited Review

(Bt) resistance to alleviate insect attack. This work is being carried out by numerous groups, at both the contained and field trial stages e.g. the 'Collaboration on Insect Management for *Brassicas* in Asia and Africa (CIMBAA) (http://www.cimbaa.org/); while field trials of Bt cabbage, cauliflower, broccoli and forage kale are currently being conducted in New Zealand (www.ermanz.govt.nz).

However, poor public perception of GM technology continues to hinder advancement. In the developed world it is likely to be the 'next generation traits,' offering increased nutritional and health benefits to the consumers that will potentially help gain better public acceptance of this technology. *Brassica* transformation is now being used not only to introduce commercially attractive agronomic traits into elite cultivars, but is increasingly being exploited as a powerful research tool to test gene function.

Despite the considerable advances in methodologies the routine transformation of *Brassica* is still hindered by genotype restrictions, with some genotypes remaining recalcitrant to transformation. Recent advances in our understanding of the genetics behind transformation have enabled researchers to develop simple screening methods to identify more readily transformable genotypes for use in routine high-throughput systems for testing gene function (Sparrow *et al*. 2004a, 2006a). However, routine high-throughput transformation is still likely to be limited to a number of key genotypes e.g. Westar is a commonly reported Spring variety for *B. napus* transformation (Moloney *et al*. 1989; Cardoza and Stewart 2006).

There may however be occasions when a particular genotype needs to be transformed and where only a few transgenic plants are required. Under these circumstances, a high transformation efficiency is less critical. Lessons can be learnt from the range of published papers that have added to our knowledge of tissue culture conditions favoured by different *Brassica* genotypes and species. In this paper we will review the advances made to *Brassica* transformation methods and discuss how this technology can be further exploited to better understand gene function.

TRANSFORMATION METHODS

The first reports of successful transformation emerged in the late 1980's / early 90's for all six of the major economically important *Brassica* species (*B. juncea* (Barfield and Pua 1991), *B. napus* (Moloney *et al*. 1989), *B. rapa* (Radke *et al*. 1992), *B. oleracea* (de Block *et al*. 1989), *B. nigra* (Gupta *et al*. 1993) and *B. carinata* (Narasimhulu *et al*. 1992). Several publications followed, reporting improvements and developments to culture conditions and the use of reporter genes to determine transformation efficiencies (discussed later). The first major reviews of *Brassica* transformation were published more than 10 years ago (Puddephat *et al*. 1996; Poulsen *et al*. 1996). In these reviews the basic methodologies were evaluated; use of *Agrobacterium*-mediated transformation (both *A. tumefaciens*, *A. rhizogenes*), biolistics and PEG-mediated transformation. They highlighted the species, genotype and explant-dependent nature of *Brassica* transformation success and reviewed the range of *Agrobacterium* strains; selectable markers and reporter genes, and possible clean gene technologies that could be employed. Since these reviews were published further advances have been reported leading to increased transformation efficiencies and the introduction of trait genes. These developments are outlined below.

Agrobacterium-mediated methods

Agrobacterium-mediated transformation still remains the favoured delivery approach for the introduction of transgenes into most dicotyledonous plant species, as well as an expanding range of monocots (Smith and Hood 1995; Opabode 2006). Two *Agrobacterium* species (*A. tumefaciens* and *A. rhizogenes*) have been widely exploited to transform a vast array of plant species. Both approaches involve the transfer and incorporation of T-DNA from an engineered plasmid, previously introduced into the *Agrobacterium*, into a host plant cell. Early research to improve plant transformation efficiencies focused on screening a range of *Agrobacterium* strains against plant genotypes of interest (see section *Bacterial strains and plasmids*). It subsequently became clear that susceptibility to *Agrobacterium* was highly genotype dependent, and thus critical for transformation success. The target then moved to screening *Brassica* populations to identify genotypes susceptible to these virulent strains (Sparrow *et al*. 2004a, 2004b; Zhang and Bhalla 2004; Sparrow *et al*. 2006a).

The genetic basis behind Agrobacterium susceptibility

Knowledge of the genetics behind the attraction, attachment, and transfer of genes from *Agrobacterium* into a host cell has also developed over recent years (Gelvin *et al*. 2003; Tzfira and Citovsky 2003), with an increased understanding of both the bacterial and plant genes involved in the transfer of T-DNA. Genetic variation for *in vivo A. tumefaciens* susceptibility has been observed in a wide range of plant species including *Prunus* (Bliss *et al*. 1999), soybean (Bailey *et al*. 1994; Mauro *et al*. 1995) and grape (*Vitis* sp.) (Szegedi and Kozma 1984). In *B. oleracea*, mapping populations have been screened and quantitative trait loci (QTL) associated with susceptibility to *A. rhizogenes* and *A. tumefaciens* identified (Cogan *et al*. 2004; Sparrow *et al*. 2004b). Screening substitution lines associated with these mapping populations confirmed the significance of these QTL and also demonstrated that susceptibility to *Agrobacterium* was a heritable trait and could be introduced into recalcitrant lines. Crossing genotypes with low *Agrobacterium* susceptibility to genotypes with high susceptibility resulted in an intermediate response in the hybrid plants (Sparrow *et al*. 2004b).

A. rhizogenes

A. rhizogenes is a soil bacterium responsible for the development of hairy root disease of dicotyledonous plants. In its modified form it has been used to transform over 79 plant species (reviewed in Christey 2001). It has been successfully used to transform *Brassica* where the focus has been on *B. oleracea* and *B. napus* (Christey and Sinclair 1992; David and Temp 1988; Puddephat *et al*. 2001). Overall, transformation rates were low, but in some cases, transgenic *Brassicas* were more efficiently obtained *via A. rhizogenes*-mediated transformation than *A. tumefaciens* (Christey *et al*. 1997). The main disadvantage of using *A. rhizogenes* was *rol* genes were often transferred and expressed in plants regenerated from hairy roots, often exhibiting an associated altered phenotype, such as wrinkled leaves, shortened internodes, reduced fertility and plagiotropic roots. However, these traits often segregated in the next generation facilitating the recovery of normal transgenic plants (Christey *et al*. 1999; Puddephat *et al*. 2001).

A. tumefaciens

The favoured *Agrobacterium*-based transformation systems use *A. tumefaciens* (**Fig. 1**). The simplest approaches are those described by Moloney *et al*. (1989) for *B. napus* cv. 'Westar' (efficiency 55%); Babic *et al*. (1998) for *B. carinata* breeding line C90-1163 (efficiency 30-50%); and Sparrow *et al*. (2004a, 2006b) for *B. oleracea* genotype AGDH 1012 (efficiency 15-25 %; see also www.bract.org for a tutorial guide to this method). All these methods use cotyledonary petioles dipped into a suspension of *A. tumefaciens* and co-cultivated for 72 hours on selection free media, before transferring to a basic MS basal medium supplemented with between 2 and 4 mg/l 6-benzyl-aminopurine (BAP). Explants are then subcultured to fresh media after approximately 3 weeks. Emerging shoots are isolated

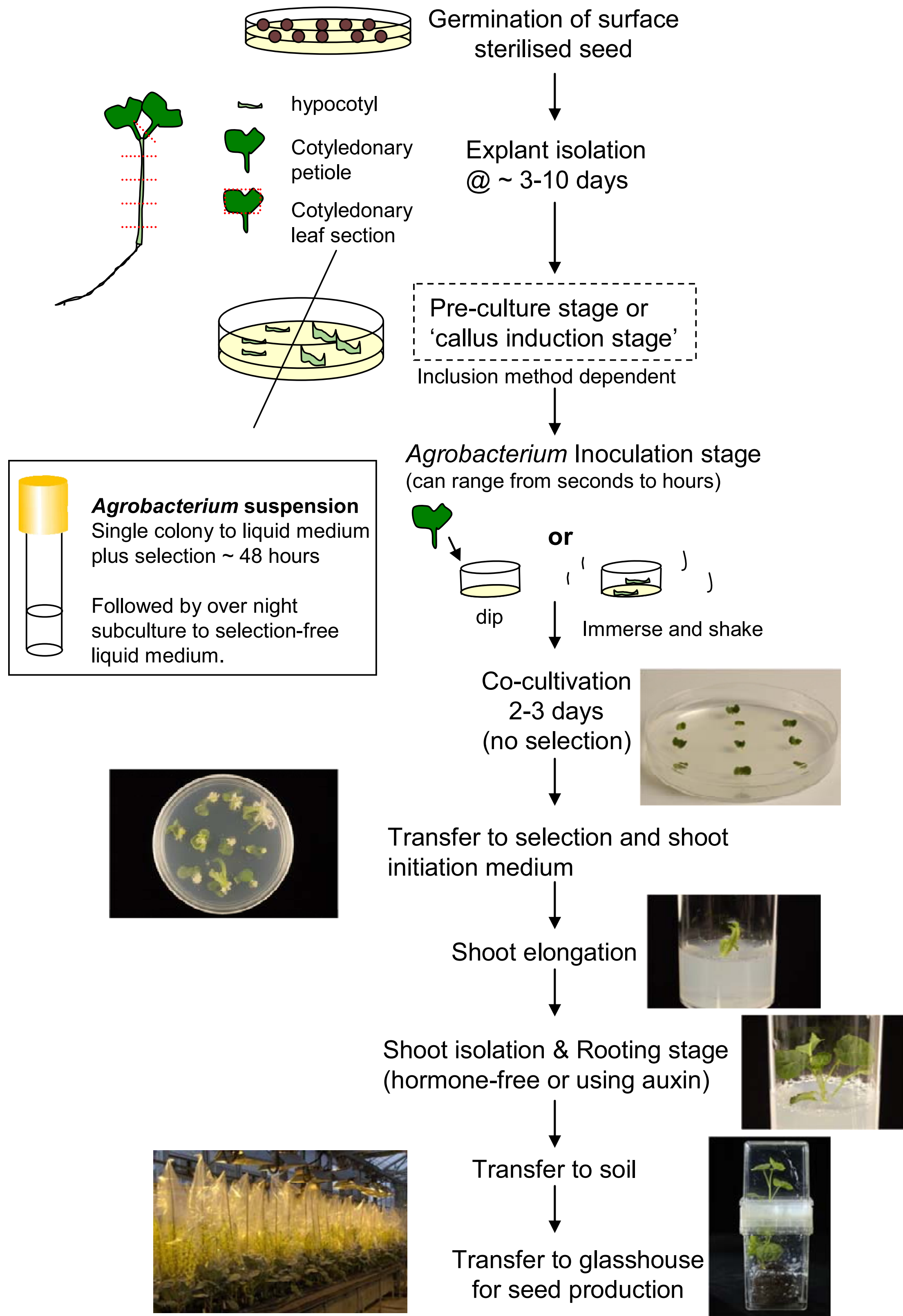

Fig. 1 General overview of *Agrobacterium*-mediated transformation.

and rooted on a hormone-free medium.

Hypocotyl methods have also been applied to *B. napus* at a reported efficiency of 25% (for an updated method see Stewart *et al.* 2006) and *B. juncea* with an efficiency of 16%[1] (Gasic and Korban 2006). These approaches use either pre-cultured hypocotyl segments (following a short callus induction period) or newly isolated hypocotyl segments, emersed in a suspension of *A. tumefaciens* for 30-60 minutes. Explants are then washed and transferred to co-cultivation medium for 48 hours, before transferring to selection medium. Both methods employ different media for callus induction, shoot induction, shoot elongation and rooting stages (see also section *Shoot regeneration*).

Brassica rapa remains the most recalcitrant of the *Brassica* species to transform. However, a limited number of successes have been reported; Radke *et al.* (1992) using hypocotyl sections; Zang *et al.* (2000) and Wahlroos *et al.*

[1] N.B. The efficiencies reported in this section are based either on the percentage of explants giving rise to viable transgenic shoots, or the percentage explants giving rise to transgenic rooted plants. All methods successfully generated fertile transgenic plants.

(2003) using cotyledonary petioles; Yang *et al*. unpublished, using cotyledonary leaf sections (as described in the regeneration paper Yang *et al*. 2004); Kuvshinov *et al*. 1999 using internodes from glasshouse grown *B. rapa*; and *Agrobacterium* infiltration of whole plants by Qing *et al*. 2000 (see also section *FLORAL DIPPING/MICRO-INJECTION*).

With the sequencing of the *B. rapa* genome currently underway (www.brassica.info.org) it is likely that efforts and interest in *B. rapa* transformation will rise over the next few years. Current studies to develop *B. rapa* transformation include those of the AdVaB consortium (http://www.brassica.info/ukbrc/advab/) where the focus is a rapid cycling genotype (RO18) which is also being used to develop a TILLING population. This will provide excellent resources to optimise the information arising from the Multinational *B. rapa* Genome sequencing project for *Brassica* functional genomics as it becomes available.

Bacterial strains and plasmids

A number of different strains of *A. tumefaciens* have been successfully used to transform *Brassica*. The most frequently used are LBA4404 (Hoekema *et al*. 1983) an octopine strain, and the nopaline strains C58 (Sciaky *et al*. 1978) and derivatives AGL1 (Lazo *et al*. 1991), EHA101 and EHA105 (Hood *et al*. 1986) which have all been used successfully and routinely. The use of GV3101 (Koncz and Schell 1986), a favourite for *Arabidopsis* transformation, has also been reported for *Brassica* (DeBlock *et al*. 1989; Mehra *et al*. 2000). It is likely that over the next few years the ability to use plasmids and *Agrobacterium* strains routinely used for *Arabidopsis* transformation will be highly desirable as researchers move findings from the model species into crops such as *Brassica*.

The type of plasmid used is thought to be less critical than bacterial strain, although choice of promoters and selectable markers is more important (see section *FLORAL DIPPING/MICRO-INJECTION*). Commonly used plasmids have been the modified pBIN19 (Bevan 1984) and its derivatives; the SLJ vectors (Jones *et al*. 1992) in particular SLJ1714 and SLJ1711; the pCAMBIA vectors (www.cambia.org/daisy/cambia/materials/overview) in particular pCAM2200; and pGreen (Hellens *et al*. 2000; www.pgreen.ac.uk). The above examples are based on the 35S promoter driving *nptII* as the selectable marker gene, but other selectable markers are available (see section *SELECTION OF TRANSGENICS*).

Direct uptake methods

The first reports of direct uptake transformation methods, such as electroporation or PEG-mediated transfer, in *Brassica* were for cauliflower protoplasts by Mukhophyay *et al*. (1991) (hypocotyls protoplasts); and Eimert and Siegemund (1992) (mesophyl protplasts). Recently, Radchuk *et al*. (2002) studied a range of factors effecting PEG mediated transformation in *Brassica* using both kanamycin and hygromycin selection. Nugent *et al*. (2006) demonstrated successful transformation in cauliflower mesophyll protoplasts using a *gus* reporter gene and hygromycin selection (see also section *Chloroplast transformation*). Regeneration from protoplasts remains the main limitation of this approach. Another direct approach is biolistics or microparticle bombardment where DNA-coated beads are fired at high speed into plant cells. These approaches again offer an alternative transformation method for genotypes that are not susceptible to *Agrobacterium* infection and are a useful approach for transient expression studies (Puddephat *et al*. 1999).

Chloroplast transformation

Plastid transformation offers a number of potential advantages over nuclear transformation (Maliga 2004). The high number of plastids per plant cell (approx. 10^5 copies per plant cell (compared to a single nucleus)) means higher expression levels can potentially be achieved. This is particularly desirable for product based transformation, where high protein yields are desirable (Dhingra *et al*. 2004). Inheritance of the introduced transgene(s) will also be maternal and therefore offers containment of the transgene, due to lack of gene flow through pollen (Daniell 2002); although observation of gene transfer from chloroplast to nuclear genomes have been reported (Stegemann *et al*. 2003) frequencies are extremely low. The successful transformation of *Brassica* chloroplasts by particle bombardment have been reported for *B. oleracea* (Hou *et al*. 2003) and for *B. napus* (Liu *et al*. 2007), both; and by Nugent *et al*. (2006) using a PEG-mediated approach.

SHOOT REGENERATION

Many factors affect the successful regeneration of shoots *in vitro*; namely genotype, media conditions and explant type and age. With the exception of the floral dip method (discussed below) the above transformation methods all rely on having a robust regeneration system in place for transformation success (recovery of transgenic plants). In this section we will focus on factors effecting *in vitro* regeneration systems for *A. tumefaciens*-mediated transformation. However, a number of the points discussed also apply to other methods.

The genetic basis of *in vitro* shoot regeneration

Extensive screening of genotypes and tissue culture conditions has improved the frequency of shoot regeneration for most *Brassica* species. Despite these advances, some genotypes remain highly recalcitrant to *in vitro* regeneration. The genotype dependent nature of *in vitro* shoot regeneration, both within and among the *Brassica* species, was first reported by Murata and Orton (1987). They observed that *B. napus* (AACC) had a higher regeneration response than *B. rapa* (AA) thereby suggesting that genes from the C genome may influence its greater regeneration response. Narasimhulu *et al*. (1988a, 1988b) studied shoot regeneration in the three diploid *Brassica* species and their synthetic amphidiploid hybrids. They found no significant difference between the B and C genomes in terms of regeneration potential, but concluded that the A genome was the most recalcitrant genome for regeneration under the conditions used. The synthetic hybrids *B. napus* (AACC) and *B. juncea* (AABB) both had lower regeneration responses than their better parent response, *B. oleracea* (CC) and *B. nigra* (BB) respectively, suggesting an inhibitory effect of the A genome. These studies suggest shoot regeneration to be a heritable trait. Hansen *et al*. (1999) reported a genetic analysis of shoot regeneration from protoplasts of *B. oleracea* by crossing a high and a low regenerating line and measuring the regeneration response in the F_2 generation. The frequency distributions observed suggested that at least three independent loci were responsible for regeneration. The finding that two or three genes control regeneration is consistent with other reports for crops such as rice (Peng and Hodes 1989; Taguchi-Shiobara *et al*. 1997) barley (Komatsuda *et al*. 1989) and tomato (Koorneef *et al*. 1987). Ono *et al*. (2000) looked at the genetic control of shoot regeneration from cotyledonary petioles in *B. napus* using a 5 × 5 diallel cross. They showed that shoot regeneration was associated with additive and dominant gene effects, with additive gene effects accounting for the majority of the variation. These findings were consistent with the findings of Sparrow *et al*. (2004c) for *B. oleracea* who screened a 12 × 12 diallel of genotypes with varying regeneration response, and also observed that *in vitro* regeneration was under strong genetic control. Genetic factors accounted for 85% of the variation, with the remainder a result of non-heritable or environmental influences. In the same study high shoot regeneration was observed to be dominant over low shoot regeneration. The production of multiple shoots (in favour

Figs 2A-C Cotyledonary petioles from 3 genotypes of *B. napus* photographed *in vitro* after 16 days in culture. Genotype (**A**) shows slight swelling to petiole base and the presence of tissue culture blackening; in (**B**) shoot regeneration is via a direct mode and associated with tissue culture blackening and genotype (**C**) regeneration is via an indirect callus mode and in the absence of tissue culture blackening.

of just a few shoots) from regenerating cotyledonary petioles was also demonstrated to be heritable with additive gene effects accounting for the majority of the variation (77%) observed within the diallel. The similarity of the inheritance patterns observed for both *B. napus* and *B. oleracea* would suggest conservation of genes for shoot regeneration within the same genome (CC). The ability to introduce or increase the *in vitro* shoot regeneration potential of a genotype, by conventional breeding, may help overcome restrictions to routine transformation programmes, where efficient shoot regeneration is a critical pre-requisite.

In theory all cells that contain a nucleus are totipotent and retain the genetic information required to regenerate a whole plant. However, some genotypes are simply unable to tolerate *in vitro* conditions, and thus regenerate shoots. Intolerance to *in vitro* conditions has been observed in *B. oleracea* and *B. napus* (Sparrow *et al.* 2004a, 2004c, 2006b). In these papers, cotyledonary petioles from a range of genotypes were screened for regeneration potential in the absence of *Agrobacterium*. A number of genotypes exhibited extreme tissue culture blackening to the petiole base (**Fig. 2A, 2B**). Genotypes that demonstrated tissue culture blackening failed to regenerate shoots, or regenerated a low number of small shoots direct from the petiole base (without a callus phase). These shoots were often non viable as they failed to develop further and could not be isolated. These genotypes made poor candidates for transformation success. By contrast, genotypes that regenerated multiple shoots, a response associated with a small callus phase, and no blackening (**Fig. 2C**) were considerably more favourable to *Agrobacterium*-mediated transformation, than genotypes that regenerated a small number of shoots direct from the petiole base.

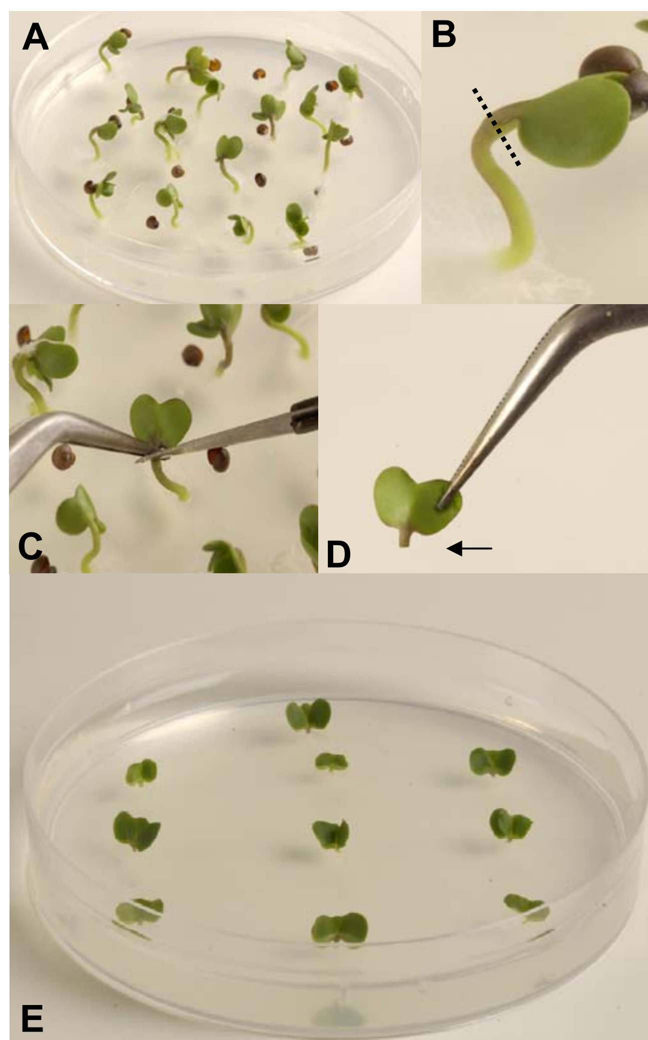

Fig. 3 Isolation of cotyledonary petioles from *B. oleracea*. (**A**) Four-day-old seedlings; (**B**) excision site (dotted line); (**C**) explant isolation; (**D**) cotyledonary petiole (arrow); (**E**) explants on co-cultivation medium. NB: Petri-dish lids not shown in photographs.

Choice of explant

In vitro shoot regeneration of *Brassica* has been achieved from an array of different explant types: cotyledonary petioles (Moloney *et al.* 1989; Ono *et al.* 1994), cotyledonary sections (Yang *et al.* 2004), hypocotyls (Yang *et al.* 1991), peduncles (Christey *et al.* 1991), leaf sections (Akaska-Kennedy *et al.* 2005), anthers, microspores (Keller and Armstrong 1977; Litchter 1982), thin cell layers of epidermal and subepidemal cells (Klimaszewska and Keller 1985), roots (Xu *et al.* 1982) and protoplasts (Glimelius 1984; Barsby *et al.* 1986). However, it is the seedling explants (cotyledons and hypocotyls) that remain favourites for transformation. Seeds can be surfaced sterilised and germinated *in vitro* to achieve sterile explant tissue. The age of the explant is also critical, with many researchers finding 3-4-day-old seedlings give optimal results (discussed below). While it is often the age of the explant that is referred to, it is actually the size of the explant that is more critical. A 3-day-old seedling in one culture room, under one light regime may be different (bigger or smaller) than the same seedling grown under different growth room conditions. For cotyledonary petioles, the optimum age/size can usually be determined by ease of isolation (see **Fig. 3**) – too early and it becomes difficult to isolate the cotyledons whilst avoiding the meristematic bundle. If left too late cotyledons will simply expand on regeneration medium rather than regenerating shoots. For hypocotyl sections, older explants (8-10 days) have also been used (Cardoza and Stewart 2006) for *B. napus*, allowing a larger number of explants to be obtained per seedling. Gasic and Korban (2006) on the other hand found hypocotyls from 3-4 day old seedlings gave optimal results in *B. juncea*. What appears critical in both these systems however, is the length of the hypocotyl section. Small sections were optimal, i.e. 5-10 mm sections (where smaller explants did better). Long hypocotyl sections had the tendency to curl and therefore loose contact with the culture media.

Media conditions

Not only is *in vitro* shoot regeneration genotype dependent, but regeneration success will also be affected by the choice of transformation protocol, hormone and other media additive levels used. The ability to regenerate from one tissue type does not guarantee regeneration success from another

explant source, and indeed it is always advisable to carry out a regeneration study with the genotype and explant of choice before selecting the transformation approach. Hypocotyl and leaf sections often require a callus phase prior to shoot regeneration (Yang *et al*. 2004; Gasic and Korban 2006) using cotyledonary leaf sections). This is normally achieved using a high cytokinin (usually BAP) to low auxin (e.g. naphthalene acetic acid (NAA)) ratio. Some transformation protocols also exploit a short callus induction phase prior to transformation, a so called preconditioning stage (Cardoza and Stewart 2006).

Shoot elongation and rooting in vitro

Once shoot regeneration has been achieved from the desired genotype, another stumbling block can be the isolation of viable shoots. This is partially true for *B. rapa* one of the more recalcitrant *Brassica* species (Sparrow and Goldsack, pers. unpublished data). Small shoots are often prone to undesirable morphology, hyperhydricity and fail to elongate and root *in vitro*. A shoot elongation step, where cytokinin levels are lowered but not removed, can often help shoots to elongate (Cardoza and Stewart 2006). Often hyperhydricity is overcome once shoots have elongated, but occasionally other supplements need to be explored (see section *Hyperhydricity and tissue necrosis*). Rooting is often achieved by simply removing the cytokinin, however in some also it may also require the addition of an auxin (such as NAA or indole-3-butyric acid (IBA)).

Hyperhydricity and tissue necrosis: Use of ethylene inhibitors

Hyperhydricity (formerly vitrification) and tissue necrosis can present a serious problem for plant tissue culturists. However, a better understanding of the underlying mechanism of hyperhydricity and its control *in vitro* can significantly contribute to improved tissue culture success (Meira 1991). In *B. rapa* and *B. juncea*, Chi and Pua (1989) and Chi *et al*. (1990) demonstrated that higher regeneration frequencies could be achieved if explants were given the appropriate media and environmental conditions.

Factors found to effect hyperhydricity and tissue necrosis are: accumulation of ethylene and high humidity in culture vessels (de Block *et al*. 1989), excessively rich media, *Agrobacterium* overgrowth/sensitivity (Jin *et al*. 2000), and high doses of exogenous cytokinin and/or auxin (Ketaeva *et al*. 1991; Kamal *et al*. 2007). In *B. napus* Cardoza and Stewart (2003) observed that increasing the percentage of gelling agent in the shoot elongation medium, and thus reducing the relative humidity of the culture vessel, allowed shoots to overcome hyperhydricity and resume normal growth within 2 weeks of transfer.

Ethylene is another key factor in optimising tissue culture conditions for some *Brassica* species (Cardoza and Stewart 2004). Silver nitrate has been used to reduce hyperhydricity in a range of *Brassica* species such as *B. rapa* (Kuvshinov 1999; Xiang *et al*. 2000; Yang *et al*. 2004) and *B. napus* (Tang *et al*. 2003). In particular *B. rapa*, one of the most recalcitrant *Brassica* species to *in vitro* culture responds positively to the addition of silver nitrate (Palmer 1992; Radke *et al*. 1992). It has also been reported that excluding silver nitrate from tissue culture media can drastically reduce regeneration frequency in some genotypes of *B. napus* (Phogat *et al*. 2000). Other ethylene inhibitors which can be used in combination, or as an alternative, include silver thiosulfate (Eapen and George 1996, 1997) and aminoethoxyvinylglycine used by Chi *et al*. (1990) for *B. rapa* and *B. juncea*; Pua and Chi (1993) for *B. juncea* and Burnett *et al*. (1994) again for *B. rapa in vitro* culture.

FLORAL DIPPING/MICRO-INJECTION

The ability to bypass the tissue culture phase associated with the above methods could overcome some of barriers to transformation success observed in *Brassica*. *In planta* transformation of *Arabidopsis* is now common practice, and involves immersing intact inflorescences in *A. tumefaciens* suspensions (Bechtold *et al*. 1993; Clough and Bent 1998; Kojima *et al*. 2006). The *Agrobacterium* targets the ovules for the transformation event (Ye *et al*. 1999), and therefore species in which the ovary remains open for an extended developmental period may be good candidates for successful *in planta* transformation (Desfeux *et al*. 2000). Transformation by infiltration of adult *Brassica* plants with A*grobacterium* was reported for *B. rapa* (Pakchoi) by Liu *et al*. (1998); and Qing *et al*. (2000). In the latter study two transgenic plants were obtained from 20 000 seed arising from the dipping of 30-50 plants. While this efficiency is low, it does demonstrate the potential to apply the method to *Brassica*. In *B. napus*, Wang *et al*. (2003) reported a success rate of 0.18% (approximately 11 putative transgenics arising from 4 dipped plants) using a double infiltration approach.

Recently, Zhandong *et al*. (2007) have reported a transformation rate of 2.35% for Chinese cabbage (*B. rapa*), using the method of Liu *et al*. (1998). The gene of interest was a *Turnip mosaic virus* (TuMV) resistance gene, and infection with TuMV was used to identify 43 transgenic plants out of 1831 seeds. At present reports of *in planta* transformation of *Brassica* are limited, and further studies on the parameters of the system will be necessary, before it can be exploited as a routine transformation method. It should be noted that the efficiencies reported to date for *Brassica* are not dramatically different to early reports in the model plant *Arabidopsis*. However floral dipping may be more amenable to *Arabidopsis*, due to its size and thus ease of handling, faster life cycle and smaller seed, which lends itself better to subsequent downstream screening for positive transgenics.

As an alternative to floral dipping, micro-injection of the bacteria directly into the ovary of flower buds before fertilisation has been investigated. This technique has been successfully applied in *B. rapa* (Chinese cabbage) by Ji-Yong *et al*. (2003, 2004). Efficiencies of up to 0.56% were obtained when the floral stage at which the micro-injection was carried out, as well as the concentration of *Agrobacterium*, sucrose and surfactant used was optimised. This efficiency was based on injecting approximately 50 flower buds with a size of 2-3 mm, from which they obtained 500-800 seeds yielding on average 3-4 transgenics. Therefore, micro-injection also provides an alternative method for *Brassica* transformation, especially where facilities for tissue culture-based techniques are unavailable.

SELECTION OF TRANSGENICS

Approximately fifty marker genes used for transgenic and transplastomic plant research or crop development have been assessed for efficiency, biosafety, scientific applications and commercialization (Miki and McHugh 2004). Selectable marker genes can be exploited by either positive or negative selection systems. Positive selectable marker genes are defined as those that promote the growth of transformed tissue, in contrast to negative selectable marker genes that result in the death of the untransformed tissue.

The majority of work with positive selection systems has centred on the use of modified sugars such as phosphomannose (for example, the PMI system – used in *Brassica* by Sonntag *et al.* 2004) or disaccharide cellobiouronic acid (CbA) (developed by the CAMBIA group (www.cambia.org)).

Negative selection systems were the first to be developed and exploited using toxic agents, such as antibiotics, herbicides or drugs. Kanamycin is the most commonly used negative selectable marker gene used for most plant transformation work (Miki and McHugh 2004). The level of antibiotic used will depend on both the genotype and the transformation method used, and has been reported in the range of 5 mg/l to over 200 mg/l. Moloney *et al*. (1989) used 15 mg/l kanamycin for the selection of transgenic

shoots arising from cotyledonary explants of Westar, while Cordoza and Stuart used 200 mg/l kanamycin when Westar hypocotyl explants were used. Hygromycin has also been successfully used in *Brassica*. Cao *et al.* (1999) and Lee *et al.* (2004) found hygromycin to be a more effective selection agent than kanamycin in the *Brassica* genotypes they tested, with very few escapes coming through the system. However, as hygromycin is a much harsher selective agent than kanamycin it may not be suitable for all genotypes. From a regulatory point of view, hygromcin as a selectable marker is unlikely to gain approval for field release, unlike Kanamycin which has now achieved GRAS status (generally regarded as safe) (EU directive 2001/18). In such cases the ability to produce marker-free transgenics is desirable (i.e. where the selectable marker is later removed). Basta or glufosinate (herbicide resistance) has also been successfully used in *Brassica* (de Block *et al.* 1989), although less desirable for cotyledonary based transformation methods, due to Basta's mode of action targeting photosynthetic material. Basta is likely to be a useful selectable marker for floral dipping or micro-injection transformation methods, as larger numbers of seeds can be soil sown and seedlings sprayed with Basta to select for positive transgenics.

TRANSFORMATION AS A RESEARCH TOOL

The *Arabidopsis*/*Brassica* relationship

Brassica is closely related to the widely used model plant *Arabidopsis thaliana* and both belong to the *Brassicaceae* family. The divergence of *Arabidopsis* and *Brassica* are reported to have occurred 14-24 million years ago (Yang *et al.* 1999; Koch *et al.* 2000; Parkin *et al.* 2005). From comparative genetic analysis *Brassicas* are believed to be ancient polyploid relatives of the model species *Arabidopsis thaliana* (Osborn *et al.* 1997; Lagercrantz 1998; Parkin *et al.* 2002; Parkin *et al.* 2005). *B. rapa* (AA genome) and *B. oleracea* (CC genome) are allopolyploids; their genomes are thought to have arisen from triplication of an ancestral genome similar to that of *Arabidopsis*. It is therefore often the case that there are three paralogous genes in a diploid *Brassica* genome for each gene in *Arabidopsis* (and up to six in the amphidiploid *Brassicas* where genomes are combined).

Comparative analysis has revealed a high degree of genetic conservation between *Arabidopsis* and *Brassica*, with an average of 86% sequence identity in the coding regions of homologous genes (Parkin *et al.* 2005). These characteristics make *Brassica* an attractive system for model-to-crop approaches, where knowledge on gene function obtained in *Arabidopsis* can be tested in *Brassica* for crop improvement or to address fundamental scientific question (King *et al.* 2006; Trick *et al.* 2007). In either case, advances in this area will be facilitated by efficient and reliable transformation methods.

Exploring gene function

The function of a particular gene is often elucidated by creating a loss-of-function mutation and then analysing the resultant phenotype compared to the wild type (Østergaard *et al.* 2004). Traditionally, forward genetic screens have been employed to create individuals with abnormal phenotypes followed by the often tedious and lengthy process of mapping the mutated gene. With the large amount of sequence information for *Arabidopsis* and increasingly for *Brassicas* it is now possible to apply reverse genetics technology. Several of the most common reverse genetics approaches are based on transformation techniques such as T-DNA insertion (Alonso *et al.* 2003), RNAi (Horiguchi 2004), artificial miRNA (Schwab *et al.* 2006), antisense (Ecker and Davies, 1986) or the newly developed "Target mimicry" technique (for knocking down miRNA function) (Franco-Zorrilla *et al.* 2007). All of these techniques have been shown to work in *Arabidopsis* and it should be possible to apply them in *Brassica*.

To data a number of examples have been published. Byzova *et al.* (2004) used an RNAi approach to modify petals into sepaloid organs in *Arabidopsis* and oilseed rape. Silencing of the BPI gene family resulted in transgenic plants producing male fertile flowers in which the petals were converted into sepals (in *Arabidopsis*) or into sepaloid petals (in *B. napus*). These novel flower phenotypes were shown to be both stable and heritable in both species. Eason *et al.* (2005) down regulated BoCP5, a cysteine protease up-regulated during harvest-induced senescence, in broccoli. Post harvest floret senescence (yellowing) was delayed in the transgenic lines produced. In addition the florets were found to contain significantly greater levels of chlorophyll during post harvest storage at 20°C when compared to wild type plants.

CONCLUDING REMARKS

A range of transformation techniques has been used to introduce a wealth of agronomically useful traits into *Brassica* (reviewed by Christey in press) and we can expect to see this continue at an even higher rate in the years to come. The knowledge that has now been obtained in *Arabidopsis* and other plant species creates exciting opportunities for testing out in crop plants such as *Brassica* whether this knowledge can usefully be exploited for improving yield.

There have already been reports to suggest that transferring knowledge and technology from *Arabidopsis* to *Brassica* will be feasible in many cases (Chandler *et al.* 2005; Østergaard *et al.* 2006; Lee *et al.* 2007). For example, pod shattering is a major problem for oilseed rape farmers with average annual losses of 11-25% experienced due to unsynchronised seed dispersal (Price *et al.* 1996). The extensive knowledge on how fruit development in *Arabidopsis* is regulated showed that it is possible to produce pod shatter resistant *Brassica* fruits by over-expressing the MADS box gene *FRUITFULL* from *Arabidopsis* in *B. juncea* (Østergaard *et al.* 2006, 2007). This manipulation resulted in loss of the highly specified valve margin tissue in fruits and consequently to pod shatter-resistance as was also observed in *Arabidopsis* (Ferrándiz *et al.* 2000).

Pod shattering is just one example of an important trait that can be manipulated based on previous knowledge from *Arabidopsis*. Since oilseed rape is a relatively young crop, in comparison to wheat, barley, rice and maize, there is plenty of room for improvement of other traits. These include flowering time, branching, canopy architecture, fatty acid composition, overall seed oil production and disease resistance just to name a few. Transformation of *Brassica* is likely to play a prominent role in obtaining the goals for crop improvement, and it is therefore important to keep optimising and refining the current protocols as well as developing new approaches.

In this review, we have attempted to cover the recent advances and developments made in the transformation of *Brassica* species. The idea here has been to provide a general description of the available techniques based on own experience and examples from the literature. We hope this review provides potential users with useful guidelines on which direction to choose.

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