Progress in Genetic Manipulation of the Brassicaceae

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Abstract With the increasing advances in Brassicaceae genetics and genomics, considerable progress has been made in the transformation of Brassicaceae. Transformation technologies are now being exploited routinely to determine the gene function and contribute to the development of novel enhanced crops. *Agrobacterium*-mediated transformation remains the most widely used approach for the introduction of transgenes into Brassicaceae. In *Brassica*, the transformation relies mainly on *in vitro* transformation methods. Nevertheless, despite the significant progress made towards enhancing the transformation efficiencies, some genotypes remain recalcitrant to transformation. Advances in our understanding of the genetics behind various transformations have enabled researchers to identify more readily transformable genotypes for use in routine high-throughput systems. These developments have opened up exciting new avenues to exploit model *Brassica* genotypes as resources for understanding the gene function in complex genomes. Although many other Brassicaceae have served as model species for improving plant transformation systems, this paper summarizes on the recent technologies employed in the transformation of both *Arabidopsis* and *Brassica*. The use of transformation technologies for the introduction of desirable traits and a comparative analysis of these as well as their future prospects are also important parts of the current research that is reviewed.

Keywords Brassica, Brassicaceae, Improvement, Transformation

Introduction

The Brassicaceae family includes many economically important edible and industrial oilseed, vegetable, condiment, and fodder crop species. Cultivated *Brassica* species are a group of crops most closely related to *Arabidopsis thaliana*, and are members of this family. Oilseed Brassicas are found within *Brassica juncea*, *Brassica carinata*, *Brassica rapa* and *Brassica napus*, and are commonly called oilseed rape. Vegetable *Brassica* are an important and highly diversified group of crops grown worldwide, and belong mainly to the species *B. oleracea*, as well as *B. rapa* and *B. napus*. This group includes plants such as broccoli, Brussels sprouts, cabbage, cauliflower, collards, kale, kohlrabi, rutabaga, and turnip. *Brassica* vegetables contain little fat, and are sources of vitamins, minerals, and fiber. Several species, e.g., *Camelina sativa*, *Crambe abyssinica*, *Eruca vesicaria*, have potential as new edible oil/protein crops, biodiesel fuel crops, or platforms for bioproducts or molecular farming (Warwick et al. 2006). The family is also known for its more than 120 weedy species, several of which are important cosmopolitan agricultural weeds (e.g., wild mustard (*Sinapis arvensis*)), stinkweed (*Thlaspi arvense*) while others form crop-weed complexes (e.g., *Raphanus sativus*-*Raphanus raphanistrum*). Several of these related weeds are able to exchange genes, including transgenes, with crops under natural field conditions (reviewed in Warwick et al. 2003, 2008), potentially increasing weediness. Although most research in *Brassica* crops has been performed on oilseed and vegetable biotypes, rapid-cycling *Brassica* biotypes of various species have gained attention.

In recent years, considerable research has been conducted in the transformation and molecular breeding of *Brassicas*. As a research tool, transformation initially focused on the overexpression of the gene(s) of interest using the genes isolated from models and then later homologous crop genes, or the use of reporter genes to assess specific

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endogenous promoters. Most of the common reverse genetics approaches (the ability to knock out or silence genes) are also 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” techniques (for knocking down miRNA function) (Franco-Zorrilla et al. 2007). All of these techniques have been shown to work in *A. thaliana* and are also now beginning to be applied in *Brassica* species. Areas of particular interest include biotic and abiotic stress tolerance (Cao et al. 2008; Purty et al. 2008), oil synthesis (Mietkiewska et al. 2008) and plant architecture (Østergaard et al. 2006). With increasing knowledge of the function of genes, as well as the development of techniques for plant transformation, the potential for further improvement through Genetic Modification (GM) of these species is also considerable. To date, GM *B. napus* is the only *Brassica* species to gain commercial regulatory approval. GM herbicide-tolerant *B. napus* (canola) was the fourth most planted GM crop in 2007 (James 2007). The other major, so-called first-generation trait introduced into *Brassica* is the *Bacillus thuringiensis* toxin (Bt) to prevent insect attack. Experimental Bt-producing *B. napus* lines (Halfhill et al. 2002) and *B. juncea* lines (Cao et al. 2008) have been developed, and in *B. oleracea* vegetable crops including broccoli (Cao et al. 1999), cabbage (Jin et al. 2000) and chinese cabbage (Cho et al. 2001) have had the Bt *cryIAC* gene incorporated to control diamondback moth. On the other hand, the public perception of GM technology still hinders its global advancement, particularly in Europe. In the developed world, so-called next generation traits, such as modified oils containing increased Omega-3 fatty acids or broccoli with increased levels of antioxidants, as well as other such consumer-targeted traits that are likely to gain public acceptance.

This review presents relevant research with a view towards improving Brassicaceae crop plants with potential and desirable traits. This includes transgenesis and the advances made to Brassicaceae transformation methods, particularly the main *Brassica* species, as well as a discussion on how this technology can be exploited further to improve of Brassicaceae crops.

Genetic transformation

The relative ease of transformation and access to complete genomic sequences in *Arabidopsis* have led to an range of forward and reverse genetic approaches that provide great

insight into the gene function. On the other hand, conservation of the gene function between *Arabidopsis* and *Brassica* can still be confirmed using transgenic approaches, such as complementation of an *Arabidopsis* mutant phenotype with a *Brassica* orthologue or mimicking over-expression phenotypes in both *Arabidopsis* and *Brassica*. Transformation systems have been developed in almost all economically important species of *Brassica*, such as *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). Poulsen (1996) reviewed a range of methods used for *Brassica* transformation and the factors affecting transformation efficiencies. *Agrobacterium*-mediated transformation (either *A. tumefaciens* or *A. rhizogenes*) still remains the favored delivery approach for the introduction of transgenes into most dicotyledonous plant species and is rapidly becoming a common practice for an expanding range of monocots (Bartlett et al. 2008).

Agrobacterium-mediated transformation

Interest in transforming *Arabidopsis* followed the adoption of *A. thaliana* as a model plant species in the 1980s (reviewed by Somerville and Koorneef 2002), with a tissue culture-based *A. tumefaciens*-mediated transformation first reported in 1986 by Lloyd et al. (1986) and An et al. (1986). Further improvements in these methods were achieved using an *in vitro* root transformation method, which was recently adapted for *Arabidopsis lyrata* (Fobis-Loisy et al. 2007). Many publications reported improvements to tissue culture conditions and the use of reporter genes to determine the transformation efficiencies but the major limiting factor in many recalcitrant genotypes was still the poor tissue culture response and insensitivity to *Agrobacterium*. Progress was later made to identify and develop protocols that worked for a wider range of genotypes and *Brassica* species (Sparrow et al. 2006a; Bhalla and Singh 2008), as well as to identify suitable genotypes for use in routine transformation studies to test the gene function (Cardoza and Stewart 2006; Gasic and Korban 2006; Sparrow et al. 2006b). The simplest approaches using hypocotyls and cotyledons for Brassicaceae crop plants are described by different authors in different times with maximum transformation efficiency are mentioned in Table 1. In cotyledon method, generally cotyledonary petioles dipped into a suspension of *A. tumefaciens* and co-cultivated for 72 h on selection-free media before transferring to a basic MS basal medium supplemented with between 2 and 4 mg/l 6-benzyl-aminopurine (BAP). The explants were then

Table 1 *Agrobacterium*-mediated maximum transformation efficiency of different Brassicaceae species using hypocotyls and cotyledons explants

Name of Species	Transformation Efficiency (%)		References
	Hypocotyls	Cotyledons	
<i>B. oleracea</i>	30	9.5	De Block et al. (1989), Bhalla and Singh (2008)
<i>B. napus</i>	30	68.1	De Block et al. (1989), Bhalla and Singh (2008)
<i>B. rapa</i>	1	9	Wahlroos et al. (2003), Takasaki et al. (2004)
<i>B. juncea</i>	16	16.2	Gasic and Korban (2006), Bhuiyan et al. (2011)
<i>B. nigra</i>	33	-	Gupta et al. (1993)
<i>B. carinata</i>	14	50	Babic et al. (1998)

sub-cultured to fresh media after 2–3 weeks. Emerging shoots are isolated and rooted on a hormone-free medium (sometimes the addition of an auxin is needed). Hypocotyl methods use either pre-cultured hypocotyl segments (following a short callus induction period) or newly isolated hypocotyl segments immersed in a suspension of *A. tumefaciens* for 30–60 min. The explants were then washed and transferred to the co-cultivation medium for 48 h before transferring them to the selection medium. Both methods employ different media for callus induction, shoot induction, shoot elongation and rooting stages. Hypocotyl and cotyledonary petioles are used for *Agrobacterium*-mediated transformation in *B. oleracea* (Fig. 1, 2). Of the cultivated *Brassica* species, *B. rapa* remains the most recalcitrant to transform.

On the other hand, a limited number of successes have been reported: hypocotyl sections (Radke et al. 1992), cotyledonary petioles (Zhang et al. 2000), cotyledonary leaf sections (Yang et al. 2004), internodes from glasshouse-grown *B. rapa* (Kuvshinov et al. 1999), *A. tumefaciens* infiltration of whole plants (Qing et al. 2000) and microinjection of *A. tumefaciens* directly into flower buds (Yan et al. 2004). With the sequencing of the *B. rapa* genome currently underway (www.brassica.info.org) it is likely that efforts and interest in a *B. rapa* transformation will increase over the next few years. This will provide excellent resources to optimize the information arising from the Multinational *B. rapa* Genome Sequencing Project for *Brassica* functional genomics as it becomes available.

Fig. 1 *Agrobacterium*-mediated transformation using cotyledon explants in cabbage. A) Callus formation, B) Shoots differentiation, C) Shoot outgrowth, D) Transgenic plant selection in high antibiotic containing medium, E) Transgenic plants in rooting medium and F) Transgenic plant in soil

A. rhizogenes is a soil bacterium responsible for the development of hairy root disease of dicotyledonous plants and has been used to transform more than 79 plant species in its modified form (reviewed in Christey 2001). This bacterium has been used successfully to transform *Brassica*, where the focus has been on *B. oleracea* and *B. napus* (Puddephat et al. 2001). Overall, the transformation rates were low, but in some cases, transgenic *Brassica* plants were obtained more efficiently via an *A. rhizogenes*-mediated transformation than *A. tumefaciens* (Christey et al. 1997). The main disadvantage of using *A. rhizogenes*, however, was that *rol* genes were often transferred and expressed in plants regenerated from hairy roots, often exhibiting the associated altered phenotype, such as wrinkled leaves, shortened internodes, reduced fertility, and plagiotropic roots. On the other hand, these traits often segregated in the next generation, facilitating the recovery of normal transgenic plants (Puddephat et al. 2001). In *Arabidopsis*, *A. rhizogenes* root transformation of wild-type shoots (composite plants) is now being used for the “in root” gene testing of transgenes and plant-microbe interaction studies (Veena and Taylor 2007). Owing to the small size of *Arabidopsis*, the procedure is carried out *in vitro* but non-tissue culture conditions can be used for larger plants. Collier et al. (2005) reported composite plant production in *Brassica*, in which the transgene expression of reporter genes in composite plant

roots has been demonstrated.

Bacterial strains and plasmids

A number of different *A. tumefaciens* strains have been used successfully to transform Brassicaceae. GV3101 is used most frequently for *Arabidopsis* (Koncz and Schell 1986), whereas other disarmed strains of the C58 background can often be substituted with similar results. LBA4404 (Hoekema et al. 1983), an octopine strain, is also commonly used but generally works less well than GV3101. This lower efficiency is sometimes desirable for obtaining a greater proportion of transgenics with a low copy number (Bent 2006). LBA4404 is also used routinely in *Brassica*, as are the nopaline strains C58 (Sciaky et al. 1978) and derivatives AGL1 (Lazo et al. 1991) and EHA101 and EHA105 (Hood et al. 1986), which have all been used successfully and routinely. The use of GV3101, which is the preferred strain for an *Arabidopsis* transformation, has also been reported for *Brassica* (Mehra et al. 2000), even though its use in crops is far from routine. It is likely that over the next few years, the ability to use the plasmids and *Agrobacterium* strains used routinely for an *Arabidopsis* transformation will be highly desirable as researchers move their findings from model species to crops, such as *Brassica*. The type of plasmid used is believed to be less critical than bacterial strains, even

Fig. 2 *Agrobacterium*-mediated transformation using hypocotyl explants in broccoli. A) Callus formation, B) Shoots differentiation, C) Shoot outgrowth, D) Transgenic plants selection in high antibiotic containing medium, E) Transgenic plant in rooting medium and F) Transgenic plant in soil

though the choice of promoters and selectable markers is important. The plasmids commonly used for *Brassica* are as follows: modified pBIN19 (Bevan 1984); SLJ vectors (Jones et al. 1992), SLJ1714 and SLJ1711; the pCAMBIA vectors, particularly pCAM2200 (www.cambia.org); and pGreen (Hellens et al. 2000, www.pgreen.ac.uk). The above examples are based on the 35S promoter driving nptII, the favored selectable marker gene for *Brassica*, but other selectable markers are available.

Direct uptake transformation methods

Direct gene transfer of mesophyll protoplasts using PEG-mediated transfer was demonstrated in *Arabidopsis* by Damm et al. (1989) as well as in *Brassica* by Mukhopadhyay et al. (1991) (using hypocotyl protoplasts) and Eimert and Siegemund (1992) (for mesophyll protoplasts) using either electroporation or PEG-mediated transfer for cauliflower protoplasts. Radchuk et al. (2002) examined a range of factors effecting a PEG-mediated transformation in *Brassica* using both kanamycin and hygromycin selection, and Nugent et al. (2006) reported a successful transformation in cauliflower mesophyll protoplasts using a gus reporter gene and hygromycin selection. Regeneration from protoplasts remains the main limitation of this approach. Another direct approach is biolistics or microparticle bombardment, where DNA-coated beads are fired into plant cells at high speed. This approach has been used successfully in *B. napus* and the target tissue was pollen (Fukuoka et al. 1998). These approaches again offer an alternative transformation method for the genotypes not susceptible to an *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 (approximately 105 copies per plant cell) means that higher expression levels can be achieved than with a nuclear transformation. This is particularly desirable for a product-based transformation, where high protein yields are desirable (Dhinrga et al. 2004). The inheritance of the introduced transgene(s) will also be maternal and therefore offers a containment of the transgene due to the lack of gene flow through pollen (Daniell 2002), even though gene transfer from chloroplasts to nuclear genomes have been reported (Stegemann et al. 2003) but with extremely low frequencies. Plastid transformants have been produced in many species including *A.*

thaliana (Sikdar et al. 1998). In addition, a *Brassica* chloroplast transformation by particle bombardment was reported in both *B. oleracea* (Hou et al. 2003) and *B. napus* (Liu et al. 2007) as well as in *B. napus* using a PEG-mediated approach (Nugent et al. 2006).

Floral dipping/microinjection

The ability to bypass the sometimes complex tissue culture phase associated with the above methods can potentially overcome some of the barriers to transformation success observed in *Brassica*. An in planta transformation of *Arabidopsis* is a common practice and involves immersing intact inflorescences in suspensions of *A. tumefaciens* (Kojima et al. 2006). The *Agrobacterium* targets the ovules for the transformation event (Ye et al. 1999). 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). Qing et al. (2000) reported a transformation by the infiltration of adult *Brassica* plants with *Agrobacterium* for *B. rapa* (Pakchoi). Although this efficiency was low, it did demonstrate the potential of this method for *Brassica*. Recently, Zhandong et al. (2007) reported a transformation rate of 2.35% infected with Turnip mosaic virus (TuMV) for Chinese cabbage (*B. rapa*) using the method reported by Liu et al. (1998). In *B. napus*, Wang et al. (2003) reported a success rate of 0.18% using a double infiltration approach. Verma et al. (2008) reported a simplified floral dip method for *B. carinata* and *B. napus* and Curtis and Nam (2001) reported a similar method for radish (*Raphanus sativus* L. *longipinnatus* Bailey). Reports of in planta transformation of *Brassica* are limited, and further studies on the parameters of the system will be needed before it can be exploited as a routine transformation method. The efficiencies reported to date for *Brassica* are similar to early reports in the model plant *Arabidopsis*. On the other hand, floral dipping may be more amenable to *Arabidopsis* owing to its size as well as its ease of handling, faster life cycle and smaller seed, which allows subsequent downstream screening for positive transgenics.

As an alternative to floral dipping, a microinjection of the bacteria directly into the flower bud (effectively flooding the bud cavity) before fertilization has been investigated. Yan et al. (2004) applied this technique successfully to *B. rapa* (Chinese cabbage). Efficiencies of up to 0.56% were obtained when the microinjection was carried out in the floral stage and when the concentration of *Agrobacterium*, sucrose and surfactant used was optimized. 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 an average of three to four transgenics. Therefore, a microinjection also provides an alternative method for a *Brassica* transformation, particularly where the facilities for tissue culture-based techniques are unavailable.

Selection of transgenics

Selectable marker genes can be classed as either positive- or negative-based selection. Positive selectable marker genes are defined as those that promote the growth of transformed tissue, where the majority of work has centered on the use of modified sugars, such as mannose (Reed et al. 2001), disaccharide, cellobiouronic acid (CbA) (developed by the CAMBIA group, www.cambia.org), or glucose using the trehalose-6-phosphate synthase (TPS) system (Leyman et al. 2006). These types of selectable markers are likely to face fewer regulatory hurdles if the overall goal is commercial transgenic plants. Negative selection systems using toxic agents, such as antibiotics, herbicides or drugs, were the first to be developed and exploited. Kanamycin is the most commonly used negative selectable marker in most plant transformation work (Miki and McHugh 2004). The level of antibiotics used will depend on both the genotype and transformation method used; kanamycin has been applied in the range of 5 mg/l to > 200 mg/l. Moloney et al. (1989) used 15 mg/l kanamycin to select transgenic shoots arising from cotyledonary explants of the *B. napus* cultivar Westar, whereas Cordoza and Stewart (2006) used 200 mg/l kanamycin on Westar hypocotyl explants. Hygromycin has also been used successfully in *Brassica*. Lee et al. (2004) reported hygromycin to be a more effective selection agent than kanamycin in the *Brassica* genotypes they tested; with very few escapes coming through the system. On the other hand, hygromycin may not be suitable for all genotypes because it is a much harsher selective agent than kanamycin. From a regulatory point of view, hygromycin, as a selectable marker, is unlikely to gain approval for field release. This is unlike kanamycin, which has achieved GRAS status (generally regarded as safe) (EU directive 2001/18). In such cases, studies to avoid marker genes or to eliminate them after use have been initiated and a growing number of methods are under development (Sundar et al. 2008; Rukavtsova et al. 2009). Recently, an *Arabidopsis* AthOL1 gene with high S-adenosyl-L-methionine-dependent methyl-transferase activity towards the thiocyanate ion was used as a positive selection gene for an efficient selectable marker for screening the transformed seedlings of *Arabidopsis* (Midorikawa et al. 2009). Upadhyaya et al. (2010) also reviewed the

development of marker-free transgenic plants. Basta or glufosinate (herbicide resistance) has also been used successfully in *Brassica* (de Block et al. 1989), even though it is less desirable for cotyledonary-based transformation methods because of mode of action of Basta in targeting photosynthetic materials. Basta is used frequently as a selectable marker for floral dipping or microinjection transformation methods, where large numbers of seeds can be soil sown and seedlings sprayed with Basta to select positive transgenics.

Recovery of transgenic plants

Plant regeneration is an indispensable tool for plant transformation. With the exception of the floral dip method, the transformation methods all rely on a robust regeneration system for transformation success. Regeneration protocols have been developed for most *Brassica* species and organogenesis is the pathway used most widely for regeneration. The regeneration of plants by organogenesis has been accomplished from a range of tissues, such as cotyledons (One et al. 1994; Yang et al. 2004), hypocotyls (Yang et al. 1991), peduncle segments (Eapen and George 1997), leaves (Radke et al. 1988), leaf sections (Alaska-Kennedy et al. 2005), anthers, microspores (Keller and Armstrong 1977; Litcher 1982), thin cell layers of epidermal and subepidermal cells (Klimaszewska and Keller 1985), roots (Xu et al. 1982), and protoplasts (Hu et al. 1999). Nevertheless, it is the seedling explants (cotyledons and hypocotyls) that remain favorites for the transformation, and have been used for most *Brassica* species because of their ability to regenerate. The following discusses many of the factors that affect the successful regeneration of shoots *in vitro*.

Genotype and explant

The genotype-dependent nature of *in vitro* shoot regeneration, both within and among *Brassica* species, was first reported by Murata and Orton (1987), who suggested shoot regeneration to be a heritable trait. Sparrow et al. (2004) examined the genetic control of shoot regeneration from cotyledonary petioles using diallel crosses in *B. napus* and *B. oleracea*, respectively. A large variation was observed in the regeneration frequency of 51 genotypes of *B. carinata* (Gil-Humanes et al. 2011). In *B. napus* there was a huge variation in the 100 cultivars tested, ranging from 0% to 91% (Ono et al. 1994). Of the 123 genotypes of Chinese cabbage (*B. rapa* ssp. *pekinensis*) tested, a large variation

in regeneration frequency was observed, ranging from 95% to 0% (Zhang et al. 1998). Therefore, genotype specificity is a limiting factor in *Brassica* tissue culture and regeneration, which severely limits the germplasm that can be manipulated or improved. The age and size of the explant is also critical for optimal results. For example, 3-day-old seedlings of *B. rapa* ssp. *oleifera* yielded better regeneration than those older than 4 days (Burnett et al. 1994). In *B. napus*, 4-day-old seedling explants proved optimal, yielding a 90% regeneration rate (Ono et al. 1994). In rapid-cycling *B. rapa*, 3-day-old explants from seedlings were best for regeneration (Teo et al. 1997). In all these cases, the regeneration capacity decreased when the age of the seedling was increased above 4 days. For hypocotyl sections, older explants (8–10 days) have also been used for *B. napus* allowing a larger number of explants to be obtained per seedling (Cardoza and Stewart 2006; Maheshwari et al. 2011). On the other hand, Gasic and Korban (2006) reported that 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 performed better). Long hypocotyl sections had a tendency to curl and therefore loose contact with the culture media.

Media constituents

Regeneration success is affected by the choice of transformation protocol, hormones and the levels of other media additives used. Hypocotyl and leaf sections often require a callus phase prior to shoot regeneration (Yang et al. 2004; Gasic and Korban 2006). This is normally achieved using a high cytokinin (usually BAP) to low auxin (e.g. NAA) ratio. Gil-Humanes et al. (2011) obtained approximately 82% shoot regeneration from a hypocotyls culture using 5 mg/l BA and 1 mg/l 2,4-D in *B. napus*. The regeneration efficiency was highest with BAP 3.0 mg/l/NAA 0.3 mg/l in *B. juncea* (Bano et al. 2010). The optimum regeneration was obtained using a combination of BAP (3.0 mg/l) and IAA (0.2 mg/l) in *B. juncea* (L) (Singh et al. 2009). Some transformation protocols also exploit a short callus induction phase prior to a transformation, a so-called preconditioning stage (Cardoza and Stewart 2006). In addition, a variety of media additives might increase the regeneration efficiencies in *Brassica*. Methylglyoxal-bis-(guanyldiazide) (MGBG), an inhibitor of spermidine biosynthesis, was reported to increase the regeneration frequencies from 7% to 63% in *Brassica* and other genera (Sethi et al. 1990). Putrescine, a polyamine,

however, was found to enhance shoot regeneration in Chinese radish when used with silver nitrate or aminoethoxyvinylglycine (Pua et al. 1996). On the other hand, putrescine was not effective when used alone, but was synergistic with ethylene inhibitors. Brassinolides, a relatively new class of compounds, have also been found to stimulate the production of adventitious shoots from cauliflower hypocotyl segments (Sasaki 2002). Infection was most effective (highest infection frequency) when Chinese cabbage explants were infected with *Agrobacterium* for 15 min and co-cultivated for 3 days in a co-cultivation medium at pH 5.2 supplemented with 10 mg/l acetosyringone (Zhang et al. 2000).

Hyperhydricity and tissue necrosis is a serious problem for plant tissue culturists and many factors including the accumulation of ethylene and high humidity in culture vessels (de Block et al. 1989), excessively rich media (Ziv 1991), *Agrobacterium* overgrowth/sensitivity (Jin et al. 2000), and high doses of exogenous cytokinin and/or auxin (Kamal et al. 2007) are found to effect it. In *B. napus* (Cardoza and Stewart 2003), the relative humidity of the culture vessel and hyperhydricity decreased with increasing percentage of gelling agent (from 0.8% phytagar to 1.2%) in the shoot elongation medium. Ethylene is another key factor in optimizing tissue culture conditions for some *Brassica* species (Cardoza and Stewart 2004). Silver nitrate has been used to reduce the hyperhydricity in a range of *Brassica* species, such as *B. rapa* (Yang et al. 2004) and *B. napus* (Tang et al. 2003). Excluding silver nitrate in the media drastically reduced the regeneration frequency of *B. napus* (Khan et al. 2002). In Chinese radish (*Raphanus sativus* var. *longipinnatus*), a combination of silver nitrate and aminoethoxyvinylglycine was found to enhance shoot regeneration significantly (Pua et al. 1996). Other ethylene inhibitors that can be used either in combination or as an alternative include silver thiosulphate (Eapen and George 1997) and aminoethoxyvinylglycine, which was used by Chi et al. (1990) for *B. rapa* and *B. juncea*, Pua and Chi (1993) for *B. juncea*, and Burnett et al. (1994) for *B. rapa*.

Rooting *in vitro*

Once shoot regeneration has been achieved from the desired genotype, another stumbling block can be the isolation of viable shoots. A shoot elongation step, where the cytokinin levels are lowered but not removed, can often help elongate the shoots (Cardoza and Stewart 2006; Bhalla and Singh 2008). Often hyperhydricity is overcome once the shoots have elongated, but occasionally, other

supplements need to be explored. Rooting is often achieved by simply removing the cytokinin (Sparrow et al. 2006b). Nevertheless, in some cases it may also require the addition of an auxin (such as NAA or indole-3-butyric acid (IBA)) (Cardoza and Stewart 2006; Verma and Singh 2007; Bhalla and Singh 2008; Singh et al. 2009).

Conclusions and future prospects

Plant cellular and molecular biology techniques have tremendous potential for plant improvement. Among these techniques transformation can be of use in the development of plants with novel desirable traits with high accuracy. Many genetic improvements with the highest impact have come from genetic transformation. A range of transformation techniques have been used to introduce a wealth of agronomically useful traits into Brassicaceae such as insect/disease/bacterial/fungal/viral and herbicide resistance, those involved in flowering control, post-harvest attributes, altered stress tolerance, and altered health benefits including altering the anthocyanin/sulphur/vitamin or amino acid levels (reviewed by Christey and Braun 2007). This is expected to 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 crop plants, such as *Brassica*. There have already been reports suggesting that transferring knowledge and technology from *Arabidopsis* to *Brassica* will be feasible in many cases (Østergaard et al. 2006; Lee et al. 2007). The extensive knowledge on how fruit development in *Arabidopsis* is regulated showed that it is possible to produce pod shatter-resistant *Brassica* fruits by overexpressing the MADS box gene *fruitful* from *Arabidopsis* in *B. juncea* (Østergaard et al. 2006). This manipulation resulted in a loss of 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 compared to wheat, barley, rice, and maize, there is considerable room for improving other traits. These include the flowering time, branching, canopy architecture, fatty acid composition, overall seed oil production, and disease resistance. The transformation of Brassicaceae is likely to play a key role in obtaining the goals for crop improvement. Therefore it is important to keep optimizing and refining the current protocols as well as developing new approaches.

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