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Genetic transformation in oilseed brassicas - A review

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ABSTRACT

Brassica is the most economically important genus in the cruciferae family, remarkable for containing agricultural and horticultural crops than any other genus. It includes over 30 wild species, their cultivars and hybrids including six most important species of *Brassicas, Brassica campestris*, *B. juncea, B. carinata* and *B. napus* which are oil yielding species. Due to their agricultural importance, *Brassicas* have been the subject of much scientific interest and considerable progress has been made in *Brassica* biotechnology. The molecular breeding and transformation technology for the introduction of desirable traits are two main strategies for its improvement. Several genes of agronomic importance have been identified in *Brassica* species and other identified genes have been transferred in *Brassica* species. Plant regeneration, transformation methods and gene of interest are the main requirements for gene transfer in any crop. Regeneration has been optimized via organogenesis and somatic embryogenesis using various explants. Transformation systems have been developed in almost all the economically important species of *Brassica* such as *B. juncea*, *B. napus*, *B. rapa*, *B. oleracea*, *B. nigra*, and *B. carinata*. Transformation has improved *Brassica* species for many traits, such as herbicide resistance, insect resistance, salt tolerance, oil quality improvement, for the production of pharmacological and industrial products, development of male-sterile lines and restoration system. Among different methods of transformation, *Agrobacterium* mediated genetic transformation is most widely used for *Brassica* and it is generally quite efficient and practical for most species in the genus. However, there is still a need for development of efficient transformation methods to overcome genotype dependency. In the present paper, the work on regeneration and genetic transformation in the *Brassica* species will be reviewed.

Key words: Genetic transformation, Oilseed brassicas

Brassica is the most economically important genus in the *Brassicaceae* family. Several species and types of *Brassicas* are significant oilseed crops, vegetables, forage crops and are used in the production of condiments, such as mustard. Among the *Brassica* crops, oilseeds have the highest economic value. The oilseed *Brassica* are found within *Brassica juncea, Brassica carinata, Brassica rapa* (syn *Brassica campestris*) and *Brassica napus* collectively and are commonly called oilseed rape. When *Brassica* oils are low in aliphatic glucosinolates and erucic acid, the varieties are increasingly commonly referred to as canola, a more pleasant-sounding name. Canola, which is most often *B. napus*, has received much attention worldwide and may soon be the most popular oilseed crop. There are now also canola-quality *B. rapa* and *B. juncea* varieties. Canola oil is widely used in cooking since it is very low in saturated fat, making it appealing to health conscious consumers. *Brassica nigra* is mainly used as a mustard condiment in addition to oil. While 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. These rapid-cycling types were genetic selections having short life cycles of 20–60 d and small sizes (Williams and Hill 1986). Rapid cycling *Brassica* are attractive model laboratory plants because of their small genome sizes, in some cases just 3–4-fold larger than *Arabidopsis thaliana* (Arumuganathan and Earle 1991). Other desirable qualities are their high female fertility, rapid seed maturation, and absence of seed dormancy. Considerable research has been conducted in tissue culture, transformation and molecular breeding of the *Brassica*. In the present paper, the work on genetic transformation in the *Brassica* species will be reviewed.

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In vitro *culture and transformation*

In vitro culture is an important tool of plant biotechnology

that exploits the totipotent nature of plant cells, demonstrated for the first time by Steward *et al.* (1958). *In vitro* culture facilitates rapid multiplication of superior clones and is a pre-requisite for improvement of plants via genetic engineering technique. Tissue culture has been exploited to create genetic variability by producing haploids, somaclonal and gametoclonal variants for crop improvement. Tissue culture, in combination with genetic engineering techniques, has been successfully used to incorporate specific traits through gene transfer.

Organogenesis

Organogenesis is an important tool for plant regeneration using tissue culture techniques and for plant transformation. *Brassica* species have been widely exploited for tissue culture purposes. Regeneration protocols have been developed for most of the *Brassica* species. Regeneration of plants via organogenesis has been accomplished from various tissues such as cotyledons (Sharma *et al*. 1990; Hachey *et al*. 1991, Ono *et al*. 1994, Gaur *et al.* 1997), hypocotyls (Das *et al*. 2006, Gaur *et al.* 1997), peduncle segments (Eapen and George 1997), leaves (Radke *et al*. 1988), thin cell layers of epidermal and sub-epidermal cells (Klimaszewska and Keller 1985), crushed apical buds (Surya Prakash *et al.* 1991), roots (Xu *et al*. 1982), and protoplasts (Glimelius 1984, Kik and Zaal 1993, Hu *et al*. 1999). However, hypocotyl segments remain the most desirable explants for tissue culture and have been used for most *Brassica* species because of their ability to regenerate. Numerous aspects of tissue culture conditions that affect plant regeneration are discussed below.

Genotype

Regeneration in *Brassica* is highly genotype dependent and has been reported in several species. In *B. napus* there was a huge variation ranging from 0% to 91% in the 100 cultivars tested (Ono *et al*. 1994). *B. napus* cultivar GSL 1 showed better regeneration efficiency than Westar (a standard cultivar for transformation) in one study (Phogat *et al*. 2000). Indian cultivars were found to have better regeneration rates than Australian cultivars in *B. juncea* (Pental *et al*. 1990). Yadav *et al.* (1991) found that regeneration frequencies significantly differed in three genotypes using cotyledonary explants. Thus, genotype specificity is a limiting factor in *Brassica* tissue culture and regeneration, which severely limits the germplasm that can be manipulated or improved.

Age of the explant

In most *Brassica* species, regeneration is dependent on the age of the explant. Young explants have been shown to give better results than older explants in most *Brassica* species. Most researchers have found that explants excised from 3–4-d-old seedlings gave optimal regeneration rates. For example, 3-d-old seedlings of *B. rapa* ssp *oleifera* yielded better regeneration than those older than 4 d (Burnett *et al*. 1994). In *B. napus*, 4-d-old seedling explants proved optimal, yielding a 90% regeneration rate (Ono *et al*. 1994). In rapid-cycling *B. rapa*, 3-d-old explants from seedlings were best for regeneration (Teo *et al*. 1997). In all the above cases, regeneration capacity declined when the age of the seedling was increased above 4 d. In *B. juncea* (Sharma *et al*. 1990) and *B. rapa* (Hachey *et al*. 1991), 3–5-days old seedlings gave optimal shoot regeneration while *Gaur et al.* (1996) found 5-6 days old seedlings optimum for plant regeneration from cotyledon and hypocotyls explants in *Brassica juncea* cv RH 30 and RH 8812.

Media constituents

Various media additives might increase regeneration efficiencies in *Brassica*. Methylglyoxal-bis-(guanylhydrazone) (MGBG), an inhibitor of spermidine biosynthesis, was reported to increase regeneration frequencies from 7% to 63% in *Brassica* and other genera (Sethi *et al*. 1990), also other researchers found a positive effect of MGBG on shoot regeneration of *B. napus* (O'Neill *et al*. 1996). Silver nitrate is routinely used in *Brassica* tissue culture. Other ethylene inhibitors such as silver thiosulfate (Eapen and George 1996, 1997) and amino-ethoxy-vinyl-glycine (Chi *et al*. 1990, Burnett *et al*. 1994) have also been reported to have a positive effect on regeneration in *Brassica* species. Excluding silver nitrate in media drastically reduced regeneration frequency in *B. napus* (Phogat *et al*. 2000, Khan *et al*. 2002).

Genetic transformation

The availability of various genetic engineering tools has opened up vast opportunities for introducing novel and useful genes of agronomical importance in various crops. The application of such technologies requires the development of reliable and reproducible methods for introducing foreign DNA in plant cells and the regeneration of normal and fertile transgenic plants from *in vitro* cultures. An essential component of plant biotechnology is genetic transformation which provides introduction of useful genes.

Why genetic modification?

Although, in past, conventional breeding has been a potent source for generating varieties with high yield and quality, very few cultivars and lines of potential crops are known that have been developed using the conventional strategy. The little success of modern breeding approaches towards the improvement of crop species is thought to be attributable to the quantitative nature of most of the processes involved, for example, the salt-tolerance trait is controlled by polygenes (Ashraf 2002, Quesada 2002). Moreover, the approach is time-consuming and labor-intensive simultaneously allowing the transfer of undesirable genes along with desirable ones. Furthermore, reproductive barriers limit transfer of favourable alleles from interspecific and

intergeneric sources. Under such circumstances, genetic engineering has proven to be a powerful means of transferring genes effectively across reproductive barriers.

Agrobacterium *mediated gene transfer*

Agrobacterium tumefaciens causes crown gall disease and used as a vector to create transgenic plants in many agronomically and horticulturally important species. It contains T-DNA which integrates into plant genome upon infection. Depending on *Agrobacterium* ability, scientist have inserted genes of interest with in the right border and left border sequences of T-DNA in place of the tumour inducing genes and subsequently into the plant cell genome. *Agrobacterium*-mediated gene transfer is a method of choice, when aiming for stable transformation in dicotyledon and monocotyledon species including cereal and vegetable crops (Hewezi *et al.* 2002). The success of first gene transfer in plants mediated by soil born pathogen *Agrobacterium tumefaciens* (De Block *et al.* 1984, Horsch *et al.* 1985) was limited to *Solanaceae*. Later on, the advancement of culture conditions, understanding of molecular biology of *vir* genes and T-DNA transfer process, it became possible to transform all the crops species using *Agrobacterium* as a vehicle of transformation. Yadav *et al.* (1996) used *Agrobacterium rhizogenes* for transforming *Brassica carinata*. Several genotypic, culture and *Agrobacterium* with target gene factors affect transformation efficiency which is summarized below in Table 1.

Effect of explants

As evident from Table 1, hypocotyl explants have been widely used for majority of the transformation studies as reported by Wu *et al.* (2009), De Block *et al.* (1989), Wang *et al.* (2005), and Moghaieb *et al.* (2006). Although, there have been successful reports of utilization of other explants such as cotyledons (Yadav *et al.* 1996,1997), cotyledonary nodes (Kong *et al*. 2009), leaf petioles (Liu *et al.* 2011) have been successfully employed for development of transgenic plants.

Effect of pre-culture

Several workers have suggested that pre-culture of explants before agro-infection enhances the transformation frequency in *Brassica,* two days by Singh *et al.* (2009); one day by Barfield and Pua (1991), whereas Das *et al.* (2006) reported 3-4 days pre culture enhances the transformation frequency.

Table 1 Summary of different factors affecting transformation efficiency in *Brassica napus* and *Brassica juncea*

Species	Explant	*Agrobacterium strain*	Trait	Selectable marker {conc. (mg/l)}	Transformation efficiency	References
Brassica juncea	Cotyledonary nodes	GV 2260	Insect resistance	Kanamycin (20)	89%	Sharma *et al.* (2004)
	Cotyledonary petioles	GV3101	Insect resistance	Kanamycin (20)	15-20%	Singh *et al.* (2009)
	Hypocotyls	LBA4404	Increased Linolenic acid content	Hygromycin (30)	6.6%	Das *et al.* (2006)
	Hypocotyls	EHA101	Tolerance to choline	Kan (25)	5%	Prasad *et al.* (2000)
	Hypocotyls	LBA4404	Pest resistance	Hyg (30)		Dutta *et al.* (2005)
Brassica napus	Hypocotyl and Cotyledon	LBA4404	chitinase gene	PPT (15)	Hypocotyl (1.14%) Cotyledon (0.01%)	Longdou *et al.* (2005)
	Hypocotyls	EHA105		Kan (10)		Wu *et al.* (2009)
	Cotyledonary petioles	EHA101	Sulfonylurea resistance	Kan (15)		Blackshaw *et al.* (1994)
	Leaf petioles	GV3101	Pest resistance	Hyg (3)	4.5%	Liu *et al.,* (2011)
	Hypocotyls	LBA4404	Pest resistance	Kan (30)	8.44%	Wang *et al.* (2005)
	Hypocotyls	GV3850		Kan (200)	25%	Cardoza & Stewart (2003)
	Floral dip method	LBA 4404		Kan (300)	3%	Li *et al.* (2010)
	Hypocotyls	EHA 101		Kan (25)		Lee *et al.* (1991)
	Hypocotyls	LBA4404		Kan (50)	31%	Moghaieb *et al.* (2006)
	Cotyledonary nodes	LBA4404		Hyg (10)	18.93%	Kong *et al*. (2009)
	Hypocotyls	EHA105	Fungal resistant	Kan (10)		Wu *et al.* (2009)

Agrobacterium *concentration and duration of infection*

Agrobacterium concentration is one of the key factors that affect genetic transformation frequency. Various workers have worked on optimization of bacterial concentration used for infection of explants. For *Brassica* transformation, *Agrobacterium* concentration usually range between 0.01 and 1.0 at $OD_{600\ nm}$ (Wu *et al.* 2009, De Block *et al.* 1989, Longdou *et al.* 2005, Das *et al.* 2006, Wang *et al.* 2005, and Moghaieb *et al.* 2006) and overnight grown *Agrobacterium* cultures are usually diluted prior to infection. Duration of infection of explants with *Agrobacterium* has been reported to be highly variable, from a few minutes to 30 minutes. Li *et al.* (2009) gave infection to the explants only for 1-2 minutes, whereas Singh *et al.* (2009) extended the agro infection step for 30 minutes.

Effect of acetosyringone

Certain phenolic compounds induce expression of *vir* genes and acetosyringone is frequently used in genetic transformation experiments as reported by several researchers. Moghaib *et al.* (2006) and Wu *et al.* (2009) used 100 µM acetosyringone for transformation in *Brassica* while Li *et al*. (2009) reported 200 µM acetosyringone concentration to be suitable for transformation experiments. Therefore, the plants which do not naturally produce sufficient amounts of *vir*-inducing phenolic compounds, addition of acetosyringone, at the time of pre-culture or co-cultivation has been found to improve the transformation efficiency in such plants.

Antibiotics for killing excess Agrobacterium *after co-cultivation*

Growth of the *Agrobacterium* after co-cultivation reduces the efficiency of transformation, hence removal of *Agrobacterium* is necessary (Jabeen *et al.* 2009). So, after co-cultivation of explants with *Agrobacterium* cells, it is important to arrest their further growth and multiplication on plant tissue culture media. Antibiotics used to inhibit and arrest the *Agrobacterium* growth, should be highly effective, inexpensive, stable and have no negative effect on plant regeneration. Carbenicillin, cefotaxime and timentin (100-500 mg/l) are the most commonly used antibiotics for suppressing *Agrobacterium* growth as reported by Sharma *et al.* (2004), Singh *et al.* (2009), Das *et al.* (2006), Wang *et al.* (2005), Moghaieb *et al.* (2006) and Wu *et al.* (2009).

Development of selection system

Selection is an essential component of any plant transformation strategy and accomplished by using selectable marker genes. Selectable marker genes confer resistance to an antibiotic. Efficient selection depends on kind of antibiotics employed and their concentration used. Resistance to the aminoglycoside antibiotic kanamycin has been used extensively in plant transformation experiments. Other antibiotic resistance genes that have been used successfully for plant transformation include, hygromycin resistance gene and gentamycin resistance gene.

Genetically modified brassicas

Transformation systems have been developed in almost all the economically important species of *Brassica* such as *B. juncea* (Barfield and Pua 1991, Gaur *et al.* 1997), *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, Yadav *et al.* 1997).

Transformation has improved *Brassica* species for many traits, but the most prominent trait has been for herbicide resistance (HR) and HR canola is the fourth most planted transgenic crop in the world. Canola tolerant to herbicides such as imidazoline, glufosinate, and glyphosate is now available commercially in the USA and Canada. Other examples of herbicide resistance include glufosinate resistance in *B. rapa* (Qing *et al*. 2000), sulfonylurea resistance in *B. napus* (Blackshaw *et al*. 1994), and bromoxynil resistance in *B. napus* (Zhong *et al*. 1997).

Oil quality improvement has been another target for *Brassica* transformation. *Brassica* oil is in great global demand and technology is available to custom-tune fatty acid profiles in seeds. Silencing the endogenous oleate desaturase increased oleic acid levels in *B. napus* and *B. juncea* (Stoutjesdijk *et al*. 2000). Canola with high g-linolenic acid was produced by transformation of d12-desaturase genes from the fungus *Mortierella alpina* (Liu *et al*. 2001). By expressing Garm FatA1, an acyl-acyl carrier protein (ACP) thioesterase isolated from *Garcinia mangostana* in canola, Hawkins and Kridl (1998) have been able to produce canola with elevated levels of stearate.

In addition to oil improvements, *Brassica* transformation can convert a crop to biochemical factories for the production of pharmacological and industrial products, such as the biodegradable polymer poly(b-hydroxybutyrate) (PHB). Oilseed *Brassica* species are ideal candidates for the commercial production of PHB since acetyl-CoA, the substrate required for the first step of PHB biosynthesis, is prevalent during oilseed fatty acid biosynthesis. Three genes (phbA or bktB, phbB and phbC) that are required for the production of the biopolymer were engineered in *B. napus* and the polymer was produced in the leucoplasts (Houmiel *et al*. 1999).

B. carinata was used in the production of a blood anticoagulant protein, hirudin (Chaudhary *et al*. 1998). An oleosin–hirudin fusion protein was engineered using *Agrobacterium*. The advantage of using *Brassica* oilseeds for the production of biologically active components fused with proteins such as hirudin is the ease of purification of the protein by floatation centrifugation. *B. napus* has also been used in the production of carotenoids (Shewmaker *et al*. 1999), which act as antioxidants in the human body. Insect resistance is a target trait since there are increasing pest

problems in *Brassica* crops. Since *Brassica* crops are susceptible to the diamondback moth, a good approach to control it is to overproduce a *Bacillus thuringiensis* endotoxin crystal protein such as *Bt Cry1A(c).* This gene has been introduced in *B. napus* (Liu *et al.* 2011, Wang *et al.* 2005), Chinese *B. napus* cultivars (Li *et al*. 1999) and rutabaga (Li *et al*. 1995).

Another important advancement in the transformation of *Brassica* crops is the development of male-sterile lines and the development of a restoration system. In *B. juncea,* it was possible to obtain male sterile plants by introducing the barnase gene with tapetum-specific promoters (Jagannath *et al*. 2001). The fertility of the male-sterile line was restored by crossing it with a barstar-containing transgenic line (Jagannath *et al*. 2002). This male sterility/fertility restorer system has tremendous potential in hybrid breeding.

CONCLUSIONS

Great advances have been made in molecular genetics of brassicas. Development of genome maps and the identification of molecular markers for genes of interest to breeders, particularly those which are important across the range of different crop types, should greatly facilitate future crop improvement. Transgenic techniques for the transfer of DNA sequences so identified and refined will have immediate value as research tools to gain knowledge of genes and their regulation and expression.

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