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REVIEW

Biotechnological advancement in genetic improvement of broccoli (*Brassica oleracea* L. var. *italica*), an important vegetable crop

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Abstract With the advent of molecular biotechnology, plant genetic engineering techniques have opened an avenue for the genetic improvement of important vegetable crops. Vegetable crop productivity and quality are seriously affected by various biotic and abiotic stresses which destabilize rural economies in many countries. Moreover, absence of proper post-harvest storage and processing facilities leads to qualitative and quantitative losses. In the past four decades, conventional breeding has significantly contributed to the improvement of vegetable yields, quality, post-harvest life, and resistance to biotic and abiotic stresses. However, there are many constraints in conventional breeding, which can only be overcome by advancements made in modern biology. Broccoli (*Brassica oleracea* L. var. *italica*) is an important vegetable crop, of the family Brassicaceae; however, various biotic and abiotic stresses cause enormous crop yield losses during the commercial cultivation of broccoli. Thus, genetic engineering can be used as a tool to add specific characteristics to existing cultivars. However, a pre-requisite for transferring genes into plants is the availability of efficient regeneration and transformation techniques. Recent advances in plant genetic engineering provide an opportunity to improve broccoli in many aspects. The goal of this review is to summarize genetic transformation studies on broccoli to draw the attention of researchers and scientists for its further genetic advancement.

Keywords *Agrobacterium* · Abiotic · Biotic resistance genes · Direct gene transfer · Genetic transformation · Plant biotechnology · Transgenic

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Introduction

Vegetables play an important role in human nutrition and health by providing minerals, micronutrients, vitamins, antioxidants, phytosterols and dietary fiber. Broccoli (*Brassica oleracea* L. var. *italica*) is an important vegetable crop of the family Brassicaceae ($2n = 18$). In India, it is still cultivated on a limited scale but its cultivation holds promise throughout the temperate and tropical regions. It is recognized as one of the most nutritious crops, especially in calcium, antioxidants, vitamin A, vitamin K, β-carotene, riboflavin and iron content (Vallejo et al. 2003; Abdel-Wahhab and Aly 2003). It has anti-cancer properties which are contributed by sulforaphane glucosinolate (Keck et al. 2003), quinone reductase, glutathione-*S*-transferase (Zhang et al. 1992; Fahey et al. 1997) and a high selenium content (Finley et al. 2001; Finley 2003). Environmental stress, pests and diseases cause enormous yield losses because of a limited gene pool.

Genetic manipulation is becoming an important method for broccoli improvement. Classical breeding techniques are insufficient for the genetic improvement. Plant breeders in the past several decades have used inter-specific hybridization to transfer genes between species. Sexual incompatibility barriers severely limit the possibilities for gene transfer between species, although some of the *Brassica* can be easily crossed using plant breeding techniques and through somatic hybridization (Puddephat et al. 1996). Thus, genetic engineering can be used to add specific characteristics to existing cultivars. However, a prerequisite for transferring genes into plants is the availability of efficient regeneration and transformation methods.

Plant regeneration studies in broccoli have been reported by various researchers using different explants such as the peduncle (Christey and Earle 1991), anther (Chang et al. 1996), protoplasts (Kaur et al. 2006) hypocotyls (Zhong and Li 1993; Puddephat et al. 2001; Kim and Botella 2002; Ravanfar et al. 2009; Huang et al. 2011; Kumar and Srivastava 2015a; Kumar et al. 2015a), leaf tissues (Robertson and Earle 1986; Cao and Earle 2003; Farzinebrahimi et al. 2012; Kumar and Srivastava 2015a), cotyledons (Qin et al. 2006; Ravanfar et al. 2011, 2014; Kumar and Srivastava 2015a, b; Kumar et al. 2016) and petioles (Kumar et al. 2015a, b). Metz et al. (1995a, b) were the first to report *Agrobacterium*-mediated gene transfer studies in broccoli, followed by others (Puddephat et al. 1996, 2001; Chen et al. 2004, 2007; Huang et al. 2005; Higgins et al. 2006; Bhalla and Singh 2008; Ravanfar and Aziz 2014; Gaur 2015). Plant regeneration efficiency and transformation of the broccoli is highly dependent on genotype and need to be established for each cultivar. In this paper different aspects of genetic improvement of broccoli using plant genetic engineering techniques have been reviewed to provide insight into its potential applications (Tables 1, 2, 3, 4).

Genetic transformation studies in broccoli

Being an economically important crop, genetic improvement programmes can be a reliable way to tackle the cultivation problems of the broccoli. Recent advances in plant biotechnology have enabled the introduction of various foreign genes into plant cells and regeneration of plants from the transformed cells. Broccoli and other cole crops are susceptible to several insect pests and diseases. Insect pests of agricultural importance include the cutworm (*Spodoptera littoralis*), cabbage looper (*Trichoplusia ni*), cabbage aphid (*Brevicoryne brassicae*), cabbage fly (*Delia brassicae*), imported cabbage worm (*Pieris rapae*), cabbage butterfly (*Pieris canidia*), cabbage moth (*Crocidolomia binotalis*) and the diamondback moth (*Plutella xylostella*). The diamondback moth is considered to be the major pest of the crucifers worldwide, and has become resistant to all major categories of insecticides (Tabashnik et al. 1991).

Broccoli is highly susceptible to the diamondback moth (*Plutella xylostella*) for which no source of resistance is available among crossable germplasm. Massive use of synthetic insecticides in chemical control has given rise to major concerns about food safety and environmental pollution, apart from high chemical and labour costs. Emergence of insect resistance to chemical pesticides (Georghiou and Tajeda 1991) requires alternative methods of pest control. Important diseases include bacterial black rot (*Xanthomonas campestris* pv. *campestris*) and soft rot (*Erwinia carotovora*), downy mildew (*Peronospora parasitica*), clubroot (*Plasmodiophora brassicae*), *Alternaria* blight (*A. brassicae*), cabbage yellows (*Fusarium oxysporum*) and cauliflower and turnip mosaic virus. While some cultivars are available with resistance to some of the diseases listed above, the majority of present day cultivars are still susceptible (Siemonsma and Piluek 1993).

Plant genetic engineering could be used to develop insect- and disease-resistant varieties by utilizing genes that cannot be transferred through traditional plant breeding techniques. The cloning of genes expressing the insecticidal proteins of *Bacillus thuringiensis* (Hiifte and Whiteley 1991) has allowed development of transgene strategies for incorporation of resistance to a variety of lepidopteran and coleopteran insect pests in many crop plants (Fischhoff et al. 1987; Perlak et al. 1990; Bottrell et al. 1992; Fujimoto et al. 1993; Fromm et al. 1994; Sharma and Srivastava 2014a, b). Genetic engineering of broccoli for insect resistance offers a safe and attractive strategy to achieve a long lasting solution against insect/pest attack. Biotechnological improvements in crops offer many advantages, such as decreased pesticide use, environment friendly nature,

Table 1 *Agrobacterium*-mediated gene (reporter and marker) transfer studies in broccoli (*Brassica oleracea* L. var. *italica*)

Plant sp./cv.	Gene(s) transferred	References
Brassica oleracea L. var. *italica*	*npt-II*	Christey et al. (1997a, b)
Brassica oleracea L. var. *italica*	*npt-II* and *gus*	Hosoki et al. (1989)
Brassica oleracea L. var. *italica*	*Gus* and *bar*	Christey and Earle (1989)
Brassica oleracea L. var. *italica*	*npt-II* and *gus*	Toriyama et al. (1991)
Brassica oleracea L. var. *italica*	*npt-II*	Metz et al. (1995a)
Brassica oleracea L. var. *italica*	*npt-II* and *gus*	Li et al. (1999)
Brassica oleracea L. var. *italica*	*npt-II*	Henzi et al. (1999)
Brassica oleracea L. var. *italica*	*npt-II*	Henzi et al. (2000)
Brassica oleracea L. var. *italica*	*gus* and *rol*	Puddephat et al. (2001)
Brassica oleracea L. var. *italica*	*gfp* gene	Cogan et al. (2001)
Brassica oleracea L. var. *italica*	*npt-II*	Gapper et al. (2002a, b)
Brassica oleracea L. var. *italica*	*npt-II* and *gus*	Suri et al. (2005)
Brassica oleracea L. var. *italica*	*npt-II* and *gus*	Bhalla and Singh (2008)
Brassica oleracea L. var. *italica*	*npt-II* and *gus*	Verma et al. (2014)
Brassica oleracea L. var. *italica*	*npt-II* and *gus*	Dhiman et al. (2014)

Table 2 Direct gene transfer technique-mediated desirable gene transfer in broccoli (*Brassica oleracea* L. var. *italica*) and related *Brassica* species

Plant species/cultivar	Technique of gene transfer	Gene transferred	Explants used	Reference
B. oleracea L. var. *botrytis*	Leaf disc transformation, PEG-mediated gene transfer and Electroporation	*npt-II*	Protoplast	Eimert and Siegemund (1992)
B. campestris L. var. *italica*	Particle gun	*gus*	Cotyledon disc	Puddephat et al. (1994)
B. campestris L. subsp. *pekinensis*	Particle gun	*gus*	Leaf sics	Cho et al. (1994)
Brassica species	Particle Bombardment	*gus*	Cotyledon and hypocotyl	Tuan and Garg (2001)
B. oleracea L. var. *botrytis*	PEG-mediated gene transfer	*npt-II, hpt and gus*	Mesophyll protoplast	Radchuk et al. (2002)
B. campestris L. subsp. *chinensis*	Microinjection	*Sucrose and Surfactant concentaration*	Floral bud	Yan et al. (2004)

decreased input costs to farmers, season-long protection independent of weather conditions, high effectivity against burrowing insects that are difficult to reach through insecticide sprays, and control at all stages of development. Resistance to fungal and viral infection has also been demonstrated in transgenic plants containing fungal resistance genes (Mora and Earle 1999, 2004), virus-resistance genes (Lomonossof 1995), including coat protein genes (Beachy et al. 1990) and, more recently, viral movement genes (Cooper et al. 1995; Tacke et al. 1996). Other traits of interest include delay of post-harvest senescence and improved quality and yield. Post-harvest senescence is quite rapid in broccoli and leads to a deterioration of crop quality. Attempts have been made by various scientists to increase the yield and quality of broccoli via genetic transformation techniques (Henzi et al. 1999; Huang et al. 2005; Higgins et al. 2006; Deng-Xia et al. 2011; Ravanfar and Aziz 2014) of agronomically important traits like aphid resistance, disease resistance, herbicide tolerance, salinity, drought tolerance and quality improvement.

Genetic transformation is a potential tool for the improvement of crop plants. Recombinant DNA techniques for introducing foreign genes into plants involve the use of dominant selectable markers that are

Table 3 Desirable gene transfer studies in broccoli (*Brassica oleracea* L. var. italica)

Plant sp./cv.	Gene(s) transferred	Trait Improved	References
Brassica oleracea L. var. *italica*	*SLG* gene	Flowering control	Toriyama et al. (1991)
Brassica oleracea L. var. *italica*	Sadenosylmethionine hydrolase *(SAD, EC-3.3.1.2)*	Prevent post-harvest losses and quality improvement	Wagoner et al. (1992)
Brassica oleracea L. var. *italica*	*npt-II* and *cryIA(c)*	Insect resistance	Metz et al. (1995a)
Brassica oleracea L. var. *italica*	*cryIA(c)* and *npt-II*	Insect resistance	Metz et al. (1995b)
Brassica oleracea L. var. *italica*	*Bar gene*	Herbicide resistance	Christey et al. (1997a, b)
Brassica oleracea L. var. *italica*	*Antisense pollen specific gene*	Flowering control	Bhalla and smith (1998b)
Brassica oleracea L. var. *italica*	*cry1C*	Insect resistance	Cao et al. (1999)
Brassica oleracea L. var. *italica*	Antisense 1amino cyclopropane-1 carboxylic acid oxidase and *npt-II*	Prevent post-harvest losses and quality improvement	Henzi et al. (1999)
Brassica oleracea L. var. *italica*	*npt-II and Trichoderma harzianum* endochitinase gene	Fungal resistance	Mora and Earle (1999)
Brassica oleracea L. var. *italica*	*Bar gene*	Herbicide resistance	Waterer et al. (2000)
Brassica oleracea L. var. *italica*	*npt* and *ipt*	Prevent post-harvest losses and quality improvement	Chen et al. (2001)
Brassica oleracea L. var. *italica*	ACC oxidase gene	Prevent post-harvest losses and quality improvement	Gapper et al. (2002a, b)
Brassica oleracea L. var. *italica*	*cry1A, cry1C,hpt* and *npt-II*	Insect resistance	Cao and Earle (2002)
Brassica oleracea L. var. *italica*	*FCA and CONSTANS*	Flowering control	Irwin et al. (2002)
Brassica oleracea L. var. *italica*	*ers* (ethylene-response-sensor), *npt-II* and *hpt*	Prevent post-harvest losses and quality improvement	Chen et al. (2004)
Brassica oleracea L. var. *italica*	Pathogenesis-related (PR-1 and PR-2)	Fungal resistance	Mora Avieles and Earle (2004)
Brassica oleracea L. var. *italica*	*cryIA* (b) and *gus*	Insect resistance	Viswakarma et al. (2004)
Brassica oleracea L. var. *italica*	Anti- gene *CYP86MF*	Flowering control	Huang et al. (2005)
Brassica oleracea L. var. *italica*	ACC synthase 1 and ACC oxidase 1 and 2	Prevent post-harvest losses and quality improvement	Higgins et al. (2006)
Brassica oleracea L. var. *italica*	Antisense chlorophyllase (BoCLH1) and hpt	Prevent post-harvest losses and quality improvement	Chen et al. (2007)
Brassica oleracea L. var. *italica*	*AtHSP 101*	Abiotic stress tolerance, heat stress	Ravanfar and Aziz (2014)

expressed at the cell and whole-plant levels (Mathews and Litz 1990). Selection and recovery of transformed cells or tissues and elimination of *Agrobacterium* from the cultures require the use of selective agents and/or antibiotics which is a critical feature in obtaining transgenic plants. Genes encoding antibiotic resistance and herbicide tolerance are widely employed as selective markers to identify the rare transformed cells (Vetten et al. 2003; Miki and McHugh 2004). Most of the transgenic plants produced to date were created through the use of the *Agrobacterium* system (Gasser and Fraley 1989; Jain

Table 4 Factors effecting transformation frequency in different plant species

Plant species/hybrid	Technique	Factors studied	Reference
Nicotiana tabacum	*Agrobacterium*-mediated gene transfer	Effect of antibiotics	Naureby et al. (1997)
Nicotiana tabaccum and *Ulmus pumila* L.	*Agrobacterium*-mediated gene transfer	Effect of antibiotics	Cheng et al. (1998)
Solanum lycopersicum	*Agrobacterium*-mediated gene transfer	Effect of antibiotics	Ling et al. (1998)
Brassica oleracea L. var. *botrytis*	*Agrobacterium*-mediated gene transfer	Effect of kanamycin	Dixit and Srivastava (1999)
Brassica campestris L.	Tissue culture and genetic transformation	Effects of kanamycin, cefotaxime, carbenicillin and ampicillin	Song et al. (2000)
Brassica campestris L.	*Agrobacterium*-mediated gene transfer	Age of seedlings, growth conditions and status of *Agrobacterium* suspension, preculture of explants, cocultivation time, acetosyringone and concentration of kanamycin	Zhao et al. (2006)
Fragaria X ananassa Duch. Chandler	*Agrobacterium*-mediated gene transfer	Effect of co-cultivation conditions, acetosyringone and antibiotics	Husaini (2010)
Lycopersicon esculentum Mill.	*Agrobacterium*-mediated gene transfer	Effect of kanamycin and cefotaxime	Sharma (2010)
Solanum lycopersicum	*Agrobacterium*-mediated gene transfer	Petiole	Sharma et al. (2011)
Populus ciliata Wall.	*Agrobacterium*-mediated gene transfer	Effect of cefotaxime, kanamycin and acetosyringone	Aggarwal (2011)
Solanum tuberosum L.	*Agrobacterium*-mediated gene transfer	Co-cultivation time, cefotaxime concentration, and days to pre-selection	Ahmad et al. (2012)
Brassica oleracea L. var. *capitata*	*Agrobacterium*-mediated gene transfer	Effect of kanamycin, cefotaxime and acetosyringone	Gambhir (2013)
Solanum lycopersicum L.	*Agrobacterium*-mediated gene transfer	Effect of kanamycin, cefotaxime and acetosyringone	Sharma (2014)
Brassica oleracea L. var. *botrytis*	*Agrobacterium*-mediated gene transfer	Effect of kanamycin, cefotaxime and acetosyringone	Gaur (2015)

1993; Singh and Sansavini 1998; Dunwell 2000; Ranjekar et al. 2003; Cardoza and Stewart 2004; Srivastava 1997; 1998, 2003; 2012a, b, 2013). The success of this approach resulted from improvement in tissue culture and genetic engineering methods.

The first transgenic plants expressing engineered foreign genes were tobacco plants produced by the use of *Agrobacterium tumefaciens* as a vector (Horsch et al. 1984; De Block et al. 1984). An efficient method for introducing cloned gene(s) into plant cells was given by Fraley et al. (1983); Herrera-Estrella et al. (1983) and Zambrysky et al. (1983). Genetic transformation of plants to confer resistance to insect pests offers an ecofriendly method of crop protection. Considerable progress has been made in developing transgenic crops with resistance to the target insect-pests over the past two decades (Vaeck et al. 1987; Chakrabarty et al. 2002). Impressive results have been obtained with the expression of *Bt* (*cry*) genes, the cowpea trypsin inhibitor gene, serine proteinase inhibitor gene and cysteine proteinase inhibitor genes in various crops (Gatehouse et al. 1980; Wolfson and Murdock 1987; Houseman et al. 1989; Johnson et al. 1989; Liang et al. 1991; Bai et al. 1992; Ding et al. 1998; Jin et al. 2000; Cho et al. 2001; Awasthi 2003; Chakrabarty et al. 2002; Zhang et al. 2004; Paul et al. 2005; Lingling et al. 2005; Zhao et al. 2006; Hua et al. 2009; Deng-Xia et al. 2011; Srivastava 2013; Sharma and Srivastava 2014a, b). Such transgenic plants have shown considerable promise in reducing insect damage, both under laboratory and field conditions, thus reducing the need to use pesticides for crop pest

management. To protect the crop plants from insect pest attack, a massive application of pesticides not only leaves harmful residues in the food, but also causes adverse effects on non-target organisms and the environment.

Agrobacterium-mediated gene (reporter and marker) transfer studies in broccoli

Genetic transformation of broccoli has been achieved using both *Agrobacterium tumefaciens* and *A. rhizogenes*. Hosoki et al. (1989) utilized a mannopine strain of *A. rhizogenes* to induce hairy root cultures from inoculated leaf explants. Plants regenerated from two hairy root clones demonstrated the presence of mannopine. Toriyama et al. (1991) demonstrated genetic transformation of broccoli with *A. tumefaciens*. Metz et al. (1995a) described the use of *A. tumefaciens* to transfer a *Bacillus thuringiensis* insecticidal protein gene to broccoli, with the recovery of 144 insect-resistant plants. Christey et al. (1997a, b) carried out regeneration of transgenic vegetable brassicas (*B. oleracea* and *B. campestris*) via Ri-mediated transformation. Leaf explants or petioles of intact cotyledons were co-cultivated with *Agrobacterium rhizogenes* strain A4T harbouring various binary vectors. The T-DNA region of all binary vectors contained *npt-II* gene for kanamycin resistance, in addition to other genes. Hairy root lines grew on hormone-free medium containing kanamycin. Transgenic shoots were regenerated from all cultivars either spontaneously or after transfer of hairy roots to a hormone-containing medium. Southern analysis confirmed that plants were transgenic. Plants from all brassica types were successfully transferred to greenhouse conditions. Li et al. (1999) reported transformation of *B. oleracea* L. var. italica cotyledon and hypocotyl protoplasts using *Agrobacterium tumefaciens* EHA105 harbouring Ti-plasmid pMOG410 containing *gus* and *npt-II* genes. Transformation was confirmed by growth on medium containing kanamycin sulphate and by β-glucuronidase assay. Puddephat et al. (2001) recovered phenotypically normal transgenic plants of *Brassica oleracea* L. var. italic by using hypocotyl explants with *A. rhizogenes* strain A4T harbouring the bacterial plasmid pRiA4 and a binary vector pMaspro: β-glucuronidase (GUS) whose T-DNA region carried the *gus* reporter gene. pRiA4 transfers TL sequences carrying the *rol* genes that induce hairy root formation. Transgenic hairy root production was increased in a difficult to transform cultivar by inclusion of 2,4-D in the medium used to resuspend *A. rhizogenes* prior to inoculation. Transgenic hairy roots could be selected from inoculated explants by screening root sections for GUS activity; this method eliminated the use of antibiotic resistance marker genes for selection. Cogan et al. 2001; Suri et al. 2005; Bhalla and Singh 2008; Verma et al. 2014; Dhiman et al. 2014 reported an effective protocol for genetic transformation in broccoli with *npt-ll* and *gus* genes. The putative transformants have shown amplification of *npt-ll* and *gus,* thereby indicating the presence/integration of *npt-II* and *gus* genes into the genome of transgenic broccoli. The expression of the *gus* gene was analyzed by using biochemical and histochemical techniques of GUS assay. The *gus* gene was expressed in the PCR positive transgenic plantlets of broccoli.

Direct gene (reporter and marker) transfer studies in broccoli

Direct gene transfer studies in *Brassica* species were initiated by Eimert and Siegemund (1992). There are only a few reports on direct gene transfer technique-mediated reporter/marker genes in *Brassica* species. Puddephat et al. (1994) carried out biolistic transformation of broccoli for transient expression of the β-glucuronidase gene. Efficient transient expression of the beta-glucuronidase (*gus*) gene had been obtained following transformation of broccoli cv. Marathon F1 cotyledons via microparticle bombardment. Cho et al. (1994) carried out transformation of Chinese cabbage (*Brassica campestris* L. var. *pekinensis*) by particle bombardment with a tungsten particle size of 0.7–1.1 mm at a target of 3 cm. Transgenic plantlets were regenerated from leaf discs on minimal MS medium with 1 mg NAA l^{-1} and 5 mg BAP l^{-1}. Tuan and Garg (2001) reported gene transformation in cotyledon and hypocotyls explants of *Brassica* sp. using particle bombardment. Mesophyll protoplasts of *Brassica oleracea* L. var. *botrytis* were successfully transformed using polyethylene glycol (PEG) by Radchuk et al. (2002). The success of plant transformation depended on both gene transfer and plant regeneration. Parameters such as PEG and vector concentrations and heat-shock conditions were tested in experiments on transient expression of the β-glucuronidase gene and the most

suitable conditions for DNA uptake were determined. Yan et al. (2004) analyzed the effect of the developmental stage of floral buds, and sucrose and surfactant concentrations on the transformation of Chinese cabbage (landrace variety Shanghaiqing) by microinjection of *Agrobacterium* into flower buds.

Desirable gene transfer studies in broccoli (*Brassica oleracea* L. var. *italica*)

A number of desirable genes have been introduced into the genome of broocoli for the development of transgenic plants with desirable traits. Wagoner et al. (1992) developed superior regeneration and *Agrobacterium* infectability of broccoli tissues and the identification of a procedure for the generation of transgenic plants. They developed a method for the transfer of a gene encoding *S*-adenosylmethionine-hydrolase into inbred broccoli (*Brassica oleracea* L. var. *italica*) germplasm, the morphogenic competence and *Agrobacterium* susceptibility of a wide range of tissues of varied source were examined.

Biotic stress

Insect resistance Metz et al. (1995a, b) have generated a large number of transgenic broccoli lines carrying the *cry1A*(*c*) gene, most of them causing 100 % mortality of first instar larvae of the diamond moth, a major insect pest of crucifers. Southern blots of some resistant transformants confirmed the presence of the *cry1A*(*c*) gene. Selected plants that gave 100 per cent mortality of susceptible larvae allowed the survival of a strain of diamondback moth that had evolved resistance to *Bt* in the field. Cao et al. (1999) developed transgenic broccoli with high levels of *Bacillus thuringiensis cry1C* protein to control diamondback moth larvae resistant to *cry1A* or *cry1C*. They demonstrated that a high production of *cry1C* protein can protect transgenic broccoli not only from susceptible DBM larvae but also from DBM selected for moderate levels of resistance of *cry1C*. The *cry1C* transgenic broccoli was also resistant to two other lepidopteran pests of crucifers (cabbage looper and imported cabbage worm). Cao and Earle (2002) studied transgene expression in broccoli (*Brassica oleracea* L. var. *italica*) clones propagated in vitro via leaf explants by using *cry1Ac* and *cry1C* genes from *Bacillus thuringiensis* associated with kanamycin and hygromycin selectable markers, respectively. PCR analysis indicated that the *cry1Ac* and *cry1C* genes were both maintained. ELISA assays showed that all of the clones produced a high level of *cry1Ac* protein similar to the original transgenic plant; however, most clones had significantly lower levels of *cry1C* protein than the original plant. Viswakarma et al. (2004) studied insect resistance of transgenic broccoli expressing a synthetic *cryIA*(*b*) gene. Broccoli has no suitable genes for insect resistance within the gene pool for transfer through conventional breeding. They optimized a protocol for the transformation of hypocotyl explants of broccoli based on transient GUS expression. A synthetic *cryIA*(*b*) gene coding for an insecticidal crystal protein of *Bacillus thuringiensis* (*Bt*) was transferred to the broccoli cultivar Pusa Broccoli KTS-1 by co-cultivating hypocotyl explants with *Agrobacterium tumefaciens*. Transformed plants resistant to kanamycin were regenerated.

Fungal resistance Mora and Earle (1999) developed transgenic lines with a *Trichoderma harzianum* endochitinase gene in broccoli. An endochitinase gene from *Trichoderma harzianum* was introduced into broccoli via *Agrobacterium tumefaciens*-mediated transformation using *npt-II* (neomycin phosphotransferase-II) as the selectable marker. Twenty-four transgenic plants were obtained in a single transformation experiment, 19 of which were assayed for endochitinase activity and fungal control. The transgenic lines had endochitinase activity 15–30 times higher than control lines. However, Mora Avieles and Earle (2004) studied expression of genes encoding two pathogenesis-related plant proteins (PR-1 and PR-2) in transgenic broccoli plants expressing the *Trichoderma harzianum* endochitinase gene and in the control plants. mRNA accumulation in 2-month-old plants inoculated with *Alternaria brassicicola* and in non-inoculated plants was assessed by RNA hybridization, using PR-1 and PR-2 DNA sequences of *Arabidopsis thaliana* as probes. The non-transgenic and transgenic controls carrying a different transgene showed accumulation of PR-1 mRNA only after inoculation. In contrast, endochitinase transgenic plants produced PR-1 mRNA with and without inoculation. These results indicated that PR-1 genes normally induced by fungal infection were constitutively expressed in transgenic plants expressing heterologous endochitinase genes.

Bacterial and virus resistance Broccoli is susceptible to bacterial and viral diseases. Important diseases include bacterial black rot (*Xanthomonas campestris* pv. *campestris*) and soft rot (*Erwinia carotovora*), clubroot (*Plasmodiophora brassicae*), *Alternaria* blight (*A. brassicae*) and cauliflower and turnip mosaic virus (Siemonsma and Piluek 1993). There is not any literature on bacterial and viral resistance gene transfer in broccoli. So broccoli can be further improved by the transfer of bacterial and viral resistance genes using plant genetic engineering techniques.

Abiotic stress

Broccoli cultivars are commonly grown in cool climate regions of the world. The cultivar, which could also be grown on the highlands of the tropics, responds adversely to extreme temperatures and high humidity in lowlands; thus gene transformation is essential for improving its tolerance to heat stress. Heat-shock proteins (HSPs) are functionally related proteins involved in the folding and unfolding of other proteins. Their expression is increased when cells are exposed to elevated temperatures or other stresses (Narberhaus 2010; Omar et al. 2011). *HSP101* appears to play a major and specific role in conferring acquired thermotolerance (Wahid et al. 2007; Su and Li 2008). Transgenic broccoli plants have shown improved tolerance to dehydration, as well as to other types of stresses (salt, heavy metals, and hydrogen peroxide) (Vinocur and Altman 2005). Ravanfar and Aziz 2014 developed heat-tolerant transgenic broccoli via *Agrobacterium*-mediated transformation with *Arabidopsis thaliana HSP 101 (AtHSP101)* cDNA. A transformation efficiency of 5 % was achieved based on the positive PCR results using the optimized procedure. The expression of the luciferase reporter gene in the transformed cells and the transcription of *AtHSP101* using RT-PCR confirmed the transgenic status of the regenerated plants.

Herbicide resistance

The incorporation of herbicide resistance into vegetable brassicas would enable growers to control weeds more efficiently. Basta-resistant broccoli has been produced and field-tested by Christey et al. (1997a, b) and Waterer et al. (2000). Glufosinate is a non-selective herbicide that acts by inhibiting the enzyme glutamine synthetase, resulting in the accumulation of ammonium leading to the death of the cell and ultimately the plant. Waterer et al. (2000) field tested six transgenic lines and noted that herbicide application had little effect on head quality and marketable yield of most lines. Christey et al. (1997a) field-tested four transgenic lines and also noted a normal phenotype, although plants were not sprayed in the field. Greenhouse application of Basta to seedlings demonstrated they were resistant.

Flowering control

In brassicas, the transition from vegetative growth to flowering is influenced by day length and low temperature, with some vegetables requiring a period of vernalization to induce flowering. Irwin et al. (2002) studied the control of the transition from vegetative growth to flowering mode in vegetable brassicas by insertion of genes from *A. thaliana* known to be important in the control of flowering. In *Arabidopsis thaliana,* overexpression of *FCA* and *CONSTANS* (*CO*) promotes early flowering and overrides the vernalization requirement. The *FCA* and *CO* genes of *Arabidopsis thaliana* were introduced into seven genotypes of *B. oleracea* and lines assessed for changes in flowering response and vernalization treatment by growth under long and short days (with and without vernalization). No major changes in flowering response were observed. Considerable variation in days to flowering was noted both with and among transgenic lines. While insertion of *FCA* or *CO* does not override the vernalization requirement, it appears to be modulated in some way. The introduction of male sterility into vegetable brassicas would aid production of hybrid seed. The only report of alteration of male sterility is by Bhalla and Smith (1998a, b), who introduced an antisense pollen-specific gene linked to a pollen-specific promoter into cauliflower and obtained the expected sterility of 50 % of pollen. Self-incompatibility prevents self-fertilization and promotes outcrossing.

In brassicas, self-incompatibility is used for hybrid seed production but has a number of drawbacks, including breakdown of incompatibility, labor intensiveness and genetic complexity of the system. In brassicas, self-incompatibility is sporophytically controlled by multiallelic genes at the *S*-locus. Two genes

have been identified at the *S*-locus, *SLG* and *SRK*. SLG encodes a secreted glycoprotein in the wall of the stigma papillar cells and SRK encodes a transmembrane receptor kinase. A self-incompatible response occurs when the same *S*-allele is expressed in pollen and stigma. Toriyama et al. (1991) introduced an *SLG* gene from the *B. campestris* S8 homozygote and were able to alter the self-incompatibility phenotype of pollen and stigma. The self-incompatible Chinese kale and partially compatible broccoli plants were fully compatible upon self-fertilization.

Plant male sterility induced by anti- gene *CYP86MF* in broccoli (*Brassica oleracea* L. var. *italica*) reported by Huang et al. (2005). They introduced an anti-gene *CYP86MF* into hypocotyls of broccoli via *Agrobacterium tumefaciens*-mediated transformation and transgenic plants were obtained by kanamycin selection. The results of PCR, southern blot and northern blot indicated that anti-gene *CYP86MF* has been integrated into the chromosome of the transgenic plant. Hypo genetic stamina or ungerminated pollen was observed.

Post-harvest attributes and quality improvement

For enhancing shelf-life to prevent postharvest losses and quality improvement, Henzi et al. (1999) carried out *Agrobacterium rhizogenes*-mediated transformation of broccoli with an antisense 1-aminocyclopropane-1 carboxylic acid oxidase gene. Leaf explants or intact cotyledons of broccoli cultivars Green Beauty, Shogun and Green Belt were co-cultivated with *Agrobacterium rhizogenes* strain A4T harbouring the binary vector pLN35. The T-DNA of this binary vector contains genes encoding antisense-1-aminocyclopropane-1-carboxylic acid (ACC) oxidase and neomycin phosphotransferase-II. Shogun and Green Beauty were successfully transformed with a transformation efficiency of 35 and 17 %, respectively. Chen et al. (2001) developed a protocol for transformation of broccoli with the *ipt* (isopentenyl transferase) gene via *Agrobacterium tumefaciens* for post-harvest yellowing retardation. The *ipt* gene was constructed under control of senescence-associated gene promoters from *Arabidopsis* in the forms of pSG529 (+) and pSG766A. Evidence of transgene integration was confirmed by assays on *npt- II* activity of selection markers, PCR and Southern hybridization. Based on chlorophyll retention rate after 4 days of post-harvest storage at 25 °C, 31 % of transformants exhibited an effect of retarding yellowing in detached leaves with 16 % having an effect on florets and 7.2 % on both leaves and florets. RT-PCR revealed that *ipt* gene expression occurred early on the day of detachment. Similar results were reported by Gapper et al. (2002a, b) to alter ethylene and cytokinin biosynthesis in broccoli. Chen et al. (2004) studied ethylene-insensitive and post-harvest yellowing retardation in mutant ethylene response sensor gene transformed broccoli. Over a hundred transformants have been obtained on the selected cotyledon and hypocotyls explants. PCR and Southern analysis demonstrated integration of transgenes in the transformants. Higgins et al. (2006) produced marker-free genetically engineered broccoli with sense and antisense *ACC synthase 1* and *ACC oxidase 1 and 2* to extend shelf-life. An *A. rhizogenes* Ri vector, pRi1855: GFP was constructed to allow expression of green fluorescent protein to identify insertion of Ri T_l—DNA into plant cells. The *Brassica oleracea ACC synthase 1 and ACC oxidase 1 & 2* cDNAs in sense and antisense orientations were co-transformed into GDDH33, a double haploid calabrese-broccoli cultivar. Transformation efficiency was 3.3 % producing 150 transgenic root lines, of which 18 were regenerated into mature plants. Buds from T_0 lines transformed with *ACC oxidase 1* and *2* constructs produced significantly less post-harvest ethylene at 20 °C than untransformed plants, and chlorophyll loss was significantly reduced in a 96 h post-harvest period. Chen et al. (2007) produced transgenic broccoli (*Brassica oleracea* var. *italica*) with an antisense chlorophyllase gene (*BoCLH1*) to delay post-harvest yellowing. Southern blot analyses were conducted to eliminate the possible non-independent transformants and investigate the insertion patterns and copy numbers.

Factor affecting transformation frequency

Various factors determine the rate of transformation frequency in different species. All these factors needed to be optimized for the establishment of an effective genetic transformation protocol. Different factors which are known to affect the transformation frequency include plant species, explant type, *Agrobacterium* strain, preculturing time, cocultivation period, concentration of bacterial inoculum, effect of different inducers like acetosyringone and lethal dose of each

agent (antibiotic). Effects of all these factors were studied by various researchers during the genetic transformation studies of different plant species (Naureby et al. 1997; Cheng et al. 1998; Ling et al. 1998; Dixit and Srivastava 1999; Awasthi 2003; Zhao et al. 2006; Thakur 2008; Husaini 2010; Sharma 2010, 2014; Sharma et al. 2011; Ahmad et al. 2012; Aggarwal 2011; Gambhir 2013; Gaur 2015). Naureby et al. (1997) investigated the influence of 150 mg timentin l^{-1} on the regeneration potential of *Nicotiana tabacum* Petit Havana SRI leaf discs and cotyledon explants and compared with the effects of 500 mg cefotaxime l^{-1} or 1000 mg carbenicillin l^{-1}. They found a positive influence of timentin on shoot regeneration from leaf discs (27 % after 1 month of culture). There was no influence on shoot production from cotyledons after 1 month and generally no influence on the rooting ability. Cefotaxime did not affect shoot production from leaf discs, but had an inhibitory effect on cotyledon explant regeneration (81 % after 2 months of culture). Carbenicillin influenced shoot formation negatively as the shooting frequency for leaf disc explants was reduced to 56 % and for cotyledons to 51 % after 2 months of culture. In general, it was demonstrated that shoots arising from cotyledon explants rooted more efficiently than shoots arising from leaf disc explants. It could be concluded that the influence of timentin on tissue culture was negligible or positive as compared to those of cefotaxime or carbenicillin. Similar results have been reported by Cheng et al. (1998), who determined the effects of timentin on shoot regeneration of tobacco (*Nicotiana tabaccum*) and Siberian elm (*Ulmus pumila* L.) and its use for the suppression of *Agrobacterium tumefaciens* in *Agrobacterium*-mediated genetic transformation and concluded that timentin may be an alternative antibiotic for the effective suppression of *A. tumefaciens* in genetic transformation.

Dixit and Srivastava (1999) studied the resistance level of kanamycin in cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Pusa Snow Ball K1). They gave increasing doses of kanamycin (10, 20, 30, 40, 50 mg l^{-1}) to hypocotyl and cotyledon explants to find the minimum concentration of kanamycin required for the selection of putative transformed cells during transformation. A decrease in fresh weight in both hypocotyl and cotyledon tissues was observed with increasing kanamycin concentrations. Even the cauliflower tissues were sensitive to 10 mg kanamycin l^{-1}. The non-transformed tissues did not survive on the selective medium containing kanamycin. Song et al. (2000) studied effects of kanamycin, cefotaxime and carbenicillin on morphogenesis of Chinese cabbage in tissue culture. Results showed that the 4 antibiotics had little effect on callus induction, but they had a great influence on shoot and root differentiation. Even at a very low concentration, kanamycin inhibited the differentiation of roots and shoots. Cefotaxime inhibited redifferentiation at lower concentrations and postponed morphogenesis of roots and shoots. Carbenicillin had no obvious effect on shoot and root differentiation with a frequency over 70 %. Similar results were reported by Sharma (2010, 2014), Husaini (2010), Sharma et al. (2011), Aggarwal (2011), Ahmad et al. (2012), and Gambhir 2013.

Conclusion

Several published reports demonstrate genetic transformation in broccoli. All accounts of transformation within this crop utilize direct gene transfer and *Agrobacterium*-mediated gene transfer techniques. In general, most published accounts of transformation have indicated somewhat limited success. With depleting natural resources and a changing global climate, conventional agricultural practices alone are unable to sustain the quality and quantity of the produce. With advent of modern molecular biotechnology, newer tools permitting gene transfer across the species: transgenics, have opened an avenue for solving an age-old problem. Some of the limitations in transgenic applications need to be resolved for wider application and acceptance of transgenic technology. Environmental risks such as cross-pollination with closely related wild relatives of the crop plants and effect of transgene products on human health and environment need to be assessed carefully on a case-by-case basis. Moreover, there are widespread concerns about the use of antibiotic- and herbicide-resistance genes as selectable markers from the point of view of ecological and human safety. Use of alternative methods to obtain marker-free transgenic plants may enhance public acceptance of transgenic crops (Vetten et al. 2003). Development of binary vectors or mini-chromosomes for multiple gene transfer (Goderis et al. 2002) and improvement in transformation technology for vegetable crops may

further increase our capability to introduce traits with long lasting value. This review provides an insight into the various plant genetic transformation studies carried out by researchers in broccoli for biotic and abiotic stress tolerant genes, herbicide resistance genes, flowering control genes, post harvest attributes and quality improvement genes along with the various factors affecting the transformation frequency.

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