Journal of Applied Horticulture, 15(1): 3-9, 2013

Genetic transformation of cabbage (*Brassica oleracea* var. capitata) with synthetic *cry*1F gene to impart resistant to diamondback moth (*Plutella xylostella*)

H.M. Mahadeva Swamy[1]*, S.N. Nagesha[1], Prakash M. Navale[1], T.K.S. Gowda[1,] R. Asokan[2] and Riaz Mahmood[3]

*[1]Department of Biotechnology, University of Agricultural Sciences, GKVK, Bangalore 560 065. Karnataka, India. [2]Division of Biotechnology, Indian Institute of Horticultural Research, Hessarghatta lake post, Bangalore 560089, Karnataka, India. [3]Post-Graduate Department of Studies and Research in Biotechnology and Bioinformatics, Kuvempu University, Jnanasahayadri, Shankaraghatta, Shimoga 577451, Karnataka, India. *E-mail: clintonbio@gmail.com*

Abstract

Insect-resistant crops have been one of the major successes of applying plant genetic engineering technology to agriculture. There is an urgent need for improvement in vegetable production, especially in developing countries where the economic, health and environmental benefits of bioengineered vegetables could be of great importance. In this view a synthetic *cry*1F gene coding for an insecticidal crystal protein of *Bacillus thuringiensis* (*Bt*) was transformed to cabbage cultivar '*Hare Krishna*' by co-cultivating hypocotyls explants with *AAgrobacterium tumefaciens* mediated transformation. The transformed plants resistant to kanamycin were regenerated on selection medium. Confirmation of transgene in putatively transformed plants was carried out by using *nptII* and *cry*1F gene specific primers. Multiple shoot regeneration of hypocotyl and shoot tip explants of cabbage after co-cultivation with *Agrobacterium* was optimized and medium containing 2 mg/L BAP was observed to be the best for shoot regeneration after co-cultivation. In this study, 45 and 32.5% transformation efficiencies were achieved for hypocotyl and cotyledonary leaf explants, respectively using the optimized procedure.

Key words: Synthetic *cry*1F gene, *Agrobacterium tumefaciens*, cabbage, transformation, regeneration, kanamycin

Introduction

Plant defence against insect pests plays an important role in agricultural systems. Direct defence, having direct effects on the performance of herbivores has been exploited in conventional breeding for host plant resistance. In recent years plant breeding for insect resistance has been further enhanced by the adoption of genetically engineered plants with genes conferring direct resistance (Fernandez-Cornejo and McBride, 2000; Groot and Dicke, 2002; Babu *et al.,* 2003; Rice, 2004; Wu and Guo, 2005; Ferry *et al.,* 2006). Among these especially *Bacillus thuringiensis (Bt)* transgenes have been commercialized and *Bt* crops have been planted on a large-scale by now, reaching a world total of *ca.* 148 million hectares (365 million acres) in 2010 (James, 2010). The incorporation of transgenic crops in integrated approaches to pest management may be used to ensure their long-term sustainability and maximize their environmental and human health benefits. It is important to understand the impact of incorporating a single transgene and even more so of combining two transgenes on pest insects and their natural enemies in the context of integrated pest management.

India is the second largest producer of vegetables in the world with the production of 13.37376 million tones of vegetables (National Horticultural Board, 2009-10). But the per capita availability of vegetables is 183 g/capita/day (2005 (provisional) - FAOSTAT (2007)) and 146 g/capita/day (2004-05 calculated from NSS data (NSS, 2007a)). In fact, majority of the Indian population is vegetarian and banks on the vegetables to carter their body requirement of vitamins, minerals and fibers. Cabbage is one of the fresh leafy vegetables rich in carbohydrates, proteins and fibers, used both as salad and cooked. One of the major limitations of cabbage production is damage due to insect pests. The most important one among them, the lepidopteron diamondback moth (*Plutella xylostella*) alone causes a worldwide damage of up to one billion dollars annually. The larvae bore into the cabbage head and even a little damage into the head reduces marketability of the crop. Synthetic insecticides used to control the pest have raised concerns about food safety and environmental pollution. Evolution of insecticide resistance further adds to the problem of chemical control (Bhattacharya *et al.,* 2002). The increasing failure of insecticides to control DBM has stimulated the development of alternative strategies, among which transgenic approaches (Christou *et al.,* 2006) to enhance host plant resistance to this pest using *Bt* genes (Schuler *et al.,* 2003; Zhao *et al.,* 2003; Zhao *et al.,* 2000) and protease inhibitors (De Leo *et al.,* 2001a; Ferry *et al.,* 2005) are promising in near future.

The mode of action for Cry1F in insect protection is generally similar to other *Bt* insecticidal crystal proteins. Like other *Bt* Cry proteins, Cry1F is a delta endotoxin that must be ingested to kill the insect, and acts by selectively binding to specific sites localized on the lining of the midgut of susceptible species. Following binding, pores are formed that disrupt midgut ion flow, causing gut paralysis and eventual death. Cry1F is lethal only when eaten by the larvae of certain lepidopteran insects, and

its specificity of action is directly attributable to the presence of specific binding sites in the target insects. There is variation in the specific binding between different Bt Cry proteins that affects the efficacy spectrum and cross resistance between different Bt's within the same species of insect.

Genetic improvement programme can be a reliable way to tackle the cultivation problems of the cabbage plants. *Agrobacterium*-mediated transformation system is the most widely applied method for cabbage genetic transformation (Cardoza and Stewart, 2004). However, the available reports on *Agrobacterium*-mediated transformation of cabbage indicated a low efficiency of transformation. The presence of an efficient transformation protocol is one of the basic needs in genetic improvement of a crop *via Agrobacterium*-mediated transformation. To achieve a high frequency of *Agrobacterium*-mediated transformation, several important factors that affect the transformation efficiency need to be optimized (Opabode, 2006). Pre-treatment of explants with centrifugation (Hiei *et al.,* 2006), osmotic treatment of explants (Jin *et al.*, 2000), inoculation conditions such as bacterial concentration (Zhao *et al.,* 2001) and immersion time (Xing *et al.,* 2007), co-cultivation condition such as period of cocultivation (Vasudevan *et al.,* 2007), and temperature conditions (Salas *et al.,* 2001) are some of the important factors that need to be optimized in *Agrobacterium*-mediated transformation system. Another essential requirement is a capable regeneration system after co-cultivation of inoculated explants (Lawrence and Koundal, 2001). Keeping this in view, an attempt has been made to develop an efficient regeneration and transformation protocol for cabbage against diamondback moth.

Materials and methods

Plant materials: Seeds of cabbage (*Brassica oleracea* subsp. capitata) cv. Hare Krishna were obtained from a private seed company and were surface sterilized by first placing under running tap water for 15 min, immersed in 70% ethanol for 2 min and rinsed once with sterile distilled water. The seeds were next immersed in a 1.05% (w/v) sodium hypochlorite solution (20%, v/v commercial bleach) added with 1-2 drop(s) of Tween 20 for 15 min, rinsed with sterile distilled water thrice and lastly blotted on a autoclaved filter paper. The surface sterilized seeds were germinated under aseptic condition on a hormone-free half strength MS salts medium. Hypocotyl and cotyledonary leaf segments (both 5-10 mm in length) were excised from 9- day-old seedlings and used as explants.

***In vitro* media and conditions:** The basal medium used in this study consisted of MS (Murashige and Skoog, 1962) salts, vitamins, 30 g/L sucrose, 2.5 g/L phytagel and pH was adjusted to 5.8. For seed germination, the MS salts were reduced to half their concentrations while the sucrose concentration was reduced to 10 g/L. The callus induction medium (CIM) for preculture treatments was prepared by adding 1 mg/L 6-benzylaminopurine (BAP) and 0.1 mg/L naphthalene acetic acid (NAA) to the basal medium. The shoot regeneration medium (SRM) consisted of the basal medium supplemented with only 1 mg/L BAP. All cultures were incubated at 25 $\pm$ 3^{0}C, 16 h (light)/8 h (dark) photoperiod, and a light intensity of 60 µmol m^{-2} s^{-1} except for the co-cultivation of inoculated explants which was carried out in the dark.

Induction of somatic embryogenesis through callus: For induction of somatic embryos from callus, following combinations were studied:

T1: MS_0; T2: MS_0 + 0.25 mg/L 2, 4-D+0.5 mg/mL kinetin; T3: MS_0 + 0.5 mg/L 2, 4-D+0.5 mg/mL kinetin; T4: MS_0 + 1.00 mg/L 2, 4-D+0.5 mg/mL kinetin; T5: MS_0 + 1.00 mg/L 2, 4-D+0.75 mg/mL kinetin; T6: MS_0 + 1.00 mg/L 2, 4-D+1.00 mg/mL kinetin

Induction of multiple shoots: The explants were initially screened on MS medium with 2 mg/L BAP, for their ability to form multiple shoots. Following observations were recorded 30 days after incubation with subculture interval of 10 days.

***In vitro* shoot elongation:** To optimize an efficient shoot elongation medium (SEM), several hormonal concentrations and their combinations were tired.

Shoot elongation medium: Following teatment combinations were used for shoot elongation:

T1: MS_0; T2: MS_0 + 0.25 mg/L IAA +0.5 mg/mL kinetin; T3: MS_0 + 0.75 mg/L IAA +0.5 mg/mL kinetin; T4: MS_0 + 0.5 mg/L IAA+1.0 mg/mL BAP; T5: MS_0 + 0.5mg /L IAA +1.5 mg/mL kinetin; T6: MS_0 + 0.5mg /L IAA +2.00 mg/mL kinetin

Observations were recorded after 2 weeks on number of explants showing elongation of at least one shoots, number of shoots elongated on a explant, shoot length (cm), percent shoot induction, average number of shoots elongated per explant, and average shoot length of shoots elongated.

***In vitro* root elongation:** To optimize an efficient root elongation medium (REM), several hormonal concentrations and their combinations were tired.

T1: MS_0; T2: MS_0 + 0.5 mg/L IAA; T3: MS_0 + 1.00 mg/L IAA; T4: MS_0 + 1.25 mg/L IAA; T5: MS_0 + 1.50 mg/L IAA.

Observations were recorded after 3 weeks on number of shoots showing root induction, number of roots and root length at the time of hardening (cm), percent root induction, average number of roots and average root length per plantlet.

***Agrobacterium* construct**

pBinAR containing *cry* 1F gene: Plasmid pBinAr (the pBinAr binary vector containing CaM35 S promoter, the coding region of synthetic *Cry* 1F gene, *ocs* terminator and *nptII* selectable marker cassette) was obtained from Dr. Udaya Kumar, Department of Crop Physiology, GKVK, Bangalore and it was mobilized into *Agrobacterium* tumefactions EH 105. It was used for transformation protocol. The *npt II* gene conferring kanamycin resistance served as a selectable marker (Data not shown).

Growth of *Agrobacterium*: Single bacterial colony was transferred from the glycerated culture containing *Agrobacterium* into 25 mL ABMM liquid medium containing 50 mg/L kanamycin and was kept in a shaking water bath overnight at 28 ^{0}C. The overnight grown culture was centrifuged at 10,000 rpm for 5 minutes at 4 ^{0}C. The supernatant was discarded and the bacterial pellet was dissolved with half strength MS basal medium.

Maintenance of bacterial culture: The *Agrobacterium* culture was maintained on the semi solid YEM (yeast extract mannitol) medium containing 50 mg/L kanamycin. Sub culturing was done every one month on fresh medium containing kanamycin.

Plant transformation protocol: Hypocotyl segments of approximately 5 mm length from four-day-old seedlings were

used for plant transformations. The incubation conditions for germination and *in vitro* culture were 25 ± 1°C and 16 h photoperiod of approximately 28 m Em^{-2} s^{-1}. One mL glycerated culture was inoculated in ABMM broth10 containing appropriate antibiotics and grown to saturation. A secondary culture, initiated from the saturated primary culture, was grown in the same selective medium supplemented with 50 mM acetosyringone. The culture was grown to OD600 = 0.6. Cells from 1.5 mL of culture were harvested by centrifugation at 5000 rpm for 10 min at 22°C and resuspended in 15 mL CI liquid medium (MS basal salts with 2% sucrose + 1 mg/L 2,4-D + 0.5 mg/L kinetin). This suspension was used for infection of hypocotyl explants, preconditioned for one day on solid CI medium (CI liquid medium + 0.8% agar) and co-cultivated for three days at 24°C in dark. The explants were incubated on SI medium (MS basal medium with 2% sucrose + 0.5 mg/L IAA + 2 mg/L BA + 3.5 mg/L silver nitrate + 250 mg/L Cefotaxime + 0.25% phytagel) for a delay period of one week, and transferred on S2 selection medium (SI medium + 25 mg/L kanamycin). The regenerated shoots were grown on root induction medium (MS basal medium + 0.1 mg/L IAA + 250 mg/L Cefotaxime +25 mg/L kanamycin). The rooted plants were transplanted into small pots containing vermiculite. After initial establishment, the plants were shifted to pots for hardening.

Hardening of putative transformants: The rooted shootlets first removed from the media bottles and washed thoroughly with sterile double distilled water to remove agar and then dipped in an antifungal solution (Bavistin 0.1%). The plants were then transferred to clean plastic cover containing different proportion of peat and red soil along with control containing natural soil. 3 mL of $1/10^{th}$ of half MS liquid media was added to each cup. The cups were then covered with thin transparent polythene cover and kept in culture room for a week. The temperature and humidity of the culture room were maintained at $25^{\pm}2$ ^{0}C and 80 percent, respectively. The plants were provided with half strength MS solution as and when required. Care was taken not to let the plants dry. Later the plants were transferred to mist chamber and subsequently into field conditions. Observations were recorded for the percent survival of the multiple shoot regenerated plants on different hardening media.

Molecular analysis of putative transformants and statistical analysis: The genomic DNA of putative transformants (on the sixteenth week after co-cultivation) and the control plants (regenerated from non-inoculated explants) were extracted from 2 g of their fresh leaves using a simplified CTAB mini-prep method (Doyle and Doyle, 1987). Based on the *npt II* and *Cry* 1F (Sigma) primers for gene specific amplification *npt II*: Froward primers- 5'GATGGATTGCACGCAGG 3' Reverse primers- 5' GAAGGCGATAGAAGGCG 3'*Cry* 1F**:** forward primer- 5' GGAGTGGGAGTGGCGTTTGGCCTG 3' reverse primer-5' CCAGTTTGTTGGAAGGCAACTCC 3'PCR amplification was carried out in 50 mL reaction volume containing 10X PCR buffer (5 mL), 10 mM dNTP mixture (1 mL), 25 mM $MgCl_2$ (4 mL), 25 mM forward primer (2 mL), 25 mM reverse primer (2 mL), genomic DNA (5 mL), Taq enzyme (0.2 mL)and autoclaved distilled water (30.8 mL). PCR conditions were set for an initial denaturation step of 3 min at 94 ^{0}C and subsequent 25 cycles at 94 ^{0}C for 30 s, 55 ^{0}C for 30 s and 72 ^{0}C for 1 min followed by a final extension step at 72 ^{0}C for 10 min. PCR amplified products were analysed by electrophoresis in a 1% (w/v) agarose-ethidium bromide gel.

Data were analyzed using one-way ANOVA and the differences contrasted using Duncan New Multiple Range Test (DNMRT) performed at 5% level.

Results

Choice of explants source: We choose cotyledons with petiole as explant. The advantage is obvious: we can get large quantities of homogenous materials easily and cheaply at any time. The hypocotyl explants showed early callus initiation (5-6 days) and regeneration (15-20 days) responses than cotyldonary leaf explants (Fig. 1A, B and C). After 21 days most hypocotyl explants showed regeneration response. But no visible shoot regeneration was evident from cotyledonary explants. Regeneration from hypocotyl was much more rapid than the regeneration from cotyledonary leaf explants. The rapidity may be due to the ability of young undifferentiated cells of hypocotyl explant to redirect

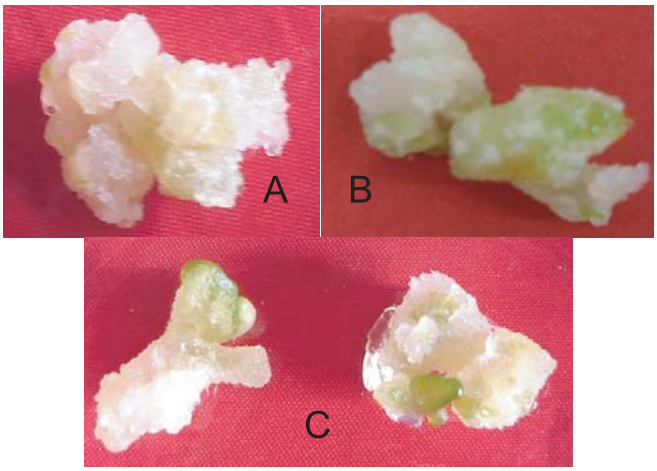

Fig. 1. The regeneration of Brassica hypocotyle from cv 'Hare Krishna' (*B. oleracea* var. capitata). A&B: Intiation of callus induction from hypocotyl explants, C: Initiation of somatic embryo from hypocotyle explants.

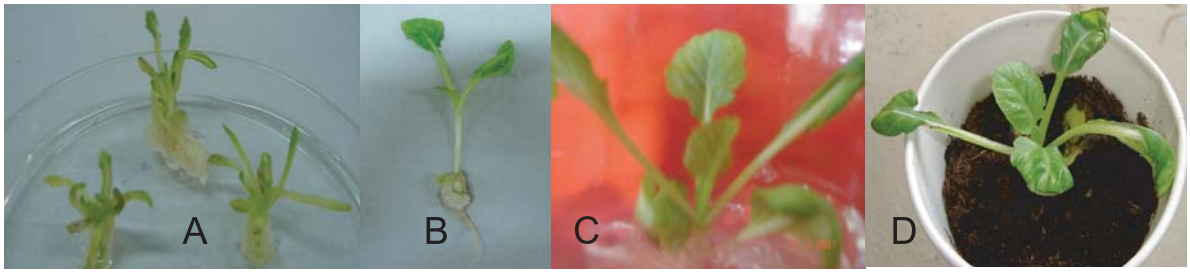

Fig. 2. The regeneration of Brassica hypocotyle from cv 'Hare Krishna' (*B. oleracea* var. capitata). A: formation of the regenerated bud, B: root formation in the regenerant, C: Matured putative transgenic plant, D: The transplantation of a regenerated *Brassica* seedling and hardening of putative transgenics.

their developmental programme and thereby produce new shoots faster than in leaf explants (Fig. 2 A, B). Moreover, intermediate callus phase might also be a reason for such delayed response in cotyledonary leaf explants. In studies of transformation of *Brassica* sp., the cotyledons have often been used as explants for *Agrobacterium* infection. Although the cotyledonary explants had high regeneration abilities, the success of transformant production was limited. Hence only hypocotyl explants were used for transformation.

Standardization of regeneration protocol of cabbage (*Brassica oleracea* Var. *capitata*) cultivar 'Hare Krishna': The procedure followed for regeneration by Bhattacharya *et al.* (2002) was used to standardize best combination of hormones for callus induction. The effect of 2, 4-D and kinetin in different combinations was studied. The data obtained is given in Table 1. From the results it was found that treatment 4 consisting MS_0+1.00 mg/L2,4-D+0.5 mg/mL kinetin was found to produce large amount of callus in 14 days. This was followed by T6 consisting MS_0+1.00mg/L2,4-D+1.0 mg/mL kinetin.

Table 1. Effects of different combinations of phytohormone for callus induction from hypocotyl segment of *B. oleracea* var. capitata.

Treatment	Extent of callus production	Colour of callus	Type of callus	Days taken for callus induction
T1	-	Golden yellow with greenish tint	compact	14
T2	+	Golden yellow with greenish tint	compact	14
T3	++	Golden yellow with greenish tint	compact	14
T4	++++	Golden yellow with greenish tint	compact	14
T5	++	Golden yellow with greenish tint	compact	14
T6	+++	Golden yellow with greenish tint	compact	14

Legend: - no callus; + poor callus growth; ++ moderate callus growth; +++ good callus growth; ++++ very good callus growth.

Regeneration of callus: Among six different combinations of IAA and BAP studied, T6 consisting MS_0+0.5mg/L IAA+2.0 mg/mL BAP (81.50%) was found to show maximum number of shoot production in 20 days. This was followed by T4 consisting MS_0+0.5mg/L IAA+1.0 mg/mL BAP (85.00%) (Table 2). Callus giving multiple shoots was studied under treatments with different combinations of IAA and BAP hormones data given in Table 3. From the data it was observed that treatment six showed highest number of explants giving multiple shoots 76% (Table 3). The maximum number of multiple shoots per explants was observed in the treatment six consisting MS_0+0.5 mg/L IAA + 2.00 mg/mL BAP. The number of shoots which showed the root regeneration was found to be maximum in T4 consisting MS_0+1.00mg/L IAA (45%) and this was followed by T6 consisting MS_0+1.50mg/L IAA (39%) (Table 4).

Transformation and regeneration after co-cultivation of explants with *Agrobacterium*: We have used the binary vector which is transformed the insecticidal gene of *Bacillus thuringiensis* to cabbage genome using cotyledon with petiole as explants and *Agrobacterium tumefaciens* cured strain from which the oncogenes were deleted as mediator. Although the level of insecticidal gene expression in cabbage cell was very low, it was sufficient to cause the *Plutella xylostella* larvae poisoned and died

Table 2. Maintenance of calli derived from hypocotyls of cabbage in different concentrations and combinations of phytohormone

Treatment	Total number of calli placed	Average number of explants	Calli producing shoots (%)
T1	20	0.00	0.00
T2	20	13.67	68.33
T3	20	14.33	71.65
T4	20	17.00	85.00
T5	20	15.00	75.00
T6	20	16.30	81.50

SEM± 0.3944; CV=4.5950; CD (*P*=1.283)

Table 3. Maintenance of calli derived from hypocotyls of cabbage in different concentrations and combinations of phytohormone for multiple shoot production

Treatment	Total number of calli placed	Explants giving multiple shoots	Calli producing multiple shoots(%)	Number of shoots per explants
T1	10	0.0	0.0	0.0
T2	10	4.4	44	8.0
T3	10	6.0	60	6.4
T4	10	5.0	50	10.4
T5	10	6.3	63	6.6
T6	10	7.6	76	14.0

SEM± 0.3944; CV=4.5950; CD (*P*=1.283)

Table 4. Maintenance of shoots derived from calli hypocotyls of cabbage in different concentrations and combinations of phytohormone for root initiation

Treatment	Number of shoots inoculated	Average number of explants	Calli producing roots (%)
T1	05	0.0	0.0
T2	05	2.3	23
T3	05	3.1	31
T4	05	4.5	45
T5	05	3.6	36
T6	05	3.9	39

SEM± 0.4082; CV13.3978; CD (*P*=1.2580)

when they took up a small amount of leaf because of powerful and specific toxicity of insecticidal protein to the larvae (Bhattacharya *et al.,* 2004). Therefore the vector used for transformation is useful for cabbage in pest control.

The shoots originated from the cut ends of hypocotyl segments within two weeks of incubation. Regeneration of transformation was initiated by *A.tumefaciens* mediated transformation. The rooted plants were transplanted into small pots containing vermiculite. After initial establishment, putative transformants were analyzed for the integration of the target gene by using PCR confirmation then the plants were shifted to earthen pots in the greenhouse. In the present study, 45 and 32.5% transformation efficiencies were achieved for hypocotyl and cotyldonary leaf explants, respectively using the optimized procedure.

PCR, which is the easiest and fastest assay for confirmation of foreign DNA fragment integration, was applied in this study. PCR conditions were optimized and no amplification was found in negative control (Untransformed plant). Amplification products of expected size of 800bp and 750bp were obtained respectively for

nptII and *cry*1F gene in both transformants and positive control (Plasmid). Based on the result, some of the putative transformants were produced from non-transformed escape shoots and therefore the expected PCR product band in the agarose gel from their DNA was not seen. The most important reason for the differential expression could be the different number of gene copies in the transgenic plants. It showed that PCR conditions were optimum and it also confirmed that the amplification products obtained were neither due to error in PCR conditions nor due to presence of *Agrobacterium* in transformed plants (Data not shown).

Discussion

The establishment of a regeneration system is a major step in the development of transgenic technology for *Brassica* vegetables. The objective of this study was to develop an efficient, reproducible transformation system for Indian cabbage. Transformation of *B.a oleracea* (*e.g.* cabbage, broccoli, kohl rabi, cauliflower, brussels sprouts and kale) using *Agrobacterium tumefaciens* has been reported by a number of groups (Srivatsava *et al.,* 1988, DeBlock *et al.,* 1989, Metz *et al.,* 1995, Pius *et al.,* 2000, Kuginuki and Tsukazaki *et al.,* 2001, Tsukazaki *et al.,* 2002, Sparrow *et al.,* 2004). Despite considerable advances in methodologies over recent years, the routine transformation of *B. oleracea* is still hindered by genotype restrictions, with some genotypes remaining recalcitrant to transformation. For gene testing to become a routine procedure it is important to identify easy to transform genotypes, with reproducible and reliable transformation efficiencies, that respond well when handled by different users.

Hypocotyl and cotyledon are the two most common explants employed in cabbage genetic transformation studies. Hypocotyls and cotyledons have been used as explants in this study. Some of these explants have a high regeneration frequency and are easily infected by *Agrobacterium*, making them good transform explants. Cotyledons plus petioles are usually chosen as explants during *Brassica* regeneration because, when cut from a growth point, they have some primordial meristematic cells and possess higher regeneration frequencies (Birch, 1997; Kim and Botellam, 2002; Murata and Orton, 1987; Puddephant *et al.*, 1996). It was apparent that hypocotyls were the best explants for cabbage regeneration in this study as they exhibited higher differentiation ability and the plant growth regulator combination was suitable for callus and regeneration induction.

Pre-culture treatment as an osmotic practice has been reported to enhance the transformation efficiency in different plants such as in *Arabidopsis thaliana* (Sangwan *et al.,* 1992), rice (Hiei *et al.,* 1994), maize (Zhao *et al.,* 2001; Frame *et al.,* 2002), Chinese cabbage (Park *et al.,* 2005), cabbage (Metz *et al.,* 1995; Jin *et al.,* 2000), *Cucumis melo* (Awatef *et al.,* 2007), and sweet potato (Xing *et al.,* 2007). However, osmotic treatment was not effective or beneficial in a few studies such as in wheat transformation (Uze *et al.,* 2000; Cheng *et al.,* 2003). This study revealed that osmotic treatment enhancement of *Agrobacterium*-mediated transformation was dependent upon the type of explants used. Hypocotyl explants needed a 3-day-period of pre-culture on CIM. The result showed that the application of BAP + NAA in CIM enhanced the transformation efficiency of hypocotyls segments more than the application of only BAP in SRM. The reason might be the application of growth hormones to explants without preexisting meristems such as hypocotyls, induces the production of meristematic cells and thereby enhances the transformation efficiency. Hence, our study reveals that the application of various plant growth regulators in the osmotic treatment medium give different effects on transformation efficiency and therefore, there is a need to study the effect of different hormones supplemented in the pre-culture medium for effective T-DNA delivery.

In this study, the beneficial effects of *Agrobacterium* concentration and period of inoculation, another two important transformation factors (Cheng *et al.,* 2004; Opabode, 2006) were confirmed. Exposure of both hypocotyl and shoot tip explants to a high concentration (1:5 dilution) of both strains of *Agrobacterium* and long period of inoculation (20 min) decreased the transformation frequency causing severe necrosis of the explants. Although, De Bondt *et al.* (1994) showed that the *Agrobacterium* concentration for transformation was strain dependent, our results showed that the highest transformation frequency in both hypocotyl and shoot tip explants were obtained using the same concentration (1:10 dilution) of both C58 and GV2260 strains of *A. tumefaciens*. The optimal bacterial concentration and inoculation time obtained in this study are in agreement with the *Agrobacterium* dilution and immersion period of Metz *et al.* (1995) whereby they applied a dilution of 1:10 of an *A. tumefaciens* strain ABI culture (OD600 = 1.6-1.8) and 5-10 min inoculation time for transformation of cabbage. The optimized transformation factors obtained in this study resulted in improved transformation efficiencies (45% and 32.5% for hypocotyl and shoot tip explants, respectively) compared to previous *Agrobacterium*-mediated transformation researches on cabbage.

One of the major problems faced in *Agrobacterium*-mediated transformation of plants is the poor regeneration of explants after co-cultivation (Godwin *et al.,* 1991; Berthomieu *et al.,* 1994) and the same problem has been reported in the Brassicas (Takasaki *et al.,* 1997; Park *et al.,* 2005). In this study, the most common regeneration system in the Brassicas which is organogenesis (Cardoza and Stewart, 2004) was applied. The importance of BAP in inducing shoot formation and multiplication is clear (Pierik, 1987) and it is one of the most used plant growth regulator for shoot regeneration of plants. Mahmoudian *et al.* (2002) applied 1mg/L BAP for shoot regeneration of lentil cotyledonary nodes while Jin *et al.* (2000) used a combination of 8.9 mM BAP and 0.3 mM NAA for regeneration of cabbage hypocotyl explants co-cultivated with *A. tumefaciens*. Rashid *et al.* (2003) showed that BAP is a suitable cytokinin for shoot formation from hypocotyl and shoot tip explants of cabbage cv. KY Cross and 1 mg/L BAP induced the highest percentage of shoot formation and mean number of shoots from both explants.

Cabbage explants in tissue culture often brownized and died. We supplemented the culture medium with 10-30 µmol/L $AgNO_3$ (Pua *et al.,* 1987). This can eliminate explants brownization greatly and facilitates callus regeneration and transformant screening. The cabbage tissues are more sensitive to kanamycin than most other plants. For the purpose of *Agrobacterium* mediated transformation the sensitivity of callus to different concentrations of kanamycin ranging from 0 to 100 mg/L was studied. After three days media containing 75 and 100 mg/L showed the complete inhibition of callus (data not shown). The three days

co-cultivation period found to be optimum for controlling the over growth of *Agrobacterium* and to get regeneration of putatively transformed callus on the selective media containing kanamycin. Out of 50 calli subjected to transformation, 40 number of calli showed regeneration. The putative transformants were studied further for the presence of *cry*1F gene using specific primers for PCR amplification. Zhang *et al.* (2000) reported that even at very low concentrations kanamycin inhibited the differentiation of shoots and roots. Cefotaxime inhibited redifferentiation at lower concentration and postponed morphogenesis of root and shoot. In our studies, kanamycin 50 mg/L was identified as stringent selection pressure for selection of transformants. Cefotaxime promoted slight callus production on explants without marked effect on regeneration response.

Our results confirmed that the optimization of transformation factors resulted in the improvement of transformation efficiency. Our results show that by fine-tuning conditions of transformation, even a recalcitrant crop like *B. oleracea* can be transformed. Although the frequency of transformation was still low as compared to model species, the protocol is repeatable and can be used to mobilize genes of agronomic importance into elite cultivars. The techniques used to standardize for regeneration and transformation will be useful for large scale production of transgenic plants to obtain efficient transgenic crops for the target insect pest. *Agrobacterium* mediated transformation using the binary vector containing *cry*1F gene has successfully transformed the hypocotyl derived calli and the transformants could be screened PCR positive based on *npt II* and *cry*1F gene amplification. While the tolerance of transgenics has still to be demonstrated under field conditions, transgenics in combination with other IPM strategies has the potential of preventing a significant part of yield losses and quality deterioration caused by diamondback moth.

Acknowledgements

Authors express sincere gratitude to Plant Molecular Biology Unit funded by Krikhouse Trust (UK), Department of Biotechnology, Bangalore, Karnataka, India for providing the facilities to carry out the research, and Prof. M. Udaykumar, Head, Division of Crop Physiology, University of Agricultural Sciences, Bangalore for technical advice and help rendered. Dr. M. Manamohan, Senior Scientist, Division of Biotechnology, IIHR, Bangalore, India and Dr. B.T. Krishan Prasad, Associate Professor (Biotechnology), Department of Agriculture Biotechnology, Agriculture College, Hassan, Karnataka, India are gratefully acknowledged for reviewing the paper.

References

Anderson Paul., S.R. Sharma, T.V.S. Sresty, Shantibala Devi, Suman Bala, P.S. Kumar, Pardha P. Saradhi, Roger Frutos, L. Altossar, P. Anand Kumar, 2005. Transgenic cabbage (*Brassica oleracea* Var. *capitata*) resistant to Diamondback moth (*Plutella xylostella). Indian J. Biotech.,* 4:72-77.

Awatef, R., H. Michel and B. Mohamed, 2007. *Agrobacterium* mediated transformation of *Tunisian Cucumis melo* cv. maazoun. *Afr. J. Biotechnol*., 6: 2162-2165.

Babu, R.M., A. Sajeena, K. Seetharaman and M.S. Reddy, 2003. Advances in genetically engineered (transgenic) plants in pest management - an overview. Crop Protec., 22**:** 1071-1086.

Berthomieu, P., C. Beclin, F. Charlot, C. Dore and L. Jouanin, 1994. Routine transformation of rapid cycling cabbage (*Brassica oleracea*)—molecular evidence for regeneration of chimeras. *Plant Sci*., 96: 223-235.

Bhattacharya, R.C., M. Maheswari, V. Dineshkumar, P.B. Kirti, S.R. Bhat and V.L. Chopra, 2004. Transformation of *Brassica oleracea* var. capitata with bacterial beta gene enhances tolerance to salt stress. *Sci. Hortic.* 100: 215-227.

Bhattacharya, R.C., N. Viswakarma, S.R. Bhat, P.B. Kirti and V.L. Chopra, 2002. Development of insect-resistant cabbage plants expressing a synthetic *cry1Ab* gene from *Bacillus thuringiensis*. *Curr. Sci.,* 83: 146-150.

Birch, R.G. 1997. Plant transformation: problems and strategies for practical application. *Annu. Rev. Plant Physiol. Plant Mol. Biol.,* 48: 297-326.

Cardoza, V. and C.N. Stewart, 2004. Brassica biotechnology: progress in cellular and molecular biology. *In Vitro Cell Dev. Biol. Plant.,* 40: 542-551.

Cheng, M., T. Hu, J.I. Layton, C.N. Liu and J.E. Fry, 2003. Desiccation of plant tissues post-*Agrobacterium* infection enhances T-DNA delivery and increases stable transformation efficiency in wheat. In *Vitro* Cell. *Dev. Biol. Plant.,* 39: 595-604.

Cheng, M., B.A. Lowe, M. Spencer, X. Ye and C.L. Armstrong, 2004. Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. *In Vitro cell. Dev.Biology-Plant.,* 40: 31-45.

Christou, P., T. Capell, A. Kohli, J.A. Gatehouse and A.M.R. Gatehouse, 2006. Recent developments and future prospects in insect pest control in transgenic crops. *Trends in Plant Science.,* 11: 302-308.

De Block, M., D. De Brouwer and P. Tenning, 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium* tumefaciens and the expression of the bar and neo genes in the transgenic plants. *Plant Physiol.,* 9: 694-701.

De Bondt, A., K. Eggermont, P. Druart, M. De Vil, I. Goderis, J. Vanderleyden and W.F. Broekaert, 1994. *Agrobacterium*-mediated transformation of apple (*Malus* × *domestica* Borkh.): an assessment of factors affecting gene transfer efficiency during early transformation steps. *Plant Cell Rep.,* 13:587-793.

De Leo, F., M. Bonade-Bottino, L.R. Ceci, R. Gallerani and L. Jouanin, 2001. Effects of a mustard trypsin inhibitor expressed in different plants on three lepidopteran pests. *Insect Biochem. Mol. Bio.,* 31: 593-602.

Doyle, J.J. and J.L. Doyle, 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bullettin*., 19: 11-15.

Fernandez-Cornejo, J. and W.D. McBride, 2000. Genetically engineered crops for pest management in U.S. agriculture. Farm-level effects. Economic Research Service/USDA. Geneti. Engin. Crop. Pest Manag./AER.,-786.

Ferry, N., M.G. Edwards, J. Gatehouse, T. Capell, P. Christou and A.M.R. Gatehouse, 2006. Transgenic plants for insect pest control: a forward looking scientific perspective. *Transg. Resea.,* 15: 13-19.

Ferry, N., L. Jouanin, L.R. Ceci, A. Mulligan, K. Emami, J.A. Gatehouse and A.M.R. Gatehouse, 2005. Impact of oilseed rape expressing the insecticidal serine protease inhibitor, mustard trypsin inhibitor-2 on the beneficial predator *Pterostichus madidus*. *Mol. Ecol.,* 14: 337-349.

Frame, B.R., H. Shou, R.K. Chikwamba, Z.I. Zhang, C.I. Xiang, T.M. Fonger, S.E.K. Pegg, B. Li, D.S. Nettleton, D. Pei and K. Wang, 2002. *Agrobacterium* tumefaciens-mediated transformation of maize embryos using a standard binary vector system. *Plant Physiol.,* 129: 13-22.

Godwin, I., G. Todd, B. Ford-Lloyd and H.J. Newbury, 1991. The effects of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. *Plant Cell Rep.,* 9: 671-675.

Groot, A.T. and M. Dicke, 2002. Insect-resistant transgenic plants in a multi-trophic context. *The Plant Journal,* 31: 387-406.

Hiei, Y., Y. Ishida, K. Kasaoka and T. Komari, 2006. Improved frequency of transformation in rice and maize by treatment of immature embryos with centrifugation and heat prior to infection with *Agrobacterium* tumefaciens. *Plant Cell Tissue Org. Cult.,* 87: 233-243.

Hiei, Y., S. Ohta, T. Komori and T. Kumashiro, 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.,* 6: 271-282.

James, Clive. 2011. Global Status of Commercialized Biotech/GM Crops: 2011. *ISAAA Brief* No. 43. ISAAA: Ithaca, NY.

Jin, R.G., Y.B. Liu, B.E. Tabashnik and D. Borthakur, 2000. Development of transgenic cabbage (*Brassica oleracea* var. capitata) for insect resistance by *Agrobacterium tumefaciens*-mediated transformation. *In Vitro Cell Dev. Biol. Plant* 36: 231-237.

Kim, J.H. and J.R. Botellam, 2002. Callus induction and plant regeneration from broccoli (*Brassica oleracea* var. *italica*) for transformation. *J. Plant Biol.,* 45(3): 177-181.

Kuginuki, Y. and H. Tsukazaki, 2001. *Agrobacterium*-mediated transformation of Brassica vegetables. *J Jpn Soc Hortic Sci.,* 70: 682-690.

Lawrence, P.K. and K.R. Koundal, 2001. *Agrobacterium* tumefaciens-mediated transformation of pigeon pea (*Cajanus cajan* L. Millsp.) and molecular analysis of regenerated plants. *Curr. Sci.,* 80: 1428-1432.

Mahmoudian, M., M. Yucel and H.A. Oktem, 2002. Transformation of lentil (*Lens culinaris* M.) cotyledonary nodes by vacuum infiltration of *Agrobacterium tumefaciens*. *Plant Mol. Biol. Rep.,* 20: 251-257.

Metz, T.D., R. Dixit and E.D. Earle, 1995. *Agrobacterium* tumefaciens-mediated transformation of broccoli (*Brassica oleracea* var. italica) and cabbage (*B. oleracea* var. capitata). *Plant Cell Rep*., 15: 287-292.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.,* 15: 473-497.

Murata, M. and T.J. Orton, 1987. Callus initiation and regeneration capacities in *Brassica* species. *Plant Cell Tissue Organ Cult.,* 11: 111-123.

Opabode, J.T. 2006. *Agrobacterium*-mediated transformation of plants: emerging factors that influence efficiency. *Biotechnol. Mol. Biol. Rev.,* 1: 12-20.

Park, J., D.H. Koo, C.P. Hong, S.J. Lee, J.W. Jeon, S.H. Lee, P.Y. Yun, B.S. Park, H.R. Kim, J.W. Bang, P. Plaha, I. Bancroft and Y.P. Lim, 2005. Physical mapping and microsynteny of Brassica rapa ssp. Pekinensis genome corresponding to a 222 kbp gene-rich region of Arabidopsis chromosome 4 and partially duplicated on chromosome 5. *Mol. Genet. Geno.,* 274: 579-588.

Pierik, R.L.M. 1987. *In vitro* Culture of Higher Plants. Martinus Nijhoff Publishers, Dordrecht.

Pius, P.K. and P.N. Achar, 2000. *Agrobacterium*-mediated transformation and plant regeneration of *Brassica oleracea* var. *capitata. Plant Cell Reports.*,19: 888-892.

Pua, E.C., A. Mehra Palta, F. Nagy and N.H. Chua, 1987. Transgenic plants of *Brassica napus* L. *Biotech.,* 5: 815-817.

Puddephat, I.J., T.J. Riggs and T.M. Fenning, 1996. Transformation of *Brassica oleracea* L.: a critical review. *Mol. Breed.,* 2: 185-210.

Rashid, A.A., M.A. Aziz, M.A. Kadir and D. Bahrun, 2003. Shoot induction from hypocotyl explants of cabbage cultivars 88 and KY Cross as affected by BAP. *Trans. Malays. Soc. Plant Physiol.,* 12: 161-164.

Rice, E.M. 2004. Transgenic rootworm corn: assessing potential agronomic, economic, and environmental benefits. Plant Management Network. Published 1 March 2004.

Salas, M.G., S.H. Park, M. Srivatanakul and R.H. Smith, 2001. Temperature influence on stable T-DNA integration in plant cells. *Plant Cell Rep.,* 20: 701-705.

Sangwan, R.S., Y. Bourgeois, S. Brown, G. Vasseur and B.S. Sangwan-Norreel, 1992. Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation of Arabidopsis thaliana. *Planta,* 188: 439-456.

Schuler, T.H., R.P.J. Potting, I. Denholm, S.J. Clark, A.J. Clark, C.N. Stewart and G.M. Poppy, 2003. Tritrophic choice experiments with Bt plants, the diamondback moth (*Plutella xylostella*) and the parasitoid *Cotesia plutellae*. *Transgenic Research*., 12: 351-361.

Sparrow, P.A.C., P.J. Dale and J.A. Irwin, 2004. The use of phenotypic markers to identify *Brassica oleracea* genotypes for routine high-throughput *Agrobacterium*-mediated transformation. *Plant Cell Reports.,* 23: 64-70.

Srivastava, V., A.S. Reddy and S. Guha-Mukherjee, 1988. Transformation of *Brassica oleracea* mediated by oncogenic *Agrobacterium tumefaciens*. *Plant Cell Reports.,* 7: 504-507.

Takasaki, T., K. Hatakeyama, K. Ojima, M. Watanabe, K. Toriyama and K. Hinata, 1997. Factors influencing *Agrobacterium*-mediated transformation of *Brassica rapa* L. *Breeding Sci.,* 47: 127-134.

Tsukazaki. H., Y. Kuginuki, R. Aida and T. Suzuki, 2002. *Agrobacterium*-mediated transformation of a doubled haploid line of cabbage. *Plant Cell Reports.,* 21: 257-262.

Uze, M., I. Potrykus and C. Sautter, 2000. Factors influencing T-DNA transfer from *Agrobacterium* to pre cultured immature wheat embryos (*Triticum aestivum* L.). *Cereal Res. Commun.,* 28: 17-23.

Vasudevan, A., N. Selvaraj, A. Ganapathi and C.W. Choi, 2007. *Agrobacterium*-mediated genetic transformation in cucumber (*Cucumis sativus* L.). *Am. J. Biotechnol. Biochem.,* 3: 24-32.

Wu, K.M. and Y.Y. Guo, 2005. The evolution of cotton pest management practices in China. *Annual review of Entomo.,* 50: 31-52.

Xing, Y., Q. Yang, Q. Ji, Y. Luo, Y. Zhang, K. Gu and D. Wang, 2007. Optimization of *Agrobacterium*-mediated transformation parameters for sweet potato embryogenic callus using b-glucuronidase (GUS) as a reporter. *Afr. J. Biotechnol.,* 6: 2578-2584.

Zhang, F.L., Y. Takahata, M. Watanabe and J.B. Xu, 2000. *Agrobacterium*-mediated transformation of cotyledonary explants of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*). *Plant Cell Rep.,* 19: 569-575.

Zhao, J.Z., J. Cao, Y.X. Li, H.L. Collins, R.T. Roush, E.D. Earle and A.M. Shelton, 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. *Nature Biotech.,* 21: 1493-1497.

Zhao, J.Z., H.L. Collins, J.D. Tang, J. Cao, E.D. Earle, R.T. Roush, S. Herrero, B. Escriche, J. Ferre and A.M. Shelton, 2000. Development and characterization of diamondback moth resistance to transgenic broccoli expressing high levels of Cry1C. *Appl Environ Microbio.,* 66: 3784-3789.

Zhao, Z.Y., W. Gu, T. Cai, L. Tagliani, D. Hondred, D. Bond, S. Schroeder, M. Rudert and D. Pierce, 2001. High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. *Mol. Breed.,* 8: 323-333.

Received: January, 2013; Revised: January, 2013; Accepted: March, 2013