Genetic transformation of cabbage (Brassica oleracea var. capitata) with synthetic cry1F gene to impart resistant to diamondback moth (Plutella xylostella)

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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 cry1F gene coding for an insecticidal crystal protein of Bacillus thuringiensis (Bt) was transformed to cabbage cultivar ‘Hare Krishna’ by co-cultivating hypocotyl explants with Agrobacterium 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 cry1F 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 cry1F 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 cater 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 lepidopteron 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 cocultivation 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 cotyledonal 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 concentration 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 grown culture was centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was discarded and the bacterial pellet was dissolved with half strength MS basal medium.

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 shoot induction, average number of shoots elongated per explant, and average shoot length of shoots elongated.

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

- T1: MS0; T2: MS0 + 0.25 mg/L 2, 4-D+0.5 mg/mL kinetin; T3: MS0 + 0.5 mg/L 2, 4-D+0.5 mg/mL kinetin; T4: MS0 + 1.00 mg/L 2, 4-D+0.5 mg/mL kinetin; T5: MS0 + 1.00 mg/L 2, 4-D+0.75 mg/mL kinetin; T6: MS0 + 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 tried.

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

- T1: MS0; T2: MS0 + 0.25 mg/L IAA +0.5 mg/mL kinetin; T3: MS0 + 0.75 mg/L IAA +0.5 mg/mL kinetin; T4: MS0 + 0.5 mg/L IAA+1.0 mg/mL BAP; T5: MS0 + 0.5mg /L IAA +1.5 mg/mL kinetin; T6: MS0 + 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 tried.

- T1: MS0; T2: MS0 + 0.5 mg/L IAA; T3: MS0 + 1.00 mg/L IAA; T4: MS0 + 1.25 mg/L IAA; T5: MS0 + 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°C. The overnight grown culture was centrifuged at 10,000 rpm for 5 minutes at 4°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 acetyl-saline. 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. The plants were transferred to mist chamber and kept in culture room for a week. The temperature and humidity of the culture room were maintained at 25 ± 1°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: Foward primers- 5’ GATGGATTGCACGCAGGA 3’ reverse primer- 5’ GAAGGGCATAGAAGGGCG 3’ Cry 1F: forward primer- 5’ GGAGTTGGAGTGGCGTTTGGCCTG 3’ reverse primer- 5’ CCAGTTTTTGGGAAGGCAACTCC 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 MgCl2 (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°C and subsequent 25 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min followed by a final extension step at 72°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 cotyledonary 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 its metabolic powers to eliminate the regenerative signal to regenerate shootlike structures. The roots were induced at 25°C ± 1°C and 16 h photoperiod of approximately 28 m Em^-2 s^-1. For the analysis, the plants were transferred to pots containing vermiculite with 2% sucrose + 1 mg/L IAA + 0.5 mg/L kinetin. After initial establishment, the plants were shifted to pots for hardening.
their developmental programme and thereby produce new shoots faster than in leaf explants (Fig. 2A, B). Moreover, intermediate callus phase might also be a reason for such delayed response in cotyledonal leaf explants. In studies of transformation of *Brassica* sp., the cotyledons have often been used as explants for *Agrobacterium* infection. Although the cotyledonal 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. From the data obtained is given in Table 1. From the results it was found that treatment 4 consisting MS0+1.00 mg/L 2,4-D+0.5 mg/mL kinetin was found to produce large amount of callus in 14 days. This was followed by T6 consisting MS0+1.00mg/L 2,4-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*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Extent of callus production</th>
<th>Colour of callus</th>
<th>Type of callus</th>
<th>Days taken for callus induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>-</td>
<td>Golden yellow with greenish tinct</td>
<td>compact</td>
<td>14</td>
</tr>
<tr>
<td>T2</td>
<td>+</td>
<td>Golden yellow with greenish tinct</td>
<td>compact</td>
<td>14</td>
</tr>
<tr>
<td>T3</td>
<td>++</td>
<td>Golden yellow with greenish tinct</td>
<td>compact</td>
<td>14</td>
</tr>
<tr>
<td>T4</td>
<td>+++</td>
<td>Golden yellow with greenish tinct</td>
<td>compact</td>
<td>14</td>
</tr>
<tr>
<td>T5</td>
<td>+++</td>
<td>Golden yellow with greenish tinct</td>
<td>compact</td>
<td>14</td>
</tr>
<tr>
<td>T6</td>
<td>+++</td>
<td>Golden yellow with greenish tinct</td>
<td>compact</td>
<td>14</td>
</tr>
</tbody>
</table>

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 MS0+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 MS0+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 MS0+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 MS0+1.00mg/L IAA (45%) and this was followed by T6 consisting MS0+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 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 transfectants 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 cotyledonal 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

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of calli placed</th>
<th>Average number of calli producing shoots (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>20</td>
<td>0.00</td>
</tr>
<tr>
<td>T2</td>
<td>20</td>
<td>13.67</td>
</tr>
<tr>
<td>T3</td>
<td>20</td>
<td>14.33</td>
</tr>
<tr>
<td>T4</td>
<td>20</td>
<td>17.00</td>
</tr>
<tr>
<td>T5</td>
<td>20</td>
<td>15.00</td>
</tr>
<tr>
<td>T6</td>
<td>20</td>
<td>16.30</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of explants giving multiple shoots</th>
<th>Calli producing multiple shoots (%)</th>
<th>Number of shoots per explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>10</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>T2</td>
<td>10</td>
<td>4.4</td>
<td>8.0</td>
</tr>
<tr>
<td>T3</td>
<td>10</td>
<td>6.0</td>
<td>6.4</td>
</tr>
<tr>
<td>T4</td>
<td>10</td>
<td>5.0</td>
<td>10.4</td>
</tr>
<tr>
<td>T5</td>
<td>10</td>
<td>6.3</td>
<td>6.6</td>
</tr>
<tr>
<td>T6</td>
<td>10</td>
<td>7.6</td>
<td>14.0</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of shoots inoculated</th>
<th>Average number of explants</th>
<th>Calli producing roots (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>05</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>T2</td>
<td>05</td>
<td>2.3</td>
<td>23</td>
</tr>
<tr>
<td>T3</td>
<td>05</td>
<td>3.1</td>
<td>31</td>
</tr>
<tr>
<td>T4</td>
<td>05</td>
<td>4.5</td>
<td>45</td>
</tr>
<tr>
<td>T5</td>
<td>05</td>
<td>3.6</td>
<td>36</td>
</tr>
<tr>
<td>T6</td>
<td>05</td>
<td>3.9</td>
<td>39</td>
</tr>
</tbody>
</table>

SEM+= 0.4082; CV13.3978; CD (P=1.2580)
**Genetic transformation of cabbage with synthetic cry1F gene**

*npII* and *cry1F* 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, kohlrabi, 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., 1997). 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 AgNO₃ (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 cry1F 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 cry1F gene has successfully transformed the hypocotyl derived calli and the transformants could be screened PCR positive based on nptII and cry1F 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.

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References


Genetic transformation of cabbage with synthetic cry1F gene


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