

Transformation And Light Inducible Expression of *cry1Ab* Gene in Oilseed Rape (*Brassica napus* L.)

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Abstract

Rapeseed (*Brassica napus* L.) is the third most important oil crop in global productions. One of the major limiting factors for oilseed rape production is lepidopteran pests of the *Brassicaceae* family. Transgenic plants expressing *Bacillus thuringiensis* (Bt) genes are powerful tools in the integrated pest management of crop plants. In the present study, we used a synthetic Bt insecticidal crystal protein gene (*cry1Ab*) under the control of the *phosphoenolpyruvate carboxylase* (PEPC) promoter for genetic transformation of *B. napus* L. (var. SLM046) using *Agrobacterium tumefaciens*-mediated transformation. PEPC-*cry1Ab*-nos cassette cloned in a binary vector pCAMBIA3300 containing *bar* gene as a selection marker. The new vector (pCAMBIAPEPCry) introduced to AGL01 strain of *A. tumefaciens*, which used in transformation of hypocotyl explants of *B. napus*. Putative transgenic rapeseed plants were regenerated in selection media containing phosphinothricin (PPT) as selection agent. Polymerase Chain Reaction (PCR) confirmed the integration of *cry1Ab* and *bar* genes at putative transgenic plant genome. Furthermore, transcription (mRNA production) and protein expression of *cry1Ab* gene was confirmed using RT-PCR and immune-strip methods, respectively. Transgenic *B. napus* plants expressed Cry1Ab protein in the shoots and not in the roots. We concluded that C4 maize PEPC promoter can induce the expression of Cry1Ab recombinant protein only in light treated (green) tissues in rapeseed plants. It recommended as a light inducible promoter for targeted expression of transgene in the rapeseed plants.

Keywords: *Agrobacterium*, *bar*, *Brassica napus*, *cry1Ab*, Lepidopteran.

Introduction

Oilseed rape (*Brassica napus* L.), a cruciferous (*Brassicaceae*) plant, is a widely cultivated plant species in the world [1]. It is predominantly used for oil

production due to its high seed oil content. Besides their protein content (45%), the quality of these crops fatty acids (40%), mostly dependent on the environment, fertilization, agrotechnics and genotype. This crop with more than 40% fatty acids is today the world's third-

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leading source of oil after soybean and date palm [2, 3, 4].

Like many other crops, the production of rapeseed crops is affected by biotic stresses such as insect pests and weeds [5]. Ubiquitous lepidopteran *Brassica* special pests, such as Diamondback moth (*Plutella xylostella* L.) and cabbage looper (*Trichoplusia ni* Hübner), and common lepidopterans, such as the beet armyworm (*Spodoptera exigua* Hübner) and corn earworm (*Helicoverpa zea* Boddie), may be important where canola is grown in warmer regions [6]. Diamondback moth is the major pest affecting a variety of cruciferous crop plants. In some years, it causes enormous crop losses in the world [7]. Therefore, to increase the rapeseed yield, it is necessary to improve its resistance to pests.

Transgenic cultivars expressing insecticidal properties may play a major role in integrated pest management strategies for canola pests [8, 9]. Although there is no commercialized lines of *Bt*-*B. napus* L., experimental transgenic *Bt*-producing *B. napus* lines have been generated confer resistance to *Brassica*-defoliating insects, including diamondback moth, cabbage looper and corn earworm [7, 10, 11, 12, 13, 14]. Moreover, a few number of transgenic *Bt*-*Brassica* species being developed, including broccoli (*B. oleracea* L. var. *botrytis*) [15, 16], cabbage (*B. oleracea* var. *capitata*) [17], and Chinese cabbage (*B. rapa* L. subsp. *chinensis*) [18, 19], using the *cry1Ac* gene designed to control diamondback moth.

The selection of an appropriate promoter sequences is critical for targeting expression of the transgene. The CaMV35S from Cauliflower Mosaic Virus and the maize ubiquitin promoter represent a group of promoters have been used for constitutive expression of *Cry* proteins in many crops [20, 21]. It is often desirable to couple high-level expression of the transgene in rapeseed foliage with no expression in other organs. One candidate for this approach is the light inducible promoters such as C4 maize *phosphoenolpyruvate carboxylase* (PEPC). PEPC, is a ubiquitous cytosolic enzyme located in mesophyll cells of C4 and CAM

plants, catalyzes the irreversible b-carboxylation of PEP to yield oxaloacetate and inorganic phosphate (Pi) [22]. PEPC is activated by light in C4 plants, however, in C3 plants, the activation of PEPC by light is limited [22].

In the present study, we described genetic transformation of *B. napus* using *cry1Ab* gene under the control of PEPC promoter and the expression of the *Cry1Ab* recombinant protein in transgenic rapeseed lines.

Materials and Methods

Plant transformation vector

The 4486 bp fragment containing sequence encoding the insecticidal protein *Cry1Ab* regulated by the maize PEPC promoter, was digested from the pCIB4421 plasmid with *Hind*III and *Eco*RI restriction enzymes [23]. This fragment was inserted into the binary vector pCAMBIA3300, which was cut with *Hind*III and *Eco*RI. The new construct, pCAMBIAPEPCry (Fig. 1), containing selectable marker gene *bar*, was then transferred into the *A. tumefaciens* strain AGLO1 via the freeze–thaw method [24].

Plant materials and transformation

Seeds of *Brassica napus* var. SLM046 were surface sterilized in 96 % ethanol for 2 minutes, 3% sodium hypochlorite (containing 1 drop of Tween20) for 15 minutes. The seeds were washed three times in sterile distilled water and were germinated on MS [25] basal medium containing 30 gr/l sucrose and 8 gr/l agar for 6 days in glass bottles at 25°C in a 16 h 4000 lux light/8 h dark photoperiod [26].

The hypocotyl explants, about 1 cm length, were excised from 6 days old seedlings and were pre-cultured for 3 days on callus inducing medium, MS containing 3% (w/v) sucrose and 0.7% (w/v) agar enriched with 1 mg/l 2,4-D. Single colonies of the *A. tumefaciens* strain AGL01 harboring pCAMBIAPEPCry were grown overnight at 28°C in LB medium supplemented with 50 mg/l kanamycin and 50 mg/l rifampicin. The cultures

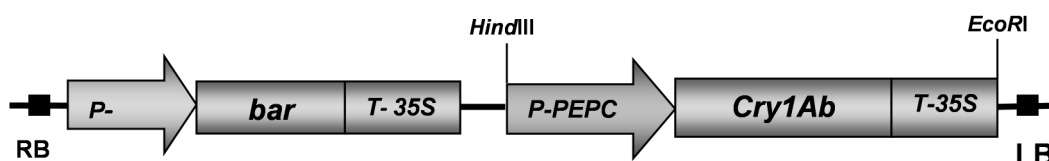


Figure 1. Schematic representation of the T-DNA region of the binary vector pCAMBIAPEPCry. LB, RB, left and right T-DNA borders, respectively; P-35S, *CaMV35S* promoter; *bar*, *phosphinothricin acetyl transferase* gene; T-35S, *CaMV35S* terminator; P-PEPC, *Phosphoenolpyruvate carboxylase* promoter; *cry1Ab*, *Bt* gene; *Hind*III, *Eco*RI, restriction enzymes.

were centrifuged and the cells were re-suspended in half-strength MS medium (pH 5.2) plus 0.05 mM Acetosyringone. Explants were then inoculated with *A. tumefaciens* for 5 min, blotted on sterile filter paper and co-cultivated on the same medium at 25°C under above light conditions. After 2 days, explants were transferred to callus-inducing medium containing 500 mg/l carbenicilin and 5 mg/l phosphinothricin (PPT) for selection of transgenic plant cells. After a 14-day selection, explants were transferred to organogenesis medium, MS containing 30 gr/l sucrose, 4 mg/l BAP, 2 mg/l zeatin, 5 mg/l AgNO₃, 500 mg/l carbenicilin and 8 mg/l phosphinothricin and cultivated for 2 - 3 weeks at 22°C under a 16/8 h (light/dark) photoperiod. For shoot regeneration, the explants were sub-cultured on MS medium containing 3 mg/l BAP, 2 mg/l zeatin, 8 mg/l phosphinothricin and 500 mg/l carbenicilin at 14 days intervals.

Then the initial shoots were transferred to root inducing medium, 1/2 MS medium containing 5 mg/l IBA, 3 mg/l phosphinothricin and 500 mg/l carbenicilin for rooting. The fully rooted shoots were transferred to a mixture soil containing pith, perlite and soil (1:1:1) and maintained in greenhouse conditions (25°C and 16 h light).

Molecular Analysis of transgenic plants

Polymerase chain reaction (PCR)

Genomic DNA was extracted from leaves and roots of putative transformed plants using CTAB method [27]. PCR analysis was performed using the primers F-cry: 5'-GGCGGCGAGAGGATCGAGAC-3' and R-cry: 5'-TCGGCGGGACGTTGTTGTTTC-3', to amplify a 1190-bp fragment of the Cry1Ab coding region and the primers 5'- ATC TCG GTG ACG GGC AGG AC -3' and 5'- CGC AGG ACC CGC AGG AGT G -3', to amplify a 500-bp fragment of the *bar* coding region. PCR reactions were performed using 25 ng of template DNA and by Roche Co Taq DNA polymerase. The initial denaturing was done at 94°C for 5 min and followed by 35 cycles of 1 min at 94°C, 1 min at 60 °C (for *cry1Ab*) or 58 °C (for *bar*) and 1 min at 72 °C. A final extension step at 72 °C for 5 min was included. The amplified products were separated by electrophoresis on a 1% (w/v) agarose gel and visualized by GelRed staining.

RT-PCR

RT-PCR was performed in order to confirm *cry1Ab* gene expression at the transcriptional level. Total RNAs were extracted using Tripure isolation reagent (Roche, Germany). RNA was treated extensively with RNase free DNase (Roche, Germany) in order to remove any

contaminating genomic DNA. The same *cry1Ab* primer set for PCR reactions was also used for the RT-PCR. Titan One-step RT-PCR reaction was used according to the manufacturer's instructions (Titan, Roche, Germany). The amplified cDNAs were visualized as PCR products.

Cry1Ab protein detection

The presence of Cry1Ab recombinant protein in the leaves and roots of transgenic rapeseed was immunologically determined using Lateral flow test strip (Bt-Cry1Ab/1Ac Immuno- Strip, Agdia Inc., IN). Sample preparation and test were carried out according to manufacture instructions (Agdia Inc. USA).

Rapeseed leaves and roots first were powdered using liquid nitrogen and then homogenized 1 ml extraction buffer (SEB4 buffer; Agdia) at 1.5 ml tubes. The solid materials were allowed to settle for 1–2 min. The strips tips were immersed in the supernatant and allowed to develop bands for 10 min before making a final interpretation of the results.

Results and Discussion

The development of genetic engineering for production of transgenic pest resistant crops is becoming a valuable component of integrated pest management (IPM) programs [28]. Transgenic BT crops producing insecticidal Cry proteins which interact with specific protein receptors present in the epithelial cells of the insect midgut and causing death of the target pest [29]. Different types of the Cry protein have been isolated and have *specific* activities against insect species. The present study has investigated the transformation and expression of *cry1Ab* gene in rapeseed crop.

Plant transformation

In one experiment, out of 114 hypocotyl explants 27 independent shoots regenerated on selection medium containing 5 mg/l PPT (Fig. 2). Transferring of the green shoots to elongation medium containing 8 mg/l PPT conducted for the complementation and stringency of the selection stage. The rooted shoots were transferred to soil and were grown under greenhouse conditions (Fig. 2).

PCR and RT-PCR analysis of putative transgenic plants

PCR analysis using *cry1Ab* and *bar* primers was performed on the putative transformed lines grown on selection medium. Overall, 23 lines were positive for both *cry1Ab* and *bar* genes in the shoots and roots (Fig.

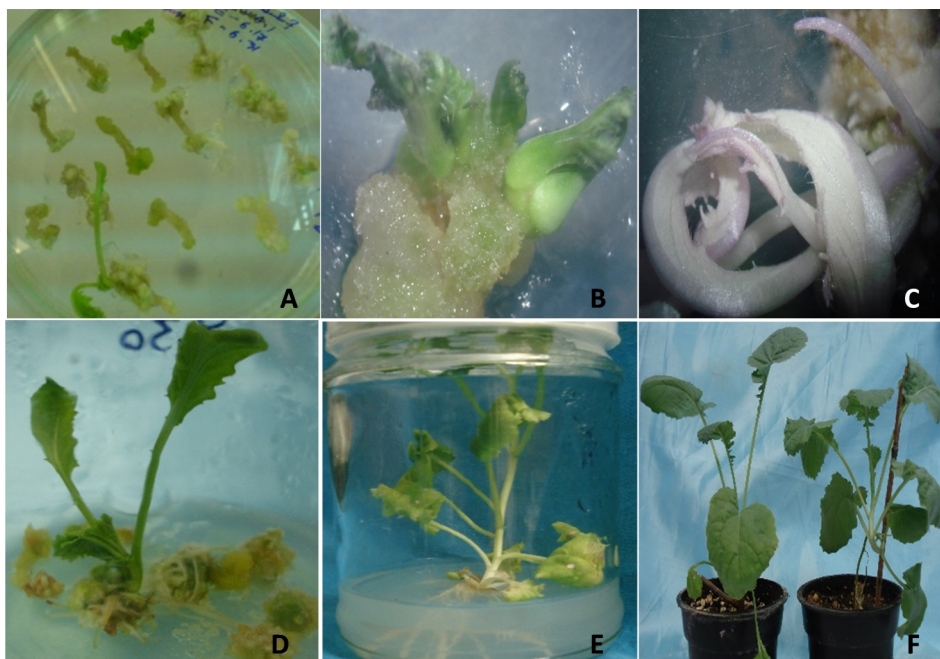


Figure 2. *In vitro* regeneration and growing of transgenic canola plants in greenhouse. A, B- Callus formation on the hypocotyl explants and plant regeneration; C- non-transgenic shoot regenerated on the selection medium; D, Putative transgenic shoot grown on selection medium; E- Root induction in putative transgenic plant; F- Putative transgenic plants in greenhouse conditions.

3; Table 1). Four lines were PCR negative for both *bar* and *cry1Ab* genes. These lines were considered as “escapes” from the initial selection of transgenic plants and therefore removed away from the other putative transgenic lines.

PCR positive plants were used for RT-PCR analysis. RT-PCR analysis confirmed the expression of *cry1Ab* gene at transcript level in the leaves of putative transgenic lines (Fig. 4; Table 1). We could not detect *cry1Ab* transcripts in the transgenic lines’ roots and in the control non-transgenic lines. This indicated that the expression of Cry1Ab inhibited in the roots of transgenic line grown in dark conditions. Therefore, PEPC promoter can control the expression of *cry1Ab* gene at the transcription level.

***Cry1Ab* protein detection**

The expression of Cry1Ab protein was assayed in the leaves and roots of PCR-positive transgenic lines. Immunostrips showed the band related to Cry1Ab protein in the leaves of 21 out of 23 transgenic lines.

The control band appeared in transgenic and non-transgenic plants, but Cry1Ab protein bands just appeared in the leaves, not in the roots of transgenic lines (Fig. 5; Table 1). The results indicated that light can induce the expression of Cry1Ab protein by regulation of PEPC promoter in different tissues of transgenic lines.

Genetic transformation in *B. napus* is highly dependent on genotype and explant. Mashayekhi et al [26] reported 4.7 and 8.96% transformation frequency in SLM046 genotype for cotyledon and hypocotyls explants, respectively. In the present work, 20.2% and 16.6% transformation frequency was obtained for the SLM046 var. respectively by PCR and protein expression analysis. However, Sakhno et al [30] reported 86.3% transformation frequency for Kalinoskiy var. by PCR analysis. Moreover, there are different reports for genetic transformation frequency *B. napus* using different genotype and explants: 11.4% for PF and Maplus hypocotyls [31, 32]; 17-25% for hypocotyls explants [33]; 25% in Maplus cv. [34].

Table 1. Regeneration frequency of *cry1Ab* expressing plants derived from hypocotyls explants of *B. napus* cv. SLM046.

No. of explants	No. of regenerated shoots	No. PCR positive plants	No. of positive expressed plants	Transformation frequency (%) by PCR	Transformation frequency (%) by immunostrip
114	27	23	21	20.2	18.4

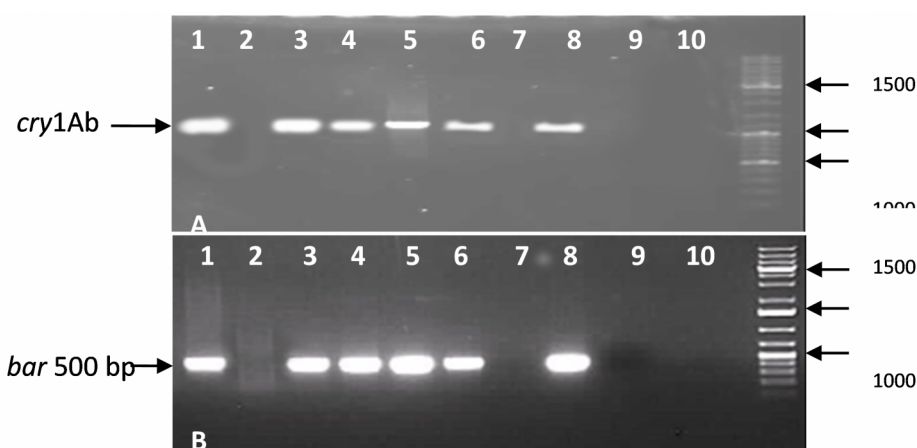


Figure 3. PCR analysis of putative transgenic canola lines for *cry1Ab* (A) and *nptII* (B) genes. 1- Positive control; 2- Negative control (water); 3-6 (leaves), 8 (root)- transgenic lines; 9-10- Negative control (Non transgenic plant); 11- 1kb ladder.

In this study, out of 27 kanamycin resistant shoots 23 were transgenics carrying the *cry1Ab* and *bar* genes by PCR analysis (Table 1). We concluded that two step selection (Callus inducing medium with 3 mg/l PPT and Organogenesis medium with 8 mg/l PPT) can highly prevent the ‘escape’ of PPT sensitive regenerants during selection.

In the present work we observed that out of 23 PCR positive plants only 21 have *cry1Ab* gene expression

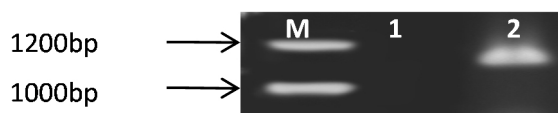


Figure 4. RT-PCR analysis for one selected PCR positive transgenic line by *cry1Ab* primers. M- 1kb ladder; 1- Negative control; 2- Transgenic line.

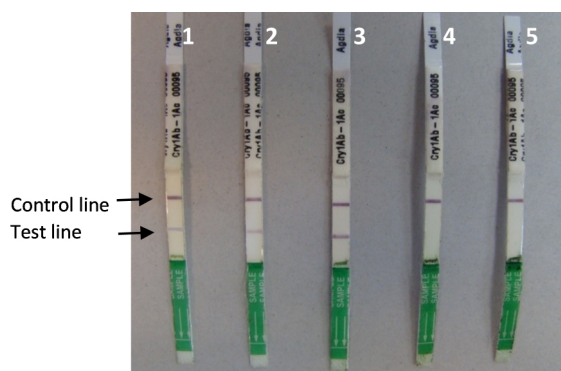


Figure 5. Immunostrip detection of Cry1Ab protein in PCR-positive transgenic plants. 1-3-Transgenic plant expressing Cry1Ab protein; 4- PCR-positive transgenic plants un-expressing Cry1Ab protein in the roots; 5- non-transgenic plant.

(Table 1). The lack of *cry1Ab* expression in some of PCR positive plant may be due to alteration or loss of *cry1Ab* gene resulting from rearrangement of the coding sequence or methylation of the gene [35].

The *cry1Ac* gene has been previously transformed into *B. napus*, exhibiting the strong resistance to the diamondback moth and the cabbage looper [36]. Recently Wang et al [34] developed transgenic *B. napus* using an optimized *cry1C** for resistant to diamondback moth. They found that transgenic rapeseed plants expressing *cry1C** gene showed a high efficacy against the pests.

Constitutive expression of a transgene may increase the risk of resistant pest development, and there may also be yield penalties as the plant directs more resources than necessary to its defense [37]. Therefore, the development of tissue-specific promoters to drive transgene expression has helped fulfill that need. It is particularly important for the future development of transgenic crops because the public may be more likely to accept ‘less intrusive’ expression of the transgene [38].

The PEPC promoter has been shown to drive green tissue specific expression of Bt protein in rice [39] and potato [23]. In the present work we developed transgenic *B. napus* plants producing Cry1Ab protein under the control of a light inducible promoter (PEPC). We concluded that C4 maize PEPC promoter can effectively express Cry1Ab protein in the light treated (green) tissues (leaves) of rapeseed plants. We can use PEPC promoter for targeted expression of transgene in the rapeseed plants.

References

- Pipan B., Sustar-Vozlic C. and Meglic V. Cultivation, varietal structure and possibilities for cross-pollination of *Brassica napus* L. in Slovenia. *Acta Agr. Slov.* **97**: 247-258 (2011).
- Laaniste P., Joudu J. and Eremeev V. Oil content of spring oilseed rape seeds according to fertilization. *Agron. Res.* **2**: 83-86 (2004).
- Snowdon R, Luhs H. and Friedt W. Genome mapping and molecular breeding in plants, volume 2. Oilseed Rape. Eds C.kole. Springer-verlag berlin Heidelberg, pp. 55-83, (2007).
- Friedt W. and Snowdon R. Oilseed rape. in: Handbook of Plant Breeding, vol. 4. Oil Crops, Vollman, J. & Rajcan, J. (eds.), Springer-Verlag, Dordrecht, Heidelberg, London, New York, pp. 91-126, (2009).
- Wang Y., Zhang Y., Wang F., Liu C. and Liu K. Development of transgenic *Brassica napus* with an optimized *cry1C** gene for resistance to diamondback moth (*Plutella xylostella*). *Can. J. Plant Sci.* **94**: 1501-1506 (2014).
- Buntin G. D. and Raymer P. L. Pest status of aphids and other insects in winter canola in Georgia. *J. Econ. Entomol.* **87**: 1097-1104 (1994).
- Masson P., Braun L., Warwick S, Zhu B, and Stewart C.N. Transgenic *Bt*-producing *Brassica napus*: *Plutella xylostella* selection pressure and fitness of weedy relatives. *Environ. Biosafety Res.* **2**: 263-276 (2003) .
- Talekar N. T. and Shelton A. M. Biology, ecology and management of the diamondback moth. *Annu. Rev. Entomol.* **38**: 275-301(1993).
- Evans K. A. and Scarisbrick D. H. Integrated insect pest management in oilseed rape crops in Europe. *Crop Prot.* **13**: 403-412 (1994) .
- Halfhill M. D., Richards H. A., Mabon S. A. and Stewart C. N. Expression of GFP and Bt transgenes in *Brassica napus* and hybridization with *Brassica rapa*. *Theor. Appl. Genet.* **103**: 659-667 (2001).
- Stewart C. N., Adang M. J., All J. A., Raymer P. L., Ramachandran S. and Parrott W. A. Insect control and dosage effects in transgenic canola containing a synthetic *Bacillus thuringiensis cry1AC* gene. *Plant Physiol.* **112**: 115-120 (1996).
- Ramachandran S., Buntin G. D., All J. N. Tabashnik B. E., Raymer P. L., Adang M. J., Pulliam D. A. and Stewart Jr C. N. Survival, development, and oviposition of resistant diamondback moth (Lepidoptera: Plutellidae) on transgenic canola producing *Bacillus thuringiensis* toxin. *J. Econ. Entomol.* **91**: 1239-1244 (1998).
- Zhu B., Lawrence J. R., Warwick S. I., Mason P., Braun L., Halfill M. D. and Stewart J. R. Stable *Bacillus thuringiensis* (Bt) toxin content in inter-specific F1 and backcross populations of wild *Brassica rapa* after Bt gene transfer. *Mol. Ecol.* **13**: 237-241(2004).
- Le Y., Stewart J. C. N., Shi H., Wei W., Mi X. and Ma K. Expression of Bt *cry1Ac* in transgenic oilseed rape in China and transgenic performance of intraspecific hybrids against *Helicoverpa armigera* larvae. *Ann. Appl. Biol.* **150**:141- 147 (2007).
- Cao J., Tang J. D., Strizhov N., Shelton A. M. and Earle E. D. Transgenic broccoli with high level of *Bacillus thuringiensis* protein control diamondback moth larvae resistant to Cry1A or Cry1C. *Mol. Breeding.* **5**:131-141 (1999).
- Metz T. D., Roush R. T., Tang J. D., Shelton A. M. and Earle E. D. Transgenic broccoli expressing a *Bacillus thuringiensis* insecticidal crystal protein: implications for pest resistance management strategies. *Mol. Breeding.* **1**:309-317 (1995).
- Jin R. G., Liu Y. B., Tabashnik B. E. and Borthakur D. Development of transgenic Cabbage (*Brassica oleracea* var. Capitata) for insect resistance by *Agrobacterium tumefaciens* mediated transformation. *In Vitro Cell Dev. Biol. Plantarum.* **36**: 231-237 (2000).
- Cho H. S., Cao J., Ren J. P. and Earle E. D. Control of Lepidopteran insect pests in transgenic Chinese cabbage (*Brassica rapa* ssp. pekinensis) transformed with a synthetic *Bacillus thuringiensis cry1C* gene. *Plant Cell Rep.* **20**: 1-7 (2001).
- Xiang Y., Wong W. K. R., Ma M. C. and Wong R. S. C. *Agrobacterium* mediated transformation of *Brassica campestris* ssp. *Parachinensis* with a synthetic *Bacillus thuringiensis cry1Ab* and *cry1Ac* genes. *Plant Cell Rep.* **19**:251-256 (2000) .
- Ramesh S., Nagadhara D., Pasalu I. C., Kumari A. P., Sarma N. P., Reddy V. D. and Rao K. V. Development of stem borer resistant transgenic parental lines involved in the production of hybrid rice. *J. Biotechnol.* **111**: 131-141 (2004).
- Abbas Zaidi M., Mohammadi M, Postel S., Masson L. and Altosaar I. The Bt gene *cry2Aa2* driven by a tissue specific ST-LS1 promoter from potato effectively controls *Heliothis virescens*. *Trans. Res.* **14**: 289-298 (2005).
- Chinthapalli B., Raghavendra A. S., Rishi A. S., and Goyal A. Phosphoenolpyruvate Carboxylase from C4 Plants: Properties and Regulation. *Rev. Plant Biochem. Biotech.* **1**: 143-159 (2002).
- Ghasimi Hagh Z., Rahnama H., Mahna N., Panahandeh J., Baghban Kohneh Rouz B., and Arab Jafari K. M. Green-tissue-specific, C4-PEPC- promoter-driven expression of *Cry1Ab* makes transgenic potato plants resistant to tuber moth (*Phthorimeae operculella*, Zeller). *Plant Cell Rep.* **28**:1869-1879 (2009).
- Hofgen R. and Willmitzer L. Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Res.* **16**: 9877 (1988).
- Murashige T. and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plantarum.* **15**: 473497 (1962).
- Mashayekhi M., Shakib A. M., Ahmad-Raji M. and Ghasemi Bezdi K. Gene transformation potential of commercial canola (*Brassica napus* L.) cultivars using cotyledon and hypocotyl explants". *Afr. J. Biotechnol.* **7**: 4459-4463 (2008).
- Doyle J. J. and Doyle J. L. Isolation of plant DNA from fresh tissue. *Focus*, **12**: 13-15 (1990).
- Meiyalaghan S., Barrell P. J., Jacobs M. E. and Conner A.J. Regeneration of multiple shoots from transgenic potato events facilitates the recovery of phenotypically normal lines: assessing a *cry9Aa2* gene conferring insect resistance. *BMC Biotechnol.* **11**: 93 (2011).
- Pelletier Y. and Michaud D. Insect pest control on potato:

- genetically-based control. In: Duchesne R-M, Boiteau G, editors. *Potato insect pest control: development of a sustainable approach*. Gouvernement du Québec; 69-79 (1995).
30. Sakhno L. A., Gocheva E. A., Komarnitskii I. K. and Kuchuk N. V. Stable Expression of the Promoterless *bar* Gene in Transformed Rapeseed Plants. *Cytol. Genet.* **42**: 16-22 (2008).
 31. Jonoubi P., Mousavi A., Majd A., Salmanian A. H., Jalali Javaran M. and Daneshian J. Efficient regeneration of *Brassica napus* L. hypocotyls and genetic transformation by *Agrobacterium tumefaciens*. *Biol. Plantarum.* **49**: 175-180 (2005).
 32. Zebarjadi A., Jalali Javaran M., Karimzadeh G., Moeini A., Mousavi A. and Salmanian A. H. Transformation of rapeseed (*Brassica napus* L.) plants with sense and antisense constructs of the fatty acid elongase gene. *Iran. J. Biotechnol.* **4**: 79-87 (2006) .
 33. Cardoza V. and Stewart C. N. Brassica Biotechnology: Progress in cellular and molecular biology. *In Vitro Cell Dev Biol Plant.* **40**: 542-551 (2004).
 34. Wang J., Chen Z., Du J., Sun Y and Liang A. Novel insect resistance in *Brassica napus* developed by transformation of chitinase and scorpion toxin gene. *Plant Cell Rep.* **24**: 549-555 (2005).
 35. Saker N. N., Salama H. S., Salama M., El-Banna A. and Abdel Ghany N. M. Production of transgenic tomato plants expressing *Cry2Ab* gene for the control of some lepidopterous insects endemic in Egypt. *J Gen. Engin. Biotechnol.* **9**: 149-155 (2011).
 36. Stewart C. N., All J. N., Raymer P. L. and Ramachandran S. Increased fitness of transgenic insecticidal rapeseed under insect selection pressure. *Mol. Ecol.* **6**: 773-779 (1997).
 37. Perera M. R. and Jones M. G. K. Expression of the peroxidase gene promoter (*Shpx6b*) From *Stylosanthes humilis* in transgenic plants during insect attack. *Entom. Experimen. App.* **111** : 165-171 (2004).
 38. Potenza C., Aleman L. and Sengupta-Gopalan C. Targeting transgene expression in research, agricultural, and environmental applications: promoters used in plant transformation. *In Vitro Cell. Dev. Biol.-Plant*, **40**:1-22 (2004).
 39. Ghareyazie B., Alinia F., Menguito C., Rubia L., De Palma J., Liwanag E., Cohen M., Khush G.S. and Bennett J. Enhanced resistance to two stemborers in an aromatic rice containing a synthetic *cry1Ab* gene. *Mol. Breed.* **3**: 401-414 (1997).