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Inheritance of Transgene and Resistance to a Lepidoptran Pest, *Spodoptera littoralis*, in Transgenic Sugar Beet Plants Harboring a Synthetic *cry1Ab* Gene

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ABSTRACT

Two transgenic sugar beet T_0 lines, 7233-3 and 7233-8, carrying *cry1Ab* gene were selfed and crossed with a single cross cytoplasmic male sterile line. One hundred and ninety F_1 plants derived from the transgenic lines were screened using polymerase chain reaction analysis. Statistical test confirmed the 1: 1 Mendelian ratio for the transgene in F_1 progenies indicating a single locus insertion into the nuclear genome. Stable expression of the transgene under the maize-phosphoenol pyruvate carboxylase promoter in F_1 progenies was confirmed by western blot analysis. The approximate quantity of protein expressed in the progenies was estimated to be about 0.1% of total leaf soluble protein. Insect bioassay in some progenies of the transgenic plants revealed an enhanced resistance to the prodenia pest (*Spodotera littoralis*).

Keywords: bioassay, insect, Mendelian ratio, prodenia, western blot
Abbreviations: Bt, *Bacillus thuringiensis*; **CaMV**, cauliflower mosaic virus; **CMS**, cytoplasmic male sterile; **PCR**, polymerase chain reaction; **PEPC**, phosphoenol pyruvate carboxylase; χ^2, chi-square

INTRODUCTION

So far, various genes have been transferred into sugar beet using several different transformation protocols (Lathouwera *et al*. 2005). However, compared with other methods of transgenic plants production, *Agrobacterium*-mediated transformation has some advantages such as, the ease of work, the lower levels of DNA rearrangements, high possibility of the integration of transgenes as a single copy, and consistent expression of transgenes in subsequent generations (Dai *et al*. 2001; Gelvin 2003). Characterization of transgenic plants including the determination of copy number or transgene, its regular inheritance and stability of expression and function in subsequent generations is a necessary step in risk assessment that is required prior to the field release and commercialization (Hiei and Komari 1996). Determination of the integrated positions of the transgene and the analysis of Mendelian segregation of the encoded trait in progenies of subsequent generation can provide a better understanding of the nature of the inheritance of the transgene (Yin *et al*. 2004). Integration of transgenes in a single locus, regardless of the number of copies, has been reported in transgenic plants obtained by both direct and indirect (*Agrobaterium*-mediated) methods of gene transfer (Spencer *et al*. 1992; Register *et al*. 1994). Also, it has been shown that the inheritance of multiple complete or incomplete copies of transgene could be similar to di- or trigenic traits (Cluster *et al*. 1996). It has been reported that the genes introduced into dicotyledonous plants *via Agrobacterium* were highly stable and inherited in a Mendelian manner in subsequent generations (Chyi *et al*. 1986; Muller *et al*. 1987). Reported frequency of non-Mendelian inheritance of transgene varies between 10 to 50% of transgenic lines (independent transgenic events). However, these data come from studies conducted on a limited number of lines (Yin *et al*. 2004). Position of integration and localized effects are among the important factors influencing the transmission and expression of the transgene (Kohli *et al*. 1999; Yin and Malepszy 2003). Therefore, an understanding of the frequency of distortions from Mendelian ratio and the causes are of high importance in transgenic plants. In recent years, the *cry* genes have been transferred into different plants for evaluation of Bt (*Bacillus thuringiensis)* protein toxicity against insect pests such as *Heliothis armigera* in cotton (Tohidfar *et al*. 2008), diamondback moth in chinese cabbage (Zhang *et al*. 2009), *Chilo suppressalis* in rice (Kiani *et al*. 2009), *Spodotera littoralis* in sugar beet (Jafari *et al*. 2009a, 2009b) and *Phythorimaea operculella* in potato (Kumar *et al*. 2010). An efficient method for *Agrobacterium*-mediated transformation of sugar beet has already been reported (Norouzi *et al.* 2005) and the high transformation efficiency and repeatability of the method in generating insect resistant transgenic plants of sugar beet was confirmed by Jafari *et al.* (2009a). However, they did the PCR analysis on a limited number of F_1 plants without doing western blot and bioassay analyses. Jafari *et al* (2009b) used synthetic *cry1Ab* gene being under the control of CaMV (cauliflower mosaic virus) 35S promoter for *Agrobacterium*-mediated sugar beet transformation. However, they did their study on the plants of T_0 generation only.

We are now reporting the Mendelian inheritance, transmission and expression stability of the *cry1Ab* gene under the control of a PEPC (phosphoenol pyruvate carboxylase) promoter in a large number of transgenic F_1 plants of sugar beet derived from crossing of T_0 plants with a single cross CMS (cytoplasmic male sterile) line against Egyptian leaf worm or prodenia insect pest *Spodoptera littoralis*.

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MATERIALS AND METHODS

Plant material

Lines No. 7233-3 and 7233-8 of transgenic sugar beet carrying *cry1Ab* gene under PEPC promoter (unpublished data) and a single cross CMS line (428*KWS) provided by the Sugar Beet Seed Institute (Karaj, Iran) were used to produce F_1 hybrid seeds.

Segregation analysis of transgene

In order to study the inheritance of *cry1Ab* gene, the sugar beet T_0 transgenic plants were adapted to greenhouse conditions and well-grown and then transferred to a cold room (4°C). After 2 months, the cold-treated plants were transferred to pots containing a mixture soil and peat moss in a 2: 1 ratio in a greenhouse with a temperature of 24 ± 2°C for flowering stalk production. Two T_0 transgenic lines were either selfed or crossed with a single cross CMS line. The collected seeds were grown under greenhouse conditions and the F_1 transgenic plants were examined for the presence of the transgene and determination of its heritability. The data derived from PCR were analyzed using Chi-square (χ^2) test.

PCR analysis

Genomic DNA was isolated from leaves of F_1 transgenic plants based on the method described by Dellaporta *et al*. (1983) and PCR analysis for DNA samples was conducted according to Jafari *et al.* (2009a). According to Ghareyazie *et al*. (1997), the sequences of the primers specific for the *cry1Ab* gene were as follows:

Forward primer: 5′-GGCGGCGAGAGGATCGAGAC-3′ and
Reverse Primer: 5′-TCGGCGGGACGTTGTTGTTC-3′.

The PCR products were electrophoretically separated on a 1% agarose gel. After staining with ethidium bromide, gels were photographed using a GelDoc system (Syngen Co., UK).

Western blot analysis

Total soluble protein was extracted from fresh leaves of transgenic F_1 the T_0 plants (as positive control) as well as non-transgenic plants (as negative control), as described by Jafari *et al.* (2009a). Protein concentration was determined by the CB dye binding-protein assay method (Bradford 1976) and using bovine albumin protein as standard. Western blotting was carried out by the method of Pham (2003) with minor modifications. In summary, about 50 µg protein was mixed with an equal volume of 1X sample loading buffer and the protein samples were denatured by placing in boiling water for 3 min and immediately transferred to ice. Then, the samples were loaded in a 10% SDS-PAGE gel. Electrophoresis was conducted with Protean II (Bio-Rad) equipment at 60V. After electrophoresis, the proteins were transferred from the gel onto a nitrocellulose membrane (Bio-Rad) at 4°C overnight by wet transblot (Bio-Rad) equipment. Immunological detection of the target protein was conducted using rabbit anti-Cry1Ab polyclonal antibody (2000X diluted). NBT/BCIP (Bio-Rad) was used as substrate for chromogenic detection. The quantity of the expressed Cry1Ab protein was determined using protoxin Bt (132 kDa) at 50 ηg as standard.

In vitro insect bioassay

Clusters of eggs of the insect *Spodoptera littoralis* were collected from the fields of Gorgan and Dezful, Iran and, were placed in Petri dishes containing the necessary nutrients prior to bioassay. Seven neonate larvae (max 24 hrs old) were placed onto the leaves of the transgenic and non-transgenic sugar beet plants in Petri dishes on sterile, wet tissue paper. Petri dishes were transferred into a growth chamber at temperature of 25 ± 2°C, 70% relative humidity and a 16-h photoperiod at 3000 lux light intensity. Bioassays were conducted for each line in four replicates. The number of dead larvae, the weight of surviving larvae and the percentage of damage were recorded at the third and seventh days after inoculation (Gallie *et al*. 1988). Data derived from the bioassay were analyzed using SAS V.9 (SAS Inc.). Mean comparisons of data were carried out by Duncan's multiple range tests at the 1% probability level.

Analysis of morphological traits of F_1 transgenic plants

Morphological traits such as leaf length, root length, leaf area, taproot diameter and number of leaves from F_1 transgenic and non-transgenic plants (as control) were measured three months after they were grown in pots under greenhouse conditions. All the data were collected from two experiments in which the plants were arranged in a randomized complete block design with three replications. Data were analyzed using SAS V.9 (SAS Inc.) and comparisons of means were performed using Fisher's protected LSD test.

RESULTS

Production of F_1 plants

Having vernalized transgenic plants (see Materials and Methods), about 99% of the transgenic plants produced a flowering stem (**Fig. 1A**). The seeds derived from selfing were small and unfilled. Therefore, no T_1 transgenic progenies were reproduced through selfing of T_0 parents. In contrast, seeds produced from crosses of the lines 7233-3 and 7233-8 with the CMS line (**Fig. 1B**) were large and viable. By planting these seeds in pots, the F_1 putative transgenic plants were readily produced (**Fig. 1C**) and well grown (**Fig. 1D**).

PCR analysis

One hundred F_1 plants derived from line 7233-3 and 90 F_1 plants from line 7233-8 were studied in PCR analysis using primers specific to the *cry1Ab* gene. The expected 1194-bp band amplified from a segment of the *cry1Ab* gene indicated the inheritance of a transgene to more than 50% of the F_1 progenies (**Fig. 2**). This again confirms the integration of the transgene into the sugar beet genome and its transmission to the subsequent generation.

Inheritance of *cry1Ab* gene in F_1 progeny

Inheritance of the *cry1Ab* gene in F_1 progeny derived from two T_0 transgenic lines were evaluated based on PCR data. According to **Table 1**, the transgene was transferred into the progeny in both lines with a 1: 1 Mendelian ratio. This ratio corresponds to the Mendelian segregation ratio for a single copy gene in the nuclear genome of F_1 progenies derived from an outcross between a T_0 transgenic line and a non-transgenic CMS.

Expression of *cry1Ab* gene in F_1 progenies

Immunoblot analysis of PCR-positive plants showed a high level of production of Cry1Ab protein in the leaves of transgenic plants (**Fig. 3**). The size of the Cry1Ab protein accumulating in leaves of PCR-positive progeny plants was

Table 1 Segregation of *cry1Ab* STS marker in F_1 progenies from two T_0 lines.

Line	Total number of analyzed progenies	Number of plants[1]	Expected ratio	χ^2 value	P value
		cry+; cry−			
7233-3-F_1	100	58; 42	1:1	2.6 [ns]	0.11
7233-8-F_1	90	46; 44	1:1	0.04 [ns]	0.83

[1] Cry^+ shows the existence of PCR band.
[ns] Non-significant

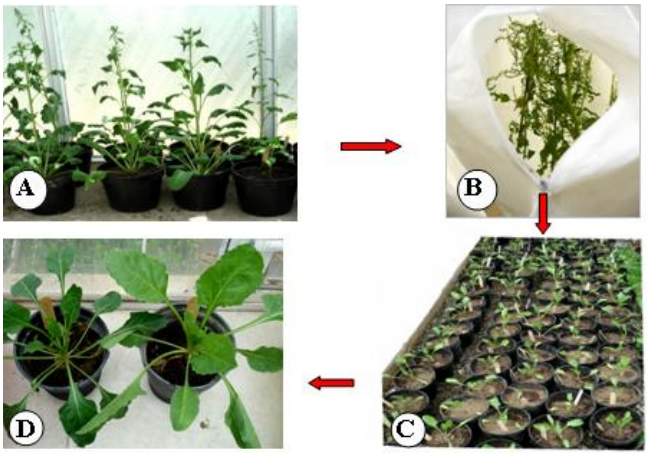

Fig. 1 Production of sugar beet F_1 plants. (**A**) Induction of flowering stem in transgenic T_0 plants following cold treatment. (**B**) Pollination stage of transgenic plants and crosses with a CMS line. (**C**) The F_1 plants derived from crosses of transgenic plants with the CMS line. (**D**) Well-grown F_1 transgenic plants.

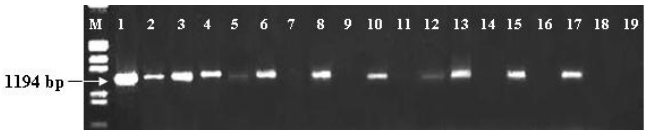

Fig. 2 PCR analysis of DNA extracted from F_1 plants using primers specific to *cry1Ab* gene. M: marker Lambda DNA/*Eco*RI+*Hin*dIII (Fermentas Co.). Lane 1: plasmid pCIB4421 as positive control. Lane 18, DNA extracted from non-transgenic plant of 7233 as the 1st negative control; lane 19, sample without DNA as the 2nd negative control; lanes 2 to 17, DNA extracted from F_1 test plants. Arrow shows the amplified 1194-bp band of a segment of *cry1Ab* gene.

67 kDa compared to 132 kDa for purified Cry1Ab protoxin; the small size of the translation product was expected because the truncated gene of the used construct pCIB4421 (Koziel *et al*. 1993) encodes only the active N-terminal half of the protoxin (Hofte and Whiteley 1989). With 50 ng protein loaded on the gel, the level of Cry1Ab protein was estimated to be approximately 0.1% of total soluble protein of the leaf. No such immunological reaction was detected for the leaf protein of PCR-negative plants (**Fig. 3**). The band intensities of F_1 plants and therefore the level of target protein expression in these plants were almost the same.

Performance analysis of F_1 transgenic plants

Results of the bioassay for F_1 transgenic plants carrying the *cry1Ab* gene under the control of a maize-PEPC promoter against prodenia pest (*Spodoptera littoralis*) is shown in **Table 2**. The mortality rate of the larvae in non-transgenic (check) plants was 4-8% while that of transgenic plants was approximately 30-46%. The weight of surviving larvae collected from the leaves of transgenic plants 3 days after infection was 0.38-0.92 mg and reached a maximum of 3.75 mg at the 7th day of infection, while it was 11-13 mg in check plants. This indicates the continued growth and development of larvae fed on the leaves of non-transgenic control leaves and negligible or ceased growth of larvae fed on the leaves of transgenic plants. The level of leaf damage in non-transgenic plants three days after infection was 28-47% and increased to 100% seven days after infection (**Fig. 4A, 4B**). However, the level of leaf damage in transgenic plants three days after infection was 3-11% and reached a maximum of 23-50% 7 days after infection. In general, the transgenic plants differed significantly from non-transgenic plants for the three studied traits and showed enhanced resistance to the sugar beet prodenia pest.

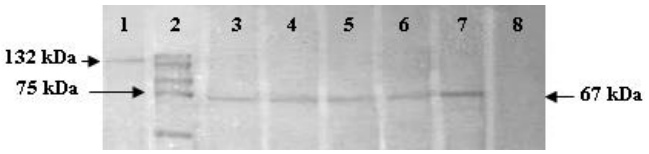

Fig. 3 Expression of *cry1Ab* gene in F_1 progenies of crosses made between a CMS line and transgenic sugar beet containing a *cry1Ab* gene under the control of maize-PEPC promoter. Protein immunoblot to detect truncated cry1Ab toxin (67 kDa) in total soluble leaf protein (50 ng) from four F_1 lines compared with a non transgenic plant and purified protoxin (132 kDa). Lane 1: purified prototoxin of cry1Ab as the standard (132 kDa). Lane 2: Precision plus protein kaleidoscope standards (Bio-Rad). Lanes 3-6, the F_1 transgenic plants of 7233-3-29, 7233-3-42, 7233-3-45 and 7233-8-12, respectively; lane 7, T_0 transgenic line (7233-3) as the positive control; lane 8, non-transgenic plant of 7233 as the negative control. The 67 kDa band indicates the protein expressed by the *cry1Ab* gene in transgenic plants.

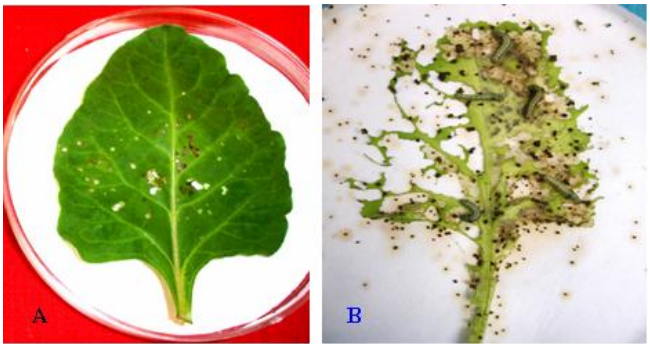

Fig. 4 Bioassay of F_1 transgenic plants carrying *cry1Ab* gene against the pest *Spodoptera littoralis*. (**A**) A leaf from a transgenic plant showing the dead larvae of the pest with only a minor leaf damage; and (**B**) A leaf from a non-transgenic (control) plant showing the well developed larvae of the pest and the significant leaf damage.

Morphological traits of F_1 transgenic plants

As shown in **Table 3**, F_1 transgenic plants were not significantly different in morphological traits compared with the control plants. All the transgenic plants were found to be morphologically similar to the control plants. This indicates that introduction of *cry1Ab* gene did not affect the overall morphological growth of the host plants.

DISCUSSION

Analysis of F_1 progenies indicated that the *cry1Ab* gene has been transferred to the subsequent generation *via* meiosis with a Mendelian inheritance expected for one locus. Similarly, Hall *et al.* (1996) studied the inheritance of *pat* gene in the progenies obtained from the crosses of sugar beet transgenic plants (PEG-mediated production) with non-transgenic plant and reported the expected 1: 1 Mendelian segregation ratio. Yang *et al*. (2005) reported T_1 progenies of AtNHX1-transgenic sugar beet, developed by *Agrobacterium*-mediated transformation, showed Mendelian 3: 1 segregation ratio. However, they observed the single locus segregation only in 5 out of 32 lines investigated and concluded that it is possible but not highly efficient to produce homologous transgenic plants through the explants of the apical buds obtained from sugar beet immature inflorescence. Hisano *et al.* (2004) also reported Mendelian 3: 1 segregation ratio in the progenies of two T_0 lines (AC4-1 and AC4-2) produced by *Agrobacterium*-mediated transformation. The progenies derived from crosses of homozygous transgenic AC4-5 line with a CMS line, though they showed a slight deviation from the expected Mendelian 1: 1 ratio for the transgene segregation. This was attributed to the limited number of lines studied.

Zhang *et al.* (2009) studied the inheritance patterns of

Table 2 Summarized results obtained from bioassay of F_1 transgenic sugar beet plants carrying *cry1Ab* gene against the pest *Spodoptera littoralis*.

Line	Number of dead larvae[♦] DAI 7;[†] DAI 3	Weight of live larvae (mg)[♦] DAI 7; DAI 3	Leaf damage (%)[♦] DAI 7; DAI 3	Mortality (%)[♦] DAI 7
*7233	0.16 ± 0.06 c; 0.00 ± 0.00 d	11.12 ± 0.38 b; 3.07 ± 0.20 b	97.43 ± 5.32 a; 28.66 ± 1.51 b	4.16 ± 1.50 c
*HM1990	0.16 ± 0.10 c; 0.00 ± 0.00 d	13.05 ± 0.86 b; 3.90 ± 0.54 b	100.00 ± 0.00 a; 47.49 ± 0.83 a	8.39 ± 1.30 c
7233-3-29	2.80 ± 0.03 a; 1.41 ± 0.01 a	3.50 ± 0.20 a; 0.38 ± 0.04 a	23.71 ± 0.86 e; 3.23 ± 0.39 d	43.89 ± 1.62 a
7233-3-42	2.61 ± 0.02 a; 1.11 ± 0.40 b	3.15 ± 0.43 a; 0.60 ± 0.14 a	32.49 ± 0.62 de; 11.20 ± 0.45 c	39.47 ± 1.74 a
7233-3-45	1.81 ± 0.03 a; 0.91 ± 0.03 bc	4.37 ± 0.24 a; 0.45 ± 0.09 a	50.00 ± 0.58 c; 9.33 ± 0.63 c	30.31 ± 1.17 b
7233-8-12	0.74 ± 0.02 b; 0.72 ± 0.02 c	3.75 ± 0.48 a; 0.92 ± 0.09 a	42.44 ± 0.85 dc; 5.57 ± 0.74 d	45.95 ± 0.67 a

[*] Non-transgenic plants as control.
[♦] Mean ±SE, [†] Days after infestation.
Mean values with different letters in each column compared by Duncan's multiple range test differ significantly at the 1% probability level.

Table 3 Morphological traits of transgenic sugar beet plants carrying the *cry1Ab* gene grown under greenhouse conditions.

Line	Traits				
	Leaf length[a] (cm)	Root length[a] (cm)	Leaf area[a] (cm^2)	Taproot diameter[a] (cm)	Leaf number[a]
Non-transgenic plants	14.2 ± 0.1 ab	11.9 ± 0.4 ab	67 ± 0.02 ab	3.10 ± 0.6 a	13 ± 0.7 a
F_1 transgenic plants[b]	13.6 ± 0.6 ab	12.4 ± 0.8 a	69 ± 0.03 ab	3.20 ± 0.4 a	12 ± 0.5 ab
F_1 transgenic plants[c]	15.4 ± 0.2 a	11.7 ± 0.9 ab	62 ± 0.01 a	2.90 ± 0.2 ab	13 ± 0.3 a

[a] Values are means±standard error obtained from two experiments, each including three replications.
Values followed by the same letters within the column are not significantly different at 5% level (Fisher's protected LSD test).
[b] F_1 Transgenic plants derived from T_0 transgenic line 7233-3.
[c] F_1 Transgenic plants derived from T_0 transgenic line 7233-8.

the *cry1Ac* transgene in T_1 offspring of transgenic cabbage using PCR analysis and kanamycin resistance test on young seedling leaves and found that progeny segregation followed Mendel's fashion with a ratio of 3: 1.

Jafari *et al*. (2009a) crossed T_0 transgenic sugar beet lines containing *cry1Ab* gene under CaMV (cauliflower mosaic virus) promoter with a single cross CMS and produced F_1 plants. They observed a deviation from the expected Mendelian 1: 1 segregation ratio for the *cry1Ab* gene which could be due to small number of examined progenies too.

In western blot analysis, expression of the integrated *cry1Ab* gene was confirmed. Therefore maize-PEPC promoter in upstream of the *cry1Ab* gene could express Bt toxin in transgenic sugar beet lines that considered as C3 plants.

Insect bioassay analysis of some transgenic progenies indicated enhanced levels of resistance to the prodenia pest as there was a significant difference between transgenic and non-transgenic (check) plants in resistance. The enhanced resistance was observed only for the plants expressing the Cry1Ab protein. However, the level of resistance may not be considered as high enough for these to be used as "high dose" plants for commercial purpose. Using high dose plants is a necessary step to delay the resistance generation in target insect pests (Tabashnik 1994). Ghareyazie *et al*. (1997) have reported the production of a "high dose" lepidopteran-pest resistant rice plants using the same construct we used in this study. The level of expression of Cry1Ab protein was similar in our plants and the rice plant reported by these investigators. Their transgenic plants are performing well (Alinia *et al*. 2001) even in commercial fields after 15 generations (pers. comm. with Ghareyazie). They reported up to 100% mortality on the larvae of four different insect species fed on transgenic leaves. Tohidfar *et al*. (2008) used *cry1Ab* gene under the control of CaMV 35S promoter in cotton (*Gossypium hirsutum*) transformation and found that the homozygous T_2 plants for the *cry1Ab* gene were of significantly higher levels of insect resistance against *Heliothis armigera* larvae compared with the control plants. Kumar *et al.* (2010) developed a potato tuber moth (*Phythorimaea operculella* Z.) transgenic potato cultivar expressing synthetic, modified cry1Ab gene under the control of tuber-specific GBSSi promoter. They found that the insect bioassay of stored transgenic tubers resulted in significant retardation and mortality in neonate tuber moth larvae. However, in our observation the maximum mortality rate was 46%.

These differences could be explained as follows: A) our plants are hemizygous for the transgene and it is expected that in homozygous state the expression will increased, B) it is possible that there is inherent difference in resistance of the insects used in the two studies and C) it is also possible that the period of seven days used in our study was not sufficient to cause the higher levels of mortality expected from the observed expression level of Cry1Ab.

The transgenic F_1 sugar beet plants were morphologically normal and indicated no abnormal morphological characteristics (**Fig. 1D**). The similarity of morphological growth of the transgenic plants carrying the *cry1Ab* gene to the wild-type plants has been reported by some authors (e.g. Ho *et al*. 2006; Liu *et al*. 2008).

However, more investigation in advanced generations is necessary to determine the stability of the target gene transmission and expression. Also, other evaluations such as the establishment of substantial equivalence of the transgenic progenies to their traditional counterparts and tissue specificity of the expression of the transgene deserve further research.

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