

Full Length Research Paper

Genetic analysis of *cry1Ab* gene in segregating populations of rice

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Three transgenic *Bt* lines (Tarom Molaii, Neda and Nemat), all containing a synthetic *cry1Ab* gene were crossed with conventional rice varieties. The mode of resistance to striped stem borer (*Chilo suppressalis*) in these lines was investigated through field studies and PCR analysis of progenies in F₂ and BC₁ populations. The monogenic segregation of 3:1 and 1:1 were observed in segregating populations of F₂ and BC₁, respectively, either phenotypically under natural infestation of SSB as well as at molecular level by means of multiplexing PCR analysis using *cry1Ab* and RG100 primers. These results acquired from phenotypic and genotypic analyses of 1346 and 243 individual plants at field and laboratory conditions imply stable transmission of *cry1Ab* gene through sexual generations in *indica/indica* genetic backgrounds. Therefore, rapid development of new elite *Bt* rice cultivars with sufficient insect resistance and ideal agronomic properties can be achieved through combination of conventional breeding with marker assisted selection.

Key words: Rice, *cry1Ab*, segregation, insect resistance.

INTRODUCTION

Rice is the most important food crop and the staple food for 40% of the world population. More than 90% of rice is produced and consumed in Asia (Khush and Brar, 2002). The global population exceeded 6 billion in 2000 and it is necessary to produce 50% more food to meet the increasing needs of growing population by 2025 (Khush, 2001).

Rice productivity is severely affected by several abiotic and biotic stresses, including damage caused by insect pests. Stem borers are chronic pests in all rice-growing environments in Asia. They cause more yield loss than any other group of rice insect pests (Savary et al., 2000), and 50% of insecticides employed in rice fields are targeted at *lepidopteran* insects (Heong et al., 1994; Huesing and English, 2004). Use of chemicals not only increase the rice production cost but also causes health

harm to rice farmers, non target organisms and deteriorates environment (Litsinger et al., 2005).

Recent advances in genetic engineering of crop plants have opened new avenues for production of transgenic plant with new genetic properties. Insecticidal crystal proteins that are produced by *Bt* genes are highly toxic to *lepidopteran*, *dipteran* and *coleopteran* insects (Hofte and Whiteley, 1989) and there are several successful reports on effective control of insect species by transgenic rice plants (Ghareyazie et al., 1997; Shu et al., 2000; Tu et al., 2000; Ye et al., 2003; Ramesh et al., 2004; Bashir et al., 2005). Such *Bt* rice plants represent a promising opportunity to make an important contribution to integrated pest management (IPM) programs (Mohan et al., 2003).

Stable inheritance and expression of transgenes in transgenic crop plants has important role on successful employment of genetic engineering in traditional breeding programs (Wu et al., 2002). Transformation of rice with genes from a soil bacterium *Bacillus thuringiensis* (*Bt*) is a common approach to confer resistance to insect infes-

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Table 1. List of plant materials used in this study.

Lines/varieties	Breeding methodology	Quality status	Subspecies classification
Tarom Molaii- <i>Bt</i>	Transformation	Aromatic	Indica
Neda- <i>Bt</i>	Backcrossed with Tarom Molaii	Non-aromatic	Indica
Nemat- <i>Bt</i>	Backcrossed with Tarom Molaii	Non-aromatic	Indica
Sang Tarom	Selection	Aromatic	Indica
Tarom Deylamani	Selection	Aromatic	Indica
Khazar	Introduction (IRRI)	Non-aromatic	Indica
Nemat	Pedigree	Non-aromatic	Indica
DN-33-18	Backcross-Pedigree	Non-aromatic	Indica
DN-32-6	Backcross-Pedigree	Non-aromatic	Indica

tations providing an opportunity for plant breeders to utilize genes from other species. Once a gene is stably introduced into a rice plant, it could be utilized for development of new varieties through classical breeding programs, especially in developing countries that modern techniques is not always available (Wang et al., 2002).

The inheritance and stable expression of foreign genes has been widely studied in different transgenic plants including rice. Gahakwa et al. (2000) studied the inheritance and expression of multiple marker and insecticidal transgenes in diverse genetic backgrounds. They demonstrated faithfully transmission of all transgenes over three generations in Mendelian fashion, suggesting integration at a single locus. Studies of Wang et al. (2002) and Wu et al. (2002) showed that segregation of *cry1Ab* gene in crosses between *japonica/japonica* displaying Mendelian 3:1 ratio but distorted segregation occurred in crosses between *indica/japonica*.

The segregation pattern of *cry1Ab* gene in Iranian *Bt* rice lines is discussed in the present study under field conditions as well as at molecular level. Such studies on inheritance of *cry1Ab* gene in crosses between transgenic and non-transgenic plants are important for integrated pest management and sustainable utilization of *cry1Ab* gene in rice.

MATERIALS AND METHODS

Plant materials and crossing method

The genetic materials used in this study are presented in Table 1. In cropping season of 2006, the crosses between transgenic lines carrying *cry1Ab* gene with non-transgenic varieties were made using transgenic lines as pollen parents. Also, crosses between promising lines DN-33-18 and DN-32-6 with transgenic Neda were made. The pollinated panicles were bagged properly. The resultant F_1 seeds were planted on next year (2007) in the same way for obtaining F_2 seeds. The F_1 plants of crosses DN-33-18/Neda-*Bt* and DN-32-6/Neda-*Bt* backcrossed to their respective recurrent parents for obtaining BC_1 progenies. Field management was traditional except that no pesticide was applied throughout the experiments. NPK fertilizers applied with the amounts of 250, 100 and 50 Kg ha⁻¹, respectively.

Field evaluation of resistance

Field resistance of individual F_2 and BC_1 progenies against SSB was evaluated based on the identification of plants with dead hearts and white head, symptoms for sensitivity to stem borer at vegetative and grain filling phases, respectively (Ghareyazie et al., 1997). Dead hearts and white heads caused by SSB were recorded for each plant of the whole populations. To confirm the dead hearts and white heads resulted from damage by SSB, the stems of tillers with dead heart or white head were dissected and examined for the presence of larvae or channels due to SSB damage (Shu et al., 2000).

Polymerase chain reaction (PCR) analysis

DNA was extracted from leaves of rice plants as described by Dellaporta et al. (1983). PCR analysis was performed using the *cry1Ab* and RG100 primers following Ghareyazie et al. (1997). A 25 μ l mixture was prepared for the PCR assay which containing 50 ng template DNA, 2.5 μ l of 10X buffer, 0.3 μ l of 10 mM dNTPs, 1 μ l of 50 mM MgCl₂, 1 μ l of each of the primers, and 1 unit of *Taq* polymerase. The PCR reaction was performed at 94°C for 5 min (initial denaturation); then for 40 cycles of 94°C for 1 min; 55°C for 1 min; 72°C for 3 min followed by 72°C for 5 min. The primers for locus RG100 were 5'-GCT GGA CGT GCC AAA GAG AG-3' (forward) and 5'-CGA ACC ACA GCC ACA GCA TG-3' (reverse) the expected size of PCR product was 0.95 Kb. The primers for the *cry1Ab* gene were 5'-GGC GGC GAG AGG ATC GAG AC-3' (forward) and 5'-TCG GCG GGA CGT TGT TGT TC-3' (reverse). The expected size of the PCR product was 1.2 Kb (Ghareyazie et al., 1997). PCR products were then analyzed by 1.5% agarose gel electrophoresis in TAE buffer. Chi-square test was performed for goodness-of-fit analysis using the expected Mendelian 3:1 or 1:1 ratios.

RESULTS

The results of phenotypic evaluations under natural infestations of SSB at field conditions are presented in Table 2. Individual plants in different populations were evaluated for their reactions against SSB infestations based on identification of plants with dead heart or white head. In all types of crosses, except aromatic \times aromatic, the segregation pattern of resistant to susceptible plants do not significantly differ from the expected 3:1 ratio which

Table 2. Segregation of resistance to SSB in different segregating populations (F₂ and BC₁) based on field data.

Cross	Population	Cross type†	No. of tested plants	No. of resistant plants	No. of susceptible plants	Resistant:susceptible ratio	(χ^2) ^a P= 0.05
Tarom Deylamani/ Tarom Molaii-Bt	F ₂	AR/AR	161	108	53	2.04:1	6.385 * (3:1)
Sang Tarom/ Tarom Molaii-Bt	F ₂	AR/AR	200	131	69	1.90:1	9.627 ** (3:1)
Nemat/ Tarom Molaii-Bt	F ₂	NA/AR	178	123	55	2.24:1	3.303 ^{ns} (3:1)
Khazar/ Tarom Molaii-Bt	F ₂	NA/AR	70	44	26	1.69:1	3.840 ^{ns} (3:1)
Sang Tarom/ Nemat-Bt	F ₂	AR/NA	209	146	63	2.32:1	2.949 ^{ns} (3:1)
Khazar/ Nemat-Bt	F ₂	NA/NA	284	210	74	2.84:1	0.169 ^{ns} (3:1)
Sang Tarom/ Neda-Bt	F ₂	AR/NA	192	141	51	2.76:1	0.25 ^{ns} (3:1)
DN-33-18/Neda-Bt // DN-33-18	BC ₁	NA/NA	28	15	13	1.15:1	0.143 ^{ns} (1:1)
DN-32-6/ Neda-Bt // DN-32-6	BC ₁	NA/NA	24	12	12	1:1	0.00 ^{ns} (1:1)
Total			1346				

†AR: Aromatic, NA: non-aromatic. ^a χ^2 (0.05) = 3.84, χ^2 (0.01) = 6.64 at 1 degree of freedom.

^{ns}, * and ** are non-significant, significant at 5% and 1% probability levels for deviation from expected ratios, respectively.

Table 3. Segregation of *cry1Ab* gene in different F₂ and BC₁ progenies based on multiplexing PCR analysis.

Cross	Population	Cross type†	No. of tested plants	No. of PCR ⁺ plants	No. of PCR ⁻ plants	PCR ⁺ /PCR ⁻	(χ^2) ^a
Tarom Deylamani/ Tarom Molaii-Bt	F ₂	AR/AR	32	20	12	1.67:1	2.67 ^{ns} (3:1)
Sang Tarom/ Tarom Molaii-Bt	F ₂	AR/AR	32	23	9	2.56:1	0.167 ^{ns} (3:1)
Khazar/ Tarom Molaii-Bt	F ₂	NA/AR	30	23	7	3.26:1	0.044 ^{ns} (3:1)
Sang Tarom/ Nemat-Bt	F ₂	AR/NA	29	22	7	3.14:1	0.011 ^{ns} (3:1)
Khazar/ Nemat-Bt	F ₂	AR/NA	36	25	11	2.27:1	0.592 ^{ns} (3:1)
Sang Tarom/ Neda-Bt	F ₂	AR/NA	32	25	7	3.57:1	0.167 ^{ns} (3:1)
DN-33-18/Neda-Bt // DN-33-18	BC ₁	AR/NA	28	15	13	1.15:1	0.143 ^{ns} (1:1)
DN-32-6/ Neda-Bt // DN-32-6	BC ₁	NA/NA	24	12	12	1:1	0.00 ^{ns} (1:1)
Total			243				

†AR: Aromatic, NA: non-aromatic. ^a χ^2 (0.05) = 3.84, χ^2 (0.01) = 6.64 at 1 degree of freedom.

^{ns}, * and ** are non-significant, significant at 5% and 1% statistical levels for deviation from expected ratios, respectively.

implied that the resistance to SSB was controlled by a single dominant gene. The results of such evaluations in BC₁ progenies showed 1:1 ratio also supporting this conclusion that resistance to SSB in transgenic lines is controlled by one single locus (Table 2).

PCR-based methods were used to follow the inheritance of the transgene in all of the populations including aromatic × aromatic, aromatic × non-aromatic and non-aromatic × non-aromatic crosses (Table 3). The results of multiplexing PCR analysis using RG100 and *cry1Ab* primers clearly confirmed monogenic segregation pattern for *cry1Ab* gene in all types of crosses even in aromatic × aromatic crosses (Figure 1) which apparently deviated from expected 3:1 ratio in phenotypic assays in field trial. These results indicated that different genetic backgrounds do not affect the inheritance pattern of *cry1Ab* gene in the crosses between *indica* and *indica* rice.

DISCUSSION

When a foreign gene is inserted into the host genome often leads to the expected 3:1 segregation ratio in selfed (Nayak et al., 1997; Cheng et al., 1998; Datta et al., 1998; Chen et al., 2005; Ho et al., 2006) or F₂ populations (Wang et al., 2002) and 1:1 in the backcross progenies (Peng et al., 1992; Hiei et al., 1994; Nayak et al., 1997; Wang et al., 2002). Under particular circumstances, the segregation of transgene in transgenic plants can be deviated from expected Mendelian ratio (Datta et al., 1990; Goto et al., 1993; Peng et al., 1995; Husnain et al., 2002). Some mechanisms are responsible for this phenomenon, including the lower viability and fertilization ability of transgenic pollen (Wu et al., 2002), low fertility of inter-species cross (Wang et al., 2002), transgene silencing (Kohli et al., 1999) and gamete selection (Lyttle,

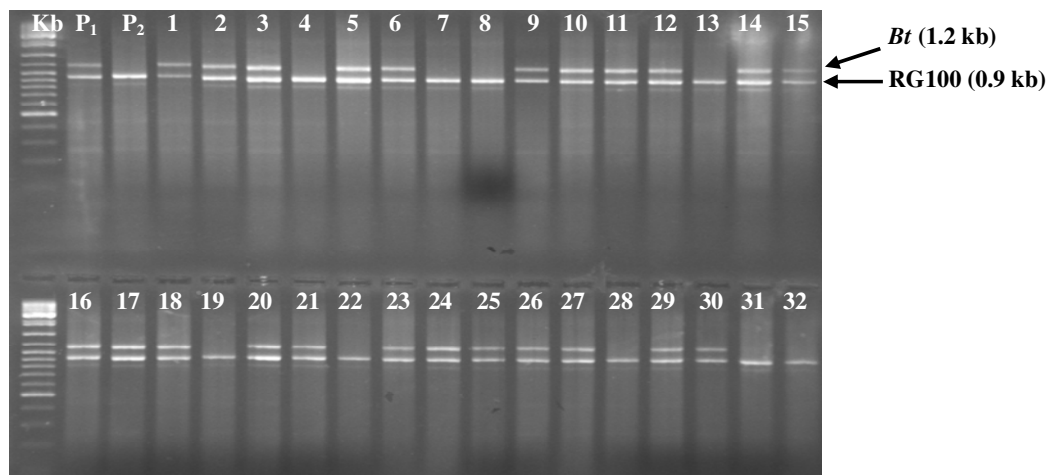


Figure 1. Multiplexing PCR analysis using *cry1Ab* and RG100 primers in F₂ population of Sang Tarom/Tarom Molaii-Bt cross. Kb ladder (SM0333), P₁: Tarom Molaii-Bt, P₂: non-transgenic Sang Tarom and numbers 1 to 32 are individual F₂ plants.

1991). In this experiment, no deviation from the expected 3:1 or 1:1, in F₂ and BC₁ populations was observed. PCR analysis also clearly supported this conclusion. However, only in the crosses type of aromatic × aromatic the deviation from monogenic segregation apparently observed at field conditions. None of the mechanisms mentioned above were identified to be responsible for this event. Nevertheless the results of PCR analyses clearly showed monogenic segregation patterns in all type of crosses and the observed deviation could be attributed to the inherent erroneous nature of phenotyping for SSB resistance according to the manifestation of insect damage in the field. This is also possible that the over estimation of susceptible plants in phenotypic assays at field conditions may be resulted from declining of resistance in transgenic Tarom Molaii at grain filling stage (Alinia et al., 2000).

Combining results from field data and PCR analysis acquired from 1346 and 243 individual plants in different populations demonstrated single copy integration of *cry1Ab* gene in the genomes of three transgenic lines Tarom Molaii, Neda and Nemat. Such single copy integrations were found to show desirable features of predictable patterns of transgene inheritance and negligible gene silencing in transgenic plants (Finnegan and McElroy, 1994).

In conventional breeding for resistance improvement for insect resistance, segregating populations derived from crosses between resistant sources with desirable genotypes are subjected to natural or artificial infestations of pests or diseases. Although these procedures have given excellent results, they are time consuming and expensive. Using PCR primers designed to amplify *cry1Ab* gene will help in identifying plants carrying the gene without subjecting them to insect attack in early generations. It needs little amounts of DNA from each of individual plants to be tested without destroying the plant. Thus it is

possible for the breeder to conduct many rounds of molecular screening in a year without depending on natural occurrence or even with existence of the pest. In different F₂ populations, after selecting of superior individual plants based on their important agronomic traits such as early maturity, plant height, panicle length, etc. (data not shown) we are now focusing on molecular screening of them for aroma (Bradbury et al., 2005) to developing new aromatic *Bt* rice lines through combination of classical and molecular breeding in Iran.

The information obtained in this study has confirmed stable integration of *cry1Ab* into the genomes of Tarom Molaii, Neda and Nemat. These transgenic *indica* rice lines can be used not only for production of stem borer resistant hybrids, but also as an exotic donor source in conventional recombination breeding. In addition, combination of conventional rice breeding with marker assisted selection will allow the most rapid development of elite *Bt* rice cultivars with sufficient insect resistance and ideal agronomic traits.

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REFERENCES

- Alinia F, Ghareyazie B, Rubia L, Bennett J, Cohen MB (2000). Effect of plant age, larval age, and fertilizer treatment on resistance of *cry1Ab*-transformed aromatic rice to *lepidopterous* stem borers and foliage feeders. *J. Econ. Entomol.* 93: 484-493

- Bashir K, Husnain T, Fatima T, Riaz N, Makhdoom R, Riazuddin S (2005). Novel *indica* basmati line (B-370) expressing two unrelated genes of *Bacillus thuringiensis* is highly resistant to two *Lepidopteran* insects in the field. *Crop Prot.* 24: 870-879.
- Bradbury LMT, Henry RJ, Jin QS, Reinke RF, Waters DLE (2005). A perfect marker for fragrance genotyping in rice. *Mol. Breed.* 16: 279-283
- Chen H, Tang W, Xu CG, Li XH, Lin YJ, Zhang QF (2005). Transgenic *indica* rice plants harboring a synthetic *cry2A** gene of *Bacillus thuringiensis* exhibit enhanced resistance against *lepidopteran* rice pests. *Theor. Appl. Genet.* 111: 1330-1337.
- Cheng X, Sardana R, Kaplan H, Altosaar I (1998). *Agrobacterium*-transformed rice plants expressing synthetic *cryIA(b)* and *cryIA(c)* genes are highly toxic to striped stem borer and yellow stem borer. *Proc. Natl. Acad. Sci. USA*, 95: 2767-2772.
- Datta K, Vasquez A, Tu J, Torrizo L, Alam MF, Oliva N, Abrigo E, Khush GS, Datta SK (1998). Constitutive and tissue-specific differential expression of *cryIA(b)* gene in transgenic rice plants conferring enhanced resistance to insect pests. *Theor. Appl. Genet.* 97: 20-30.
- Datta SK, Peterhaus A, Datta K, Potrykus I (1990). Genetically engineered fertile *indica* rice recovered from protoplasts. *Biol. Tech.* 8: 736-740.
- Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA minipreparation: version II. *Plant Mol. Biol. Rep.* 1: 19-21.
- Finnegan J, McElroy D (1994). Transgene inactivation: Plants fight back! *Biol. Tech.* 12: 883-888.
- Gahakwa D, Maqbool SB, Fu X, Sudhakar D, Christou P, Kohli A (2000). Transgenic rice as a system to study the stability of transgene expression: multiple heterologous transgenes show similar behavior in diverse genetic backgrounds. *Theor. Appl. Genet.* 101: 388-399.
- Ghareyazie B, Alinia F, Menguito CA, Rubia LG, de Palma JM, Liwanag EA, Cohen MB, Khush GS, Bennett J (1997). Enhanced resistance to two stem borers in an aromatic rice containing a synthetic *cry1Ab* gene. *Mol. Breed.* 3: 401-414.
- Goto F, Toki S, Uchiyama H (1993). Inheritance of a co-transferred foreign gene in the progenies of transgenic rice plants. *Transgen. Res.* 2: 300-3005.
- Heong KL, Escalada MM, Mai V (1994). An analysis of insecticide use in rice: case studies in the Philippines and Vietnam. *Int. J. Pest Mgmt.* 40: 173-178.
- Hiei Y, Ohta S, Komari T, Kumashiro T (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.
- Ho NH, Baisakh N, Oliva N, Datta K, Frutos R, Datta SK (2006). Translational fusion hybrid *Bt* genes confer resistance against yellow stem borer in transgenic elite Vietnamese rice (*Oryza sativa* L.) cultivars. *Crop Sci.* 46: 781-789.
- Hofte H, Whiteley HR (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* 53: 242-255.
- Huesing J, English L (2004). The impact of *Bt* crops on the developing world. *Ag. Biol. Forum* 7: 84-95.
- Husnain T, Jan A, Maqbool SB, Datta SK, Riazuddin S (2002). Variability in expression of insecticidal *cry1Ab* gene in *indica* basmati rice. *Euphytica* 128: 121-128.
- Kohli A, Gahakwa D, Vain P, Laurie DA, Christou P (1999). Transgene expression in rice engineered through particle bombardment: molecular factors controlling stable expression and transgene silencing. *Planta*, 208: 88-97.
- Litsinger JA, Bandong JP, Canapi BL, Dela Cruz CG, Pantua PC, Alviola AL, Batay-An EH (2005). Evaluation of action thresholds for chronic rice insect pests in the Philippines. I. Less frequently occurring pests and overall assessment. *Int. J. Pest Manage.* 51: 45-61.
- Lyttle TW (1991). Segregation distorters. *Ann. Rev. Genet.* 25: 511-557.
- Mohan BR, Sajeena A, Seetharaman K, Reddy MS (2003). Advances in genetically engineered (transgenic) plants in pest management- an over view. *Crop Prot.* 22: 1071-1086.
- Nayak P, Basu D, Das S, Basu A, Ghosh D, Ramakrishnan NA, Ghosh M, Sen SK (1997). Transgenic elite *indica* rice plants expressing *cryIAc* δ -endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). *Proc. Natl. Acad. Sci. USA*, 94: 2111-2116.
- Khush GS, Brar DS (2002). Biotechnology for rice breeding: Progress and Potential impact. In: Proceeding of the 20th Session of the International Rice Commission 23-26th July, Bangkok, Thailand.
- Khush GS (2001). Green revolution: the way forward. *Nat. Rev. Genet.* 2: 815-822.
- Peng J, Kononowicz H, Hodges TK (1992). Transgenic *indica* rice plants. *Theor. Appl. Genet.* 83: 855-863.
- Peng J, Wen F, Lister RL, Hodges TK (1995). Inheritance of *gusA* and *neo* genes in transgenic rice. *Plant Mol. Biol.* 27: 91-104.
- Ramesh S, Nagadhara D, Pasalu IC, Kumari AP, Sarma NP, Reddy VD, Rao KV (2004). Development of stem borer resistant transgenic parental lines involved in the production of hybrid rice. *J. Biotech.* 111: 131-141.
- Savary S, Willocquet L, Elazegui FS, Castilla NP, Teng PS (2000). Rice pest constraints in tropical Asia: quantification of yield losses due to rice pests in a range of production situations. *Plant Dis.* 84: 357-369.
- Shu Q, Ye G, Cui H, Cheng X, Xiang Y, Wu D, Gao M, Xia Y, Hu C, Sardana R, Altosaar I (2000). Transgenic rice plants with a synthetic *cry1Ab* gene from *Bacillus thuringiensis* were highly resistant to eight *lepidopteran* rice pest species. *Mol. Breed.* 6: 433-439.
- Tu J, Zhang G, Datta K, Xu C, He Y, Zhang Q, Khush GS, Datta SK (2000). Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* δ -endotoxin. *Nat. Biotech.* 18: 1101-1104.
- Wang Z, Shu Q, Ye G, Cui H, Wu D, Altosar I, Xia Y (2002). Genetic analysis of resistance of *Bt* rice to stripe stem borer (*Chilo suppressalis*). *Euphytica*, 123: 379-386.
- Wu G, Cui H, Ye G, Xia Y, Sardana R, Cheng X, Li Y, Altosar I, Shu Q (2002) Inheritance and expression of the *cry1Ab* gene in *Bt* (*Bacillus thuringiensis*) transgenic rice. *Theor. Appl. Genet.* 104: 727-734.
- Ye GY, Yao HW, Shu QY, Cheng X, Hu C, Xia YW, Gao MW, Altosar I (2003). High levels of stable resistance in transgenic rice with a *cry1Ab* gene from *Bacillus thuringiensis* Berliner to rice leaf folder, *Cnaphalocrocis medinalis* (Guenée) under field conditions. *Crop Prot.* 22: 171-178.