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Evaluation of Bt (*Bacillus thuringiensis*) Rice Varieties Against Stem Borer (*Chilo suppressalis*)

¹Ghaffar Kiani, ¹Ghorban Ali Nematzadeh, ²Behzad Ghareyazie and ³Majid Sattari

¹Department of Agronomy and Plant Breeding,
Sari University of Agricultural Sciences and Natural Resources,
Rice and Citrus Research Institute, Sari, Iran

²Agricultural Biotechnology Research Institute, Karaj, Iran

³Rice Research Institute, Amol, Iran

Abstract: Three transgenic rice varieties namely Khazar, Neda and Nemat, all containing a cry1Ab gene, were evaluated through PCR analysis and field examinations for their resistance at natural infestation of insect pests during 2007. The results showed that all transgenic varieties produced 1.2 kb PCR product derived from application of cry1Ab gene. In field conditions, transgenic varieties exhibited high levels of resistance against natural infestation of stem borer and the damaged plants based on dead heart or white heat for them were less than 1%. Moreover, in stem-cut bioassay 100% of released larvae died within four days after infestation. These results demonstrate that expression of cry1Ab gene in the genome of transgenic varieties provided season-long protection from the natural infestation of lepidopteran insects.

Key words: Bt-rice, cry1Ab, PCR analysis, insect bioassay

INTRODUCTION

Rice productivity is severely affected by several abiotic and biotic factors, including damage caused by pests and diseases. Larvae of lepidoptera are the major pests in the world (Khan *et al.*, 1991). In Asia, stem borers cause about 5% grain losses (IRRI, 1996) and 50% of insecticides employed in rice fields are targeted at lepidopteran insects (Heong *et al.*, 1994). Use of chemicals not only increase the rice production cost but also causes health harms to rice farmers as well as deteriorates the rice field environment (Pingali and Roger, 1995).

Bacillus thuringiensis (Bt) is a soil bacterium used for more than 50 years as a biological insecticide. The insecticidal activity resides in crystalline inclusion bodies that are produced during sporulation of the bacteria and are composed of δ -endotoxin (Macintosh *et al.*, 1998). The mode of action of the δ -endotoxin involves solubilization of the crystal in the insect midgut, proteolytic processing of the protoxin by midgut proteases, binding of δ -endotoxin to midgut receptors and insertion of the toxin into the apical membrane to create ion channels or pores that lead to disruption of osmotic processes (Adang *et al.*, 1985; Gill *et al.*, 1992;

Schnepf *et al.*, 1998). Bt insecticidal activity is highly specific in that the endotoxins are nontoxic to non target insects, birds and mammals (Perlak *et al.*, 1990; English and Slatin, 1992; McClintock *et al.*, 1995).

Recent advances in genetic engineering of crops opened new avenues for production of transgenic plant with new genetic properties. Transformation of rice with Bt genes is a common approach to confer resistance to insect infestations. Such Bt rice plants represent a promising opportunity to make an important contribution to Integrated Pest Management (IPM) programs (Mohan Babu *et al.*, 2003).

Recently three transgenic Bt rice varieties are produced in Iran. The resistance reaction of these lines against insect pests is discussed in this paper at molecular level as well as field conditions.

MATERIALS AND METHODS

Plant materials and experimental design: The genetic materials used in this study were three transgenic Bt lines namely Khazar, Neda and Nemat, all containing a synthetic cry1Ab gene. These lines improved through backcross method using transgenic Tarom molaii (Ghareyazie *et al.*, 1997) as non-recurrent parent. Seeds

provided from Rice Research Institute, Armol, Iran. These transgenic varieties along with a control transplanted with a delay of one month on early June (normal transplantation is on early May) to ensure that maximum tillering and booting stages coincide with the peak damage of stem borer that occurs on late August in North of Iran. Experiments carried out in RCBD layout with three replications at research farm of Sari University of Agricultural Sciences and Natural Resources during 2007. Each plot was 6 m² with 25×25 cm planting pattern. Cultural practices followed normally during the experiments except that no chemicals applied against pests. Field resistance to insect pests was investigated by observation of white head and dead heart, symptoms for sensitivity to stem borer, during the whole growth period of genotypes.

Insect bioassay: The Entomology Division of Sari University of Agricultural Sciences and Natural Resources provided Striped Stem Borer (SSB) neonate larvae. Their moths were collected from rice fields in Sari, Iran. The moths were caged on rice plants covered by muslin cloths at greenhouse conditions for three days with the temperature of 28±2°C, the relative humidity of 70-90% and 14 h photo phase. Stems were cut into 5 cm long sections from the middle portion of each genotype, at maximum tillering stage and placed in Petri plate with moistened filter paper. The stems were infested with 5 neonate larvae and the plates were sealed with Para film. Petri dishes were incubated at 28°C in the dark. After 2, 3 and 4 days, the stems were dissected and mortality and developmental stage of larvae were recorded.

Data analysis: The data for white head, dead heart and bioassay analysis were arcsine-transformed and subjected to analysis of variance using MSTATC software. The mean comparisons performed using Least Significant Difference (LSD) test at 5% statistical level.

Polymerase Chain Reaction (PCR) analysis: PCR analysis was performed using the cry1Ab Bt and RG100 primers. A 25 µL mixture was prepared for the PCR assay which containing 50 ng template DNA, 2.5 µL of 10x buffer, 0.3 µL of 10 mM dNTPs, 1 µL of 50 mM MgCl₂, 1 µL of each 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 is 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 is 1.2 kb. PCR products were then analyzed by 1.5% agarose gel electrophoresis.

RESULTS AND DISCUSSION

Figure 1 shows PCR analysis of transgenic varieties along with a non-transgenic control. The 0.95 kb product derived from application of RG100 locus obtained in all transgenic genotypes as well as control indicating accuracy of PCR reaction. All of transgenic lines were positive for presence of cry1Ab gene and produced 1.2 kb product derived from application of cry1Ab gene while it was absent in control. These results indicated that there is stable integration of the cry1Ab gene in the genome of Bt varieties.

To determine the entomocidal activity of the cry1Ab insecticidal protein in the transgenic lines, insect feeding bioassays were performed. The analysis of variance showed significant differences between treatments (Table 1). The results showed that all of transgenic varieties were highly toxic to SSB larvae and 100% of released larvae died within four days after infestation (Table 2, Fig. 2a), while the rate of natural mortality in

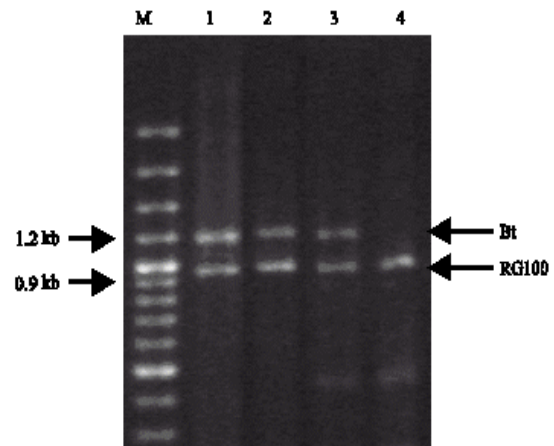


Fig. 1: PCR analysis of transgenic rice lines for presence of synthetic cry1Ab gene. Lanes 1, 2 and 3 are transgenic Khazar, Neda and Nemat, respectively. Lane 4 is non-transgenic control, M: Molecular weight

Table 1: Analysis of variance for stem-cut bioassay data

Sources of variation	df	SS	MS	F	Probability
Varieties	3	16524.76	5508.25	174.10	0.0000
Days after infestation	2	10487.14	5243.57	165.73	0.0000
Interaction	6	2428.04	404.67	12.79	0.0000
Error	24	759.32	31.64		

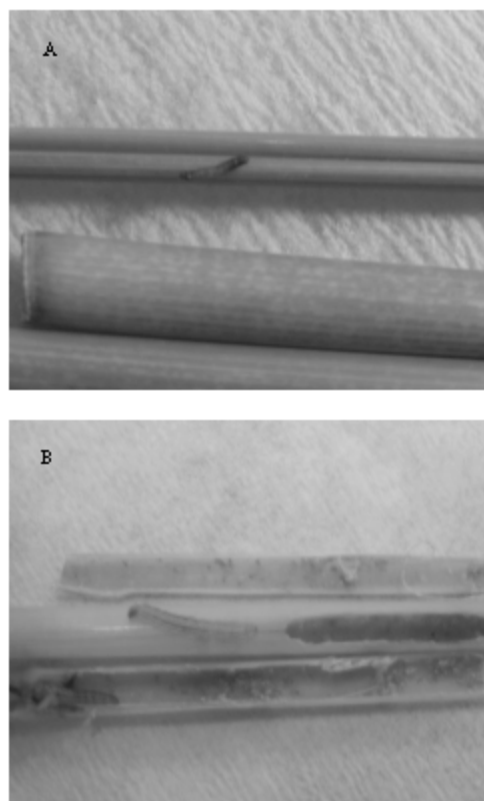


Fig. 2: Stem-cut bioassay for transgenic Bt rice. A: Larval mortality on a transgenic cultivar. B: Survival and feeding of larva on non-transgenic control

control was less than 10%. The larvae infesting the stem segments of the control grew normally and completed their developmental stages (Fig. 2b).

Infestation during vegetable growth damages growing tillers and produces symptoms known as dead hearts. Infestation during the reproductive phase causes damage that blocks the transport of assimilates from the stem to the panicle, resulting in the characteristic symptom of stem borer infestation known as white heads (Ghareyazie *et al.*, 1997). In this base, field evaluations for resistance of transgenic cultivars against insect pests evaluated and the result is shown in Table 3. All transgenic varieties exhibited effective resistance against natural breakout of rice stem borer. Transgenic lines were statistically better than control ($p < 0.05$) regarding dead heart and white head. The mean rates of dead heart and white head for them were 0 and less than 1%, respectively. While the mean rates of dead hearts and white heads for control variety were 3.65 and 26.74, respectively (Table 3). There are several reports on effectiveness of Bt

Table 2: Larval mortality on stem segments of Bt varieties in comparison with control

Varieties	Larval mortality (%)		
	2 DAI	3 DAI	4 DAI
Khazar-Bt	36.4 [†]	67.2 ^a	100.0 ^a
Neda-Bt	20.0 ^a	52.2 ^a	100.0 ^a
Nemat-Bt	34.5 ^a	61.1 ^a	100.0 ^a
Control	0.0 ^b	0.0 ^b	6.7 ^b

†: Significantly different than control using LSD test at 5% statistical level
DAI: Days After Infestation

Table 3: Yield and resistance reaction of transgenic rice varieties under field conditions

Varieties	Yield (t ha ⁻¹)	Dead heart (%)	White head (%)
Khazar-Bt	5.84 ^a	0.00 ^b	0.69 ^b
Neda-Bt	6.35 ^a	0.00 ^b	0.35 ^b
Nemat-Bt	4.83 ^b	0.00 ^b	0.00 ^b
Control	4.08 ^b	3.65 ^a	26.74 ^a

Means followed by the similar letter(s) refer to non-significant difference at 5% statistical level

technology in controlling rice pests (Shu *et al.*, 2000; Tu *et al.*, 2000; Ye *et al.*, 2003; Ramesh *et al.*, 2004; Bashir *et al.*, 2005).

The paddy yield of transgenic lines was more than control. The maximum yield belonged to Neda (6.35 t ha⁻¹) followed by Khazar (5.84 t ha⁻¹) and Nemat (4.83 t ha⁻¹) (Table 3). It is obvious that Bt rice has the potential to increase yield and could greatly reduce the chemical use in rice fields.

Considering that the field trial was conducted without the use of chemicals after transplanting and test materials transplanted with a delay of one month than normal cultivation, the results demonstrate that the integration of cryIAb gene in rice genome is stable and the expression of the gene in tissues of transgenic varieties provided season-long protection for them from the natural infestation of lepidopteran insects.

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