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Pyramiding of *cry1Ab* and *fgr* Genes in Two Iranian Rice Cultivars Neda and Nemat

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ABSTRACT

Pyramiding genes related to grain quality and resistance through marker assisted selection (MAS) is an important approach in rice breeding programs. Marker-assisted selection can be used for monitoring the presence or absence of these genes in breeding populations and can be combined with conventional breeding approaches. This study is a part of cultivar development program in Iran through integration of conventional breeding with marker assisted selection. Crosses between two high yielding transgenic lines carrying an insect resistance gene (*cry1Ab*, from *Bacillus thuringiensis*) with a local aromatic variety were made followed by selection for incorporation of insect resistance and aroma (*fgr*) genes in desirable single F_2 plants. Finally, plants homozygous for aroma and carrying *cry1Ab* genes with good agronomic performance were identified. Further analyses are underway on these plants in F_3 generation. These plants promise to develop new aromatic *Bt* rice lines through integration of classical and molecular breeding in the near future in Iran

Keywords: Aroma, Insect resistance, Marker aided selection, Transgenic rice.

INTRODUCTION

Rice is the most important food crop, providing the staple food for nearly half of the global population, especially in Asia, Africa, and Latin America (FAO, 2004). In Iran, rice is a second main staple crop after wheat. National breeding programs in Iran depend mainly on pedigree breeding method for cultivar development. Efforts have been focused on development of varieties with high yield potential and acceptable quality through hybridization of local aromatic varieties with high yielding commercial cultivars (Nematzadeh and Kiani, 2007).

The fragrance in rice has been associated with increased levels of 2-acetyl-1-pyrroline (2AP) (Widjaja *et al.,* 1996; Yoshihashi, 2002). There are some traditional methods adopted to assist breeders in selecting fragrant rice cultivars. These methods are based on tasting individual grains, chemical analysis, and gas chromatography: however, evaluation of fragrance using these methods is time consuming, difficult, and unreliable. Recently, molecular markers have been developed for the selection of fragrant plants in rice that have the advantages of being inexpensive, simple, rapid, requiring small amounts of tissue, and highly reliable (Bradbury *et al.,* 2005). Genetic studies carried out on the inheritance of aroma in rice have revealed that a recessive nuclear gene controls aroma in rice (Dong *et al.,* 2000). Molecular marker that is closely linked to the aroma gene can be used to facilitate early selection for the presence or absence of scent, and to identify the nature of the locus (homozygous or heterozygous

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condition). It may also be useful for the rapid incorporation of the scent character into breeding lines.

Transformation of rice with genes from a soil bacterium *Bacillus thuringiensis* (*Bt*) is a common approach to confer resistance to insect infestations. Insecticidal crystal proteins produced by *Bt* genes are highly toxic to lepidopteran, dipteran and coleopteran insects (Hofte and Whiteley, 1989).

Stem borers are chronic pests in all rice-growing environments in Asia and they cause more yield loss than any other group of rice insect pests (Savary *et al*., 2000). Limitations in conventional breeding arise because of the lack of resistance genes in cultivated rice germplasm (*Oryza sativa* L.) and inadequate understanding of phenotypic variability. Hence, transgenic research offers unique opportunities to overcome these problems and to produce improved cultivars with reduced yield gaps (Datta, 2004).

Pyramiding genes related to grain quality and resistance through marker assisted selection (MAS) is an important approach in rice breeding programs. Marker-assisted selection can be used for monitoring the presence or absence of these genes in breeding populations and can be combined with conventional breeding approaches. The use of cost-effective DNA markers for important agronomic traits provides opportunities for breeders to develop high-yielding, stress-resistant, and better-quality rice cultivars (Jena and Mackill, 2008). At present, PCR based markers are available for aroma (Bradbury *et al.,* 2005) and *cry1Ab* (Ghareyazie *et al.,* 1997) genes.

PCR markers can offer the advantages of convenience, cost efficiency, and high throughput (Williams *et al.,* 1991). 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.

This study aimed to pyramid *fgr* and *cry1Ab* genes in individual F_2 plants with superior agronomic attributes derived from the crosses between high yielding transgenic lines and a local high quality variety.

MATERIALS AND METHODS

Plant Materials and Field Evaluations

We have transferred *cry1Ab* gene to high yielding rice cultivars 'Neda' and 'Nemat' by back crossing these two cultivars with transgenic Tarom Molaii (Ghareyazie *et al.,* 1997). Neda and Nemat are high-yielding *indica* cultivars developed through pedigree breeding method. These cultivars were then used as male parents and crossed with a high quality local variety, 'Sang Tarom', in 2006. F_1 seeds of these crosses were grown in field to obtain F_2 seeds, in 2007. Subsequently, segregating populations were planted in cropping season of 2008 with a delay of one month to allow natural breakout of striped stem borer (SSB) under field conditions. Cultural practices were those practiced traditionally in the region, except that no chemicals were applied for insect control. Field resistance of individual F_2 plants against stem borer was evaluated based on the identification of plants with dead heart and white head symptoms for sensitivity to stem borer in vegetative and grain filling stages, respectively. Data on plant height, tiller number, panicle length, filled grains/panicle, and 1000-grain weight were recorded on selected individual plants.

Polymerase Chain Reaction (PCR) Analysis for *cry1Ab* and *fgr* Genes

DNA was extracted from leaves of rice plants as described by Dellaporta *et al.* (1983). PCR analyses were performed using *cry1Ab* and RG100 primers (Table 1) as described by Ghareyazie *et al.* (1997). RG100 amplification product is a control reaction that denotes successful amplification for the *cry1Ab* marker reaction. A 25 µl mixture was prepared for

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Table 1. List of primers used for insect resistance and fragrance genotyping in rice (Ghareyazie *et al.,* 1997; Bradbury *et al.*, 2005).

Primer name	Primer sequence
Bt	*ggC ggC gAg Agg ATC gAg AC* (Forward) *TCg gCg ggA CgT TgT TgT TC* (Reverse)
RG100	*gCT ggA CgT gCC AAA gAg Ag* (Forward) *CgA ACC ACA gCC ACA gCA Tg* (Reverse)
External sense primer (ESP)	*TTg TTT ggA gCT TgC TgA Tg*
Internal fragrant antisense primer (IFAP)	*CAT Agg AgC AgC TgA AAT ATA TAC C*
Internal non-fragrant sense primer (INSP)	*CTg gTA AAA AgA TTA Tgg CTT CA*
External antisense primer (EAP)	*AgT gCT TTA CAA AgT CCC gC*

the PCR assay that contained 50 ng template DNA, 2.5 µl of 10X buffer, 0.3 µl of 10 mM dNTPs, 1 µl of 50 mM $MgCl_2$, 1 µl of each of the primers [2 µM], and 1 unit of *Taq* polymerase (5 units). The PCR reaction was performed at 94°C for 5 minutes; then, for 40 cycles of 94°C for 1 minute; 55°C for 1 minute; 72°C for 3 minutes, followed by 72°C for 5 minutes. The expected sizes of PCR products for RG100 and *cry1Ab* were 0.95 and 1.2 Kb, respectively.

Fragrance genotyping was carried out using allele specific amplification (ASA) method described by Bradbury *et al.* (2005). PCR was performed using 0.2 µl Taq DNA Polymerase (5 units), 1 µl of genomic DNA 10 ng μl^{-1}, 2.5 µl of 10X buffer, 1 µl of 50 mM MgCl2, 1 µl of dNTPs (5 mM), 2.5 µl of each primer (ESP, IFAP, INSP and EAP; Table 1) (2 µM), in a total volume of 25 µl. PCR was performed using ABI, 2720. Cycling conditions were an initial denaturation of 94°C for 2 minutes followed by 30 cycles of 5 seconds at 94°C, 5 seconds at 58°C, 5 seconds at 72°C; concluding with a final extension of 72°C for 5 minutes. Primers ESP and EAP acted as internal controls and produced a 580 bp band in fragrant as well as non-fragrant genotypes. These primers in combination with IFAP and INSP primers related to genotype of individual plants produced 355 bp (homozygote non-fragrant), 257 bp (homozygote fragrant) or both 355 and 257 bp bands (heterozygote non-fragrant) (Bradbury *et al.,* 2005). PCR products were then fractioned by ethidium bromide-stained 1.5% agarose gel electrophoresis in TAE buffer and photographed. A 100 bp ladder molecular weight standard (Roche) was used to estimate PCR fragment size.

RESULTS AND DISCUSSION

Two F_2 populations were developed and three rounds of selection, one phenotypic and two genotypic, were carried out on these populations to obtain agronomically desirable plants with *cry1Ab* and *fgr* genes. Stringent phenotypic selection was conducted based on field resistance and phenotypic preference to reduce the population size for further PCR analyses (Table 2).

The first round of screening the selected genotypes was carried out through

Table 2. Phenotypic and genotypic screening of F_2 segregating populations.

Cross	Total plants	No. of phenotypic selected plants	No. of *cry1Ab*$^+$ plants	No. of aromatic plants	No. of aromatic and *cry1Ab*$^+$ plants
Sang Tarom×Neda *(Bt)*	192	64	50	7	3
Sang Tarom×Nemat *(Bt)*	209	69	52	10	8

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multiplexing PCR analysis using *cry1Ab* and RG100 primers. Insect resistant individual plants produced 1.2 and 0.95 kb fragments (Figure 1-A) from amplification of *cry1Ab* gene and RG100 locus, respectively, while susceptible plants produced only 0.95 kb fragment. As judged by these results, 78.12 and 75.36% of the selected F_2 plants were positive for presence of *cry1Ab* gene in 'Sang Tarom'×'Neda' *(Bt)* and 'Sang Tarom'×'Nemat' *(Bt)* crosses, respectively (Table 2).

Fragrance genotyping was carried out using a PCR marker as described by Bradbury *et al.* (1997). The PCR marker allowed efficient screening of F_2 plants. Fragrant plants produced both 580 and 257 bp bands (Figure 1-B). Seven out of 64 and 10 out of 69 selected plants were fragrant in 'Sang Tarom'×'Neda' *(Bt)* and 'Sang Tarom'×'Nemat' *(Bt)* crosses, respectively (Table 2).

Gene pyramiding was successfully employed for crop improvement. For example, Datta *et al.* (2002) pyramided three genes *Bt* (insect resistance), *Xa21* (resistance to bacterial blight), and Kitinase (resistance to sheet blight) in a rice line using marker assisted selection. Also, Wei *et al.* (2008) pyramided *cry1Ab*, *Xa21* and *bar* (herbicide resistance) genes in genetic background of a restorer line through backcross and marker assisted selection. In this study, for insect resistance and quality improvement, two segregating F_2 populations were developed from crosses between each transgenic variety with an aromatic local variety. In these populations, superior individual plants were selected based on their desirable morphological characteristics such as early maturity, panicle length, etc. In this way, stringent phenotypic selection reduced the population size for further PCR analyses. Then, we focused on their molecular screening through identifying plants carrying the *cry1Ab* and *fgr* genes simultaneously using PCR-based markers. In this regard, marker assisted selection showed that 13.64% and 11.59% of phenotypically selected plants were homozygous for aroma status and carried the *cry1Ab* insect resistant gene in 'Sang Tarom'×'Neda' *(Bt)* and 'Sang Tarom'×'Nemat' *(Bt)* crosses, respectively. Important agronomic characteristics of these selected plants are presented in Table 3. Further analyses are underway on these plants on subsequent generations. These are

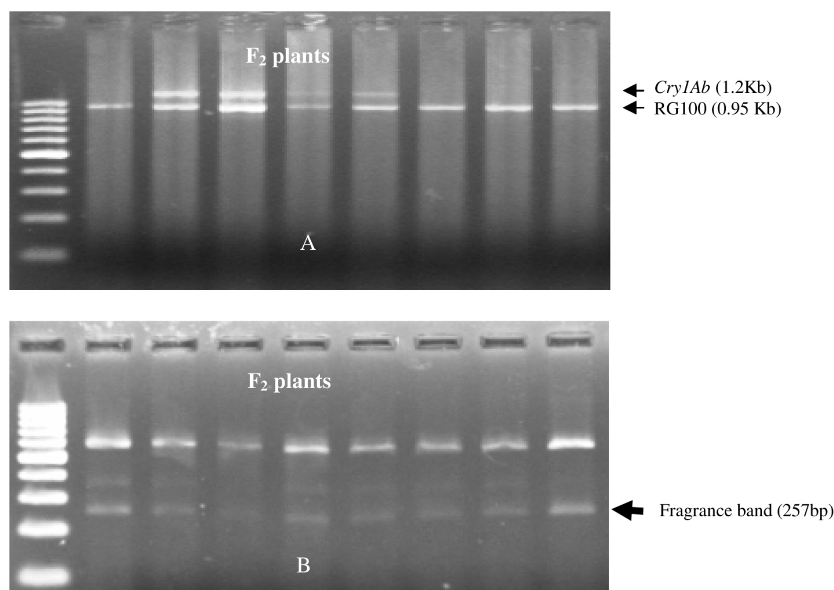

Figure 1. PCR analysis in some of selected individual F_2 plants from a cross between 'Sang tarom'×'Neda' (*Bt*) for presence of insect resistance (*Bt*) (A) and aroma (*fgr*) (B) genes. Lane 1 is 100 bp ladder.

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Table 3. Agronomic attributes of selected plants from F_2 populations carrying *cry1Ab* and *fgr* genes.

Plant	Plant height (cm)	Tiller number	Panicle length (cm)	Filled grains/Panicle	1000-grain weight
1	163	20	34	138	29.0
2	170	15	34.7	158	25.8
3	131	31	33	128	31.0
4	129	20	26.5	85	23.7
5	152	17	33.5	136	23.9
6	129	18	28	106	26.9
7	138	30	30	148	34.0
8	145	13	37	152	29.0
9	140	32	30.7	85	22.9
10	130	29	26	116	25.5
11	146	19	30	116	22.7
'Sang Tarom'	140 ± 2.1*	21 ± 1.9	28.2 ± 0.17	108 ± 9.0	22.6 ± 0.9
'Neda' (*Bt*)	122 ± 2.1	24 ± 2.2	30.8 ± 0.44	127 ± 3.5	26.0 ± 0.4
'Nemat' (*Bt*)	117 ± 0.9	16 ± 2.1	33.0 ± 0.76	120 ± 11.7	23.4 ± 1.3

* Mean±Standard error.

promising plants for development of new aromatic *Bt* rice lines through integration of classical and molecular breeding in the near future in Iran.

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هرمی کردن ژن‌های *cry1Ab* و *fgr* در دو رقم ایرانی برنج ندا و نعمت

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چکیده

هرمی کردن ژن‌های مرتبط با کیفیت دانه و مقاومت از طریق انتخاب به کمک نشانگر مولکولی (MAS) روش مهمی در برنامه‌های اصلاحی برنج می‌باشد. از انتخاب به کمک نشانگر مولکولی برای ردیابی حضور یا عدم حضور این ژن‌ها در جمعیت‌های اصلاحی استفاده می‌شود و با روش‌های اصلاحی کلاسیک می‌تواند تلفیق شود. این مطالعه بخشی از برنامه تولید رقم در ایران از طریق تلفیق اصلاح کلاسیک با انتخاب به کمک نشانگر مولکولی می‌باشد. تلاقی‌ها بین دو لاین تراریخت پرمحصول واجد ژن مقاومت به آفات (*cry1Ab*، *Bacillus thuringiensis*) با یک رقم محلی انجام و متعاقب آن انتخاب برای ادغام ژن‌های مقاومت به آفات و عطر (*fgr*) در تک بوته‌های مطلوب F2 صورت گرفت. در نهایت بوته‌های هموزیگوت از نظر عطر و واجد ژن *cry1Ab* با خصوصیات زراعی مطلوب شناسائی شدند. سایر مطالعات روی این بوته‌ها در نسل F3 در جریان می‌باشد. این بوته‌ها، نویدبخش تولید لاین‌های جدید برنج *Bt* معطر از طریق تلفیق اصلاح نباتات کلاسیک و مولکولی در آینده‌ای نزدیک در ایران می‌باشند.

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