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## Chitinase Polymorphism in Some Iranian Rice (*Oriza Saiva* L.) Cultivars using RFLP-PBR

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### Abstract

Sheath blight disease, caused by *Rhizoctonia solani*, is a major fungal disease of rice, occurring mostly in Asian rice. The present study was performed to identify the chitinase polymorphism in 19 Iranian rice cultivars by using RFLP-PBR, with Rsa I, Taq I, Sac I and Ava I restriction enzymes. Digestion of the chitinase gene RICCH-3 by its specific digesting enzymes, i.e. RsaI and TaqI, produced a uniform banding pattern in all the rice cultivars studied. However RICCH-1 digestion showed banding pattern polymorphism in the two cultivars 1205 and 1206 and digestion of RICCH-2 showed banding pattern polymorphism in the cultivars 665 and 667. The cultivars studied showed no polymorphism for the sites of the AvaI and SacI enzymes. In total, 13 bands were obtained by the digesting action of RsaI and TaqI enzymes among 19 rice cultivars studied, 4 bands were common in all the cultivars while one unique band was observed in the cultivar 326. There were 4 other bands present in all other cultivars but missing in the cultivar 326, making this cultivar significantly different from the other rice cultivars studied. Data obtained suggested the possible occurrence of intra-geneic deletion in the RICCH-1 site and intra-genic duplication in RICCH-2 site. Grouping of the rice cultivars on the basis of the RFLP bands obtained did not correlate with their geographical regions of cultivation. This is the first report on chitinase polymorphism in Iranian rice cultivars.

**Keywords:** chitinase, Iran, RFLP, rice.

### بررسی پلی مورفیسم ژن کیتیناز در چند رقم برنج ایرانی با استفاده از روش RFLP-PBR

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### چکیده

بیماری زنگ برگ در برنج یک بیماری قارچی عمده است که توسط *Rhizoctonia solani* پدید می آید. تحقیق حاضر برای شناسایی تنوع ژن کیتیناز در ۱۹ رقم برنج ایرانی و با استفاده از روش RFLP-PBR و آنزیم های محدود کننده RsaI، TaqI، SacI و AvaI انجام گرفت. هضم ژن RICCH-3 توسط آنزیم های اختصاصی RsaI و TaqI باندهای یکسانی را در تمامی ارقام برنج مطالعه شده پدید آورد در حالی که هضم ژن RICCH-1 وجود پلی مورفیسم را در دو رقم ۱۲۰۵ و ۱۲۰۶ نشان داد. از طرفی هضم ژن RICCH-2 باندهای پلی مورف در ارقام ۶۶۵ و ۶۶۷ بوجود آورد. ارقام مطالعه شده هیچ تنوعی را برای جایگاه های AvaI و SacI نشان ندادند. بطور کلی تعداد ۱۳ باند از هضم آنزیم های RsaI و TaqI در ارقام برنج بدست آمد که از این میان تعداد ۴ باند در تمامی ارقام مشترک بودند. یک باند اختصاصی در رقم ۳۲۶ وجود داشت در حالی که ۴ باند در تمامی ارقام بجز رقم ۳۲۶ مشاهده شدند. نتایج بدست آمده احتمال وقوع حذف درون ژن RICCH-1 و نیز مضاعف شدگی درون ژن RICCH-2 را نشان می دهند. گروه بندی ارقام برنج بر اساس باندهای حاصل از RFLP ارتباطی را با محل جغرافیایی ارقام نشان نداد.

کلیدواژه ها: کیتیناز، ایران، چند شکلی طولی قطعات محدود شده، برنج.

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## Introduction

Rice (*Oryza sativa* L.) is an erect annual grass, up to 1.2m tall, producing tiny oblong grains (28,000 to 44,000 per kg, depending on the variety). Native to the tropics and subtropics of Southeast Asia (where it has been cultivated since at least 5000 B.C.), it is now grown in many localities throughout the world with favorable climatic conditions and is the major source of human food. More than 90% of the world rice production is in Asia, with China and India being the largest producers.

Genetic diversity as observed in landraces of rice and its wild relatives enables the plants to evolve and differentiate into various cultivars to adapt to different environments. Recently, however, the diversity of the gene pool has become depleted, mainly on account of extension of modern high-yielding varieties. Natural habitats of wild rice are also threatened by various farmland development projects. In many rice growing countries, landraces carrying a vast amount of genetic diversity were distributed in remote villages. The number of landraces began to decline with the introduction of high-yielding varieties. Most of the old landraces are now available in certain gene banks only and not in the hands of farmers. Therefore it is necessary to study the available diversity and introduce new variability as well.

In general, the perennial populations of wild rice are more variable within each than annual populations, while the annuals are more variable from each other. This trend was recognized in both isozymes and phenotypic characters (Oka, 1988). The perennial wild-rice outcrosses more (30-50%), while the annual wild rice has relatively lower out crossing rate (10-20%). This difference in the breeding system results in different population structures. However, the within-population diversity in the reaction to races of bacterial blight was greater for the annual than for the perennial populations (Morishima, 1994). The reason for this is not clear.

Sheath blight disease, caused by *Rhizoctonia solani*, is a major fungal disease of rice, occurring

mostly in the Asian rice. Cultivars resistant to this disease have not been identified so far (Sridevi *et al.*, 2003). An important plant defense mechanism against pathogen attack is the synthesis of 'pathogenesis-related' (PR) proteins. The PR proteins are of host origin and are synthesized under specific pathological and stress conditions (Sridevi *et al.*, 2003). In most parts of a plant, the natural defense mechanism consists of a number of genes that are induced to action upon infection. In some parts of a plant, some of these genes are expressed constitutively. A variety of such genes has been identified. In most cases, their precise mode of action is not understood, but the most obvious functions of the encoded proteins allow some deductions to be made. Defense genes encode: 1) hydrolases (chitinases and glucanases), that are thought to act by digesting fungal cell walls and/or by producing elicitors from fungal or plant cell walls that induce the plant's defense reaction; 2) ribosome-inactivating proteins (Rips), which may inactivate fungal ribosomes; 3) proteins that alter the plant's cell wall; 4) proteins that may detoxify compounds present in the infected cell; 5) proteins that may interfere with fungal or cellular membrane stability (thaumatin-like proteins, thionins, etc.); 6) proteins that are involved in the synthesis of antifungal compounds, 7) inhibitors of fungal proteases, and 8) a number of proteins with even less well-known functions (Joosten and De Wit 1989; Peg, 1976; Linthorst, 1991).

Genetic engineering of rice using antifungal genes offers an opportunity to develop sheath blight resistance in rice; in fact, antifungal activities of some of these proteins have been proven using in vitro assays in microinjected plant cells and recently in transgenic plants. In a variety of transgenic dicot plants, constitutive expression of a single defense gene strengthened the plant's defense against fungal infection (Alexander *et al.*, 1991; Broglie *et al.*, 1991; Hain *et al.*, 1993).

Chitinase (PR-3) has been extensively studied for its antifungal role (Sridevi *et al.*, 2003) and several studies have been performed for identification and

characterization of chitinase genes in rice genome (Zhu and Lamb, 1991; Huang *et al.*, 1991; Nishizawa and Kawakami, 1999). (Nishizawa *et al.*, 1999) isolated three class-I chitinase genes from rice and that their expression was activated by chitin oligomers and showed that transgenic plants (tobacco, strawberry and cucumber) constitutively express the rice chitinase gene with an increased resistance to fungal diseases.

The present study reports the chitinase gene allelic polymorphism in 19 Iranian rice cultivars for the first time by using the PCR based RFLP (PBR) molecular technique.

## Material and Methods

### *Plant material and DNA extraction*

Nineteen rice cultivars available in the rice gene bank of Iran Rice Research Institute were used for molecular studies. Fresh leaves were selected randomly from 3-5 plants of each cultivar and DNA extraction was done according to the method of Dellaporta (1983).

### *Primer Construction and DNA Amplification*

The construction of specific primers for the chitinase genes (RICCH-1, RICCH-2 and RICCH-3) was done by the use of BLAST (NCBI, <http://www.ncbi.nlm.nih.gov/>). The PCR reaction mixture consisted of 75 ng template DNA, 10 x PCR buffer (100 mM Tris-HCL pH 8.3, 500 mM KCL, 0.1% gelatin), 1.92 mM MgCl<sub>2</sub>, 100 μM dNTPs, 1 μM primer and 2 μl of Taq polymerase, in a total volume of 25 μl.

DNA amplification was performed on a palm cyclor. Template DNA was initially denatured at 94°C for 3 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 1 min at 94°C, primer annealing for 1 min at 60°C and primer extension for 2 min at 72°C. A final incubation for 5 min at 72°C was performed to ensure that the primer extension reaction proceeded to completion. The PCR amplified products were separated by electrophoresis on a 1.5% agarose gels using 0.5 X TBE buffer (44.5 Mm Tris/Borate, 0.5 Mm EDTA, pH 8.0).

### *Digestion Procedure*

The restriction digestion to detect chitinase polymorphism was performed by using Rsa I, Taq I, Sac I and Ava I. Twenty-five μl of digestion reaction was made by using 10 μl DNA, 2.5 μl buffer, 1 μl enzyme, and 11.5 μl distilled water. The digestion reaction was incubated for 4 hours to overnight at an appropriate incubation temperature for the enzyme used. The PCR products or the DNA fragments produced by restriction digestion were resolved electrophoretically on 1.5 % agarose gel in 1X TAE buffer.

### *Statistical Analysis*

For grouping of the rice cultivars studied, different clustering methods including UPGMA (Unweighted Paired Group with Arithmetic Averages), WPGMA (Weighted Paired Group with Arithmetic Averages) and Single Linkage as well as Principal Coordinate Analysis (PCO) was performed (Chatfield and Collins, 1995; Sheidai *et al.*, 2007).

The RFLP bands obtained were treated as binary characters and coded accordingly (presence =1, absence = 0). Simple matching and Jaccard coefficients were determined and used for cluster analysis (Chatfield and Collins, 1995; Sheidai and Noormohammadi, 2005). In order to check the fit of clustering results to the original data, cophenetic correlation was determined. In order to identify the most variable RFLP bands among the rice cultivars studied, Factor Analysis (FA) based on Principal Components Analysis (PCA) was performed. NTSYS Ver. 2.02 (1998) and SPSS ver. 10.0.5 (1999) was used for clustering and PCA analyses.

### *Results and Discussion*

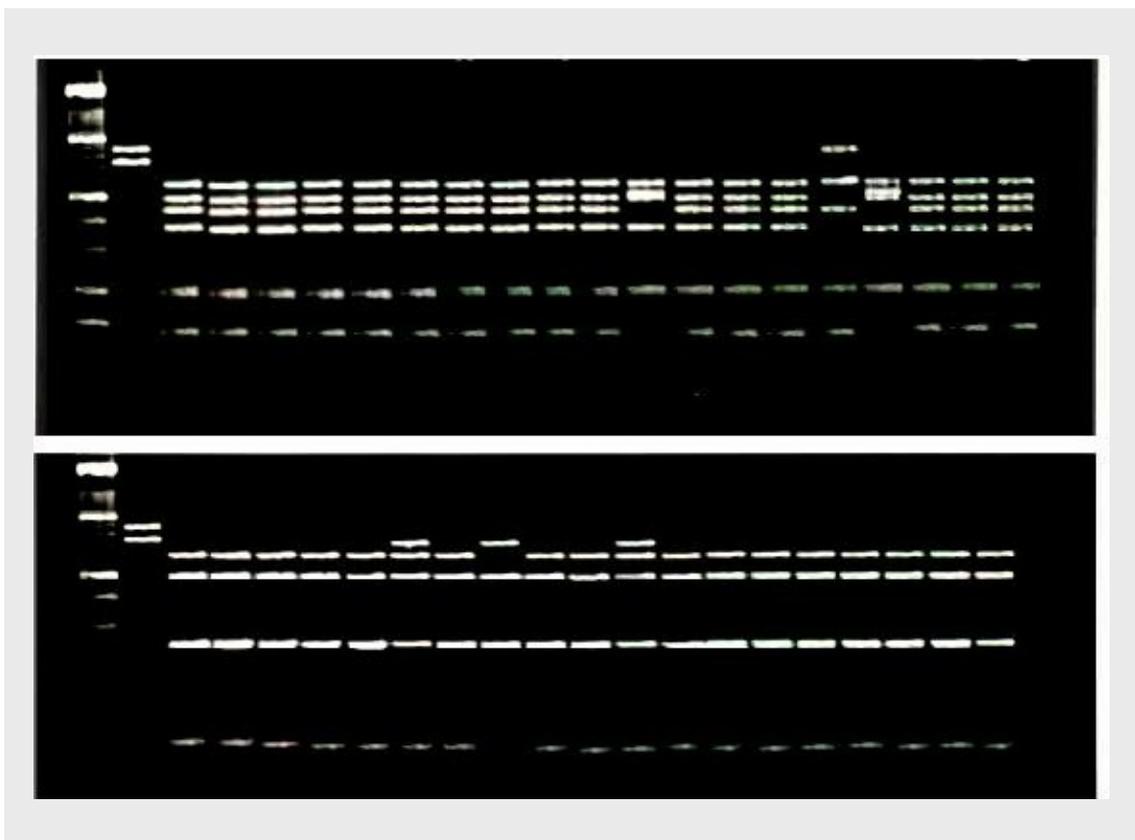
Digestion of the chitinase gene RICCH-3 by its specific digesting enzymes i.e. RsaI and TaqI, produced a uniform banding pattern in all the rice cultivars studied. However RICCH-1 digestion showed banding pattern polymorphism in the two cultivars 1205 and 1206 and digestion of RICCH-2

showed banding pattern polymorph in the cultivars 665 and 667.

The cultivars 908, 519 and 3265 showed polymorphism for the site of RsaI, while the cultivars 80161, 326 and 80089 were polymorphic for the cutting sites of the TaqI enzyme (Figs. 1 and 2). The cultivars studied showed no polymorphism for the sites of AvaI and SacI enzymes. In total, 13 bands were obtained by digesting action of RsaI and TaqI enzymes among 19 rice cultivars studied, producing a data matrix of 11 X 19 for further statistical analysis. Out of 13 bands obtained, 4 bands were common in all the cultivars while one unique band was observed in the cultivar 326. There were 4 other bands present in all other cultivars but missing in the cultivar 326, making this cultivar much different from the other rice cultivars studied.

Studying the rice data base concerned with chitinase genes in the rice cultivars of Indica and Japonica showed that the size of RICCH-1, RICCH-2 and RICCH-3 is 765, 880 and 760 bp respectively. Since digestion of RICCH-3 showed no polymorphism in the rice cultivars studied, the presence of a band with approximate size of 700 bp in the cultivars 1205 and 1206 is related to the RICCH-1 site which may have been produced by intra-genetic deletion. Moreover the presence of a band with the approximate size of 900 bp is related to the RICCH-2 site possibly produced by intra-genetic duplication.

A better understanding of digestion profiles may be obtained by considering the sequences of the genes studied and their restriction (cutting) sites for the enzymes used in the present work. It is expected that digestion of the site RICCH-1 by RsaI produces



**Figures 1 and 2.** Digestion profiles of RICCH -1, RICCH -2 and RICCH -3 sites by RsaI and TaqI enzymes in the rice cultivars studied. (The lanes from left to right: Ladder (100 bp), uncut DNA, Amol2, 1663, 461, 80161, 388, 1517, Haraz, 2, 80089, 731, 908, 1553, 1058, 541, 326, 519, 558, Sefid and 580.

fragments with 80, 230 and 450 bp length, while digestion of the site RICCH-2 produces fragments with 380 and 500 bp length. Digestion of the site RICCH-3 by RsaI is expected to produce fragments with 210 and 550 bp length. Therefore absence of the bands with 380 and 500 bp in the cultivar 326 indicates the possible occurrence of a point mutation in the recognition site of RsaI in RICCH-2, while absence of fragments with the size of 80 and 450 bp in this cultivar may indicate the possible deletion or the occurrence of a mutation in a recognition site of RsaI in RICCH-1.

It is expected that digestion of the site RICCH-1 by TaqI produces fragments with 260 and 500 bp lengths, while digestion of the site RICCH-2 produces fragments with 80, 250 and 650 bp length. Digestion of the site RICCH-3 by TaqI is expected to produce fragments with 250 and 510 bp lengths, which were observed in most of the rice cultivars studied. However variation observed in the TaqI digestion profile of the cultivars 800161 and 80089, may be

related to the loss of the recognition site (750 bp length) in RICCH-1 and the absence of 260 and 500 bp fragments in these cultivars. Similarly the absence of fragments with 80 and 650 bp lengths and the presence of a fragment with about 730 bp length in the cultivar 326 may be related to the loss of recognition site in RICCH-2.

The present results indicate the use of PBR technique in showing the allelic polymorphism in the chitinase genes of rice, however it is only capable of showing the occurrence of mutations in the cutting sites of the enzymes used and can not show the occurrence of other possible DNA mutations.

Grouping of the rice cultivars studied by different clustering methods as well as PCO ordination produced similar results (Fig. 3). The highest value of cophenetic correlation ( $r = 0.99$ ) was obtained for UPGMA clustering, therefore UPGMA dendrogram and PCO plotting is discussed. In general, four major clusters or groups are formed showing the distinctiveness of the cultivars 80161 and 800169, 908

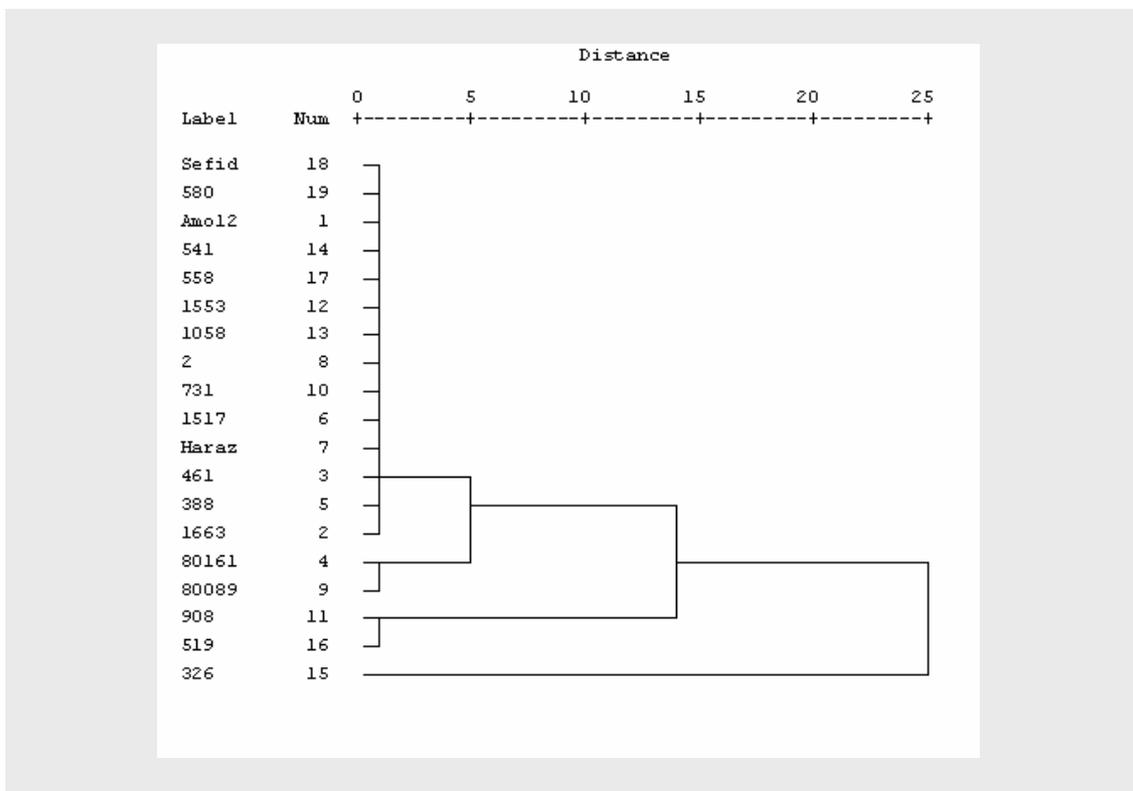


Figure 3. UPGMA clustering of the rice cultivars.

and 519 as well as the cultivar 326 which have been placed in separate clusters or groups due to their genetic differences, while the other cultivars show a high amount of genetic similarity and are placed in one cluster.

Although the geographical collection regions of some of the rice cultivars studied from the gene bank are missing but considering those available, clearly shows that the grouping of the rice cultivars does not seem to be related to geographical region; for example the cultivars Amol2 and 908 are from Amol city but show different mutations and are placed in different clusters far from each other. The same holds true for the three cultivars of Sefid, 80089 and 519, all collected from Gilan Province which also stand in different clusters.

Factor analysis revealed that the first two components comprise about 92.80% of total variation. In the first factor with about 59% of total variation, RFLP bands No. 5, 7, 11 and 14, possessed the highest positive correlation ( $r = 0.98$ ), while band No. 2, possessed the highest negative correlation ( $r = -0.98$ ), and are the most variable RFLP bands of the first component. These bands are produced by digesting action of RsaI and separate the cultivars 908 and 519 from the other rice cultivars.

In the second factor with about 32% of total variance, the bands No. 6 and 9, possessed the highest positive correlation ( $r = 0.97$ ), while band No. 4, possessed the highest negative correlation ( $r = -0.97$ ). Therefore these are the most variable RFLP bands of the second component, produced by digesting action of TaqI, separating the cultivars 326, 80089 and 80161 from the other rice cultivars. In order to plan a better breeding program for Sheath blight disease resistance in Iranian rice cultivars, similar studies should be performed in other cultivars.

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