Pak. J. Bot., 43(3): 1607-1612, 2011.

DIVERSITY IN MAJOR SEED STORAGE PROTEINS OF RICE LANDRACES OF PAKISTAN

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Abstract

Grain proteins from 173 rice landraces (*Oryza sativa* L.) were electrophoretically separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Densitometric scanning of the electrophoretic profiles permitted the estimation of the relative concentration of 25 polypeptide fractions, which were used as variables for the calculation of similarity coefficients between these accessions. A considerable variation was observed in glutelin subunits and Wx gene product. Very low variation was observed in prolamin subunits. Lower genetic distance based on total seed protein profile may be attributed to narrow genetic base. This electrophoretically detectable protein polymorphism in rice grain can be used in further breeding proposes and variety development. Our results suggest that screening of landraces on the basis of variation in seed storage protein profile using SDS-PAGE could be highly effective tool to identify valuable rice genetic resources.

Introduction

Rice, *Oryza sativa* (2n = 24) belonging to the family Poaceae and subfamily Oryzoidea occupies almost one-fifth of the total land area covered under cereals. It is grown under diverse cultural conditions and over wide geographical range. About 23% of the total foreign exchange earnings is shared by rice and thus called as "Golden Grain of Pakistan" (Shah *et al.,* 1999). Hence one third of total production is annually exported and two third is locally consumed to meet food needs (Sagar *et al.,*1988). Pakistan is the third largest rice exporting country. However, it's per hectare yield is the lowest among the rice producing countries (Abbas, 2000).

Pakistan is famous for growing and exporting long grain aromatic Basmati rice. Rice exports hovering around three million tonnes per annum have accounted for 5% of the foreign exchange from merchandize exports. Rice cultivation area occupies 2.96 million hectares that is 12% of the total cultivated area. Its production is 6.95 million tones and 2347kg yield per hectares (Anon., 2009). Among Asian rice growing countries, Pakistan is a key producer of many rice varieties such as aromatic rices and old landraces. In alarming situation of global biodiversity loss, Pakistan lost several rice varieties. Conservation as well as exploration of landrace genotypes is essential to develop a gene-pool of aromatic rice for breeding purposes of high yielding varieties in the country (Pervaiz *et al*., 2009).

The quality preferences of rice consumers have resulted in a wide diversity of varieties specific to different localities. Although the exact diversity cannot be gauged, it is estimated to be around 140,000 different genotypes. Around 100,000 accessions of rice are preserved in International Rice Research Institute, Philippines (Anon., 1995). In Pakistan, Gene-bank at National Agricultural Research Centre has more than 2900 accessions. Conventional breeding techniques have increased the diversity of Pakistani rices in terms of morphological and quality traits, especially grain size, shape and colour, as well as aroma and endosperm properties.

Seed protein electrophoresis has been used as a tool to resolve taxonomic and evolutionary problems, varietal and species identification in many crops and has a range of applications in breeding programs (Nisar *et al*., 2007; Hameed *et al*., 2009; Nisar *et al*., 2009a). On average protein content of Rice vary from 5.9-11.9% in which major seed protein glutelin accounts for 70% of total proteins on weight basis (Ogawa *et al*., 1987; Li & Okita 1993). Damerval *et al*., (1987) hypothesized that the quantitative variations in gene product levels revealed by electrophoretic techniques is a more important basis for detection of morphological and adaptive change than classical variability (presence/absence). They found that genetic variation in proteins (more or less intense spots), revealed by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), in five maize lines was significantly correlated to morphological distances.

Numerous seed protein profile studies have been done with various plant species including rice (Cheema *et al*., 2010; Khan *et al*., 2010). This strongly supports the hypothesis that molecular polymorphisms are neutral in natural selection. Seed material or powder is relatively easy to handle with respect to protein extraction and more important, the seed may be regarded as a fixed physiological state. In taxonomic studies, it is critical to compare organs at the same stage as well as morphology. In this sense, the seed, and its proteins, may be regarded as a "conservative" unit, little affected by the environment, geographic origin, seasonal fluctuations and chromosomal rearrangements (Ladizinsky & Hymowitz, 1979; Vaughan, 1983). Various techniques have been used for analysis of genetic variability at the molecular level in plants: isozymes, DNA restriction fragment length polymorphism (RFLPs) and random amplified polymorphic DNA (RAPDs). However, seed protein profiles are still powerful tools for determining genetic homology at the molecular level and for solving problems in systematic methodology.

SDS-PAGE is particularly reliable and cheap method for polypeptides analysis (Nisar *et al*., 2009b). The present study is focused on the determination of genetic diversity based on Wx (waxy) gene product, glutelin, globulin and prolamin subunits in Pakistani rice landraces.

Materials and Methods

Plant materials: A total of 173 rice accessions were obtained from the PGRP Gene-bank, National Agricultural Research Centre, Islamabad. Passport number, geographical location and seed morphology of the samples used in present study are given in Table 1. These accessions were collected from different agro ecological zones (I-IV) of Pakistan (Table 1). Two varieties IR36 (Indica type) and Kinmaze (Japonica type) were used as standard.

SDS-PAGE electrophoresis: De-husked seeds of each accession were finely grinded by using mortar and pestle. Seed flour (0.01g) was taken and mixed with 400µl of extraction buffer (0.05M Tris-HCl pH 8.0, 0.2%SDS, 5M Urea, 1% beta marcaptoethanol and 0.05% bromophenol blue as tracking dye). Centrifugation was done at 15,000rpm for 5 min at room temperature. Extracted samples were analyzed through 15% SDS-polyacrylamide gel electrophoresis (Laemmli, 1990). A protein marker (Fermantas S# 0431) was loaded as molecular weight standard.

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Table 1. Geographical, morphological and passport detail of Pakistani rice landraces used in study.

Geographical locations	Accession numbers	Japonica/Indica
Punjab	6505, 6506, 6507, 6508, 6509, 6512, 6514, 6515, 6516, 6517, 6521, 6522, 6523, 6524, 6525, 6526, 6527, 6529, 6530, 6531, 6532, 6535, 6536, 6537, 6538, 6540, 6541, 6542, 6545, 6546, 6547, 6549, 6550, 6551, 6552,6553, 6554, 6556, 6557, 6558, 6559, 6560, 6562, 6563, 6565, 6570, 6571, 6572, 6578, 6580, 6581, 6582, 6585, 6588, 6589, 6590, 6593, 6595, 6596, 6597, 6599, 6603, 6605, 6606, 6608, 6610, 6611, 6613, 6614, 6615, 6616, 6620, 6621, 6622, 6626, 6627, 6633, 6634, 6636, 6640, 6641, 6642, 6645, 6646, 6647, 6649, 6650, 6651, 6652, 6654, 6655, 6658, 6659, 6661, 6663, 6664, 6665, 6666, 6667, 6668, 6670, 6674, 6675, 6676, 6677, 6680, 6681, 6682, 6683, 6684, 6685, 6686, 6690, 6693, 6694, 6695, 6697, 6698, 6703, 6705, 6706, 6708, 6711, 6712, 6717, 6718, 6719, 6720, 6722, 6724, 6725, 6728, 6729, 6731, 6732, 6733, 6734, 6737, 6738, 6739, 6740, 6744, 6745, 6746, 6751, 6753, 6754, 6755, 6756, 6757, 6758, 6759	Indica type *(3.1-4.0)
Sindh	6760, 6761, 6765, 6766, 6769, 6770, 6771, 6774, 6775, 6779	Indica type*(3.3-4.0)
NWFP	6519, 6520, 6564, 6672, 6638, 6628, 6629, 6569, 6623, 6624, 6574	Japonica type*(2.2-3.5)

*Data collected from separate seed morphological studies not provided in this article

Staining and data analysis: Gels were stained with 0.2% (w/v) commassie brilliant blue (R250) for about 1 hour and de-stained overnight. Gels were preserved by using gel dryer. Scoring the bands as 1 for their presence and as 0 for their absence across the genotypes generated a rectangular matrix. Genetic similarity was computed based on Nei & Li's (1979) coefficient of similarity. It was calculated as:

$$\text{Similarity} = \frac{2N_{xy}}{N_x + N_y}$$

where 'Nx' and 'Ny' represent the number of bands present in landrace 'x' and 'y', respectively. Nxy the number of bands shared by the landrace 'x' and 'y'. The data was subsequently used to construct a dendrogram using the un-weighted pair group method of arithmetic averages (UPGMA) employing sequential, agglomerative hierarchic and non-overlapping clustering (SAHN) (Sneath & Sokal, 1979). All the computations were carried out using the software NTSYSpc, version 2.1 (Applied Biostatistics Inc., USA).

Results

Seed protein polymorphism: In present study SDS-PAGE of total seed protein was performed to evaluate the genetic diversity among landraces collected from different areas of Pakistan. The electrophorogram showing polypeptide banding pattern of different rice landraces are given in Fig. 1. A total of 25 bands were observed which are divided into six groups on the basis of major seed proteins. The group I consisted of high molecular weight five polypeptides that are most conserved peptides and showed no variation in almost all landraces (Table 2). Group II consisted of one band of 60kDa of Wx gene product showing 12% polymorphism. Group III is further sub-divided into three classes A, B and C. Two bands of 55kDa and 57kDa (a precursor polypeptide, expressed form of glu-p gene) which showed 5.3% and 17.9% of genetic polymorphism, respectively constitute group III (A). Group III (B) consisted of four glutelin acidic subunits alpha 1, 2, 3 in the range of 34-40kDa. No variation was observed in alpha-4 glutelin polypeptide subunit (28kDa). A band of 32 kDa was also observed, which was expressed in only eight accessions. Group III (C) composed of 20, 21, 22kDa of beta polypeptides of glutelin among which only β-20kDa showed highest polymorphism, while other two subunits showed very low variation in Pakistani genotypes. Group IV was composed of two albumin peptide subunits (42kDa and 47kDa), which showed no polymorphism in Pakistani rice landraces. A 26kDa globulin which was conserved in almost all accessions constituted Group V and Group VI consisted of one 16kDa, three 13kDa (a, b, c) and one 10kDa prolamin polypeptides showing very low variation.

Among the 25-polypeptide bands, the bands 65, 47, 42 and 26kDa were highly conserved and monomorphic. The band 32 and 20kDa showed 82.2% of genetic polymorphism. While the product of Wx gene (60kDa), the precursor of glutelin polypeptide (57kDa) and the 16kDa subunit of prolamin gave 12, 17 and 11.50% of protein polymorphism in banding profile (Table 2).

Protein markers: Based on protein markers, some elite lines were selected. The grouping was based on the reproducibility of the some unique bands (protein marker). The accessions, which reproduced the unique bands, were considered in the present study. Twenty-one accessions without Wx gene product (60kDa polypeptides absent) were selected along with one japonica check variety Kinmaze. The accessions 6755, 6756, 6757, 6750 were without glutelin (acidic and basic peptide subunit) and were placed in group-II. In the group-III eight accessions 6695, 6685, 6626, 6611, 6582, 6563, 6549, 6623 cluster having a novel protein marker of 32kDa peptide subunit.

Wx gene product is positively correlated with amylose content. So these accessions were with no or very low amylose content. Four elite accessions with no acidic (37, 38, 39kDa) and basic glutelin peptide subunits were selected from whole landraces. These lines showed very high staining intensity at precursor glutelin peptide level (Table 3).

Group construction: Landraces with same banding profile were assembled into 12 groups and named group A to Group L, alphabetically, while other with unique profile were allocated with accession number (Table 4). A UPGMA dendrogram was constructed on the Nei's unbiased genetic similarity coefficients among landraces on the basis of protein profile (Fig. 2). The dendrogram revealed genetic variation among the landraces with similarity coefficients varying between 0.81 and 1.00. It also revealed four distinct major clusters at similarity value of 0.91. The first cluster consisted of 4 accessions with no or very low glutelin (both acidic & basic subunits are absent). Second cluster consisted of single accession 6708 that has no Wx gene product which shows that it has very low amylose contents, while alpha-2 and 3 of glutelin subunits and 16 kDa subunit of prolamin polypeptide was absent. Third cluster also consisted of nine accessions along with Kinmaze (Japonica standard) with no Wx gene product, only alpha-1 subunit of glutelin polypeptide and 32 kDa peptide were absent, while 'c' subunit of 13kDa prolamin was also missing. Fourth cluster consisted of 160 accessions, accounting for 91% of total accessions including IR36 (Indica standard).

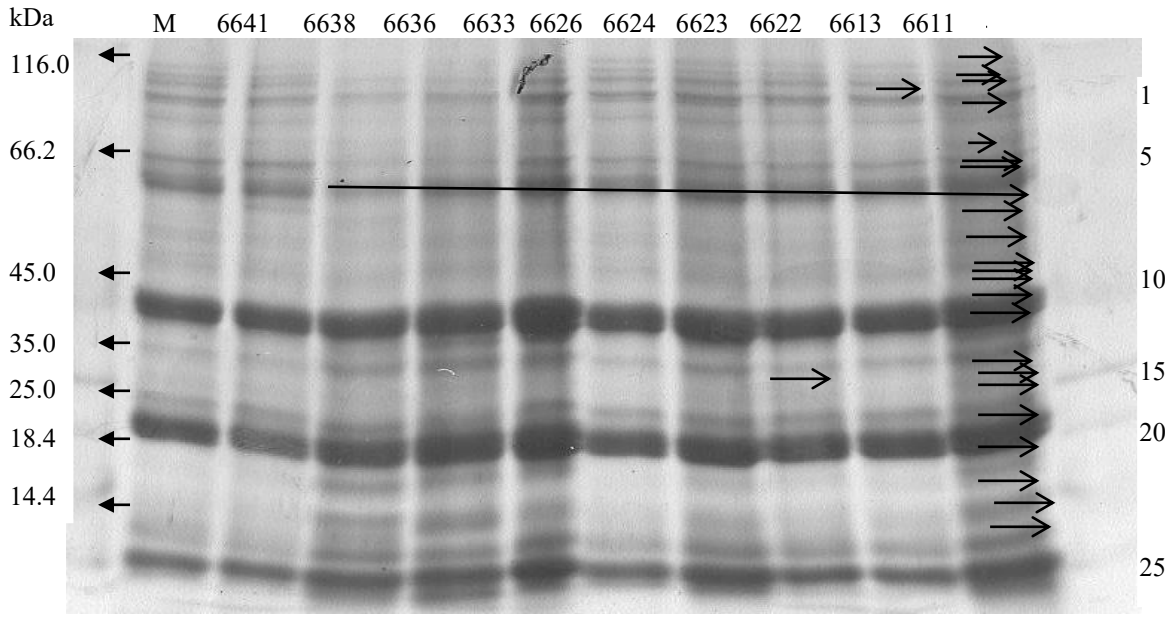

Fig. 1. Electrophorogram showing types of protein banding pattern separated by 15% separation gel in different Pakistani rice landraces (arrows indicate band number as mention in Table 2).

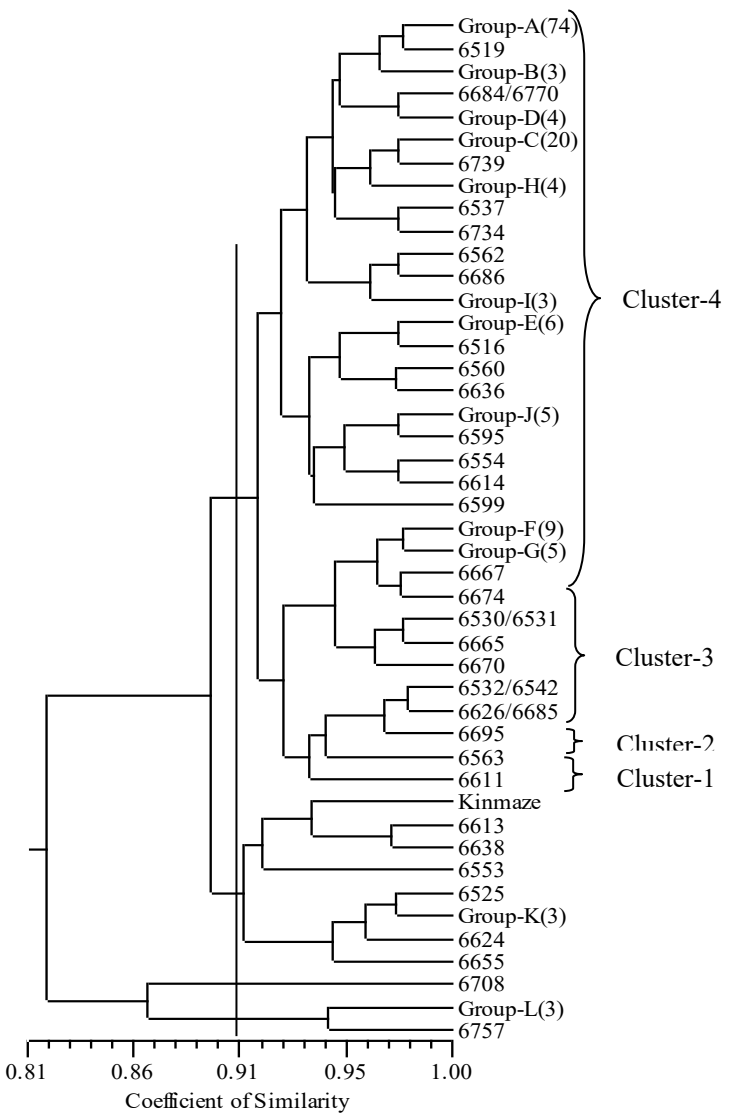

Fig. 2. A dendrogram showing relationship of 176 rice landrace genotypes of Pakistan divided into common groups on the basis of presence of common bands profile.

Discussion

In present investigation, maximum 25 bands were observed in accession 6685, while 24 in accessions 6695, 6542 and 6532. Minimum 17 bands were observed in accession 6757 and 18 in accessions 6655 and 6613. Habib *et al.,* (2000) evaluated 15 genotypes of rice using SDS-PAGE, showed a total of 32 bands. Sharief *et al.,* (2005) reported maximum17 bands in basic, registered, certified and commercial seed of rice 'Giza 177' cultivar using SDS-PAGE and also observed minimum 12 bands in commercial seed of Sakha 101 using SDS-PAGE. This variation in number of polypeptide subunits may be due to gel percentage, preference of major subunits for evaluation and size of the gel.

No significant association of geographical location and variation in seed protein profile was observed. The landraces from different geographical zones were grouped together in same clusters. This shows that expression profile is least affected by environment reported by various studies on rice as well as other cereal crops (Sanni *et al*., 2008). Lower genetic distance (similarity 0.81) may be attributed to the same genetic background, traditional framing practices and consumer preference (Javaid *et al*., 2004).

Our study revealed that storage protein polymorphism in rice can be used to discriminate rice accessions on the basis of amylose contents (high or low on the basis of presence or absence of Wx gene product in protein banding profile) and glutinous rice varieties from non-glutinous ones on the basis presence of acidic and basic peptide subunits of glutelin. In present study one japonica variety Kinmaze also showed absence of Wx gene product in addition to other twenty-one Pakistani landraces. Pakistani germplasm is not japonica type because Kinmaze clustered with few landraces and showed no 100 percent similarity with any accession. Majority of the accessions grouped with indica type check variety IR36, with 100 percent similarity, which clearly indicates that rice landraces of Pakistan are almost all indica type. So protein-banding profile can be used to differentiate japonica and indica subspecies of *Oryza sativa*. Variation in 13 and 16 kDa prolamin peptide subunits is limited to Asian rice (Hilu & Sharova, 2002) and our study is again a confirmation of this conclusion, 16 kDa peptide subunit showed considerable polymorphism in Pakistani germplasm.

Another important thing observed in Pakistani germplasm accessions protein banding profile, was that four accessions (6755, 6756, 6757, and 6758), which showed no acidic and basic glutelin peptide subunit, took least days to heading and maturity (data based on morphological studies, not included in this article). This gives a clue to a correlation between glutelin markers and days to heading and maturity in rice landraces. Further study is required to link protein markers with agronomic traits.

Table 2. Detailed profile of protein banding pattern of Pakistani rice landraces.

Groups	Band#	Molecular weight	Polymorphic/ conserved	Major/ minor	Protein type	Accessions expressing (%)	Polymorphism (%)
G-1	1,2,3,4,5	More than 65kDa	Conserved	Minor	High molecular weight peptide subunits	100	0
G-II	6	60kDa	Polymorphic	Major	Waxy protein	87.90	12
G-III (A)	7,8	55-57kDa	Polymorphic	Major	Precursor polypeptide of glutelin	94.8, 82.1	5.3, 17.9
	9	52kDa	Highly polymorphic	Minor	Rapidly moving peptide subunit of glutelin	4	86.20
G-IV	10,11	42kDa, 47kDa	Conserved	Minor	Albumin polypeptide sub units	100	0
G-III (B)	12,13,14	34, 38and 39kDa	Polymorphic	Major	Alpha (acidic) polypeptides of glutelin	91.9, 97.1, 91.3	8.1, 2.9, 8.7
	15	32kDa	Highly polymorphic	Minor	A unique peptide	4.59	95.41
	16	28kDa	Conserved	Major	Alpha-4 subunit of glutelin	100	0
G-V	17	26kDa	Conserved	Major	Globulin	100	0
G-III (C)	18,19,20	20,21,22kDa	Polymorphic	Major	Beta (basic) polypeptides of glutelin	17.8, 97.1, 97.7	82.2, 2.9, 2.3
G-VI	21	16kDa	Polymorphic	Major	Prolamin subunit	88.50	11.50
	22,23,24	13kDa (a, b, c)	Polymorphic	Major	Prolamin subunit	100, 98.8, 92.5	0, 1.2, 7.5
	25	10kDa	Polymorphic	Minor	Prolamin subunit	92.50	7.50

G-group; Band #-band/bands number/numbers; %-percentage; kDa- kilo Dalton

Table 3. Identification of elite Pakistani rice landraces on the basis of protein.

Qualities/ individuality	Selected accessions
Accessions without Wx gene product (60kDa polypeptide absent)	6509, 6516, 6520, 6530, 6531, 6550, 6553, 6554, 6560, 6562, 6599, 6608, 6613, 6633, 6636, 6638, 6645, 6665, 6670, 6739, 6708
Accessions without glutelin (acidic and basic peptide subunits)	6755, 6756, 6757, 6758
Accessions with 32 kDa peptide subunit	6695, 6685, 6626, 6611, 6582, 6563, 6549, 6623

Table 4. Grouping of landraces/accessions on the basis of same protein profile.

Groups	Accession number
Group-A	6505, 6506, 6507, 6508, 6779, 6774, 6512, 6765, 6766, 6759, 6761, 6514, 6515, 6517, IR36, 6521, 6522, 6523, 6524, 6526, 6535, 6536, 6538, 6541, 6547, 6775, 6552, 6556, 6557, 6558, 6564, 6565, 6569, 6570, 6571, 6572, 6664, 6661, 6659, 6658, 6652, 6650, 6647, 6760, 6641, 6654, 6634, 6629, 6627, 6649, 6646, 6620, 6615, 6640, 6610, 6607, 6605, 6769, 6596, 6593, 6589, 6588, 6585, 6621, 6581, 6580, 6651, 6616, 6663, 6628, 6590, 6771, 6606
Group-B	6549,6582,6623
Group-C	6697, 6753, 6738, 6729, 6725, 6722, 6719, 6717, 6711, 6737, 6733, 6731, 6728, 6724, 6720, 6732, 6712, 6751, 6705, 6698
Group-D	6740, 6744, 6746, 6745
Group-E	6509, 6520, 6550, 6608, 6633, 6645
Group-F	6545, 6677, 6546, 6551, 6666, 6680, 6676, 6597, 6603
Group-G	6668, 6681, 6675, 6672, 6682
Group-H	6703, 6706, 6718, 6754
Group-I	6690, 6693, 6694
Group-J	6527, 6529, 6540, 6574, 6578
Group-K	6622, 6642, 6683
Group-L	6755, 6756, 6758

Two novel peptide markers (52kDa and 32kDa peptide bands) that were observed in Pakistani germplasm needed further exploration. It may be concluded that hybridization between accessions from two groups (one with all bands and other with missing band is suggested to be conducted with the expectation that missing or extra band might be linked with some agronomic traits.

In the present study 173 Pakistani landraces were evaluated at protein expression level. SDS-PAGE separated the whole landraces into two groups i.e. indica type and japonica and concluded that majority of Pakistani rice landraces are indica type. In the study Wx gene product, glutelin subunit showed significant level of polymorphism, while low level of variation was recorded in prolamin peptide subunit. It was also identified that Pakistani rice landraces have important genes, which needed further study by using Serial Analysis of Gene Expression.

Acknowledgments

The authors gratefully acknowledge the financial support for this project through AREF under the Agricultural Linkages Program (ALP) of Pakistan Agricultural Research Council, Islamabad, Pakistan. This work was also supported in part by Higher Education Commission, Islamabad in the form of PhD scholarship to the first author. We are also indebted to the Institute of Agri-Biotechnology & Genetic Resources, National Agricultural Research Center, Islamabad for providing the seed material of collected germplasm and extending laboratory facilities for the conduct of given study.

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(Received for publication 15 February 2010)