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Null Mutation in Puroindoline A is Prevalent in Indian Wheats: Puroindoline Genes are Located in The Distal Part of 5DS

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PCR amplification and protein analysis of pinA and pinB, the two components of friabilin, a marker protein for grain softness, were carried out in one hundred varieties from India and forty varieties from Kansas State, USA. Glycine to serine change in pinB or null mutation in pinA (absence of gene) has been reported linked with grain hardness. Here we report that majority of Kansas State hard wheats possess glycine to serine mutation in pinB and few have null mutation in pinA. In contrast, majority of varieties released in India are hard and have null mutation in pinA. There are some exceptions where some hard wheats in India do not exhibit either null mutation in pinA or glycine to serine change in pinB. There might be some additional mutations that are to be characterised in elucidating the molecular basis of hardness for usage in genetic engineering. PinA and pinB genes have been assigned on the distal part of 5D short arm using deletion lines of chinese spring wheat where hardness gene *Ha* is reported to be present.

Key words : grain hardness, puroindolines, friabilin, deletion lines, PCR.

Wheat endosperm texture is one of the primary determinants of end product quality. Based on grain texture wheat is classified into two distinct classes, hard and soft. Hard wheats are used for bread and *chapati* making and soft wheat for biscuit, cakes, pretzels and noodles (1-4). Genetic basis of hardness is relatively well established. One major gene for grain hardness (*Ha*) has been located on short arm of 5D chromosome (5-7). However, the physicochemical nature of the components responsible for hardness/softness is still not clear. Electron microscopic and staining techniques indicated the presence of substances between the starch granules and protein bodies in the endosperm (8, 9). The amount of the material adhering to the surface of the starch granules differs between the hard and soft wheat grains. Greenwell and Schofield (10) demonstrated abundant presence of friabilin, a 15 kD starch surface protein, on the surface of starch grains of soft wheat washed with water, which is scarce in hard wheat and absent in durum wheat. They hypothesized that friabilin might influence the degree of adhesion between starch granules and protein matrix surrounding the starch and thus variation in grain texture.

Subsequently friabilin was shown to be composed of two components, puroindoline A (pinA) and puroindoline B (pinB) whose transcripts are controlled by genes on 5D chromosome (11-13). Puroindolines are Triton X-114 soluble basic cystine-rich proteins with the tryptophan-rich domain having affinity with lipid binding proteins (14). Two classes of bound polar lipids (glyco and phospholipids) follow the same pattern of occurrence as friabilin (15). An RFLP clone corresponding to pinA has been found closely linked with Ha gene (16). Recently glycine to serine change in pinB at 46 position, a null mutation in pinA (17, 18) and leucine to proline change at 60 position in pinB (19) have been found associated with grain hardness. However both pinA and glycine type pinB have been reported to be present in hard wheats (20). Therefore, screening of a wide range of germplasm is required for puroindoline genes to establish their role in grain hardness. In this study, wheat lines have been analysed from India and Kansas, USA, for pinA and pinB genes to observe the variability in puroindolines and their relationship with grain hardness and position assigned to the distal part of 5D short arm.

Materials and Methods

Plant material and grain hardness measurements — One hundred varieties of wheat released in India and forty varieties from Kansas State, USA were used for PCR amplification and protein analysis for puroindolines and

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hardness measurements. Grain hardness was measured using single kernel characterisation system (SKCS) (Perten model-4100) and OSK 8055 hardness tester. In OSK the force applied in Kg until the kernel is broken measures grain hardness. Twenty kernels were taken from each sample varying in size. The mean value of all these kernels is reported here. In SKCS around 300 kernels from a sample are used for the measurement of grain hardness. Ten Indian varieties available in KSU were analysed for grain hardness using both the systems. Both the readings were found correlated with each other.

Isolation and electrophoretic separation of puroindolines — Triton soluble proteins were isolated by phase partitioning of Triton X 114 (21). 100 mg of finely ground kernels were homogenised in 1.0 ml of Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5) with 1% (v/v) TX-114 and mixed for 2 h at 4°C. The mixture was spined at 13,000 × g for 1 min at 4°C and the supernatant was transferred to a fresh tube and incubated at 37°C for 45 min. Again the mixture was spined for 2 min at room temperature and the aqueous upper phase was discarded. One ml of cold TBS was added into the remaining detergent rich phase and incubated at 37°C for 30 min. The aqueous upper phase was removed and the protein in the lower detergent rich phase was precipitated with 1.0 ml acetone. The pellet was dried and dissolved in sample buffer and 10 µl was loaded per lane on 13% SDS- PAGE gel. The gel after 3 h run at 150 volts was stained with silver staining (11, 22).

DNA isolation and PCR amplification — DNA was extracted from single kernel using modified method (23) with extraction buffer (100 mM Tris pH8, 50 mm EDTA pH 8.0, 500 mM NaCl and 2% SDS). The mixture was gently homogenised and maintained at 65°C for 10 min. The extracts were centrifuged at 4°C and the DNA in the supernatant was precipitated with ethanol, dissolved in TE buffer and used for PCR amplification. The DNA from all the lines was analysed for puroindoline genes using sequence specific primers. Amplification of pinA and pinB was performed as described (17). The 3'primer specific for glycine type pinB was the reverse complement of 5'GGCGGCTGTGAGCATGAG3'. The 3'primer specific for serine type pinB was the reverse complement of 5'AGCGGCTGTGAGCATGAG3'. The 5' primer for both the glycine and serine type pinB was ATGAAGACCTTATTCCTCCTA3'. To amplify puroindoline A gene(s) the sense strand primer was 5'ATGAAGGCCCTCTTCCTCA3' and the antisense strand primer was 5'TCACCAGTAATAGCCAATAGTG3'. The reaction mixture contained 1X PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.1µg of primers and 1 unit of Taq Polymerase. The temperature cycling was done 1 cycle at 94°C for 4 min, 35 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1.5 min and 1 cycle at 72°C for 8 min. The PCR products were separated on a 1.5 % agarose gel to visualise products.

Gene mapping — The mapping strategy was used as described by Gill *et al* (24). Deletion stocks of Chinese Spring wheat for 5D short arm developed and maintained at WGRC, Kansas State University Manhattan were used to locate pinA and pinB genes. The PCR amplification of puroindoline genes was done using forward and reverse primers for pinA and glycine specific primers for pinB. For homozygous deletion lines the scoring was done for the presence/ absence of the PCR amplification using primer for both pinA and pinB genes and compared with heterozygous and normal lines.

Results and Discussion

Grain hardness — Hardness measurements showed that most of the wheat varieties released in India are hard to medium hard and most of the varieties from Kansas State are hard. Ten hard Indian varieties available in US were evaluated for grain hardness using both the systems (SKCS and OSK). The data indicated high correlation between both the readings (r=0.91). Hardness measurement of wheat varieties in India was done using OSK system. In this system soft wheat has less than 9 Kg and hard wheat more than 10 Kg. There was a variation in grain hardness values ranging from 10 to 18 in OSK system in Indian wheats with the mean value of 14.1. This indicates the presence of some additional modifying genes influencing the level of hardness. Since *chapati* (unleavened flat bread) is the staple diet in India and hard wheats are used for it, selection has been made for developing hard wheats. Out of the forty four lines studied in Kansas State five lines namely Chinese Spring, Downy, Houser, Caldwell and IL90 were soft and the rest were hard. The hardness value of the hard lines varied from minimum of 46 to maximum of 88 in SKCS with the average value of 76. The soft varieties showed hardness value less than 40 in SKCS. This indicates large variability in the range of hardness. Since grain hardness is a major component which determines its technological quality it has become imperative to understand molecular basis of grain hardness in wheat.

PCR amplification and puroindoline analysis — Friabilin, a 15 kD starch surface protein in the wheat endosperm, referred as 'GSP' by Jolly *et al* (25), has been reported to be linked with grain softness (10) along with polar lipids (15). Subsequent work showed that friabilin is composed of two components as puroindoline A (pinA) and puroindoline B (pinB) whose transcripts are controlled by gene on 5D chromosome (11-13). The sequence analysis of puroindoline genes showed the relationship between glycine to serine change in pinB at 46th position near tryptophan rich domain or null mutation in pinA with grain hardness in US material (17, 18). Glycine to serine change was due to presence of point mutation from adenine to guanine in the coding sequence. This information was used to design and synthesize glycine or serine specific primers (17). The full-length pinA and pinB were amplified using pinA specific and pinB specific primers, respectively, and produced 450 bp length PCR fragment. Partial pinB sequence was amplified using glycine specific or serine specific forward primers and generated 250 bp long PCR fragment differentiating some soft and hard varieties. As expected there was no amplification of both the puroindoline genes in durum wheats analysed since durum wheats do not have D genome on which puroindoline genes are present.

Protein analysis was performed on all the varieties and it was found that there was perfect correspondence of the presence/absence of pinA/pinB with PCR amplification pattern of these genes. The protein band corresponding to pinA was absent in wheat lines where PCR amplification using pinA specific primers was absent. The data showed that there were only two types of mutations in hard wheats in Kansas State as either glycine to serine change in pinB or null mutation in pinA. The glycine to serine change was common in hard varieties in Kansas State as 36 varieties had glycine to serine mutation and four varieties namely Pioneer, Jagger, Arlin and Coronado showed null mutation in pinA (Fig. 1). All the soft varieties studied were having pinA and glycine type of pinB. Hardness level varied between the two mutations. Grains with null mutation in pinA were harder (5.3 points) than those having serine type pinB. The mean value of hardness was 77.6 in the varieties having null mutation in pinA and 72.3 in varieties with serine type of pinB. This indicates that both the puroindolines together might influence the level of grain hardness as reported earlier by Morris *et al* (26) using different set of varieties.

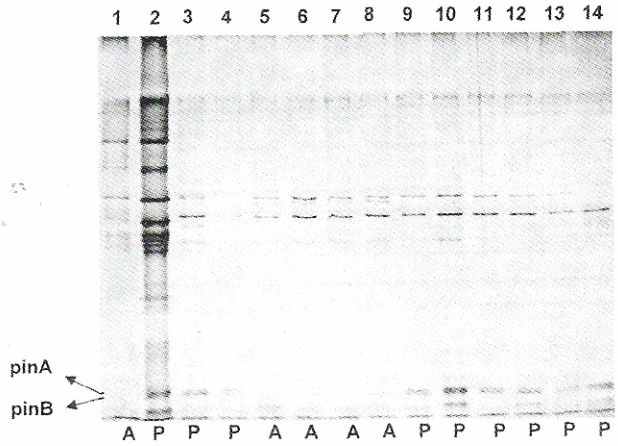

Fig.1. Gel photo of silver stained puroindolines (A and B) from some hard wheat varieties from Kansas State showing null mutation in four varieties. Here Langdon was used as check along with its substitution line (5D)5B. Lanes: 1-Landon, 2-Langdon(5D)5B, 3-Tam107, 4-Plainsman, 5-Pioneer, 6-Jagger, 7-Arlin, 8-Coronado, 9-Vora, 10-Agseco, 11-Newton, 12-Arkan, 13-Dominator, and 14-Custor. A-pinA absent, P-pinA present.

The analysis of Indian wheat varieties indicated that majority of the varieties were hard and had null mutation in pinA prevalent (Fig. 2). Full-length pinB (450 bp) was present in all the varieties studied (Fig. 3). This is in contrast to wheat varieties in Kansas State where glycine to serine change in pinB was found common. Only two lines (Sonalika and NP846) showed glycine to serine mutation in pinB and all others had glycine type of pinB (Fig. 2). This indicates that the source of major gene for grain texture

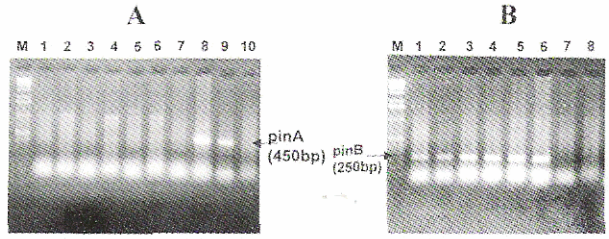

Fig.2. PCR amplification products of pinA (**A**) and glycine type pinB (**B**) of some Indian wheat varieties showing prevalence of null mutation in pinA. Glycine type of pinB was present in all the varieties except NP846 and Sonalika .
(**A**) Lanes: M – Molecular Marker, 1 – NP4, 2 – K68, 3 – SUJATA, 4 – HD2329, 5 – PBW343, 6 – UP262, 7 – RAJ3765, 8 – HYB65, 9 – NI5439 and 10 – HP1731.
(**B**) Lanes: M – Molecular Marker, 1 – NP4, 2 – K68, 3 – HD2329, 4 – PBW343, 5 – HYB65, 6 – NI5439, 7 – NP846, and 8 – Sonalika.

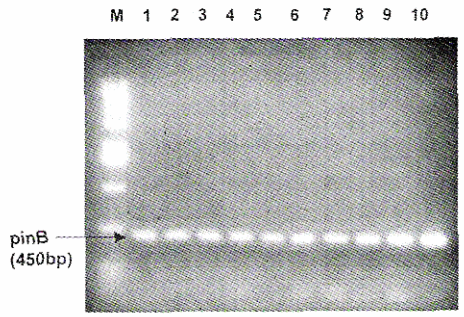

Fig.3. PCR amplification products of full length pinB of some Indian wheat varieties showing the presence of pinB in all the varieties. Lanes: M – Molecular Marker, 1 – NP4, 2 – K68, 3 – SUJATA, 4 – HD2329, 5 – PBW343, 6 – UP262, 7 – RAJ3765, 8 – HYB65, 9 – NI5439, and 10 – HP1731.

used for developing varieties in these countries had been different. All wheat cultivars with glycine to serine mutation in pinB or null mutation in pinA were indeed hard confirming the earlier findings of Giroux and Morris (18). Sourdille *et al* (16) have also shown the close linkage between *Ha* gene and Xmta locus corresponding to pinA. Ninety-two varieties out of one hundred released varieties had null mutation in pin A. Eight hard varieties namely HP42, Hyb65, HS207, HP1633, NI5439, HD1949, HD2402 and HD2380 showed exceptions by possessing both glycine type of pinB (soft type) and wild type of pinA and their protein products. There might be additional yet unknown mutations in these exceptional varieties the characterisation of which is being done in our laboratory to further elucidate the mechanism of hardness at molecular level. Turnbull *et al* (20) also found two hard cultivars (Cook and Diaz) out of 15 cultivars surveyed in Australia with normal amount of pin A and without glycine to serine change in pin B. Recently Lillemo and Morris (19) reported two new mutations in pinB, involving a leucine to proline change at 60 position and tryptophan to arginine change at 44 position, associated with grain hardness in wheat genotypes of mostly the North European origin.

Gene mapping — The grain hardness (*Ha*) and puroindoline genes are reported to be present on 5D chromosome. To further localize puroindoline genes on sub-arm, deletion stocks of Chinese Spring for 5DS developed at WGRC, KSU were utilized. The deletion lines of Chinese Spring for 5D chromosomes having break points at FL 0.78 (2), 0.67 (5), 0.63 (1) and 0.20 (4) were characterised using primers for both pinA and glycine type pinB. Three of the deletion lines (FL 0.78, 0.67, 0.63) were homozygous and one (FL 0.20) was heterozygous. There was no amplification in all the homozygous deletion lines (Fig. 4). The heterozygous and normal lines exhibited PCR amplification using primers for both glycine type pinB and pinA. This demonstrates that pinA/B genes are tightly linked and present at the distal part of short arm of 5D chromosome (Fig. 5) where *Ha* gene is reported to be present (7). This supports the view (18) that puroindolines may be the part of *Ha* gene itself.

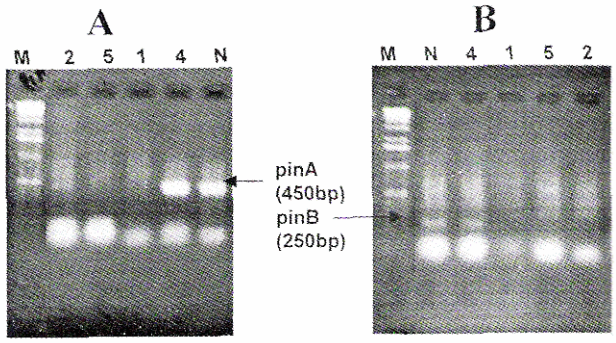

Fig.4. PCR amplification product of puroindoline genes from normal (N), heterozygous (4) and homozygous (2, 5, 1) deletion lines of chinese spring using pinA (**A**) and glycine type pinB (**B**) specific primers. The amplification product is absent in homozygous deletion lines. The figures on top of the gel photo indicate the no. of deletion line in the genetic stock.

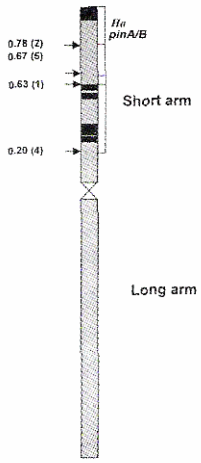

Fig.5. Deletion map of 5D short arm of chinese spring showing the location of pinA/B genes in the distal segment of the chromosome where grain hardness gene (*Ha*) is reported to be present. Figures in bracket indicate the number of the deletion line in stock.

Thus, the data demonstrate that majority of wheat varieties developed in India are medium hard to hard and have null mutation in pinA gene. In contrast, hard wheats from US have glycine to serine mutation in pinB gene. There are some exceptions where hard wheats do not possess

either mutation. There might some other mutations in puroindoline genes of these varieties. These exceptions have to be analysed to elucidate further the mechanism of grain hardness at molecular level.

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