

Molecular characterization of diverse wheat germplasm for puroindoline proteins and their antimicrobial activity

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Abstract: Thirty different wheat genotypes, including landraces, cultivars, and related wild species, were analyzed for puroindoline genes and proteins. Full-length *Pina* and *Pinb* genes were amplified using gene-specific markers where 13 genotypes amplified both genes, while 17 genotypes had either one or none of the puroindoline genes. None of the puroindoline genes were detected in any of the *Triticum durum* cultivars. Genotypes with both puroindoline genes had a soft grain texture, while other genotypes had hard grains. Related nonprogenitor species with A, C, M, U, and S genomes also showed the presence of either one or both the puroindoline genes, indicating that these species can also be utilized for development of extra-soft wheat. SDS-PAGE analysis of selected wheat genotypes revealed that puroindoline proteins are approximately 13–14 kDa and the protein results were in agreement with the PCR results. Puroindoline proteins of selected 3 soft wheat genotypes HPW 89, Naphal, and IITR-9 also exhibited antimicrobial activity against gram-positive and gram-negative bacteria. Both genes of a soft wheat cultivar, HPW 89, were successfully cloned in the *Escherichia coli* DH-5α strain. Introgression and molecular characterization of diverse *Pina* and *Pinb* genes to develop extra-soft wheat is in progress.

Key words: *Triticum aestivum*, puroindolines, soft wheat, PINs, wheat wild relatives, antimicrobial activity

1. Introduction

Grain hardness is an important quality parameter determining market classification and end-use properties of common wheat (*Triticum aestivum* L. subsp. *aestivum*) (Yücel et al., 2009). Grain hardness refers to whether the endosperm is physically hard or soft. Most of the world's wheat production and trade is conducted on the basis of endosperm texture, i.e. soft or hard (Pomeranz and Williams, 1990). Due to its soft endosperm, soft wheat requires less energy to mill, yielding smaller flour particles with less starch damage and thus absorbing less water for dough preparation as compared to hard wheat (Symes, 1965, 1969). Hard wheat is typically used to make bread, while soft wheat is generally used for making cookies and pastries (Morris and Rose, 1996). The expression of one major gene, designated the *Ha* (Hardness) locus on the short arm of chromosome 5D, is responsible for controlling grain texture (Law et al., 1978). However, durum wheat (*Triticum turgidum* L. subsp. *durum*) (AABB, 2n = 4x = 28) without the D genome generally has a harder grain texture (Morris, 2002). Friabilin has been found on water-washed starch granules from soft wheat, while little or no friabilin

has been found on water-washed starch granules from hard wheat (Greenwell and Scholfield, 1986). It was later shown that genes encoding friabilin were closely linked to the *Ha* locus on chromosome 5D (Jolly et al., 1996). N-terminal sequencing of friabilin revealed that it is composed mainly of 2 proteins, puroindoline A (PIN A) and puroindoline B (PIN B), which are the main components of friabilin (Jolly et al., 1993; Morris et al., 1994). However, *Pina* and *Pinb* genes are also present and expressed in other diploid species, including those with genomes related to the A and B genomes of bread wheat (Chantret et al., 2004; Chen et al., 2005; Gazza et al., 2006; Massa and Morris, 2006; Simeone et al., 2006), and in at least some tetraploid and hexaploid taxa of *Triticum* (Chen et al., 2005; Massa and Morris, 2006). This is relevant not only to evolutionary mechanisms but also to crop improvement, as genes from many of these species can be introgressed into bread or durum wheats by conventional genetic approaches. We have therefore analyzed a number of diploid, tetraploid, and hexaploid genotypes of *Triticum* and *Aegilops* species in order to identify diversity and evolutionary relationships among *Pin* genes and proteins that could be exploited in improvement of cultivated wheats.

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The term “puroindoline” is derived from Greek word “puro”, meaning wheat, and “indoline” for the indoline ring of tryptophan. Research on PINs over the last decade has focused on their possible role in controlling the endosperm texture (hardness or softness) of wheat grain, an important characteristic that has profound effects on the industrial processing performance of wheat (Pomeranz and Williams, 1990). A strong link has been shown between allelic differences among *Pin* genes, largely through analysis of genomic DNA sequences and variation in wheat grain endosperm texture (Ikeda et al., 2005; Xia et al., 2005). Soft endosperm texture is considered to be the wild phenotype, containing both PIN A (genetic allele *Pina-D1a*) and wild-type PIN B (allele *Pinb-D1a*) proteins (Giroux and Morris, 1998; Lillemo and Morris, 2000; Morris and Konzak, 2001). To date, all the characterized hard wheats carry a mutation in one of their puroindoline genes, with the known mutations being single nucleotide changes in the coding sequence of *Pina* and *Pinb* and null mutations (Giroux and Morris, 1998; Lillemo and Morris, 2000; Morris et al., 2001).

In addition, puroindolines are also reported to exhibit antimicrobial and antifungal activity which may play some role in plant defense (Douliez et al., 2000; Igrejas et al., 2001; Capparelli et al., 2007). As plant seeds are very sensitive to viruses, bacteria, and fungi, they contain high concentrations of various antimicrobial proteins, particularly during maturation and germination, thus constituting a potential reservoir of antimicrobial agents. A large number of plant proteins are already known for their antimicrobial activity in vitro (Garcia-Olmedo et al., 1998; Boman, 2003). Puroindolines are small 13-kDa basic and cysteine-rich proteins belonging to a protein superfamily that includes alpha-amylase/trypsin inhibitors, nonspecific lipid-binding proteins, and a mixture of puroindoline-like polypeptides and grain softness proteins (Douliez et al., 2000). The spatial and temporal localization of PIN, nonspecific lipid transfer protein, and purothionin in starchy wheat endosperm suggests that the synergistic effects of these antimicrobial proteins observed in vitro may also occur in vivo (Dubreil et al., 1998). Hence it is very important to characterize the wheat germplasm for puroindoline proteins for their potential role in plants' quality traits and defense mechanisms. Keeping in view the above points, the present study was undertaken to characterize puroindoline proteins in a diverse wheat germplasm at the molecular and biochemical levels. This study also provides a survey of puroindoline variants in common wheat and related wild species for understanding the genetic diversity and evolutionary relationships of puroindoline genes for improvement of wheat processing quality.

2. Materials and methods

2.1. Plant materials

The germplasm of cultivated wheat, landraces, and related wild species was obtained from Punjab Agricultural University (PAU), Ludhiana, and the Indian Institute of Technology, Roorkee, India. All the genotypes surveyed in this study were planted at the experimental farms of Eternal University, Himachal Pradesh, India, during the December 2012–April 2013 wheat season, employing the local cultural practices for wheat, which involved the use of special measures for cultivation of related wild species. The germplasm evaluated included hexaploid soft wheat cultivar HPW 89; landraces Naphal, IITR-9, IITR-21, IITR-26, IITR-27, IITR-29, IITR-30, IITR-31, IITR-34, and IITR-67 from the Uttarakhand hills of India; wild related species *Ae. kotschy* 387, *Ae. peregrina* 3477, *T. araraticum* 4692, *Ae. longissima* 28, *Ae. variabilis* 115.1.1, *Ae. cylindrica* 3472, *Ae. ovata* 3547, *T. dicoccoides* 4630, *T. boeoticum* V-21, *Ae. squarrosa* KBS3784, *Ae. ventricosa* 3520, and *Ae. speltoides* 3804; domesticated diploid wheat *T. monococcum* 4087; and durum wheat cultivars PDW 314, PDW 233, PDW 274, PDW 291, Aconchi 89, and HighProDurum (Table 1).

2.2. DNA extraction and PCR amplification

Genomic DNA was isolated from young leaves using a modified cetyltrimethylammonium bromide method (Murray and Thompson, 1980). The *Pina-D1* and *Pinb-D1* genes were amplified using specific primers, details of which are given in Table 2. PCR was carried out in an Applied Biosystems ABI Veriti thermocycler according to the method of Ayala et al. (2013) with some modifications. Reactions were performed in a 20-μL volume containing 50 ng of DNA, 0.2 μM of each primer (forward and reverse of *Pina-D1* and *Pinb-D1*, respectively), 0.2 mM dNTPs, 1.5 mM MgCl₂, 2 μL of 10X PCR buffer, and 0.75 U of DNA polymerase (Promega). The PCR conditions included an initial denaturation step of 4 min at 94 °C followed by 35 cycles as follows: 1 min at 94 °C, 1 min at 56 °C, and extension at 72 °C for 1.5 min. After 35 cycles, there was a final extension step for 10 min at 72 °C. The PCR products were resolved on 2.5% agarose gel. The gels were visualized and photographed using a gel documentation system (Syngene GBOX).

2.3. Purification and cloning of puroindoline genes

The desired puroindoline genes' PCR products from selected wheat cultivars were purified by the NucleoSpin gel and PCR clean-up kit developed by Macherey-Nagel. Cloning was achieved from the eluted PCR product through the Thermo Scientific InsTAclone PCR Cloning Kit. It is a TA system for direct one-step cloning of PCR products with 3'-dA overhangs. The high-quality TA cloning vector pTZ57R/T was used for efficient ligation

Table 1. Presence/absence of puroindoline genes in wheat landraces, related wild species, and durum cultivars characterized through PCR using *Pina*- and *Pinb*-specific primers and their grain textures.

| S. no. | Samples | Genome | Pedigree | Procured from | <i>Pina</i> | <i>Pinb</i> | Grain hardness (kg/grain) (mean \pm SD) | Grain texture* |
|----------------------------|-------------------------------|-------------------------------------------------------------|----------------------------------------------------------------------------|-------------------------|-------------|-------------|-------------------------------------------|-----------------|
| Hexaploid wheat cultivar | | | | | | | | |
| 1. | HPW 89 | AABBDD | Intermedio Rodi/HD 2248 | Himachal Pradesh, India | + | + | 4.7 \pm 0.15 | Soft |
| Wheat landraces | | | | | | | | |
| 2. | NAPHAL | AABBDD | ----- | Uttarakhand, India | + | + | 4.5 \pm 0.25 | Soft |
| 3. | IITR-9 | AABBDD | ----- | Uttarakhand, India | + | + | 6.1 \pm 0.3** | Soft |
| 4. | IITR-21 | AABBDD | ----- | Uttarakhand, India | + | + | 6.4 \pm 0.35** | Soft |
| 5. | IITR-26 | AABBDD | ----- | Uttarakhand, India | + | + | 6.6 \pm 0.3** | Soft |
| 6. | IITR-27 | AABBDD | ----- | Uttarakhand, India | + | + | 6.1 \pm 0.3** | Soft |
| 7. | IITR-29 | AABBDD | ----- | Uttarakhand, India | + | + | 6.6 \pm 0.45** | Soft |
| 8. | IITR-30 | AABBDD | ----- | Uttarakhand, India | - | + | 10.6 \pm 0.95** | Moderately hard |
| 9. | IITR-31 | AABBDD | ----- | Uttarakhand, India | - | + | 10.7 \pm 0.81** | Moderately hard |
| 10. | IITR-34 | AABBDD | ----- | Uttarakhand, India | - | + | 9.4 \pm 0.35** | Moderately hard |
| 11. | IITR-67 | AABBDD | ----- | Uttarakhand, India | + | + | 6.6 \pm 0.25** | Soft |
| Wild related species | | | | | | | | |
| 12. | <i>Ae. kotschyii</i> 387 | US ^s | ----- | ICARDA, Syria | + | - | 11.2 \pm 0.48** | Moderately hard |
| 13. | <i>Ae. peregrina</i> 3477 | US ^s | ----- | USDA, USA | + | - | 11.0 \pm 0.36** | Moderately hard |
| 14. | <i>T. araraticum</i> 4692 | AAGG | ----- | USDA, USA | + | - | 10.8 \pm 0.66** | Moderately hard |
| 15. | <i>Ae. longissima</i> 28 | S ^l | ----- | USDA, USA | + | - | 9.9 \pm 0.9** | Moderately hard |
| 16. | <i>Ae. variabilis</i> 115.1.1 | UUS | ----- | USDA, USA | + | - | 11.3 \pm 0.71** | Moderately hard |
| 17. | <i>Ae. cylindrica</i> 3472 | CCDD | ----- | USDA, USA | + | + | 5.8 \pm 0.16** | Soft |
| 18. | <i>Ae. ovata</i> 3547 | C ^U C ^U M ⁰ M ⁰ | ----- | USDA, USA | + | + | 5.9 \pm 0.22** | Soft |
| 19. | <i>T. dicoccoides</i> 4630 | AABB | ----- | USDA, USA | - | - | 11.6 \pm 0.15** | Ultrahard |
| 20. | <i>T. boeoticum</i> V-21 | A ^b A ^b | ----- | USDA, USA | + | + | 5.2 \pm 0.3** | Soft |
| 21. | <i>Ae. squarrosa</i> KBS3784 | DD | ----- | USDA, USA | + | + | 5.1 \pm 0.4** | Soft |
| 22. | <i>Ae. ventricosa</i> 3520 | D ^N | ----- | USDA, USA | - | - | 11.8 \pm 0.39** | Ultrahard |
| 23. | <i>Ae. speloides</i> 3804 | S (\approx B or G) | ----- | USDA, USA | + | - | 10.5 \pm 0.2** | Moderately hard |
| Domesticated diploid wheat | | | | | | | | |
| 24. | <i>T. monococcum</i> 4087 | A ^m | ----- | USDA, USA | + | + | 6.9 \pm 0.44** | Soft |
| <i>T. durum</i> cultivars | | | | | | | | |
| 25. | PDW 314 | AABB | Ajaia 12/F3 Local (Sel. Ethio.135.85)/Plata 13/3/Somat3/4/Sooty9/Rascon 37 | PAU, Ludhiana, India | - | - | 12.8 \pm 0.89** | Ultrahard |
| 26. | PDW 233 | AABB | Yavaros(SIB)/(SIB)Ten; Yavaros(SIB)/(SIB) Tezontle | PAU, Ludhiana, India | - | - | 12.2 \pm 0.66** | Ultrahard |
| 27. | PDW 274 | AABB | DWL-6018/Karpasia | PAU, Ludhiana, India | - | - | 12.1 \pm 0.38** | Ultrahard |
| 28. | PDW 291 | AABB | Boomer 21/ Mojo2 | PAU, Ludhiana, India | - | - | 12.3 \pm 0.3** | Ultrahard |
| 29. | Aconchi 89 | AABB | Altar 84/Araos | PAU, Ludhiana, India | - | - | 12.4 \pm 0.56** | Ultrahard |
| 30. | HighProDurum | AABB | Langdon with GPC-6BI from <i>T. turgidum</i> L. var. dicoccoides | PAU, Ludhiana, India | - | - | 12.0 \pm 0.6** | Ultrahard |

*Results presented are the mean of 10 grains crushed separately; SD is standard deviation.

**Significant at P = 0.05.

Table 2. PCR primers used for generating puroindoline-D1 alleles.

| Primer name | Primer sequences | Corresponding gene | Reference |
|--------------------------------|-----------------------------------------------------|--------------------|-----------------------|
| <i>Pina-D1</i> specific primer | Forward 5'-CATCTATTCATCTCCACCTGC-3' | <i>Pina-D1</i> | Lillemo et al. (2006) |
| | Reverse 5'-GTGACAGTTTATTAGCTAGTC-3' | | |
| | Forward 5'-AATACATATGCAATATAGCGAAATTGTTGGCAGTTAC-3' | | Tiwari et al. (2011) |
| | Reverse 5'-AATACTCGAGTCACCAGTAATAGCCAATAGTG-3' | | |
| <i>Pinb-D1</i> specific primer | Forward 5'-ATGAAGACCTTATTCCTCCTA-3' | <i>Pinb-D1</i> | Gautier et al. (1994) |
| | Reverse 5'-TCACCAGTAATAGCCACTAGGGAA-3' | | |
| | Forward 5'-AATACATATGCAATACTCAGAAAGTTGGGGC-3' | | Tiwari et al. (2011) |
| | Reverse 5'-AATACTCGAGTCACCAGTAATAGCCATAGG-3' | | |

with PCR products, providing high cloning yields and low background.

2.4. Grain hardness

Grain hardness was measured using the grain hardness tester supplied by M/S Ogawa Seiki Co. Ltd. (Japan) by individually crushing 10 randomly selected grains from the lot (Ram et al., 2013). The mean force (kg) required to crush the grains was recorded and the results are presented as mean \pm standard deviation.

2.5. Extraction of puroindoline proteins and SDS-PAGE

Total puroindoline proteins were extracted according to the method of Bordier (1981) using Triton X-114 (TX-114) detergent. About 100 mg of ground kernels or flour was extracted with 1 mL of Tris-buffered saline (TBS, 10 mM Tris/150 mM NaCl, pH 7.5) and 0.15 mL of 12% TX-114 (in TBS) and was vortexed for 1 h at 4 °C. After centrifugation at $10,000 \times g$ for 1 min at 4 °C, the supernatant was transferred to a fresh tube and 0.5 mL of cold TBS was added to the pellet, and the tubes were again vortexed. Samples were once again centrifuged for 1 min and both the supernatants were combined after centrifugation. The samples were incubated at 37 °C for 45 min and then centrifuged for 2 min. The upper nondetergent phase was then aspirated off. A portion (40:1) of the remaining detergent-rich phase was transferred to a fresh tube, avoiding the pellet that consisted of starch and protein contaminants insoluble in TX-114. To the detergent-rich phase, 1 mL of cold TBS was added and vortexed. Tubes were placed at 37 °C for 30 min and centrifuged for 2 min. The upper nondetergent phase was discarded and 1 mL of acetone (–20 °C) was added to the detergent phase. Samples were again vortexed and centrifuged for 5 min at 4 °C, and the supernatant was discarded. Proteins that were soluble in TX-114 were sequentially washed with 1 mL of acetone (–20 °C) and ethyl ether (–20 °C) and allowed to air dry.

For gel fractionation, 60:1 of SDS sample buffer without β -mercaptoethanol was added. Prior to loading of gels, samples were incubated at 70 °C for 10 min with

occasional mixing. Fractionation of proteins was carried out at 5% stacking gel and 15% resolving gel as described by Laemmli (1970).

2.6. Assessment of antimicrobial activity

Cultures of 2 gram-negative bacterial strains (*Escherichia coli* and *Serratia marcescens*) and 1 gram-positive bacterium (*Staphylococcus aureus*) were used in the present study. The disk diffusion method was used to study the antimicrobial activity of the puroindoline proteins (Bauer et al., 1966). Sterile paper disks of 1 mm in diameter (Whatman filter paper no. 1) were impregnated with the proteins isolated by TX-114 and were put on a well prepared in a Luria Bertani agar plate. The plates were incubated at 37 °C for 24 h for further study. Microbial growth was determined by measuring the diameter of the zone of inhibition. For each bacterial strain, controls were maintained where pure solvents were used instead of the protein extract. All PIN peptides were tested in triplicate and mean values are presented.

2.7. Statistical analysis

Grain hardness values are presented as mean \pm standard deviation of 10 replicates. The relationship between puroindoline composition and grain hardness values was statistically analyzed by Student's t-test using GraphPad software.

3. Results

3.1. PCR amplification and cloning of *Pina-D1* and *Pinb-D1* genes

In the present study, a collection of wheat landraces, wheat and durum cultivars, and related wild species were analyzed and characterized at the molecular and biochemical levels for puroindoline composition. The full-length *Pina* and *Pinb* genes were amplified using *Pina*- and *Pinb*-specific primers, respectively; PCR fragments of the puroindoline genes of around 447 bp in length were resolved (Figure 1). Among the wheat genotypes, 7 landraces (Naphal, IITR-9, IITR-21, IITR-26, IITR-27, IITR-29, IITR-67), 1 hexaploid

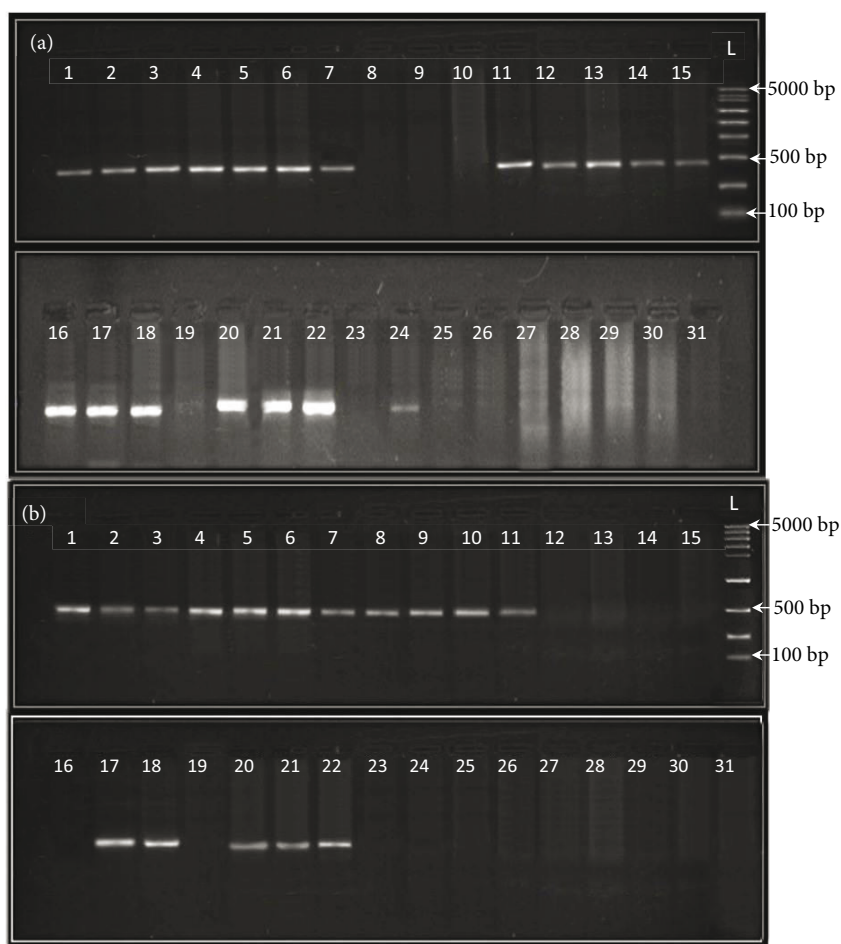


Figure 1. PCR amplification of *Pina* (a) and *Pinb* genes (b). Loading sequence is: 1. HPW 89, 2. Naphal, 3. IITR-9, 4. IITR-21, 5. IITR-26, 6. IITR-27, 7. IITR-29, 8. IITR-30, 9. IITR-31, 10. IITR-34, 11. IITR-67, 12. *Ae. kotschy* 387, 13. *Ae. peregrina* 3477, 14. *T. araraticum* 4692, 15. *Ae. longissima* 28, 16. *Ae. variabilis* 115.1.1, 17. *Ae. cylindrica* 3472, 18. *Ae. ovata* 3547, 19. *T. dicoccoides* 4630, 20. *T. boeoticum* V-21, 21. *T. monococcum* 4087, 22. *Ae. squarrosa* KBS3784, 23. *Ae. ventricosa* 3520, 24. *Ae. speltoides* 3804, 25. PDW 314, 26. PDW 233, 27. PDW 274, 28. PDW 291, 29. Aconchi 89, 30. HighProDurum, 31. control, L. gene ruler express ladder (Thermo Scientific).

cultivar (HPW 89) and 4 accessions of related wild species (*T. boeoticum* V-21, *Ae. squarrosa* KBS3784, *Ae. cylindrica* 3472, *Ae. ovata* 3547), and 1 domesticated diploid wheat (*T. monococcum* 4087) showed the amplification of both the *Pina* and *Pinb* genes, while 9 genotypes, including 3 landraces (IITR-30, IITR-31, IITR-34) and 6 accessions of related wild species (*Ae. kotschy* 387, *Ae. peregrina* 3477, *T. araraticum* 4692, *Ae. longissima* 28, *Ae. variabilis* 115.1.1, *Ae. speltoides* 3804) showed the amplification of either the *Pina* or *Pinb* gene. In 8 genotypes, including 2 wild related species (*T. dicoccoides* 4630, *Ae. ventricosa* 3520) and 6 durum cultivars (PDW 314, PDW 233, PDW 274, PDW 291 Aconchi 89, HighProDurum), neither puroindoline gene was amplified.

The 447-bp purified products of HPW 89 were ligated into the pTZ57R/T easy vector and transformed into cells of the *Escherichia coli* DH-5 α strain (Figure 2a). Transformed colonies were identified based on the blue-white screening method, using X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and isopropyl β -D-1-thiogalactopyranoside. More than 90% positive recombinant clones were observed. Plasmid DNA was isolated from the cloned bacterial strains. When the isolated plasmid was run along with uncloned vector pTZ57R/T, it was observed that the vector cloned with HPW 89 *Pina* and *Pinb* had a higher molecular weight compared to the uncloned vector, confirming the successful cloning of the genes in the DH-5 α strain (Figure 2b). Strains with both cloned genes were stored for future studies.

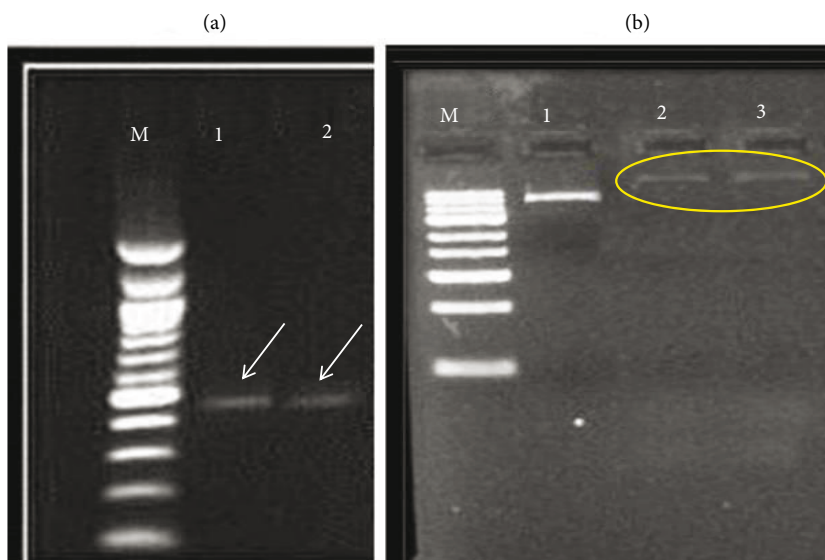


Figure 2. (a) Purified puroindoline genes of HPW 89 after PCR amplification. 1. HPW 89 (*Pina*), 2. HPW 89 (*Pinb*). (b) Cloning of *Pina* and *Pinb* gene in *E. coli* DH-5α where M is marker ladder (100 bp). Lane 1: uncloned vector pTZ57R/T, lane 2: HPW 89 *Pina*, lane 3: HPW 89 *Pinb*.

3.2. Estimation of grain hardness

Grain hardness tests revealed that all the genotypes with the amplification of both the alleles had a crushing force in the range of 4.5–6.9 kg/grain, whereas wheat genotypes with the presence of either one of the puroindoline genes had a crushing force in the range of 9.4–11.2 kg/grain (Table 1). Genotypes lacking both puroindoline genes had the highest crushing force, in the range of 11.6–12.8 kg/grain. On the basis of the grain hardness data and PCR pattern, the genotypes were classified as soft (both alleles present), moderately hard (only one allele present), and ultrahard (both alleles absent) (Table 1). Statistical analysis through Student's t-test also revealed that the moderately hard and ultrahard genotypes were significantly different from soft-textured wheat genotypes.

3.3. SDS-PAGE electrophoresis

On the basis of PCR results, 10 wheat genotypes differing in their endosperm texture (Table 1), including 1 hexaploid cultivar (HPW 89), 6 landraces (Naphal, IITR-9, IITR-21, IITR-26, IITR-31, IITR-34), and 3 *T. durum* cultivars (PDW 314, PDW 233 and PDW 291), were selected for SDS-PAGE analysis to check the presence of puroindoline proteins. Upon SDS fractionation, puroindolines (PIN A and PIN B) appeared as 2 sharp bands with a molecular weight of approximately 13–14 kDa (when compared with the broad-range protein ladder [TaKaRa] applied along with the samples) in HPW 89, Naphal, IITR-9, IITR-21, and IITR-26 (Figure 3). In contrast, these specific protein bands were absent in all the *T. durum* cultivars (PDW 314,

PDW 233, PDW 291), whereas only a single PIN B band appeared in wheat landraces IITR-31 and IITR-34 (Figure 3). The SDS-PAGE results completely coincided with the PCR results of all the genotypes tested.

3.4. Antimicrobial activity

On the basis of the results of PCR and SDS-PAGE, 3 soft wheat genotypes (HPW 89, Naphal, and IITR-9) showing the presence of both the puroindoline proteins were selected to study the antimicrobial activity of these proteins (1 mg/mL). The inhibitory effect of the puroindoline proteins was studied against 2 gram-negative bacteria and 1 gram-positive bacterium, and the zone of inhibition was measured. Puroindolines isolated from the aforementioned soft wheat genotypes exhibited substantial antimicrobial activity against both the bacterial strains (Figure 4). Streptomycin was used as a positive control, which showed the maximum zone of inhibition. To ensure that observed antimicrobial activity was due to a particular treatment only, TBS was used as a negative control and no inhibition zone was observed with it (Figure 4). Among the 3 wheat genotypes, HPW 89 showed a maximum inhibition of 50% with respect to streptomycin against *E. coli*, whereas against *Staphylococcus aureus*, an inhibition zone of 0.6 cm (42% of the control) was recorded with the puroindoline proteins isolated from IITR-9 (Figure 4; Table 3). All 3 genotypes showed a comparable inhibition against *Serratia marcescens*, with a maximum inhibition of 47% by HPW 89 PINs. At a 10^{-2} dilution, HPW 89 showed 11% inhibition against *Serratia marcescens*, while at a

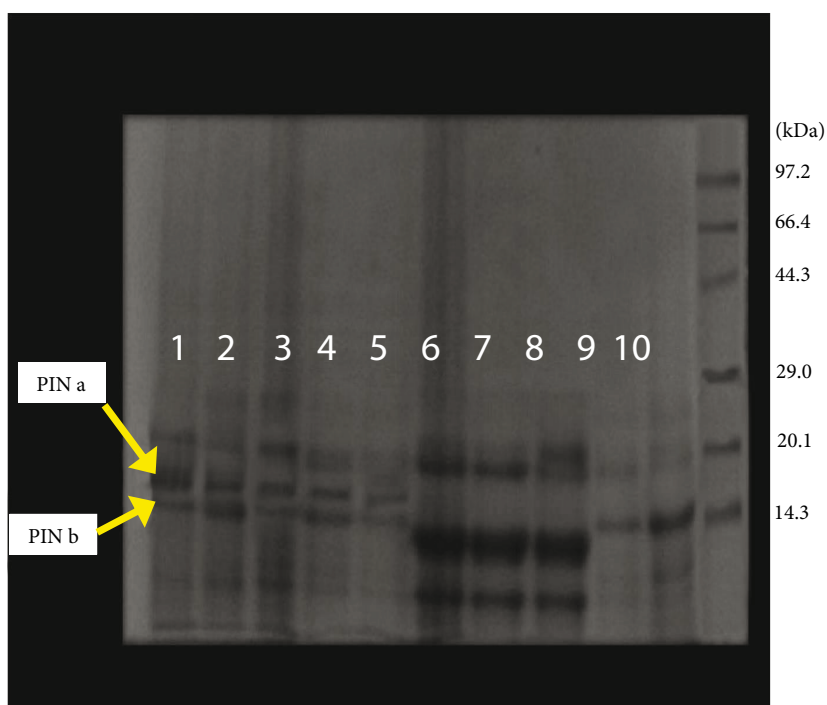


Figure 3. SDS-PAGE of Triton X-114-extracted puroindoline proteins. **1.** HPW 89, **2.** Naphal, **3.** IITR-9, **4.** IITR-21, **5.** IITR-26, **6.** PDW 314, **7.** PDW 233, **8.** PDW 291, **9.** IITR-31, **10.** IITR-34.

10^{-3} dilution only a 5.8% inhibition was observed against the same bacterium. The higher concentration tested for puroindolines activity was comparable to that of the antibiotic used as the positive control.

4. Discussion

Soft wheat genotypes are associated with the presence of wild type alleles of both *Pin* genes, whereas single or double mutant alleles at one or both PIN loci develop hard grains (Morris and Bhavé, 2008). In this study, a collection of wheat cultivars, landraces, and related wild species were characterized at the molecular level for puroindoline composition. Out of the 30 genotypes tested, 13 genotypes were characterized as soft where both the puroindoline genes were present. However, 17 genotypes were classified as moderately hard and ultrahard, and they had the absence of either *Pina-D1* or *Pinb-D1* or both, which is in agreement with the results published earlier (Giroux and Morris, 1997, 1998; Ikeda et al., 2005; Chen et al., 2006, 2007). Amplification of both the puroindoline genes in wheat landraces similar to the already established soft wheat landrace Naphal and soft-textured hexaploid cultivar HPW 89 indicates that these could also have soft grain texture and hence could be used directly or as donors in breeding programs for the development of soft wheat, as both the puroindoline genes (*Pina* and *Pinb*) have been associated with grain softness (Xia et al., 1996; Giroux and

Morris, 1997, 1998). The results at DNA level were also validated at the protein level isolated by phase partitioning using nonionic detergent Triton X-114. Genotypes in which both the *Pin* genes were amplified also showed the presence of both the proteins on SDS-PAGE gel. Similar were also reported by Tiwari et al. (2011), who correlated the different soft-textured wheat landraces with the PCR amplification of both *Pina* and *Pinb*. Our results are also in agreement with the study of Ayala et al. (2013), in which Mexican wheat landraces containing both puroindoline genes were considered as soft. Results observed at the DNA and protein levels were also in concurrence with the results of the grain hardness testing. Hence, these landraces can be used in wheat quality improvement programs, as some of these landraces have shown potential for increasing grain iron and zinc concentrations (Tiwari et al., 2011).

All the durum cultivars showed no amplification for either puroindoline gene and consequently no PIN proteins were detected in SDS-PAGE analysis. Other reports also suggest that durum wheat cultivars lack the D-genome and, consequentially, the *Ha* locus localized on 5DS (Mattern et al., 1973; Law et al., 1978). Our failure to detect PIN proteins in durum cultivars with the A and B genomes agrees with the reports of Gautier et al. (2000) and Rakszegi et al. (2009), who used PCR of genomic DNA and Southern blot analysis to show that *dicoccoides*, *dicoccum*, and *durum* lacked *Pin* genes. The most likely

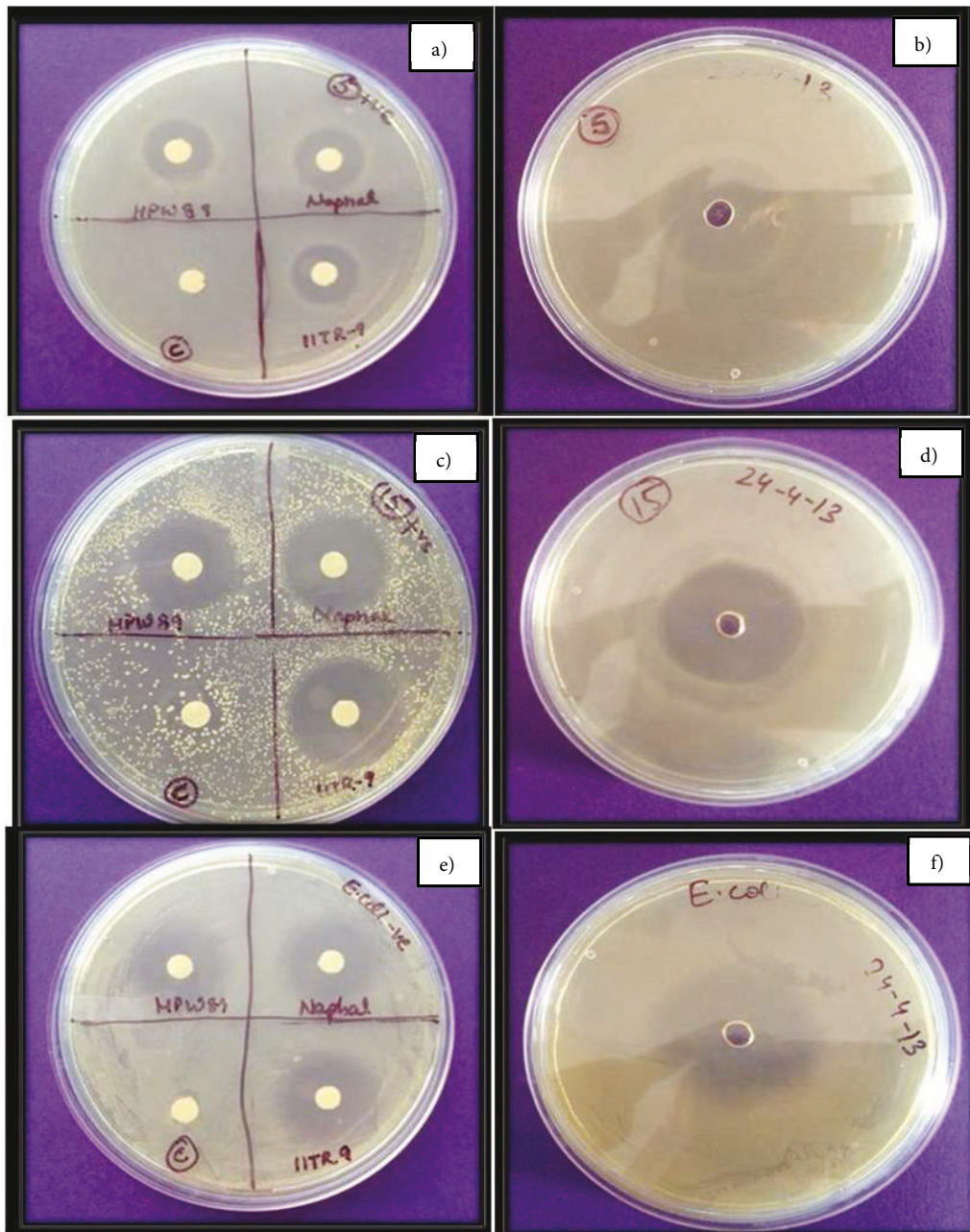


Figure 4. Antibacterial activity of puroindoline proteins isolated from HPW 89, Naphal, and IITR-9 (a) against gram-positive *Staphylococcus aureus* with respect to (b) streptomycin control, (c) gram-negative *Serratia marcescens* with respect to (d) streptomycin control, and (e) gram-negative *E. coli* with respect to (f) streptomycin control. C denotes the negative control (TBS).

explanation for the absence of *Pina* and *Pinb* sequences in tetraploid wheat is that the *Pin* genes were lost during evolution, which is plausible considering the rapid elimination of low-copy DNA sequences during the early stages of allopolyploidization observed in wheat (Feldman et al., 1995; Chantret et al., 2005).

The presence of *Pina*, *Pinb*, or both in several other related wild species with progenitor and nonprogenitor

genomes other than the D-genome strongly suggests that the *Pin* genes must have evolved in their ancestral species before their divergence. These genes can be mobilized onto *T. durum* and *T. aestivum* to develop ultrasoft wheat cultivars for superior biscuits and pastries. Li et al. (2008) also reported that all the C-, M-, U-, and N-genome species also had one copy of the *Pin* genes. Chen et al. (2005) analyzed kernel texture using scanning electronic

Table 3. Inhibition of puroindoline proteins with respect to streptomycin for gram-positive and gram-negative bacterial strains.

| Bacterial strain | Sample (1 mg/mL) | Radius of zone of inhibition (cm) | % inhibition with respect to streptomycin |
|------------------------------|------------------|-----------------------------------|-------------------------------------------|
| <i>Escherichia coli</i> | Streptomycin | 1.6 ± 0.3 | Positive control |
| | Naphal | 0.6 ± 0.1 | 37.5 |
| | HPW 89 | 0.8 ± 0.1 | 50 |
| | IITR-9 | 0.7 ± 0.2 | 43 |
| <i>Staphylococcus aureus</i> | Streptomycin | 1.4 ± 0.3 | Positive control |
| | Naphal | 0.5 ± 0.2 | 35 |
| | HPW 89 | 0.5 ± 0.2 | 35 |
| | IITR-9 | 0.6 ± 0.1 | 42 |
| <i>Serratia marcescens</i> | Streptomycin | 1.7 ± 0.4 | Positive control |
| | Naphal | 0.7 ± 0.2 | 41 |
| | HPW 89 | 0.8 ± 0.2 | 47 |
| | IITR-9 | 0.7 ± 0.1 | 41 |

microscopy and reported that all *Aegilops* species of various genomes and ploidy levels were soft. Pogna et al. (2002) also tested the hardness of 67 accessions of *T. monococcum* and found all of them to be soft. As evident by the grain hardness results of the present study, *T. araraticum* 4692, *Ae. kotschy* 387, *Ae. peregrina* 3477, *Ae. longissima* 28, *Ae. variabilis* 115.1.1, and *Ae. speltoides* 3804, showing the presence of *Pina* gene, may be considered harder than IITR-30, IITR-31, and IITR-34, which had *Pinb* genes, since some investigators have suggested that genotypes with only *Pina* genes are harder than genotypes with only the *Pinb* gene (Giroux et al., 2000; Martin et al., 2001).

A perfect correspondence for the presence/absence of PIN A and PIN B proteins in SDS-PAGE analysis with the PCR amplification patterns of puroindoline genes in different tetraploid and hexaploid wheat cultivars indicates that either approach could be effectively used for analyzing grain hardness or screening a large collection of germplasm for the presence of puroindolines.

The mode of action of many antimicrobial peptides is usually attributed to the perturbation of the bacterial cytoplasmic membrane, which directly leads to the release of cellular components and cell death (Nguyen et al., 2011). In the present investigation, puroindoline proteins isolated from selected soft wheat genotypes exhibited significant antimicrobial activity against gram-negative and gram-positive bacteria. The most plausible explanation for this could be the presence of a hydrophobic, tryptophan-rich region with net cationic charge in both PIN A and PIN B proteins that could strongly bind with the negatively charged lipids present in the bacterial membrane. This may

be partially responsible for the antimicrobial properties of these 2 PIN proteins. Expression of puroindolines in transgenic rice has also been reported to confer enhanced resistance to pathogens (Krishnamurthy et al., 2001). It is also well accepted that proteins that are structurally amphiphilic, hydrophobic, or capable of binding to lipids may have inherent antimicrobial properties (Garcia-Olmedo et al., 1995; Broekaert et al., 1997). Jing et al. (2003) also found that a 13-residue fragment of puroindoline A (FPVTWRWWKWK-NH₂) (PuroA) exhibited activity against both *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) bacteria and suggested that PuroA may be a bactericidal domain of PIN A. They further reported that PuroA interacted strongly with negatively charged phospholipid vesicles and induced efficient dye release from these vesicles, suggesting that the microbicidal effect of PuroA may be due to interactions with bacterial membranes. Hence, binding and permeabilization of membranes is thought to be the generalized mechanism by which puroindolines cause cell death. However, in a very recent study, a tryptophan-rich region in the wheat PIN A was found to possess potent antimicrobial activity against gram-positive and gram-negative bacteria, which was attributed to the peptide-inducing membrane instability. However, the PIN B tryptophan-rich peptide (PuroB) was relatively inactive compared to the related PIN A peptide (PuroA), despite their strong sequence similarity (Haney et al., 2013). The study further revealed that all of the puroindoline-derived peptides bind DNA and block macromolecular synthesis in vivo, leading to their antimicrobial activity.

In conclusion, the results presented here confirm the presence of full-length *Pina* and *Pinb* genes in soft wheat cultivar HPW 89 and several Indian wheat landraces, suggesting that these genes can be used as the marker for the early screening of soft-textured endosperm wheat genotypes in soft wheat improvement programs. It is also evident from the study that puroindoline genes are not only present in the D genome but also on the other genomes like A, C, M, U, and S. PCR-amplified *Pina* and *Pinb* genes of soft wheat cultivar HPW 89 were also successfully cloned in the *E. coli* DH-5 α strain, which can be used for further studies. A complete concurrence

was observed in the protein banding pattern, with the PCR results further substantiating the molecular marker validation. The observed antimicrobial activity of puroindoline proteins suggests that these proteins could be used as food preservatives in baking products. Given the increasing problem of resistance to conventional antibiotics, puroindolines could also be investigated for use as possible therapeutic agents. Our results are expected to guide and facilitate the application of genetic engineering and molecular breeding to produce new types of cereal crops in which hardness is optimized for specific end-uses for the benefit of manufacturers and consumers.

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