

## Characterization of Genes Encoding Wheat Grain Hardness from Chinese Cultivar GaoCheng 8901

Z. Pan,<sup>1,2</sup> W. Song,<sup>1</sup> F. Meng,<sup>1</sup> L. Xu,<sup>1</sup> B. Liu,<sup>1</sup> and J. Zhu<sup>3,4</sup>

### ABSTRACT

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The puroindoline-b (*Pinb-D1*) gene from Chinese hard wheat cultivar GaoCheng 8901 (*Triticum aestivum* L.) was obtained using two pairs of primers designed based on the known *Pinb-D1* gene sequence and polymerase chain reaction (PCR) amplification. The PCR amplification was made using the genomic DNA of the wheat as a template and the specific fragment ≈450 bp in size was screened. The results indicated that the *Pinb-D1* gene in GaoCheng 8901 shared 99.78% and 99.32% homology in nucleotide acid sequence and amino acid sequence, respectively, compared

with the *Pinb-D1* gene from hard wheat cultivars Wanser and Cheyenne. A new mutation in this *Pinb-D1* gene, different from the six known mutations in the *Pinb-D1* gene, was characterized with a change of a lysine to glutamic acid at position 45 in its protein sequence. This mutation, designated as *Pinb-D1l* in this study, might contribute to the formation of grain hardness in GaoCheng 8901. The characterization of *Pinb-D1* gene would be helpful in manipulating grain hardness of wheat through genetic engineering.

Wheat (*Triticum aestivum* L.) grain hardness is one of the important quality characteristics of wheat affecting milling, baking, and end-use quality. Soft wheats are preferred for cookies, cakes, and pastries, while hard wheats are better suited for yeast-leavened bread baking (Morris and Rose 1996). However, the genetic study of grain hardness in Chinese wheat is very limited. Therefore, it would be significant to characterize the genes controlling grain hardness in Chinese wheat cultivars. Kernel hardness is inherited simply, and controlled by one major gene and perhaps one or more minor genes (Anjum and Walker 1991). The major gene controlling grain hardness, *Ha*, is located on the short arm of chromosome 5D (Law et al 1978). It recently has been reported that the *Ha* locus is functionally composed of genes encoding two proteins, puroindoline-a (*Pina-D1*) and puroindoline-b (*Pinb-D1*). *Pina-D1* and *Pinb-D1* both have been cloned and sequenced from wheat cultivar Capitole (Gautier et al 1994). Giroux and Morris (1997) have previously reported that hard wheat is associated with a glycine (GGC) to serine (AGC) mutation at position 46 in *Pinb-D1* gene (*Pinb-D1b*). Two more mutations in the *Pin-D1* gene were reported from Northern European wheats, which involved a leucine (CTG) to proline (CCG) mutation at position 60 (*Pinb-D1c*) and a tryptophan to arginine at position 44 (*Pinb-D1d*), respectively (Giroux and Morris 1997, 1998). Recently, three new hardness alleles, all with single nucleotide changes to stop codons in puroindoline b, designated as *Pinb-D1e*, *Pinb-D1f*, and *Pinb-D1g*, were identified (Morris et al 2001; Morris 2002). Pins are specific for *Triticeae* and have highly conserved characteristics with their genes found in cereals closely related to wheat (barley, oat, and rye) (Gautier et al 2000). Different from other plant proteins, Pins are basic cysteine-rich proteins and contain a tryptophan-rich domain, which has high lipid-binding abilities that could contribute to the formation and stability of dough foams, as well as to the structure of bread crumb and bread volume (Dubreil et al 1997). In addition, Pins have antifungal properties in vitro (Dubreil et al 1998). Therefore, Pin genes could be manipulated to improve cereal grain quality and to increase plant antifungal diseases ability.

Increasing yield has been a focus of Chinese wheat breeding programs for some time, while wheat quality was neglected until the late 1980's. With the market need for better quality products, improving wheat quality has become a primary target in Chinese wheat breeding programs. Studies on wheat quality have increased significantly since then. However, no study has been reported on the genetic control of grain hardness at molecular levels in Chinese wheat cultivars. Therefore, it would be of interest to elucidate the mechanism of grain hardness in Chinese wheats, and to effectively use the diversified Chinese wheat germplasm (Sambrook et al 1989).

### MATERIALS AND METHODS

#### Materials

A winter wheat cultivar, GaoCheng 8901, developed by Institute of Gaocheng Agricultural Academy, identified during a survey of mutations for puroindoline grain hardness, was used in this study.

#### Isolation of Plasmid DNA

Plasmid DNA was detected, isolated, and purified as described (Sambrook et al 1989).

#### Isolation of Wheat Genomic DNA

After being soaked, wheat seeds were planted in plant incubator house until that the seedlings reached the stage of four or five leaves. The new fresh leaves were then isolated and put into a cold mortar and ground with a pestle using liquid nitrogen. The ground powder was then transferred into a 50-mL centrifuge tube and the DNA extraction solution was added. The genomic DNA was isolated with CTAB method (Rogers and Bendich 1985).

#### Amplification of *Pinb-D1* Gene

Oligonucleotide primers were designed based on the sequence of *Pinb-D1* cDNA isolated from a wheat seed cDNA library (Gautier et al 1994).

Forward primer:

5' - ATG AAG ACC TTA TTC CTC CTA GCT - 3'.

Reverse primer:

5' - TCA CCA GTA ATA GCC ACT AGG GAA - 3'.

The PCR reactions were made with 200 ng of isolated wheat genomic DNA in a final volume of 50  $\mu$ L containing 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM MgCl<sub>2</sub>, 0.1%, w/v, Triton X-100, 10% glycerol, 0.2 mM of each dNTP, 0.25  $\mu$ M of each primer, and 1.5 units of *Taq* DNA polymerase. The samples were denatured

<sup>1</sup> Institute of Crop Breeding and Cultivation, Chinese Academy of Agricultural Science, Beijing, 100081, China.

<sup>2</sup> Corresponding authors. E-mail: panzhm@mail.caas.net.cn; jzhu@chgventher.com

<sup>3</sup> Dept. of Cereal & Food Sciences, North Dakota State University, Fargo, ND 58105.

<sup>4</sup> Grain Science and Industry Program, Zhengzhou Institute of Technology, Zhengzhou 450052, China.

at 94°C for 5 min, followed by 35 cycles of 1-min denaturation at 94°C, 1.5-min primer annealing at 60°C and 2-min extension at 72°C, with a final extension at 72°C for 10 min. All PCR cycles were made using a Perkin-Elmer Cetus Thermocycler 9600.

### Cloning of *Pinb-D1* PCR Products

The product (5 µL) of amplification was analyzed using electrophoresis of 1.5% agarose gel. Photographs were taken under UV transilluminator after staining with ethidium bromide using Gel Doc 1000 video gel documentation system (Bio-Rad, Hercules, CA). Amplified specific fragments were gel purified and cloned into the pGEM-T vector (Promega Co., Madison, WI). Positive clones were isolated and sequenced on both strands by the method of Sanger et al (1977) with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and an ABI Prism 377XL DNA Sequencer (Applied Biosystems, Foster City, CA).

### Sequencing and Analysis of *Pinb-D1* Gene

Positive clones were sequenced in-house using DNA automatic sequencer (ABI Prism 377). DNA sequence analysis was performed using the Promie Primer 5 software package. The EMBL/Genbank database was used for DNA sequence searching.

## RESULTS AND ANALYSIS

### Amplification, Cloning, and Characterization of *Pinb-D1* Gene

A 450-bp specific fragment was obtained with primers designed and a template from wheat cultivar GaoCheng 8901 genomic DNA. The fragment was cloned into the pGEM-T vector after purification from the gel. Positive recombinants (pTPB) were isolated and determined using blue/white plaque screening and restrictive enzyme digestion.

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1  CCGCGGGATT ATGAAGACCTTATTCTCTAGCTCTCCTTGCTCTGTAG
      M K T L F L L A L L A L V
51  CGAGCACAACCTTCGCGCAATACTCAGAAGTTGGCGGCTGGTACAATGAA
      A S T T F A ▲ Q Y S E V G G W Y ▲ E
101 GTTGGCGGAGGAGGTTGTTCTCAACAATGTCCGCAGGAGC GGCCGAAGCT
      V G G G G G S Q Q □ P Q E R P K L
151 AAGCTCTTGCAAGGATTACGTGATGGAGCGATGTTTACAATGAAGGATT
      S S □ K D Y V M E R □ F T M K D
201 TTCCAGTCACCTGGCCACAAAAATGGTGGGAGGGCGGCTGTGAGCATGAG
      F P V T W P T K W W ▲ E G G □ E H E
251 GTTCGGGAGAAGTGTGCAAGCAGCTGAGCCAGATAGCACCACAATGTCC
      V R E K □ □ K Q L S Q I A P Q □ R
301 CTGTGATTCTATCCGGCAGTGATCCAAGGCAGGCTCGGTGGCTTCTTGG
      □ D S I R R V I Q G R L G G F L
351 GCATTGGCGGAGGTGAGGTATTCAACAACCTCAGAGGGCCAGAGCCTC
      G I W R G E V F K Q L Q R A Q S L
401 CCCTCAAAGTGCAACATGGGCGCCACTGCAAGTTCCTAGTGGCTATTA
      P S K □ N M G A D □ K F P S G Y Y
481 CTGGTGAATCACTAGTGGCGCCCTGCAGGTCGACCATATGGGAGAGC
      W

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**Fig. 1.** Map of nucleotide sequence analyzed from wheat cultivar GaoCheng 8901. Arrow indicates position of point mutation: base A was mutated to G at position 230. The first closed arrowhead indicates the putative cleave site of the signal peptide and the second closed arrowhead indicates the N-terminal end of puroindoline b isolated from wheat.

### Sequencing and Analysis of *Pinb-D1* Gene

The nucleotide sequence of pTPB and deduced primary structure of the *Pinb-D1* gene are shown in Fig. 1. The pTPB was 447 bp in length and the open reading frame encoded a polypeptide of 149 amino acids with a theoretical molecular mass of 16,387 Da, which contained a tryptophan-rich domain (WPTKWW) that could bind easily with lipids, and 10 cysteine residues, whose position was conserved and organized in a cysteine skeleton (Giroux and Morris 1997, 1998). The comparison of the deduced primary structure of *Pinb-D1* with the N-terminal sequence of the mature puroindoline-b (Blochet et al 1991) revealed that the deduced sequence contains an N-terminal cleavable polypeptide. The putative cleavage site of the N-terminal polypeptide of 29–30 residues was identified from comparison with the N-terminal sequence of puroindolines isolated from wheat. The first 19 amino acids of *Pinb-D1* exhibit the characteristics of many eukaryotic signal peptides, mainly the lysine residue next to the initiating methionine and the hydrophobic-rich domain. Compared with the reported sequences of *Pinb-D1* genes controlling grain hardness of wheat from cultivars of Capitole, Wanser, and Cheyenne (Giroux and Morris 1997, 1998) and soft wheat cultivars Chinese Spring and Hill 81, the *Pinb-D1* gene (pTPB) from Chinese wheat cultivar GaoCheng 8901 exhibited 99.78% homology in nucleotide sequence and 99.32% in amino acid sequence. These results suggested that pTPB was a wheat *Pinb-D1* clone. However, Gautier et al (1994) reported a single nucleotide change in the coding sequence of pTPB near the tailing end of the tryptophan-rich domain. The change occurred at the position 45 from lysine (AAG) to glutamic acid (GAG), indicating the uniqueness of the *Pinb* gene from GaoCheng 8901.

## DISCUSSION

Wheat grain hardness is inherited simply (Baker 1977; Anjum and Walker 1991). Most mutations in hardness have been associated with a single locus, *Ha*, on the short arm of chromosome 5D (Law et al 1978; Anjum and Walker 1991; Giroux and Morris 1997). Wheat grain texture would be changed from softness to hardness if a mutation occurs at any one of the three known amino acid positions. These mutations included a change of tryptophan (TGG) to arginine (AGG) at position 44, glycine (GGC) to serine (AGC) at position 46, and leucine (CTG) to proline (CCG) at position 60 (Giroux and Morris 1997, 1998). However, no change was found in these three positions of the *Pinb-D1* gene from the cultivar GaoCheng 8901 under this study. Instead, a single nucleotide change was observed near the tailing end of the tryptophan-rich domain in the coding sequence of the *Pinb-D1* gene (Fig. 1), resulting in a mutation from lysine (AAG) to glutamic acid (GAG) at position 45 of its protein sequence. This might be a new mutation *Pinb-D1* allele in wheat as the cultivar is a typical of hard wheat. Interlaboratory tests also confirmed the results. Therefore, the formation of GaoCheng 8901 wheat grain hardness might be different from the previously discovered mutations in *Pinb-D1*, which have been designated *Pinb-D1b* through *Pinb-D1g* among hard wheats (Giroux and Morris 1997, 1998; Morris 2002). According to the revised guidelines for nomenclature of Biochemical/Molecular Loci in Wheat and Related Species (McIntosh et al 1995), this new mutation could be designated *Pin b-D1l*.

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