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# A Puroindoline Mutigene Family Exhibits Sequence Diversity in Wheat and is Associated with Yield-Related Traits

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## 1. Introduction

Kernel texture (grain hardness) is a leading quality characteristic of bread wheat (*Triticum aestivum* L.) as it dramatically influences the milling and processing properties, and consequently determines the classification and marketing of grain (Bhave and Morris 2008a, b). The word puroindoline is derived from the Greek word "*puros*" meaning wheat and "*indoline*" describing the indole ring of tryptophan (Gautier et al. 1994). Puroindolines, composed of puroindoline a and b, are amphipathic proteins of ca. 13,000 Da, and share homology with grain softness protein (GSP), purothionins, lipid transfer proteins, and other members of the prolamin super-family of proteins (Shewry and Halford 2002). Puroindoline proteins possess a characteristic tryptophan-rich domain and cysteine backbone; isoforms occur in the starchy endosperm of the Triticeae. Their secondary structure, determined by infrared and Raman spectroscopies, is comprised of approximately 30% *a*-helices, 30% *β*-sheets, and 40% unordered structure at pH 7.4 in solution (Bihan et al. 1996). Puroindoline genes are present throughout the Triticeae tribe of the Poaceae (Gramineae), including wheat (*Triticum* sp.), rye (*Secale* sp.), barley (*Hordeum* sp.), and the wild relatives of wheat (*Aegilops* sp. and *Triticum* sp.). In *Triticum aestivum*, puroindolines exist as two expressed genes, Puroindoline a and Puroindoline b, and are located on the distal end of the short arm of chromosome 5 (5DS). An exception to this general situation lies with the tetraploid (AABB) wheats (*T. turgidum*), which include cultivated durum (ssp. *durum*). Apparently during the allotetraploidization formation of T. *diccocoides*, the wild ancestor of cultivated durum, both the A- and B-genome Puroindoline loci were eliminated due to transposable element insertion and two large deletions in the *Hardness* loci caused by illegitimate DNA recombination (Chantret et al. 2005). Consequently, hexaploid wheat, *T. aestivum*, possesses

puroindoline a and b on only the D-genome (contributed by *Ae. tauschii* during the allohexaploidization of this taxon), but lacks homoeologous loci on the A- and B-genomes.

The expression of puroindoline genes is mainly associated with 'soft' and 'hard' grain texture of bread wheat, whereas durum wheat is 'very hard' due to the lack of puroindoline genes (Capparelli et al. 2003). Extensive genetic surveys, cytological analysis, and transformation experiments in wheat and rice (*Oryza sativa*) have demonstrated that puroindoline a and b act to create soft kernel texture (Bhave and Morris 2008a, b, Morris 2002, Morris and Bhave 2008). However, puroindolines have also been extensively studied regarding their multiple roles in the development and resistance of plants against biotic stress, and their profound influence on the breeding and processing quality of food crops (Luo et al. 2008; Giroux and Morris 1997, 1998; Ragupathy and Cloutier 2008; Chen et al. 2007a, b). Considerable allelic variation in puroindoline a and b have been identified in a great number of genotypes stemming from different geographies. Gene sequence polymorphism and allele designations have been recently reconciled and reviewed by Morris and Bhave (2008) and Bhave and Morris (2008a, b).

Although clearly exerting a major role in wheat kernel texture variation, puroindolines do not account for all of the variation observed in bread wheat. Several minor quantitative trait loci (QTLs) for kernel texture have been mapped on different chromosomes (Sourdille et al. 1996; Turner et al. 2004). Four additional regions located on chromosomes 2A, 2D, 5B, and 6D were shown to have single-factor effects on hardness, while three others located on chromosomes 5A, 6D and 7A had interaction effects (Sourdille et al. 1996).

Three new puroindoline gene-like sequences in hexaploid wheat were reported by Wilkinson et al. (2008). Chen et al. (2010) renamed them *Pinb-2v1*, *Pinb-2v2* and *Pinb-2v3*, and reported the discovery of a fourth novel puroindoline variant in bread wheat, designated *Pinb-2v4*. Physical mapping results of Chen et al. (2010) were not fully consistent with the results reported by Wilkinson et al. (2008). Interestingly, *Pinb-2v1* and *Pinb-2v4* were present in all of the surveyed cultivars whereas *Pinb-2v2* and *Pinb-2v3* were reciprocally present in different wheat cultivars (Chen et al. 2010). This result indicates that *Pinb-2v2* and *Pinb-2v3* are likely allelic. In this study, we remapped the four known *Puroindoline b-2* variants using aneuploids of bread wheat and durum wheat in order to confirm their physical location on the chromosomes of wheat, and report the discovery of a novel *Pinb-2v3* allele and a new *Puroindoline b-2* variant, designated *Pinb-2v5*. Also, we investigated the association of *Puroindoline b-2* variants with yield-related traits. This study provides useful information for illustrating the molecular and genetic basis of kernel hardness and gene duplication events in wheat.

## 2. Materials and methods

### 2.1 Wheat germplasm, DNA extraction and PCR amplification

A total of 109 bread wheat lines developed for the Yellow and Huai Valleys of China were planted at the Zhengzhou Scientific Research and Education Center of Henan Agricultural University during the 2009-10 cropping seasons according to local management practices. Analysis of agronomic traits and measurement of SKCS hardness as well as identification of puroindoline b-2 variants were performed. Each plot was comprised of four 200 cm-long rows with 23 cm between neighboring rows and 10 cm between neighboring plants. All surveyed cultivars grew well and no lodging was present in the trial. After harvest, all wheat samples were cleaned.

Durum wheat cultivar Langdon (LND), bread wheat cultivar Chinese Spring (CS), two disomic substitution lines of Langdon 7D(7A) and Langdon 7D(7B) with substituted 7D chromosomes of Chinese Spring and six nullisomic-tetrasomic lines of Chinese Spring involving group 7 chromosomes (CS N7A-T7B, CS N7A-T7D, CS N7B-T7A, CS N7B-T7D, CS N7D-T7A and CS N7D-T7B) were used in this study. DNA was extracted from two seeds each of the 109 bread wheat lines, genetic stocks, and 15 Chinese, CIMMYT and Italian durum wheat cultivars following the rapid extraction method of genomic DNA derived from Chen et al. (2006).

PCR primer sequences were designed using Primer Premier 5.0 software. Reactions were performed in 25 µL containing 100 ng of genomic DNA, 10 pmol of each primer, 250 µM of each dNTP, 1x Taq DNA polymerase reaction buffer containing 1.5 µM of $MgCl_2$ and 0.5 unit of Taq DNA polymerase (Promega, Madison, WI). The cycling conditions were 94°C for 5 min followed by 35 cycles of 94°C for 50 s, 45°C to 65°C for 50 s (primer-specific annealing temperatures, see Table 1), 72°C for 1 min, followed by a final 10-min extension at 72°C. An aliquot (8 µL) of the PCR products was analyzed on 1.5% (w/v) agarose gels, stained with ethidium bromide, and visualized with UV light. Forty-eight clones from three independent PCR reactions using the universal primers on durum cv. Langdon were sequenced from both strands by SinoGenoMax Co., Ltd.

Multiple alignments of sequences and translations of nucleotide into amino acid sequence were performed by software DNAMAN Version 6.0. Sequence chromatograms were analyzed by Chromas Version 1.4.5 and FinchTV Version 1.4.0.

### 2.2 Measurement of agronomic traits and hardness index

Before harvesting, ten plants of each genotype were randomly selected in each plot to determine spikelet number per plant, flag leaf length and flag leaf width, as well as flag leaf area calculated from the product of leaf length and maximum leaf width × 0.75. Grains per spike, grain weight per plant and grain weight per spike were determined after harvesting.

Kernel hardness, grain diameter and thousand-kernel weight were measured on 300-kernel samples of each genotype using the Perten Single Kernel Characterization System (SKCS) 4100, following the manufacturer's operation procedure (Perten Instruments North America Inc., Springfield, IL). Mean, standard deviation, and distribution of SKCS hardness data were used to classify the genotypes tested into soft, mixed, and hard types.

## 3. Results

### 3.1 Physical mapping of *Pinb-2* variants

Physical mapping results with variant-specific primers (Table 1) showed that *Pinb-2v1* was present in LND 7D(7A), LND 7D(7B), Chinese Spring, CS N7A-T7B, CS N7A-T7D, CS N7B-T7A and CS N7B-T7D, but absent in wild-type Langdon, CS N7D-T7A and CS N7D-T7B, indicating that *Pinb-2v1* is located on 7D of Chinese Spring. *Pinb-2v2* was present in wild-type Chinese Spring, CS N7A-T7B, CS N7A-T7D, CS N7D-T7A and CS N7D-T7B, but absent in wild-type Langdon, LND 7D(7A), LND 7D(7B), CS N7B-T7A and CS N7B-T7D, indicating that *Pinb-2v2* is located on 7B of Chinese Spring. *Pinb-2v3* was present in wild-type Langdon, LND 7D(7A), but absent in LND 7D(7B), wild-type Chinese Spring and all of its six nullisomic-tetrosomic lines, indicating that *Pinb-2v3* is located on 7B of Langdon. *Pinb-2v4* was present in Langdon, LND 7D(7B), CS N7B-T7A, CS N7B-T7D, CS N7D-T7A and CS

N7D-T7B, but absent in LND 7D(7A), CS N7A-T7B and CS N7A-T7D, indicating that *Pinb-2v4* is located on 7A of Langdon and Chinese Spring.

Therefore, it is concluded that *Pinb-2v1* is on 7D of Chinese Spring, *Pinb-2v2* is on 7B of Chinese Spring, *Pinb-2v3* is on 7B of Langdon and *Pinb-2v4* is on 7A of Langdon and Chinese Spring. The results of mapping *Pinb-2v* in Chinese Spring and its derived nullisomic-tetrosomic lines are consistent with the locations of the four *Puroindoline b-2* variants reported by Chen et al. (2010a).

### 3.2 Variation in *puroindoline b-2* variants and the discovery of a new *puroindoline b-2* variant

Based on PCR amplification with four specific primers (Table 1), 35 of 36 durum wheat cultivars surveyed possessed variant combination *Pinb-2v3*/*Pinb-2v4*, whereas the durum wheat cultivar Norba, introduced from Italy, possessed combination *Pinb-2v2*/*Pinb-2v4*. None of the surveyed durum cultivars possessed the *Pinb-2v1* gene. Further sequencing of PCR products with specific primers showed that all of the sequences of *Pinb-2v4* were exactly the same as the *Pinb-2v4* sequence of bread wheat reported by Chen et al. (2010), whereas the coding region of *Pinb-2v3* in durum wheat cultivar Langdon had a single nucleotide change from G to T at the 6th position compared with the *Pinb-2v3* sequence reported by Wilkinson et al. (2008) and Chen et al. (2010). Furthermore, 18 durum wheat cultivars with the *Pinb-2v3* variant were sequenced and all of them uniformly contained a single nucleotide change from G to T at the 6th position. Sequencing of bread wheat cultivar Wichita showed the same change at the 6th position, compared with a previous *Pinb-2v3* sequence of Wichita reported by Wilkinson et al. (2008) and Chen et al. (2010). The discrepancy may have resulted from the previously used primers due to a single nucleotide change present in the forward primer sequence. However, after we modified the *Pinb-2v3* sequence from G to T at the 6th position of Wichita, a one base-pair change from T to C at the 311th position was found in the coding region of *Pinb-2v3* in 12 durum wheat cultivars, resulting in the deduced amino acid change from valine to alanine at position 104 in PINB-2v3. According to the gene nomenclature for puroindoline a and b, this *Pinb-2v3* allele in wheat cultivars containing the sequence of AM99733 and GQ496618 with the modification of G to T at the 6th position will be designated as *Pinb-2v3a*, and the *Pinb-2v3* allele with a nucleotide change from T to C at the 311th position in durum wheat cultivars will be designated as *Pinb-2v3b*.

Three repeated PCR amplifications of the durum cultivar Langdon were performed with the universal primer in order to reduce the influence of mismatching amplification of *Taq* polymerase. Cloning and sequencing results showed that 40 sub-clones belonged to either *Pinb-2v3* or *Pinb-2v4* whereas 7 other sub-clones did not belong to any known *Puroindoline b-2* variant. Further analysis indicated that a novel variant, designated as *Pinb-2v5* (HM245236), shared high homology and was most closely related to *Pinb-2v4* with only 5 SNPs (Fig. 1).

The overall alignment with *Pinb-D1a* with the five *Pinb-2* variant genes showed that *Pinb-2v5* has 74.3%, 95.8%, 94.0%, 92.3% and 98.8% identity with *Pinb-D1a*, *Pinb-2v1*, *Pinb-2v2*, *Pinb-2v3* and *Pinb-2v4*, respectively, at the DNA level. Moreover, all four puroindoline b variant sequences were at least 91.8% homologous amongst themselves. Full alignment of deduced amino acid sequences of *Pinb-D1a* and the five *Puroindoline b-2* variant genes indicated that

*Pinb-2v5* has 57.8%, 96.0%, 91.3%, 87.9% and 96.5% identity with *Pinb-D1a*, *Pinb-2v1*, *Pinb-2v2*, *Pinb-2v3* and *Pinb-2v4*, respectively, at the amino acid level (data not shown).

| Gene | Forward primer | Reverse primer | PCR annealing Temp °C | Fragment size (bp) |
|---|---|---|---|---|
| *Pinb-2vU* | ATGAAGACCTTATTCCTCCTA | TCASTAGTAATAGCCATTAKTA | 54 | 453 |
| *Pinb-2v1* | GGTTCTCAAAACTGCCCAT | ACTTGCAGTTGGAATCCAG | 57 | 319 |
| *Pinb-2v2* | CTTGTAGTGAGCACAACCTTTGCA | GTATGGACGAACTTGCAGCTGGAG | 65 | 401 |
| *Pinb-2v3* | GCAGAAAAAGCCATTGCACCTA | CATTAGTAGGGACGAACTTGCAGCTA | 65 | 528 |
| *Pinb-2v4* | CCTTTCTCTTGTAGTGAGCACAACCA | GACGAACTTGCAGTTGGAATCCAA | 65 | 403 |
| *Pinb-D1b* | ATGAAGGCCCTCTTCCTCA | CTCATGCTCACAGCCGCT | 58 | 250 |
| *N-Pinb-D1b* | ATGAAGGCCCTCTTCCTCA | CTCATGCTCACAGCCGCC | 58 | 250 |

Table 1. PCR primers used in generating *Puroindoline b-2* variant gene sequences in durum wheat

### 3.3 Association *of puroindoline b-B2* variants with SKCS grain hardness, other grain traits, grain yield components, and flag leaf size

In order to investigate the influence of *puroindoline b-2* variants on kernel hardness independent of puroindoline a and b alleles, the average SKCS hardness index of two different *Puroindoline b-2* variant combinations, *Pinb-2v1/Pinb-2v2/Pinb-2v4* and *Pinb-2v1/Pinb-2v3/Pinb-2v4*, were compared by sub-setting the lines according to *Pin-D1* haplotype. Puroindoline alleles are associated with dramatic effects on kernel hardness (reviewed in Morris, 2002; Morris and Behave, 2008; Behave and Morris, 2008a, b). Results indicated that in soft wheat cultivars with *Pina-D1a/Pinb-D1a,* the average of SKCS hardness index of cultivars with *Pinb-2v3* was 24.6, which was significantly higher than that of cultivars with *Pinb-2v2* (14.2). No significant difference was observed among cultivars with *Pinb-D1b*, possibly suggesting that the *Puroindoline b-2* variants had a larger impact in soft wheat than in hard.

No significant association between the puroindoline D1 alleles and the 9 agronomic traits relating to wheat yield were observed in the surveyed Chinese wheat cultivars of the Yellow and Huai Valleys, suggesting that the puroindoline genes have no apparent relationship with wheat grain yield. However, when the agronomic traits of cultivars with different *Puroindoline b-2* variant combinations were compared, the averages of grain number per spike, grain weight per spike, width of flag leaf and flag leaf area of cultivars with *Pinb-2v3* were significantly higher than those cultivars with *Pinb-2v2*. Differences in thousand kernel weight, grain length and spikelet number per spike were not different between these two *Pinb-2v2* and

*Pinb-2v3* genotypes (Table 2). The results indicated that *Pinb-2v3* cultivars possessed superior grain yield traits compared to *Pinb-2v2*, possibly suggesting that the *Puroindoline b-2* gene could modulate wheat grain yield to some extent, and that the grain yield of cultivars with *Pinb-2v3* is slightly higher than that of cultivars with *Pinb-2v2*. Another likely possibility is that these *Pinb-2* variants are identifying germplasm pools or founder effects.

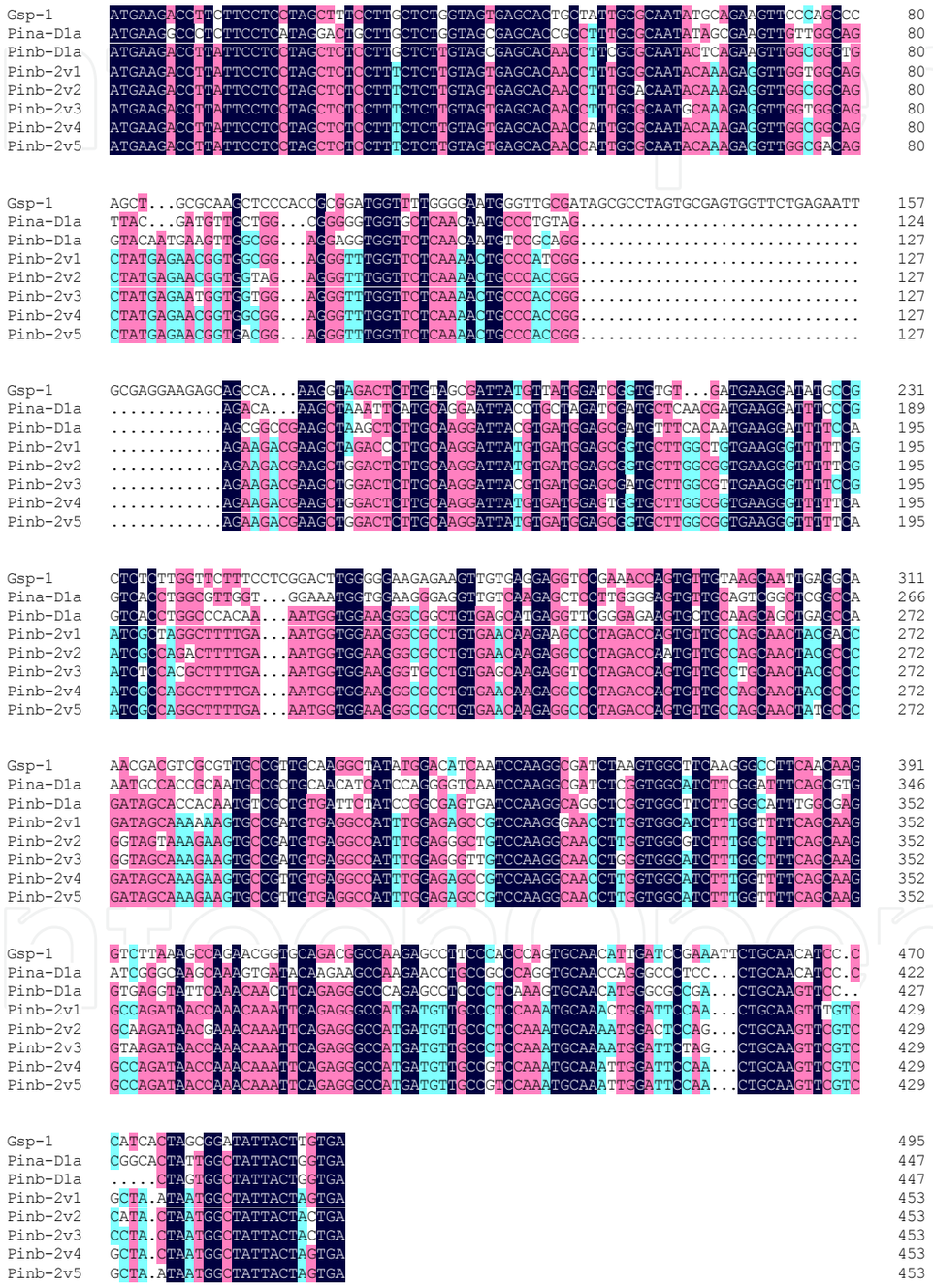


Fig. 1. DNA sequence alignment of *Puroindoline b-2* gene variants *Pinb-2v1*, *Pinb-2v2*, *Pinb-2v3*, *Pinb-2v4*, *Pinb-2v5, Pinb-D1a, Pina-D1a* as well as *Gsp-1* from bread wheat.

| | Wild type | | *Pinb-D1b* | | Total | |
|---|---|---|---|---|---|---|
| | *Pinb-2v2* | *Pinb-2v3* | *Pinb-2v2* | *Pinb-2v3* | *Pinb-2v2* | *Pinb-2v3* |
| No. of sample | 2 | 23 | 19 | 48 | 21 | 71 |
| SKCS Hardness | 14.2a | 24.6b | 67.5a | 60.7b | - | - |
| 1000-kernel weight | 59.2a | 54.7b | 50.9a | 52.2b | 51.7a | 52.9a |
| Grain length (mm) | 6.85a | 6.8a | 6.61a | 6.73a | 6.63a | 6.66a |
| Grain width (mm) | 3.65a | 3.57a | 3.47a | 3.55a | 3.49a | 3.50a |
| Spikelet number per spike | 20.1a | 20.83a | 20.09a | 20.59a | 20.10a | 20.67a |
| Grain number per spike | 42.1a | 45.18b | 40.7a | 43.7b | 40.86a | 44.17b |
| Grain weight per spike (g) | 2.11a | 2.27a | 1.95a | 2.17b | 1.96a | 2.21b |
| Length of flag leaf (cm) | 16.9a | 17.4a | 15.7a | 17.9b | 15.77a | 17.70b |
| Width of flag leaf (cm) | 1.85a | 1.96a | 1.85a | 1.92a | 1.85a | 1.93a |
| Area of flag leaf ($cm^2$) | 23.6a | 25.6b | 21.7a | 25.7b | 21.89a | 25.69b |

Different letters indicate significant difference at 5% probability level

Table 2. Comparison of two *Puroindoline b-B2* variants on grain hardness and yield-related traits

## 4. Discussion

Even though physical mapping of *Puroindoline b-2* variants was conducted using aneuploids of Chinese Spring by Chen et al. (2010), we re-mapped the *Puroindoline b-2* variants in the durum wheat cultivar Langdon and bread wheat cultivar Chinese Spring. The re-mapping was performed using different stocks than those reported by Chen et al. (2010) due to the controversial difference between the results of Chen et al. (2010) and Wilkinson et al. (2008). According to this study and the previous report (Chen et al. 2010), *Pinb-2v1* is located on chromosome 7D and is present in all the bread wheat cultivars surveyed. Therefore, in this study, the absence of *Pinb-2v1* in durum wheat is expected. *Pinb-2v2* and *Pinb-2v3* are reciprocally present on chromosome 7B in all of the bread wheat cultivars surveyed. Only one durum cultivar possessed the *Pinb-2v2/Pinb-2v4* haplotype combination, whereas 35 durum cultivars possessed the *Pinb-2v3/Pinb-2v4* combination, suggesting that *Pinb-2v3* and *Pinb-2v2* were likely allelic. In this set of durum germplasm, the *Pinb-2v3/Pinb-2v4* combination was the predominant haplotype.

Wilkinson et al. (2008) mapped *Puroindoline b-2* variant 2 to chromosome 7AL in three doubled haploid populations and amplified sequences of *Pinb-2v3* and *Pinb-2v1* from the genomic DNA of the durum wheat cultivar Ofanto. We did not find *Pinb-2v1* in any of the durum cultivars surveyed, including 48 sequencing results of cloned PCR amplicon in the

durum wheat Langdon. The reason for this discrepancy is possibly due to primer sequence and specificity (or lack thereof) for the various variant genes used in the two studies.

According to Chen et al. (2010), *Pinb-2v1, Pinb-2v2, Pinb-2v3* and *Pinb-2v4* are located on chromosome 7DL, 7BL, 7B and 7AL in bread wheat, respectively. Coincidently, the strongest QTL effects controlling grain yield, especially for grain weight, were found on chromosomes 7AL and 7BL in the report of Quarrie et al. (2005), and a QTL associated with grain yield has also been identified on chromosome 7D in the study of Kuchel et al. (2007). More recently, several QTLs including QTLs on 7A and 7B associated with grain yield and yield components have been discovered in a recombinant inbred line population (McIntyre et al. 2009). However, further studies are required to determine if this is 'cause and effect' or simply linkage occurring.

Even though many QTLs controlling wheat grain yield and its components have been studied for many years, no specific gene with detailed sequence has been reported so far. Therefore, the possibility of the *Puroindoline b-2* gene possessing some function in modulating grain yield traits may provide useful information for MAS (Marker Assisted Selection). Future studies should define the function of the *Puroindoline b-2* genes by using populations with defined genetic background.

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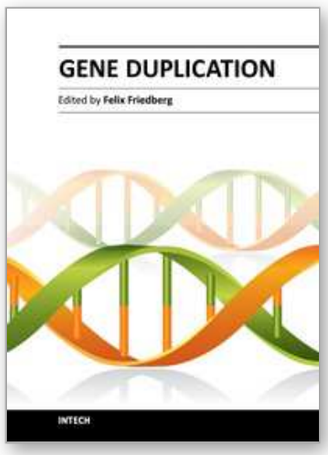


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