Occurrence of Puroindoline Alleles in Chinese Winter Wheats

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ABSTRACT

Grain hardness is one of the most important characters that determine the end-use quality of bread wheat (*Triticum aestivum* L.). Mutations in genes encoding either puroindoline a (*Pina*) or b (*Pinb*) have been associated with hard grain texture, i.e., *Pina* null at *Pina-D1* or seven mutations at *Pinb-D1*. In this study, the diversity of puroindoline alleles in 251 Chinese winter wheat cultivars and advanced lines from four major autumn-planted wheat regions were investigated. Among the examined cultivars, 79 were classified as soft, while 53 were mixed in hardness, and 119 were uniformly hard. Of these hard winter wheats, three of the seven reported mutation types were observed, with *Pina-D1a*/*Pinb-D1b* being the dominant type for hard texture; 91 genotypes carried this allele. Sixteen genotypes had the *Pina-D1b* allele, and two genotypes had the *Pinb-D1d* allele. A new mutation, designated as *Pinb-D1p*, was detected in 10 hard genotypes, with a single nucleotide (A) deletion corresponding to position 42 in the amino acid sequence of puroindoline b, involving a lysine (K) to asparagine (N) change, and leading to a shift in the open reading frame (ORF). This deletion disrupts the last part of the tryptophan-rich domain, changing it from KWWK to NGGR, which is considered essential for the lipid-binding activity of this protein, and results in a stop codon corresponding to position Pro-60 in the amino acid sequence. The characterization of different hardness alleles provides useful information in understanding the mechanism underlying the formation of endosperm hardness while providing breeders the means of manipulating this important trait.

Grain hardness is an important wheat quality trait because it determines the end use and marketing classification of bread wheat. Hard grain cultivars with high protein content and high elasticity are essential for good breadmaking quality, while soft grain cultivars with low protein content are preferred for cakes, cookies, and pastries (Morris and Rose 1996). Grain hardness is controlled by one major gene (*Ha*) located on the short arm of chromosome 5D, and one on several minor genes (Mattern 1973; Baker 1977; Anjum and Walker 1991). A deeper understanding of grain hardness came with the discovery of friabilin (Greenwell and Schofield 1986). The presence of friabilin on the surface of water-washed starch has been associated with soft grain texture, and friabilin is composed primarily of puroindoline a and b (PINA and PINB). Morris (2002) provided a thorough review of friabilin, puroindolines, and grain hardness from the molecular genetic basis. Puroindolines are lipid-binding proteins rich in tryptophan and cysteine residues (Gautier et al 1994; Giroux and Morris 1997). Genes coding for PINA and PINB were located at the *Ha* locus and assigned as *Pina-D1* and *Pinb-D1*, respectively (Giroux and Morris 1997). When both *Pina* and *Pinb* are in their functional wild-type allelic state, grain texture is soft. When either of the puroindolines is absent or altered by mutation, then the result is hard texture. Up to now, seven single nucleotide mutations in *Pinb* and a *Pina* null mutation have been reported in bread wheat, which result in a kernel hardness change from soft to hard (Giroux and Morris 1998; Lillemo and Morris 2000; Morris et al 2001). However, in *Aegilops tauschii* accessions, five different *Pina* mutations were detected and assigned as *Pina-D1c*, *Pina-D1d*, *Pina-D1e, Pina-D1f*, and *Pina-D1g*, and three unique *Pinb* sequences were found and assigned as *Pinb-D1h*, *Pinb-D1i*, and *Pinb-D1j*, respectively (Massa et al 2004), although none of these alleles lead to any changes in endosperm texture. Interaction of PINA and PINB with starch is mediated by the residual polar lipids present at the surface of starch granules (Greenblatt et al 1995). Full functional friabilin requires both PINA and PINB, and the absence of PINA will result in the lack of starch association (Capparelli et al 2003). Krishnamurthy and Giroux (2001) transferred the *Pina* and *Pinb* genes from bread wheat into rice and softened the kernel texture. The causal relationship between puroindolines and grain hardness has been further tested through transformation in bread wheat, where expression of the wild-type allele of *Pinb* restored the soft endosperm in a hard wheat cultivar with the Gly to Ser mutation at position 46 (Beecher et al 2002). With the study of different transgenic lines harboring foreign *Pina*, *Pinb*, or both, Hogg et al (2004) showed that wheat puroindolines interact to form friabilin and control wheat grain hardness.

In addition, different puroindoline alleles show different frequency in various regions due to the origin of their progenitor. For example, *Pina-D1b* and *Pinb-D1b* were prevalent in winter wheat cultivars, whereas the other types seldom appeared (Lillemo and Morris 2000; Morris et al 2001). But for spring wheat, *Pina-D1b* is mainly found in wheat germplasm from Northern and Latin America; *Pinb-D1b* is present mostly in wheats from North America and Europe; *Pinb-D1c* and *Pinb-D1d* are mainly distributed in Northern Europe; while *Pinb-D1e, Pinb-D1f*, and *Pinb-D1g* were only detected in a few North American cultivars (Lillemo and Morris 2000; Morris et al 2001).

China is the largest wheat-producing country in the world with a harvested area of ≈23 million ha. Wheat is planted in 10 agro-ecological zones, with winter, facultative, and spring wheats sown both in autumn and spring (He et al 2001). Chinese wheat germplasm differs from that of other countries in several aspects. China is the secondary original place for wheat with broad diversity in germplasm resources, there is a wide distribution of different wheats in diverse environments, early maturity to suit the double cropping system, and unique products such as steamed bread and noodle are the main end uses of wheat. In fact, Chinese wheat is a mixed and unselected population in terms of processing quality because selection for yield performance and early maturity with disease resistance has been the top priority due to the high pressure of feeding a large population for the last 50 years (He et al 2002). Our previous work indicated that hard, medium, or mixed, and soft types are present in various parts of China, while hard types are dominant in north China and soft types have a relatively high frequency in south China (Zhou et al 2002). More importantly, grain texture is not matched with protein content and quality, i.e., soft kernel with high protein content or hard kernel with high protein content, but weak gluten quality is commonly observed in Chinese wheat. As the living standard improves, consumers and processors demand higher quality wheat and more diverse products. Therefore, quality improvement has become a top priority in Chinese wheat-breeding programs. Understanding the distribution of various puroindoline alleles in Chinese wheat

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germplasm could provide crucial information for genetic improvement of processing quality. In this study, we evaluate the distribution of puroindoline alleles in 251 winter wheat cultivars and advanced lines, and discover a novel single nucleotide deletion in puroindoline b in 10 Chinese cultivars. In addition to those mutations characterized previously, this mutation also confers hard texture in bread wheat.

MATERIALS AND METHODS

Wheat Germplasm

In total, 251 winter wheat cultivars and advanced lines collected from the breeding programs across the autumn-sown wheat regions were used in this study. These genotypes represent some of the historical landmark cultivars from the 1980s and 1990s, current commercial cultivars comprising >85% of crop acreage, and the most promising lines in the regional yield trials. The autumn-sown wheat regions include North China Plain Winter Wheat Region (Zone I), facultative wheat in the Yellow and Huai Valley (Zone II), autumn-sown spring wheat in Mid- and Lower-Yangtze Valley (Zone III), and Southwestern Region (Zone IV). All tested genotypes were sown at Anyang Experimental Station of the Chinese Academy of Agricultural Sciences in the 2001-02 and 2003-04 crop seasons according to local management practices. After harvest, all wheat samples were cleaned. Falling number tests indicated that they were free of sprouting damage.

Hardness Measurement

Kernel hardness was measured on 300-kernel samples of each genotype using the 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 tested genotypes into soft, mixed, and hard types. The SKCS produces a four-class frequency distribution of hardness data for each cultivar with class limits of ≤33, 34–46, 47–59, and ≥60. For grain hardness classification, class 1 is hard type, class 2 and class 3 are mixed types, and class 4 and 5 are soft types.

DNA Isolation and PCR Amplification to Detect the Puroindoline b Alleles

DNA was isolated from wheat seedlings at the two- to three-leaf stage using the procedure described by Dellaporta et al (1983). For each genotype, three samples, that is, two from individual seedlings and one from a composite sample of three to six seedlings, were amplified to verify the purity of the sample and to detect the bona fide alleles. Full-length puroindoline b was amplified with the primers described by Gautier et al (1994), and specific PCR products of ≈450 bp were sequenced using the amplification primers. The allele-specific primers used for detection of *Pinb-D1b* (Gly46 to Ser46) were: 5'-ATGAAGGCCCTCTTCCTCA-3' (up primer), 5'-CTCATGCTCACAGCCGCT-3' (down primer for mutation type), 5'-CTCATGCTCACAGCCGCC-3' (down primer for wild type). SDS-PAGE of Triton X-114 soluble proteins was used to detect the puroindoline a null as described below. For the remaining hard genotypes, full-length sequencing of the PCR products was used to identify puroindoline alleles. The PCR amplification conditions were one cycle of 94°C for 3 min; followed by 35 cycles of 94°C for 30 sec, 50°C for 1 min, and 72°C for 1 min;and a final 10 min elongation at 72°C. All PCR products were visualized on 1–1.5% (w/v) agarose gels.

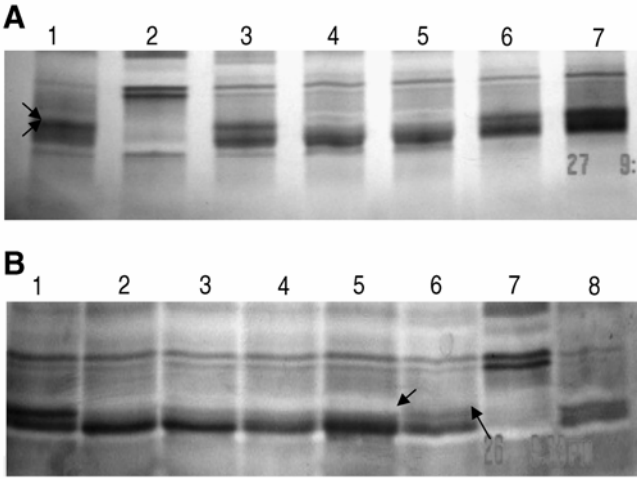

Fig. 1. SDS-PAGE of Triton X-114 extracted protein from single kernel. **A,** 1. Chinese spring, 2. Durum wheat cv. Stewart, 3. Falcon, 4. Nongda 3213, 5. Nongda 3395, 6. Gaocheng 8901, 7. Gaocheng 8901. **B,** 1. Soft wheat Jing 9428, 2. Nongda 3213, 3. Nongda 3213, 4. Nongda 3395, 5. Nongda 3395, 6. Falcon, 7. Durum wheat cv. Stewart, 8. Chinese Spring. Arrows showed expected bands of PINA and PINB.

TABLE I
Puroindoline Alleles and Their Frequency in 198 Chinese Winter Wheat Cultivars and Advanced Lines

Puroindoline a	Puroindoline b	Phenotype	Molecular Change	No. of Genotypes	Frequency (%)
Pina-D1a	*Pinb-D1a*	Soft	Wild type	79	39.9
Pina-D1b	*Pinb-D1a*	Hard	No expression of *Pina*	16	8.1
Pina-D1a	*Pinb-D1b*	Hard	Gly46 to Ser46 in *Pinb*	91	46.0
Pina-D1a	*Pinb-D1d*	Hard	Trp44 to Arg44 in *Pinb*	2	1.0
Pina-D1a	*Pinb-D1p*	Hard	A deletion in Lys42 in *Pinb*	10	5.0

TABLE II
Distribution of Puroindoline Alleles in Different Wheat Regions

Genotype	Phenotype	No.	Zone[a]				SKCS Hardness[b]
			I	II	III	IV	
Pina-D1a/Pinb-D1a	Soft	79	10	38	15	16	25c
Pina-D1b/Pinb-D1a	Hard	16	3	7	1	5	76a
Pina-D1a/Pinb-D1b	Hard	91	27	52	9	3	66b
Pina-D1a/Pinb-D1d	Hard	2	2	0	0	0	67b
Pina-D1a/Pinb-D1p	Hard	10	4	5	1	0	67b
Total		198	46	102	26	24	

[a] Zones: I, North China Plain winter wheat region; II, Yellow and Huai Valleys facultative wheat region; III, Mid and Lower Yangtze Valleys autumn-sown spring wheat region; IV, Southwestern autumn-sown spring wheat region.
[b] Single kernel characterization system (SKCS) hardness data was averaged from two years. Different letters indicate significance at 5% probability level.

Isolation of Triton-Soluble Proteins and SDS-PAGE to Detect the Puroindoline a Null

Triton X-114 soluble proteins were extracted from single wheat kernels following the procedure described by Giroux and Morris (1998). Each sample was repeated three times to ensure the purity of the seeds. SDS-PAGE and its visualization were performed following the method of Morris et al (1994).

Statistical Analysis

SAS software was used to compute the average grain hardness from two seasons and LSD multiple comparison was used to examine the hardness difference among various hardness alleles.

RESULTS AND ANALYSIS

Soft and Mixed Genotypes

In total, 79 genotypes were classified as soft and 53 as mixed according to the SKCS hardness index. For the soft types, both *Pina* and *Pinb* genes should be in the wild-type allelic state according to the current model of the relationship of puroindoline genes and kernel texture. Sequencing of both *Pina* and *Pinb* in 10 randomly selected soft wheat genotypes confirmed the hardness model (data not shown). Further analysis was not performed for the mixed genotypes.

Screening for Known Puroindoline Mutations

Puroindoline alleles and their frequency in 198 Chinese winter wheat genotypes, excluding the 53 mixed genotypes, are presented in Table I. For the 119 hard wheat genotypes, the primers for detecting *Pinb-D1b* identified 91 hard wheats with the *Pinb-D1b* genotype. This indicated that over half of hard wheats belong to the *Pinb-D1b* type. This proportion is similar to that found in North America (Morris et al 2001).

Hard genotypes without the reported *Pinb-D1b* mutation were further evaluated for the expression of puroindoline a protein using SDS-PAGE of Triton X-114 soluble proteins from single kernel. Among the tested genotypes, 16 genotypes, including Gaocheng 8901, possessed the *Pina-D1b* allele (Fig. 1). Although it was tested several times, we could not confirm the presence of *Pinb-D1l* mutation in Gaocheng 8901, as reported by Pan et al (2004).

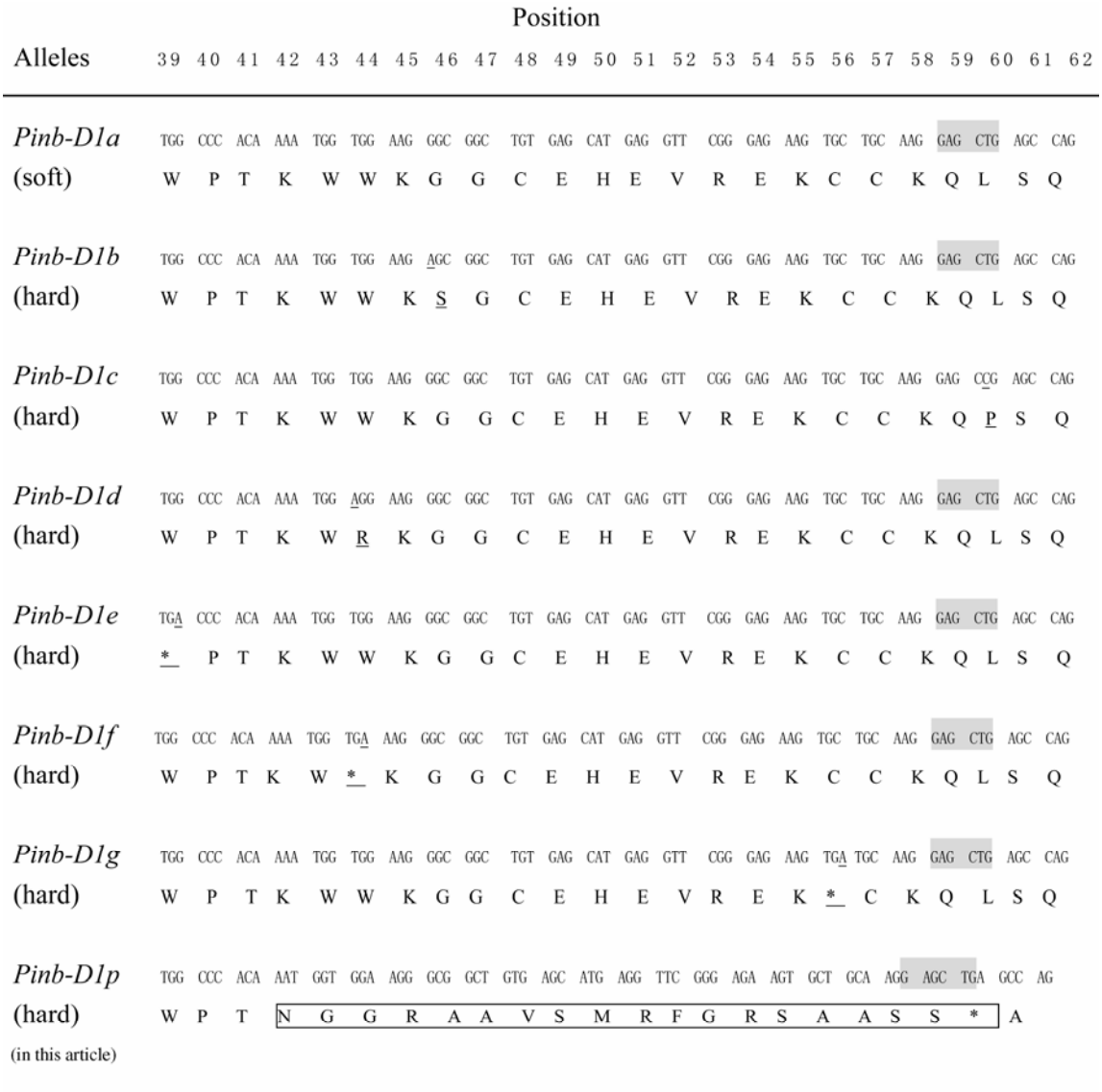

Fig. 2. Nucleotide and deduced amino acid sequence changes in the currently reported puroindoline b alleles in bread wheat. Mutation sites are underlined and in bold. Denotations for the reported alleles and the new mutation type in this article are shown at the left. Shadowed areas in the DNA sequence indicate the restriction site for *Pvu*II. Boxed areas indicate the shifted open reading frame after the single A deletion. * indicates stop codon. *Pinb-D1l* has not been detected.

Efforts are currently underway to obtain identical germplasm as used by Pan et al (2004). For other alleles, they were detected by whole length sequencing of the PCR products. The results indicated that only two genotypes (CA9648 and Dongfeng 9801 from Zone I) carried the *Pinb-D1d* mutation, and no other *Pinb-D1* mutation type was found in this set of Chinese germplasm.

TABLE III
SKCS Hardness and Puroindoline Alleles of Important Chinese Winter Wheat Cultivars

				Frequency Distribution (%)				Puroindoline Allele[b]	
Cultivar	**Origin[a]**	**Class**	**Mean ± SD**	**≤33**	**34–46**	**47–59**	**≥60**	***Pina-D1***	***Pinb-D1***
Nongda 139	Zone I	5	14 ± 15	92	5	1	2	A+	G+ S–
Jing 411	Zone I	5	24 ± 15	78	15	5	2	A+	G+ S–
Jing 9428	Zone I	5	11 ± 16	92	5	1	2	A+	G+ S–
Jimai 24	Zone II	5	15 ± 13	94	4	1	1	A+	G+ S–
Yumai 2	Zone II	5	26 ± 17	71	17	7	5	A+	G+ S–
Yumai 18	Zone II	5	12 ± 15	94	3	2	1	A+	G+ S–
Yumai 35	Zone II	5	10 ± 12	97	3	0	0	A+	G+ S–
Yumai 49	Zone II	5	25 ± 15	73	21	3	3	A+	G+ S–
Yumai 70	Zone II	5	14 ± 14	95	4	0	1	A+	G+ S–
Yangmai 5	Zone III	5	31 ± 14	66	26	5	3	A+	G+ S–
Chuanmai 107	Zone IV	5	20 ± 16	80	15	4	1	A+	G+ S–
Mianyang 26	Zone IV	5	19 ± 13	86	11	2	1	A+	G+ S–
Mianyang 11	Zone IV	4	26 ± 24	62	19	12	7	A+	G+ S–
Dongfanghong 3	Zone I	3	46 ± 16	17	31	29	23	· · ·	· · ·
Fengkang 2	Zone I	3	46 ± 16	21	32	31	16	· · ·	· · ·
Jingdong 6	Zone I	3	48 ± 16	12	26	40	22	· · ·	· · ·
Jingdong 8	Zone I	3	47 ± 14	11	37	33	19	· · ·	· · ·
Jingning 13	Zone II	3	48 ± 19	19	19	32	30	· · ·	· · ·
Fengkang 8	Zone I	2	50 ± 13	8	28	45	19	· · ·	· · ·
Xiaoyan 6	Zone II	2	54 ± 16	9	20	35	36	· · ·	· · ·
Lumai 22	Zone II	2	54 ± 14	9	18	35	38	· · ·	· · ·
Lumai 23	Zone II	2	55 ± 14	9	15	33	43	· · ·	· · ·
Yumai 21	Zone II	2	56 ± 18	9	15	37	39	· · ·	· · ·
Beijing 10	Zone I	1	66 ± 60	2	7	23	68	A+	G– S+
Beijing 837	Zone I	1	66 ± 14	1	8	21	70	A+	G– S+
CA9648	Zone I	1	63 ± 14	0	11	33	56	A+	G+ S– R44+
Dongfeng 9801	Zone I	1	66 ± 12	0	5	21	74	A+	G+ S– R44+
Jinnong 218	Zone I	1	75 ± 13	0	2	4	94	A+	G+ S– N42+
Nongda 3213	Zone I	1	66 ± 13	2	5	25	68	A+	G+ S– N42+
Nongda 3291	Zone I	1	70 ± 13	1	1	15	83	A+	G+ S– N42+
Nongda 3395	Zone I	1	61 ± 12	0	1	6	93	A+	G+ S– N42+
Zhongmai 9	Zone I	1	62 ± 14	1	13	27	59	A+	G– S+
Jingnong 8318	Zone I	1	59 ± 12	2	11	37	50	A+	G– S+
Jinmai 45	Zone II	1	60 ± 14	3	7	37	53	A+	G– S+
Baiyu 149	Zone II	1	54 ± 13	5	26	35	34	A+	G– S+
PH82-2-2	Zone II	1	61 ± 16	4	9	30	57	A+	G– S+
Ji 5219	Zone II	1	69 ± 13	0	3	19	78	A+	G+ S– N42+
Ji Z76	Zone II	1	66 ± 15	4	4	17	75	A+	G+ S– N42+
HS97-1	Zone II	1	63 ± 13	3	7	30	60	A+	G+ S– N42+
Gaocheng 8901	Zone II	1	79 ± 18	1	4	5	90	A–	G+ S–
Yumai 34	Zone II	1	60 ± 12	3	9	33	55	A+	G– S+
Yumai 47	Zone II	1	64 ± 16	3	5	24	68	A+	G– S+
Yumai 63	Zone II	1	63 ± 14	3	7	23	67	A+	G+ S– N42+
Zhengzhou 974	Zone II	1	61 ± 15	1	12	34	53	A+	G+ S– N42+
Zhoumai 13	Zone II	1	60 ± 15	4	8	37	51	A+	G– S+
Yanyou 361	Zone II	1	67 ± 13	1	7	18	74	A+	G– S+
Jinan 17	Zone II	1	87 ± 17	2	4	0	94	A+	G– S+
Jimai 19	Zone II	1	69 ± 18	0	1	0	99	A+	G– S+
Jimai 20	Zone II	1	70 ± 14	1	3	15	81	A+	G– S+
Shaan 160	Zone II	1	65 ± 14	1	5	26	68	A+	G– S+
Shaan 229	Zone II	1	81 ± 16	1	0	5	94	A+	G– S+
Shaanyou 225	Zone II	1	63 ± 13	1	5	39	55	A+	G– S+
Shaan 253	Zone II	1	80 ± 13	0	2	3	95	A–	G+ S–
Xuzhou 25	Zone II	1	61 ± 15	5	8	27	60	A+	G– S+
Huaimai 16	Zone II	1	67 ± 14	3	3	17	77	A+	G+ S– N42+
Yangmai 158	Zone III	1	69 ± 14	0	7	14	79	A+	G– S+
Wanmai 18	Zone III	1	76 ± 16	1	1	8	90	A+	G– S+
Wanmai 33	Zone III	1	70 ± 13	1	3	18	78	A+	G– S+
Wanmai 38	Zone III	1	71 ± 14	1	2	8	89	A+	G– S+
Yunmai 42	Zone IV	1	69 ± 17	6	1	8	85	A–	G+ S–
Yunmai 44	Zone IV	1	75 ± 16	3	1	7	89	A–	G+ S–

[a] Zones: I, North China Plain winter wheat region; II, Yellow and Huai Valleys facultative wheat region; III, Mid and Lower Yangtze Valleys autumn-sown spring wheat region; IV, Southwestern autumn-sown spring wheat region.

[b] Puroindoline alleles, where A+ and A– indicate the presence or absence of PINA protein, respectively, based on detection of puroindoline a protein by SDS-PAGE. By inference, these results indicate the presence of the *Pina-D1a* or *Pina-D1b* alleles, respectively. G+ and G– indicate presence or absence of *Pinb-D1a*, respectively, based on presence or absence of a 250-bp PCR product using Gly-46 specific primers; S+ and S– indicate the presence or absence of *Pinb-D1b*, respectively, based on presence or absence of a 250-bp PCR product using Ser-46 specific primers; R44+ and N42+ indicate the presence of *Pinb-D1d* and *Pinb-D1p,* respectively, based on sequencing the entire *Pinb* PCR product; · · · , indicates that data are not available.

The distribution of puroindoline alleles varied in different regions, for example, 27 of the 46 genotypes in Zone I and 52 of the 102 genotypes in Zone II carry *Pinb-D1b*, respectively. However, only three genotypes in Zone IV carry *Pinb-D1b* (Table II). This could be due to the various parents used in the crossing programs in different regions. The hardness data and the occurrence of puroindoline alleles in some historical landmark cultivars, current cultivars, and some advanced lines are presented in Table III. Only SKCS data from the 2003-04 season were included in Table III because the grain hardness classification in the two years was consistent. Detailed information of hardness alleles and SKCS distributions in 251 Chinese winter wheat germplasm is available in the Puroindoline Genotype Database at http://wheat.pw.usda.gov/ggpages/germplasm.shtml (submitted).

A New Mutation Type—A Single Nucleotide Deletion in the Trp-Rich Domain of Pinb Also Associated with Hard Texture

Ten hard wheat genotypes including Nongda 3213, Nongda 3291, and Nongda 3395 derived from the same cross Nongda 3338/S108 by the wheat program at the China Agricultural University, expressed puroindoline a protein but not puroindoline b protein (SDS-PAGE) (Fig. 1). None of the previously identified mutations in puroindoline b were present in these genotypes. Consequently, PCR products from both single plant and composites were further analyzed by DNA sequencing. The sequence results of puroindoline a and b revealed a new type of mutation in puroindoline b, characterized as a single nucleotide (A) deletion at position 42, changing Lys (K) to Asn (N), leading to a shift in the open reading frame (ORF) thereafter, and resulting in a stop codon corresponding to position Pro-60 and, finally, no production of PINB (Fig. 2). This deletion disrupted the Trp-domain, which is considered essential for the lipid-binding activity of this protein, changing it from KWWK to NGGR. No mutation was found in puroindoline a. SKCS data of these 10 genotypes as presented in Table III, indicated that they all belong to the hard class. These results suggested that this single nucleotide deletion in the Trp-domain of puroindoline b was also associated with hard texture. According to the 2003 Supplement of the Wheat Gene Catalogue (McIntosh et al 2003), this deletion was designated as *Pinb-D1p*. It was also interesting to observe that all 10 genotypes carrying *Pinb-D1p* originated from Zones I and II (Table II). As indicated in Table II, the grain hardness for *Pina-D1b*/*Pinb-D1a* was significantly higher than the other three hard genotypes, and *Pina-D1a*/*Pinb-D1b* and *Pina-D1a*/*Pinb-D1p* have similar performance in grain hardness.

DISCUSSION

Puroindoline a and b form the molecular-genetic basis of grain texture in wheat. To date, eight hardness mutations have been reported, involving either no expression of puroindoline a or single nucleotide changes in the coding sequence of puroindoline b, all of which are associated with hard kernel texture (Morris 2002; Pan et al 2004). Our survey of Chinese winter wheat germplasm, including the new mutation type discovered in 10 cultivars, has further confirmed the current model. The impact of different alleles on wheat end-use quality in the same genetic background needs to be evaluated, and specific puroindoline alleles may suit certain end-use requirements (Morris 2002).

It remains unclear how the puroindolines affect the endosperm texture of wheat. One possible role of the puroindolines in determining grain texture could be through the stabilization of the amyloplast lipid bilayer membrane during desiccation (Lillemo and Morris 2000). The Tryptophan-rich domain in puroindolines determines the lipid-binding activity of these proteins (Gautier et al 1994). Among the mutations in puroindoline b reported so far, four occur within or near this domain, i.e., the glycine to serine change at position 46 (*Pinb-D1b*), Trp-44 to Arg-44 (*Pinb-D1d*), and Trp-44 to stop codon (*Pinb-D1f*). All these mutations might change the tertiary structure of the protein, and cause a loss of function. Therefore, it is reasonable that the single nucleotide A deletion, located in the Tryptophan-rich domain, which shifts the reading frame of the protein in these two Chinese genotypes and results in no production of PINB protein, is associated with hard texture. Further characterization of different hardness alleles will provide useful information in understanding the mechanism underlying the formation of endosperm texture.

Wheat puroindolines interact to form friabilin and control wheat grain hardness (Hogg et al 2004). The absence of PINA (*Pina-D1b*) will result in a dramatic decrease in the association of PINB with the starch granule surface (Capparelli et al 2003). Hardness results from the complete or partial loss of functionality of either puroindoline a or b. Dubreil et al (1997) showed that puroindoline a binds phospholipids and glycolipids tightly, whereas puroindoline b only binds negatively charged phospholipids and forms lipoprotein complexes with glycolipids. Because mutations in either gene will result in a loss of endosperm softness, there must be some kind of interaction between them, either directly or indirectly. When either has a mutation, the interaction becomes weak and their binding activity to lipids will also be changed. Therefore, the two-hybrid yeast system could be used to look for any direct interaction between puroindoline a and puroindoline b, and the possible effects of different hardness mutations on this interaction. Furthermore, RNAi, antisense RNA, and overexpression strategies combined together to knock-out or knock-in the function of these two genes would be helpful in improving the grain hardness of cereal plants through genetic engineering.

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