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Development of SNP Assays for Genotyping the Puroindoline b Gene for Grain Hardness in Wheat Using Pyrosequencing

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Grain hardness is one of the most important quality characteristics of cultivated bread wheat (*Triticum aestivum* L.) and has been reported to result from either a failure to express puroindoline a (*Pina*) or single-nucleotide mutations in puroindoline b (*Pinb*). Up to now, seven alleles from *Pinb-D1a* to *Pinb-D1g* were identified in bread wheat. Compared to the DNA coding region of *Pinb-D1a* (allele for softness), six single-nucleotide polymorphisms (SNPs) were detected in six alleles for *Pinb-D1*. In this study, we used pyrosequencing technology to develop two SNP assays for identification of the seven *Pinb* alleles and characterized SNP variations in the *Pinb* of 493 European wheat varieties. Of the three hardness alleles *Pinb-D1b*, *Pinb-D1c*, and *Pinb-D1d* detected in this study, *Pinb-D1b* was the most predominant hardness allele in European hard wheats. The hardness genotypes of partial German wheat varieties available confirmed the reliability and validation of the SNP assays developed for the *Pinb* locus. Therefore, pyrosequencing technology offers an efficient, precise, and reliable concept for high-throughout genotyping to assist selection of grain hardness genes in wheat quality breeding programs.

KEYWORDS: Grain hardness; puroindoline b; pyrosequencing; single nucleotide polymorphism; *Triticum aestivum*; wheat

INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is one of the most important food crops in the world. On the basis of the endosperm texture of the grain, wheat is classified into hard and soft classes. Hard wheats are used for making breads, whereas soft wheats are suitable for cakes and pastries (*1*). Grain hardness is simply inherited and is primarily controlled by the *Hardness* (*Ha*) locus (softness being dominant), which is located on the short arm of chromosome 5D of hexaploid wheat (*2*, *3*).

Biochemical studies indicated that a starch surface-associated protein (15 kDa) was found to be associated with grain softness and referred to as either friabilin (*4*) or grain softness protein (GSP) (*5*). Three related lipid-binding proteins, puroindolines a (*Pina*) and b (*Pinb*) (*6*) and *GSP-1* (*7*), were found to be components of friabilin, *Pina* and *Pinb* of which are the main components. The genes for *Pina-D1*, *Pinb-D1*, and *Gsp-D1* mapped in the distal region of chromosome 5D and are tightly linked to the *Ha* locus (*8*−*11*). All hard wheats characterized to date have a sequence alteration in either *Pina* or *Pinb* relative to soft wheats (*12*). Up to now, two alleles (*Pina-D1a* and *Pina-D1b*) were found for *Pina*, whereas seven alleles (*Pinb-D1a*−*Pinb-D1g*) were identified for *Pinb* in bread wheat. A deletion in *Pina-D1b* resulting in a complete absence of *Pina* protein and single-nucleotide mutations of DNA sequences encoding *Pinb* protein are associated with grain hardness (*13*). Compared to the DNA sequence of the coding region of *Pinb-D1a* (allele for softness), six single-nucleotide polymorphisms (SNPs) were identified in six alleles for *Pinb-D1* (**Figure 1**) (*8*, *12*, *14*, *15*), namely, *Pinb-D1b* ($G^{223} \rightarrow A^{223}$, Gly to Ser), *Pinb-D1c* ($T^{266} \rightarrow C^{266}$, Leu to Pro), *Pinb-D1d* ($T^{217} \rightarrow A^{217}$, Trp to Arg), *Pinb-D1e* ($G^{204} \rightarrow A^{204}$, Trp to stop codon), *Pinb-D1f* ($G^{219} \rightarrow A^{219}$, Trp to stop codon), and *Pinb-D1g* ($C^{255} \rightarrow A^{255}$, Cys to stop codon). Either allele-specific polymerase chain reaction (PCR) primers (*8*) or site-specific cleavage of PCR-amplified *Pinb* with restriction enzyme (*14*) were used for genotyping different *Pinb-D1* alleles. Each assay was used only for a single allele and was time-consuming. These methods are not amenable for a high-throughout screening of a large number of genotypes.

Pyrosequencing (Biotage) is a new method of obtaining short segments of DNA sequence, typically up to 20 nucleotides, simultaneously from 96 different templates (*16*, *17*). It has been successfully applied for disease studies in human (*16*, *18*) and for identification of β-amylase alleles in barley (*19*). Comparison of the coding sequences of six *Pinb-D1* alleles for hardness to that of *Pinb-D1a* for softness indicates that four SNPs exist within 20 nucleotides from position 204 to position 223, whereas two SNPs are observed within 12 nucleotides from position 255 to position 266 (**Figure 1**). The objectives of the present study were to develop SNP assays for identification of the puroindoline

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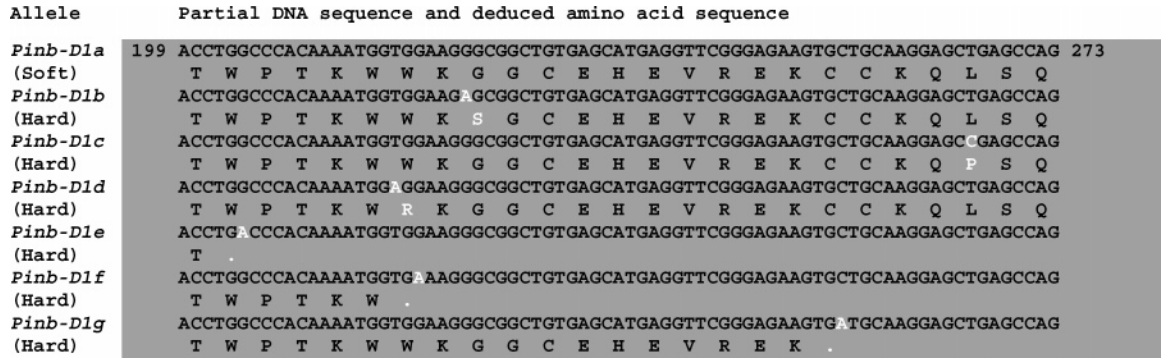

Figure 1. Alignment of partial DNA and deduced amino acid sequence of the coding region of puroindoline b.

Table 1. Origin of 493 European Wheat Varieties and Their Genotype for Puroindoline b

country of origin	no. of varieties analyzed	*Pinb-D1a*	*Pinb-D1b*	*Pinb-D1c*	*Pinb-D1d*
Austria	53	17	22	8	6
Belgium	6	2	1		3
Bulgaria	2		2		
Denmark	2	1			1
France	82	42	20	3	17
Germany	113	35	35	19	24
Greece	24	18	5		1
Italy	30	20	10		
Netherlands	9	2	7		
Poland	2	2			
Portugal	6	5	1		
Spain	17	16	1		
Sweden	2	1	1		
Switzerland	10	2	4	3	1
Turkey	18	17	1		
U.K.	64	29	18	1	16
Yugoslavia	38	7	30		1
others and unknown	15	6	4		5
total (%)	493	222 (45%)	162 (33%)	34 (7%)	75 (15%)

b alleles and to characterize SNP variation in the puroindoline b among 493 European wheat varieties.

MATERIALS AND METHODS

Plant Materials and DNA Isolation. Seeds of 493 European wheat varieties used in this study were obtained from different sources and breeding companies. **Table 1** lists the number of varieties from different countries.

Three nullitetrasomic lines (N5AT5B, N5BT5A, and N5DT5A) and two ditelosomic lines (DT5DS and DT5DL) of Chinese Spring, which were originally obtained from the late Dr. E. R. Sears, Columbia, MO, were used for chromosome arm assignment of PCR products.

Genomic DNA was extracted from bulked leaves of six plants of each variety and provided by TraitGenetics GmbH, Gatersleben, Germany. DNA was isolated as described in ref *20* and diluted to a final concentration of 50 ng/μL.

Primer Design and PCR Amplification. Primer design for the puroindoline b was based on the coding region of cDNA clone pTa19B2 (Genbank X69912) (*6*). For SNP genotyping by pyrosequencing, three primers are required: two PCR primers for PCR amplification and one sequencing primer for pyrosequencing. Primer pairs flanking six SNPs were designed using the program Primer 0.5, which was kindly provided by E. Lander (Massachusetts Institute of Technology). The sequences of primers used were forward 5′- TGCAAGGATTACGTGATGGA-3′ and reverse 5′- TCACCAGTAATAGCCACTAGGGAA-3′ to amplify a 300-bp fragment containing all six SNPs. The reverse primer was biotinylated at the 5′ end and also HPLC-purified. The sequencing primer should be very close (one or two nucleotides) to the SNP position and was designed using SNP Primer Design Software v 1.01 on the Web site of Pyrosequencing (Biotage, Uppsala, Sweden): http://techsupport.pyrosequencing.com/v2/AssayDesign/index.asp. The sequencing primers for 4-SNP and 2-SNP detection were forward 5′-GGATTTTCCAGTCACCT-3′ and forward 5′-GGTTCGGGAGAAGTG-3′, respectively.

The PCRs were performed according to refs *21* and *22* with modifications. PCR reactions contained 100 ng of template DNA, a 0.2 μM concentration of each biotinylated and unlabeled primer, 1 U of *Taq* DNA polymerase, 2.5 μL of 10× PCR buffer, 1.5 mM $MgCl_2$, and 0.2 mM dNTPs in a total volume of 25 μL. The PCR reaction was carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). After 3 min of denaturation at 94 °C, 45 cycles were performed with 1 min at 94 °C, 1 min at 60 °C, 2 min at 72 °C, and a final extension step of 10 min at 72 °C.

PCR products of Chinese Spring, three nullitetrasomic lines (N5AT5B, N5BT5A, and N5DT5A), and two ditelosomic lines (DT5DS and DT5DL) were checked on 1.5% agarose gel for determining their chromosomal arm location.

Template Preparation for Pyrosequencing. An 8 μL sample of biotinylated PCR products was transferred into the new PCR plate, and then for each sample, 2 μL of streptavidin Sepharose beads (Amersham Biosciences AB, Uppsala, Sweden), 40 μL of binding buffer (10 mM Tris−HCl, pH 7.6, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20), and 30 μL of H_2O were added. The PCR plate was covered and shaken for 10 min. During shaking, biotinylated PCR products were immobilized onto streptavidin Sepharose beads. For preparing sequencing plates, 11.64 μL of annealing buffer (20 mM Tris−acetate, pH 7.6, 2 mM $MgAc_2$) and 0.36 μL of the sequencing primer (10 μM) were required for each sample. After shaking, PCR products immobilized on the beads were transferred from the PCR plate to the sequencing plate using Vacuum Prep Tool (Pyrosequencing, Biotage). During the transfer, single-stranded DNA was obtained by incubating the immobilized PCR product in 0.2 M NaOH for 8 s and subsequently in washing buffer (10 mM Tris−acetate, pH 7.6) for 8 s. Hybridization between the sequencing primer and the single-stranded template was performed by incubation at 80 °C for 2 min and then cooling to room temperature.

Pyrosequencing and Data Analysis. A 96-well plate containing single-stranded DNA template with the annealed sequencing primer was placed inside the pyrosequencer instrument, PSQ HS 96A system (Pyrosequencing, Biotage). The cartridge was filled with an enzyme mixture (DNA polymerase, ATP-sulfurylase, luciferase, apyrase), a substrate mixture (luciferin, adenosine 5′-phosphosulfate), and nucleotides (dATP, dCTP, dGTP, dTTP) contained in PSQ HS 96A SNP Reagent Kit 50×96. The volumes required are indicated in the run setup. Pyrosequencing was performed for 18 min for 2-SNP assay and 24 min for 4-SNP assay.

Genotypes were analyzed with PSQ 96MA SNP Software v 2.0 from Pyrosequencing (Biotage).

RESULTS AND DISCUSSION

Partial Amplification of the Coding Sequence of the Puroindoline b. The following criteria should be considered for PCR primer design: (1) the fragment length is as short as possible for the optimal SNP identification by means of

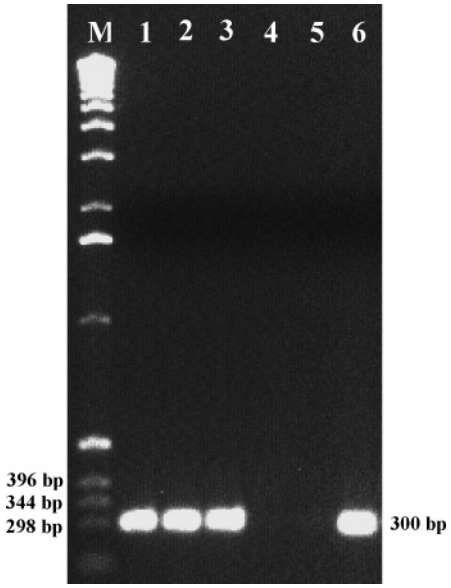

Figure 2. Genome-specific amplification of the partial coding region of puroindoline b containing six SNPs analyzed using Chinese Spring (lane 1), three nullitetrasomic lines (N5AT5B (2), N5BT5A (3), and N5DT5A (4)), and two ditelosomic lines (DT5DL (5) and DT5DS (6)). M = 1-kb ladder.

pyrosequencing; (2) the potential amplicon must contain six SNPs; (3) the amplified fragment must be genome-specific. On the basis of the coding sequence of puroindoline b, three forward primers and four reverse primers including original primers published in ref *6* flanking six SNPs were designed using Primer 0.5. A total of 12 primer pairs were used to amplify DNA of CS, three nullitetrasomic lines (N5AT5B, N5BT5A, and N5DT5A), and two ditelosomic lines (DT5DS and DT5DL) for checking genome specificity of amplified fragments (fragment sizes from 214 to 454 bp). The 454-bp PCR fragment amplified with original primers published in ref *6* was genome-specific and contained six SNPs, but was likely too long for the optimal SNP identification by use of pyrosequencing. Nonspecific signals in the SNP positions were found when we started to detect SNPs using original primers and pyrosequencing (data not shown). This was the reason we redesigned the PCR primers for amplification of the coding region of puroindoline b. The 300-bp PCR fragment produced using the primers described in the present study was present in CS, nullitetrasomic lines N5AT5B and N5BT5A, and ditelosomic line DT5DS, but absent in N5DT5A and DT5DL (**Figure 2**). This indicated that this fragment is located on the short arm of chromosome 5D. Fragments amplified with the remaining 10 primer pairs were not genome-specific.

Pyrosequencing for SNP Genotyping. After one PCR amplification, two SNP assays were performed, one for 4-SNPs and the other for 2-SNPs. Parts **A** and **D** of **Figure 3** present the G, T, G, and G alleles of 4-SNPs at positions 204 (*Pinb-D1e*), 217 (*Pinb-D1d*), 219 (*Pinb-D1f*), and 223 (*Pinb-D1b*), and the C and G alleles of 2-SNPs at positions 255 (*Pinb-D1g*) and 266 (*Pinb-D1c*) for softness in CS, respectively. The grain hardness A allele of SNP at position 217 (*Pinb-D1d*) in the French variety Soissons, the A allele of SNP at position 223 (*Pinb-D1b*) in the German variety Toronto, and the C allele of SNP at position 266 (*Pinb-D1c*) in the German variety Aristos are shown in parts **B**, **C**, and **E** of **Figure 3**, respectively.

Among 493 European wheat varieties investigated in the present study, 222 (45%) varieties possess the *Pinb-D1a* allele.

Table 2. *Pinb* Genotype and Phenotype of 41 German Winter Wheat Varieties

variety	*Pinb-D1a*	*Pinb-D1b*	*Pinb-D1c*	*Pinb-D1d*	phenotype for hardness[a]
Alidos	–[b]	+	–	–	H
Altos	–	+	–	–	H
Ares	+	–	–	–	S
Aristos	–	–	+	–	H
Aron	–	–	–	+	H
Aspirant	–	–	–	+	H
Astron	–	+	–	–	H
Atlantis	+	–	–	–	S
Batis	–	–	+	–	H
Biscay	–	+	–	–	H
Bold	–	+	–	–	H
Borneo	–	–	–	+	H
Bussard	–	+	–	–	H
Cardos	–	+	–	–	H
Carolus	–	+	–	–	H
Certo	–	–	–	+	H
Contra	+	–	–	–	S
Convent	–	–	–	+	H
Dekan	–	–	–	+	H
Dream	–	+	–	–	H
Drifter	–	–	–	+	H
Ebi	–	–	+	–	H
Exsept	–	+	–	–	H
Flair	–	+	–	–	H
Florida	–	+	–	–	H
Glockner	–	+	–	–	H
Habicht	–	–	–	+	H
Kanzler	+	–	–	–	S
Kontrast	–	+	–	–	H
Kris	–	–	–	+	H
Maltop	+	–	–	–	S
Mikon	–	+	–	–	H
Moldau	–	–	–	+	H
Pegassos	–	–	–	+	H
Ritmo	–	–	–	+	H
Skater	–	+	–	–	H
Tambor	–	+	–	–	H
Tarso	–	+	–	–	H
Toronto	–	+	–	–	H
Transit	–	–	+	–	H
Zentos	–	+	–	–	H
total	5	20	4	12	

[a] Phenotypes were available from BSA (2003); H = hardness, and S = softness. [b] +/– = presence/absence of allele.

Totals of 162 (33%), 34 (7%), and 75 (15%) varieties carry the hardness alleles *Pinb-D1b*, *Pinb-D1c*, and *Pinb-D1d*, respectively (**Table 1**). Morris et al. (*15*) reported that three other hardness alleles (*Pinb-D1e*, *Pinb-D1f*, and *Pinb-D1g*) were identified in the North American wheat varieties. All three alleles involved a single-nucleotide change from G to A, from G to A, and from C to A at positions 204, 219, and 255 of the DNA sequence that resulted in changes from Trp, Trp, and Cys to a stop codon, respectively, at amino acid levels. These three hardness alleles were not detected in the 493 European wheat varieties. Among 217 European wheat varieties carrying the hardness alleles, 60%, 12%, and 28% of the varieties were found to possess the hardness alleles *Pinb-D1b*, *Pinb-D1c*, and *Pinb-D1d*, respectively, indicating that the *Pinb-D1b* allele was the most predominant hardness allele in European hard wheats. This result is coincident with that of Morris et al. (*15*), who found that *Pinb-D1b* was the most prevalent hardness mutation in North American wheats, but in contrast with the result of Lillemo and Morris (*14*), who reported that the *Pinb-D1c* was frequently present in hard wheats from Northern Europe. The haplotype for each variety at the *Pinb* gene locus will be

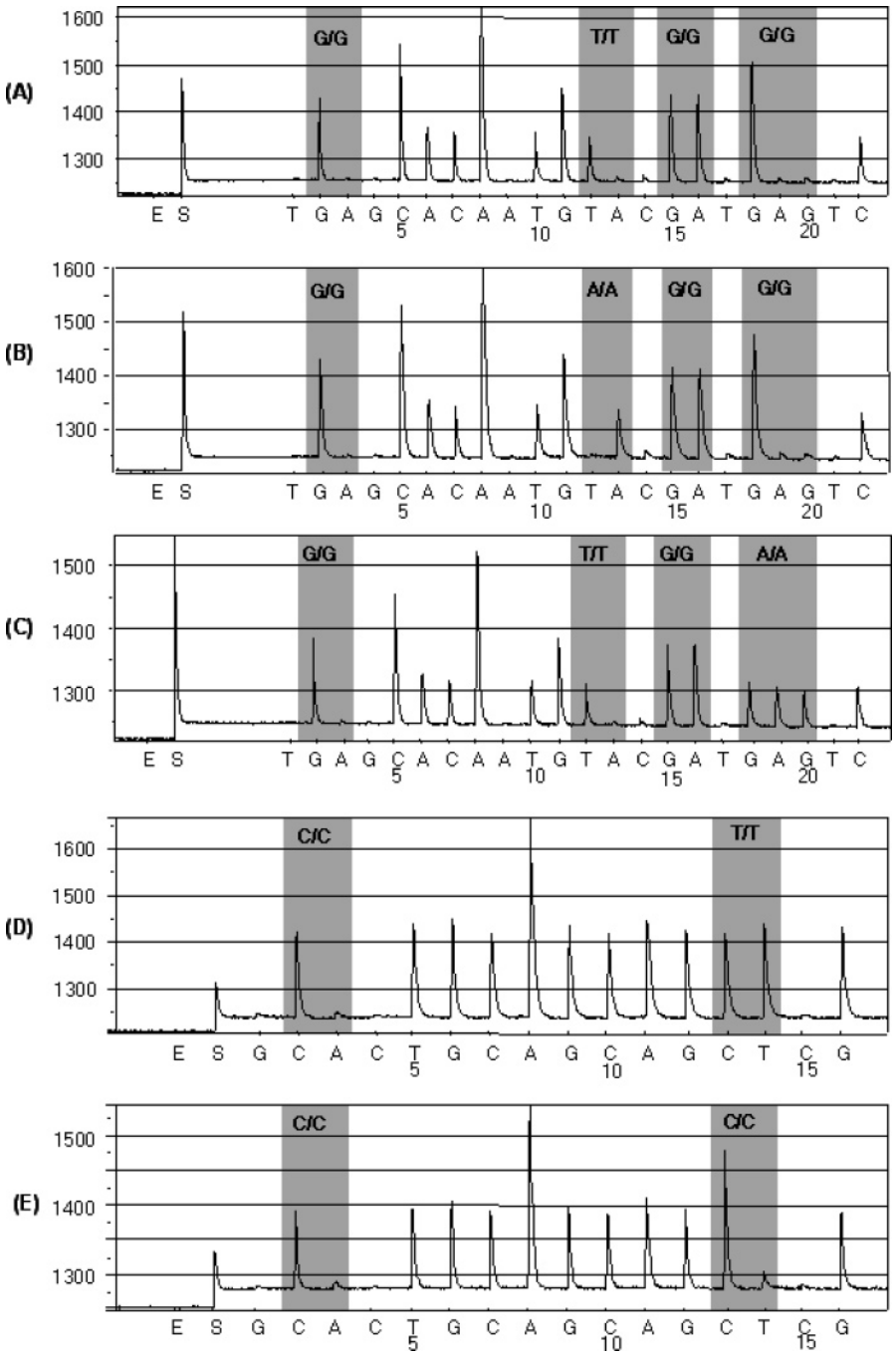

Figure 3. Pyrograms of six SNP positions (204, 217, 219, 223, 255, and 266) for the puroindoline b gene alleles *Pinb-D1e*, *Pinb-D1d*, *Pinb-D1f*, *Pinb-D1b*, *Pinb-D1g*, and *Pinb-D1c*, respectively: (**A**) G, T, G and G alleles of four SNPs at positions 204, 217, 219 and 223 in CS; (**B**) A allele of SNP at position 217 in Soissons; (**C**) A allele of SNP at position 223 in Toronto; (**D**) C and G alleles of two SNPs at positions 255 and 266 in CS; (**E**) C allele of SNP at position 266 in Aristos. Variable positions are highlighted.

published on the Web site of the Plant Genome Resources Center of IPK (http://pgrc.ipk-gatersleben.de/puroindoline/).

Validation of SNP Assays by Pyrosequencing. Of 113 German wheat varieties, the hardness phenotypes of 41 varieties were available from BSA (*23*). Their *Pinb* genotype and phenotype are listed in **Table 2**. Five soft wheat varieties (Ares, Atlantis, Contra, Kanzler, and Maltop) carry the *Pinb-D1a* allele, whereas 20, 4, and 12 hard varieties possess the alleles *Pinb-D1b*, *Pinb-D1c*, and *Pinb-D1d*, respectively. This confirmed the reliability and validation of SNP assays developed for the *Pinb* locus using pyrosequencing.

SNPs are very useful genetic markers for linkage disequilibrium and association studies of quantitative trait loci (QTLs) (*24*, *25*) and have been used in diploid plants such as maize (*26*). Due to duplication of genes in polyploid plants such as hexaploid wheat, analysis of SNPs becomes more difficult. By SNP analysis using pyrosequencing in combination with nullitetrasomic lines of CS, Mochida et al. (*27*) could assign EST contigs to each of the homologous chromosomes of hexaploid wheat. It was found that pyrosequencing is superior to other systems such as allele-specific amplification (*28*), DHPLC (*29*), microarray-based SNP assay (*30*), and SnapShot (*31*) for SNP analysis, because of its linear dose−response curve and high throughput. In the present study, two SNP assays from single PCR were completed within 42 min and detected six SNPs at the *Pinb* locus for 96 samples, indicating that pyrosequencing

is a very efficient method for SNP analysis. A heterozygous SNP can be easily identified by pyrosequencing. Polakova et al. (*19*) detected heterozygous SNPs at the β-amylase gene locus in barley varieties using pyrosequencing technology. In the present study we did not discover any heterozygous SNPs at the *Pinb* locus.

More recently, Massa et al. (*32*) reported that three new alleles from *Pinb-D1h* to *Pinb-D1j* at the *Pinb* locus, five new alleles from *Pina-D1c* to *Pina-D1g* at the *Pina* locus, and seven new alleles from *Gsp-D1b* to *Gsp-D1h* at the *Gsp-D1* locus were detected in the 50 accessions of *Aegilops tauschii* Coss, the donor of the D-genome of hexaploid wheat. Now it is possible to use pyrosequencing technology to characterize SNP genotypes of a large number of accessions of *Ae. tauschii* to discover more genetic variations at these three loci for improving cultivated bread wheat. In conclusion, pyrosequencing technology offers an efficient, precise, and reliable concept for high-throughout genotyping to assist selection of grain hardness genes in wheat quality breeding programs.

ABBREVIATIONS USED

DHPLC, denaturing high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; GSP, grain softness protein; PCR, polymerase chain reaction; Pin, puroindoline; SNP, single-nucleotide polymorphism; Tris, tris(hydroxymethyl)aminomethane.

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