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Brief Report

Genotyping the High Protein Content Gene *NAM-B1* in Wheat (*Triticum aestivum* L.) and the Development of a KASP Marker to Identify a Functional Haplotype

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Abstract: Protein content is one of the main factors determining the end-use quality of wheat. *NO APICAL MERISTEM-B1* (*NAM-B1*) is a major gene regulating wheat grain protein content. The present study aimed to identify new genetic resources using the wild-type *NAM-B1* allele to breed high-protein-content wheat cultivars. We genotyped the *HIGH GRAIN PROTEIN CONTENT-B1* (*GPC-B1*) locus and *NAM-B1* allele in 165 wheat cultivars. A kompetitive allele-specific polymerase chain reaction (KASP) marker was designed for functional *NAM-B1* allele screening. The results revealed that 41 out of 165 cultivars carried the *GPC-B1* locus. Among the 41 *GPC-B1*-carrying cultivars, the wild-type *NAM-B1* allele was identified in only 3 cultivars, none of which were Korean. The remaining 38 cultivars showed a 1-bp insertion in *NAM-B1*, resulting in a stop codon in the middle of the gene, rendering it nonfunctional. Overall, this study reveals that the utilization of the three selected cultivars possessing the wild-type *NAM-B1* gene, in conjunction with the developed KASP assay, could increase the protein content in Korean wheat cultivars.

Keywords: wheat; protein content; *GPC-B1*; *NAM-B1*; KASP marker; genotyping

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1. Introduction

Protein content is a crucial determinant for assessing the quality and applicability of wheat flour [1]. In wheat, *NO APICAL MERISTERM-B1* (*NAM-B1*) is located on the short arm of chromosome 6B and plays a crucial role in regulating the protein content of the grains [2]. *NAM-B1* was identified as a quantitative trait locus (QTL) in 1997 using recombinant inbred lines derived from a cross between wild emmer wheat (*Triticum turgidum* subsp. *dicoccoides*, AABB) and durum wheat (*Triticum turgidum* subsp. *durum*, AABB) [3]. Olmos et al. [4] identified it as *HIGH GRAIN PROTEIN CONTENT-B1* (*GPC-B1*) and discovered that it follows Mendelian inheritance. Distelfeld et al. [5] and Uauy et al. [2] performed fine mapping and identified five genes within the *GPC-B1* QTL region. Among these genes, *NAM-B1* is associated with the increased protein content in wheat grains (Figure 1a). Moreover, *NAM-B1* exhibits three haplotypes: the functional wild-type, *NAM-B1*, derived from wild emmer wheat; the mutated-type, *NAM-B1*, with a 1-bp insertion that introduces a stop codon in the middle of the gene, rendering it nonfunctional; and the gene deletion variant [2,4,6] (Figure 1b–d). Only the wild-type *NAM-B1* allele retains normal gene function; it increases the grain protein content by accelerating leaf senescence and remobilizing nutrients from leaves to developing grains [7,8].

In common wheat (*Triticum aestivum* subsp. *Aestivum*, AABBDD), the effect of wild-type *NAM-B1* on increasing the grain protein content is observed across various genotypes and environmental conditions [9]. Notably, a negative correlation exists between protein content and grain yield. However, these findings are based on correlation analyses between agronomic traits and grain protein content, without considering the genetic diversity of the

evaluated cultivars [10–12]. Uauy et al. [2] confirmed the function of wild-type *NAM-B1* by comparing transgenic lines that showed a difference of over 30% in grain protein content without a significant difference in thousand kernel weight. Moreover, the wheat protein content in near-isogenic lines (NIL) or backcrossed lines containing wild-type *NAM-B1* was 2.1–72 g/kg of grain, higher than that in the recurrent parents lacking wild-type *NAM-B1* [13–18]. The introduction of wild-type *NAM-B1* does not result in significant differences in grain yield compared to that from recurrent parents [13–18]. In addition to the grain protein content, wild-type *NAM-B1* increases the thousand-grain weight and grain yield, depending on the genotypes of the recurrent parents [19].

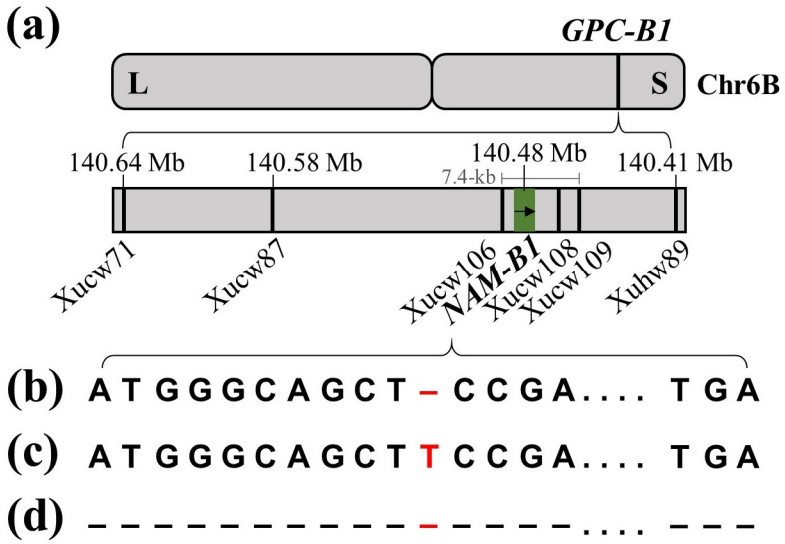


Figure 1. Three haplotypes of the *NO APICAL MERISTEM-B1* (*NAM-B1*) in *HIGH GRAIN PROTEIN CONTENT-B1* (*GPC-B1*) quantitative trait loci (QTL) region of the wheat chromosome 6BS. (**a**) *NAM-B1* between the markers used for detecting the *GPC-B1* QTL region on wheat chromosome 6B in Uauy et al. [2] and Yang et al. [20]. S and L indicate the short and long arms of the chromosome, respectively. The orientation of *NAM-B1* is indicated by a black arrow. (**b**) Functional wild-type *NAM-B1*. (**c**) Nonfunctional 1-bp inserted type *NAM-B1*. (**d**) Gene deletion type.

The effect of wild-type *NAM-B1* on the development of high-protein-content wheat lines was evaluated against the hard red spring wheat cv. Glupro (PI 592759), the original source of the wild-type *NAM-B1* allele, or against Glupro-derived inbred lines, which are the commonly-used donor parents in several studies [14,19,21–23]. In studies that developed and evaluated wild-type *NAM-B1* NIL or backcrossed lines, marker-assisted selection (MAS) commonly used markers such as Xuhw89, Xucw87, and Xucw108, which are closely linked to *NAM-B1* [2,5]. However, these markers can only assist in determining the presence or absence of the gene, making it difficult to confirm whether a new source possesses the functional wild-type *NAM-B1* allele or the nonfunctional 1-bp inserted *NAM-B1* allele. Therefore, it is challenging to discover and utilize new wild-type *NAM-B1*-allele-harboring genetic resources, which could reduce the genetic diversity of the wild-type *NAM-B1*-allele-harboring wheat cultivars.

The use of a limited number of wild-type *NAM-B1*-allele-harboring cultivars, such as cv. Glupro, in wheat breeding has resulted in a considerable decrease in the frequency of this allele during the development of high-yield and environmentally-stable cultivars [6,17,18]. Among the 367 global wheat accessions exhibiting genetic diversity, only five harbored the wild-type *NAM-B1* allele [9]. Similarly, only two wild-type *NAM-B1*-allele-harboring cultivars were reported when 51 Australian wheat cultivars were investigated [20]. Furthermore, in 218 Chinese wheat cultivars, no cultivar harbored the wild-type *NAM-B1*, only 53 cultivars exhibited nonfunctional *NAM-B1*, and the remaining cultivars showed gene deletion [6]. Moreover, whole-gene sequencing analysis, encompassing 1544 bp,

was conducted on these 53 cultivars, revealing eight regions showing genetic variation. However, the cDNA sequence analysis of these cultivars did not reveal any change in gene function.

The present study aimed to identify new genetic resources harboring the wild-type *NAM-B1* allele to enable the breeding of new cultivars with a high protein content and increased genetic diversity. The *GPC-B1* locus has been identified in Korean cultivars and genetic resources from other countries and wild-type *NAM-B1*-allele-harboring cultivars were identified through DNA sequencing. Moreover, the gene-specific kompetitive allele-specific polymerase chain reaction (KASP) marker was developed to enable rapid and efficient MAS for identifying the wild-type *NAM-B1* allele for potential use in wheat breeding programs.

2. Materials and Methods

2.1. Wheat Materials

We analyzed a total of 165 wheat cultivars, including 63 Korean cultivars and 102 genetic resources from foreign countries. All genetic resources were obtained from the Gene Bank of the National Institute of Agricultural Sciences, Rural Development Administration, Republic of Korea.

2.2. Genotyping the GPC-B1 Locus and NAM-B1 Allele

For genotyping *GPC-B1* and *NAM-B1*, DNA was extracted from the fresh seedling leaves of each cultivar using a MagMAX DNA Multi-Sample Kit (Applied Biosystems, Woburn, MA, USA) and Kingfisher DNA extraction machine (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using Xucw108 and Xucw109 primers (Table 1) to confirm the presence of the *GPC-B1* QTL region using the following thermal cycling conditions: initial denaturation at 95 °C for 5 min; followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 40 s; and a final extension at 72 °C for 7 min. The PCR products were separated through electrophoresis on a 3% agarose gel at 200 V for 45 min.

Table 1. Primer sequences and PCR conditions for genotyping *GPC-B1* and *NAM-B1*.

Locus	Marker Type	Primer Name	Primer Sequence (5′-3′)	Product Size (bp)	Annealing Temperature	Reference
GPC-B1	SSR	Xucw108	AGCCAGGGATAGAGGAGGAA	217	58 °C	Uauy et al. [2]
			AGCTGTGAGCTGGTGTCCTT			
		Xucw109	ATCTGCAATTCCAGGCACAC	212	58 °C	
			CCAGCAGATCAAGGAGAATTG			
NAM-B1	Sequencing	*NAM-B1*-specific pair 2	GGAAGAATATAAAAATACTACTTGTGC	555	56 °C	
			CTCCGTTCCTTCCTTCACAC			
NAM-B1	KASP	FAM	GAGCCGGAAGATGAGTCGGAG	-	65→55 °C (−1 °C/cycle), 57 °C	This study
		HEX	GAGCCGGAAGATGAGTCGGAA			
		Common	GGGAAGAAGATCTGATGAGGTCCAT			

GPC-B1, *HIGH GRAIN PROTEIN CONTENT-B1*; *NAM-B1*, *NO APICAL MERISTEM-B1*; KASP, kompetitive allele-specific polymerase chain reaction; SSR, simple sequence repeats; FAM, fluorescein amidite; HEX, hexachlorofluorescein; and bp, base pair.

Subsequently, the *NAM-B1*-specific pair two primers were used for the PCR amplification of *NAM-B1* in *GPC-B1*-carrying cultivars. The PCR conditions were as follows: initial denaturation at 94 °C for 10 min; 40 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min. The PCR products corresponding to the 555-bp band were extracted from the agarose gel and subjected to Sanger sequencing using the reverse *NAM-B1*-specific pair two primers. Gel extraction and Sanger sequencing were performed using services provided by the Bioneer Corporation (Daejeon, Korea).

2.3. Development and Evaluation of a KASP Marker for Identifying the NAM-B1 Allele

A KASP marker was designed to genotype the *NAM-B1* allele, using the reference *NAM-B1* sequence (accession number MG587691) obtained from the National Center for Biotechnology Information. The KASP marker was designed using the KASP assay design service provided by LGC Genomics (LGC Ltd., Teddington, UK). The PCR conditions were as follows: denaturation at 95 °C for 15 min; 10 touchdown cycles at 95 °C for 20 s; annealing starting at 65 °C and decreasing by 1 °C per cycle for 25 s; additional 30 annealing cycles at 95 °C for 10 s and 57 °C for 1 min; and a final extension at 72 °C for 5 min. Fluorescence detection and data analysis were performed using a QuantStudio 3 real-time PCR instrument and the QuantStudio Design & Analysis Software v1.5.1 (Applied Biosystems, Carlsbad, CA, USA), respectively.

3. Results and Discussion

3.1. Selecting GPC-B1-Carrying Cultivars and Analysis of NAM-B1 Alleles

A total of 165 wheat cultivars were screened using Xucw108 and Xucw109 markers to identify the presence of the *GPC-B1* QTL locus. Both markers exhibited polymorphism among the cultivars, and the presence or absence of the 217-bp band for Xucw108 and the 212-bp band for Xucw109 was consistent across the cultivars (Figure 2A,B). Among the 165 cultivars, 41 harbored *GPC-B1* (Table 2 and Table S1). Among those 41 cultivars, only 9 Korean cultivars, cv. Ariheukchal, Arijinheuk, Joah, Olgeuru, Saekeumkang, Saeol, Joongmo2013, Jeonju410, and Jeonju416, harbored *GPC-B1*. The remaining 125 cultivars did not possess the *GPC-B1* locus, indicating that >85% of the Korean cultivars and >75% of the 165 cultivars lacked this region.

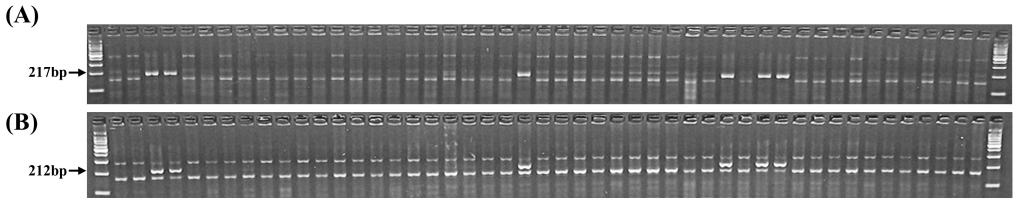


Figure 2. Evaluation of genetic resources with molecular markers (**A**) Xucw108 and (**B**) Xucw109 for detecting the *HIGH GRAIN PROTEIN CONTENT-B1* locus.

Table 2. The *HIGH GRAIN PROTEIN CONTENT-B1* (*GPC-B1*) locus distribution among the 165 wheat cultivars evaluated in this study.

GPC-B1	Korean		Others		Total	
	Number of Cultivars	Frequency (%)	Number of Cultivars	Frequency (%)	Number of Cultivars	Frequency (%)
Presence	9	14.3	32	31.1	41	24.7
Absence	54	85.7	71	68.9	125	75.3

Xucw108 and Xucw109 were positioned within the flanking region (approximately 7.4 kb) of *NAM-B1*. The gene reportedly loses its functionality even with a 1-bp insertion within the gene [2,24]. Therefore, *NAM-B1* sequences from all 41 *GPC-B1*-containing cultivars were analyzed (Table S1) to identify the cultivars carrying the functional wild-type *NAM-B1* allele. Sanger sequencing using the *NAM-B1*-specific pair two primers showed that three cultivars, cv. Benhur (IT15821), BETHLEHEM (IT176584), and PI 350731 (IT336105), possessed the functional wild-type *NAM-B1* allele (Table 3 and Table S2). The results for PI350731 were consistent with those reported by Uauy et al. [2] and Lundström et al. [25]. The findings for BETHLEHEM were similar to those previously reported by Yang et al. [20]. Benhur was identified as a novel cultivar that possessed wild-type *NAM-B1*;

this could contribute to increasing the genetic diversity of wheat cultivars during breeding programs.

Table 3. List of cultivars harboring the wild-type or 1-bp-inserted *NAM-B1* verified using Sanger sequencing and KASP assay analysis. Detailed information is presented in Table S2.

NAM-B1	SNP [1]	Number	Cultivars
Wild-type	-	3	Benhur, BETHLEHEM, PI 350731
1-bp insertion	T	38	Chinese Spring, Ariheukchal, etc.

[1] Located on the 11th SNP from the start codon "ATG" of *NAM-B1*. *NAM-B1*, *NO APICAL MERISTEM-B1*; SNP, single nucleotide polymorphism; and KASP, kompetitive allele-specific polymerase chain reaction.

The remaining 38 cultivars exhibited a genotype characterized by a 1-bp insertion at the 11th single nucleotide polymorphism (SNP) region from the start codon ATG, resulting in a loss of gene function. The cv. Chinese spring showed a 1-bp insertion, consistent with previous studies by Uauy et al. [2] and Lundström et al. [25]. Moreover, the identification of only three cultivars harboring the wild-type *NAM-B1* allele among the 165 cultivars aligned with the results of previous studies suggesting the gene loss of wild-type *NAM-B1*, which was originally introduced from wild emmer wheat, during the breeding process [17].

3.2. Development of a NAM-B1-Specific KASP Marker for the Efficient Breeding of Wheat with High-Protein-Content

The Xucw108 and Xucw109 markers (Section 3.1) can only be used to determine the presence or absence of the *GPC-B1* locus. Therefore, DNA sequencing was required to identify the functional wild-type *NAM-B1* gene. We designed a KASP marker and evaluated it for the rapid and efficient selection of wild-type *NAM-B1*-carrying wheat lines in wheat breeding programs. The results showing wild-type *NAM-B1*-carrying cultivars are presented on the *X*-axis, whereas those with the 1-bp-inserted nonfunctional *NAM-B1* genotype are presented on the *Y*-axis (Figure 3, Table S2). These findings were consistent with the DNA sequencing results (Table 3 and Table S2). Therefore, wild-type *NAM-B1* and nonfunctional *NAM-B1* were easily classifiable using the developed KASP marker. However, *NAM-B1* gene deletion was also observed on the *X*-axis when evaluating cultivars derived from the cross between the wild-type *NAM-B1*-carrying and *NAM-B1* deletion cultivars. Therefore, the use of the Xucw108 or Xucw109 markers can be more beneficial than the use of the developed KASP marker when determining the presence or absence of the *GPC-B1* locus in new genetic resources.

Although some genetic resources harbor valuable genes such as *NAM-B1*, their introduction into leading cultivars is necessary to ensure the stability of the cultivation characteristics in specific environments. MAS facilitates the fixation of desirable genes in early generations during breeding programs [26]. Moreover, a recently established speed vernalization system, which is a wheat generation acceleration system, has been shown to reduce the breeding cycle period [27]. Therefore, integrating MAS using the novel KASP marker with the wheat generation acceleration system will accelerate the breeding of *NAM-B1*-harboring cultivars, rendering it more efficient.

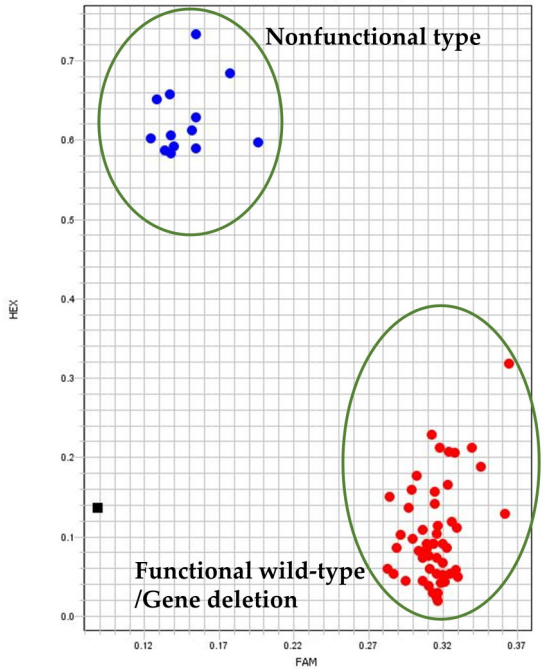


Figure 3. Evaluation of the kompetitive allele-specific polymerase chain reaction assay for identifying the *NO APICAL MERISTEM-B1* (*NAM-B1*) genotype. Genotypes on the *X*-axis represent *NAM-B1* deletion or functional wild-type. Genotypes on the *Y*-axis are nonfunctional types (T insertion). The black square indicates the No Template Control (NTC).

4. Conclusions

NAM-B1 is a major factor affecting the protein content of wheat grains. In this study, the *GPC-B1* QTL and *NAM-B1* genes were evaluated in 165 wheat cultivars collected worldwide. Among the 165 wheat cultivars examined, 41 harbored the *GPC-B1* QTL region and 124 exhibited the *GPC-B1* deletion. Among the 41 *GPC-B1*-harboring cultivars, 3 cultivars (cv. Benhur, BETHLEHEM, and PI 350731) harbored the wild-type *NAM-B1* allele, with cv. Benhur being a novel cultivar reported in this study. The remaining 38 cultivars harbored a 1-bp-inserted nonfunctional *NAM-B1* allele. The KASP marker developed in this study enabled the distinction between wild-type and nonfunctional *NAM-B1* alleles, allowing the identification of wild-type *NAM-B1*-harboring cultivars which can be used to introduce wild-type *NAM-B1* into the wheat lines. These results have important implications for the rapid and efficient introduction of the wild-type *NAM-B1* allele into new breeding lines and the development of high-protein-content wheat cultivars.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13081977/s1. Table S1: List of genetic resources evaluated for the presence of *HIGH GRAIN PROTEIN CONTENT-B1*. Table S2: Genotyping results of sequencing and kompetitive allele-specific polymerase chain reaction assay for *NO APICAL MERISTEM-B1*.

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