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Variation and their relationship of *NAM-G1* gene and grain protein content in *Triticum timopheevii* Zhuk.

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ABSTRACT

NAM is an important domestication gene and valuable to enhance grain protein contents (GPCs) of modern wheat cultivars. In the present study, 12 *NAM-G1* genes in *Triticum timopheevii* Zhuk. (AAGG, $2n=4x=28$) were cloned. These genes had the same length of 1546 bp including two introns and three exons, and encoded a polypeptide of 407 amino acid residues which contained a N-terminal NAC domain with five sub-domains, and a C-terminal transcriptional activation region (TAR). They were highly similar to the previously published functional *NAM-B1* gene DQ871219 from *T. turgidum* ssp. *dicoccoides* Körn. (AABB, $2n=4x=28$) in both the nucleotide and protein sequences, with a very high identity of 99.5%. The differences among the 12 *NAM-G1* genes resulted from 17 SNPs including 14 transitions and 3 transversions. They had outstandingly different expression levels in qRT-PCR. And, their relative expression quantities were significantly positively correlated with GPC of the accessions. In addition, the difference in amino acid sequences of the *NAM-G1* genes may also affect the GPC variation.

Introduction

NAC is an acronym from the three genes including NAM (no apical meristem) in *Petunia*, ATAF (*Arabidopsis thaliana* transcription factor) and CUC2 (cup-shaped cotyledon) in *Arabidopsis* (Souer et al., 1996; Aida et al., 1997). NAC family proteins are plant-specific transcriptional regulators (Duval et al., 2002), which play many important roles in abiotic and biotic stress adaptation and plant development regulation (Olsen et al., 2005; Guo and Gan, 2006; Uauy et al., 2006; Mitsuda et al., 2007; Zhong et al., 2007). Based on sequences, *NAC* genes can be divided into different subfamilies, such as the NAM, ATAF, and OsNAC3 (Kikuchi et al., 2000).

In the *NAM* gene subfamily (Waters et al., 2009), the ancestral *NAM-B1* gene on chromosome 6B was firstly located in a quantitative trait locus (QTL) for wheat grain protein content (GPC) (Joppa et al., 1997). It was expressed in tetraploid wheat *T. turgidum* ssp. *dicoccoides* and ssp. *dicoccum* Schrank, but non-functional in *T. turgidum* ssp. *durum* Desf. and hexaploid bread wheat (*T. aestivem* ssp. *aestivum* L.) (Uauy et al., 2006). The gene non-function was attributed to 1 bp insertion within it, generating a frame-shift mutation (Uauy et al., 2006; Dubcovsky and Dvorak, 2007; Asplund et al., 2010). This gene is of much interest to modern-day breeders because it was considered as a domestication gene affecting GPC in wheat (Dubcovsky and Dvorak, 2007).

The genome G of tetraploid wheat *T. timopheevii* (AAGG, $2n=4x=28$) and the genome B of *T. turgidum* ssp. *dicoccoides* have been deemed as being derived from genome S of *Aegilops speltoides* Tausch (SS, $2n=2x=14$) (Shands and Kimber, 1973; Kimber, 1974). The molecular structure of *NAM* gene from the G genome in *T. timopheevii* has been unknown. The objectives of the present study were to characterize the *NAM* gene in *T. timopheevii*, to probe the association between the gene and GPC of *T. timopheevii*, and to analyze their phylogenetic relationships with previously reported ones, for better exploiting genetic resources with high GPC in quality improvement of modern wheat cultivars.

Materials and methods

Biological materials

In this study, 12 *Triticum timopheevii* accessions were used, which were provided by the NPGS (http://www.ars-grin.gov). PI94761 and PI94760 are from Georgia, CItr15205 from Greece, PI119442 from Turkey, PI190974 from Spain, PI221421 from Yugoslavia, PI251017 from Russian Federation, PI266850 from United Kingdom, PI272523 and PI272530 from Hungary, PI282932 and PI282933 from Argentina. All the accessions were grown in the

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Table 1
Primer sequence, amplification efficiency and coefficient of determination in qRT-PCR.

Gene name	Forward and reserved primers[a]	Amplification efficiency (%)	Coefficient of determination (R^2)
NAM-G1	F: TTGTCCACTGCGCCAGC R: GGCTCCGACCAAACAGTTTC	96.4	0.997
Actin	F: ACCTTCAGTTGCCCAGCAAT R: CAGAGTCGAGCACAATACCAGTTG	97.6	0.990

[a] All the primer sequences of these genes were from Uauy et al. (2006).

experimental field of Triticeae Research Institute, Sichuan Agricultural University at Wenjiang, Chengdu, Sichuan, PR China.

DNA extraction, NAM-G1 gene amplification and sequencing

Genomic DNA was extracted from young leaves using CTAB method described by Yan et al. (2002). According to *NAM-B1* gene sequence DQ871219 from *Triticum turgidum* ssp. *dicoccoides* (Uauy et al., 2006), a pair of primers P1 and P2 was designed and used for amplifying the full DNA sequences of *NAM* gene from the 12 *T. timopheevii* accessions. PCR was carried out using the high-fidelity polymerase *ExTaq* (TaKaRa, Dalian, China). The PCR reaction was programmed at 94 °C for 5 min to denature the DNA, followed by 35 cycles of 94 °C for 50 s, 65 °C for 1 min and 72 °C for 2 min. The final extension step was for 7 min at 72 °C. Amplified products were separated in 1.0% agarose gel, and the anticipated band was excised from the agarose gel and purified using Puex Gel DNA Recovery Kit (Bioche, Beijing, China). DNA fragments were ligated into the pMD-19T vector (TaKaRa, Dalian, China) and then sequenced by the commercial Company BGI, Shenzhen, China. The cloning and sequencing were repeated three times to exclude sequencing errors.

RNA extraction and cDNA synthesis

The flag leaves during the developmental stage from earing to anthesis of the 12 *T. timopheevii* accessions were collected for RNA extraction. They were collected for 3 biological replicates, and were snap frozen in liquid nitrogen and stored at −80 °C.

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Beijing, China) according to the user manual. Extracted RNA concentrations and quality were determined using the DU 800 UV/Vis Spectrophotometer (Beckman Coulter, USA). High quality RNA samples were selected for further cDNA synthesis. A total of not exceeding 1 μg RNA was used for synthesizing first cDNA strand by cDNA Synthesis Kit (ReverTra Ace, Toyobo, Japan) in a 20 μL reaction volume following the manufacturer's instruction. After inactivation of the reverse-transcriptase, the cDNA products were diluted to a final volume of 100 μL by adding 80 μL of ddH_2O, which then served as a template for PCR and quantitative real-time PCR (qRT-PCR) analyses.

Expression analysis of NAM-G1 genes

qRT-PCR was performed with the iQ™5 Real-Time PCR Detection Systems (Bio-Rad, CA, USA). Each reaction was with a 25 μL final volume, including 5 μL cDNA, corresponding to 50 ng of total RNA, 12.5 μL SYBR® *Premix Ex Taq*™ II (Prefect Real Time) (TaKaRa, Dalian, China), 1.0 μL (10 μM) of each primer, and 5.5 μL ddH_2O. A control contained 5 μL ddH_2O instead of the cDNA (no template). All reactions were carried out in duplicate for each cDNA sample. The PCR conditions were 50 °C for 2 min, 95 °C for 3 min, 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C, followed by the

Table 2
The *NAM* genes from this study and previous reports in Triticeae.

Gene	bp	Chromosome[c]	Species (accessions)	Reference	GenBank no.
NAM-A1	1549	6A	*Triticum turgidum* ssp. *durum*	Uauy et al. (2006)	DQ869672
NAM-B1	1542	6B	*T. turgidum* ssp. *dicoccoides*	Uauy et al. (2006)	DQ869673
NAM-B1[a,b]	1308	6B	*T. turgidum* ssp. *durum*	Uauy et al. (2006)	DQ869674
NAM-B1	1542	6B	*T. turgidum* ssp. *dicoccoides*	Uauy et al. (2006)	DQ871219
NAM-D1	1550	6D	*Aegilops tauschii*	Uauy et al. (2006)	DQ869675
NAM-H1	1585	6H	*Hordeum vulgare* ssp. *vulgare*	Uauy et al. (2006)	DQ869678
NAM-H1	1585	6H	*H. vulgare* ssp. *vulgare*	Distelfeld et al. (2008)	EU368851
NAM-H1	1585	6H	*H. vulgare* ssp. *vulgare*	Distelfeld et al. (2008)	EU368852
NAM-H1	1585	6H	*H. vulgare* ssp. *spontaneum*	Jamar et al. (2010)	EU908210
NAM-H1[a,b]	1586	6H	*H. vulgare* ssp. *vulgare*	Jamar et al. (2010)	EU908209
NAM-H1[a,b]	1549	6H	*H. bulbosum* ssp. *nodosum*	Jamar et al. (2010)	EU908211
NAM-B2	1498	2B	*T. turgidum* ssp. *durum*	Uauy et al. (2006)	DQ869676
NAM-D2	1458	2D	*Ae. tauschii*	Uauy et al. (2006)	DQ869677
NAM-H2	1528	2H	*H. vulgare* ssp. *vulgare*	Uauy et al. (2006)	DQ869679
NAM-G1	1546	6G	*T. timopheevii* (PI94761)	This study	HQ843865
NAM-G1	1546	6G	*T. timopheevii* (PI94760)	This study	HQ843866
NAM-G1	1546	6G	*T. timopheevii* (CItr15205)	This study	HQ843867
NAM-G1	1546	6G	*T. timopheevii* (PI119442)	This study	HQ843868
NAM-G1	1546	6G	*T. timopheevii* (PI190974)	This study	HQ843869
NAM-G1	1546	6G	*T. timopheevii* (PI221421)	This study	HQ843870
NAM-G1	1546	6G	*T. timopheevii* (PI251017)	This study	HQ843871
NAM-G1	1546	6G	*T. timopheevii* (PI266850)	This study	HQ843872
NAM-G1	1546	6G	*T. timopheevii* (PI272523)	This study	HQ843873
NAM-G1	1546	6G	*T. timopheevii* (PI272530)	This study	HQ843874
NAM-G1	1546	6G	*T. timopheevii* (PI282932)	This study	HQ843875
NAM-G1	1546	6G	*T. timopheevii* (PI282933)	This study	HQ843876

[a] Partial sequence.
[b] Nonfunctional NAC transcription factor.
[c] Chromosome location of *NAM-G1* was predicted according to homology.

Table 3
Variation of nucleotide and deduced amino acid sequences from the full-length coding regions of *NAM-G1* genes from 12 *T. timopheevii* accessions.

Accessions	GenBank no.	Nucleotide base			Amino acid		
		Substitution	Position	Exon	Variation	Position	Domain[a]
PI94761	HQ843865	C→T	294	Exon II	/		
		T→C	442	Exon II	F→L	148	NAC (D)
		A→G	963	Exon III	/		
PI94760	HQ843866	A→G	358	Exon II	T→A	120	NAC (C)
CItr15205	HQ843867	A→G	176	Exon I	D→G	59	NAC
		A→T	871	Exon III	R→W	291	TAR
		G→T	1139	Exon III	G→V	380	TAR
PI119442	HQ843868	T→C	16	Exon I	S→P	6	NAC
		C→T	323	Exon II	P→L	108	NAC (C)
		A→G	963	Exon III	/		
PI190974	HQ843869	–					
PI221421	HQ843870	–					
PI251017	HQ843871	G→T	66	Exon I	Q→H	22	NAC
		A→G	680	Exon III	E→G	227	TAR
		G→A	1058	Exon III	G→D	353	TAR
		C→T	1070	Exon III	A→V	357	TAR
PI266850	HQ843872	A→G	1210	Exon III	N→D	404	TAR
PI272523	HQ843873	G→A	115	Exon I	G→S	39	NAC (A)
		A→G	918	Exon III	/		
PI272530	HQ843874	–					
PI282932	HQ843875	G→A	75	Exon I	/		
PI282933	HQ843876	–					

Note: Dashes (–), not nucleotide base substitution; Biases (/), nucleotide base substitution not resulted in variation of deduced amino acids. HQ843865 and HQ843868 had an identical variation site for A→G.

[a] The letters in parenthesis indicate the sub-domain of aa variation.

generation of a dissociation curve by increasing temperature from 65 to 95 °C to check amplification specificity.

In order to analyze qRT-PCR of *NAM-G1* genes, the pair of specific primers used for the *NAM-B1* gene designed by Uauy et al. (2006) was directly adopted, and *actin* gene (Uauy et al., 2006) was used as reference (Table 1). The efficiency and standard deviation of each primer were given by Bio-Rad iQ™5 ver. 2.1 on a standard curve generated from a two-fold dilution series of one sample at five dilution points for two technical replicates. Baseline and threshold cycles (Ct value) were automatically determined with Bio-Rad iQ™5 ver. 2.1 using the default parameters. The relative quantities of *NAM-G1* genes were presented as normalized linearized values using $2^{-\Delta\Delta Ct}$ method.

Sequence comparison and phylogenetic analysis

Multiple alignments were performed by DNAMAN (ver. 6.0.3.48). A phylogenetic tree was constructed using nucleotide sequences by MEGA ver. 4.0 software (Tamura et al., 2007) using the neighbor joining method (NJ), and the bootstrap values were estimated based on 1000 replications.

GPC determination

According to Pettersson and Eckersten (2007), the grain protein content (GPC) in mature seeds from the *T. timopheevii* accessions was measured by Infratec® 1241 Grain Analyzer (FOSS, Denmark). Three replications were made for each tested accession.

Statistical analysis

The JMP ver. 9.1 statistical package (SAS Institute, Cary, NC, USA) was used for statistical analysis. The data on both the relative expression levels of *NAM-G1* gene and GPCs were subjected to variance analysis. Correlation analysis was performed to investigate the relationship between expression levels of *NAM-G1* gene and GPCs in *T. timopheevii* accessions.

Results

Nucleotide sequence analysis of NAM-G1 genes

Amplified products about 1500 bp in PCR using the primers P1 and P2 (Fig. 1A), were obtained from the genomic DNA of all the 12 *T. timopheevii* accessions. The full-length coding regions were further obtained from cDNA, which were about 1200 bp (Fig. 1B). All the 12 nucleotide sequences possessed 1546 bp including two introns with 322 bp and three exons with 1224 bp. They had highly similar length and structure to the previously reported functional *NAM-B1* gene sequence DQ871219 from *T. turgidum* ssp. *dicoccoides*, with an identity of 99.5% (Table 2). However, some differences existed among the 13 sequences: 2 insertions occurred in exon I and intron II, and 1 deletion in intron I, as well as 22 single nucleotide polymorphisms (SNPs) such as C 54, A 191, A 468, etc. in the 3 exons (Fig. 2).

Among the 12 sequences from *T. timopheevii* accessions, there were 17 SNPs, including five, four, and eight in exon I, II and III, respectively. Three transversions involving two G→T and one A→T were observed in the two sequences HQ843871 (PI251017)

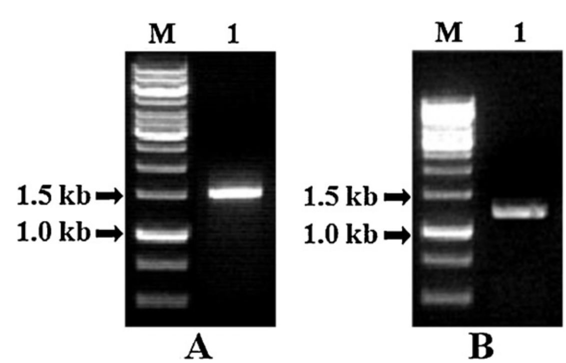

Fig. 1. PCR amplifications of *NAM-G1* gene from the accession PI94761 being representative of *T. timopheevii*. Lane M is the DNA ladder; Lane 1 is PCR amplification products using genomic DNA (A) and cDNA templates (B), respectively.

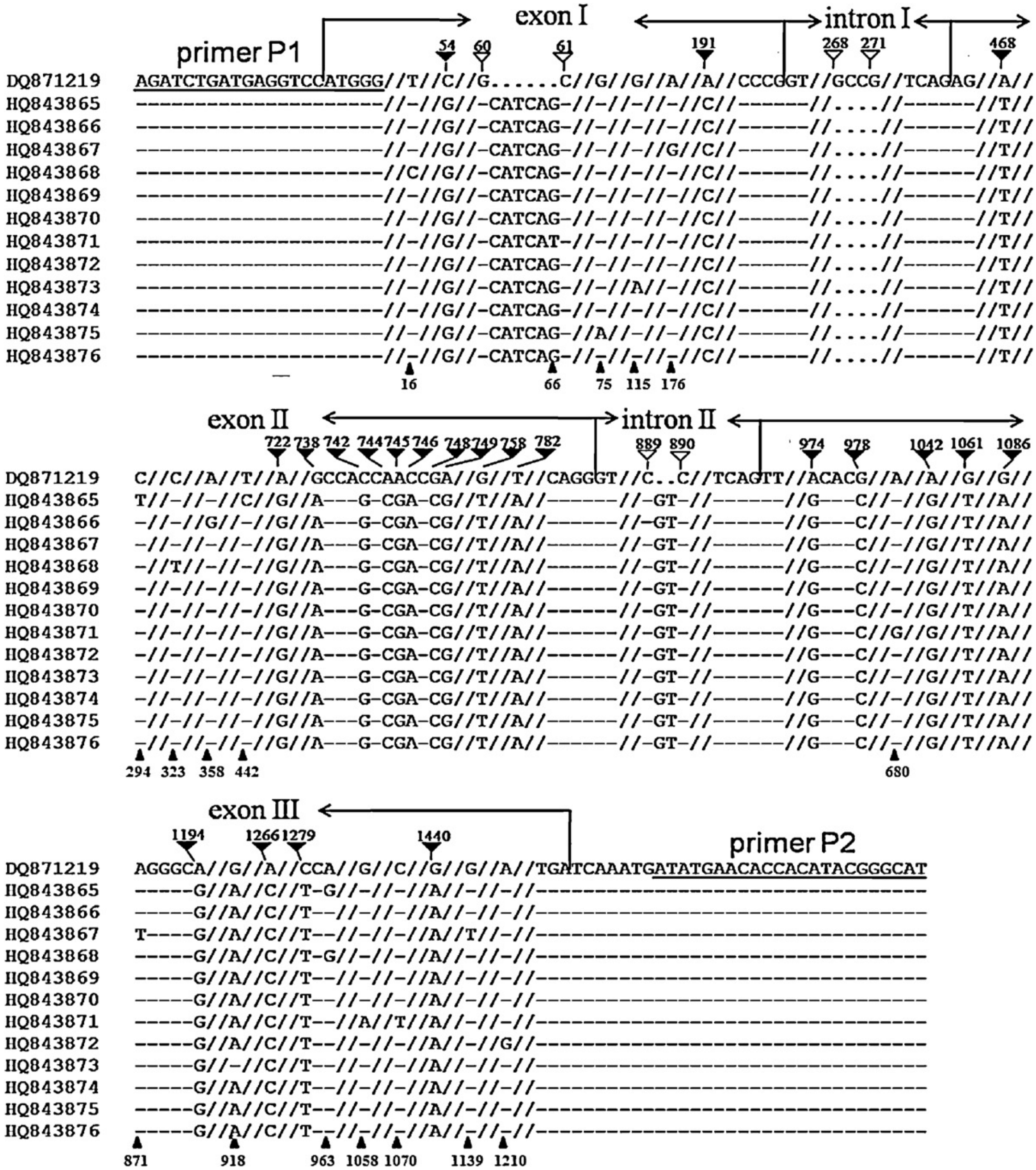

Fig. 2. Comparison of the nucleotide sequences between the 12 *NAM-G1* (HQ843865–HQ843876) genes from *T. timopheevii* and the previously reported functional *NAM-B1* gene (DQ871219) from *T. turgidum* ssp. *dicoccoides*. Identical sequences are not listed and represented as double biases (//) or dashes (–). Dots indicate deletions. The positions of different nt as well as start and end nt of insertion/deletion segments between *NAM-G1* and *NAM-B1* genes are marked by solid and hollow inverted triangular with number, respectively. Single nucleotide polymorphisms (SNPs) among the full-length coding regions of 12 *NAM-G1* genes are indicated by upward no-tailed arrowheads with number.

and HQ843867 (CItr15205). The other transitions included two T→C, three G→A, three C→T, and six A→G, occurring in the eight sequences HQ843865 (PI94761), HQ843866 (PI94760), HQ843867 (CItr15205), HQ843868 (PI119442), HQ843871 (PI251017), HQ843872 (PI266850), HQ843873 (PI272523), and HQ843875 (PI282932) (Fig. 2 and Table 3).

Amino acid sequence analysis

The amino acid (aa) sequences deduced from the 12 *NAM-G1* genes had an identical polypeptide length with 407 aa. Similar to the protein encoded by the functional *NAM-B1* gene DQ871219, they possessed a typical structure of a NAC protein, containing a highly conserved N-terminal NAC domain with five sub-domains A, B, C, D, and E, and a highly divergent C-terminal transcriptional activation region TAR (Fig. 3). Yet, between the 12 *NAM-G1* genes and the *NAM-B1* gene DQ871219, there were two insertion/deletion aa between Q 20 and H 21, as well as 11 aa substitutions like H 18, N 64, I 85, etc., resulting from 14 non-synonymous mutations of 22 SNPs (Fig. 2). The former 7 substitutions occurred in the outer of A, B, C, D, and E sub-domains of the N-terminal NAC domain, and the others in the C-terminal TAR (Fig. 3).

Among the 12 NAM-G1 protein sequences, there were 13 variations of amino acids (Fig. 3 and Table 3). The aa polymorphisms resulted from base substitutions including base transitions and transversions without insertion/deletion. Seven occurred in the

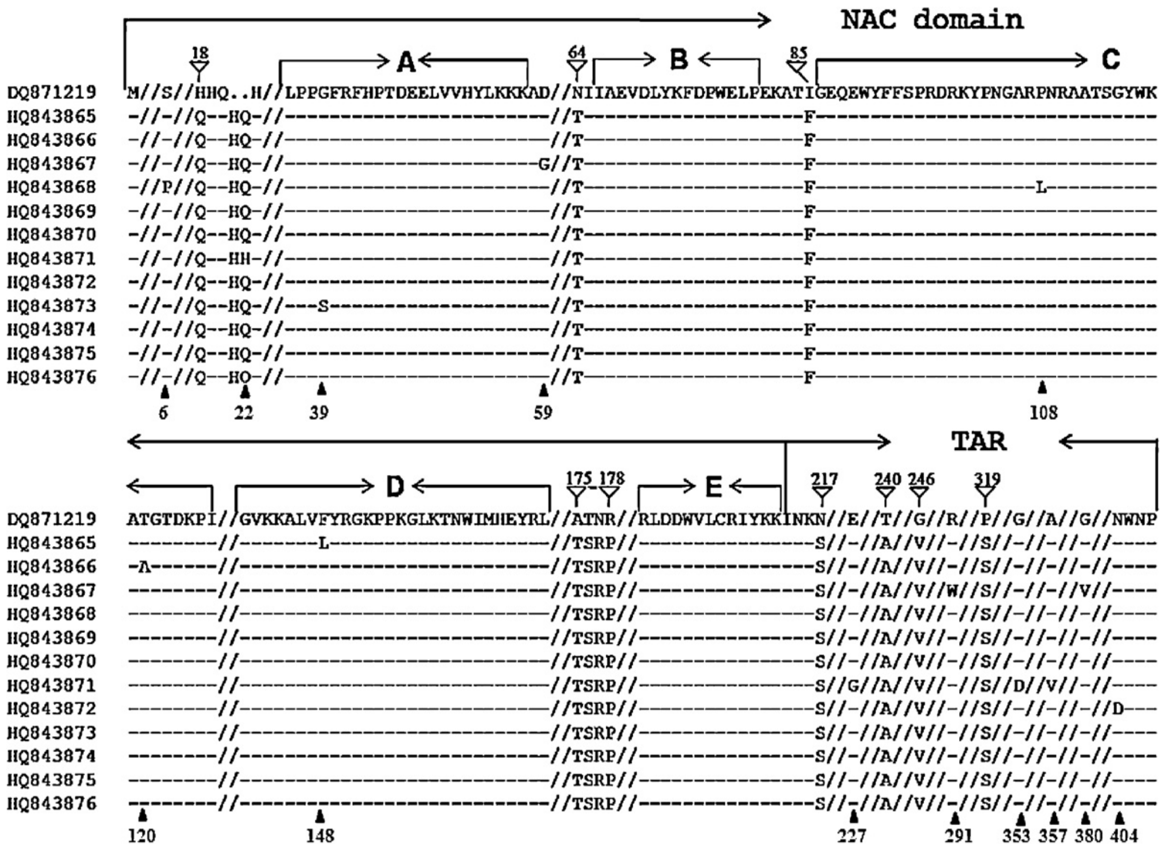

Fig. 3. Comparison of the deduced amino acid sequences between the 12 *NAM-G1* (HQ843865–HQ843876) genes of *T. timopheevii* and the previously reported *NAM-B1* (DQ871219) gene of *T. turgidum* ssp. *dicoccoides*. The corresponding NAC domain (sub-domain A, B, C, D, and E) and TAR (transcriptional activation region) are indicated. Identical amino acids are not listed and are represented as double biases (//) or dashes (–). Dots indicate deletions. The positions of different amino acids are marked between the 12 *NAM-G1* genes and the *NAM-B1* gene by hollow inverted triangular with number. Amino acid variations among the 12 *NAM-G1* genes are indicated by upward no-tailed arrowheads with number.

sub-domains A with its two sides, C, D of NAC, and six in the TAR. However, not any amino acid was changed in both the B and E sub-domains (Fig. 3 and Table 3). These protein polymorphisms were brought from the seven *NAM-G1* genes HQ843865, HQ843866, HQ843867, HQ843868, HQ843871, HQ843872, and HQ843873. HQ843867 of CItr15205 and HQ843871 of PI251017 had more variation among amino acids than others, which were with the three substitutions like D→G (59), R→W (291) and G→V (380), and the four ones, like Q→H (22), E→G (227), G→D (353) and A→V (357), respectively. The variation among amino acids mainly occurred in the TAR, with the two R→W, G→V and the three E→G, G→D, A→V substitutions, respectively, being different from those of the others (Fig. 3 and Table 3).

Expression analysis of the NAM-G1 genes

The real-time PCR efficiencies of *NAM-G1* and *actin* genes were for 96.4% and 97.6%, respectively, and the coefficients of determination (R^2) were high for 0.997 and 0.990, respectively (Table 1). The amplification specificities were confirmed by the melting curve that had a single peak. The amplicons checked with 1.5% agarose gel electrophoresis with GoldenView^{TM} staining revealed a single band with the expected size (Fig. 4A).

The relative expression levels of *NAM-G1* genes in qRT-PCR were significantly different among the 12 *T. timopheevii* accessions ($F = 85.25$, $P < 0.01$) (Fig. 4B and C). The relative expression quantities of the genes ranged from 0.63 to 1.41. The genes from PI272530, PI272523, PI94760, PI119442, and PI282932 possessed a higher expression quantity, while those of the two genes from CItr15205 and PI251017, were at a lower level.

GPC and the relationship between it and NAM-G1 gene

Grain protein contents (GPCs) among the tested 12 *T. timopheevii* accessions were significantly different ($F = 2.36$, $P < 0.05$). The 6 accessions PI272530, PI272523, PI94760, PI119442, PI282932, and PI190974 possessed the first high GPC, about 18%. The 4 accessions PI221421, PI94761, PI266850, and PI282933 had the medium-high GPC, ranging from 15.97% to 17.13%. The 2 accessions PI251017 and CItr15205 had the lowest GPC, from 14.57 to 14.80% (Fig. 4D).

Correlation analysis showed that the GPC was highly positively correlated with the *NAM-G1* expression level ($r = 0.957$, $P < 0.01$). Comparative analysis discovered that the variation trend of GPC and gene expression levels was highly accordant (Fig. 4C and D).

Phylogenetic analysis of NAM-G1 gene

The phylogenetic tree constituted of the present 12 and other previously published 14 *NAM* genes was clearly separated into the two independent groups I and II, belonging to the homoeologous group-6 and -2 chromosomes, respectively. Within the group I, twenty-three *NAM* sequences were further divided into *NAM-H1*, *NAM-A1*, *NAM-D1*, *NAM-B1*, and *NAM-G1* sub-groups. The *NAM-G1* sub-group from *T. timopheevii* shared a high bootstrap value of 99 with *NAM-B1* gene from *T. turgidum* ssp. *dicoccoides* (Fig. 5 and Table 2).

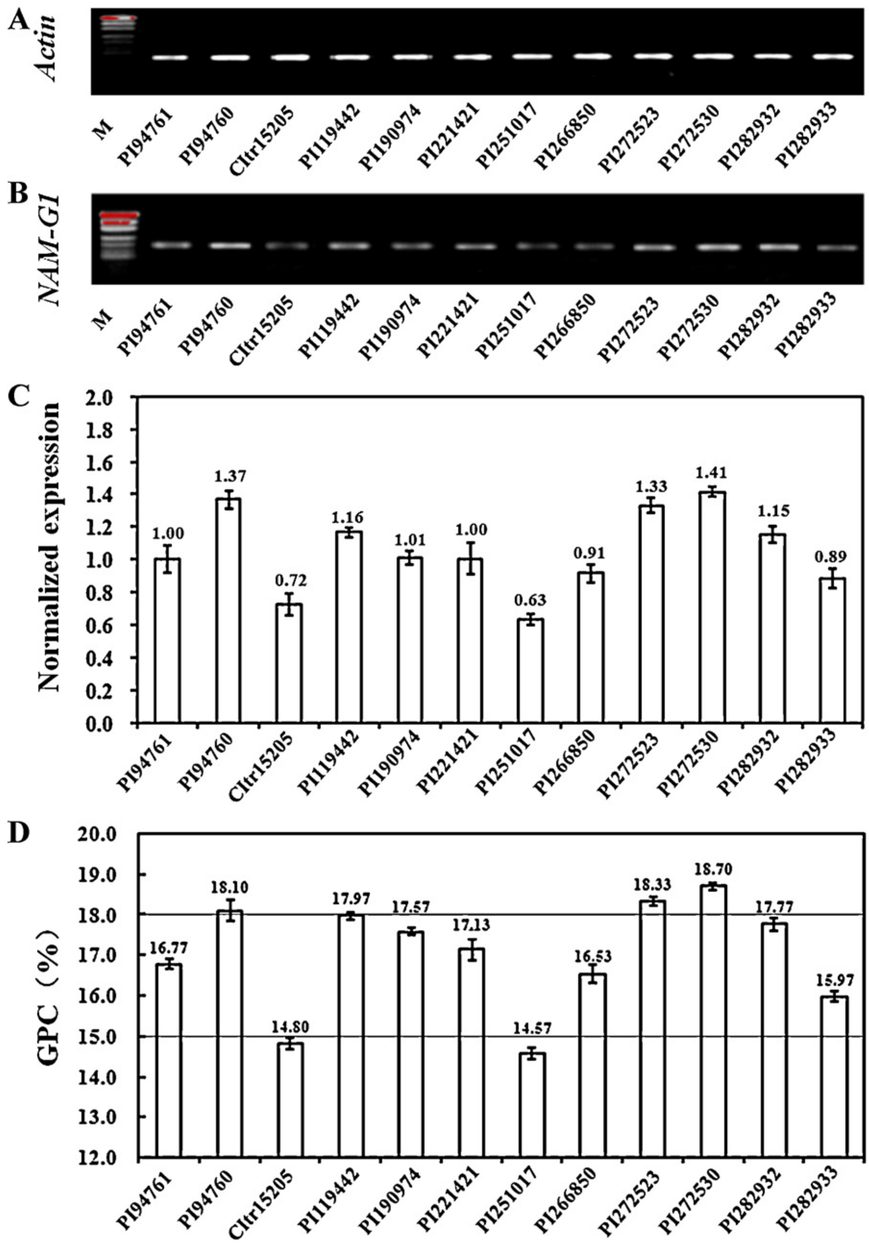

Fig. 4. Expression pattern of *NAM-G1* gene in qRT-PCR and GPC from twelve *T. timopheevii* accessions. A and B are the amplicons of *actin* and *NAM-G1* genes, respectively, and Lane M is the DNA ladder; C is the expression pattern of *NAM-G1* gene; D is the representation of GPC, and the horizontals lines represent the first high GPC (18.0%) and medium-high GPC (15.0%).

Discussion

NAC family proteins encoded by *NAC* gene are identifiable by the typical structure including both a highly conserved N-terminal NAC domain (Souer et al., 1996; Aida et al., 1997), and a highly divergent C-terminal transcriptional activation region (TAR) (Ren et al., 2000; Xie et al., 2000; Duval et al., 2002; Ernst et al., 2004). The NAC domain, which is associated with DNA-binding ability, generally contains the five sub-domains A, B, C, D, and E (Kikuchi et al., 2000; Apweiler et al., 2001; Duval et al., 2002; Ooka et al., 2003; Olsen et al., 2005; Guo and Gan, 2006; Uauy et al., 2006; Liu et al., 2009; Jamar et al., 2010). Of them, B and E are highly conserved, which is responsible for maintaining the correct structure of NAC domain in dimerization or DNA attachment (Duval et al., 2002; Xue et al., 2006). In this study, all the 12 *NAM-G1* genes from *T. timopheevii* possessed the typical structural characteristics of *NAC* family genes, containing the complete encoding sequence for both NAC domain and TAR. Their sequences were highly similar to that of the ancestral functional *NAM-B1* gene DQ871219 (Uauy et al., 2006) and had very high identity of 99.5% with it (Figs. 2 and 3). The differences among the 12 *NAM-G1* genes resulted from some base substitutions involving both transitions and transversions (Figs. 2 and 3).

Grain protein content (GPC) is associated with both nutrient and process qualities of wheat. In this study, GPCs among the 12 *T. timopheevii* accessions were significantly different, and ranged from 14.57% to 18.70% (Fig. 4D). According to the criterion of protein content over 15% for processing first-class quality bread (Xu et al., 2006), the 10 accessions PI272530, PI272523, PI94760, PI119442, PI282932, PI190974, PI221421, PI94761, PI266850, and PI282933 possessed high levels of GPC. Comparatively, GPCs of the 2 accessions PI251017 and CItr15205 were lower, but also arrived at the level for making second-class quality bread (Xu et al., 2006). These results also revealed, to a certain extent, that *T. timopheevii* wheat possesses abundant germplasm resources of high protein content.

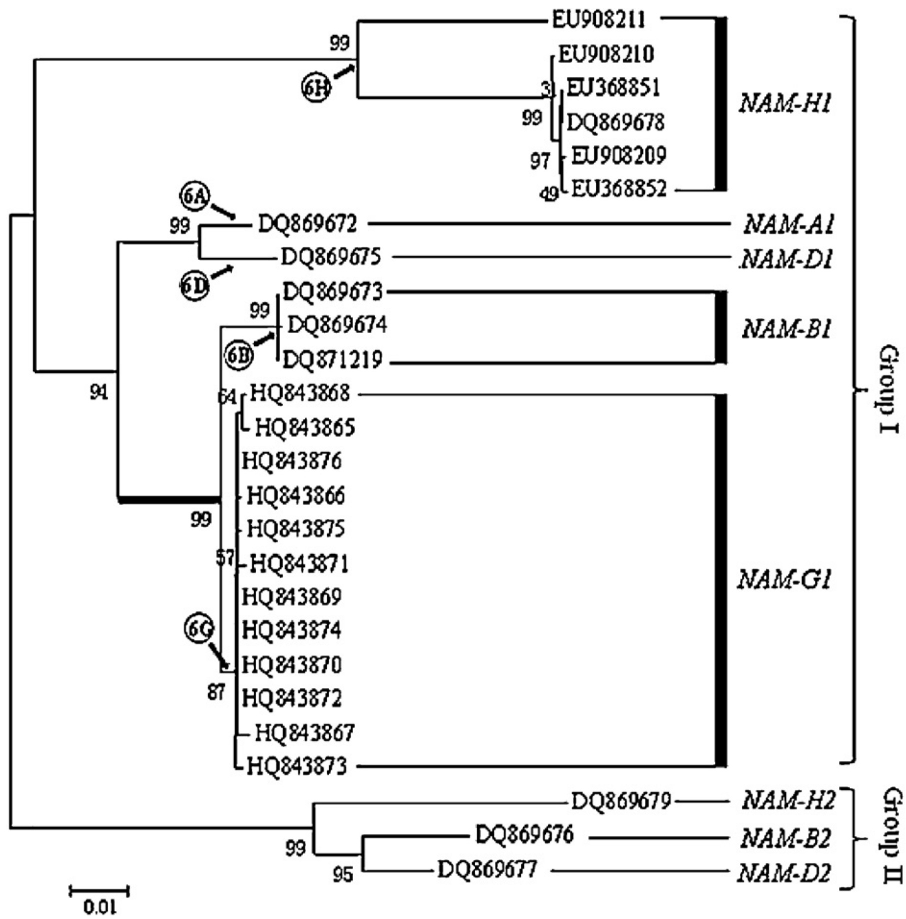

Fig. 5. Phylogenetic relationships among the 26 *NAM* genes, including 12 *NAM-G1* from this study and 14 previously reported genes.

A deletion or a non-functional copy of the ancestral *NAM-B1* gene as a result of a frame-shift mutation like the non-functional *NAM* gene DQ869674 in *T. turgidum* ssp. *durum* (Table 2), is associated with the low GPC of modern wheat varieties (Uauy et al., 2006). This is also supported by other authors (Distelfeld et al., 2008; Jukanti and Fischer, 2008; Jamar et al., 2010). Nevertheless, the *HvNAM-1* gene without polymorphisms cannot explain the GPC differences among 11 *Hordeum vulgare* varieties (Jamar et al., 2010). Jamar et al. (2010) conjectured that expression difference of the *HvNAM-1* gene or other genes should play a role in GPC regulation. In the present research, the 12 *NAM-G1* genes from 12 *T. timopheevii* accessions showed significantly different expression levels in qRT-PCR. The variation trend of *NAM-G1* gene expression levels was highly accordant with that of GPCs (Fig. 4C and D). Correlation analysis suggested that the relative expression quantity was significantly positively correlated with the GPC. Comparison analysis revealed that the two genes from CItr15205 and PI251017 with the lowest GPC had the lowest expression quantity (Fig. 4). Compared with others, they contained more base substitutions in Exon III, leading to more amino acid variations in the C-terminal TAR, with the two R→W, G→V, and the three E→G, G→D, A→V substitutions, respectively (Figs. 2 and 3 and Table 3). The highly divergent TAR has been considered as affecting the function of NAC protein (Ooka et al., 2003; Olsen et al., 2005). The impact of aa modification on GPC variation is also supported by the studies on barley and wheat *NAM* genes (Distelfeld et al., 2008; Jamar et al., 2010). Therefore, GPC variation among the *T. timopheevii* accessions may be attributed to the difference of expression levels of *NAM-G1* genes. The difference of NAM-G1 protein sequences might be also responsible for GPC variation.

Nitrogen (N) content in leaf is highest during the whole wheat development, which contributes the most N accumulated mainly before flowering to GPC (Jing et al., 2004; Barneix, 2007). The *NAM-B1* gene plays an important role in regulating the N remobilization from leaf to grain (Waters et al., 2009). Uauy et al. (2006) confirmed that the functional *NAM-B1* gene DQ871219 from *T. turgidum* ssp. *dicoccoides* with multiple pleiotropic effects, which accelerates senescence and increases nutrient remobilization from leaves to developing grains, resulting in increase of Zn, and Fe concentrations besides GPC in the grain. This gene was also associated with bread and pasta quality parameters like water absorption, mixing time, loaf volume and wet gluten content (Brevis et al., 2010). The present study showed, that the 12 *NAM-G1* genes from *T. timopheevii* were closely clustered with all the previously reported *NAM-B1* genes (Fig. 5), and had a very high identity of 99.5% with the functional gene DQ871219 (Uauy et al., 2006). So, the *NAM-G1* genes regulating high GPC might be also used to improve quality of modern wheat cultivars, just as the orthologous *NAM-B1* gene with the pleiotropic effects.

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