Haynaldia villosa NAM-V1 is linked with the powdery mildew resistance gene Pm21 and contributes to increasing grain protein content in wheat

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Abstract

Background: The 6AL/6VS translocation lines, carrying the wheat powdery mildew resistance gene Pm21, are planted on more than 3.4 million hectares. The NAM-A1 gene, located on chromosome 6AS of hexaploid wheat, has been implicated with increased wheat grain protein content (GPC). However, the NAM-A1 gene was removed from the 6AL/6VS translocation lines after the original chromosome 6AS was replaced by chromosome 6VS of Haynaldia villosa. The present study aimed to clone the NAM homologous gene from chromosome 6VS, to analyze the changes of GPC in the 6AL/6VS translocation lines, and to develop related molecular markers for wheat molecular breeding.

Results: A new NAM family gene, NAM-V1, was cloned from 6VS of H. villosa (GenBank ACC. no. KR873101). NAM-V1 contained an intact open reading frame (ORF) and putatively encodes a protein of 407 amino acids. Phylogenetic analysis indicated that NAM-V1 was an orthologous gene of NAM-A1, B1, and D1. The determination of GPC in four Pm21 F2 segregation populations demonstrated that the replacement of NAM-A1 by NAM-V1 confers increased GPC in hexaploid wheat. Multiple sequence alignment of NAM-A1, B1, B2, D1, D2, and V1 showed the single nucleotide polymorphism (SNP) sites for each of the NAM genes, allowing us to develop a molecular marker, CauNAM-V1, for the specific detection of NAM-V1 gene. Our results indicate that CauNAM-V1 can be used as a novel DNA marker for NAM-V1, and can also be used for selecting Pm21 in wheat breeding programs. Further, we developed a marker, CauNAM-ABD, for the amplification and simultaneously distinguish among the NAM-A1, NAM-B1, NAM-B2, NAM-D1, and NAM-D2 genes in a single step. CauNAM-ABD enabled us to develop an efficient "one-marker-for-five-genes" procedure for identifying genes and its copy numbers related with grain protein content.

Conclusion: Here, we report the isolation of the NAM-V1 gene of H. villosa. This gene contributes to increasing GPC in 6AL/6VS translocation wheat lines. We developed a molecular marker for the specific detection of NAM-V1 and a molecular marker that can be used to simultaneously distinguished among the NAM-A1, NAM-B1, NAM-B2, NAM-D1, and NAM-D2 genes in a single step.

Keywords: Wheat, Haynaldia villosa, NAM gene, Grain protein content, Pm21

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Background
Common wheat (*Triticum aestivum*) is one of the most important crops in the world, accounting for about 20% of the world’s total calorie consumption and providing about 70 million tons of protein every year. Grain protein concentration (GPC) is an important agronomic trait in wheat. Wheat varieties with high gluten and GPC > 12% are suitable for making bread. Wheats with low gluten content and GPC < 9% are suitable for making cookies and cakes [1]. It has been established that the GPC of wheat is a quantitative trait that is affected by environmental conditions [2, 3]. Many wild relatives of wheat, including wild emmer wheat (*Triticum turgidum* L. var. *dicoccoides*), have high GPC. In 1991, a complete set of disomic substitution lines were developed by the introgression of each of the chromosomes of wild emmer wheat with high GPC (DIC) into the durum cultivar ‘Langdon’ (LDN). The substitution line in which the chromosome LDN-6B was completely replaced by DIC-6B, showed the highest protein yield [1, 4]. Later, a quantitative trait locus (QTL) for wheat GPC was mapped onto the short arm of chromosome (6BS) using the recombinant inbred lines DIC and LDN [5], and later mapped within a 2.7 cM region as a single Mendelian locus, *Gpc-B1* [6]. Uauy et al. (2006) positionally cloned *Gpc-B1* and established that it is a member of the NAC transcription factor; they renamed it *NAM-B1* [7].

The NAC transcript factors are a plant-specific family of transcription factors with a variety of biological functions, including roles in the development of embryos and flowers and responses to biotic and abiotic stress [8–12]. The name NAC is related to the NAM (No Apical Meristem) gene of *Petunia*, the *ATAF1* and *ATAF2* (*Arabidopsis* transcription activation factor) of *Arabidopsis*, and the CUC2 (cup-shaped cotyledon) gene of *Arabidopsis*.

*NAM-B1* in wheat is a typical NAC transcription factor gene; these genes are highly conserved in maize, rice, barley, and other cereal crops [7]. In addition to the *NAM-B1* gene on 6BS, its orthologous genes *NAM-A1* on 6AS and *NAM-D1* on 6DS, and its homologous genes *NAM-B2* on 2BS and *NAM-D2* on 2DS have also been identified. The *NAM* genes of wheat are associated with increasing wheat grain protein, zinc, and iron content. The function of *NAM-B1*, *B2*, *A1*, *D1*, and *D2* are thought to be largely redundant. The silencing of *NAM* genes resulted in decreases of 30%, 36% and 38% for GPC, iron, and zinc, respectively [7]. Recently, NAM orthologous genes have been identified in *Hordeum vulgare* and *Triticum timopheevii* Zhuk; these have been shown to have the same function [1, 13, 14].

*Haynaldia villosa* (2n = 2 × 14, V genome), belonging to the tribe *Triticeae*, is an annual or perennial diploid plant [15]. As one of the important genetic resources for wheat genetic improvement, *H. villosa* contains many excellent traits, including of resistance to cold, salt, drought, and various wheat diseases, winter hardiness, and vigorous tillering ability, multi-spikelet morphology, and high grain protein content [16]. Considerable success has been made in transferring beneficial genes from *H. villosa* into wheat via the development and use of substitution and translocation lines. For example, the translocation lines 6AL/6VS carry the powdery mildew resistant gene *Pm21* and showing strong resistance to most of the powdery mildew isolates. The varieties derived from the 6AL/6VS translocation lines are now planted more than 3.4 million hectares [17], The *Gli-V2* gene for k-type sulfur-rich prolamins was also identified from 6VS of *H. villosa* [18, 19]. However, to date, no NAM genes have been reported in *H. villosa*. For 6AL/6VS wheat translocation lines, it is unknown about the changes of GPC when the functional *NAM-A1* gene on 6A chromosome was removed.

In this study, we report the isolation of the *NAM-V1* gene from *H. villosa*. We developed a molecular marker, *CauNAM-V1*, which was specific to *NAM-V1* and is linked to the powdery mildew resistance gene *Pm21*. In addition, our results showed that *NAM-V1* contributes to increasing GPC in hexaploid wheat. We also developed a marker, *CauNAM-ABD*, which can amplify and simultaneously distinguish among *NAM-A1*, *NAM-B1*, *NAM-B2*, *NAM-D1*, and *NAM-D2* in a single step. *CauNAM-ABD* enabled the development of an efficient “one-marker-for-five-genes” procedure for identifying genes related with grain protein, zinc, and iron content.

Results
Cloning of the *NAM-V1* gene
The genomic DNA and cDNA of the *NAM-V1* gene were amplified from *H. villosa* using two pairs of primers (Fig. 1a). Sequence alignment with known NAM genes cataloged in GenBank confirmed that *NAM-V1* is a new NAM homologous gene (NCBI GenBank ACC. no. KR873101). The *NAM-V1* gene had 92% identity with *NAM-A1*, 91% identity with *NAM-D1*, and 90% identity with *NAM-B1*. *NAM-V1* encodes a NAM superfamily domain protein (Fig. 1b). The full-length of *NAM-V1* gene is 1,528 bp, and contains three exons and two introns. It is predicted to encodes a protein of 407 amino acids with a molecular weight of 43 KDa (Fig. 1c).

Phylogenetic analysis of NAM proteins
A neighbor-joining phylogenetic tree was deduced using MEGA 6.0 based the predicted amino acid sequences of *NAM-V1* and NAM family proteins of other species (Fig. 2). A total of nineteen proteins were classified into four groups. *NAM-V1* belongs to group I, the largest group (10 genes). Group I also includes NAM-A1 encoded by a gene on chromosome 6A and NAM-B2 from chromosome 2B of durum wheat (*T. turgidum* var. *durum*), NAM-B1 from chromosome 6B of wild emmer wheat (*T. turgidum* L. var. *dicoccoides*), NAM-D1 from chromosome 6D and
NAM-D2 from chromosome 2D of Aegilops tauschii, HV-NAM1 and HV-NAM2 from the H genome of Hordeum vulgare, and NAM-G from the G genome of Triticum turgidum Zhuk. The phylogenetic tree showed that NAM-VI belonged to the same group with NAM-A1, B1 and D1, the orthologous genes from the sixth chromosomes of the A, B, and D sub genomes, respectively. NAM-B2 and NAM-D2, encoded by genes on the second chromosomes of the B and D sub genomes, respectively, were also close to NAM-VI. It has been shown that NAM-A1, B1, B2, D1, and D2 all function in the regulation of grain protein content, iron, and zinc. Therefore, it is reasonable to speculate that the intact NAM-VI gene might encode a protein with a similar function. There were three proteins in group II; all three are from Arabidopsis. Among these AtNAC2 is associated with lateral root development [20]. Group III included three proteins. In this group, OsABA91266 and OsABA95705 come from Oryza sativa. TaNAC69 come from wheat, which responds to cold, drought and salt stress, and being associated with the adaptability of wheat under stress conditions [21]. Group IV also included three genes, that was TaNAC2 of wheat, OsN_NP 912423 of Oryza sativa and AtNAC3 of Arabidopsis. Both TaNAC2 and OsN_NP 912423 were related to stress tolerance [22, 23].

**Sequence alignment and molecular maker development**

In order to develop specific markers for the detection of the NAM-VI gene and other NAM genes in hexaploid common wheat, a multiple sequence alignment was conducted (Fig. 3, Additional file 1: Figure S1). Multiple sequence alignment of NAM-A1, B1, B2, D1, D2, and V1 showed that there was a specific “ATGTC” insert at the 247th nucleotide of NAM-VI. The “G to T” single nucleotide polymorphism (SNP) was only observed in the NAM-VI gene at 785th nucleotide (Fig. 3a). These polymorphic sites were introduced into the 3’ region of the forward and reverse primers, allowing us to develop a specific molecular marker, “CauNAM-VI”, for the NAM-VI gene.

We also found a region rich in polymorphism from the 240th -430th nucleotide of the NAM genes; this region contained six obvious polymorphic sites (Fig. 3b). A pair of primers, “CauNAM-ABD”, for the simultaneous detection and discrimination among all of these NAM genes, was designed according to the sequence alignment results. For NAM-A1, B1, B2, D1, D2, and V1, the
expected lengths of the amplification products were 294 bp, 290 bp, 265 bp, 283 bp, 270 bp and 270 bp, respectively.

**Molecular marker CauNAM-V1 is specific for the NAM-V1 gene and is linked with powdery mildew resistance gene Pm21**

To test if the molecular marker CauNAM-V1 was specific for the NAM-V1 gene, it was used with DNA from common wheat Chinese Spring (CS), Aegilops tauschii, T. urartu, T. monococcum, Chinese Spring nullisomic-tetrasomic lines CS N2B-T2D and CS N6A-T6B, susceptible and resistance individuals from F2 a segregation population of Pm21, and a wheat cultivar carrying Pm12. CauNAM-V1 was able to amplify a product only in the materials carrying Pm21 that contained the 6VS chromosome of H. villosa (Fig. 4a). According to the powdery mildew resistance identification results (Fig. 4b), ten resistant individuals and ten susceptible individuals were used for amplification via CauNAM-V1. Using CauNAM-V1, a product was amplified from all of the tested resistant individuals; no product was amplified from any of the tested susceptible individuals (Fig. 4c). Thus the marker CauNAM-V1 is linked to Pm21. These experiments also indicate that NAM-V1 comes from chromosome 6 V, not from 6A or 6D.

**Detection of NAM-A1, B1, D1, D2, and B2 using CauNAM-ABD**

Using CauNAM-ABD, five specific products with different sizes were amplified from common wheat Chinese Spring (CS); these products represented NAM-A1 (294 bp), B1 (290 bp), D1 (283 bp), D2 (270 bp) and B2 (265 bp), respectively (Fig. 5). For Pm21 and Pm12, there were no bands for NAM-A1 or NAM-B1, owing to the deletion of 6AS and 6BS, respectively. Only one specific amplification band could be detected with T. monococcum (A^m^ genome). In the Chinese Spring nullisomic-tetrasomic lines CS N6A-T6B, the band representing the NAM-A1 product were not observed. The band for the NAM-B1 product was brighter than the other bands because there are two copies of the NAM-B1 gene in CS N6A-T6B. The same phenomenon was also observed in CS N2B-T2D, suggesting the CauNAM-ABD can also measure the copy number of NAM genes. Thus, CauNAM-ABD can amplify and distinguish NAM-A1, NAM-B1, NAM-B2, NAM-D1, and NAM-D2 in hexaploid common wheat.

**Correlation analysis of GPC and genotype**

In order to analyze the contribution of NAM-V1 and NAM-A1 to GPC, four Pm21 F2 segregation populations (W50200, W50175, W50156, and W50176) were constructed. The average GPC for the NAM-V1/NAM-A1 genotypes in W50200, W50175, W50156, and W50176 were 13.94 %/13.42 %, 17.99 %/16.88 %, 13.33 %/13.31 %
and 15.41 %/14.33 %, respectively (Fig. 6). The GPC of the individuals containing the NAM-V1 gene were higher than those containing the NAM-A1 gene in all four of the segregation populations. The average increasing of GPC were 0.52 %, 1.11 %, 0.02 % and 1.08 % in four populations. These results suggest that NAM-V1 contributes to increasing GPC in 6AL/6VS translocation lines of hexaploid wheat.

**Discussion**

In developing countries, malnutrition caused by the lack of one or many kinds of trace elements is affecting more than 20 million people. In some areas, as many as 47 % of preschool children suffer from iron deficiency, resulting in poor physical and mental development. Malnutrition caused by zinc deficiency affects about 10 million people worldwide. Zinc deficiency can also cause retarded growth and can destroy the body’s immune system [24]. The NAM-B1 gene from wild emmer wheat and the NAM-A1, NAM-D1, NAM-B2, and NAM-D2 genes from durum wheat not only affect the protein content of wheat grain, the expression levels of these genes are also positively correlated with the iron and zinc levels in grain [7]. In this

![Fig. 4 Specific molecular marker for the NAM-V1 gene.](image1)

a) PCR amplification of the NAM-V1 gene using a specific molecular marker in different wheat materials. b) Identification of the resistance of Pm21 segregation population to powdery mildew. c) PCR detection using the CauNAM-V1 specific molecular marker in the segregation population resistant to powdery mildew.

![Fig. 5 The amplification of primer CauNAM-ABD in different species.](image2)

Line 1 to line 9 represented Chinese Spring (CS), 6AL/6VS translocation of wheat carrying powdery mildew resistant gene Pm21, 6BL/6SS translocation of wheat carrying powdery mildew resistant gene Pm12, Aegilops tauschii, T. urartu, T. monococcum, CS (N2B-T2D) and CS (N6A-T6D), respectively.
study, a new homologous gene of NAM-B1, NAM-V1, was cloned from H. villosa. Gene structure analysis showed that the gene had a complete open reading frame, suggesting that NAM-V1 is a functional gene.

Molecular marker-assisted selection plays an important role in current crop breeding methods, especially in plant disease-resistance breeding. To date, about 70 powdery mildew resistance genes have been identified. One of these is Pm21, an effective disease resistance gene for most of the physiological races of the fungal pathogen Blumeria graminis f. sp. Triticil [17]. Pm21 and NAM-V1 were all identified from 6VS of H. villosa. Because chromosome synopsis did not occur between 6AS of H. villosa and 6AS of common wheat during meiosis [25], the specific marker CauNAM-V1 can be used to detect Pm21 and loci on 6VS that may be associated with other agronomic traits such as the k-type sulfur-rich prolamins gene Gli-V2. Previous studies have shown that NAM-A1, NAM-B1, NAM-B2, NAM-D1, and NAM-D2 are all functional genes that are highly conserved in hexaploid common wheat [7]. Additionally, owing to sequence similarity, it has been difficult to discriminate the genotype of NAM genes. Here, we developed a molecular marker that can simultaneously amplify NAM-A1, NAM-B1, NAM-B2, NAM-D1, and NAM-D2. The genotype and gene copy numbers can be estimated according the electrophoresis results, providing a useful method for screening high grain protein, zinc, and iron content wheat varieties.

Pm21 is one of the most effective resistance genes against powdery mildew. The 6AL/6VS translocation lines of hexaploid wheat, which carry Pm21, has been widely applied in wheat breeding programs. However, it is unclear whether the changes in GPC that occur following the introgression of chromosome segments of H. villosa, when original function gene NAM-A1 in 6A chromosome was removed. Here, we isolated the NAM-V1 gene from H. villosa and showed that NAM-V1 is an intact and likely functional gene in 6AL/6VS translocation lines of hexaploid wheat. In common wheat, NAM-A1 is known to be a functional gene. Our results demonstrate that the replacement of NAM-A1 by NAM-V1 confers increased grain protein content, implying that NAM-V1 is more efficient than NAM-A1 in increasing the GPC. The differences in the efficiency between NAM-V1 and NAM-A1 might be affected by many factors such as gene structure, gene expression levels, and/or promoter sequences. In addition, this study showed that the powdery mildew resistant genes Pm21 and NAM-V1 are responsible for co-segregating traits in wheat 6AL/6VS translocation lines. Therefore, the specific marker CauNAM-V1 can also be used for selecting both disease resistance and high GPC genotypes in wheat breeding programs. CauNAM-V1 is a dominant molecular marker that can be easily detected by agarose gel electrophoresis. CauNAM-V1 should help efforts to utilize disease resistance and high protein genes from 6VS of H. villosa in wheat improvement programs.

Conclusions

Here, we report the isolation of the NAM-V1 gene of H. villosa. This gene contributes to increasing GPC in 6AL/6VS translocation wheat lines. We developed a molecular marker for the specific detection of NAM-V1 and a molecular marker that can be used to simultaneously distinguished among the NAM-A1, NAM-B1, NAM-B2, NAM-D1, and NAM-D2 genes in a single step.

Methods

Plant material and fungal isolates

The einkorn wheat cultivars T. urartu (A\textsuperscript{u}) and T. mononoceccum (A\textsuperscript{m}) were obtained from the Plant Germplasm Insitute of Kyoto University (Japan). Powdery mildew isolate E09 was provided by Prof. Xiaoyu Duan of the Institute of Plant Protection of the Chinese Academy of Agricultural Sciences. The wheat lines 2 N1862 (containing the powdery mildew resistance gene Pm12) and W50200 (containing the powdery mildew resistance gene Pm21), as well as the common wheat cultivars Chinese Spring and Xuezao are kept at our laboratory. Two Chinese Spring nullisome-tetrasomic lines for homeologous group 2 (CS N2B-T2B) and homeologous group 6 (CS N6A-T6B) were kindly provided by Drs. W. J. Raupp and B. S. Gill of the Wheat Genetics Resource Centre of Kansas State University, USA.

Powdery mildew resistance identification

Powdery mildew resistance identification was performed as described in a previous study [26]. The reaction of seedlings to powdery mildew were scored on 0 (no visible symptoms), 1 (necrotic flecks), 1 (necrosis with low sporulation),
Table 1 Primers used in this study

| Primer name  | Primer sequence (5’-3’) | Product length | Annealing Temperature | Purpose                        |
|--------------|-------------------------|----------------|-----------------------|-------------------------------|
| NAMORF1      | F: GATGAGGTCCATGGGCAG   | 1528 bp        | 60 °C                 | Genomic DNA cloning           |
|              | R: TCTATTTCCTAGGGATTCC  |                |                       |                               |
| NAMORF2      | F: ATGGGCAAGCTGCACTCA   | 1540 bp        | 60 °C                 | cDNA cloning                  |
|              | R: TCAGGGATCCAGTTACG    |                |                       |                               |
| CauNAM-V1    | F: TCCCCGGTATGCCATGTC   | 575 bp         | 58 °C                 | Specific molecular marker for NAM-V1 |
|              | R: AAGATACCGGACTACGTA   |                |                       |                               |
| CauNAM-ABD   | F: TACAAGTTCGACCCATGGGA | 265 -294 bp    | 58 °C                 | Molecular marker for NAM-A1, B1, D1, B2 and D2 |
|              | R: GGCAGGGCTGAAAGGTA    |                |                       |                               |

Bioinformatics analysis of the NAM-V1 gene

The open reading frame (ORF) of the NAM-V1 gene was predicted using the NCBI online ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weight and isoelectric point were predicted using DNastar software (http://www.dnastar.com). The conserved domain(s) were predicted by alignment with NCBI CDD database (http://www.ncbi.nlm.nih.gov/cdd). Multiple sequence alignments were analyzed using ClustalW software (http://www.ch.embnet.org/software/ClustalW.html). Multiple-alignment files were shaded using BOXSHADE 3.2 (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic analysis conducted with MEGA 6 (www.megasoftware.net/).

Additional file

Additional file 1: Figure S1. Multiple alignments of deduced amino acid sequences of the NAM proteins. (RTF 117 kb)

Abbreviations
6AS, short arm of chromosome 6A; 6BS, short arm of chromosome 6B; CS, common wheat cultivar Chinese Spring; GPC, grain protein content; NAM, no apical meristem; QTL, quantitative trait locus; SNP, single nucleotide polymorphism

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Availability of supporting data
The genomic and cDNA sequences of the NAM-V1 gene discussed in this manuscript have been deposited in NCBI’s GenBank and are accessible with the accession number KR873101.

Authors’ contributions
CX and QS designed the study. CX, XL, YL, FL, MG, YM, XW, and ZN carried out most of the experiments and data analysis, and wrote the Materials and Methods section of the manuscript. CX and CZ wrote the manuscript, generated the figures, and finalized the table. All authors have read and approved the manuscript.

Competing interests
The authors declare that they have no competing interests.
Consent to publish
Not applicable.

Ethics and consent to participate
Not applicable.

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