RESEARCH ARTICLE

NAM gene allelic composition and its relation to grain-filling duration and nitrogen utilisation efficiency of Australian wheat

Zaid Alhabbar1,2, Rongchang Yang1, Angela Juhasz1, Hu Xin1,2, Maoyun She1, Masood Anwar1, Nigarin Sultana1, Dean Diepeveen4,5, Wujun Ma1*, Shahidul Islam1*

1 Australia China Centre for Wheat Improvement, School of Veterinary and Life Sciences, Murdoch University, Murdoch, Western Australia, Australia, 2 Department of field crops, College of Agriculture and Forestry, Mosul University, Mosul, Iraq, 3 College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei, China, 4 Department of Primary Industries and Regional Development, South Perth, Western Australia, Australia, 5 School of Veterinary and Life Sciences, Murdoch University, Murdoch, Western Australia, Australia

* W.Ma@murdoch.edu.au (WM); s.islam@murdoch.edu.au (SI)

Abstract

Optimising nitrogen fertiliser management in combination with using high nitrogen efficient wheat cultivars is the most effective strategy to maximise productivity in a cost-efficient manner. The present study was designed to investigate the associations between nitrogen utilisation efficiency (NUtE) and the allelic composition of the NAM genes in Australian wheat cultivars. As results, the non-functional NAM-B1 allele was more responsive to the nitrogen levels and increased NUtE significantly, leading to a higher grain yield but reduced grain protein content. Nitrogen application at different developmental stages (mid-tillering, booting, and flowering) did not show significant differences in grain yield and protein content. The NAM-A1 allelic variation is significantly associated with the length of the grain-filling period. While the NAM-A1 allele a was associated with a short to moderate grain-filling phase, the alleles c and d were related to moderate to long grain-filling phase. Thus, selection of appropriate combinations of NAM gene alleles can fine-tune the duration of growth phases affecting sink-source relationships which offers an opportunity to develop high NUtE cultivars for target environments.

1. Introduction

Breeding cultivars with high nitrogen use efficiency (NUE) is essential for sustainable wheat production. Generally, NUE comprises two aspects, including nitrogen uptake efficiency (NUpE) that represents the capacity of plants to absorb nitrogen (N) from soil and nitrogen utilisation efficiency (NUtE) that represents the plant’s ability to use absorbed N to produce grain [1, 2]. Understanding the mechanisms regulating these two processes is essential to improve NUE in crop plants. The NUpE, and NUtE metabolic pathways are strongly influenced by genetic variation and environmental factors [3, 4]. Modern wheat cultivars have been
generally selected under non-limiting N fertilisation levels in breeding programs, resulting in sub-optimal NUE [5, 6]. Unless NUE is improved in modern cultivars, the application of N fertilisers is expected to increase more than three-fold in the next 30 years to meet the increasing food demand [7, 8]. High N fertiliser usage means higher environmental pollution and production costs [9]. Thus, developing cultivars with high NUE becomes highly important in modern agriculture. High NUE cultivars can be described as being able to produce greater than average yields in low N environments [10]. They have also been defined as genotypes that can produce higher yields when additional N is provided. Timing and dosage of N addition is crucial when determining NUE. While additional N can usually increase grain yield, excess N has a deleterious effect on NUE [11, 12].

The Gpc-B1 or NAM-B1 gene commonly present in wild emmer wheats facilitates efficient translocation of nutrients to the grain [13] and is considered as a genetic factor influencing NUE. Gpc-B1 has been reported to improve grain protein content (GPC) in bread wheat without reducing grain yield [14]. The identification and practical utilisation of Gpc-B1 started when Avivi (1978) evaluated wild emmer wheat (Triticum turgidum ssp. dicoccoides) from Israel and found that it was associated with higher protein content and large grain size [15]. The functional NAM-B1 allele, which encodes a transcription factor of the NAC family, accelerates senescence and increases nutrient remobilisation from leaf tissues into the developing grain [16]. NAM-A1 is a gene with similar function to NAM-B1, with beneficial effects on grain nutritional quality and several bread-making properties [17]. However, the modern wheat cultivars that carry a non-functional NAM-B1 allele result in delayed senescence and reduced wheat GPC by over 30% [18]. Leaf senescence can influence crop production in two ways, i.e. by modifying nutrient remobilisation efficiency or by affecting the duration of photosynthesis. Leaf senescence is a plant growth-dependent process that allows the transfer of nutrients from areas of lower nutrient requirement to areas of higher nutrient requirement driven by active cell development such as developing grains.

In contrast to the NAM gene studies, there are many reports arguing that stay-green cultivars with delayed senescence provide a longer grain-filling period through continued N uptake and translocation [19, 20]. These cultivars have greater N uptake, accumulation, and translocation capabilities which provide further metabolic gains in NUE [21]. In addition, delayed leaf senescence also provides further carbon and nitrogen to the plant roots during grain-filling, which increases the capacity to extract more N from the soil compared to shorter grain-filling cultivars [22–24]. In order to understand the impact of NAM-1 gene alleles on the NUE and its components of wheats grown in environments with no limiting factors, we conducted the current study using a suite of Australian wheat cultivars that differ in NAM-1 allelic compositions. Allelic effects on grain yield, protein content, and NUE components were studied based on various N application timing and dosage.

2. Materials and methods

2.1 Field trial design

A field trial was conducted at Broomehill, Western Australia consisting of 19 Australian wheat cultivars with different allelic combinations of NAM-A1, B1 and D1 genes (Table 1) [25]. Field trial was managed by the Department of Primary Industries and Rural Development (DPIRD) on a private crop field under a formal agreement between DPIRD and the owner. Soil nutrient composition was analysed before conducting the experiment (S1 Table) that showed the soil N content was lower than the usual which made the site N responsive. The Nitrogen treatment included three levels: 0 kg N ha⁻¹, 50 kg N ha⁻¹ and 100 kg N ha⁻¹. The timing of the N application was synchronised to several Zadoks growth stages: T1 = 100% of N rate was applied at
mid-tillering (Z22-Z24); T2 = 100% of N rate was applied at booting (Z43- Z45); and T3 = 50% of N rate was applied at mid-tillering, and 50% of N rate was applied at booting [26]. Flexi-N (42.2% of N) was applied as a source of N and includes three types of N: 50% urea, 25% nitrate, and 25% ammonium [27].

The plot size was 3 m × 1.25 m with a 0.5 m gap between plots. Sowing date was mid June, which is the recommended date for this part of Western Australian. Field trial was carried out as a split-plot design, with cultivars randomised as main plots, N treatment randomised as the subplots, and each treatment replicated three times.

### 2.2 Phenotyping and sample collection from field trial

Grain and straw samples were harvested when all plants were completely matured by visual inspection. Before mechanical harvesting, a quadrate of 0.44 m² of plant material was cut off at ground level using a small hand harvester for yield component measurement. Grain and straw yield was estimated, and the grain protein content and residual N in straw were analysed using a FOSS XPS Near-infrared reflectance (NIR) equipment with a model 5000 spinning cup. NIR data analysis was collected using WinISI software (FOSS NIR Systems Inc., Laurel, MD, USA). The residual N concentration in straw was calculated using both free nitrogen and protein/amino-acid bound divided by 4.43 [28].

| Cultivars  | NAM-A1 allele | NAM-B1 allele | NAM-D1 allele | Maturity | GY Kg ha⁻¹ | GPC % | NUtE kg grain kg N⁻¹ | NUpE kg N kg N⁻¹ | NUE kg grain kg N⁻¹ |
|------------|---------------|---------------|---------------|----------|------------|------|---------------------|------------------|-------------------|
| Alsen      | c             | Deletion      | a             | Mid      | 1467 e     | 15.90 a| 3.20 e              | 9.50 c-f         | 30.70 gh          |
| Baxter     | a             | Non-functional| a             | Early—mid| 1443 e     | 14.30 bc| 3.70 d              | 9.20 d-f         | 35.30 e-g         |
| Bonnie-Rock| a             | Non-functional| a             | Early    | 1989 cb    | 12.70 g-j| 3.90 cd             | 11.70 ab         | 46.70 b-d         |
| Chara      | a             | Non-functional| a             | Early—mid| 1520 de    | 13.60 de| 3.80 cd             | 9.10 d-f         | 35.20 e-g         |
| Drysdale   | c             | Mixed         | a             | Early    | 1152 f     | 13.20 d-g| 3.20 e              | 8.50 e-g         | 26.30 h           |
| Excalibur  | b             | Non-functional| a             | Early    | 1960 cb    | 12.90 e-i| 4.10 bc             | 11.20 a-c        | 49.20 bc          |
| Gladius    | c/d           | Non-functional| a             | Mid      | 1432 e     | 13.10 e-h| 3.80 cd             | 8.80 e-g         | 34.20 fg          |
| Gregory    | c             | Non-functional| a             | Mid—long | 1787 cd    | 12.40 h-k| 4.00 cd             | 10.30 b-e        | 42.60 c-e         |
| H45        | a             | Non-functional| a             | Early    | 1628 de    | 11.90 k  | 4.40 ab             | 8.10 fg          | 36.00 e-g         |
| Kukri      | a             | Non-functional| a             | Early    | 1640 de    | 13.50 d-f| 3.70 d              | 10.60 a-d        | 38.90 ef          |
| Livingston | a             | Non-functional| a             | Early    | 1560 de    | 13.00 e-i| 4.00 cd             | 8.70 e-g         | 34.40 fg          |
| Mace       | d             | Non-functional| a             | Early—mid| 2295 a     | 11.90 k  | 4.60 a              | 12.20 a          | 56.40 a           |
| Pastor     | c             | Non-functional| a             | Early—mid| 1594 de    | 12.60 g-j| 3.70 d              | 9.60 c-f         | 38.20 e-g         |
| RAC875     | c             | Non-functional| a             | Mid—long | 1755 cd    | 12.40 i-k| 3.90 cd             | 9.80 c-f         | 38.10 e-g         |
| Spitfire    | a             | Mixed         | a             | Early    | 1468 e     | 13.90 cd| 3.80 cd             | 8.80 e-g         | 35.50 e-g         |
| Volcani    | c             | Functional    | a             | Early    | 1064 f     | 14.60 b  | 3.30 e              | 7.30 g           | 24.30 h           |
| Westonia   | a             | Deletion      | a             | Early—mid| 2057 ab    | 12.20 jk | 4.30 ab             | 11.30 a-c        | 50.80 ab          |
| Wyalkatchem| a             | Non-functional| a             | Early—mid| 2088 ab    | 12.90 f-j| 4.30 ab             | 11.20 a-c        | 51.60 ab          |
| Yitpi      | d             | Non-functional| a             | Mid—long | 1641 de    | 13.50 d-f| 3.70 d              | 10.30 b-e        | 40.90 df          |

Grain yield (GY), grain protein content (GPC), N utilisation efficiency (NUtE), N uptake efficiency (NUpE), and N use efficiency (NUE). Within the columns in each factor, means followed by the same letter are not significantly different according to LSD (P = 0.05). Note: maturity data adapted from Bioplatforms Australia. Retrieved from [https://data.bioplatforms.com/organization/about/bpa-wheat-cultivars](https://data.bioplatforms.com/organization/about/bpa-wheat-cultivars)

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2.3 Glasshouse experiment design

Based on the field trial results, four cultivars: Westonia, Spitfire, Bethlehem, and Mace were selected for a glasshouse experiment. Three cultivars, Westonia, Spitfire, and Mace, showed contrasting NUE in the field trial and also comprised different NAM-1 allelic compositions. One cultivar originated from Israel, namely Bethlehem was included in this study since it has the functional NAM-B1 allele that is different from the other three cultivars. The unique characteristics of each cultivar were also considered for cultivar selection: Mace (high-yielding), released in 2008 and rapidly became the dominant cultivar in Western Australia and accounted for 66.7% of the total area sown to wheat in 2016 [29]; Spitfire, known as a high protein content and N remobilisation cultivar; Westonia, high in both protein content and grain yield [30]; Bethlehem, high protein content and moderate grain yield [31].

Soil collected from the field trial was used in the glasshouse experiment. Plants were grown in a controlled temperature and light environment. The experiment was laid out in a complete randomised block design. The pot has a dimension of 190 mm height × 200 mm top diameter × 180 mm bottom diameter without holes to avoid leaching. The pots were watered manually. A base N dose of 20 N kg ha\(^{-1}\) was applied at sowing, coupled with P and K fertilisers. Three N rates 0, 50 and 100 kg N ha\(^{-1}\) were applied at mid-tillering, booting and flowering stages, as shown in Table 2. In order to achieve an adequate statistical power for data analysis and trait dissection, each treatment contains 12 replications in the glasshouse, making a total of 432 pots being planted.

2.4 Phenotyping and sample collection from glasshouse experiment

The anthesis time was estimated by the appearance of anthers on approximately 50% of all heads (Z61-Z65). Leaf tissue samples were collected at mid-tillering (Z22-Z24), booting (Z43-Z45), and flowering (Z65), just before the N application according to Zadoks' scale of cereal growth stages [26]. The Leaves were frozen in liquid nitrogen and then stored at -80°C. The frozen leaves were ground to a fine powder in liquid nitrogen and used for RNA extraction and N content measurements of leaf tissue.

The vegetative phase duration was estimated as the period from sowing to flowering, and the grain-filling phase duration was estimated as the period from flowering to the physiological maturity [32, 33]. The grain, straw and root samples were harvested when all plants were considered completely mature by visual inspection. Plants were hand-harvested to measure yield components. The number of heads was counted in each plant. The heads were cut off and the remaining straw was cut at ground level. Roots were washed thoroughly with water using a 1

| Nitrogen rates and timing of application in the glasshouse experiment. | Tiller| Booting | Flowering |
|---------------------------------------------------------------|-----|-------|---------|
| 0 kg N ha\(^{-1}\)                                            | 0%  | 0%    | 0%      |
| 50 kg N ha\(^{-1}\)                                           | T1  | 100%  | 0%      |
|                                                              | T2  | 0%    | 100%    |
|                                                              | T3  | 50%   | 50%     |
|                                                              | T4  | 40%   | 20%     |
| 100 kg N ha\(^{-1}\)                                          | T1  | 100%  | 0%      |
|                                                              | T2  | 0%    | 100%    |
|                                                              | T3  | 50%   | 50%     |
|                                                              | T4  | 40%   | 20%     |

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mm mesh sized sieve until totally free of soil. Roots of the two plants grown in the same pot were weighed together. The grain and straw yields were measured and the grain number was counted for each head. The TGW was measured by multiplying the weight of grains by 1000 divided by the number of grains per sample. The grain, straw and root samples were oven-dried separately in a forced air circulating dryer at 70°C for 72 hours. The total nitrogen content of straw and grains was analysed following the same procedure of field samples as detailed in the previous section. Harvest index (HI) and N harvest index (NHI) were obtained by calculating the ratio of grain or N at harvest to total above-ground biomass or N, respectively [34, 35]. N uptake efficiency (NUpE) was calculated as the ratio of aboveground N content to total N supply. N utilisation efficiency (NUtE) was calculated as the ratio of grain yield to aboveground N content. N use efficiency (NUE) was calculated as the ratio of grain yield to total N supply or multiplying NUpE by NUtE [4, 8].

2.5 Leaf N content analysis
Plant samples were dried at 40 °C, and 0.1 g leaf tissue was analysed for total nitrogen using the Dumas high-temperature combustion method (CSBP Laboratory, Western Australian). Samples were loaded into a combustion tube at 950 °C and flushed with oxygen. Gases generated from this process were measured using a thermal conductivity cell for nitrogen (according to the instruction manual).

2.6 Gene expression analysis
2.6.1 Digital droplet PCR (ddPCR) for the cDNA standards in the qRT PCR. The gene copy number of total NAM-1 genes (NAM-A1, -B1 and -D1) in wheat cultivars was calculated based on the standards generated by a ddPCR. RNA was extracted from the flag leaf of wheat cultivar Spitfire using a Qiagen RNeasy mini kit. The cDNA was then synthesised using the SensiFAST cDNA Synthesis Kit (BioLine, Alexandria, NSW, Australia). The ddPCR was conducted as described by Yang et. al. [36] with a slight modification using 1 μl cDNA as template instead of DNA. Forward primer 5’ - TCA CTG CTC CAT CAT CAG GA, reverse primer 5’ - GGC GTC GTC TGC TGT GAA C, and probe 6xFAM - 5’ CAG CCA TTT CCT GGA GGG CCT were used in the ddPCR.

2.6.2 qRT PCR for the quantification of NAM gene expression in four wheat cultivars at three growth stages. The RNA extraction and the synthesis of cDNA followed the protocols described above. The purified RNAs were quantified by NanoDrop ND-1000 spectrophotometer and their concentration adjusted to 50 ng/μl for qPCR, which was carried out in a Rotor-Gene Q (Qiagen, Hilden, Germany). The qPCR reaction contained 7.5 μl 2x qPCR master mix, 1 μl cDNA, 0.4 μl 10 pmol/μl forward and reverse primers, and 0.5 μl 10 pmol probe, as described above. A 1: 10 dilution dilution series (5) of the standards generated from ddPCR was included in each run to generate the standard curves and calculate the number of NAM gene expressed copies.

2.7 Statistical analysis
Analysis of variance (ANOVA) was performed using the Genstat statistical software (Genstat Eighteenth Edition; 18.1.0.17005, 2015, UK) to determine genotype and nitrogen treatment effects at different times of application. In the case of significant differences based on ANOVA and F-values for treatment effects, LSD (p<0.05) test and standard deviation/error of means were used to identify significant means. Correlation analysis was conducted to investigate the relationship between the NAM1 gene alleles and NUE components using Genstat.
3 Results

3.1 Influence of NAM genes under field conditions

3.1.1 Grain yield, protein content, NUE, and its components were influenced by NAM gene allelic composition. A total of 19 wheat cultivars included in the field trial were compared to examine the influence of NAM gene allelic combinations on plant maturity type, grain yield, grain protein content, nitrogen use efficiency and its components (Table 1). Significant grain yield differences ($P<0.001$) were found among the 19 cultivars. Mace, Wyalkatchem, and Westonia produced the highest grain yield (Table 1). Results also indicated that the grain yield was significantly influenced by N rate ($P<0.013$). The maximum yield (1705 kg ha$^{-1}$) was achieved at the highest level of N (100 kg N ha$^{-1}$); while the lowest yield (1578 kg ha$^{-1}$) was obtained from the control treatment (0 kg N ha$^{-1}$, S2 Table). Similarly, significant differences ($P<0.001$) were observed for grain protein content among the cultivars. The grain protein content increased with the increase of N rate. Application of N at booting stage led to achieve maximum grain protein content (13.40% average). The highest grain protein content was obtained in cultivar Alsen, being 33.61% higher than that of H45 and Mace (Table 1). NUE and its components NUtE and NUpE were significantly influenced ($P<0.001$) by the cultivars and N rate. Cultivars Mace, Wyalkatchem, Westonia, Excalibur, and Bonnie-Rock attained the highest NUE, NUtE, and NUpE. The control treatment which only had the base N application (20 kg ha$^{-1}$) produced the highest NUE, NUtE, and NUpE followed by 50 and 100 kg N ha$^{-1}$.

3.1.2 Correlation of NAM-A1 and -B1 alleles with phenotypes. Correlation analysis of NAM-A1 and -B1 alleles with phenotypes is presented in Table 3. A significant correlation was observed between NAM-B1 allelic variation and yield, protein content, and NUtE. The functional NAM-B1 allele produced a lower grain yield and NUtE but higher protein content. The NAM-B1 deletion allele was positively correlated with grain protein content (Table 1). On the other hand, there was no significant correlation between NAM-A1 allelic variation with yield or protein content alone. However, NAM-A1 allelic variation was significantly correlated with plant maturity and NUtE (Table 3). NAM-A1c and d alleles were associated with low NUtE and longer maturity. It is worth pointing out that the NAM-A1a allele had both positive effects on grain yield and protein content even though such effect was not statistically significant based on the field trial data used in the current study.

| NAM-A1 | NAM-B1 | Maturity | GPC | GY | NUpE | NUtE | NUE |
|--------|--------|----------|-----|----|------|------|-----|
| 1      |        |          |     |    |      |      |     |
| -0.15  | 1      |          |     |    |      |      |     |
| 0.58** | 0.37***| 1        |     |    |      |      |     |
| 0.07   | -0.23**| 0.04     | 1   |    |      |      |     |
| -0.16  | 0.48***| 0.06     | -0.47***| 1 |
| -0.02  | 0.10   | 0.02     | -0.38***| 0.02 | 1 |
| -0.29***| 0.39***| -0.07   | -0.78***| 0.65***| 0.44***| 1 |
| -0.06  | 0.15   | 0.01     | -0.47***| 0.14 | 0.98***| 0.56***| 1 |

*“, ***, ***” Significant at the 0.05, 0.01 and 0.001 probability level, respectively. Grain protein content (GPC), Grain yield (GY), N uptake efficiency (NUpE), N utilisation efficiency (NUtE), and N use efficiency (NUE).

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3.2 Influence of NAM genes under controlled environmental conditions

Four cultivars: Westonia, Spitfire, Bethlehem, and Mace had been selected for further investigation under controlled environment in glasshouse based on their contrasting responses to nutrient remobilisation in the field trial and NAM gene composition. As being investigated by this research group in another study [25], Bethlehem contain a functional NAM-B1 gene, while Westonia has deletion and Mace has a non-functional allele. On the other hand Spitfire has been identified with the combination of both the functional and non-functional NAM-B1 alleles. In the case of NAM-A1 gene, Westonia and Spitfire contain allele a, while Bethlehem and Mace contain allele d (Table 4).

3.2.1 Grain yield and its components, aboveground biomass, HI and NHI. There were significant differences (P < 0.001) in grain yield and yield components among the cultivars and N treatments. Interactions of N rate with both of the cultivars and time of application significantly influenced the grain yield and its components. The mean values for the cultivars across all N rates and times of application showed a 13.97% higher grain yield of Mace compared to Bethlehem and Spitfire (Table 5). Increasing N rates led to grain yield increases for all cultivars and 100 kg N ha⁻¹ application produced the highest average yield (7.08 g plant⁻¹). The interaction between cultivars and N rate had a highly significant impact on grain yield, demonstrated by the highest yield of Mace at 100 kg N ha⁻¹ being 43.88% higher than that of Spitfire at

| Cultivar   | NAM-A1 allele | NAM-B1 allele | NAM-D1 allele | Vegetative phase duration (days) | Grain-filling phase duration (days) | Days to maturity |
|------------|---------------|---------------|---------------|----------------------------------|-------------------------------------|-----------------|
| Spitfire   | a             | Mixed         | a             | 73                               | 37                                  | 110             |
| Mace       | d             | Non-functional| a             | 77                               | 48                                  | 125             |
| Westonia   | a             | Deletion      | a             | 72                               | 40                                  | 112             |
| Bethlehem  | d             | Functional    | a             | 69                               | 53                                  | 122             |

Table 4. Wheat cultivars carrying different NAM-1 genes has various of vegetative, grain filling and total duration.

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| Cultivars | AGB g plant⁻¹ | GY g plant⁻¹ | HI % | NHI % | NUgE g grain g N plant⁻¹ | NUgE g N plant⁻¹ g N⁻¹ | NUE g grain g N⁻¹ |
|-----------|----------------|--------------|------|-------|--------------------------|------------------------|-------------------|
| Bethlehem | 11.31 b        | 6.37 b       | 0.56 | 0.89 a | 7.17 b                   | 17.95 b                | 128.70 b          |
| Mace      | 12.27 a        | 7.26 a       | 0.59 | 0.89 a | 7.66 a                   | 19.21 a                | 147.15 a          |
| Spitfire  | 11.46 b        | 6.39 b       | 0.56 | 0.87 b | 6.88 b                   | 17.99 a                | 129.28 b          |
| Westonia  | 11.77 ab       | 6.63 b       | 0.56 | 0.87 b | 7.05 b                   | 19.03 a                | 134.16 b          |

Table 5. Effects of four wheat cultivars, N application, and time of N application on NUE components.

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Aboveground biomass (AGB), grain yield (GY), harvest index (HI), N harvest index (NHI), N utilisation efficiency (NUgE), N uptake efficiency (NUgE), and N use efficiency (NUE). Within the columns in each factor, means followed by the same letter are not significantly different according to LSD (P = 0.05). Means with no letter are not statistically different (F > 0.05).
0 kg N ha\(^{-1}\) (S1 Fig). The N application time and rate were also interacting significantly, with the highest average grain yield (7.50 g plant\(^{-1}\)) achieved from 100 kg N ha\(^{-1}\) applied at mid-tillering while the lowest average (6.20 g plant\(^{-1}\)) obtained from the 0 kg N ha\(^{-1}\) in control treatment (S2 Fig). The highest number of grain head\(^{-1}\) was recorded from Mace (49.31 grain) while the lowest number was recorded from Spitfire (42.77 grain, Table 6). The time of N application also influenced the number of grain head\(^{-1}\). The highest number of grain head\(^{-1}\) (47.08 grain) was achieved by applying N at booting stage.

On the other hand, TGW was significantly influenced by cultivar and time of N application (p < 0.001). However, there was no evidence that N rate had an influence on TGW. The maximum TGW (44.06 g) was achieved when N was applied at the latest stage (flowering). The result of interaction between cultivar and N rate showed that Bethlehem produced the highest TGW (45.96 g) at 0 kg N ha\(^{-1}\) treatment (Data not shown). HI and NHI were significantly influenced by cultivars and by the interaction of cultivars and N rates (p < 0.001) where Mace produced the highest in both the cases. The aboveground biomass increased significantly with the increase of N rates (p < 0.001).

Correlation analysis of NAM-A1 and -B1 alleles with phenotypes (Table 7) shows that the non-functional NAM-B1 allele was positively correlated with both the grain yield and HI, while negatively correlated with TGW. The NAM-A1a cultivars showed an earlier senescence while NAM-A1d cultivar demonstrated a longer green period. The higher HI, NHI and TGW were achieved by the NAM-A1d allele compared to that of allele NAM-A1a, indicating a longer green period in glasshouse is desirable for achieving higher grain yield.

### 3.2.2 Nitrogen content in leaf tissue

Analysis of the data revealed significant effects of cultivar, N rate, and the time of N application on leaf N content. N content in leaves at three developmental stages (tillering, booting, and flowering) was significantly different (p < 0.01). Fig 1 showed that all cultivars’ maximum N content in leaves was achieved at tillering stage (5.48%), while the lowest was at flowering stage (3.26%). Cultivar Bethlehem showed higher (p < 0.01) N-content (4.30%) compared to Spitfire and Westonia (3.95 and 3.98%, respectively). The control treatment (0 kg N ha\(^{-1}\)) recorded the highest (p < 0.01) N content in leaf tissue.

### Table 6. Effects of four wheat cultivars, N application, and time of N application on agronomic traits.

| Cultivars | Anthesis date Day | Grain number Seed head\(^{-1}\) | Head number Head plant\(^{-1}\) | TGW g | DRW g | GPC % | RNS % |
|-----------|-------------------|-------------------------------|-------------------------------|-------|-------|-------|-------|
| Bethlehem | 68.42 d           | 45.16 b                       | 3.28 b                        | 42.54 a | 5.91 b | 8.98 a | 1.44 c |
| Mace      | 76.56 a           | 49.31 a                       | 3.58 a                        | 41.69 b | 7.65 a | 8.42 c | 1.53 b |
| Spitfire  | 72.50 b           | 42.77 b                       | 3.46 ab                       | 41.76 b | 6.07 b | 8.73 b | 1.61 a |
| Westonia  | 71.33 c           | 45.41 b                       | 3.61 a                        | 41.10 b | 6.57 b | 8.92 a | 1.52 b |

N Rates

| Time N app. |
|-------------|
| 0           |
| 50          |
| 100         |

| T1          | 72.53  | 44.83 ab | 3.64  | 41.62 b | 6.29  | 8.64  | 1.53  |
|-------------|--------|----------|-------|---------|-------|-------|-------|
| T2          | 72.08  | 47.08 a  | 3.45  | 41.37 b | 6.36  | 8.79  | 1.53  |
| T3          | 71.97  | 46.99 a  | 3.42  | 41.74 b | 6.50  | 8.75  | 1.53  |
| T4          | 72.22  | 43.75 b  | 3.42  | 44.06 a | 7.05  | 8.86  | 1.52  |

Anthesis date, grain number, head number, thousand grain weight (TGW), dry root weight (DRW), grain protein content (GPC), residual N in straw (RNS). Within the columns in each factor, means followed by the same letter are not significantly different according to LSD (P = 0.05). Means with no letter are not statistically different (F > 0.05).
compared to 50 and 100 kg N ha$^{-1}$. The majority of the interactions between stages and other factors were significant for N content in leaf tissue.

### 3.2.3 Grain protein content and residual N in straw

There was a significant difference (P<0.001) in the grain protein content and residual N in the straw between the cultivars and the N treatments. Cultivar Bethlehem produced the highest grain protein which was 7.98% more than the lowest (Mace), while it resulted the lowest residual N in the straw which was 6.15% less than the highest (Mace) (Table 6). Both grain protein content and residual N content in the straw increased when higher N rates were applied. Maximum grain protein content

| NAM-A1 | NAM-B1 | D Vet | DGF | DRW | TGW | GPC | GY | HI% | NHI% | NUpE | NUtE |
|--------|--------|-------|-----|-----|-----|-----|----|-----|------|------|------|
| NAM-A1 | 1      |       |     |     |     |     |    |     |      |      |      |
| NAM-B1 | 0.01   | 1     |     |     |     |     |    |     |      |      |      |
| D Vet  | 0.09   | 0.86*** | 1   |     |     |     |    |     |      |      |      |
| DGF    | 0.92*** | -0.03 | -0.20 | 1   |     |     |    |     |      |      |      |
| DRW    | 0.13   | 0.45** | 0.29 | 0.12 | 1   |     |    |     |      |      |      |
| TGW    | 0.31*  | -0.38* | -0.38* | 0.22 | 0.01 | 1   |    |     |      |      |      |
| GPC    | -0.12  | -0.46** | -0.50** | 0.00 | -0.07 | 0.32 | 1 |     |      |      |      |
| GY     | 0.24   | 0.57*** | 0.48** | 0.07 | 0.48** | -0.31 | -0.48** | 1 |
| HI%    | 0.39** | 0.46*  | 0.47** | 0.12 | 0.15 | -0.15 | -0.42* | 0.65*** | 1 |
| NHI%   | 0.47*** | 0.16   | -0.12 | 0.53*** | 0.07 | 0.40** | 0.22 | 0.27 | 0.72*** | 1 |
| NUpE   | -0.01 | 0.03   | 0.16 | -0.08 | -0.20 | 0.01 | -0.46*** | -0.52*** | -0.06 | -0.06 | 1 |
| NUtE   | 0.48*** | 0.35*  | 0.38** | 0.34* | 0.12 | 0.08 | -0.68*** | 0.41** | 0.81*** | 0.55*** | 0.37* | 1 |

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability level, respectively. Duration of vegetative phase (D Vet), grain-filling phase (DGF), dry root weight (DRW), thousand-grain weight (TGW), grain protein content (GPC), grain yield (GY), harvest index (HI), N uptake efficiency (NUpE), N utilisation efficiency (NUtE), and N use efficiency (NUE).
and residual N in the straw were obtained from 100 kg N ha\(^{-1}\), followed by 50 kg N ha\(^{-1}\), and the lowest from the control. The non-functional NAM-B1 allele was negatively correlated with grain protein content (\(r = -0.46, P < 0.01\)). Allelic variation of NAM-A1 did not show any significant correlation with grain protein content.

### 3.2.4 Dry root weight (DRW)

DRW showed significant variations depending on the cultivars and N treatment (\(P < 0.001\)). Cultivar Mace had the maximum DRW (7.65 g) while the lowest was with Bethlehem (5.91 g) (Table 6). DRW at 100 kg N ha\(^{-1}\) treatment was significantly higher than that of 0 and 50 kg N ha\(^{-1}\) (\(P < 0.001\)). However, the effect of time of N application was not significant at \(P = 0.05\). N applied at the latest stage (flowering) resulted in the highest DRW (7.05 g). DRW had a significant positive correlation with the non-functional NAM-B1 allele (\(r = 0.45, P < 0.01\)).

### 3.2.5 Nitrogen use efficiency (NUE) and its components

There were significant differences (\(P < 0.001\)) in NUpE, NUtE, and NUE among the cultivars, N rate, and the interaction of cultivars with N rate. Cultivars Mace, Spitfire and Westonia exhibited significantly higher NUpE than Bethlehem. Similar to the field trial results, the control treatment (0 kg N ha\(^{-1}\)) produced significantly higher NUpE than that of the 50 and 100 kg N ha\(^{-1}\) treatment. Mace at 0 kg N ha\(^{-1}\) had the highest NUpE (\(P < 0.01\)). Mace also had the highest NUtE and overall NUE regardless of N application rate and timing of application (\(P < 0.01\)). Mace at 0 kg N ha\(^{-1}\) had the highest NUE and NUtE (\(P < 0.01\)). Among NUE and its components, only NUtE had a significant correlation with NAM-A1 and -B1 alleles (Table 7). NUtE was significantly correlated with the non-functional NAM-B1 allele (\(r = 0.35, P < 0.05\)), and the NAM-A1d allele (\(r = 0.48, P < 0.001\)). Both the non-functional NAM-B1 allele and NAM-A1d allele increased the NUtE.

### 3.3 NAM-1 gene expression is influenced by cultivars and N treatments

Expression levels of total active NAM-1 genes (NAM-B1, -A1 and -D1) as determined by RT-PCR were significantly different (\(p < 0.001\)) across cultivars, N rates, times of N application, and developmental stage (Fig 2). Averaged across cultivars, N rate, and time of N application, the highest total NAM-1 gene expression level (3107) was occurred at flowering stage, followed by booting stage (1080), and mid-tillering stage (689, Fig 2). Averaged across growth stages, N rate, and time of application, Bethlehem (1868) and Spitfire (1864) exhibited higher total NAM-1 gene expression levels than Mace (1393) and Westonia (1377). Gene expression level of the combined NAM-1 genes increased (1389, 1608, and 1879) with the increase of N rates (0, 50 and 100) kg N ha\(^{-1}\), respectively. Averaged across cultivars, N rate, and growth stages, the highest combined NAM-1 gene expression (1840) was observed when N was applied 50% at mid-tillering and 50% at booting stage, while the lowest NAM-1 gene expression was observed when N was applied at once either at mid-tillering or at booting stage. NAM-1 gene expression was significantly influenced by most interactions (S3 Fig).

### 4. Discussion

NUE is a complex trait that results from an interaction of several component traits such as grain and protein yield, NUtE, and NUpE. Recent studies have confirmed that around 60–95% of wheat grain N comes from the remobilisation of N stored in plant parts such as roots and shoots before anthesis (Kong et al. 2016, Barraclough et al. 2010, Hirel et al. 2007). The genetic factors involved in the absorption and utilisation of nutrients, such as differences in morphological, physiological and biochemical processes, have a large impact on NUE and its components. The NAM-B1 transcription factor increases nutrient remobilisation and accelerates monocarpic senescence coupled with a slight yield penalty [16, 37], and is being considered as a genetic factor influencing NUE. NAM-A1 is a gene with a similar function to NAM-B1.
involved in remobilising nutrients and accelerating senescence, with beneficial effects on protein content and yield [17]. However, NAM-D1 has never been reported with any influence on NUE. This study was able to include only one cultivar in the field trial that harboring functional NAM-B1. This is because of the fact that functional NAM-B1 is not common in bread wheat cultivars [13, 38, 39] which can be grown in Western Australian conditions. Only one Australian wheat cultivar has been identified having functional NAM-B1 in the screening of 51 cultivars [25]. Thus to improve the strength of the correlation analysis we have included another cultivar (Bethlehem) from overseas (Israel) with functional NAM-B1 in the glasshouse experiment.

In general, the non-functional NAM-B1 allele showed a significant association with higher yield as evident by correlation analyses. For examples, Mace, Wyalkatchem, and Bonnie-Rock, which produced the highest grain yield, NUtE, NUpE, and NUE, carry a combination of non-functional NAM-B1 and NAM-A1a or d alleles. However, independently from the presence of a functional gene or complete lack of NAM-B1, the presence of the NAM-A1c allele resulted in higher protein content, as seen in cultivars Volcani (functional NAM-B1, NAM-A1c), and Alsen and Drysdale (deletion NAM-B1, NAM-A1c).

The correlation analysis results showed a significant relationship between NAM-1 allelic variation and maturity type. However, the interaction effect of NAM-A1 and NAM-B1 genes on determining the maturity of wheat cultivars also has been noticed. NAM-A1 alleles a and b were exclusively characteristic of early and early to mid-maturing cultivars regardless of their NAM-B1 allelic composition. On the other hand, NAM-A1 alleles c and d were related to mid and late maturity only in combination with non-functional NAM-B1 allele (Table 1), which is in concordance with the senescence-promoting role of the functional NAM-B1 allele. In contrast, NAM-A1 alleles c and d combined with functional/deletion NAM-B1 alleles showed early
and early to mid maturity in general. Based on the glasshouse results which represent wheat growing conditions without any limiting factors, we conclude, that the non-functional NAM-B1 coupled with NAM-A1 alleles c or d can be associated with high-yielding potential might be due to positive relation to the length of the grain-filling period. However, in most of the field conditions across the world with a range of complex environmental factors exist; different allelic effects have been detected. Under Western Australian conditions, our results have shown that the non-functional NAM-B1 allele and the NAM-A1a alleles are favourable alleles for achieving higher grain yield and NUtE.

Based on the expression profiles shown at expVIPs (www.wheat-expression.com) [40], NAM-B1 and -A1 show the highest expression in the flag leaf and stamens, but is also expressed in the spikelet while NAM-D1 is expressed in the spikelet and stamens during anthesis. The qRT-PCR analysis showed that the NAM gene expression reached the peak at flowering (Fig 2) in accordance with previously published articles confirming that the NAM gene function relates to senescence which ultimately influencing the maturity of the cultivars [13, 16–18, 41]. This was a total NAM gene expression which does not allow us to interpret the expression level of individual NAM gene or allele. However, the relative comparison indicated the total NAM gene expression was influenced by the types of alleles. For example, cultivars with functional NAM-B1 allele (spitfire and Bethlehem) showed higher expression of total NAM gene than the cultivars with non-functional or deletion NAM-B1 allele (Mace and Westonia). As presented in the Table 4 cultivar Spitfire and Westonia had the similar composition of NAM-A1 and D1 and also Mace and Bethlehem had the similar NAM-A1 and D1 composition. Thus the variation in the total gene expression between Spitfire and Westonia and also between Mace and Bethlehem is contributed by the variation at NAM-B1 allele. On the other hand, comparison between the NAM gene expression of Mace and Westonia both of which carry similar type of NAM-B1 allele showed that the variation of NAM allele doesn’t show much difference in total gene expression. It is worth mentioning that the less influence of NAM-A1 allelic variation on the total NAM gene expression variation doesn’t disprove its function of determining maturity. This expression analysis also clearly demonstrated that NAM-B1 functional allele boosted the gene expression when N fertilizer was added.

Although the four cultivars used in the glasshouse experiment represented early and early to mid-ripening types, we observed large differences in their development. The duration of the entire cycle was different in the four cultivars. The life cycle of Mace and Bethlehem was around two weeks longer than that of Spitfire and Westonia. Likewise, there was a difference between Mace and Bethlehem in the duration of vegetative and grain-filling periods. Based on these observations, we conclude that the four cultivars represent three different developmental mechanisms.

The first mechanism can be seen in Mace, which had a long vegetative phase (77 days) combined with a long grain-filling period (48 days). Accordingly, this cultivar had more chance to accumulate further N in the vegetative parts, which could then be remobilised to the grains during the grain-filling period. Mace, as a representative of high-yielding cultivars, carries a non-functional NAM-B1 combined with the NAM-A1d allele (Table 4), which delayed the senescence and resulted in a low grain protein content. In contrast, this allelic combination produced higher grain yield, above-ground biomass, DRW and NUE and its components (Tables 5 and 6). Increasing the duration of the vegetative phase has the potential to improve the accumulation of total dry matter [42–44]. On the other hand, delayed leaf senescence can extend the duration of grain filling phase and thus enhance grain yield due to the addition in photosynthesis rate and grain filling capacity [45–47]. In the current study, the total dry matter and entire life cycle were significantly higher (P<0.01) in Mace resulted in higher grain yield, NUPe, NUtE, and NUE. Several studies have shown differences among cultivars in the pre-
anthesis and post-anthesis build-up of grain yield and yield components [5, 48, 49]. The second mechanism can be observed in Bethlehem. This cultivar takes nearly the same number of days to reach the end of the grain-filling stage as Mace. However, Bethlehem had a shorter vegetative phase (69 days) coupled with a long grain-filling period (53 days) to uptake and remobilise the N during the grain-filling period. The potential storage capacity of the grain is determined during the initial stage of endosperm cell division that is within the first 15 days after anthesis [22, 50]. The low grain yield and high grain protein content of Bethlehem, Alsen, Drysdale and Volcani can be explained by the different combinations of NAM gene alleles.

Short vegetative phase related to the functional NAM-B1 combined with low DRW, number of tillers (Heads plant$^{-1}$), and number of grain head$^{-1}$ (low total dry matter) resulted in decreased grain yield and yield components. However, a long grain-filling period is associated with the NAM-A1d allele, which resulted in an increased N uptake and remobilisation during the grain-filling period, led to an increased grain protein content and TGW. Our results are in consistent with the finding by Martre et al. (2007) ie., an increase in the duration of grain-filling phases is a strategy to improve grain protein yield and NUE [22].

The third mechanism can be seen in Spitfire and Westonia, which represented similar growth types. Both cultivars have a medium vegetative phase (73 and 72 days respectively) followed by a very short grain-filling period (37 and 40 days, respectively). Medium vegetative phase was linked to absent or mixed NAM-B1 allele and short grain-filling period related to NAM-A1a allele. Accelerated senescence might improve N remobilisation and grain protein content but it resulted in reduced TGW and lower grain number, thus resulting in decreased grain yield in cultivars carrying the NAM-A1a allele [17].

Differences between the three mechanisms can be explained in terms of the different combinations of NAM-B1 and -A1 alleles. The non-functional NAM-B1 allele delayed leaf senescence [13, 39]. Increased duration of grain-filling due to the presence of NAM-A1c and d might provide more carbon and nitrogen, resulting a higher grain yield [17, 23]. At the same NAM-A1 background, a negative correlation between the non-functional NAM-B1 allele and grain protein content was observed. Remarkably, both functional NAM-B1 and the deletion of NAM-B1 gene correlate to the higher protein content.

The glasshouse result from the leaf tissue N analyses provides further support for the differences between the three mechanisms described above. In Fig 1, we can see that the maximum N accumulation in leaf tissues was at the early stage (mid-tillering) for all cultivars. At later stages (booting and flowering) the N was translocated to the developing grains. However, Spitfire and Westonia translocated the N earlier, because they have a shorter grain-filling period. The NHI data for the four cultivars provides further support regarding the negative impact of the short grain-filling period on grain yield. Spitfire and Westonia, which had a short grain-filling period, showed a low NHI compared with the other two cultivars (Table 5). NHI is an important parameter to measure the translocation efficiency of absorbed N from vegetative parts to the grain [20, 51]. To summarise, an increase in the duration of N accumulation on the leaves or the duration of N remobilisation to grains can both be good strategies to improve NUE.

Allele NAM-A1a is prevalent in early-ripening cultivars. The combinations of NAM-A1c/d with a functional NAM-B1 gene result in higher protein content, as for example in Volcani and Bethlehem. However, it is important to measure gene expression levels, not only allelic presence or absence when determining the effect of NAM-1 genes on yield, protein content, and NUTE.

5. Conclusions

Based on a large scale glasshouse experiment, three different developmental mechanisms involving the different duration of the grain-filling period were interpreted with the different
combination of NAM-1 genes. Presence of the non-functional NAM-B1 allele is related to delayed leaf senescence meaning a longer grain-filling period and generally higher NUtE. On the other hand, functional NAM-B1 allele and the NAM-A1a allele were associated with a shorter grain-filling period, making it useful in regions with a short rainfall season like Western Australia. A negative correlation between the non-functional NAM-B1 allele and grain protein content was observed. In contrast, presence of functional NAM-B1 allele or deletion of the NAM-B1 gene are correlated with higher protein content. NAM-A1 gene allelic composition was also strongly associated with maturity types. Cultivars with NAM-A1a and b alleles demonstrated early to mid maturity, while the cultivars with NAM-A1c and d alleles showed mid to late maturity at the same (non-functional) NAM-B1 allele background.

The allelic effects are highly dependent on environmental conditions. The selection of specific combinations of NAM-1 alleles offers an opportunity to develop new high NUtE cultivars for target environments. The higher N application had a positive effect on grain yield and its components as well as dry matter and dry root weight, while the timing and splitting of N applications had no obvious effect on NUE or its components. Late N application only increased grain protein content.

Supporting information

S1 Table. General characteristics of the soil used in the experiments.
(DOCX)

S2 Table. The effects of N rates and the time of N application on grain yield and protein content.
(DOCX)

S1 Fig. The interaction between the four cultivars and N rates on grain yield plant\(^{-1}\).
(TIF)

S2 Fig. The interaction between N rates and time of N application on grain yield plant\(^{-1}\).
(TIF)

S3 Fig. Effect of the time of N application on total NAM gene expression at different growth stage.
(TIF)

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Author Contributions

Conceptualization: Zaid Alhabbar, Dean Diepeveen, Wujun Ma, Shahidul Islam.

Data curation: Zaid Alhabbar, Masood Anwar.

Formal analysis: Zaid Alhabbar.

Funding acquisition: Wujun Ma, Shahidul Islam.

Investigation: Zaid Alhabbar.

Methodology: Zaid Alhabbar, Dean Diepeveen, Wujun Ma, Shahidul Islam.
Project administration: Zaid Alhabbar, Wujun Ma, Shahidul Islam.

Resources: Dean Diepeveen, Wujun Ma, Shahidul Islam.

Supervision: Rongchang Yang, Maoyun She, Dean Diepeveen, Wujun Ma, Shahidul Islam.

Validation: Zaid Alhabbar, Dean Diepeveen, Shahidul Islam.

Visualization: Zaid Alhabbar, Hu Xin, Nigarin Sultana.

Writing – original draft: Zaid Alhabbar.

Writing – review & editing: Angela Juhasz, Wujun Ma, Shahidul Islam.

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