The association of the genes HvNAM1 and HvNAM2 with grain protein content in Nordic barley

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In barley, the GPC (Grain Protein Content) has proved to be of great importance for both feed, food and beer production. When it comes to feed and food, a high GPC is desirable since it indicates good nutritional values, while in beer production a low and stable GPC is needed to avoid beer chill haze. In previous studies a decrease in the GPC has been seen in different accessions of barley developed at different time periods during the last 100 years. The gene family HvNAM, including the genes HvNAM1 and HvNAM2, has in previous studies been shown to be important for the remobilization of nutrients towards the grains during the senescence and thus also for the GPC. In this study, 40 Nordic accessions from different improvement groups from the end of the 19th century until today have been analyzed for polymorphism in those genes. Statistical analyses has been conducted to investigate if there are any associations between the polymorph nucleotide positions and the nutritional values of grain protein, iron and zinc contents. However, no such associations were found. Instead some correlations could be seen between the nutrient content and thousand grain weight, a relative measurement of the grain size. In conclusion, since no polymorphisms were found to be associated to the nutritional value there might instead be a correlation between the gene expression and the nutritional value. Future work should thus focus on the gene expression of HvNAM1 and HvNAM2 in Nordic accessions of barley.

Keywords:
barley, HvNAM1, HvNAM2, GPC
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1 Abstract

In barley, the GPC (Grain Protein Content) has proved to be of great importance for both feed, food and beer production. When it comes to feed and food, a high GPC is desirable since it indicates good nutritional values, while in beer production a low and stable GPC is needed to avoid beer chill haze. In previous studies a decrease in the GPC has been seen in different accessions of barley developed at different time periods during the last 100 years. The gene family HvNAM, including the genes HvNAM1 and HvNAM2, has in previous studies been shown to be important for the remobilization of nutrients towards the grains during the senescence and thus also for the GPC. In this study, 40 Nordic accessions from different improvement groups from the end of the 19th century until today have been analyzed for polymorphism in those genes. Statistical analyses have been conducted to investigate if there are any associations between the polymorph nucleotide positions and the nutritional values of grain protein, iron and zinc contents. However, no such associations were found. Instead some correlations could be seen between the nutrient content and thousand grain weight, a relative measurement of the grain size. In conclusion, since no polymorphisms were found to be associated to the nutritional value there might instead be a correlation between the gene expression and the nutritional value. Future work should thus focus on the gene expression of HvNAM1 and HvNAM2 in Nordic accessions of barley.

2 Introduction

The improvement of crops has long been important for agriculture, to increase the yields and to get adequate nutritional values. When it comes to barley several path of breeding developed, for two-row and six-row barley separately (Persson, 1997). These are the two subtypes of barley evolved under different environmental conditions, where six-row barley is adapted to shorter growing seasons (Persson, 1997). One path of breeding has been developed for barley with good properties for feed and food production and one for barley with appropriate brewing qualities. Barley for feed and food production preferably has a high GPC (Grain Protein Content) to provide good nutritional values. Barley for malt production on the other hand preferably has a low GPC which allows for an increase in malt extract and a reduction of beer chill haze (Distelfeld et al., 2008), that otherwise would have made the beer turbid at fridge temperature. However, if the GPC becomes too low in barley for malt and beer production, the fermentation process might be affected since the growth of yeast decreases (Emebiri et al., 2005). Taken together barley for malt production needs a stable level of GPC.
Since the nutritional value is of great importance several genetic studies have been carried out, to detect which genes are correlated with GPC. Studies in wheat have revealed that the NAC transcription factors are associated with the remobilization of micronutrients towards the grains during senescence, but also the extent of the senescence period and therefore the GPC (Uauy et al., 2006a). NAM-B1 is a NAC transcription factor found in wheat that except from remobilization of nitrogen also has shown to be important for remobilization of iron and zinc (Waters et al., 2009). As a transcription factor the NAM gene helps to regulate the genes that are carrying out the physiological processes in translocations of the nutrients to the grain (Waters et al., 2006). An ortholog sequence to NAM-B1, designated HvNAM1, controlling the GPC was found on chromosome 6H in barley, together with its paralogue sequence, designated HvNAM2 on chromosome 2H (Uauy et al., 2006a). In a study between Tibetan wild barley (Hordeum spontaneum) and cultivated barley (Hordeum vulgare) from different areas around the world, a significant correlation could be found between different haplotypes of the HvNAM1 gene and the GPC (Cai et al., 2013). In the same study one SNP (Single Nucleotide Polymorphism), nucleotide position 798, on the HvNAM2 gene was shown to be associated to GPC and the gene also showed a correlation between its haplotypes and GPC (Cai et al., 2013). However, this study did not consider any differences between two-row and six-row barley nor in any Nordic barley material.

Tibetan wild barley is considered an original version of the barley that is used today, and thus it is of interest that the study showed Tibetan wild barley to have higher GPC than the cultivated barley (Cai et al., 2013). This indicates that the nutritional values of barley have decreased during the industrial breeding. What would be an interesting prospect to study is whether the same correlations and associations between haplotypes, polymorphism and nutritional values over time can be seen in Nordic accessions. This since the functional NAM-B1 allele in wheat has been shown to not only increase GPC but also accelerate senescence (Uauy et al., 2006a). It would therefore be reasonable to suspect that during the cultivation of Nordic barley, shorter growing seasons might have contributed to the preservation of the HvNAM wildtype allele. Such preservation has previously been found in the NAM-B1 gene in wheat (Hagenblad et al., 2012). Also the differences between the two subtypes of barley, two-row and six-row, would be an interesting prospect studying. This because they are developed under different ecological niches, where six-row barley is adapted to shorter and more intense senescence periods than two-row barley (Persson, 1997).
An earlier study of HvNAM1 in Nordic accessions, according to grain protein, iron and zinc content, could not detect any association between previously identified polymorphisms and the nutrient content (Madhavan, 2011). However, this study only considered two regions in HvNAM1 (Madhavan, 2011), nucleotide position 102 and 357, where Distelfeld et al. (2008) previously had found polymorphism to occur in barley. I have therefore expanded the study of Madhavan (2011) by searching for polymorphism within the entire sequence of HvNAM1 and additionally, within the sequence of HvNAM2.

In this study, I have investigated the correlations and associations between the nutritional values (grain protein, iron and zinc content) and the improvement status group, subtype and the thousand grain weight. More importantly I have also investigated whether different haplotypes of HvNAM1 and HvNAM2 in Nordic accessions of barley can be correlated to its nutritional value. Moreover the study tested whether single SNPs in the two genes caused mutations that can be correlated to changes in the nutritional content of barley.

3 Material and methods

3.1 Plant material

DNA used for the PCR optimization was extracted from plant tissue of Hordeum vulgare (Accession number: HOR11177) grown in a greenhouse from 20th to 25th March 2015. The soil was kept moist during the whole period. After harvesting, the plant materials was frozen and stored at -80 °C until used for DNA extraction.

The DNA used for the remaining study was provided by Linköping University and Nordiska Museet in Stockholm together with data on the accessions’ nutritional values and kernel sizes. The DNA had previously been extracted and the data collected by Madhavan (2011). The DNA-samples provided were from Hordeum vulgare of Nordic accessions, were 11 samples were landrace accessions, 6 samples from cultivars developed 1890 - 1940, 11 samples from cultivars developed 1941 – 1970 and 12 samples from cultivars developed 1971 – present. Two control samples were also obtained from accessions with low GPC and an inactive NAM-allele (Karl, accession number: Clho15487) respectively high GPC and an active NAM-allele (Lewis, accession number: Clho15856) (table 1).
3.2 DNA-extraction

The DNA used for the PCR optimization was extracted using the DNeasy Plant Mini Kit (QIAGEN, Netherlands) according to DNeasy Plant Handbook (QIAGEN, Netherlands, October 2012), with some modifications. The frozen tissue was first disrupted by grinding with a pestle in a 2 ml microcentrifuge tube together with the lysisation buffer, AP1. The centrifugation steps were carried out at a maximal speed of 17000 x g. During each of the two elution steps, 75 μl of buffer AE was added to the column and the eluates were collected in one 1.5 ml microcentrifuge tube.

3.3 PCR-amplification

The PCR-amplifications were conducted using S1000 Thermal cycler (Bio-Rad Laboratories, USA). The PCR reaction mixtures were at a total volume of 25 μl and contained 10 x DreamTaq buffer (containing 20 mM MgCl₂) (Thermo Scientific, USA), 0.2 mM dNTP Mix (Thermo Scientific, USA), 1.25 U DreamTaq DNA polymerase (Thermo Scientific, USA), 0.1 μM forward primer, 0.1 μM reverse primer and 1.25 μl DNA template. The sequences of the primers used are given in appendix table 1.

During the amplification of HvNAM1, the PCR-program was set as follows: initial denaturation at 94 °C for 2:30 min, 35 cycles of denaturation at 94 °C for 0:30 min, annealing at 63 °C for 0:30 min and elongation at 72 °C for 1:30 min and finally 72 °C for 10 min as a final elongation step. For samples where it was difficult to get a quality product a Touch-down PCR was performed where the 35 cycles from the original program was exchanged to; denaturation at 94 °C for 0:30 min, annealing at a starting temperature of 72 °C for 0:30 min and elongation at 72 °C for 1:30 min. The annealing temperature was decreased with 0.5 °C for each cycle until reaching a temperature of 62 °C where it remained for 30 cycles.

During the amplification of HvNAM2 the same setups were used as for the original program for HvNAM1, except for the annealing temperature that was set to 58 °C.

3.4 ExoTAP-purification

The PCR-products were purified enzymatically with the Exonuclease Thermosensitive Alkaline Phosphatase (ExoTAP) purification method. To 20 μl PCR-product, 0.4 U Exonuclease I (Fermentas, USA), 0.2 U Thermosensitive alkaline phosphatase (Thermo scientific, USA) and milliQ-water was added to a final volume of 28 μl. Thereafter the
samples were incubated, first for 30 minutes at 37 °C and then for 5 minutes at 95 °C, using a S1000 Thermal cycler (Bio-Rad Laboratories, USA).

3.5 DNA sequencing

The samples were sent to Macrogen Europe (The Netherlands) for DNA sequencing with the fluorescent dye terminator Sanger sequencing method. Since the fragments to sequence during this study were more than 1500 bp, too long for sequencing in a single reaction, each fragment was sequenced in two parts. This was conducted by use of four primers for each gene, first the primers used during the amplification and also two internal primers, one forward and one reverse. The sequences of the primers are presented in appendix table 1.

3.6 Statistical analysis

The sequences received from Macrogen Europe were aligned and manually cleaned using visual inspection in the software Geneious. Using the same software the sequences were also analyzed for SNPs and translated into amino acid sequences. Thereafter, they were analyzed for amino acid substitutions in the variable nucleotide positions.

The statistical analyses were conducted using SPSS-statistics. One of the Nordic accessions, NGB13068, was excluded from the statistical analyses since no data over its nutritional values were available. Since GPC in barley cannot be measured directly it is calculated from the content of nitrogen with a linear correlation. Therefore, for all statistical analyses during this study, the nitrogen content is used to represent the correlations with GPC. Additionally the contents of iron and zinc were used as indicators for the nutritional value in barley.

3.6.1 One-way ANOVA

To test whether the different haplotypes found among the Nordic accessions were correlated to the nutritional values a one-way ANOVA was conducted with the significance level set to 0.05. A one-way ANOVA was also conducted to test for correlations between nutritional values and improvement status groups of the Nordic accessions.

3.6.2 Pearson correlation test

To test for correlations between nutritional values and the thousand grain weight, the weight in grams of 1000 grains, Pearson correlation tests were conducted with the significance level set to 0.05. The test was conducted
for the different subtypes separately, since the grain size of 2-row barley is usually bigger than those of 6-row barley.

3.6.3 Independent samples T-test

Independent sample T-tests, with the significance level set to 0.05, were used in those cases when only two variants were obtained for one parameter, such as for the correlation between nutritional value and the different subtypes of barley, i.e. two-row or six-row. Thus the correlation between the nutritional value and the variation in a nucleotide position where only two SNPs occurred was also tested by the same method.

4 Results

4.1 Sequence analysis

4.1.1 HvNAM1

To test whether variations in the sequences of HvNAM1 and HvNAM2 could be correlated to the nutritional values in Nordic barley, the genes were first sequenced and polymorphic regions detected. After sequencing the HvNAM1 gene two of the Nordic accessions, NGB4641 and NGB2105, were removed for being of too low quality for comparison to the other sequences. The remaining 40 sequences provided 1509 bases of good quality sequence, from nucleotide position 55 and forth of the total gene sequence. Within that region five SNPs were found, as can be seen in table 1. However, neither of the Nordic accessions had any SNP in common with the control accessions, Karl and Lewis with low respectively high GPC. All the SNPs in the low GPC control sample led to amino acid substitutions. The SNP at nucleotide position 1167 in one of the Nordic accessions caused no amino acid exchange. However, the deletion at nucleotide position 1559 caused a shift in the reading frame and thus amino acid substitutions following that position.

No significant correlations were found between the SNP at position 1559 in the Nordic accessions and the nutritional value, (N: p = 0.22, t = 1.24, Fe: p = 0.66, t = -0.45 and Zn: p = 0.98, t = 0.02), for either measure of nutrient quality (independent sample T-test).
Table 1. All accessions used during the study, their country of origin, subtype and improvement status group. Variable positions are presented for HvNAM1 and HvNAM2 respectively, and the bases at those positions for each accession. Polymorphic bases are defined by squares. Empty spaces indicate the accessions for which no reliable sequence was obtained and a dash indicates a deletion at the position. Amino acid (AA) substitutions caused by the mutations are presented for non-synonymous mutations.

<table>
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<th>accession</th>
<th>origin</th>
<th>subtype</th>
<th>improvement status group</th>
<th>nt position:</th>
<th>HvNAM1</th>
<th>HvNAM2</th>
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<td>6rw</td>
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AA-substitutions: W→S A→K C→Y C→T N→I S→Stop

4.1.2 HvNAM2

After sequencing the HvNAM2 gene one of the Nordic accessions, NGB468, was removed for being of too low quality. The remaining 41 sequences provided 1352 bases of good quality sequence, from nucleotide position 158 and forth of the total gene sequence. However, no polymorphisms were encountered within the Nordic accessions and the sequences were identical. Also the sequence of the control accession Lewis, with high GPC, was identical to the Nordic accessions. The only sequence that showed polymorphism within the gene was Karl. It contained three transversions at the nucleotide positions 307, 798 and 979, only one of which, 798, led to a change in amino acid sequence by introducing a stop-codon (table 1).
4.2 Nutritional data

For 2-row barley, the nitrogen content but also the zinc content were significantly negatively correlated to the thousand grain weight (N: p = 0.00, r = -0.64 and Zn: p = 0.01, r = -0.56) (figure 1A and B). However, no correlation could be seen between the iron content and the thousand grain weight (Fe: p = 0.77, r = 0.07) (figure 1C). The analyses were conducted by Pearson correlation test (N = 20).

For 6-row barley, the result showed a significant positively correlation between the zinc content and the thousand grain weight (Zn: p = 0.03, r = 0.50) (figure 1D). A tendency towards a positive correlation could be seen also between the iron content and the thousand grain weight (Fe: p = 0.08, r = 0.42) (figure 1E). However, between the nitrogen content and the thousand grain weight no correlation could be seen (N: p = 0.80, r = 0.06) (figure 1F). The analyses were conducted by Pearson correlation test (N = 18).
Figure 1. Scatterplots, including fitted line with spikes, over the results from the correlation tests between the thousand grain weight (gms) of two-row barley and; A) N (% of DMB (Dry Matter Basis)), B) Zn (mg/kg DMB) and C) Fe (mg/kg DMB) and between the thousand grain weights (gms) of six-row barley and; D) Zn (mg/kg DMB), E) Fe (mg/kg DMB) and F) N (% of DMB). A star (*) indicates for a significant correlation (p < 0.05) and (tr) indicates for a trend towards a correlation (p < 0.10).

No significant differences was found between the nutritional values for the improvement status groups (landrace (N = 11), cultivar developed 1890-1940 (N = 6), cultivar developed 1941-1970 (N = 11) and cultivar
developed 1971-present (N = 11)) (N: p = 0.10, F = 2.24, Fe: p = 0.17, F = 1.76 and Zn: p = 0.48, F = 0.83) (ANOVA).

No significant associations between nutritional values of two-row (N = 20) or six-row (N = 18) barley were seen either (N: p = 0.60, F = 2.55, Fe: p = 0.67, F = 2.78 and Zn: p = 0.45, F = 2.04) (independent sample T-test).

When comparing the content of nitrogen between the Nordic accessions, Karl and Lewis the result shows a big part of the Nordic accessions to have a higher GPC than Lewis, which barely reaches the median value of the Nordic accessions (figure 2A). Only a small portion of the Nordic accessions has nitrogen content as low as Karl does. For the iron content the comparison is the opposite, were the Nordic accessions to a large extent has much lower iron content than both the control accessions (figure 2B). Only for the zinc content, the values are more evenly distributed with the control samples at opposite sides of the Nordic accessions sample median (figure 2C).

![Figure 2. Boxplots over the nutritional data for the Nordic accessions and the comparison with its variation to the control samples Karl (low GPC) and Lewis (high GPC) for the nutritional values of; A) N (% of DMB), B) Fe (mg/kg DMB) and C) Zn (mg/kg DMB). The boxes show the median and the upper and lower quartile at 25 %. The T-bars show the upper and lower limit within the samples.](image)

5 Discussion

5.1 Social and ethical aspects

Malnutrition is still a problem for many countries around the world, according to the “2015 world hunger and poverty facts and statistics” (May 2015) and one major problem, except from the lack of cultivable soils, is the lack of micronutrients. An increasing knowledge about the genes involved in the nutrient remobilization in barley, but also in other
cereals, is therefore important. This is due to the possibilities it will give the breeding industry to develop new varieties with better nutritional values, but still a high yield. In turn it would be an important step towards solving the worlds hunger issues. The GPC is additionally important for the malt and beer production, where stable low protein content is required. This might not be as much of a world issue, but of great financial interest to the developed countries.

5.2 HvNAM1

The HvNAM1 gene of the Nordic accessions contained three different haplotypes, with no SNP shared with the ones found in the control accessions, Karl and Lewis. This result might match the hypothesis that Nordic accessions have suffered shorter growing seasons, causing other influences on the HvNAM1 gene. This would be in correlation to the study by Hagenblad and colleagues (2012), which showed how a shorter growing season caused the preservation on NAM-B1 in wheat. In this study, one of the Nordic haplotypes was found only in the accession NGB6929 and therefore its correlation to the nutritional values could not be tested statistically. On the other hand, the SNP did not cause any change in the amino acid sequence and it is very unlikely that it would have caused any changes in the function of the gene. The other SNP found within the Nordic population was present in seven accessions. However, it did not show any correlation to the nutritional values even though it was the cause of a shift in the reading frame. How the shift might have changed the end region of the gene could not be analyzed, since the shift was located at the very end of the part of the gene that was successfully sequenced. Notably, however, is that the SNP, and the ensuing shift in reading frame, was situated at the end of the gene meaning a big part of the gene was still intact. This might explain why no effect was found on the nutritional value from the SNP, but also it might be an indication that the exact sequence of the C-terminal of the protein is not of great importance for the gene function. This is supported by that all NAC domain proteins have a highly variable C-terminal region (Distelfeld et al., 2008).

The HvNAM1-gene in Karl contained 3 different SNPs which were the same as Cai with colleagues (2013) found in cultivated barley from different areas around the world. However, those SNPs were without any associations to the nutritional value (Cai et al., 2013). In this study, all the SNPs in Karl led to amino acid substitutions. However, these results differ from the SNPs that Distelfeld and colleagues (2008) found to cause amino acid substitutions in barley. An explanation for such varying results might be that there is genetic variation within the Karl accession.
5.3 HvNAM2

In the Nordic accessions, the HvNAM2 gene was completely invariant. The Karl accession on the other hand showed similar polymorphism as previously presented by Cai and colleagues (2013). Moreover only one of the SNPs, at nucleotide position 798, led to a change in the codon. However, this nucleotide position is, according to the study by Cai and colleagues (2013), located within the second intron of the gene and thus, it will not affect the amino acid sequence. However, the SNP is associated to the GPC in barley (Cai et al., 2013). Introns have proved to be important for the gene expression in several studies before (Brinster et al., 1987; McKenzie & Brennan, 1996; Martínez-Salazar et al., 2014). One explanation might be that the nucleotide position is involved in the splicing of the gene, for instance as a binding site for the spliceosome. Another explanation could be that the intron site is somehow important for the gene expression.

5.4 Gene expression

Only a small degree of polymorphism and no correlations between the SNPs and the nutritional values in the Nordic accessions could be found in this study. This might be due to a preserved function of the HvNAM genes in Nordic accessions and thus that the difference in nutritional value in this case is not correlated to the sequence of those genes at all. Instead the nutritional values might be correlated to the expressions of the genes, which can be caused by differences in the regulatory regions. For instance there could be mutations at regulatory elements, promoters or genes that control the gene expression of HvNAM1 and/or HvNAM2. A natural next step for research would therefore be studying the expressions of the genes.

5.5 Nutritional correlations

When comparing the content of nitrogen between the Nordic accessions, Karl and Lewis it is clear that even Lewis, that is considered to have a high GPC, barely reach the median value for the Nordic accessions, (figure 2A). This suggests that the Nordic accessions generally have a high GPC. For the iron content the comparison is the opposite, were the Nordic accessions seem to have generally low iron content (figure 2B). For the zinc content, however, the differences between the Nordic accessions, Karl and Lewis are not as obvious (figure 2C). These results indicate that there are other genes affecting the nutritional contents in Nordic barley, such as genes that are correlated to utilization of the micronutrients from the soil or other metabolic processes. This since it seems unlikely that a mutation in a NAM gene should affect different
micronutrients in different ways. It has previously been found by Uuay and colleagues (2006a) that the NAM genes in wheat have a pleiotropic effect on the remobilization of nitrogen, iron and zinc towards the grains during senescence.

The comparison of the nutritional data against the thousand grain weight showed a significant negative correlation for two-row barley, while for six-row barley it showed a significant positive correlation. For the two-row barley the results might be explained that the nutrients, grain protein and zinc, are diluted with the increase in yield. Such occasions could be caused by the preservation of the gene function and expression even though the cultivation process is increasing the yield. Such a dilution effect has previously been suggested in wheat (Fan et al., 2008; Oury et al., 2006). The question then would be why such a dilution did not occur also for the iron content?

Six-row barley is developed for different niches, with shorter growing seasons, compared to two-row barley and thus, a shorter senescence period has been of an evolutionary greater importance than a high yield, which has caused the six-row barley to get a lower thousand grain weight than two-row barley (Persson, 1997). The reason for the positive correlation between the zinc content and the thousand grain weight, and the trend towards a positive correlation between the iron content and the thousand grain weight, might therefore not be due to a more efficient NAC transcription factor but due to the consistency of the NAC transcription factor even when the senescence period and therefore also yield has been decreasing. However, the question will be why such a positive correlation did not occurred also for the nitrogen content? It has previously been found that the NAM-B1 gene in wheat is correlated to both the senescence period and the remobilization of nitrogen to the grain (Uuay et al., 2006b). The study shows how a shorter senescence period is correlated to a decrease in grain size but an increase in GPC (Uuay et al., 2006b) which support the positive correlations found between thousand grain weight and nutrient content from this study. However, it does not support the lack of correlation between the GPC and the thousand grain weight.

It has previously been shown for both wheat (Garvin et al., 2006; Fan et al., 2008) and barley (Cai et al., 2013) how the nutritional values have decreased along with the cultivation process. However, none of the nutrients, nitrogen, iron or zinc, showed any significant correlations to neither the improvement status groups nor the subtypes during this study. This indicates that for the Nordic accessions, the nutritional values has not changed much over time or differ between the subtypes. This is
despite that several of the nutritional values were correlated to the thousand grain weight, which differed between the two subtypes. This suggests that the overall nutritional values are similar between two-row and six-row barley but within each subtype it differs depending on the thousand grain weight, and again, the divergent results might have been an indication of the involvement of other genes.

5.6 Conclusion

Neither the HvNAM1 nor the HvNAM2 gene showed any genetic variation that was correlated to the nutritional values in Nordic barley, which might be due to the preservation of those genes due to short growing seasons. However, there was a significant association found between the nutritional values and the thousand grain weight in barley depending on its subtype. Two-row Nordic barley had a negative correlation between nitrogen/zinc and thousand grain weight while six-row barley had a positive correlation between the zinc content and the thousand grain weight, while for the iron content a tendency towards a correlation could be seen.

The difference in the nutritional values and thus the nutrients correlation to thousand grain weight found in this study might not be due to the gene sequence, but rather to the expression of the genes. Therefore it would be of great interest to study the gene expression, as a future prospect of investigation.

6 Acknowledgement

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7 References


Madhavan, S. (2011). Screening of HvNAM-B1 gene polymorphism, grain nutrient content and seed size in 80 Scandinavian barley cultivars. [http://urn.kb.se/resolve?urn=urn%3Anbn%3Ase%3Aliu%3Adiva-69079](http://urn.kb.se/resolve?urn=urn%3Anbn%3Ase%3Aliu%3Adiva-69079)


# Appendix

Table 1. The sequences of the primers used during the amplification of HvNAM1 and HvNAM2 together with the extra primers used during the sequencing of the genes.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HvNAM1 forward</td>
<td>5’-ATGGGCAGCCCGGACTCATCCTCC-3’</td>
</tr>
<tr>
<td>HvNAM1 reverse</td>
<td>5’-TACAGGGATTCCAGTTCACGCAGCGGAT-3’</td>
</tr>
<tr>
<td>HvNAM1 sequencing forward</td>
<td>5’-GCATGAGTACCGCTCAC-3’</td>
</tr>
<tr>
<td>HvNAM1 sequencing reverse</td>
<td>5’-GTGAGGCGGTACTCATGC-3’</td>
</tr>
<tr>
<td>HvNAM2 forward</td>
<td>5’-ATGGGCAGCTGGACTCATCTTCC-3’</td>
</tr>
<tr>
<td>HvNAM2 reverse</td>
<td>5’-TCAGGGATTCCAGTTCACGCAGCGGAT -3</td>
</tr>
<tr>
<td>HvNAM2 sequencing forward</td>
<td>5’-GCAGTAACCAGTCTCCGATTT-3’</td>
</tr>
<tr>
<td>HvNAM2 sequencing reverse</td>
<td>5’-GGAGATCGTTACTGCTTGAC-3’</td>
</tr>
</tbody>
</table>