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Suppression of C-Hordein Synthesis in Barley by Antisense Constructs Results in a More Balanced Amino Acid Composition

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Barley has for feeding purposes a shortage of essential amino acids, especially lysine, threonine, and methionine, and an excess of proline and glutamine. In the present study, we have introduced into barley an antisense construct against C-hordeins, the storage protein with the lowest nutritional quality. SDS-PAGE and reverse phase HPLC revealed a relative reduction in the amounts of C-hordeins and relative increases in the content of the other storage proteins. The five different lines analyzed had lower amounts of proline, glutamic acid/glutamine, and phenylalanine (up to 12%, 6%, and 9% reductions), while the lysine, threonine, and methionine content was increased with up to 16%, 13% and 11%. It is concluded that antisense mediated suppression of C-hordein synthesis may be a promising approach for improving the nutritional value of barley as a feed crop while at the same time reducing the environmental nitrogen load.

KEYWORDS: Essential amino acids; antisense; storage proteins; cereals; high lysine

INTRODUCTION

Cereals are the most important crops in the world with a total annual grain yield exceeding 2000 million tonnes (mt). Despite a relatively low grain protein content (8−14%), cereals provide more than 200 mt of protein for the nutrition of humans and livestock. This is about three times the amount derived from legume seeds (*1*) However, while cereals are excellent sources of metabolizable energy in the form of starch, they have a number of nutritional shortcomings, including a low content of micronutrients, low bio-availability of phosphate reserves for monogastric animals (*2*), and a far from ideal amino acid composition (*3*, *4*).

Intensive livestock production is at present largely based on cereals with a large-scale supplementation with soybean or other high protein sources as well as microbially produced or synthetic amino acids. In barley, the first and second limiting essential amino acids for feeding pigs are lysine and threonine and for broilers they are methionine, lysine, and threonine. In addition to these primary deficiencies, there is a suboptimal content of isoleucine, leucine, and valine which in contrast to lysine, threonine, and methionine are not yet commercially available at a price realistic for feed purposes (*5*). The second major issue with regard to the amino acid composition of barley is an excess of the nonessential amino acids proline and glutamine. The surplus is degraded, and the nitrogen is excreted. In consequence, the nitrogen content of animal waste is unnecessarily high and makes a significant contribution to the environmental nitrogen load in areas with intensive livestock production. The problems encountered are very similar for wheat while in maize lysine and tryptophan are the primary limiting amino acids

The unbalanced amino acid composition of maize, barley, and wheat results from the abundance of alcohol-soluble storage proteins (prolamins) that typically constitute around 50% of the total grain protein. In maize, prolamins are termed zeins; in wheat, gliadins; and in barley, hordeins. These proteins are characterized by an abundance of glutamine and proline while there is a low content of essential amino acids. The other storage protein fractions, the albumins, globulins and glutelins have from a nutritional point of view a more balanced composition.

Over the last five decades large-scale mutagenesis programs have been devoted to improving the amino acid composition of in particular maize and barley. A range of primarily recessive mutants have been identified that confer a high lysine phenotype, in particular the *opaque-2* mutants of maize and the *lys* mutants in barley (*6*). However, the high lysine trait is invariably

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associated with pleiotropic effects that affect yield, quality, and agronomic performance. The opaque phenotype in maize confers a uniform soft chalky endosperm without the hard, vitreous, and translucent peripheral endosperm of wild type maize while in barley there is a reduced starch production combined with enlargement of the embryo. In maize, it has been possible by conventional breeding to introduce so-called modifier genes that ensure starch quality, yield, and other characteristics similar to those found in the wild type (*7*). In double recessive *opaque-2* lines, the lysine content increases to 3.8% compared to 1.5% in the near isogenic line. This increase is due to a higher content of the amount of free amino acids and nonzein proteins and a reduction in the α-zein content (*8*).

In barley, substantial improvements have been achieved in the yield of *lys* mutants but so far not to a level that makes high lysine barley commercially competitive (*6*). The four best characterized barley mutants are *lys1* (Hiproly), *lys3* (Risø 1508), and *lys7* (Mutant 7) and Mutant 56 found in the Carlsberg II cultivar. Additional alleles have been found, in particular, in the *lys3* locus where the *lys3a* allele confers the highest lysine phenotype (*9*). The *lys3* gene has not yet been cloned. Mutant 56 has a large deletion covering the locus for the major group of prolamin storage proteins, the B-hordeins (*10*). In the *lys3a* mutant, there is a decrease in the amount of B- and C-hordeins due to reduced transcription but an increase in the high molecular weight D-hordeins (*11*). There are enhanced transcript and protein levels of chymotrypsin inhibitors and an α-amylase/subtilisin inhibitor, proteins that all possess a high lysine content. On the other hand, the genes encoding the so-called protein Z and β-amylase are down-regulated by the *lys3a* mutation (*12*, *13*).

A large number of gene technological approaches have launched for improving the seed lysine content in a range of species (*14*). The efforts have addressed (1) the possibilities for circumventing the feedback regulation of lysine and threonine biosynthesis in the aspartate family pathway by bacterial genes encoding feedback-insensitive aspartate kinase and dihydrodipicolinate synthase; (2) reducing lysine catabolism; and (3) the introduction of genes encoding heterologous storage proteins with high levels of, e.g., lysine and methionine including synthetic genes designed for a high lysine and methionine content. However, although significant increases in, e.g., the lysine content have been achieved, other drawbacks have been reported such as reduced seed viability.

At present, one of the most promising strategies appears to be an altering of the relative proportions of the storage protein by antisense or RNAi. In rice, it has been shown that antisense suppression of glutelin formation results in an increased synthesis of prolamins which confers improved functional properties with regard to the brewing of sake (*15*). In oilseed rape (*Brassica napus*), Kohno-Murase et al. (*16*) suppressed the synthesis of nutritionally poor storage proteins, cruciferin, by antisense technology and found a compensating increase in the other major storage protein, napin, that has a higher lysine and methionine content. In maize, RNAi inhibition of transcription of the 22 kDa α-zeins resulted in an 18.5% increase in lysine content with limited compensatory changes in the abundance of other storage proteins (*17*). Targeting of the 19 kDa α-zein gene family by an antisense construct led to significant increases in lysine, threonine, and tryptophan (*18*).

In the present study, we have taken a similar approach involving an antisense mediated suppression of the C-hordeins, the hordein subgroup that has the poorest nutritional and environmental profile. The polypeptides of this subgroup consist primarily of proline and glutamine and have a very low content of essential amino acids (**Table 1**). In contrast to the three other subgroups of storage proteins, the B-, D-, and γ-hordeins, C-hordeins are entirely devoid of cysteines (*19*, *20*). We find in the transgenic lines relative reductions in the C-hordein content and relative increases in B, D-, and γ-hordeins as well as increases in the amounts of albumins/globulins and glutelins. These changes are accompanied by reductions in proline, glutamine, and phenylalanine and increased amounts of the remaining amino acids.

Table 1. Amino Acid Composition (mol %) of D-, C-, B-, and γ-Hordeins[a]

type (code)[a]	D (Q40054)	C (Q40055)	B (P06470)	γ (P17990)
ALA	3.2	1.5	2.6	2.1
ARG	1.6	0.9	2.6	1.8
ASX	1.5	1.5	0.7	2.4
CYS	1.5	0.0	2.9	3.5
GLX	28.0	38.8	32.1	30.1
GLY	15.7	0.6	2.9	3.1
HIS	3.0	0.6	1.5	1.4
ILE	0.7	3.4	4.4	3.8
LEU	4.1	8.6	8.0	7.0
LYS	1.2	0.9	0.7	1.8
MET	0.4	0.0	1.1	1.8
PHE	1.3	7.7	4.7	5.6
PRO	10.5	29.1	19.4	16.8
SER	10.5	2.5	4.7	5.6
THR	7.3	1.2	2.2	3.1
TRP	1.2	0.6	0.7	0.7
TYR	4.2	1.8	2.6	2.1
VAL	4.1	0.3	6.2	7.3

[a] Derived from amino acid sequences (Databank Uni Prot KB/TREMBL (http://pir.georgetown.edu).

MATERIALS AND METHODS

Plant Material. Barley plants (*Hordeum vulgare* cv. Golden Promise) were grown in a greenhouse in soil beds or in pots (50:50 peat:perlite mix) under a cycle of 16 h illumination and 8 h darkness at 23 and 18 °C, respectively.

Expression Vectors. For the transformation experiment, the twin T-DNA vector pWBVec82b (*21*) was used. This vector contains a 35S promoter, the hygromycin resistance gene, and the *nos* terminator between one set of T-DNA borders and an adjacent border set for insertion of the gene of interest. A 480 bp fragment of the 3′ region of a C-hordein encoding gene (Gene Bank Accession No: S66938) was amplified from barley genomic DNA by using primers RLHordC 5′-GGTTACACATGATGGTGCACATCA-3′ and FSHordC 5′-CTG-GCAACCACAACAACCATTTC-3′. The fragment was cloned in antisense orientation into the *Sma*I site of pBluescript KS+ (Fermentas Life Sciences). A *Pst*I−*Sal*I fragment from pUBarnIII (*22*), containing the 1.4 kb ubiquitin promoter (*23*) and an *Eco*RI−*Sac*I fragment containing the *nos* terminator (*24*) were cloned into pUC19. The antisense construct was assembled by the insertion of the antisense C-hordein fragment between the ubiquitin promoter and the *nos* terminator. The antisense construct was released by *Hin*dIII-*Not*I digest and cloned into the *Hin*dIII-*Not*I sites of the binary vector pWBVec82b (*21*), located between the second set of borders. The completed construct was named pML15. The plasmid was purified by using an Eppendorf Perfectprep Plasmid kit (Eppendorf AG).

Plant Transformation. The *Agrobacterium tumefaciens* strain AGL0 (*25*) was transformed with the pML15 plasmid by the freeze−thaw method (*26*). *Agrobacterium* mediated transformations of barley immature zygotic embryos (approximately 14 days after pollination with an embryo length of 1−2 mm) were carried out using hygromycin selection as described by Mathews et al. (*21*). The regenerated lines were termed AsHorC.

Transgene Integration Analysis by Southern Blot. Transformation and transgene integration was assessed by Southern blot hybridization.

Genomic DNA was isolated as described by Guidet et al. (*27*) and digested with the restriction endonuclease EcoRV. The digested DNA was resolved by electrophoresis (0.7% agarose gels; 5−8 μg/lane) and blotted onto Hybond N^+ membranes according to the manufacturer's instructions (Amersham Pharmacia Biotech Inc). Radioactive probes were made by random priming using the Ready-To-Go DNA labeling beads according to the manufacturer's instruction (Amersham Biosciences). The T-DNA probe was a 1.4 kb fragment of the ubiquitin promoter released by an *Eco*RI/*Kpn*I digest (*23*). Filters were hybridized according to the manufacturer's instructions (Amersham Pharmacia Biotech Inc) followed by two instances of 1 h washing in 1× SSPE and 1% SDS at 65 °C.

Analysis of Antisense Gene Expression. The expression of the antisense construct was studied by reverse transcription Polymerase Chain Reaction (RT-PCR). Total RNA was prepared from leaves with TRIzol reagent (Invitrogen), and the first strand cDNA synthesis was performed (Life technology, Superscript RT enzyme). C-Hordein specific primers FSHordC 5′-CTGGCAACCACAACAACCATTTC-3′ and RSHordC 5′-AGACAATTCCTCTGGTAGATAGG-3′, producing a 300 bp fragment, were used to amplify the C-hordein transcript. The reaction mixture consisted of 200 ng template cDNA, 2 μL PCR buffer, 1 Unit Taq DNA polymerase, 0.2 mmol/L of each of the dNTP's, 1.5 mmol/L $MgCl_2$, and 10 μM of each primer in a final volume of 20 μL. Samples were heated to 95 °C for 10 min and amplified in a Biometra T3 Thermocycler by 39 cycles for 30 s at 95 °C for denaturation, 45 s at 55 °C for annealing, and 45 s at 72 °C for extension. A pair of tubulin primers TUB F 5′-GGTCATCTCATC-CCTGACTGC-3′ and TUB R 5′-CGCTTGGCGTACATGAGGTCG-3′ producing a 508 bp fragment from cDNA was used for generating an endogenous control fragment. The conditions for amplification of the tubulin fragment were as above with the exception that annealing was performed at 60 °C.

Analyses of Storage Protein Composition. The storage protein composition of the transgenic lines was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and reversed phase (RP-) HPLC. For the initial screening of seeds of the primary regenerants, embryos were dissected out of sterilized mature seed and germinated on a half-strength Murashige and Skoog medium (*28*) in a growth chamber at 24 °C. When the root system was established, the plantlets were transferred to pots in the greenhouse. The remaining part of the seeds was used for the biochemical analyses. The first screening comprised an evaluation of changes in the relative proportions of the different hordeins by using SDS-PAGE. The de-embryonated seeds were crushed, and the flour was extracted by ethanol (70%, v/v; 3 μL ethanol/mg flour). After incubation for 1 h at room temperature, the samples were centrifuged (10 min at 6.000*g*) and the supernatant with the hordeins in solution was removed. An additional round of extraction was carried out with 0.7% (v/v) acetic acid, 0.6% 2-mercaptoethanol, and 55% propan-2-ol (*29*), and the supernatants from the two extractions were combined. A 10 μL portion of the protein extract was mixed with sample buffer (20% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate, 0.02% (w/v) bromophenol blue, 200 mM Tris, 40 mM dithiothreitol, pH 7.5) and heated at 100 °C for 3 min. The hordeins were separated in a Nupage 4−12% Bis-Tris gel with a MOPS buffer (Invitrogen Life Technologies Ltd.). The protein standard was a prestained SDS-PAGE standard (BIO-RAD). The gel was run at 125 V for 1.5 h, and coomassie stained according to the work of Schägger and Vonjagow (*30*), where ethanol was used instead of methanol.

For the RP-HPLC analysis, samples of wild type barley and T_2 grains from five selected transgenic lines were crushed and extracted stepwise two times with 1.0 mL NaCl (0.4 M) + $HKNaPO_4$ (0.067 M, pH 7.6) at 20 °C (extraction of albumins and globulins), three times with 0.5 mL 60% (v/v) ethanol at ≈20 °C (extraction of prolamins), and two times with 1.0 mL 50% (v/v) 1-propanol + urea (2 M) + Tris-HCl (0.05 M, pH 7.5) + dithioerythritol (1%, w/v) under nitrogen at 60 °C (extraction of glutelins) (*31*). Each suspension was centrifuged for 15 min at 6000*g* at room temperature. After centrifugation, the corresponding supernatants were combined and diluted to 2.0 mL with the respective extraction solvent. Aliquots (200 μL) of prolamine extracts were dried under a stream of nitrogen and treated with 200 μL of the glutelin extraction solvent. RP-HPLC analysis of the filtered extracts was performed as described by Wieser et al. (*31*) and Gellrich et al. (*32*). The injection volumes were 100 μL of albumins/globulins or 50 μL of the others. Eluted proteins were detected at 210 nm and quantified using the corresponding absorbance areas at 210 nm, which have been shown to be highly correlated with the amount of protein type (*31*).

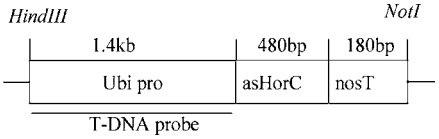

Figure 1. Schematic drawing of the antisense expression casette. The expression casette was assembled in pBluescript and cloned with *Hin*dIII/*Not*I digest into the multiple cloning site of pWBVec8.2 (*21*).

Measurement of Amino Acid Content. The total amino acids and total protein of wild type and T_2 seeds were determined on seed from material grown in soil beds. The amino acid content was analyzed according to the European standard procedure 98/64/EF. The tryptophan content was not measured. The total protein content was determined by measuring total nitrogen by the Dumas method on a Rapid N *III* instrument (Elementar, America Inc.) and multiplying by 6.25.

Evaluation of Amino Acid Content in Relation to Total Protein Content. As the protein content of the different lines were quite different, we implemented the equation developed by Boisen (*33*) for evaluating the feed value of barley at different protein concentrations. The lysine, threonine, methionine, isoleucine, leucine, and valine content as percentages of total amino acids were for each of the transgenic lines plotted against total protein content and compared with the values calculated from the equation. The general equation used for calculating percentage amino acids in relation to percentage total protein was the following:

$$\frac{\%P \times \mathrm{CF} + Y}{\%P} \times 100$$

%*P*: percentage of total protein of dry matter

CF: conversion factors for each amino acid

Y: constant for each amino acid

Lysine: CF = 0.0235, *Y* = 0.133. Methionine: CF = 0.0152, *Y* = 0.019. Threonine: CF = 0.0299, *Y* = 0.044. Isoleucine: CF = 0.0337, *Y* = 0.1. Leucine: CF = 0.0692, *Y* = 0.005. Valine: CF = 0.0417, *Y* = 0.076.

RESULTS

Transformation of Barley and T-DNA Integration. Barley immature embryos were transformed with a C-hordein antisense construct by *Agrobacterium* mediated transformation using hygromycin as the selectable marker (**Figure 1** (*21*)). From 650 infected immature embryos, 48 barley lines were regenerated and analyzed by Southern blot experiments to establish their transgenic status. Hybridizing bands were found in 35 lines of primary transformants. The transformation frequency, expressed as Southern positive lines in percentage of the total number of infected embryos, was accordingly 5.4%. **Figure 2** shows the Southern blot hybridization results of five lines that were chosen for further analysis based on RT-PCR evidence for expression of the transgene and suppression of C-hordein synthesis as revealed by SDS-PAGE. Two of the lines had single locus integration sites (**Figure 2**, lines 1 and 4), line 2 had two integration sites, and four integration sites were identified in two lines (**Figure 2**, lines 3 and 5). Lines 1, 2, 3, and 5 possessed bands of a size below that of the antisense cassette indicating rearrangements and/or deletions.

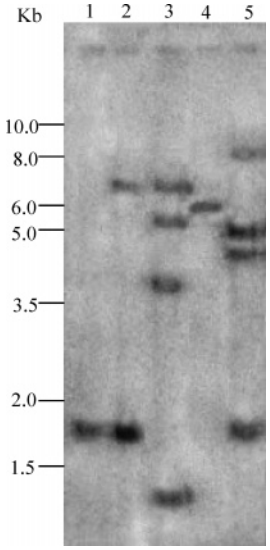

Figure 2. Southern blot demonstrating the transgenic status of the five antisense lines (1–5). A 1.4 kb fragment of the ubiquitin promoter was used as probe.

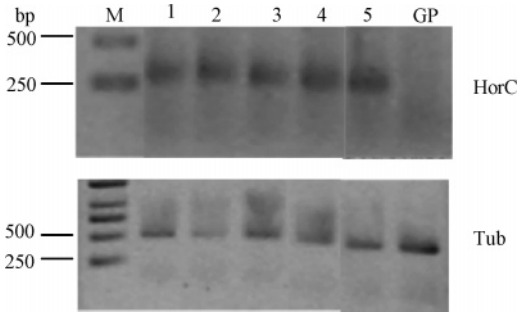

Figure 3. RT-PCR on leaves from the transgenic antisense lines using primers for the C-hordein transcript. GP = Golden Promise (mother cultivar).

Detection of Transgene Expression by RT-PCR. Expression of the antisense transcript from the Ubi-AsHorC-nosT construct (**Figure 1**) was evaluated in the five selected transgenic barley lines by performing RT-PCR reactions with RNA isolated from leaf material of the transgenic lines. Amplification of a tubulin gene was used as control. In all five plants, a 300 bp fragment was found as expected from the primer design for detection of the antisense C-hordein fragment while no bands were amplified from the mother cultivar (**Figure 3**).

SDS-PAGE Analysis of the Hordeins in the Transgenic Lines. Thirty-five Southern positive primary regenerants were grown to maturity in the greenhouse. The hordein polypeptide profile was determined by SDS-PAGE electrophoresis of three seeds from each line. As the transgenic trait is expected to segregate in the T_1 seeds, an SDS-PAGE gel was run for each seed. The embryo part was dissected from the endosperm and germinated to produce the T_1 generation that subsequently by self-pollination generated the T_2 population.

Eighteen of the 35 transgenic lines appeared to have a changed hordein profile (data not shown). Five of these lines were chosen for further analysis. The T_2 seeds from the five lines were tested again by electrophoresis in order to confirm the changes in the hordein profile (**Figure 4**). The profile was basically the same as that observed in the T_1 seeds (data not shown). As evidenced from the intensity of the hordein polypeptide bands, it appeared that all 5 lines had a reduced C-hordein content compared to the mother variety. Furthermore,

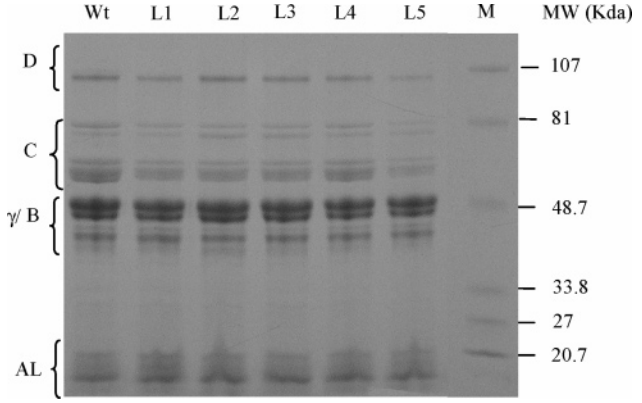

Figure 4. SDS-PAGE of the extracted endosperm proteins from single de-embryonated seeds of wild type (Wt) and T_2 seeds of antisense lines 1–5. The molecular weights are given in kilodalton. The positions of the D-, C-, and γ/B-hordeins are indicated. AL = albumins and globulins.

Table 2. Relative Amounts (Absorbance Units (AU) $\times 10^{-2}$/2 mL) and Percentages (%) of Albumins/Globulins (AL), Prolamins (PR), and Glutelins (GL) in the Five Different Antisense Lines (L1–L5) and the Mother Variety Golden Promise (Wt)

	Wt	L1	L2	L3	L4	L5
AL (AU)	205	219	197	168	193	172
AL (%)	9.2	12.3	10.9	11.2	10.9	8.6
PR (AU)	1166	819	705	563	759	828
PR (%)	52.3	46.1	38.9	37.5	42.9	41.3
GL (AU)	860	739	909	769	819	1006
GL (%)	38.5	41.6	50.2	51.3	46.2	50.1
sum (AU)	2231	1777	1811	1500	1771	2006

there appeared to be small differences in the total amounts of hordeins between the lines with number 3 being the one with the lowest hordein content.

RP-HPLC Analysis of the Storage Protein Composition of the Transgenic Lines. The storage proteins were isolated from the five selected lines as described in the Materials and Methods section and subjected to RP-HPLC analysis. The relative amounts and proportions of albumins/globulins (AL), prolamins (PR), and glutelins (GL) were determined by integration of absorbance areas at 210 nm shown to be highly correlated with the amounts of proteins (*31*). HPLC analysis of unreduced prolamins (PR) was used to determine the total amounts of prolamins (**Table 2**).

In the mother cultivar, prolamins constituted 52.3% of the total extractable proteins, while in the transgenic lines the relative prolamine content was reduced, ranging from 37.5% to 46.1% of total extractable proteins (**Table 2**). The glutelin fraction increased from 38.5% in the mother cultivar to 41.6%, 50.2%, 51.3%, 46.2%, and 50.1% in the five transgenic lines, while there were minor relative increases in the albumin/globulin fraction in the transgenic lines compared to the mother line. The chromatograms of reduced prolamins and glutelins were used for determining the relative content of the hordein subgroups (D-, C-, and γ/B-hordeins). The relative amount of the C-hordeins ranged from 26.7% to 31.4% in the five lines while in the mother line the C-hordein fraction accounted for 35.4% of the total hordeins (**Table 3**). The relative amount of γ/B-hordeins was increased in the transgenic lines (range of 60.6−66.2% compared to 58.5% in the mother line) while there were also minor relative increases in the amounts of D-hordeins in the antisense lines (**Table 3**).

Amino Acid Analysis. Seed weight, protein content, and content of the individual amino acids were measured in the T_1

Table 3. Relative Amounts (Absorbance Units (AU) $\times 10^{-2}$/2 mL) and Proportions (Percent According to Total Hordeins) of D-, C-, and γ/B-Hordeins in the Five Different Antisense Lines (L1–L5) and the Mother Variety Golden Promise (Wt)[a]

	Wt		L1		L2		L3		L4		L5	
protein	AU	%	AU	%	AU	%	AU	%	AU	%	AU	%
D-hordein	100	4.9	76	4.9	91	5.7	90	6.7	90	5.7	128	7.0
C-hordein	717	35.4	444	28.5	431	26.7	372	27.9	496	31.4	569	31.0
γ/B-hordein	1185	58.5	1013	65.0	1069	66.2	850	63.8	972	61.6	1112	60.6
residual	24	1.2	25	1.6	23	1.4	21	1.6	21	1.3	25	1.4
Σ	2026	100.0	1558	100.0	1614	100.0	1333	100.0	1579	100.0	1834	100.0

[a] The data for the mother lines are the means of two individual samples.

Table 4. Amino Acid Content in Grams per 100 g Protein in Wild Type and the Five Transgenic Lines (L1–L5)[a]

	Wt	L1	%	L2	%	L3	%	L4	%	L5	%
ALA	3.8	4.3	(13.2)	4.1	(7.9)	4.2	(10.5)	4.1	(7.9)	4.4	(15.8)
ARG	5.0	5.5	(10.0)	5.2	(4.0)	5.4	(8.0)	5.3	(6.0)	5.6	(12.0)
ASP/ASN	5.6	6.3	(12.5)	5.9	(5.4)	6.1	(8.9)	6.0	(7.1)	6.3	(12.5)
CYS	2.2	2.6	(18.2)	2.4	(9.1)	2.4	(9.1)	2.3	(4.5)	2.6	(18.2)
GLU/GLN	28.7	25.9	(–9.8)	28.2	(–1.7)	28.5	(–0.7)	27.6	(–3.8)	27.0	(–5.9)
GLY	3.9	4.3	(10.3)	4.2	(7.7)	4.3	(10.3)	4.2	(7.7)	4.5	(15.4)
HIS	2.3	2.5	(8.7)	2.5	(8.7)	2.6	(13.0)	2.4	(4.3)	2.5	(8.7)
ILE	4.0	4.0	(0.0)	4.1	(2.5)	4.2	(5.0)	4.0	(0.0)	4.2	(5.0)
LEU	7.4	7.7	(4.1)	7.9	(6.8)	8.0	(8.1)	7.7	(4.1)	8.0	(8.1)
LYS	3.4	3.8	(11.8)	3.7	(8.8)	3.8	(11.8)	3.7	(8.8)	3.9	(14.7)
MET	1.7	1.8	(5.9)	1.8	(5.9)	1.8	(5.9)	1.8	(5.9)	1.9	(11.8)
PHE	6.3	5.8	(–7.9)	6.0	(–4.8)	6.1	(–3.2)	6.1	(–3.2)	5.9	(–6.3)
TYR	3.7	3.8	(2.7)	3.8	(2.7)	3.8	(2.7)	3.8	(2.7)	3.9	(5.4)
PRO	16.5	14.3	(–13.3)	15.5	(–6.1)	15.8	(–4.2)	15.0	(–9.1)	14.5	(–12.1)
SER	4.6	4.8	(4.3)	5.0	(8.7)	5.0	(8.7)	4.8	(4.3)	5.0	(8.7)
THR	3.4	3.6	(5.9)	3.6	(5.9)	3.6	(5.9)	3.6	(5.9)	3.7	(8.8)
VAL	5.4	5.8	(7.4)	5.8	(7.4)	6.3	(16.7)	5.7	(5.6)	6.0	(11.1)
total protein	17.1	12.8		13.3		14.5		15.4		15.7	
seed weight	35.2	39.3		40.8		31		38.5		37.6	

[a] The numbers in brackets are the relative differences in percentage between the amino acid content in the transgenic line and the wild type. The seed weight is the mean of 20 seeds.

seeds of the five transgenic lines and the mother line. Line 3 showed a lower seed weight than the mother line (31.0 mg compared to 35.2 mg) while the four other lines had heavier seeds (ranging from 37.6 to 40.8 mg; **Table 4**). Apart from this weight difference, the seeds appeared very similar to the mother line in their external morphology. The protein content of the transgenic lines ranged from 12.8% to 15.7% while the mother line had a protein content of 17.1%. The amino acid content (excluding tryptophan) was determined following hydrolysis of total proteins. The glutamine and asparagine contents were accordingly included in the glutamate and aspartate fractions. A number of differences in amino acid content between the transgenic lines and the mother variety were revealed (**Table 4**). In the transgenic lines, glutamine/glutamate, proline, and phenylalanine were less abundant than in the mother line. The percentage difference ranged from −0.7% to −9.8% for glutamine/glutamate, −4.4% to −14.6% for proline, and −2.5% to −8.5% for phenylalanine. The glutamine/glutamate and proline reductions are in agreement with the general reduction in the prolamin content of the transgenic lines as evidenced by SDS-PAGE and RP-HPLC. Furthermore, the decrease in phenylalanine is in line with a specific suppression of C-hordeins as this subgroup is enriched with phenylalanine (**Table 1**). All other amino acids were more abundant per 100 g of protein in the transgenic lines than in the mother line. The sulfur containing amino acids cysteine/cystine and methionine were increased by up to 17.0% and 13.2% (range: 3.1−17.0% and 6.8−13.2%) and other essential amino acids like lysine, threonine, isoleucine, leucine, and valine were increased by up to 16.0%, 10.7%, 6.6%, 8.1%, and 17.1%.

Evaluation of the Amino Acid Profile in Relation to the Protein Content of the Grain. The changes of the amino acid profile in relation to the total protein content were evaluated using a set of regression equations developed to predict the amino acid content as a function of different protein contents (*33*). The percentages of total protein for the essential amino acids lysine, threonine, methionine, isoleucine, leucine, and valine in antisense lines 1−5 were plotted against the total protein content and compared with the predictions for wild type barley (**Figure 5**). Except for isoleucine, all six amino acids were present in higher amounts than the wild type predictions throughout the total protein range of 12−17%. In the mother line, Golden Promise, the amounts of lysine, threonine, methionine, leucine, and valine exceeded the predictions but were lower than those in the transgenic lines, whereas the amount of isoleucine in the wild type was close to the predictions.

DISCUSSION

In the current study, a large number of transgenic barley lines were generated having constitutive expression of a C-hordein gene antisense sequence. The working hypothesis was that a selective suppression of C-hordeins would result in the up-regulation of the synthesis of other storage proteins and thereby an improvement of the amino acid composition of the barley grain. From a nutritional and environmental perspective, C-hordeins are inferior to all other types of proteins in the barley grain as they consist primarily of repetitive sequences of proline and glutamine with a very low content of essential amino acids like lysine, threonine, isoleucine, leucine, valine, methionine, and cysteine.

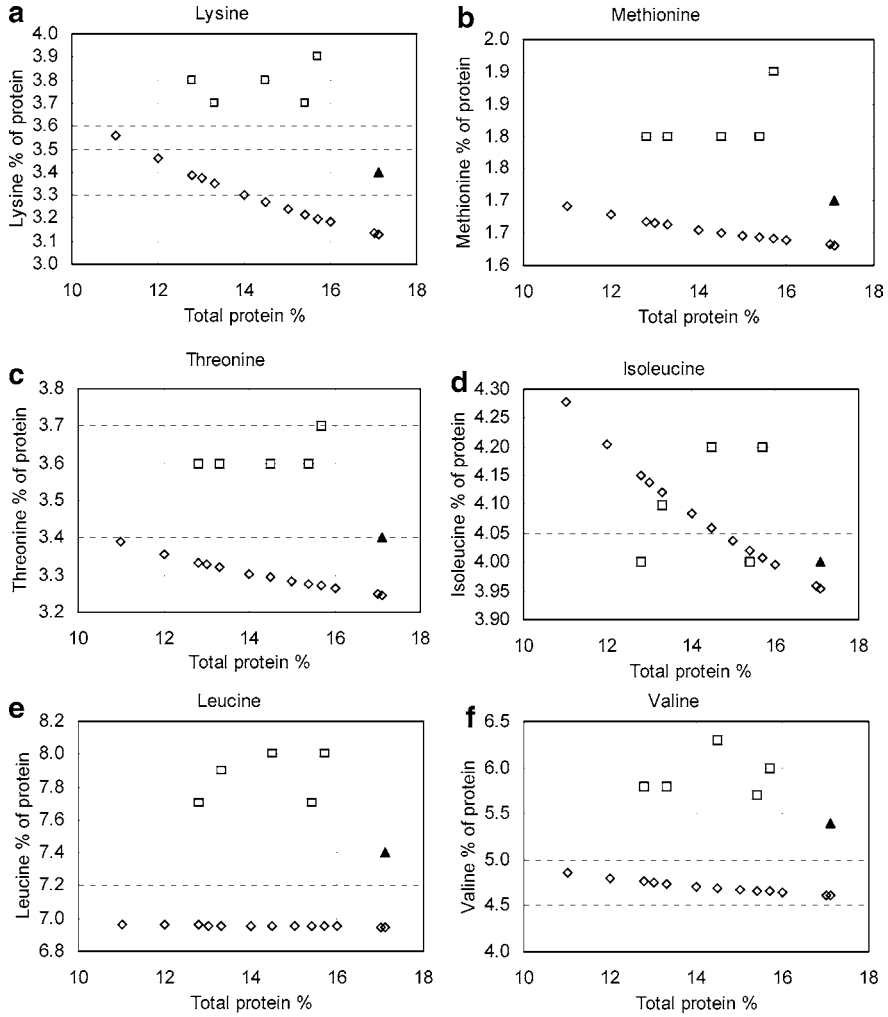

Figure 5. (**a**–**f**) Correlation between protein content and the percentage of six essential amino acids measured in wild type and the five transgenic lines compared to the predicted amino acid content as a function of the protein content: (▲) wild type; (◇) standard prediction from regression equation; (□) transgenic lines.

A total of 48 lines were generated of which 35 were shown by Southern blotting to harbor the introduced antisense construct. The transformation frequency amounted to 5.4% (number of transgenic lines regenerated divided by the number of embryos infected with *Agrobacterium*) which is very similar to that reported for other *Agrobacterium* mediated transformation experiments in barley (*21*). T_1 seeds of the 35 lines were prescreened for alterations in the abundance of the different hordein subgroups (γ/B-, C-, and D-hordein), and five lines displaying a reduced amount of C-hordein were selected for further studies. All lines expressed the antisense sequence in leaves as evidenced by RT-PCR in agreement with the ability of the maize ubiquitin promoter for driving a strong constitutive expression. In the current study, the transcription of the construct in the seeds was not analyzed, but several other studies have shown that the maize ubiquitin promoter also confers strong expression in the barley grain (*34*).

The effects of the antisense construct on the storage protein composition in the mature grain as well as the relative proportions of the different hordein subgroups were analyzed in detail by SDS-PAGE and by RP-HPLC. RP-HPLC has been found to be capable of high-resolution separations of cereal seed proteins, and the resolution is often better than that obtained by any other chromatographic or electrophoretic method (*31*). RP-HPLC resolves proteins primarily on the basis of differences in surface hydrophobicity, complementing other techniques that separate proteins on the basis of size or charge. However, because γ- and B-hordeins are similar in hydrophobicity, they could not be separated into single subgroups by RP-HPLC and were therefore quantified as a sum The analyses revealed an overall reduction in the prolamin fraction from 52.3% in the mother line to 37.5−46.1% in the transgenic lines (**Table 2** and **Figure 4**) and an increase in the albumin/globulin and glutelin fractions. Four of the five C-hordein antisense lines possessed a minor increase in the albumin fraction (line 1−4), and all five lines had more glutelin (**Table 2**). Within the hordeins, there was a reduction in the relative content of C-hordeins (range of 26.7−31%) compared to 35.4% in the mother line while the relative proportions of D- and γ/B-hordeins were increased (**Table 3**).

The amino acid analysis of the transgenic lines showed a reduction in the amino acids that are very abundant in the C-hordeins, namely glutamine, proline, and phenylalanine and increases in the other amino acids with some variance between the lines (**Table 4**). The levels of the essential amino acids, lysine, threonine, and methionine were increased together with all the other amino acids including isoleucine, leucine, and valine that are considered to be the secondary limiting amino acids. Line number 5 had among the lines the largest increase in lysine (16%), methionine (13.2%), and threonine (10.7%). Valine was increased in all the lines, where line 3 had the largest increase of 17.1% (**Table 4**).

All the analyses accordingly indicate a selective suppression of the C-hordein content with compensatory increases in other fractions of storage proteins. One complicating factor is the large variation in protein content between the lines ranging from 12.8% to 15.7% with a slightly higher protein content of 17.1% in the mother line. We attribute these differences to the fact that the plants were grown in a greenhouse where in our experience it is difficult to obtain a uniform protein content of the grain. Also, the protein content was somewhat higher than in field grown materials that under Danish cultivation and fertilization conditions is of the order of 10−11% protein. This will affect the amino acid profile as the relative proportions of the different storage proteins are affected by the amounts of nitrogen available (*35*). B-Hordeins and in particular C-hordeins act as sinks for excess nitrogen (*35*). If sulfur supply is limited under high nitrogen fertilization conditions, the proportion of the sulfur-poor prolamins (C-hordeins) will increase even more. The proportion of C-hordein can thus be increased to 70−80% of the total hordein fraction (usually ≈ 20%) under greenhouse conditions if starved for sulfur (*36*, *37*). In contrast, the glutelin content remained relatively constant indicating the limited importance of this fraction as a sink for excess N in barley (*38*).

To compensate for the differences in protein content between the lines, we implemented the protein evaluation system of Boisen (*33*) where regression equations have been developed between the total protein content and the amount of nutritionally important amino acids. For five amino acids evaluated (lysine, threonine, methionine, leucine, and valine), the transgenic lines contained a higher content of amino acids than predicted from the equation for wild type barley as well as the Golden Promise mother lines.

We have at present no conclusive explanation for the variation between the lines. It may result from different transcriptional efficiencies of the C-hordein construct. Also, it should be emphasized that due to limited sequence information on the B- and the C-hordein genes and the transcriptional activity of the individual members of the family the effects of the C-hordein antisense construct cannot be predicted. It has been estimated based on Southern blotting that there are of the order of 5 genes encoding γ-hordeins, up to 30 C-hordein genes at the *Hor1* locus, and up to 50 B-hordein encoding genes at the *Hor 2* locus, which appears to contain two subfamilies of genes. Two-dimensional electrophoresis of B-hordeins from 8 different cultivars revealed a total of 47 B-hordein polypeptides (*39*) while 34 C-hordein polypeptides were identified in a sample of 6 cultivars (*40*).

The lines generated may have a significant potential as feed crops, but the agronomic performance and quality of the transgenic lines need to be further characterized under field conditions. Likewise, a number of structural and composition parameters need to be evaluated. The seed weight of the transgenic lines grown in the greenhouse was within the normal range, and no external morphological irregularities were observed. In contrast, the conventional high lysine mutants are readily identifiable by their shrunken kernels and enlarged embryos. Avoidance of too drastic morphological changes was an important reason for using a conventional antisense approach rather than RNAi in the present work. Hence, the RNAi approach in maize on suppression of the 22 kDa α-zeins resulted in an opaque phenotype (*17*). This was also the case for several of the lines generated by Huang et al. (*18*), where the 19 kDa α-zeins were suppressed by antisense.

In summary, a series of C-hordein antisense lines was generated that via a moderate suppression of C-hordein synthesis has resulted in marked changes in the storage protein and amino acid profile. Further efforts will be devoted to field trials and assessment of the nutritional value of these lines as well as the pleiotropic effects on the transcriptome and proteome imposed by the antisense suppression.

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