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Near infrared spectra indicate specific mutant endosperm genes and reveal a new mechanism for substituting starch with (1→3,1→4)-β-glucan in barley

L. Munck[a,*,1], B. Møller[a,1], S. Jacobsen[b,2], I. Søndergaard[b,2]

[a]*Chemometric and Spectroscopic Group, Department of Food Science, Food Technology, The Royal Veterinary and Agricultural University (KVL), Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark*

[b]*Biochemistry and Nutrition Group, BioCentrum-DTU, The Technical University of Denmark, Søltoft Plads, Building 224, DK-2800 Kgs. Lyngby, Denmark*

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Abstract

Near Infrared Reflectance spectroscopy was tested as a screening method to characterise high lysine mutants from a barley collection by classification through Principal Component Analysis (PCA). Mean spectra of the samples within each cluster identified gene-specific patterns in the 2270–2360 nm region. The characteristic spectral signatures representing the *lys5* locus (Risø mutants 13 and 29) were found to be associated with large changes in percentage of starch and (1→3,1→4)-β-glucan. These alleles compensated for a low level of starch (down to 30%) by a high level of (1→3,1→4)-β-glucan (up to 15–20%), thus, maintaining a constant production of polysaccharides at 50–55%, within the range of normal barley.

The spectral tool was tested by an independent data set with six mutants with unknown polysaccharide composition. Spectral data from four of these were classified within the high (1→3,1→4)-β-glucan BG *lys5* cluster in a PCA. Their high (1→3,1→4)-β-glucan and low starch content was verified. It is concluded that genetic diversity such as from gene regulated polysaccharide and storage protein pathways in the endosperm tissue can be discovered directly from the phenotype by chemometric classification of a spectral library, representing the digitised phenome from a barley gene bank.

Keywords: (1→3,1→4)-β-Glucan/starch mutants; Barley; Chemometrics; Genetics; Near-infrared spectroscopy; Plant breeding

1. Introduction

The barley endosperm, a well-conserved imprint of the physical–chemical dynamics of a ∼35-day developmental process after anthesis, is regulated by specific genes according to a precise timetable set by the genotype and is partly independent of environment. Due to self-pollination all advanced barley lines are almost homozygotic and mutants have near isogenic backgrounds allowing their use for precise reference. The desiccated seed/endosperm system is ideal for exploration by near infrared spectroscopy because of reduced interference by water peaks. The phenome (Watkins et al., 2001) is, here, regarded as an interface expressed as patterns of chemical bonds for the expression of specific genes (Munck, 2003). These are indirectly observed by spectroscopy as chemical–physical fingerprints.

Near Infrared Transmission (NIT) measurements of spectra evaluated by PCA were used by Campbell et al. (2000) to classify maize endosperm mutants. Munck et al. (2001) demonstrated how Near Infrared Reflectance (NIR) spectra of barley flour can be used to differentiate between normal and high lysine *lys3a* barley (Risø mutant 1508, Doll, 1983),

Abbreviations: DBC, dye binding capacity; d.m., dry matter; MSC, multiplicative signal correction; NIR, near infrared reflectance; NIT, near infrared transmission; PCA, principal component analysis; PLSR, partial least square regression; BG, (1→3,1→4)-β-glucan; *A*/*P*, ratio of amide N to protein N.

* Corresponding author. Tel.: +45 35 28 33 58; fax: +45 35 28 32 45.
E-mail address: lmu@kvl.dk (L. Munck).

[1] www.models.kvl.dk

[2] www.biocentrum.dtu.dk

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which appear as two different clusters in a PCA. The genetic differences were identified as specific signatures in NIR spectra, especially at 2270–2380 nm. Environmental differences are mainly expressed as offsets from the baseline.

In this paper, it is demonstrated how the spectral screening tools originally developed in food science (Osborne et al., 1993) can be used as a link between data from the genotype and phenotype, to detect previously unknown ways of gene expression in barley endosperm mutants.

2. Material and methods

The barley mutant genes (Doll, 1983) investigated were:

1. Four alleles in the *lys3* locus in chromosome 5 (new nomenclature, see Muravenko et al., 1991) with alleles Risø mutants *a* (1508), *b* (mutant 18) and *c* (mutant 19) in a Bomi background and the Carlsberg mutant 1460 (Munck, 1992) in Minerva, here, called *lys3m*.
2. *lys5* alleles in chromosome 6 Risø mutants *5f* in Bomi (mutant 13) and *5g* in Carlsberg II (mutant 29) as well as the double recessive *lys3a5g*.

An independent test set included four high lysine mutants and two waxy lines: the Risø mutants *lys4d* (mutant 8, in chromosome 1) and mutant 16 (in chromosome 7) both induced in Bomi (Doll, 1983), the Italian mutants 95 and 449 induced in Perga by Di Fonzo and Stanca (1977) and two putative waxy (amylopectin) lines 1201 and 841878 of unknown origin previously imported to the Carlsberg collection maintained at the Royal Veterinary and Agricultural University (KVL) called w1 and w2.

The mutants and their parent varieties and segregating crosses with normal barley as well as a range of normal barley varieties were grown under different conditions (field, outdoor pots, greenhouse) in different years.

The material was stored in closed containers in the refrigerator after equilibration to laboratory temperature and moisture. Two groups of samples were obtained:

Group 1. Fifty-four normal and original mutant lines (*lys3*, *lys5*, *lys3a5g*) grown in a greenhouse in 1998–2000.
Group 2. Nine lines from the test set of six mutants defined above, grown mainly in a greenhouse.

The NIR analysis (on milled flour from ripe seeds) was carried out as described by Munck et al. (2001) together with the chemical analyses of protein, amino acids, amide, nitrogen, and starch. The apparent amylose content in starch from the two waxy lines was determined using an iodine spectroscopic method on non-defatted isolated starch (BeMiller, 1964). Two methods of BG analysis were employed. The fluorimetric BG analysis with Calcofluor (Munck et al., 1989) was used routinely and was checked with an enzymatic method specific for (1→3,1→4)-β-glucan (Anonymous, 1998). Both methods had a linear correlation with minimal offset up to at least 15% BG d.m.

For classification chemometric pattern recognition analysis of spectral data was performed by Principal Component Analysis (PCA). The Unscrambler software (Camo A/S, Trondheim, Norway) was used according to Martens and Næs (1989). The spectra were subjected to multiplicative signal correction (MSC) according to Geladi et al. (1985), using the Unscrambler.

3. Results and discussion

NIR spectra of whole milled flour of 54 barley lines grown in one environment (Group 1, greenhouse) are presented in a multiplicative signal corrected (MSC) form in Fig. 1A. These data were used to develop a PCA score plot (Fig. 1B) between the principal components PC1 (*abcissa*) and PC2 (*ordinate*). Three clusters are shown in the PCA plot (Fig. 1B). Empirically observing such interesting patterns, it is natural to try to identify their cause. From the additional information on the samples it is found that the clusters reflect four different genotypes—normal barley N, *lys3* (four alleles *a*, *b*, *c* and *m*) along the PC1 axis and *lys5* (two alleles *f* and *g*) spanning the PC2 axis and with the double recessive *lys3a5g* in between. The evaluation of the PCA score plot in Fig. 1B facilitates a reduction of the 54 spectra to four mean spectra representing the clusters of normal (N), *lys3, lys5* and *lys3a5g* barley. Guided by loadings of the PCA model, the most important wavelengths can be assigned and when inspecting these four mean spectra, we can identify an interesting small area in the NIR spectra indicated with a square in Fig. 1A between 2270 and 2380 nm and displayed enlarged in Fig. 1C,D.

The mean *lys3* and *lys5* spectral signatures in Fig. 1C are distinctly different from each other and from that of normal barley (mean), while *lys3a5g* (mean) is intermediate between those of *lys3* and *lys5*. In Fig. 1D, the same conclusion can be drawn from spectra of individual samples of the four *lys3* alleles *lys3a, lys3b, lys3c, lys3m* and from the two alleles in *lys5*, *lys5f* and *lys5g*. The similar spectral responses for the samples of all the *lys3* alleles in Bomi background *lys3a*, *lys3b*, *lys3c* show the same response as the fourth *lys3* allele mutant *lys3m* in Minerva as demonstrated in Fig. 1D. The spectra from *lys5f* and *lys5g* have similar form. However, *lys5f* has a more extreme peak at 2350 nm, which confirms the more extreme position of *lys5f*, compared to *lys5g* in the PCA in Fig. 1B. The spectral differences between the barley reference varieties Bomi and Minerva and between most of the other normal lines are small.

As shown above, NIR spectroscopy evaluated by PCA is surprisingly effective in differentiating this genetic material

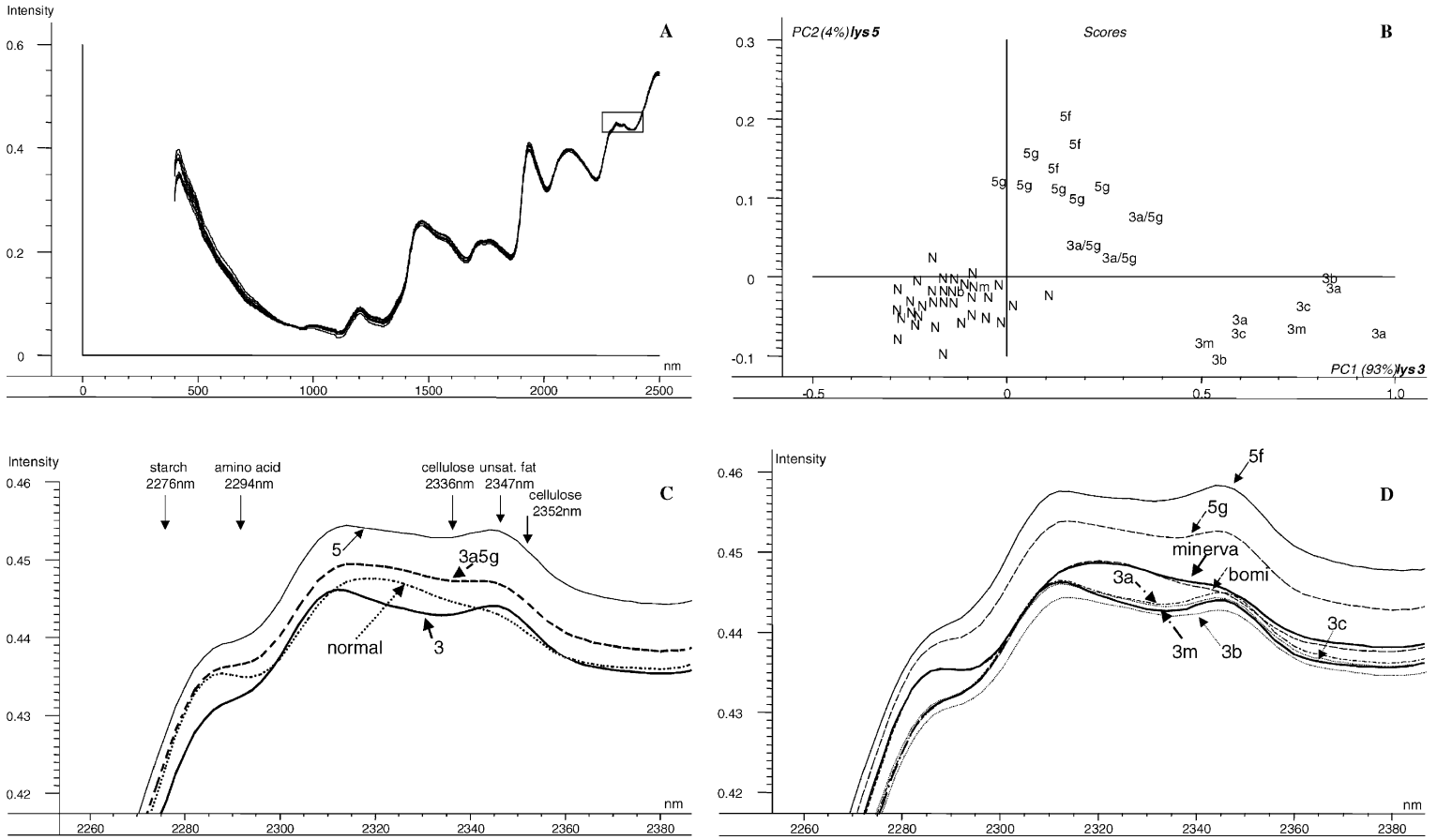

Fig. 1. (A) NIR (MSC) spectra 400–2500 nm of the 54 barley lines grown in a greenhouse (Group 1). (B) PCA scoreplot (PC1:2) of whole NIR spectra in (A). Nb = Bomi, Nm = Minerva, for mutant identification see text. (C) Mean spectra from enlarged area 2260–2380 nm (marked with a square in (A)) of the four genotype clusters (normal, *lys3*, *lys5* and *lys3a5g*) revealed from the PCA in (B). (D) Comparing spectra (2260–2380 nm) from *lys5f*, *lys5g*, *lys3a*, *lys3b*, *lys3c*, *lys3m* and the mother lines Bomi and Minerva (*lys3m*).

Table 1A
Chemical properties of the 54 samples (Group 1) and the six original mutants (Group 2) grown in a greenhouse

Greenhouse	*n*	BG (%)	Starch (*S*) (%)	BG+*S*	Protein (%)	Amide (%)	*A*/*P*	Fat (%)	Lys (mol%)	Glu (mol%)
Lys3a	3	4.73±0.98	40.4±1.0	45.2±0.1	17.7±0.9	0.32±0.03	11.41±0.56	3.51[d]	4.94[d]	14.86[d]
lys3b	2	3.05±0.78	–	–	17.1±0.7	0.32±0.01	11.51±0.22	–	–	–
lys3c	2	6.10±1.56	–	–	17.5±0.5	0.34±0.01	11.96±0.93	–	–	–
lys3m	2	2.25±0.01	39.3±1.3	41.6±1.3	17.4±0.5	0.27±0.01	9.50±0.06	–	–	–
lys5f	3	19.80±0.20	29.8±0.6[b]	54.8±6.0[b]	17.0±1.4	0.42±0.06	15.52±0.99	3.69[d]	3.32[d]	27.59[d]
lys5g	6	13.26±0.56	44.7[d]	58.2[d]	17.4±1.0	0.43±0.04	15.46±0.48	2.30±0.25[b]	3.76[d]	20.09[d]
3a5g	3	7.8±1.3	34.7±10.5[b]	43.2±10.6[b]	17.2±1.7	0.37±0.02	13.6±1.1	–	4.0[d]	20.1[d]
Normal incl. B	33	6.45±2.67[a]	47.8±1.0[c]	54.3±1.8[c]	16.2±1.3	0.44±0.01	19.95±0.62	1.94±0.16	3.05±0.15	24.13±0.83
Bomi (B)	1	6.80	48.8	55.6	14.6	0.38	16.24	1.74	3.27	22.90
lys4d	1	4.0	41.1	45.1	17.5	0.37	13.21	–	4.04	19.46
16	2	16.6±1.9	29.9[d]	45.1[d]	17.1±1.5	0.45±0.06	16.26±0.92	–	3.37[d]	22.50[d]
449	2	13.5±0	26.5[d]	40.0[d]	20.7±2.3	0.05±0.06	15.14±0.02	–	–	–
w1[e]	1	15.4	27.3	42.7	16.5	0.40	15.14	–	–	–
w2[e]	1	7.0	49.0	56.0	17.4	0.47	16.93	–	–	–

[a] n=30.
[b] n=2.
[c] n=9.
[d] n=1.
[e] Content of amylose: w1=20.3% and w2=4.2% of starch of d.m.

grown in a greenhouse. For the best genetic separation, the material should be grown in the same environment (Munck et al., 2001). The spectroscopic signatures indicative for different gene loci and normal barley are clear cut and reproducible. The method is able to differentiate between alleles in the same locus. Thus, *lys5f* seems to be a more extreme mutant than *lys5g*. Differences in genetic background within the normal barley category (Bomi and Minerva) and within mutant alleles are less important than the effects of the mutants themselves (Munck et al., 2001). The NIR spectrum contains repetitive confounded physical–chemical information throughout the NIR spectrum as primary, secondary, tertiary, etc. vibration overtones and combination bands from 2500 to 713 nm emerging from the fundamental vibrations in the infrared region, 2500–13,000 nm.

The NIR detection of the *lys3* alleles (Fig. 1B) was not surprising. The *lys3a* genotype (Munck et al., 2001) is characterised by a low amide/protein N ratio (*A*/*P*) of 11.4 compared to its mother variety Bomi (*A*/*P*=16.3) due to the low content of hordeins that are rich in amides. It was then shown that the *lys3a* gene is likely to be detected by NIR because it confers low amide content. Information for the amide bond is according to Osborne et al. (1993) distributed at 20 wavelengths in the NIR area 1430–2180 nm. We also confirmed (Munck et al., 2001) a high correlation between lysine and amide content ($r=-0.97$). As discussed below, amide detection is only a part of the definition of the *lys3a* phenotype by NIR spectroscopy.

The two *lys5* alleles *lys5f* (Risø mutant 13) and *lys5g* (Risø mutant 29) were selected by the dye-binding method (Doll, 1983), which indicates an increase in basic amino acids such as lysine. The lysine content in these mutants was only slightly increased (10%). Later, Greber et al. (2000) suggested that these mutants should be looked upon as having gene lesions in the starch synthesis pathway because they were considerably reduced in starch (50–75% compared to normal near isogenic barley controls). However, if we compare the sum of starch and BG content of these mutants, the picture changes (Table 1A). *lys5g* now even seems to exceed normal barley (58.0 versus 53.9%), and even for the extreme mutant *lys5f* the low starch content (29.8%) is compensated for with a high BG content (19.8%) to give a total starch and BG content as high as 49.4%. As far as we know, such compensating effects of BG on starch reducing genes have not previously been recognised.

It was thus surprising to note (Table 1A) that the *lys5* cluster in Fig. 1B in addition to low levels of starch, is characterised by very high BG levels that fully or partially compensate for the decrease in starch. The extreme gene *lys5f* produces BG-levels as high as 19.8% compared to 13.3% for *lys5g* and a value of 6.5% for normal barley. Although a greenhouse environment regularly produces a higher BG and protein content than in the field (compare Table 1A with Table 1B), the effect of the *lys5f* genes on BG is spectacular.

Table 1B
Chemical properties of 18 samples from Group 1 and seven samples from Group 2 field grown

Field	*n*	BG (%)	Starch (*S*) (%)	BG+*S*	Protein (%)	Amide (%)	*A*/*P*	Fat (%)	Lys (mol%)	Glu (mol%)
lys3a	1	3.1	48.5	51.6	12.7	0.23	11.36	2.63	–	–
lys3m	1	2.4	48.8	51.2	12.3	–	–	–	–	–
lys5f	1	16.5	–	–	–	–	–	3.77	3.8	19.9
lys5g	2	8.9±1.0	–	–	11.8±0.1	0.26[a]	13.7[a]	–	–	–
3a5g	1	–	–	–	15.5	0.28	11.3	–	4.8	15.6
Normal incl. B	13	4.5±0.8	55.1±2.2	49.7±.5	11.3±1.1	0.28±0.04[b]	15.4±0.6[b]	1.90±0.21	–	–
Bomi (B)	1	4.9	53.6	58.5	11.5	0.29	16.4	1.91	3.5	21.8
lys4d	1	4.1	–	–	12.9	0.29	14.0	–	4.2	19.1
16	1	12.0	–	–	13.8	0.32	14.5	–	–	–
95[c]	2	12.2	29.6[a]	41.8[a]	15.1±0.5	0.35±0.03	14.2±0.7	–	–	–
449	1	12.4	–	–	14.6	0.32	13.7	–	–	–
w1	1	15.6	–	–	–	–	–	–	–	–
w2	1	5.7	–	–	13.0	0.33	15.9	–	–	–

[a] n=1.
[b] n=11.
[c] One sample grown in outdoor pots.

The allele *lys3m* (induced in Minerva) originally selected as a low BG mutant at Carlsberg (Munck, 1992) has an extremely low *A*/*P* index of 9.5 compared to 17.5 in Minerva. It is interesting to note that the mutant allele *lys3c* in Bomi differs from the other alleles in displaying a normal barley BG value of 6.1%. The other *lys3* alleles are all low in BG (approximately 2.5%). The double recessive *lys3a5g* has *A*/*P* index and BG content intermediate between *lys3a* and *lys5g* barley verifying its intermediate position in the NIR classification by PCA between the *lys3* and *lys5* classes (Fig. 1B).

There are many chemical ways to detect barley mutants because they give a range of specific complex physical–chemical imprints on the phenotype, only detectable as a whole by multivariate pattern recognition analysis (Jacobsen et al., unpublished observations). It is surprising to note that very simple chemical plots and ratios like BG (*abscissa*) and *A*/*P* index (*ordinate*) in Fig. 2A and even starch (*abscissa*) and BG (*ordinate*) in Fig. 2B suffice for successful gene classification as compared to the PCA of corresponding NIR data in Fig. 1B. The efficiency of simple ratios and plots in mutant classification is discussed by Munck (1972).

The NIR approach is useful because the screening and classification can be performed empirically on unknown material identifying a broad physical–chemical fingerprint of the endosperm phenome also including unexpected effects such as BG. The spectra can be interpreted by PCA representing the total effects of genetic covariance (pleiotropy and linkage) of the mutant gene preferably compared against a near isogenic background with barley material grown in the same environment. This involves not only the detection of chemical bonds of obvious interest (here from amide, starch and BG), but also the important indirect physical effects of the genes, e.g. of importance for the fragmentation of the barley flour which may be detected by NIR in spite of multiple scatter correction (MSC). The indicative wavelengths for chemical bonds can be found in the spectroscopic literature (Osborne et al., 1993), suggesting which chemical analyses should be performed for validation of the assigned NIR classification. In Fig. 1C, five wavelengths for specific absorbers indicative for starch, amino acid, cellulose (2) and unsaturated fatty acids are selected from the many possible in the area 2270–2380 nm in order to characterise specific spectral areas which show significant differences. The NIR approach also picks up unexpected correlations such as with water content for which NIR spectroscopy is very sensitive. Thus, a high BG content at the expense of starch in *lys5* seems to result in an increased, ~1.5%, dry matter content (and lower content of water) (Tables 1A and B). This is presumably due to more water being bound to crystalline starch in the amyloplasts compared to water bound to BG in the walls of endosperm cells. Thus, the specific effect of water associated with the *lys5* gene is also included in the spectral classification together with a broad range of other side effects from the mutant gene.

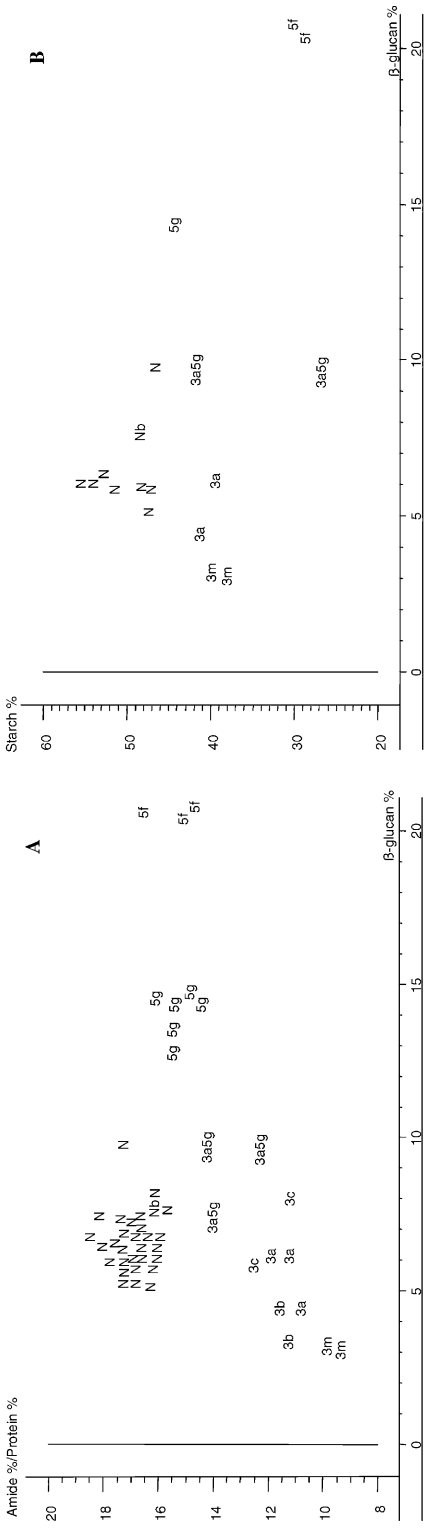

Fig. 2. (A) Variation of amide/protein ratio (ordinate) with β-glucan content in *lys5f*, *lys5g*, *lys3a*, *lys3b*, *lys3c*, *lys3m*, *lys3a5g* and normal (N). (B) Variation of starch content (ordinate) with β-glucan content (lines as in Fig. 2A).

These pleiotropic effects are automatically summed in the gene specific spectral fingerprint by a PCA which can be chemically and physically defined a posteori.

Nine samples from the six new genotypes in group 2 were measured by NIR spectroscopy and added to the 54 spectra (Fig. 1A) in a new PCA with 63 samples (Fig. 3A). Seven of the barley samples were grown in a greenhouse. The two samples of mutant 95 were grown in the field and outdoor in pots. Mutant 16, mutant 449 and w1 (a waxy line) were all located in the BG rich cluster around *lys5* with the two mutant 95 samples above to the right.

w2 (also considered waxy) was included in the upper part of the normal cluster closer to the *lys5* area, while the *lys4d* barley sample resides in the very high lysine *lys3* area below to the right. In Fig. 3B, it can be seen that mutants 95 and 449 resemble in the 2270–2380 nm region *lys5f* because they have similar scores, hence similar spectral profiles. The two mutant 95 samples grown in the field and outdoors in pots have similar profiles, but are shifted above the baseline due to the environmental difference. Mutant 16 in Bomi has a spectral form in the 2270–2380 nm area which resembles *lys5g* as seen in Fig. 3C. As expected from the classification in Fig. 3A, the spectrum of *lys4d* (also mutant in Bomi) has a similar form to that of *lys3a* (Fig. 3C), indicating a major change in amino acid composition. w1 in the *lys5* cluster has a spectral form resembling that of *lys5f*, while w2 is near to that of normal barley (Bomi) (Fig. 3D).

The result of the chemometric classification analysis of the spectral information in Fig. 3A is validated by the chemical analyses shown in Tables 1A and B. All the new mutants positioned in the *lys5* cluster (or near to it like mutant 95) have strongly increased levels of BG (individual samples), namely mutant 16 (15.2%), mutant 449 (13.5%), and w1 (15.4%). The starch contents of the new mutants were lower than those of *lys5g* and *lys5f*, and the starch plus BG level (% d.m.) were clearly below the normal lines as well the *lys5g* and *lys5f* mutants (Table 1A). Also, mutant 95 grown outdoors in the field and in pots is on the high BG side above the baseline in the PCA in Fig. 3A. It is high in BG (12.2 and 14.2%). It is clear that the position in the PCA plot has been altered because of environmental effects. Because its spectral form of mutant 95 is near to that of *lys5f* (Fig. 3B), it would belong to the *lys5* cluster in the PCA score plot in Fig. 3B if it were grown in a greenhouse.

lys4d, which was classified in the *lys3* cluster with changed amino acid pattern and low BG content, is confirmed to have a low *A*/*P* index and BG as is the case with *lys3a*, *lys3b* and *lys3m* (Tables 1A and B). This is further verified by the amino acid composition (lysine and glutamine/glutamic acid) relative to Bomi in Table 1A, which is significantly changed in the direction of *lys3a* in *lys4d*.

In evaluating the two supposedly waxy mutants (Tables 1A and B), it can be seen that w1 has a practically normal amylose content (20.3%), but is very high in BG (15.4%) and low in starch (42.7%). Therefore, w1 is not a classic

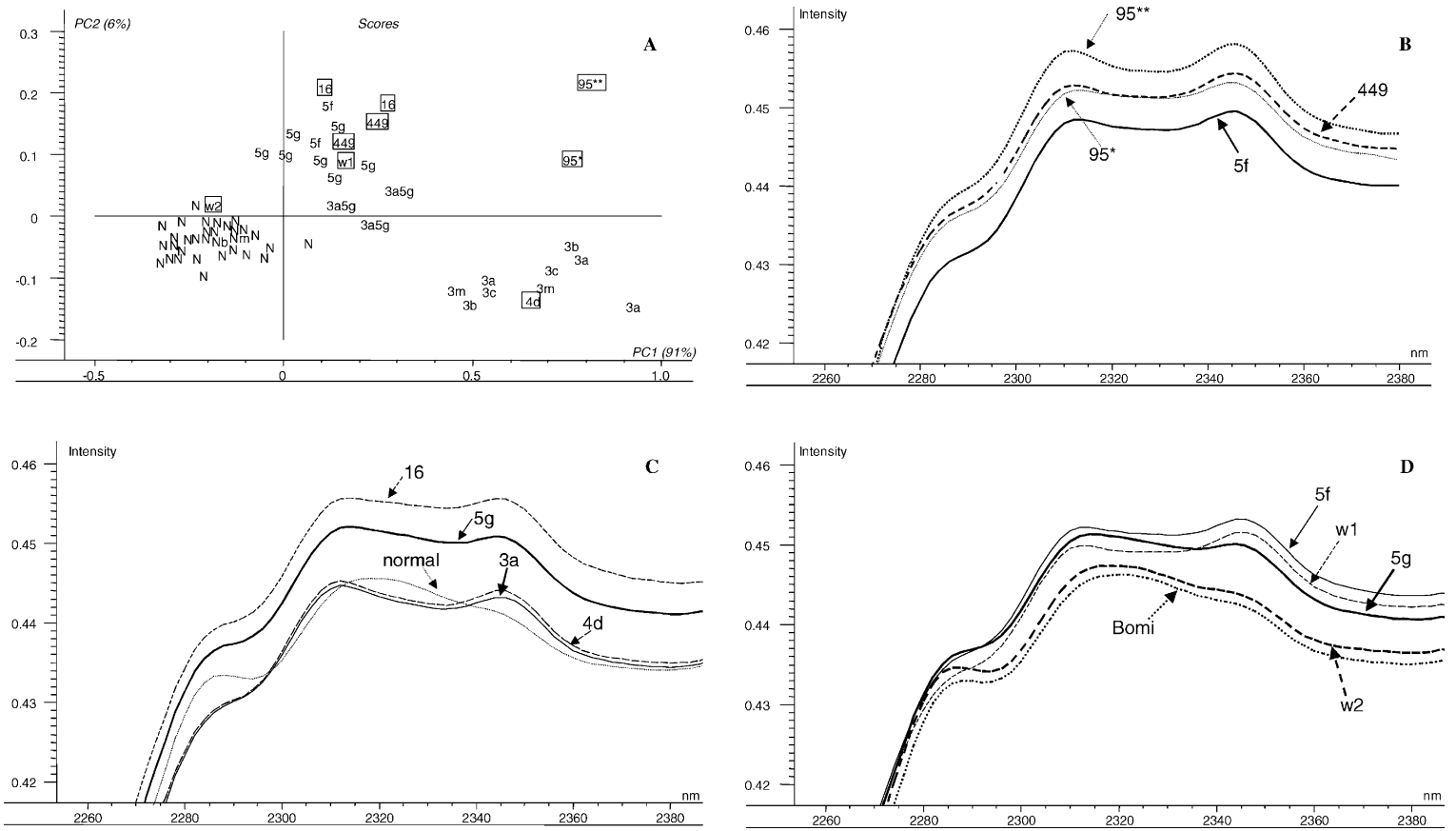

Fig. 3. (A) PCA (PC1:2) of NIR (MSC) spectra from the 54 barley samples (Group 1) grown in greenhouse and nine samples of six different mutants in the test set (Group 2) discussed in the text. (B) Comparing spectra (2260–2380 nm) from the mutants grown in a greenhouse *lys5f*, mutant 449 and mutant 95 grown in field* and in outdoor pots**. (C) Comparing spectra (2260–2380 nm) from the mutant *lys5g*, mutant 16 *lys3a, lys4d* and normal barley. (D) Comparing spectra (2260–2380 nm) from the mutants w1, w2, *lys5f*, *lys5g* and Bomi.

waxy high amylopectin low amylose mutant with slightly increased BG, but rather a mutant in the new category of low starch/high BG mutants. w2, which has a NIR spectral form (Fig. 3D) closer to normal barley (Bomi), is waxy. It has a low amylose content (4.2%) and a BG content on the high side (7.0%) compared to normal barley in Table 1A (mean 6.5%).

With a synergistic combination of spectroscopic and chemometric tools applied to cereal seeds, it has, thus, been possible to detect previously unknown endosperm genes and mutants. In such a study, it is difficult to differentiate between the two different sources of covariance, pleiotropy (biochemical gene applications) and linkage (association of adjacent genes on the chromosome). We, therefore, name the combined effect of pleiotropy and linkage 'genetic covariance'. This technology allows a truly exploratory strategy, with a minimum of hypotheses, where the chemical effects of the gene are determined after selection using PCA with the spectra as preliminary guidelines (Osborne et al., 1993) for generating new hypotheses in a dialogue with a priori knowledge.

High to moderate levels of BG and a high content of free sugars and even phytoglycogens are present in many mutant alleles associated with the amylopectin waxy gene (wax) in barley (Fujita et al., 1999; Newman and Newman, 1992). There are only slight reductions in starch level and seed size. It is interesting to note that the *lys5g* (mutant 29) and mutant 16, found here to be high in BG, have approximately normal levels of amylose (Tester et al., 1993). The sum of starch and BG in these mutants, as shown in Tables 1A and B, however, approaches normal values in percentage of dry matter with *lys5g* as the best performer. In addition, the high lysine amino-acid mutants *lys3a* and *lys4d* in our study, displayed a reduction in BG, but are unchanged in amylose levels (Tester et al., 1993).

The BG content of the six BG compensated, starch reduced barley mutants reported here is extremely high compared to the range of values of 2.8–10.7% d.m. reported by MacGregor and Fincher (1993). There are amylose free waxy (wax) genes as well as alleles which contain amylose such as the w2 line (4.2% apparent amylose) reported in this investigation (BG 7.0%, see Table 1A). The high amylose amo 1 barley genes such as in Glacier AC38 (apparent amylose 40.6%) also have a moderately increased content of BG (7.9%) compared to a normal variety (BG 4.7%, apparent amylose 33.1%) as reported by Fujita et al. (1999). Swanston et al. (1995) demonstrated that the double recessive line between a waxy (non-amylose free) and the amo 1 genes had 9.4% BG compared to 3.6% for the controls. This was confirmed by Fujita et al. (1999) with a waxy, amylose-free allele combined with the gene amo 1 in a double recessive line that reached the high BG level of 12.4%, about 2.6 times higher than the control line. In this paper, the six BG compensated, starch mutants have a range of BG between 8.9 and 16.5% when grown in the field and 13.3 and 19.8% when grown in a greenhouse, compared to a mean of 4.5 and 6.5%, respectively, for a set of control varieties (Tables 1A and B). This increase in BG is 2.0–3.7-fold.

High lysine mutants such as mutants *lys3a,b,m* and *lys4d* seem to have reduced BG contents (Table 1A). Note that the allele in the *lys3* locus *lys3c* has a normal BG content. This fact suggests that the high lysine and BG reducing traits here are controlled by adjacent genes and that the mutations involve chromosome segments of different lengths around the *lys3* locus in chromosome 5. There is a positive significant correlation between the *A*/*P* index and the BG content of *lys3* genotypes of $r=0.83$, which indicates a position effect of the different alleles. The reduced BG content should, thus, not be pleiotropic to the *lys3a*, *lys3b* and *lys3m* alleles, but rather depend on a very tight linkage (which is very difficult to break by recombination) to an adjacent gene, which retards BG synthesis in three of the four *lys3* genotypes. According to our experience, BG synthesis is active, also rather late in kernel development and is dependent on environmental factors such as heat and precipitation (Aastrup, 1979). It is inherited in normal barley by a simple genetic additive system (Powell et al., 1985). Although the BG content is higher under greenhouse conditions, the environmental differences between most mutants and normal barley are consistent (compare Table 1A with Table 1B).

We are, thus, able to propose a hypothesis for the relationship between starch and BG synthesis based on the NIR spectroscopic screening method. Genes that regulate BG synthesis appear to be closely coupled to and apparently compete with those genes that regulate starch synthesis in the developing endosperm. The sugar precursors available are shifted in their destiny accordingly, the cause of which may be found by further investigating the proteomes of the developing endosperm of the six genotypes by classic biochemical methods. Three of the six BG compensating starch mutants are confirmed to have a normal starch amylose to amylopectin composition. At least two gene loci *lys5* (alleles *lys5f* and *lys5g*) in chromosome 6 and mutant 16 in chromosome 7 are involved in the BG compensated starch mutant trait.

BG is of major importance in cereals for food because of its positive properties in human nutrition as a dietary fibre (McCleary and Prosky, 2001) for regulating the viscosity of intestinal contents, lowering the cholesterol value in the blood and reducing the risk for colon cancer (McIntosh et al., 1991). BG is also of importance for stimulating the immune system. Because BG is not readily digested in the human alimentary tract, it is not an energy resource, so that the energy value of a cereal product high in BG is correspondingly reduced implying a decrease in blood glucose as expressed in the glycemic index (GI). This is of great importance in diabetes prevention and treatment (Salmeron et al., 1997). The very high BG level of the six barley BG mutants presented here makes them well suited

as candidates for ingredients in functional foods low in energy and with a high level of soluble and insoluble fibre.

4. Conclusion

In this investigation, we have classified by NIR spectroscopy 10 of the classic 'high lysine' barley mutants of the 20–30 available. We have found that five of those genes—the *lys5f, lys5g* alleles in chromosome 6, mutant 16 in chromosome 7 (Doll, 1983) and mutants 95 and 449 (Di Fonzo and Stanca, 1977) with unknown chromosome locations—combine low starch synthesis with excessive BG synthesis completely or partly compensating for the decrease in starch formation. Two of the five high BG compensating starch mutants—Risø mutants 16 and *lys5g* originally selected as high lysine were shown to have normal amylose content by Tester et al. (1993). Additionally, a sixth high BG, low starch mutant content was found (w1; 1201), which previously had been selected erroneously by plant breeders as waxy. It has a normal amylose content. The presence of high BG levels in barley, reported previously in the literature, has been associated mainly with either low (waxy) or high amylose (amo 1) genes. To our knowledge, data combining normal amylose barley with BG levels approaching 20% (*lys5f*, Table 1A) have not been published before.

The introduction of spectroscopy and chemometrics makes it possible to reveal specific gene expression patterns as discussed here and by Munck et al. (2001) and Munck (2003) on the level of the phenome. Chemometric pattern recognition statistical methods, e.g. through PCA, is now being used more frequently in molecular biology to connect different levels of biological organisation (Fiehn, 2002). NIR spectroscopy as demonstrated in this paper and other spectroscopic screening methods such as Nuclear Magnetic Resonance (NMR) evaluated by chemometrics should be effective in revealing new metabolic mechanisms.

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