# Are GM and conventionally bred cereals really different?

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Concerns over the safety of GM crops have led to detailed comparisons of their composition and performance with those of conventionally bred crops, under both field and glasshouse conditions. Such studies of wheat have shown that it is possible to develop GM lines which are substantially equivalent to conventional varieties. This information is important to inform the debate on the future development of GM food crops.

## Introduction

The advantages of transgenesis for improving the agronomic performance of crops are clearly demonstrated by their extensive cultivation, with approximately 90 million ha of transgenic soybean, maize, cotton and canola being grown in 2005, predominantly in the USA (49.8 million ha) and Argentina (17.1 million ha) (www.isaaa.org). These crops have been engineered to provide resistance to herbicides used for weed control and/or insect pests, resulting in economic and environmental benefits from decreased use of agrochemicals. These "input" traits have proved attractive for manipulation in "first generation" transgenic crops as significant impacts can be obtained using only one or two well-characterised genes. However, although agronomic performance and crop yield remain key targets, the emphasis is now moving towards "output" traits, which include aspects of crop quality. Thus, the "second generation" of transgenic crops will be manipulated to improve their properties for the processor (eg, breadmaking performance of wheat) and consumers (eg, increased vitamins and minerals, reduced glycaemic index and increased content of soluble fibre in cereals) rather than the farmer.

Because these modifications will affect the composition of the major staple foods such as wheat they will almost certainly lead to renewed debate on the safety and acceptability of transgenic crops. It is essential that this debate is informed by sound scientific data and we are contributing to this in two ways. Firstly, by developing a suite of technologies that can be used to establish the "substantial equivalence" of GM and non-GM crops and secondly, by applying these to compare the composition and performance of GM and non-GM lines of wheat grown under field and glasshouse conditions. This work is discussed here in the context of wider studies of GM cereals.

## Technologies for comparing GM and conventionally bred crops

Wheat and most other major food crops have been consumed by humans for many years, extending to millennia in some cases, and are therefore generally regarded as safe. Because of this the main concept used to evaluate the safety of modified forms of cereals is that of substantial equivalence (OECD, 1993). However, as pointed out by Millstone, Brunner, and Mayer (1999), this concept has never been rigorously defined, resulting in a degree of "vagueness, which makes the concept useful to industry but unacceptable to consumers". In our studies, we have taken substantial equivalence to mean that the composition of the GM food is within the range of variation shown by conventionally bred cultivars when grown under similar conditions.

The levels of analysis which have been used to determine substantial equivalence are summarised in Fig. 1. At the genomic level, it is necessary to confirm that transgenes behave similarly to endogenous genes in their stability and inheritance between generations. This is of particular importance because it is not currently possible to control the sites of transgene insertion and they could possibly be inserted in regions of the genome which are not normally transcribed (which probably account for over 90% of the

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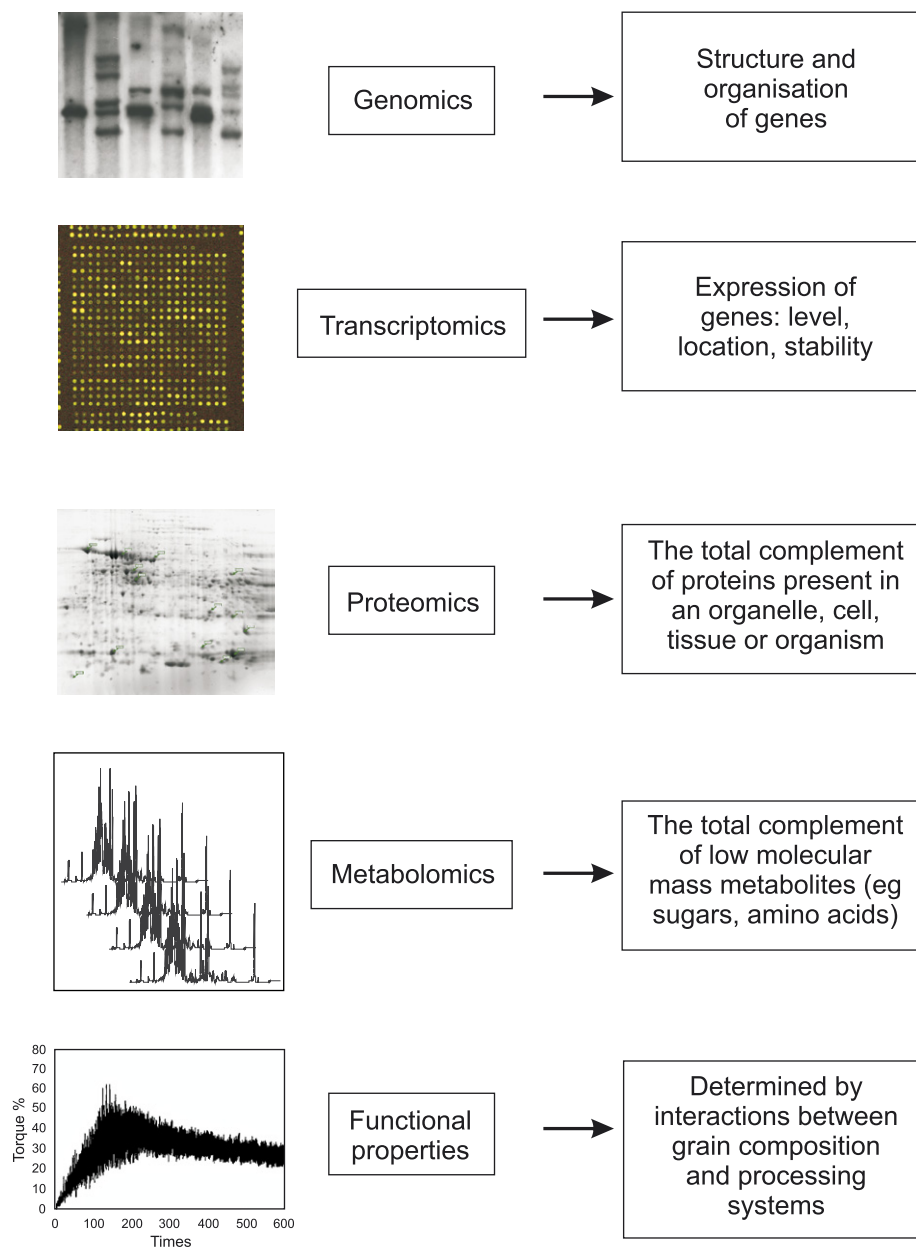
Fig. 1. Levels of analysis used to define substantial equivalence of GM crops. The figures show, from top to bottom, a "Southern blot" of wheat genomic DNA, part of a cDNA microarray, a two-dimensional gel of wheat grain proteins, an NMR trace and a Mixograph trace.

total DNA in wheat) or even within other genes. This could in theory lead to the inactivation of endogenous genes or the expression of novel transcripts encoding chimaeric proteins. Because of this there is also a requirement for transcriptome comparison. It is obvious that the GM and non-GM crops should differ in expression of the transgenes but there should be no unexpected "knock on" effects on the expression of other genes. Transcriptome comparisons are usually carried out using DNA microarray technology in which sequences corresponding to genes are attached to small glass slides and used to detect corresponding transcripts by hybridisation. For example, the Affymetrix array system for wheat can detect the expression of about 55,000 genes, approximately half of the estimated total number present in the genome.

Although it is important to compare gene expression profiles, there is no certainty that any differences which are identified during grain development will result in differences in composition of the harvested product. Consequently, it is also necessary to carry out detailed comparisons of grain composition and properties.

Clarke, Hobbs, Skylas, and Appels (2000) reported that almost 1700 proteins could be separated from wheat endosperm tissue at 8–12 days after anthesis while Skylas *et al*.

**Table 1. Characteristics of the control and transgenic lines used in the studies**

| Line | Characteristics | Endogenous HMW subunit genes | HMW subunit transgenes | Marker genes |
|------|-----------------|------------------------------|------------------------|--------------|
| L88-6 | Parental line. Derived from Australian lines Olympic × Gabo | 1Ax1, 1Bx17, 1By18, 1Dx5, 1Dy10 | None | None |
| B73-6-1 | Transgenic. L88-6 transformed with 1Dx5 as whole plasmid | 1Ax1, 1Bx17, 1By18, 1Dx5, 1Dy10 | 1Dx5 | *bar*, *uidA* |
| L88-31 | Parental line. Sister line derived from same cross as L88-6 | 1A null, 1Bx17, 1By18, 1D null | None | None |
| L88-18 | Control line. Sister line derived from same cross as L88-6 and L88-31 | 1Ax1, 1Bx17, 1By18, 1D null | None | None |
| B102-1-1 | Transgenic. L88-31 transformed with 1Ax1 gene as whole plasmid | 1A null, 1Bx17, 1By18, 1D null | 1Ax1 | *bar*, *uidA* |
| B102-1-2 | Transgenic. L88-31 transformed with 1Ax1 gene as whole plasmid. Sister line of B102-1-1 | 1A null, 1Bx17, 1By18, 1D null | 1Ax1 | *bar*[a] |
| B72-8-11a | Null transformant of L88-31 | 1A null, 1Bx17, 1By18, 1D null | None | None |
| Cadenza | Commercial cultivar | 1A null, 1Bx14, 1By15, 1Dx5, 1Dy10 | None | |
| B13554 | Cadenza transformed with 1Ax1 gene as clean fragment | 1A null, 1Bx14, 1By15, 1Dx5, 1Dy10 | 1Ax1 | *bar* |
| B1118 | Cadenza transformed with 1Ax1 gene as whole plasmid | 1A null, 1Bx14, 1By15, 1Dx5, 1Dy10 | 1Ax1 | *bar*, *uidA* |

HMW, high molecular weight.

The table is based on data in Lawrence, MacRitchie, and Wrigley (1988), Barro et al. (1997) and Rooke et al. (2003).

[a] *uidA* not detected by polymerase chain reaction; presumably lost by plasmid rearrangement during transformation.

(2000) separated about 1300 proteins from endosperm at 17 days after anthesis but slightly less (1125) from mature (45 days) endosperm tissue. However, these and other reported studies of cereal proteomes have used two-dimensional electrophoresis for protein separation and these systems are notoriously difficult to reproduce between laboratories and cannot quantify small differences in expression (see Ruebelt, Leimgruber, et al., 2006). Nevertheless, two-dimensional electrophoresis remains the most widely available method for proteomic analysis and has been used for determination of substantial equivalence.

In contrast to proteomics, metabolomic analyses can be carried out in automated high throughput systems with sophisticated data handling methods. The system used in the UK Centre for Plant and Microbial Metabolomics (MeT-RO) at Rothamsted is based on initial comparisons of total soluble metabolites by $^1$H NMR spectroscopy (Ward, Harris, Lewis, & Beale, 2003) followed by various mass spectrometry techniques.

Finally, it is also important to ensure that the transgenic crop is equivalent in its functional properties and, in particular, the stability of these from year to year and across environments.

## Production of transgenic wheat expressing additional HMW subunit genes

The high molecular weight (HMW) subunits account for about 10–12% of the total gluten proteins of wheat but have a major impact on dough strength due to their ability to form high molecular mass polymers (reviewed by Shewry et al., 2003). Different allelic forms of these proteins are associated with strong or weak dough and the former are routinely selected by plant breeders. In addition, there is a clear correlation between the number of HMW subunit genes which are expressed (three, four or five in different wheat cultivars), the amount of HMW subunit protein and dough strength, which has led to attempts to improve grain quality by the addition of extra gene copies by transgenesis (see eg, Blechl & Anderson, 1996; Altpeter, Vasil, Srivastava, & Vasil, 1996).

We have also adopted this approach by transforming several lines of wheat with genes encoding HMW subunits called 1Ax1 and 1Dx5; both of these subunits are positively correlated with improved grain quality, the latter as part of an allelic pair (1Dx5 + 1Dy10) (see Barro et al., 1997; Rooke et al., 1999; Rooke, Steele, Barcelo, Shewry, & Lazzeri, 2003). The details of all of the lines discussed here are given in Table 1 and 1D SDS-PAGE protein profiles shown in Fig. 2.

Most of these lines were co-transformed with two plasmids; one of these contained the HMW subunit transgene while the second contained two genes used as markers in the transformation process. These are the *bar* gene which allows the selection of transformed plants by providing resistance to the herbicide Basta and the *uidA* gene (encoding β-glucuronidase, GUS) which allows transgenic plants to be identified by a simple colorimetric reaction. The HMW subunit transgenes were expressed under control of their own promoters and so should only be expressed in the developing endosperm of the grain. In contrast, the *bar* and *uidA* genes were controlled by a constitutive maize promoter and should be expressed in all tissues of the plant. In addition, the transgenic plants also contain sequences derived from the bacterial plasmids used for transformation including the ampicillin resistance gene which is not expressed in the plant. These additional genes and sequences are of some concern to regulatory authorities so we have also produced lines transformed with only sequences encoding the HMW subunit and *bar* genes. These are referred to as "clean fragment" lines. Analysis of this material can
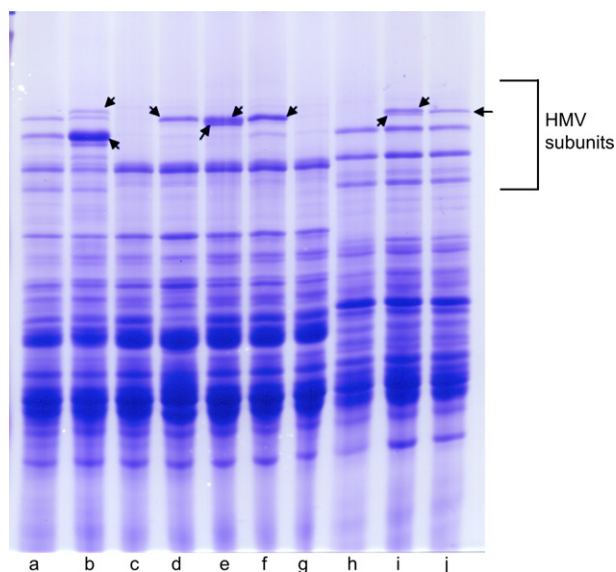
**Fig. 2**. SDS-PAGE of gluten proteins from the control and transgenic lines. a, control line L88-6; b, B73-6-1; c, control line L88-31; d, control line L88-18; e, B102-1-1; f, B102-1-2; g, B72-8-11a; h, cv Cadenza; i, B13554; j, B1118. The HMW subunits are indicated by the bracket in track j while additional bands encoded by the transgenes are indicated by arrows in tracks b, e, f, i and j. The arrow in track d indicates the endogenous HMW subunit 1Ax1 present in L88-18.

therefore provide a range of valuable comparisons: lines with transgenes compared with control lines, lines with transgenes compared with lines with the equivalent endogenous genes and lines transformed with "clean fragments" or lines transformed with whole plasmids.

## Transcriptome comparison of lines grown in the glasshouse

Transcriptomic analyses are expensive and time consuming and can only be carried out on limited numbers of samples. We therefore decided to compare six lines grown in three glasshouse experiments. In each case developing endosperms were compared at 14 and 28 days after anthesis and leaves at 8 days after germination (Baudo et al., 2006). Unfortunately, the Affymetrix array platform discussed above was not available when the work was carried out and we therefore used a smaller cDNA array comprising 9246 unique wheat sequences (Wilson et al., 2004). These may account for between 10% and 20% of the total transcribed genes in wheat. The results from these comparisons are summarised in Table 2.

Comparison of the pairs of transgenic and "parental" lines (B102-1-1 v L88-31, B13554 v Cadenza, B1118 v Cadenza) showed only a small number of differentially expressed genes, with none differing by more than 2-fold. In contrast, comparison of the two non-transgenic sister lines (L88-31 and L88-18), which were produced from a single cross between the cultivars Olympic and Gabo, showed that a larger number of genes were differentially expressed

in developing endosperms, 92 at 14 days (13 by greater than 3-fold) and 527 at 28 days (85 by greater than 2-fold). These two lines differ in that L88-18 expresses the endogenous form of the 1Ax1 transgene which was used to transform L88-31 to give B102-1-1. It is therefore not surprising that comparison of B102-1-1 and L88-18 also showed greater numbers of differentially expressed genes than the comparison of B102-1-1 and L88-31. Similar small numbers of differentially expressed transcripts were identified when Cadenza was compared with single lines transformed with *either* whole plasmids (line B1118) or clean fragments (B13554) but the number increased when the two transgenic lines were compared.

Finally, with one exception, the comparisons of leaf tissues showed smaller numbers of differentially expressed genes than the comparisons of the endosperm tissues. This may reflect the fact that only the *bar* and *uidA* genes were expressed in leaf tissues and at lower levels than the HMW subunit transgenes which were expressed with *bar* and *uidA* in the endosperms.

Although this is the most detailed study of substantial equivalence at the transcriptome level which has been reported so far, similar results were reported by Gregersen, Brinch-Pedersen, and Holm (2005) who used the same cDNA microarray to determine the substantial equivalence of transgenic wheat lines expressing a fungal phytase gene to increase mineral availability for livestock.

A striking result from our studies was the small numbers of differentially expressed genes between the transgenic and control lines when compared with sister lines (L88-31, L88-18) produced by a conventional crossing programme. This is consistent with the hypothesis that transgenesis is a highly precise and controlled method of crop improvement compared to conventional breeding in which many thousands of genes may differ between the lines.

It is possible to predict the functions of some of the small numbers of differentially expressed genes in the transgenic lines based on comparison of their sequences with characterised genes in other species (see supplementary material to Baudo et al., 2006) and none of those identified in the present study give obvious cause for concern in relation to human health or field release. Nevertheless, it is also necessary to carry out direct comparisons of the grain composition and we chose to carry these out on material grown in field trials over three years.

## Comparison of field grown transgenic and non-transgenic lines

The development of cereal varieties which are stable despite year-to-year and site-to-site variation in environmental conditions is an important, and extremely challenging, target for plant breeders. Because environmental factors can have major impacts on grain composition and end use properties it is important that transgenic lines should be tested under a range of environments and over several years.

**Table 2. Numbers and percentages of statistically significant differentially expressed genes in pairwise comparisons of various transgenic and control lines of wheat**

| Lines used for comparison | | 14-day Endosperms | | 28-day Endosperms | | 8-day Leaves | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| Line with 1Ax1 transgene (B102-1-1) | Control untransformed line without 1Ax1 gene (L88-31) | 5 | 0.05 | 2 | 0.02 | 6 | 0.06 |
| Control untransformed line without 1Ax1 gene (L88-31) | Related control line with endogenous 1Ax1 gene (L88-18) | 92 | 0.99 | 527 | 0.59 | 26 | 0.27 |
| Line with 1Ax1 transgene (B102-1-1) | Related control line with endogenous 1Ax1 gene (L88-18) | 154 | 1.63 | 118 | 1.25 | 4 | 0.04 |
| Line transformed with 1Ax1 clean fragment (B13554) | Control untransformed line (Cadenza) | 6 | 0.06 | 9 | 0.1 | 1 | 0.01 |
| Line transformed with 1Ax1 gene as whole plasmid (B1118) | Control untransformed line (Cadenza) | 7 | 0.07 | 12 | 0.13 | 2 | 0.02 |
| Line transformed with 1Ax1 gene as clean fragment clean gene (B13554) | Line transformed with 1Ax1 gene as whole plasmid (B1118) | 26 | 0.28 | 4 | 0.04 | 3 | 0.03 |

The comparison was carried out using a cDNA array comprising 9246 elements. Transcripts which differed in expression levels between pairs of lines by at least 1.5-fold were identified and expressed as percentages of the total elements present on the array. Data from Baudo *et al.* (2006).

We therefore grew a series of transgenic and "control" wheat lines in replicate field experiments over four years (1998–2000). The trials were also carried out on two sites with contrasting climates: Long Ashton (near Bristol, UK), in the west of England and Rothamsted Research (Fig. 3), which is about 50 km north of London in an area traditionally used for cereal production, and is drier than the Long Ashton site. A range of measurements were carried out on the grain including dry weight, nitrogen content, protein composition by SDS-PAGE, dough mixing properties by Mixograph and metabolite profiles. This has allowed us to draw conclusions about the relative stability of expression of the endogenous and transgenic forms of the HMW subunit genes and their impact on the grain composition and properties.

Detailed statistical analyses of HMW subunit expression levels were carried out on data from quantitative scanning of SDS-PAGE gels (Shewry *et al.*, 2006), with some of the data being presented visually by linear discriminant analysis as shown in Fig. 4A. This plot is based on the proportions of endogenous (1Dx5, 1Bx17 + 1By18) and transgenic (1Ax1, 1Dx5) subunits over all years and sites. The *x*-axis accounts for 58.9% of the total variation, which is determined mainly by differences in the proportions of subunit 1Dx5 which is highly over-expressed in the transgenic line B73-6-1. The *y*-axis accounts for 19.4% of the total variation and is mainly determined by the proportions of subunits 1Bx17 + 1By18. It should be noted that lines B102-1-1 and B102-1-2 express the same transgene (1Ax1) in the same background and hence cluster together. Furthermore, the clusters formed by the points are of similar areas for the control (L88-6) and transgenic (B73-6-1, B102-1-1/2) lines demonstrating similar stability of expression levels.

The same four lines and two additional control lines (the parental line L88-31 and the null transformant B72-8-11a)

were used to compare data for grain weight, grain %N and Mixograph peak resistance. No consistent differences in the stability of these parameters were observed between the transgenic and control lines and linear discriminant analysis of the data (Fig. 4B) confirmed this. In this plot, the *x*- and *y*-axes account for 79% and 18% of the total variation, respectively.

Although two broad groups are separated by the first discriminant vector, both comprise both transgenic and control lines. However, the right hand group comprises mainly samples from 1999 and this separation resulted from the fact that the data for peak resistance were lower and more variable for all lines in 1999 than in 1998 or 2000. Hence the major discriminant is on year not on line or site.

It can also be noted that the main outliers on the *x*-axis are the parental line L88-6 and the transgenic line derived



**Fig. 3**. The transgenic field plots at Rothamsted Research (Harpenden, UK).
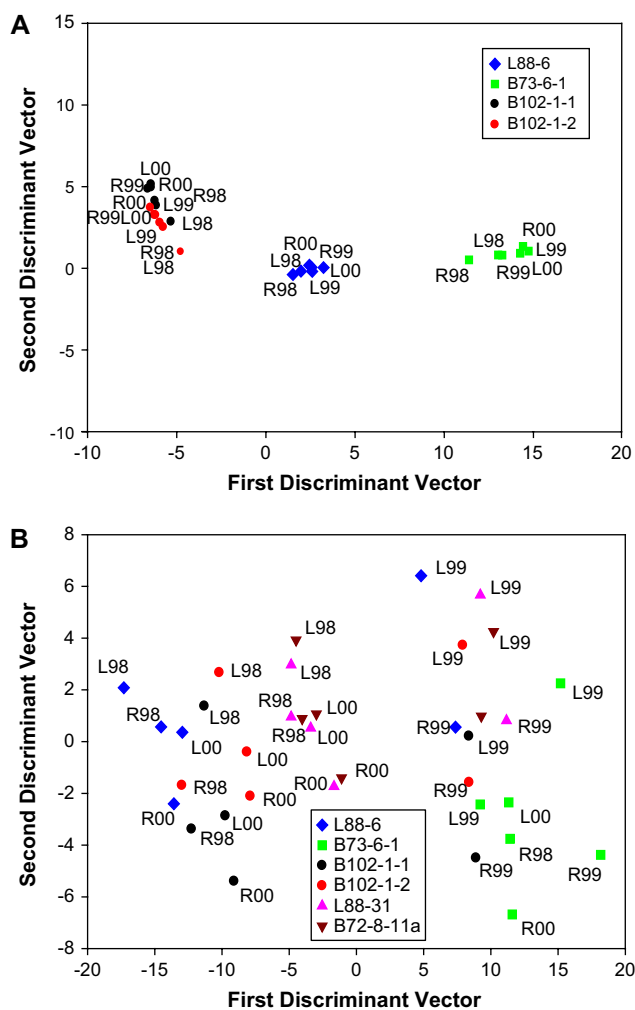
**Fig. 4**. Linear discriminant analysis (LDA) of parameters measured for transgenic (B73-6-1, B102-1-1, B102-1-2) and control (L88-6, L88-31, B72-8-11a) grown at two sites (R, Rothamsted; L, Long Ashton) over three years (1998, 1999, 2000). (A) HMW subunit expression levels. (B) Data for grain nitrogen, grain dry weight and dough peak resistance. Part (A) is taken from Shewry et al. (2006) with permission and part (B) is calculated from data presented in the same paper.

from this, B73-6-1. This separation is due to the *intended* effect of the transgenic event on the mixing properties (peak resistance) of the dough. However, there are no differences between the relative stabilities of these two lines, or between any of the other control and transgenic lines.

Bregitzer et al. (2006) have also recently reported extensive studies of 50 transgenic lines of wheat expressing HMW subunit transgenes grown at three sites for two years, determining the heading date, height and grain characteristics (yield, protein, test weight, 1000 grain weight). Although most of the transgenic lines had poorer performance than the control line, there was no clear relationship between performance and either the specific transgene or the transgene expression level. Furthermore, at least some

of the lines showed similar agronomic performance to the control line.

## Metabolite profiling

We also used metabolomic profiling to determine whether differences were present in the compositions of low molecular mass metabolites in white flour fractions from these lines, using grain from plots grown in 1999–2001 (Baker et al., 2006). Samples were extracted with $D_2O:CD_3OD$ (80:20) essentially as described by Ward et al. (2003), and analysed using a Bruker 400 MHz NMR spectrometer. The spectra were collected and aligned automatically and the data sets subjected to multivariate analysis by principal component analysis (PCA).

The PCA score plots from the full data set are shown in Fig. 5, colour coded to show differences between lines, years and sites. It is clear that the major separation relates to the year and site (Fig. 5a), with no clear discrimination between lines when the data set is considered as a whole (Fig. 5b). However, some separation between the control line L88-6 and the derived transgenic line B73-6-1 can be seen when the data are displayed on a year and site basis, as shown in Fig. 5c and d for 1999 only. In contrast, no such separation was observed for the two transgenic lines (B102-1-1/2) derived from L88-31 (Fig. 5e and f).

The differences observed between B73-6-1 and L88-6 may relate to the fact that the former line shows very high over-expression of the transgene (Fig. 2), with the combined amounts of the endogenous and transgenic forms of subunit 1Dx5 accounting for about 17% of the total grain protein compared to the endogenous subunit 1Dx5, which accounts for 4.1% of the total protein in L88-6. Nevertheless, the differences between these two lines did not exceed the overall range of variation related to site and year.

Comparisons of the loading plots from the PCA models allow the major metabolites which are responsible for the separations to be identified. In the case of B73-6-1 and L88-6, these are the levels of maltose and sucrose, both common metabolites which are present in many foods.

Similarly, the variation between sites was mainly due to two common amino acids, aspartic acid and glutamine. Hence, none of these differences could be expected to pose a problem for human health.

Obert et al. (2004) also failed to show any compositional differences between grain and forage materials from field plots of herbicide-resistant and control lines of wheat, while analyses reported for other transgenic crops (eg, peas, rice, soybean and tomato) also failed to show any greater differences than those normally present within samples of the crop (Charlton et al., 2004; Le Gall, Colquhoun, Davis, Collins, & Verhoeyen, 2003; McCann, Liu, Trujillo, & Dobert, 2005; Oberdoerfer, Shillito, De Beuckeleer, & Mitten, 2005).

We are currently carrying out proteomic comparisons of our lines to identify changes which are not related to expression of the HMW subunit transgenes and associated
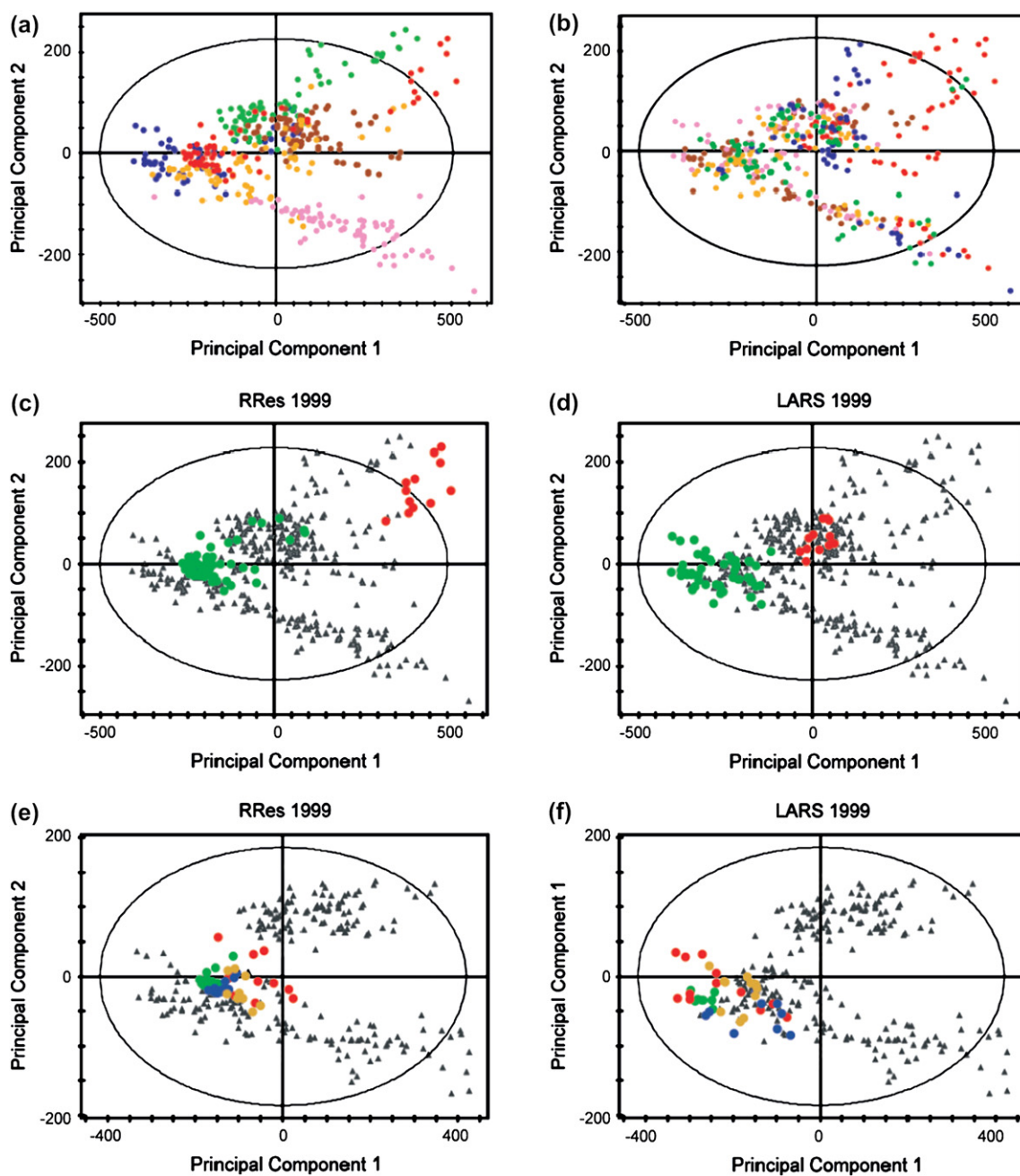
**Fig. 5**. Principal components analysis score plots for all control and transgenic lines, sites and years. (a) Coloured according to year and site: pink, RRes 2001; brown, RRes 2000; red, RRes 1998; orange, LARS 2001; green, LARS 2000, blue, LARS 1998. (b) Coloured according to line: blue, L88-6; green, L88-31; red, B73-6-1; orange, B72-8-11a; brown, B102-1-1; pink, B102-1-2. (c, d) Coloured to show 1999 data only for B73-6-1 (red) and all other lines (green), for RRes (c) and LARS (d). (e, f) Coloured to show 1999 data only for L88-31 (orange), B102-1-1 (blue), B102-1-2 (red) and B72-8-22a (green), for RRes (e) and LARS (f). In parts (c), (d), (e) and (f), all other data points are shown in grey. RRes, Rothamsted Research; LARS, Long Ashton Research Station. Taken from Baker et al. (2006) with permission.

marker genes. Little data on this are currently available for other transgenic crops but a detailed study of Arabidopsis showed that differences between the proteomes of transgenic and non-transgenic lines were either directly related to the introduced gene or within the range of variation found in 12 Arabidopsis ecotypes (Ruebelt, Leimgruber, et al., 2006; Ruebelt, Lipp, Reynolds, Astwood, et al., 2006; Ruebelt, Lipp, Reynolds, Schmuke, et al., 2006).

## Conclusions

The studies discussed here allow us to draw several important conclusions about the substantial equivalence of GM and non-GM wheat.

1. The expression of the transgenes in the lines studied is not intrinsically more or less stable than that of the corresponding endogenous genes.
2. The transgenic and control lines show similar stability in agronomic performance and grain functional properties when grown at multiple sites and years.
3. The gene expression profiles in developing grains of transgenic and control lines are much more similar to those of the parental lines than are the profiles of lines produced by conventional plant breeding.
4. The metabolite profiles of control and transgenic lines usually fall within the range of variation which is observed between genotypes of the species or samples of the same genotype grown under varying environmental conditions.

Despite our results, we would not claim that all transgenic crops are substantially equivalent to non-GM crops. However, our results (and those reported by others) clearly demonstrate that it is possible to produce transgenic wheat lines which *are* substantially equivalent to non-GM wheat at the level of analysis provided by modern "omics" technologies, except for effects that are directly attributable to the transgene. Information of this type is clearly crucial to underpin the commercial introduction of transgenic food crops, not least to assure consumers that transgenic crops are not inherently unsafe.

## Acknowledgements

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