Effects of Genotype and Environment on Free Amino Acid Levels in Wheat Grain: Implications for Acrylamide Formation during Processing

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Acrylamide forms from free asparagine and reducing sugars during cooking, with asparagine concentration being the key parameter determining the formation in foods produced from wheat flour. In this study free amino acid concentrations were measured in the grain of varieties Spark and Rialto and four doubled haploid lines from a Spark × Rialto mapping population. The parental and doubled haploid lines had differing levels of total free amino acids and free asparagine in the grain, with one line consistently being lower than either parent for both of these factors. Sulfur deprivation led to huge increases in the concentrations of free asparagine and glutamine, and canonical variate analysis showed clear separation of the grain samples as a result of treatment (environment, E) and genotype (G) and provided evidence of G × E interactions. Low grain sulfur and high free asparagine concentration were closely associated with increased risk of acrylamide formation. G, E, and G × E effects were also evident in grain from six varieties of wheat grown at field locations around the United Kingdom in 2006 and 2007. The data indicate that progress in reducing the risk of acrylamide formation in processed wheat products could be made immediately through the selection and cultivation of low grain asparagine varieties and that further genetically driven improvements should be achievable. However, genotypes that are selected should also be tested under a range of environmental conditions.

KEYWORDS: Acrylamide; asparagine; food contaminants; plant breeding; sulfur fertilization; *Triticum aestivum*; wheat

INTRODUCTION

Acrylamide is present in a variety of cooked foods in concentrations that may exceed 1 mg/kg (1000 ppb) (*1, 2*). It is formed as a result of the Maillard reaction (*3, 4*), which involves the thermal degradation of amino acids in the presence of reducing sugars and, in the case of asparagine, results in the formation of acrylamide. The major precursors for acrylamide formation, therefore, are free asparagine and reducing sugars. Acrylamide is classified as "probably carcinogenic" to humans, and two recent epidemiological studies have linked high dietary intake of acrylamide with cancer (*5, 6*); it also has effects on the nervous system and reproduction (*2*).

The acrylamide issue has become one of the food industry's most pressing problems and a general public health concern. Research on strategies to reduce its formation during cooking and processing are described in the "Acrylamide toolbox" produced by the Confederation of the Food and Drinks Industries of the European Union (CIAA) (www.ciaa.be/documents/ brochures/CIAA_Acrylamide_Toolbox_Oct2006.pdf).

Wheat (*Triticum aestivum*) has a relatively high acrylamide risk and, significantly for wheat producers, acrylamide formation in wheat-derived products is higher than that in "competitor" cereals such as maize and rice, although it is generally lower than that in potato products. It is therefore possible that food producers could switch to other cereals for products such as biscuits and breakfast cereals if they come under pressure from regulators or consumers to reduce the levels of acrylamide. It is important, therefore, that the acrylamide risk of wheat is reduced.

Asparagine rather than sugar concentration has been shown to be the key component in wheat grain, with acrylamide

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formation on heating correlating closely with free asparagine concentration (*7−9*). However, the ratio of free asparagine to other free amino acids rather than the concentration of free asparagine per se has been shown to be particularly important for potato (*10*), and it would be premature at this stage to rule this out for wheat because it may affect the rate of acrylamide formation and formation rate may be important in some food products.

The importance of free asparagine and free amino acid levels in general to acrylamide formation has renewed interest in the factors controlling free amino acid accumulation. Asparagine accumulates in different plant species in response to a variety of environmental conditions (*11, 12*); in general, its concentration increases when the rate of protein synthesis is low and there is a plentiful supply of reduced nitrogen. This occurs during normal physiological processes, such as seed germination, but can be induced by both biotic and abiotic stresses, including nutrient deprivation. Sulfur deprivation has a particularly dramatic effect on free asparagine concentration in wheat and barley grain, causing increases of up to 30-fold compared with grain from wheat grown with a plentiful sulfur supply (*7, 8, 11−15*).

The increase in free asparagine concentration in wheat grain under conditions of sulfur deprivation is so great that ensuring that wheat has a plentiful supply of sulfur could affect significantly the acrylamide risk of wheat (*8*). The current Rothamsted recommendation for sulfur application to wheat crops grown in the United Kingdom is 15−20 kg/ha (Fangjie Zhao, Rothamsted Research, personal communication), and it is important that the fertilizer is applied evenly and that total coverage is achieved (*16*). Avoiding sulfur deficiency is not the complete answer, however, because wheat grown under optimal conditions still contains a pool of free asparagine that could potentially reach relatively high concentrations. Previous work with German wheat varieties has shown differences in levels of acrylamide both between varieties and arising from different agronomic practices (*17*). The aim of the present study was therefore to determine the relative impact of genetic and environmental factors, and the interaction between the two, on free asparagine accumulation in wheat grain and to assess the likelihood of success in reducing the acrylamide risk of wheat by breeding or other genetic approaches

MATERIALS AND METHODS

Doubled Haploid Wheat Lines. A mapping population of doubled haploid lines produced from a cross between elite U.K. wheat (*T. aestivum*) varieties Spark and Rialto (*18*) was kindly provided by John Snape of the John Innes Centre, Norwich, U.K. Four lines, SR3, SR41, SR107, and SR7, were selected for study because analyses of material grown for a separate experiment showed a range of free asparagine concentrations in their grain (Peter Shewry and Claudia Underwood, Rothamsted Research, unpublished data). The doubled haploid and parental lines were grown in a glasshouse in pots containing compost (Rothamsted mixture), with nine pots per line arranged in a randomized block design in three blocks with three pots of each line per block. Day temperature was maintained at 18 °C and night temperature at 14 °C; supplementary lighting was used to provide the plants with a 16 h day. Grain was collected at maturity, and 5 g samples were milled to fine, wholemeal flour in a ball mill.

Growth of Doubled Haploid and Parental Lines under Glass in Vermiculite with and without Sulfur Feeding. The four doubled haploid lines and the parents, Spark and Rialto, were also grown in pots in a glasshouse with or without sulfur (S+ and S−, respectively) in a randomized design with 15 pots per line in each of two plots, one S+ and one S−. Six plants were grown in each pot. The pots contained vermiculite [general formula $(Na_{0.21}, K_{0.39}, Mg_{0.19}, Ca_{0.13} \cdot 6H_2O)$ $(Mg_5, Fe^{2+}_{0.2}, Fe^{3+}_{0.8}) [Si_{5.5}, Al_{2.5}, O_{20}] (OH)_4]$, and the plants were

Table 1. Sites Used for Field Production of Grain for the Study

site	location[a]	previous crop	soil type
1	NIAB site, Harper Adams College, Shropshire	sugar beet	light sand
2	NIAB site, Stoke, Hampshire	spring oilseed rape	medium
3	NIAB site, Ivychurch, Kent	peas	deep clay
4	BSPB site, Nickersons, Quidenham, Norfolk	sugar beet	light sand
5	Scottish Agricultural College, East Lothian	winter oilseed rape	medium
6	The Arable Group site, Caythorpe, Lincolnshire	linseed	medium

[a] NIAB, National Institute of Agricultural Botany; BSPB, British Society of Plant Breeders.

therefore reliant on supplied minerals. Glasshouse conditions were as described above, and feeding was started immediately after potting and continued every other day with two different treatment solutions. S+ plants were watered with medium containing sufficient amounts of potassium, phosphate, calcium, magnesium, sodium, iron, and nitrate and sulfate ions (1 mM $MgSO_4$), whereas S− plants were watered with the same medium lacking the sulfate, as described by Muttucumaru and co-workers (*7*). Grain was harvested at maturity; five independent samples, each containing grain bulked from three pots, were taken for each line, and 5 g was removed and milled in a ball mill.

Grain from Six Different Elite Wheat Varieties Grown at Different Locations around the United Kingdom in 2006 and 2007. Grain from six elite U.K. wheat varieties, Solstice, Malacca, Robigus, Einstein, Xi19, and Claire, which had been harvested in 2006 and 2007, was kindly provided by the Home Grown Cereal Authority (HGCA) of the United Kingdom. Each variety had been grown at six different sites in the United Kingdom. The locations, previous crop, and soil type of these sites are given in **Table 1**. Information on the soil types can be found in Department for the Environment, Food and Rural Affairs (DEFRA) Technical Note 52 (http://naturalengland.communisis.com/naturalenglandshop/docs/tan_52.pdf).

In each case, 1 kg of grain was provided, and 30 g of this was milled to fine, wholemeal flour in a ball mill. In this way, samples provided three replicates from the 1 kg "pool".

Concentrations of Free Amino Acids. For the wheat grown under glass in compost, measurements of free amino acids were made in fine, wholemeal flour from nine grain samples per line (three samples per block). For the S+ and S− vermiculite-grown wheat, five grain samples per line were analyzed for each treatment combination. Finally, for the field-grown wheat, three samples per variety per area per year were used.

Amino acids were extracted and quantified as described previously (*7*). Briefly, flour samples (0.5 g) were weighed into 14 mL screw-top bottles. HCl (10 mL, 0.01 M) was added to the vial, and the sample was stirred for 15 min at room temperature and then allowed to stand for a further 15 min. An aliquot (1.5 mL) was removed and centrifuged at 7200*g* for 15 min; an aliquot (100 μL) of the supernatant was then derivatized using the EZ-Faast amino acid derivatization technique for gas chromatography and mass spectrometry (GC-MS) (Phenomenex, Torrance, CA) as described previously (*19*).

Gas chromatography−mass spectrometry (GC-MS) analysis of the derivatized samples was carried out using an Agilent 5975 system (Agilent, Santa Clara CA) in electron impact mode. An aliquot of the derivatized amino acid solution (1 μL) was injected at 280 °C in split mode (40:1) onto a Zebron ZB-AAA capillary column (10 m × 0.25 mm; 0.25 μm film thickness). The oven temperature was held at 110 °C for 1 min and then increased at 30 °C/min to 310 °C. The transfer line and ion source were maintained at 320 and 230 °C, respectively; carrier gas flow rate was kept constant throughout the run at 1.5 mL/min. One analysis was performed for each sample.

Measurements of Total Grain Nitrogen and Sulfur. Measurements of total grain nitrogen and sulfur were made by the Analytical Unit of

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Table 2. Means of Free Amino Acid Concentrations (Millimoles per Kilogram) in Fine Flour from Grain of Wheat (*Triticum aestivum*) Varieties Spark and Rialto (*n* = 9) and in Doubled Haploid Lines Grown under Glass in Compost with $\log_e$ Data Means in Parentheses[a]

amino acid	SR3	SR7	SR41	SR107	Rialto	Spark	sed	lsd	significance (%)
alanine	0.48 (−0.74)	0.46 (−0.78)	0.66 (−0.42)	0.54 (−0.61)	0.63 (−0.47)	0.57 (−0.55)	0.083	0.185	0.70
asparagine	1.68 (0.52)	2.13 (0.76)	3.23 (1.17)	2.33 (0.84)	2.45 (0.89)	2.71 (0.10)	0.149	0.332	2.20
aspartate	3.26 (1.18)	3.48 (1.25)	3.95 (1.37)	2.86 (1.05)	2.81 (1.03)	2.43 (0.89)	0.125	0.278	3.30
GABA	0.20 (−1.61)	0.17 (−1.75)	0.24 (−1.43)	0.27 (−1.32)	0.31 (−1.18)	0.23 (−1.49)	0.156	0.348	4.70
glutamate	1.19 (0.17)	1.48 (0.39)	1.88 (0.63)	1.86 (0.62)	1.71 (0.54)	1.81 (0.59)	0.128	0.284	3.00
glutamine	0.06 (−2.74)	0.07 (−2.67)	0.17 (−1.78)	0.13 (−2.06)	0.18 (−1.73)	0.19 (−1.67)	0.387	0.863	NS
glycine	0.21 (−1.56)	0.21 (−1.58)	0.28 (−1.27)	0.28 (−1.26)	0.28 (−1.28)	0.26 (−1.34)	0.092	0.204	1.30
histidine	0.06 (−2.79)	0.06 (−2.89)	0.04 (−3.32)	0.07 (−2.64)	0.08 (−2.61)	0.08 (−2.55)	0.335	0.747	NS
isoleucine	0.11 (−2.20)	0.08 (−2.50)	0.11 (−2.19)	0.10 (−2.29)	0.15 (−1.87)	0.11 (−2.22)	0.138	0.308	2.40
leucine	0.15 (−1.88)	0.14 (−1.98)	0.17 (−1.80)	0.17 (−1.76)	0.21 (−1.56)	0.16 (−1.83)	0.123	0.274	NS
lysine	0.15 (−1.93)	0.16 (−1.83)	0.19 (−1.66)	0.20 (−1.60)	0.23 (−1.47)	0.16 (−1.83)	0.134	0.299	5.00
methionine	0.04 (−3.25)	0.09 (−2.40)	0.13 (−2.06)	0.08 (−2.57)	0.09 (−2.43)	0.04 (−3.34)	0.657	1.464	NS
ornithine	0.01 (−4.47)	0.01 (−4.23)	0.03 (−3.57)	0.02 (−3.80)	0.03 (−3.63)	0.02 (−3.85)	0.221	0.493	0.01
phenylalanine	0.07 (−2.72)	0.07 (−2.59)	0.07 (−2.62)	0.07 (−2.64)	0.09 (−2.35)	0.08 (−2.52)	0.150	0.335	NS
proline	0.18 (−1.74)	0.14 (−1.99)	0.28 (−1.26)	0.23 (−1.47)	0.38 (−0.98)	0.19 (−1.64)	0.205	0.457	0.70
serine	0.17 (−1.74)	0.11 (−2.23)	0.28 (−1.27)	0.30 (−1.21)	0.19 (−1.65)	0.27 (−1.31)	0.320	0.712	NS
threonine	0.08 (−2.58)	0.09 (−2.42)	0.11 (−2.17)	0.10 (−2.34)	0.11 (−2.21)	0.09 (−2.38)	0.164	0.364	NS
tryptophan	0.37 (−0.98)	0.25 (−1.37)	0.39 (−0.95)	0.45 (−0.79)	0.40 (−0.92)	0.19 (−1.66)	0.248	0.553	4.00
tyrosine	0.03 (−3.39)	0.03 (−3.40)	0.04 (−3.32)	0.03 (−3.50)	0.05 (−3.09)	0.03 (−3.44)	0.099	0.221	0.03
valine	0.23 (−1.49)	0.19 (−1.67)	0.27 (−1.29)	0.25 (−1.38)	0.34 (−1.09)	0.30 (−1.21)	0.097	0.217	0.20
total	8.73	9.42	12.52	10.34	10.71	9.92			

[a] GABA, γ-aminobutyrate; sed, standard error of difference between $\log_e$ data means; lsd, least significant difference between $\log_e$ data means (5% level, comparisons made on the $\log_e$ scale); NS, not significant at 5%. The sed and lsd are on 10 degrees of freedom.

the Soil Science Department, Rothamsted Research. Total grain nitrogen was determined according to the "Dumas" digestion method using a LECO CNS 2000 combustion analyzer. Total sulfur concentration was determined using an Accuris inductively-coupled plasma optical emission spectrometer (ICP-OES) (Applied Research Laboratories, Vallaire, Ecublens, Switzerland; supplied by Thermo Optek, Crawley, U.K.) after the samples had been digested with a mixture of HNO_3 and $HClO_4$.

Production and Analysis of Acrylamide. Acrylamide was produced and analyzed essentially as described by Muttucumaru and co-workers (7). Flour samples (0.5 g) in unsealed glass ampules (1 mL capacity) were heated for 20 min at 180 °C. Acrylamide was extracted from these samples with 25% (v/v) aqueous methanol and converted to the dibromo derivative for analysis by GC-MS, using the method of Castle and co-workers (20), with the modifications described by Elmore and co-workers (19). Labeled [$^{13}C_3$]acrylamide was used as the internal standard.

The brominated extracts (2 μL) were injected onto an Agilent 5975 GC-MS system in pulsed splitless mode at 250 °C, the splitter opening after 0.5 min. The helium carrier gas pressure was 21 psi in pulsed mode, falling to 9.6 psi for the rest of the run. A DB-5 MS capillary column was used (30 m × 0.25 m × 1 μm; Agilent). The oven temperature was 85 °C for 1 min, rising at 8 °C/min to 200 °C and then at 30 °C/min to 280 °C for 10 min. The transfer line was held at 280 °C and the ion source at 180 °C. The mass spectrometer was operated in electron impact mode with selected ion monitoring. Two ions were used to monitor brominated [$^{13}C_3$]acrylamide (*m/z* 153 and 155), and another two ions were used for brominated acrylamide (*m/z* 150 and 152). The ion *m/z* 155 was used to quantify brominated [$^{13}C_3$]acrylamide, and the ion *m/z* 150 was used to quantify brominated acrylamide. Each sample was prepared and analyzed in triplicate.

Statistical Analyses. The GenStat statistical system [GenStat, 2007, 10th ed., Lawes Agricultural Trust (Rothamsted Research), VSN International Ltd., U.K.] was used for analyses of variance (ANOVA) and canonical variate analyses (CVA) of the free amino acid concentrations with reference to Payne and co-workers (21). Prior to any analysis, data were transformed to the natural logarithm ($\log_e$) scale to account for heterogeneity of variance across the treatment combinations. CVA is a multivariate technique (22) and was used to analyze the data from all 21 amino acids together, rather than considering separate ANOVAs. It maximizes the ratio of the variation between treatment combinations to the variation within treatment combinations, thus discriminating between all treatment combinations. The fewest number of canonical

variates (CVs) are retained that take up the most variation in the data and hence make the most discrimination. The data are then visualized on the new dimensions by plotting the CV scores for each sample [i.e., each row of the original data matrix of observations by amino acids (columns)]. The mean of CV scores in each dimension, for each treatment combination, that is, the CV means, are also plotted. Making the assumption of multivariate normality for the data on the natural logarithm ($\log_e$) scale, 95% confidence circles are placed around the CV means. The radius of these circles is $\sqrt{\chi^2_{2,0.05}}/\sqrt{n}$, where *n* is the replication and $\sqrt{\chi^2_{2,0.05}} = 5.99$ is the upper 5% point of a chi-squared distribution on 2 degrees of freedom. Each new dimension is formed as a linear combination of the amino acids. The magnitude of the loadings on the amino acids is inspected to conclude which amino acids are most responsible for the discrimination.

RESULTS

Free Amino Acid Concentrations in the Grain of Wheat (*T. aestivum*) Varieties Spark and Rialto and Four Doubled Haploid Lines from a Spark × Rialto Mapping Population. A mapping population of doubled haploid lines produced from a cross between elite U.K. wheat (*T. aestivum*) varieties Spark and Rialto (*18*) was available, and four doubled haploid lines, SR3, SR41, SR107, and SR7, were selected because they had previously been shown to have differing concentrations of free asparagine in their grain (unpublished data). Plants of the four doubled haploid lines and the two parental varieties, Spark and Rialto, were grown under glass in compost, and grain was harvested at maturity and milled to a fine (wholemeal) flour. Free amino acid concentrations in the flour were determined by GC-MS and analysis of variance (ANOVA) applied to the data for each individual amino acid. The means on the $\log_e$ scale and their back-transforms are presented in **Table 2**.

The major contributors to the free amino acid pool were asparagine, aspartate, and glutamate, and all three showed significant differences ($P < 0.05$) in concentration between the lines. Of the two parents, Rialto contained a higher overall concentration of free amino acids (10.71 compared with 9.92 mmol/kg) but less free asparagine than Spark (2.45 compared with 2.71 mmol/kg), a slightly lower concentration of free glutamate (1.71 compared with 1.81 mmol/kg), and a higher

concentration of free aspartate (2.81 compared with 2.43 mmol/kg). In other words, the ratios of free asparagine and to a lesser extent glutamate to aspartate differed between the two varieties. In three of the four doubled haploid lines, SR3, SR7, and SR107, these ratios were shifted even further, with free asparagine levels being lower than in either parent (1.68, 2.13, and 2.33 mmol/kg for SR3, SR7, and SR107, respectively) and free aspartate levels being higher (3.26, 3.48, and 2.86 mmol/kg). Free glutamate levels were lower than in either parent in SR3 and SR7 (1.19 and 1.48 mmol/kg, respectively) but were similar to the levels in the parents in SR107 (1.86 mmol/kg). SR3 and SR7 also differed from the parental lines in having a smaller total pool of free amino acids (8.73 and 9.42 mmol/kg, respectively), whereas the total pool of free amino acids in SR107 was midway between those in the two parents (10.34 mmol/kg) and that in SR41 was higher than in either parent (12.52 mmol/kg).

The decreases in free asparagine concentration compared with Rialto (the parent variety with the lower asparagine concentration) were 31% for SR3 and 13% for SR7, and the fact that this coincided in both cases with a decrease in the total pool of free amino acids and a shift in the ratios of free asparagine and glutamate to aspartate suggested that it had been brought about by the same genetic mechanism.

Line SR41, in contrast, had a higher concentration of free asparagine (3.23 mmol/kg) than either parent (19% higher than Spark), but also a higher concentration of free aspartate (3.95 mmol/kg), whereas the pool of free glutamate was relatively unchanged. That both asparagine and aspartate increased while glutamate was unchanged suggested that a different genetic mechanism related to this ratio was affected in this line from that affected in SR3 or SR7.

CVA was applied to the data to show the separation of the lines graphically and to confirm which amino acids were mainly responsible for the separations. Most (70%) of the variation (discrimination) was taken up by the first two CVs (CV1 and CV2), which were retained for visualization of the data. The analysis highlighted the separation of the two parents from each other and of SR3, SR7, and SR41 from either parent, whereas SR107 was shown to be similar to Spark (**Figure 1**). The major contributors to the discrimination were free asparagine, aspartate, and glycine for CV1 and valine and aspartate for CV2.

Effect of Sulfur Deprivation on Free Amino Acid Levels. Sulfur deprivation has been shown previously to cause a dramatic increase in free asparagine accumulation in grain of pot- and field-grown wheat (*7−9, 15*), and an experiment similar to that described by Muttucumaru and co-workers (*7*) was performed with the parental varieties, Spark and Rialto, and the four doubled haploid lines being used in this study. The aim was to provide evidence for the genetic regulation of free asparagine accumulation under severe sulfur deficiency and to determine whether varieties and lines that can be considered as relatively low in acrylamide risk under normal (S+) conditions are also low risk when grown under sulfur-deficient (S−) conditions.

The plants were grown in vermiculite in pots under glass, with or without sulfur feeding (S+ and S−, respectively). For the S− treatment, no sulfur was supplied at all; the fact that the plants were able to grow and set seed was probably due to small amounts of contaminating sulfate or sulfide ions in the vermiculite. The plants showed clear symptoms of severe sulfur deprivation, including yellow leaves with reduced chlorophyll content, decreased tiller number (by up to 50%), slow growth, decreased height at maturity (by up to 50%), late anthesis, and
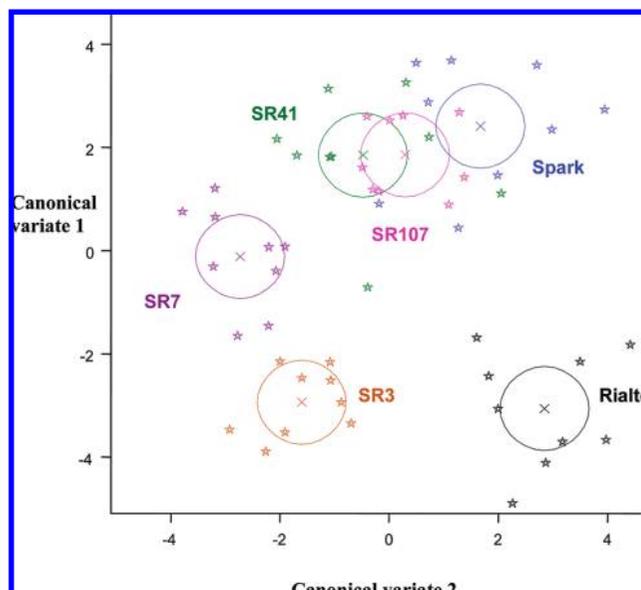

Figure 1. Canonical variate analysis plot of data on free amino acid concentrations in grain of wheat (*Triticum aestivum*) varieties Spark and Rialto and four doubled haploid lines, SR3, SR7, SR41, and SR107, from a Spark × Rialto mapping population, grown under glass in compost (**Table 2**). The plot shows the canonical variate scores (stars) and means (crosses); 95% confidence circles are shown around each mean. The major contributors to the discrimination were free asparagine, aspartate, and glycine for CV1 and valine and aspartate for CV2.

decreased grain yield. In particularly badly affected plants a sweet drop formed between the glumes, presumably resulting from sugars being transported to the ear where there was insufficient grain to utilize it. The top of the ear was light yellow to white and deteriorated earlier than any other part of the plant. Grain was harvested at maturity, milled, and analyzed as previously described. The concentrations of free amino acids are presented in **Table 3**.

Analysis of variance was performed on (log$_e$) data for each amino acid to ascertain which effects, environment (E), genotype (G), and the interaction between the two (G × E), were significant. The interaction was significant (*P* < 0.05) for glycine, valine, leucine, asparagine, aspartate, glutamate, lysine, tyrosine, and tryptophan. There were some differences between the results for the plants grown in vermiculite compared with compost. In general, the free amino acid levels were higher in the vermiculite-grown plants than in the compost-grown plants. Of the parental varieties, Rialto again had a higher concentration of free amino acids in the grain than Spark (15.40 compared with 11.15 mmol/kg) but relative concentrations of free asparagine were reversed, with Rialto having a higher concentration than Spark (3.27 compared with 2.54 mmol/kg). The doubled haploid line SR3 again had a lower concentration of total free amino acids than either parent (11.10 mmol/kg), but SR7, SR107, and SR41 were intermediate between the two parents in terms of the concentrations of total free amino acids and asparagine.

These differences were small compared with the effects of sulfur deprivation. In the sulfur-deprived plants, free amino acid concentrations were greatly increased, mainly as a result of a huge accumulation of free asparagine and glutamine, with asparagine being by far the most abundant free amino acid. The highest levels of all were measured in the grain of the parent variety Spark, which had an asparagine concentration of 62.02 mmol/kg and a total free amino acid pool of 154.13 mmol/kg, compared with 39.01 mmol/kg asparagine and 101.21 mmol/

Table 3. Free Amino Acid Concentrations (Millimoles per Kilogram) in Fine Flour from Grain of Wheat (*Triticum aestivum*) Varieties Spark and Rialto and in Doubled Haploid Lines Grown under Glass in Vermiculite either with or without Sulfur (S+ and S−, Respectively)[a]

amino acid	SR3 S+	SR3 S−	SR7 S+	SR7 S−	SR41 S+	SR41 S−	SR107 S+	SR107 S−	Rialto S+	Rialto S−	Spark S+	Spark S−
alanine	**0.70** 0.05	**3.15** 0.25	**0.99** 0.04	**6.28** 0.33	**0.89** 0.05	**6.88** 0.53	**1.00** 0.23	**5.62** 0.77	**1.04** 0.12	**6.35** 0.60	**0.85** 0.05	**7.15** 0.85
asparagine	**2.05** 0.14	**25.87** 2.09	**3.21** 0.26	**45.46** 3.63	**3.09** 0.25	**38.51** 3.44	**2.29** 0.35	**51.57** 6.75	**3.27** 0.37	**39.01** 5.04	**2.54** 0.20	**62.02** 6.77
aspartate	**3.78** 0.20	**13.08** 0.95	**3.24** 0.18	**15.49** 0.62	**5.15** 0.35	**13.09** 0.46	**3.03** 0.20	**16.60** 0.55	**3.55** 0.19	**14.93** 0.88	**2.58** 0.45	**16.68** 0.92
glutamine	**0.11** 0.02	**8.22** 1.54	**0.49** 0.08	**22.80** 2.19	**0.17** 0.07	**28.08** 4.08	**0.37** 0.18	**18.62** 5.40	**0.36** 0.09	**14.09** 2.94	**0.37** 0.09	**36.53** 5.11
GABA	**0.28** 0.03	**0.52** 0.06	**0.50** 0.07	**0.79** 0.05	**0.31** 0.04	**0.91** 0.05	**0.43** 0.08	**0.73** 0.09	**0.58** 0.04	**1.02** 0.11	**0.47** 0.15	**0.73** 0.08
glutamate	**1.70** 0.11	**4.87** 0.35	**1.73** 0.20	**8.52** 0.55	**2.07** 0.15	**7.36** 0.78	**1.72** 0.19	**7.74** 0.52	**1.91** 0.16	**8.11** 0.84	**1.60** 0.18	**10.75** 1.37
glycine	**0.25** 0.01	**1.09** 0.09	**0.36** 0.02	**2.40** 0.23	**0.24** 0.01	**2.08** 0.20	**0.31** 0.07	**1.66** 0.27	**0.34** 0.03	**1.84** 0.17	**0.31** 0.04	**2.46** 0.27
histidine	**0.12** 0.03	**0.25** 0.07	**0.09** 0.01	**0.36** 0.07	**0.10** 0.01	**0.41** 0.03	**0.10** 0.04	**0.35** 0.09	**0.13** 0.03	**0.35** 0.08	**0.16** 0.08	**0.70** 0.13
isoleucine	**0.13** 0.01	**0.44** 0.04	**0.17** 0.01	**0.62** 0.02	**0.12** 0.01	**0.66** 0.06	**0.20** 0.06	**0.66** 0.10	**0.20** 0.03	**0.47** 0.03	**0.15** 0.01	**0.83** 0.15
leucine	**0.24** 0.01	**0.57** 0.03	**0.30** 0.01	**0.82** 0.04	**0.20** 0.02	**0.88** 0.07	**0.30** 0.08	**0.74** 0.09	**0.34** 0.03	**0.71** 0.06	**0.22** 0.01	**0.99** 0.14
lysine	**0.17** 0.02	**0.93** 0.08	**0.30** 0.01	**2.04** 0.19	**0.26** 0.03	**1.71** 0.14	**0.23** 0.04	**1.79** 0.32	**0.29** 0.03	**1.83** 0.20	**0.18** 0.03	**2.60** 0.39
methionine	**0.07** 0.06	**0.41** 0.03	**0.12** 0.05	**0.46** 0.02	**0.20** 0.06	**0.44** 0.02	**0.19** 0.06	**0.40** 0.10	**0.12** 0.05	**0.43** 0.02	**0.08** 0.02	**0.51** 0.03
ornithine	**0.02** 0.00	**0.18** 0.02	**0.03** 0.01	**0.65** 0.05	**0.03** 0.00	**0.47** 0.06	**0.03** 0.00	**0.41** 0.06	**0.04** 0.02	**0.49** 0.06	**0.03** 0.01	**0.55** 0.09
phenylalanine	**0.09** 0.00	**0.19** 0.01	**0.12** 0.01	**0.22** 0.01	**0.11** 0.01	**0.24** 0.01	**0.14** 0.04	**0.22** 0.02	**0.15** 0.01	**0.24** 0.02	**0.10** 0.00	**0.28** 0.02
proline	**0.50** 0.11	**1.80** 0.22	**1.51** 0.12	**3.39** 0.27	**0.55** 0.15	**3.26** 0.46	**1.36** 0.64	**2.22** 0.45	**1.71** 0.45	**4.01** 0.31	**0.64** 0.15	**2.44** 0.15
serine	**0.21** 0.04	**1.40** 0.12	**0.45** 0.06	**4.49** 0.36	**0.21** 0.03	**4.43** 0.53	**0.37** 0.11	**3.01** 0.63	**0.39** 0.12	**4.07** 0.37	**0.21** 0.06	**4.36** 0.56
threonine	**0.06** 0.03	**0.46** 0.07	**0.12** 0.00	**1.22** 0.08	**0.12** 0.02	**1.16** 0.11	**0.12** 0.03	**1.03** 0.16	**0.13** 0.02	**1.00** 0.09	**0.09** 0.02	**1.30** 0.12
tryptophan	**0.21** 0.04	**0.18** 0.02	**0.14** 0.01	**0.14** 0.01	**0.24** 0.03	**0.22** 0.01	**0.21** 0.03	**0.27** 0.02	**0.21** 0.05	**0.21** 0.02	**0.12** 0.03	**0.18** 0.01
tyrosine	**0.05** 0.00	**0.10** 0.01	**0.05** 0.00	**0.14** 0.01	**0.04** 0.00	**0.15** 0.01	**0.06** 0.02	**0.14** 0.01	**0.06** 0.00	**0.13** 0.00	**0.05** 0.01	**0.15** 0.02
valine	**0.36** 0.02	**1.34** 0.12	**0.49** 0.04	**2.28** 0.13	**0.33** 0.04	**2.32** 0.21	**0.41** 0.09	**2.07** 0.32	**0.58** 0.07	**1.92** 0.16	**0.40** 0.03	**2.92** 0.36
total	**11.10**	65.05	**14.41**	118.57	**14.43**	113.26	**12.87**	115.85	**15.40**	101.21	**11.15**	154.13

[a] Means (*n* = 5) are given in bold, standard errors in normal type.

kg total free amino acids in Rialto. Once again line SR3 had the lowest concentration of total free amino acids (65.05 mmol/kg) and asparagine (25.87 mmol/kg).

CVA was applied to the data, which comprised 12 treatment/line combinations (2 treatments, S+ and S−, and 6 lines, SR3, SR7, SR41, SR107, Spark, and Rialto) with 5 replicates. The first two CVs accounted for 90% of the variation. The major contributors to this discrimination were free asparagine and valine for CV1 and alanine, glycine, and phenylalanine for CV2.

Figure 2 illustrates the very clear separation of the data based on treatment (environment, E) in the CV1 direction and to a lesser extent on line (genotype, G) in the CV2 direction. Note that there is also a strong G × E interaction, illustrated by the fact that the lines are in different positions relative to each other under the different treatments. The free asparagine concentrations showed this very clearly with the lines falling into two groups (**Table 3**). In the S− grain of Rialto, SR3, SR7, and SR41, the free asparagine concentrations were 12−14-fold higher than in the corresponding S+ grain. Spark and SR107, on the other hand, which were indicated to be similar to each other by the CVA of the compost-grown material, showed much larger increases of 23- and 24-fold in grain free asparagine concentrations in response to sulfur deprivation. This indicates clearly that there is genetic control of free asparagine accumulation and that genetic effects interact with environmental effects such as sulfur deprivation.

Analysis of Grain from Field-Grown Material from Different Locations in the United Kingdom and Two Harvest Years, 2006 and 2007. Grain from six different wheat varieties, Claire, Einstein, Malacca, Robigus, Solstice, and Xi19, grown at six different locations around the United Kingdom (**Table 1**) and harvested in 2006 and 2007, was provided by the Home Grown Cereals Authority (HGCA) of the United Kingdom. Free amino acid levels in the grain were measured and are given in Table S1 of the Supporting Information.

The concentrations of free asparagine in the grain samples varied widely, from 0.6 to 4.4 mmol/kg, representing a >6-fold difference (**Figure 3A**), and the variety, location, and year of harvest all affected the concentration. Robigus was the
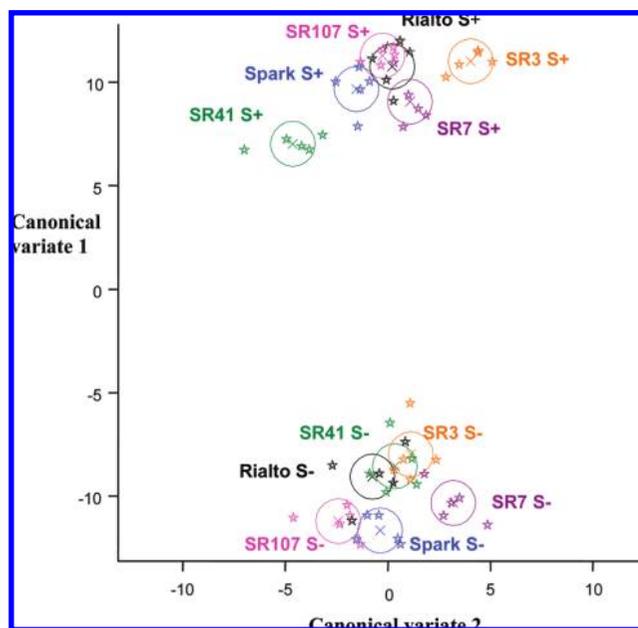

Figure 2. Canonical variate analysis plot of data on free amino acid concentrations in grain of wheat (*Triticum aestivum*) varieties Spark and Rialto and four doubled haploid lines, SR3, SR7, SR41, and SR107, from a Spark × Rialto mapping population, grown under glass in vermiculite and fed with nutrients either including sulfur (S+) or excluding it (S−) (**Table 3**). The plot shows the canonical variate scores (stars) and means (crosses); 95% confidence circles are shown around each mean. The data separate according to environment in the CV1 direction and according to genotype in the CV2 direction; the major contributors to the discrimination were free asparagine and valine for CV1 and alanine, glycine, and phenylalanine for CV2.

"worst" performer, with an asparagine concentration of 4.4 mmol/kg at a site in Kent in 2006 and an average concentration across the sites and years of 2.59 mmol/kg. In contrast, the highest concentration measured in Claire was 2.7 mmol/kg, whereas in Einstein it was 2.8 mmol/kg. Einstein had the lowest overall average of 1.89 mmol/kg. Grain from wheat grown at
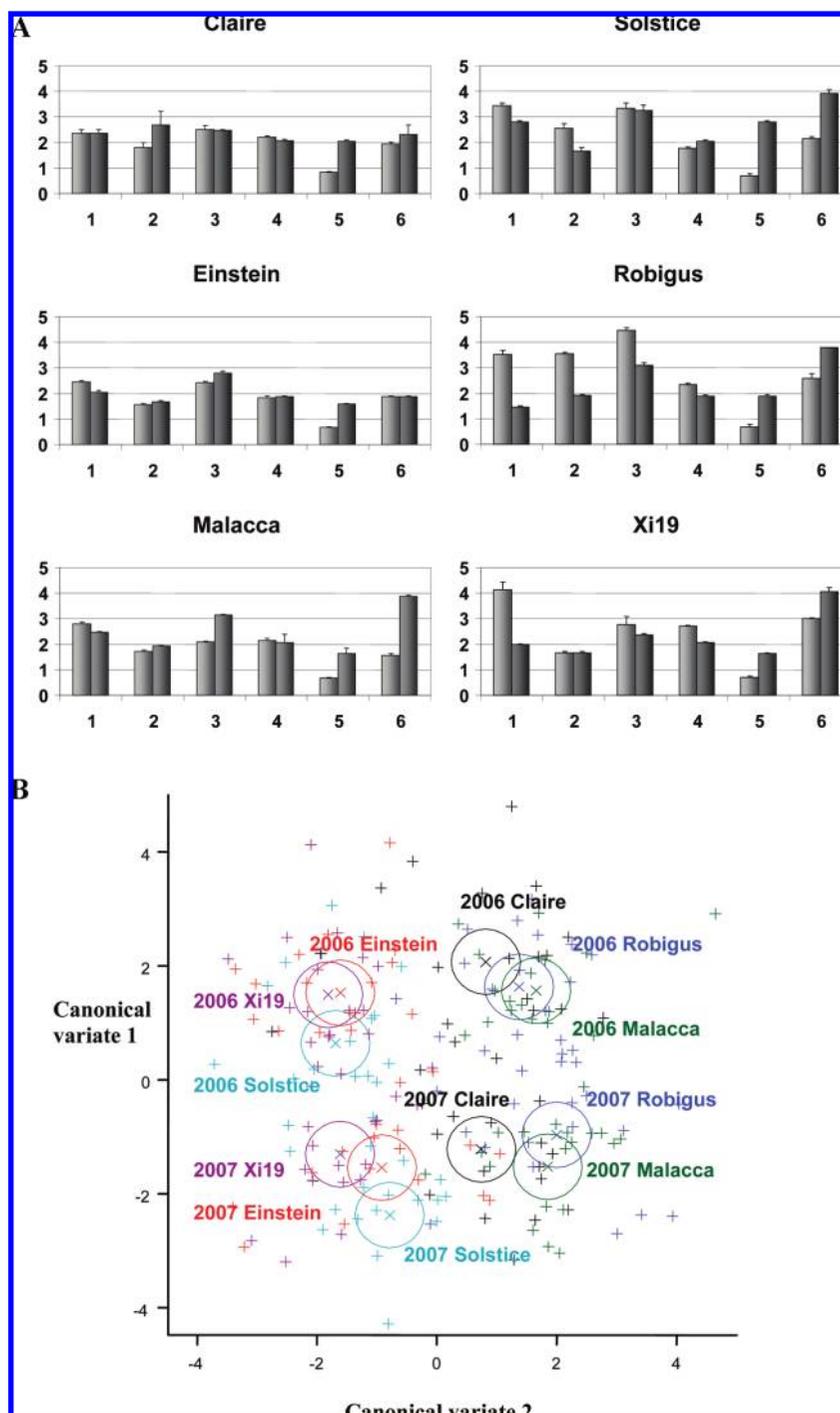
Figure 3. (**A**) Concentration of free asparagine (mmol/kg) in six wheat (*Triticum aestivum*) varieties, Claire, Solstice, Einstein, Robigus, Malacca, and Xi19, as indicated, grown at six different locations (**Table 1**) in the United Kingdom in 2006 (light gray bars) and 2007 (dark gray bars). Means and standard errors from three replicate samples taken from a 1 kg pool are shown in each case. (**B**) Canonical variate analysis plot of data on free amino acid concentrations in grain of wheat varieties as described in **A**, using variety by year combinations over all locations. The plot shows the canonical variate scores (stars) and means (crosses); 95% confidence circles are shown around each mean. Glutamate and glycine were the most discriminatory amino acids in CV1, followed by alanine and asparagine. Alanine was the most important amino acid in CV2, followed by aspartic acid, asparagine, glycine, and phenylalanine. The scattering of the points results from the variability across locations.

the sites in Lincolnshire and Kent generally contained higher concentrations of asparagine than grain from wheat grown at the other sites. G × E interactions were evident, with Claire and Einstein, for example, showing little effect of location on free asparagine concentration, whereas the other varieties contained much higher concentrations of free asparagine when grown on the Lincolnshire and Kent sites.

The free asparagine concentrations were higher in grain harvested in 2007 (mean = 2.37 mmol/kg) than in grain

harvested in 2006 (mean = 2.21 mmol/kg). This was particularly true for the East Lothian site, where all varieties contained very low levels of free asparagine in 2006 (0.6−0.8 mmol/kg), whereas in 2007 the concentrations were comparable with the other sites (1.6−2.8 mmol/kg).

CVA was applied to the free amino acid data to investigate the effects of variety and year of harvest over all locations. There were 12 variety/year-of-harvest combinations, and the first two CVs accounted for 68% of the variation. The CV scores, means,

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Table 4. Total Grain Sulfur and Nitrogen Content in a Selection of Grain Samples from Glasshouse-Grown Plants

genotype	sulfur regimen	grain sulfur (g/kg)	grain nitrogen (g/kg)
Spark	−	0.81	27.80
SR107	−	0.91	26.20
SR7	−	0.82	27.10
Rialto	−	0.98	27.20
SR41	−	0.97	26.70
SR3	−	1.06	26.00
Rialto	+	1.62	30.80
SR3	+	1.90	31.20

Table 5. Concentration of Acrylamide Formed in Fine Flour after Heating at 180 °C for 20 min[a]

genotype and sample	sulfur regimen	free asparagine (mmol/kg)	acrylamide (mg/kg)
Spark glasshouse	−	**62.02** 6.77	**16.19** 0.87
SR107 glasshouse	−	**51.57** 6.75	**16.84** 0.17
SR7 glasshouse	−	**45.46** 3.63	**15.97** 0.37
Rialto glasshouse	−	**39.01** 5.04	**16.63** 0.12
SR41 glasshouse	−	**38.51** 3.44	**14.28** 0.14
SR3 glasshouse	−	**25.87** 2.09	**12.70** 0.69
Robigus field site 3, 2006	+	**4.46** 0.11	**3.12** 0.17
Rialto glasshouse	+	**3.27** 0.37	**3.01** 0.06
Claire field site 3, 2006	+	**2.50** 0.15	**2.22** 0.05
SR3 glasshouse	+	**2.05** 0.14	**1.90** 0.05
Claire field site 5, 2006	+	**0.82** 0.04	**0.88** 0.05
Solstice field site 5, 2006	+	**0.70** 0.09	**0.83** 0.02
Einstein field site 5, 2006	+	**0.67** 0.02	**0.69** 0.01

[a] Samples of grain showing a range of free asparagine concentrations were selected for analysis. Means ($n = 5$ for asparagine, $n = 3$ for acrylamide) are given in bold, standard errors in normal type.

and 95% confidence circles were plotted (**Figure 3B**). Glutamate and glycine were the most discriminatory amino acids in CV1, followed by alanine and asparagine. Alanine was the most important amino acid in CV2, followed by aspartic acid, asparagine, glycine, and phenylalanine.

The CV scores were considerably spread for the 12 combinations, due to the variability across locations. However, a clear separation emerged for the two harvest years and for the varieties within years, with the first CV distinguishing the harvest years and the second CV the varieties. No overall interaction between years and varieties was indicated by the analysis, but within each year the varieties split into two groups, with Claire, Malacca, and Robigus in one group and Einstein, Solstice, and Xi19 in the other.

Grain Nitrogen and Sulfur Concentrations. A selection of grain samples having a range of free asparagine concentrations were analyzed for total grain nitrogen and sulfur concentration. The samples were taken from the glasshouse experiment using S+ and S− treatments. There was a clear effect of sulfur supply on grain sulfur concentration, which ranged from 1.62 to 1.90 g/kg in the grain from sulfur-fed plants and from 0.81 to 1.02 g/kg in the grain from sulfur-deprived plants (**Table 4**). The total grain nitrogen differed less both between the lines and in response to the treatment, ranging from 26.00 to 31.20 g/kg, although there was a trend for lower grain sulfur to be associated with lower grain nitrogen.

Acrylamide Levels. Acrylamide formation in flour heated for 20 min at 180 °C was determined for a selection of grain samples having a range of free asparagine concentrations (**Table 5**). A wide range of concentrations of acrylamide were determined in the heated flour, from 0.69 mg/kg (ppm) to 16.84 mg/kg, a difference of >24-fold. As reported previously (*7−9*), there was a relationship between free asparagine concentration

and the amount of acrylamide formed (**Figure 4A**; $R^2 = 0.99$). However, the best-fitting model for the data was a quadratic curve, indicating that at very high levels of free asparagine another factor or factors, probably sugars, became limiting. There was some evidence of this in the data of Muttucumaru and co-workers (*7*), but the range of concentrations in the material in this study was greater and the limiting effect much more obvious.

The data also revealed a strong negative correlation between grain sulfur content and acrylamide formation (**Figure 4B**; $R^2 = 0.95$).

DISCUSSION

We have provided evidence of genetic control of the concentration of free asparagine and other free amino acids in wheat grain. Our results should encourage plant breeders to include low grain asparagine concentration as one of their targets, and there is every possibility that breeding could play an important role in mitigating the acrylamide risk of wheat.

There were wide differences in free asparagine contents of closely related genotypes grown in compost in carefully controlled glasshouse conditions, from 1.68 to 3.23 mmol/kg. This, together with the fact that one of the doubled haploid lines, SR3, consistently contained a lower concentration of free asparagine in its grain than either parent, Spark or Rialto, indicates that it should be possible to identify quantitative trait loci (QTL) for low grain asparagine concentration by analyzing the full mapping population. Two factors contributed to low free asparagine concentration: a low overall free amino acid pool and the ratio of asparagine and glutamate to aspartate. The latter implicates the enzyme asparagine synthetase, which catalyzes the ATP-dependent transfer of the amino group of glutamine to a molecule of aspartate to generate glutamate and asparagine (*11, 12*):

$$\text{glutamine} + \text{aspartate} + \text{ATP} \rightarrow \text{glutamate} + \text{asparagine} + \text{AMP} + \text{PPi}$$
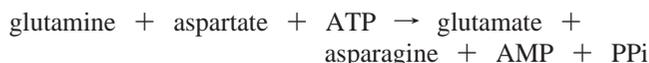
Less is known about the factors controlling the size of the free amino pool, but a regulatory protein kinase, GCN2, has been shown to be involved in the response to herbicide-induced changes in free amino acid levels in *Arabidopsis* (*23, 24*), suggesting that it could also be involved here.

Dramatic changes occur to the free amino acid pool in the grain in response to sulfur deprivation. First, the pool becomes much larger, possibly, at least in part, due to a reduction in storage protein gene expression (*13, 14*). Second, the proportions of amino acids in the pool change, with a massive accumulation of free asparagine and, to a lesser extent, free glutamine. In this case, the rise in free asparagine is not accompanied by an equal rise in glutamate, which suggests either that glutamate produced as a result of greater asparagine synthesis in the grain is recycled or that most of the free asparagine that accumulates under these conditions is made elsewhere in the plant and transported to the grain, rather than being synthesized de novo.

Total grain nitrogen levels were higher in grain from sulfur-fed than sulfur-deprived plants, but did not fall below 26 g/kg. This indicated that in sulfur-deficient conditions the plants continued to store nitrogen in the grain but that a much higher proportion was present as free asparagine and glutamine. Asparagine is attractive in this regard because it is relatively unreactive and has an N:C ratio of 2:4 compared with 2:5 for glutamine, making it less costly in terms of carbon for storing nitrogen (*12*). The total grain sulfur levels, as might be expected, were much lower in the grain from sulfur-deprived than sulfur-fed plants.
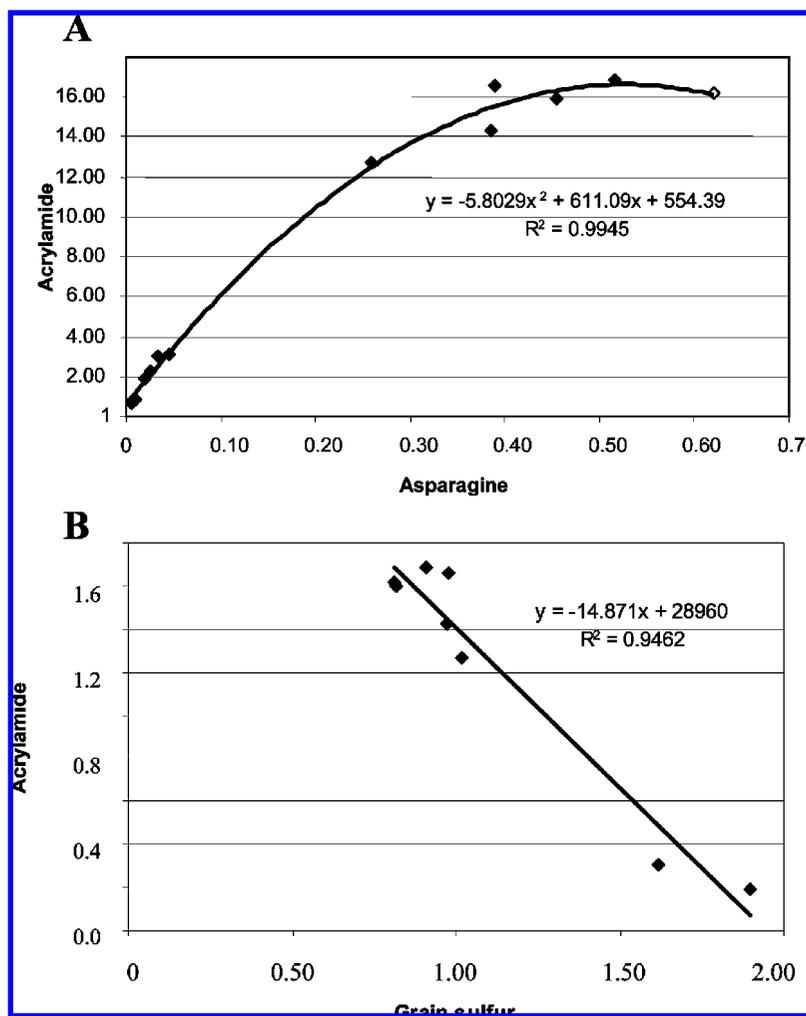
Figure 4. (**A**) Free asparagine concentration (mmol/kg) in wheat (*Triticum aestivum*) grain plotted against acrylamide formed in heated flour (mg/kg). (**B**) Total grain sulfur (mg/g) plotted against acrylamide formed in heated flour (mg/kg).

The link between low grain sulfur concentration, high free asparagine concentration, and acrylamide risk suggests that grain sulfur and/or free asparagine concentrations could be used as quality control parameters, with high-risk grain being used for low-risk products that do not require very high temperature processing and low-risk grain being used for high-risk products. Even with current technology the assays are not trivial, but the extremely high levels of acrylamide (up to almost 17 mg/kg) produced in flour derived from the grain of sulfur-deprived wheat highlight the importance of excluding even small amounts of such material from the food chain (*8*). On the other hand, if farmers can be encouraged to ensure that sulfur deficiency does not occur and wheat breeders can develop varieties that do not accumulate high levels of free asparagine in the grain, such quality control measures may not be necessary.

It should be noted that the levels of acrylamide obtained in this study reflect the potential of the material to form acrylamide, not how much would be formed in a particular food product. The amount of acrylamide that forms during food production depends not only on the raw material but also on the processing methods and, in the case of wheat, the degree of refining: products made from white flour contain considerably less acrylamide than equivalent products made from wholemeal flour (*17*).

The relationship between glutamine and asparagine is a potential target for genetic intervention, with the aim of producing varieties that accumulate glutamine instead of as-

paragine in response to sulfur deprivation and other stresses. The predominant product of the Maillard reaction from glutamine is 2-pyrrolidinone, which is not considered to be toxic (*25*), and the strategy of altering the ratios of free amino acids rather than reducing the size of the total free amino acid pool is attractive because it is likely to have less impact on flavor (*26*).

The grain from different varieties grown at different locations around the United Kingdom in 2006 and 2007 showed a wider range of free asparagine concentrations than the lines grown in the glasshouse, from 0.6 to 4.4 mmol/kg, an almost 7-fold difference. This was despite similar agronomic practice being followed at each site. Environmental factors played a large part, with significant variance arising from location and harvest year, but substantial varietal (genotype) differences were also evident. This means that progress on the acrylamide issue could be made almost immediately by selecting low free asparagine varieties that are already in commercial use.

It would be almost impossible to state with absolute certainty what specific environmental conditions contributed to the differences in free asparagine concentrations. This would be true of almost any field-based study. Soil type is an obvious candidate, but no clear trends emerged. The best site, in East Lothian, had a medium soil, but so did the worst site, in Lincolnshire. Climate may have been a factor contributing to the variance between harvest years. U.K. Meteorological Office records show that the summer of 2006 was considerably drier, warmer, and sunnier than the summer of 2007, particularly

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during the months of June and July (http://www.metoffice.gov.uk/climate/uk/index.html).

Genotype × environment (G × E) interactions were evident in the sulfur-feeding experiment, in which doubled haploid lines SR3 and SR41 were grouped closer to the parental lines by the CVA when grown under sulfur-deficient conditions than when supplied with sulfur, despite the huge changes in the free amino acid pool brought about by sulfur deprivation. G × E interactions also contributed greatly to the variance seen in the U.K. variety/location/year data, with Claire and Einstein having a consistently low free asparagine concentration but Robigus, for example, appearing to be much more sensitive to environmental differences. Clearly, it is important that apparently low free asparagine genotypes are tested at different locations and under stress conditions before incorporation into breeding programs.

ACKNOWLEDGMENT

Supporting Information Available: Free amino acid concentrations in fine flour from grain of wheat varieties grown at six different locations in the United Kingdom in 2006 and 2007. This material is available free of charge via the Internet at http://pubs.acs.org.

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