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Overexpression of GCN2-type protein kinase in wheat has profound effects on free amino acid concentration and gene expression

Edward H. Byrne[1,†], Ian Prosser[1], Nira Muttucumaru[1], Tanya Y. Curtis[1], Astrid Wingler[2], Stephen Powers[3] and Nigel G. Halford[1,*]

[1]*Department of Plant Science, Rothamsted Research, Harpenden, Hertfordshire, UK*

[2]*Research Department of Genetics, Evolution and Environment, University College London, Gower Street, London, UK*

[3]*Department of Biomathematics and Bioinformatics, Rothamsted Research, Harpenden, Hertfordshire, UK*

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**Correspondence* (Tel 44 1582 763133; fax 44 1582 763010; email nigel.halford@rothamsted.ac.uk)
†Present address: Cereal Genomics, School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, UK.
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Summary

A key point of regulation of protein synthesis and amino acid homoeostasis in eukaryotes is the phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α) by protein kinase general control nonderepressible (GCN)-2. In this study, a GCN2-type PCR product (TaGCN2) was amplified from wheat (*Triticum aestivum*) RNA, while a wheat eIF2α homologue was identified in wheat genome data and found to contain a conserved target site for phosphorylation by GCN2. *TaGCN2* overexpression in transgenic wheat resulted in significant decreases in total free amino acid concentration in the grain, with free asparagine concentration in particular being much lower than in controls. There were significant increases in the expression of eIF2α and protein phosphatase PP2A, as well as a nitrate reductase gene and genes encoding phosphoserine phosphatase and dihydrodipicolinate synthase, while the expression of an asparagine synthetase (*AS1*) gene and genes encoding cystathionine gamma-synthase and sulphur-deficiency-induced-1 all decreased significantly. Sulphur deficiency–induced activation of these genes occurred in wild-type plants but not in *TaGCN2* overexpressing lines. Under sulphur deprivation, the expression of genes encoding aspartate kinase/homoserine dehydrogenase and 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase was also lower than in controls. The study demonstrates that *TaGCN2* plays an important role in the regulation of genes encoding enzymes of amino acid biosynthesis in wheat and is the first to implicate GCN2-type protein kinases so clearly in sulphur signalling in any organism. It shows that manipulation of *TaGCN2* gene expression could be used to reduce free asparagine accumulation in wheat grain and the risk of acrylamide formation in wheat products.

Introduction

Translation initiation, the point at which a ribosome recruits an mRNA molecule, is a key control point for protein synthesis in all eukaryotic species. It is regulated by the phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α) (reviewed by Hershey and Merrick, 2000). eIF2 is a trimeric factor (subunits α, β and γ) that can bind either guanosine diphosphate (GDP) or triphosphate (GTP). Only when bound to GTP is it able to carry out its physiological function of binding Met-tRNA to the ribosome and transferring it to the 40S ribosomal subunit. Following attachment of the (eIF2.GTP.Met-tRNA) complex to the 40S subunit, the GTP is hydrolysed to GDP. Phosphorylation of eIF2α inhibits the conversion of eIF2-GDP to eIF2-GTP, preventing further cycles of translation initiation and suppressing protein synthesis (Wek *et al.*, 2006).

In budding yeast (*Saccharomyces cerevisiae*), phosphorylation of eIF2α not only causes a general reduction in protein synthesis, but also initiates a change in the expression of a large number of genes, most notably involved in amino acid biosynthesis. Thus, under conditions of amino acid starvation, yeast can switch on amino acid biosynthesis genes, helping the cell to maintain homoeostasis and survive. This 'general amino acid control' is orchestrated by the transcription factor general control nonderepressible 4 (GCN4) (Hinnebusch, 1997, 2005), the name arising from the fact that general amino acid control is in an irreversibly repressed state in *gcn4* and other *gcn* mutants. In budding yeast, GCN4 levels are regulated post-transcriptionally, the synthesis of GCN4 increasing when eIF2α is phosphorylated owing to translation proceeding from an initiation codon that is not used under normal conditions (Hinnebusch, 1992, 1994). GCN4 promotes the expression of genes encoding enzymes in every amino acid biosynthetic pathway except cysteine, as well as many other genes involved in a wide range of cellular processes (Natarajan *et al.*, 2001). In mammals, phosphorylation of eIF2α leads to an increase in the translation of ATF4, the functional orthologue of GCN4. Increased levels of ATF4 lead to the induction of additional bZIP transcription regulators, ATF3 and CHOP/GADD153 (Harding *et al.*, 2000).

The protein kinase that phosphorylates eIF2α was given the name GCN2 (Wek *et al.*, 1989). In yeast, GCN2 is a relatively large protein kinase (1659 amino acid residues; 190 kDa) that senses a reduction in cellular amino acid content through the interaction of its regulatory domain with uncharged tRNA, the

cellular concentration of which increases under conditions of amino acid starvation (Wek *et al.*, 1989, 2003; Zhu *et al.*, 1996). The GCN2 regulatory domain has some amino acid sequence similarity with histidyl-tRNA synthetases and is sometimes called the histidyl-tRNA synthetase-like domain. Activation involves a conformational change in GCN2 and autophosphorylation at two threonine residues in the conserved activation loop of the kinase domain. GCN2 may also be activated and protein synthesis inhibited in response to purine deprivation, exposure to UV-B light, oxidative and osmotic stress or glucose deprivation (Yang *et al.*, 2000; Hinnebusch, 2005; Mascarenhas *et al.*, 2008).

Three other animal protein kinases are known to be able to phosphorylate eIF2α: double-stranded, RNA-dependent protein kinase (PKR), PKR-like endoplasmic reticulum kinase and haem-regulated inhibitor (Chen and London, 1995; Kaufman, 1999; Nanduri *et al.*, 2000). The four eIF2α kinases share a highly conserved protein kinase domain but their regulatory domains differ, enabling each kinase to respond to a different stimulus.

The first plant GCN2 homologue to be identified was AtGCN2 from Arabidopsis (*Arabidopsis thaliana*) (Zhang *et al.*, 2003). It is structurally similar to GCN2 from fungi and animals, with a characteristic eIF2α kinase domain adjacent to a histidyl-tRNA synthetase-like regulatory domain, and it complements the *gcn2* mutation of yeast (Zhang *et al.*, 2003). However, it is smaller than yeast GCN2 (1241 amino acid residues; 140 kDa). Arabidopsis mutants lacking AtGCN2 grow normally in compost but are more sensitive than wild type to herbicides such as glyphosate and chlorsulphuron that interfere with amino acid biosynthesis, an effect that can be reversed by feeding the plants with the appropriate amino acids (Zhang *et al.*, 2008). These herbicides induce the phosphorylation of eIF2α in wild-type Arabidopsis but not in *gcn2* mutants (Zhang *et al.*, 2008). GCN2-like expressed sequence tags (ESTs) and genomic sequences have since been identified in a variety of plant species (Halford, 2006) but have not been characterized in any detail. In all plant species where full genome data are available, GCN2 is encoded by a single gene and is the only eIF2α kinase.

As in fungal systems, AtGCN2 may be activated in response to other stress stimuli, such as purine deprivation, UV light, cold shock and wounding (Lageix *et al.*, 2008). AtGCN2 is also activated in response to treatment with methyl jasmonate or salicylic acid, which are involved in the activation of defence mechanisms in response to insect herbivores, and aminocyclopropane carboxylic acid (ACC), which is involved in ethylene biosynthesis and therefore ripening and senescence (Lageix *et al.*, 2008).

The discovery of a plant GCN2 homologue was evidence that a general amino acid control system, similar to that of fungi and animals, might exist in plants, at least in part. Previous studies had suggested that this might be so. For example, blocking histidine biosynthesis in Arabidopsis with a specific inhibitor, IRL 1803, had been shown to increase the expression of eight genes involved not only in the synthesis of histidine but also in the synthesis of the aromatic amino acids (tyrosine, tryptophan and phenylalanine), lysine and purines (Guyer *et al.*, 1995). Genes encoding tryptophan biosynthesis pathway enzymes had also been shown to be induced by amino acid starvation caused by glyphosate application and other treatments in Arabidopsis (Zhao *et al.*, 1998). In another study, the contents of most minor amino acids had been shown to vary in concert in wheat, barley and potato leaves (Noctor *et al.*, 2000). However, although Zhang *et al.* (2008) showed that the expression of key genes of amino acid biosynthesis was affected by treatment of Arabidopsis with herbicides that affected amino acid metabolism, this response was also seen in mutants lacking AtGCN2 (Zhang *et al.*, 2008). The only exception was a nitrate reductase (NR) gene, *NIA1*, the expression of which was reduced in the mutant plants. Furthermore, no obvious candidate for a GCN4 homologue is identifiable in plants based on amino acid sequence similarity (Halford, 2006).

Wheat GCN2 has not been characterized previously but wheat eIF2α has been reported to contain a conserved GCN2 phosphorylation site, although its full amino acid sequence has not been described previously. Yeast GCN2 has been shown to phosphorylate wheat eIF2α *in vitro* at this site (Chang *et al.*, 1999) and wheat eIF2α complements eIF2α deletion mutants of yeast, restoring a fully functional general amino acid control system (Chang *et al.*, 2000).

Interest in the control of free amino acid accumulation in cereal grain and other important crop products has been stimulated in recent years because free amino acid concentrations have been shown to affect processing properties and product quality. Free amino acids react with reducing sugars in the Maillard reaction, a complex series of nonenzymatic reactions that occurs during frying, baking, roasting and high-temperature processing. The products of the Maillard reaction include melanoidin pigments and complex mixtures of compounds that impart flavour and aroma (Mottram, 2007; Halford *et al.*, 2011). However, the Maillard reaction also produces undesirable compounds, and these include acrylamide, which was discovered in many popular foods in 2002 (Tareke *et al.*, 2002). Acrylamide is formed if the amino acid that participates in the reaction's final stages is asparagine (Mottram *et al.*, 2002; Stadler *et al.*, 2002). Acrylamide is neurotoxic, carcinogenic and genotoxic in rodents and has been classified as a probable human carcinogen by the World Health Organisation (Friedman, 2003). The reduction of free amino acid and specifically free asparagine accumulation in wheat grain is therefore highly desirable. In wheat, sulphur deprivation has a dramatic effect particularly on free asparagine concentration in the grain, causing increases of up to 30-fold (Muttucumaru *et al.*, 2006; Granvogl *et al.*, 2007; Curtis *et al.*, 2009).

In this study, a polymerase chain reaction (PCR) product derived from the transcript of a GCN2-related gene (*TaGCN2*) was amplified from wheat leaf RNA and transgenic wheat plants were produced in which *TaGCN2* was overexpressed. Analysis of these plants showed dramatic effects on free amino acid levels and gene expression and placed TaGCN2 irrefutably in the sulphur signalling pathway. The study showed that manipulation of *TaGCN2* gene expression could be used to reduce free asparagine accumulation in wheat grain and therefore the risk of acrylamide formation in wheat products.

Results

Molecular cloning of a wheat (*Triticum aestivum*) GCN2 homologue, TaGCN2

A GCN2-related polymerase chain reaction (PCR) product was amplified from wheat cv. Cadenza leaf RNA. A product of approximately 3.8 kb containing an open reading frame running from bases 1 to 3741 was cloned. This open reading frame encoded a protein having 52% amino acid sequence identity with Arabidopsis (*Arabidopsis thaliana*) AtGCN2 (Zhang

et al., 2003) and 84% identity with a rice (*Oryza sativa*) GCN2-type protein kinase encoded by mRNA nucleotide sequence XM473001 from GenBank. The protein was given the name TaGCN2. Significantly higher degree of identity with the other cereal GCN2 homologue than with AtGCN2 should be noted.

Additional nucleotide sequence data from the 3′ end of the transcript were obtained by rapid amplification of the cDNA end (3′RACE). This showed the *TaGCN2* transcript to have a 658-nucleotide untranslated region prior to a poly-adenosine tail of 22 nucleotides. The entire sequence of 4439 nucleotides was submitted to the EMBL database and has been assigned the accession number FR839672.

The *TaGCN2* nucleotide sequence was used to mine the recently available wheat genomic sequence (http://www.cerealsdb.uk.net/), and three separate contigs that matched different parts of the *TaGCN2* sequence were identified. The consensus sequence of one of these contigs aligned with the 5′ end of the *TaGCN2* PCR product and extended a further 2 kb 'upstream' of the ATG translation start site. Another contig aligned with the 3′ end of the *TaGCN2* PCR and 3′RACE products, with an intron in the 3′ untranslated region. The entire nucleotide and derived amino acid sequences of the *TaGCN2* PCR product and the wheat genome sequence data that aligned with the 5′ and 3′ ends are given in Data S1.

The encoded protein consists of 1247 amino acid residues and has a molecular weight of 140 kDa. It contains a RING-finger, WD40, DEAD-box helicase domain (RWD-domain) at the N-terminus between residues 28 and 142, an eIF2α kinase domain between residues 422 and 738 and a histidyl-tRNA synthetase-like regulatory domain towards the C-terminal end of the protein between residues 799 and 1128 (Figure 1). An anticodon-binding subdomain of the regulatory domain was found at the extreme C-terminal end of the protein between residues 1129 and 1237, although it is truncated in TaGCN2 compared with yeast GCN2. The presence of these domains in the same protein is a defining characteristic of GCN2-type protein kinases (Wek *et al.*, 1995).

In Arabidopsis, *AtGCN2* has been shown to be expressed in all tissues (Zhang *et al.*, 2003). Expression of *TaGCN2* in flag leaves and grain through the period of grain development was analysed by real-time PCR. Transcripts were detectable in all of the samples, and no significant changes in transcript levels between tissues or at different developmental stages were evident.

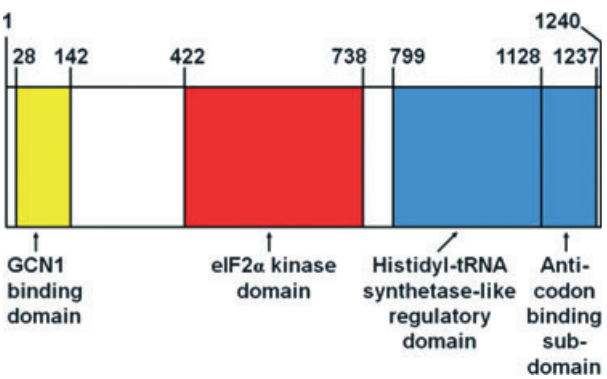

Figure 1 Schematic diagram representing the structure of wheat GCN2-related protein kinase, TaGCN2. The relative positions of the GCN1 binding domain (yellow), eIF2α kinase domain (red) and regulatory domain (including anticodon-binding subdomain) (blue) are shown.

In silico identification of a wheat (*Triticum aestivum*) eIF2α homologue and identification of a putative target site for phosphorylation by TaGCN2

A search of the wheat genome database (http://www.cerealsdb.uk.net/) was performed using a maize eIF2α nucleotide sequence, accession NP-001146159, and overlapping contigs were assembled. The derived amino acid sequence of the encoded protein is shown in Data S1; it comprised 340 amino acids with 95%–97% amino acid sequence identity with maize, sorghum and rice eIF2α proteins, accession numbers ACL53376, EER95021 and ABF95443. The putative target residue for phosphorylation by GCN2-type protein kinases is a serine residue in the N-terminal region, and it was readily identifiable at position 50 of the wheat eIF2α protein. This and the surrounding residues are absolutely conserved in organisms as diverse as yeast, humans and plants: NIEGMILF**S**ELSRRRIRSI (target serine in boldface and underlined).

Production of transgenic wheat plants overexpressing *TaGCN2*

TaGCN2 was overexpressed in transgenic wheat plants under the control of a rice actin gene promoter, which is constitutively active (McElroy *et al.*, 1990). Three independent, homozygous lines, 395, 402 and 426, were produced. These lines came from separate transformation experiments, and *TaGCN2* expression in 395 was measured using cyclophilin as a reference gene, while that in 402 and 426 was measured using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase (SDH) as reference genes. All three showed significantly higher ($P < 0.05$) levels of *TaGCN2* expression than null segregant controls (Figure 2). The transgenic lines showed no visible alteration in phenotype compared with null segregant or wild-type plants.

Free amino acid concentrations in the seeds of homozygous T3 plants were measured by gas chromatography-mass spectrometry (GC-MS), and the results are given in Table 1. Analysis of variance (ANOVA) was applied to the data for each amino acid and the total free amino acids. Following an *F*-test result indicating significant ($P < 0.05$) overall differences between the lines, specific comparisons of transgenic lines with controls were made using the standard error of the difference (SED) (Table 1) in post-ANOVA *t*-tests. This showed total free amino acid and free asparagine concentrations to be significantly reduced ($P < 0.05$) in all three transgenic lines. In line 426, free asparagine concentration was 0.955 mmol/kg, compared with an average of 3.00 mmol/kg in null segregant controls, a reduction of more than two-thirds. The total free amino acid and free asparagine concentrations are shown graphically in Figure 3.

Effects of manipulating *TaGCN2* gene expression on genes of amino acid biosynthesis under adequate nutrient supply and in response to sulphur deprivation

Transgenic wheat lines overexpressing *TaGCN2* were used to investigate the role of *TaGCN2* in regulating expression of key genes in amino acid metabolism under conditions of sulphur sufficiency and deficiency. Sulphur deprivation was used to perturb the system in this experiment because it has been shown to cause a massive increase in free amino acid accumulation in wheat, with free asparagine, which can increase 30-fold in concentration in wheat grain, and free glutamine accounting for

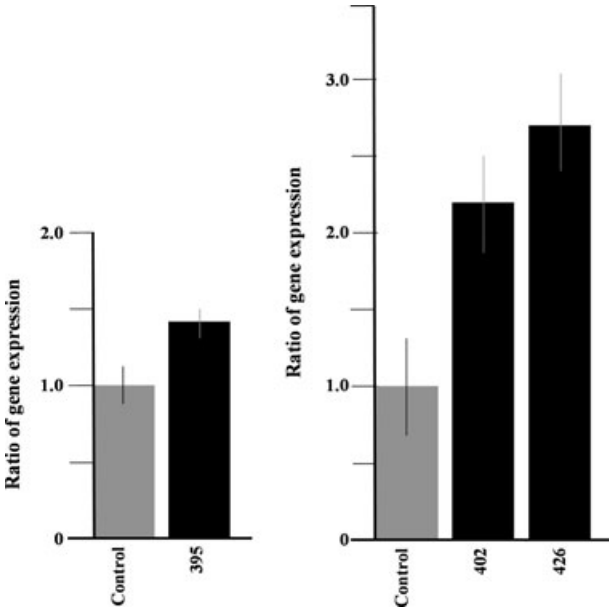

Figure 2 Relative expression of *TaGCN2* in the leaves of transgenic wheat lines in which *TaGCN2* was overexpressed under the control of a rice actin gene promoter, compared with controls. The analysis was carried out in two separate quantitative real-time polymerase chain reaction experiments using different reference genes. In each case, expression in the control lines is represented as 1. Error bars represent standard error of the mean from analyses of two biological replicates. Expression levels were significantly different between control and overexpressing lines ($P < 0.05$ for 395 and $P < 0.01$ for 402 and 426).

most of the increase (Muttucumaru *et al.*, 2006; Granvogl *et al.*, 2007; Curtis *et al.*, 2009). The plants were grown in vermiculite, which does not retain nutrients, and feeding was started 3 weeks after potting. There were two feeding regimes: one set of plants (S+) were watered with 'complete' medium containing 1.1 mM $MgSO_4$ (Muttucumaru *et al.*, 2006; Curtis *et al.*, 2009) and a second set (S–) were watered with the same medium containing one-tenth the concentration of $MgSO_4$.

The expression levels of *TaGCN2* and a suite of other genes (Table 2) in flag leaves of lines 402 and 426 were compared with those in wheat cv. Cadenza controls by real-time, quantitative PCR using genes encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and SDH as reference genes. The genes that were selected for study were those used previously in an analysis of an Arabidopsis mutant lacking GCN2 (Zhang *et al.*, 2008), with the addition of asparagine synthetase (*AS1*) and *sulphur deficiency-induced-1*, and encode enzymes in a range of amino acid biosynthetic pathways (Table 2). The target gene sequences were identified initially through searches of wheat ESTs using annotated Arabidopsis gene sequences and then checked against rice and *Brachypodium* genome data. In some cases, additional searches of the wheat genome database were carried out until the full-length gene sequence was obtained. With the exception of aspartate amino transferase (AAT) x, y and z, primers were designed to amplify a product from all three homeologues. In the case of AAT, primer pairs were designed for each different homeologue, but they were called x, y and z because it was not possible to assign the homeologues with certainty to the A, B and D genomes. Primer sequences are given in Table S1.

Analysis of variance was applied to assess the statistical significance of line and sulphur treatment as main effects and the interaction between these two factors using the *F*-test. The results are shown in Table 3A. Following an *F*-test result indicating significant ($P < 0.05$) or marginal ($0.05 < P < 0.10$) differences (genes shown in boldface in Table 3A), the least significant difference (LSD) at the 5% level of significance was used to separate pairs of means of interest in the appropriate table of means for each gene. The results are given in Table 3B for genes that showed no significant ($P > 0.05$) sulphur response and Table 3C for genes that showed a significant ($P < 0.05$) sulphur response. It should be noted that the ANOVA was applied to the log (to base 2)-transformed inverse of the normalized relative quantity (NRQ) data to ensure the homogeneity of variance across the line by sulphur treatment combinations and effectively to provide values back on the *ct*-scale. Therefore, as for *ct* values, a low $\log_2(1/\text{NRQ})$ in Table 3B,C indicates high gene expression, whereas a high $\log_2(1/\text{NRQ})$ indicates low gene expression. The expression levels of genes that showed significant differences ($P < 0.05$, LSD) between the control plants and both transgenic lines are shown graphically in Figure 4.

TaGCN2 was confirmed to be significantly ($P < 0.01$) overexpressed in both transgenic lines. Expression of translation initiation factor-2α (eIF2α) and protein phosphatase-2A (PP2A) was also significantly increased. eIF2α is the substrate for phosphorylation by GCN2-type protein kinases, while PP2A dephosphorylates eIF2α, thereby opposing the action of GCN2. The increase in the expression of these genes could therefore be interpreted as evidence of the plants compensating for the overexpression of *TaGCN2* by producing more substrate and more of the opposing phosphatase. There was also a significant ($P < 0.05$) increase in the expression of the NR gene. This is consistent with the finding of Zhang *et al.* (2008) that expression of a NR gene was reduced in an Arabidopsis mutant lacking AtGCN2 and is therefore further evidence of a role of GCN2 in regulating nitrogen assimilation in plants.

In the plants that were supplied with sulphur, there were significantly ($P < 0.05$) higher levels of expression of genes encoding phosphoserine phosphatase (PSP) and dihydrodipicolinate synthase (DHDPS) in the transgenic lines compared with controls, while the expression of an asparagine synthetase (*AS1*) gene and genes encoding cystathionine gamma-synthase (CGS) and sulphur-deficiency-induced-1 (SDI1) was significantly ($P < 0.05$) lower. SDI1 is involved in the utilization of stored sulphate pools under S-limiting conditions and is used as a marker for sulphur deficiency (Howarth *et al.*, 2009), while CGS is involved in the synthesis of the sulphur-containing amino acids, cysteine and methionine, as well as other aspects of sulphur metabolism.

This apparent link with sulphur was dramatically confirmed by the analysis of the sulphur-deprived plants. In the control lines, the expression of genes encoding SDI1 and AS1 increased significantly ($P < 0.05$) (Table 3A,C; Figure 4), whereas in the *TaGCN2* overexpressing lines, there was no increase in expression. The expression of two other genes, encoding aspartate kinase/homoserine dehydrogenase (AK/HSDH) and 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DHS) (note the alternative abbreviation of DAHP), was significantly ($P < 0.05$) lower in the transgenic lines than in controls under sulphur deprivation. AK/HSDH is a bifunctional enzyme but the phosphorylation of aspartate by its AK activity is the first step in

Table 1 Free amino acid concentrations (mmol kg^{-1}) in flour produced from grain of transgenic wheat lines in which gene *TaGCN2* was overexpressed, and null segregant controls for line 395 (control A) and for lines 402 and 426 (control B)

	Control A	Control B	Line 395	Line 402	Line 426	SED
Alanine	0.851 (−0.17)	0.682 (−0.38)	**0.488** (**−0.72**)	**0.402** (**−0.91**)	**0.369** (**−1.00**)	0.141* 0.127†
Arginine	ND	ND	ND	ND	ND	–
Asparagine	3.83 (1.34)	2.17 (0.77)	**1.62** (**0.49**)	**0.991** (**−0.01**)	**0.955** (**−0.05**)	0.137 0.122
Aspartic acid	3.07 (1.12)	2.12 (0.74)	**1.90** (**0.64**)	2.03 (0.71)	1.943 (0.57)	0.175 0.157
Cysteine	0.061 (−3.11)	0.051 (−3.04)	0.066 (−2.73)	0.087 (−2.48)	0.077 (−2.57)	0.666 0.596
Glutamic acid	1.64 (0.49)	1.13 (0.11)	**0.855** (**−0.16**)	0.898 (−0.12)	0.829 (−0.19)	0.178 0.159
Glutamine	0.891 (−0.13)	0.331 (−1.18)	**0.113** (**−2.27**)	**0.114** (**−2.21**)	**0.083** (**−2.55**)	0.460 0.412
Glycine	0.281 (−1.28)	0.246 (−1.40)	0.204 (−1.59)	**0.169** (**−1.78**)	**0.172** (**−1.76**)	0.136 0.122
Histidine	0.203 (−1.80)	0.249 (−1.55)	0.134 (−2.09)	0.128 (−2.18)	0.097 (−2.35)	0.651 0.583
Isoleucine	0.121 (−2.12)	0.132 (−2.03)	**0.060** (**−2.82**)	**0.067** (**−2.71**)	**0.063** (**−2.78**)	0.162 0.145
Leucine	0.219 (−1.52)	0.229 (−1.48)	**0.118** (**−2.14**)	**0.093** (**−2.39**)	**0.093** (**−2.38**)	0.146 0.131
Lysine	0.278 (−1.32)	0.300 (−1.29)	0.196 (−1.67)	**0.137** (**−2.05**)	**0.139** (**−1.98**)	0.349 0.312
Methionine	0.020 (−4.00)	0.032 (−3.47)	0.016 (−4.19)	0.021 (−3.86)	0.018 (−4.02)	0.374 0.334
Phenylalanine	0.114 (−2.17)	0.105 (−2.26)	**0.073** (**−2.62**)	0.095 (−2.37)	0.086 (−2.46)	0.165 0.148
Proline	0.385 (−0.958)	0.303 (−1.20)	0.129 (−2.06)	0.259 (−1.35)	**0.121** (**−2.12**)	0.150 0.134
Serine	0.872 (−0.14)	0.999 (−0.08)	0.681 (−0.47)	0.652 (−0.43)	0.479 (−0.78)	0.817 0.578
Threonine	0.875 (−0.44)	0.921 (−0.17)	0.347 (−1.48)	**0.189** (**−1.70**)	0.667 (−1.09)	0.727 0.650
Tryptophan	0.135 (−2.02)	1.34 (0.29)	**0.641** (**−0.45**)	**0.851** (**−0.17**)	1.37 (0.31)	0.171 0.153
Tyrosine	0.125 (−2.10)	0.127 (−2.09)	0.101 (−2.32)	0.108 (−2.27)	0.096 (−2.34)	0.268 0.240
Valine	0.327 (−1.12)	0.313 (−1.17)	**0.154** (**−1.88**)	**0.132** (**−2.03**)	**0.137** (**−1.99**)	0.155 0.139
Total	13.4 (2.60)	10.8 (2.38)	**7.23** (**1.98**)	**6.79** (**1.91**)	**7.32** (**1.99**)	0.169 0.152

Values are means of three replicates except for line 402 (two replicates), with (natural) log-transformed data values in parenthesis, which are used to compare lines using post-ANOVA *t*-tests based on the standard error of the difference (SED) value on 33 degrees of freedom (df). Readings in boldface indicate lines significantly different ($P < 0.05$) from the respective control.

*Comparisons with line 426.

†All other comparisons.

methionine, lysine and threonine synthesis. DHS is involved in the early stages of aromatic amino acid synthesis. Expression of PSP in the control plants increased significantly ($P < 0.05$) in response to sulphur deprivation to the levels seen in the over-expressing plants, which did not change in response to sulphur. In other words, overexpression of *TaGCN2* resulted in the expression of PSP being at the levels seen in sulphur-deprived control plants whether sulphur was supplied or not.

For the genes encoding AATx and y, there was a significant difference ($P < 0.05$) in the expression between the control and line 426, but no significant difference between the control and line 402. There was no significant difference ($P > 0.10$) in expression of AATz, alanine amino transferase (AlaAT), aceto-lactate synthase (ALS), histidinol dehydrogenase (HDH) or phos-phoribosylanthranilate transferase (PAT). The expression of a gene encoding a 14-3-3 protein that interacts with NR showed

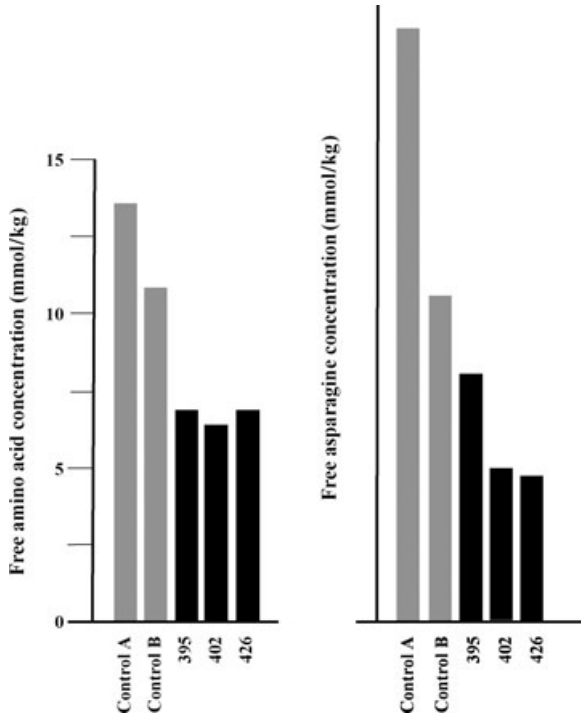

Figure 3 Concentrations (mmol kg^{-1}) of total free amino acids (left) and free asparagine (right) in the grain of transgenic wheat lines in which the expression of *TaGCN2* was increased by constitutive overexpression, compared with null segregant controls: 'Control A' for line 395 and 'Control B' for lines 402 and 426. The differences between the control and overexpressing lines were statistically significant ($P < 0.05$) for both total free amino acids and free asparagine. For statistical analyses, including the standard error of the differences for making comparisons of the lines, refer to Table 1.

a marginally significant ($P < 0.10$) response to sulphur but was not affected by *TaGCN2* overexpression.

Yield

The grain yield and 1000 grain weight of the *TaGCN2* overexpressing lines were measured and compared with wild-type (cv. Cadenza) controls (Table S2). There was a trend for the overexpressing lines to yield more grain weight per plant than the control plants, although 1000 grain weight was similar in control and transgenic lines, indicating that there was no difference in the size of individual grains. The nitrogen content of the grain from the transgenic lines was lower than that of controls (2.2 ± 0.3% dry weight compared with 2.7 ± 0.3% dry weight), while the carbon content was almost unchanged (45.6 ± 0.1% dry weight compared with 45.4 ± 0.1% dry weight), meaning that the transgenic lines had a higher ratio of carbon to nitrogen than the controls.

As genetically modified lines, European law required the plants to be kept in a containment glasshouse, and it was not possible to have sufficient replication of each line to ensure a robust assessment of the differences between them. Furthermore, trends in the yield of wheat under glasshouse conditions often disappear when the experiment is repeated under field conditions. However, it is important to note that there was no evidence of a negative effect of *TaGCN2* overexpression on grain yield.

Discussion

We have shown that overexpression of the wheat *GCN2* homologue, *TaGCN2*, has profound effects on free amino acid concentrations in wheat grain and on the expression of several genes encoding key enzymes in amino acid biosynthesis. Free amino acid concentrations in the grain of the transgenic lines were decreased, mainly as a result of substantial reductions in the concentrations of free asparagine. In one line, free asparagine concentration was reduced by more than two-thirds compared with controls. There was some evidence that *TaGCN2* overexpression could increase grain yield but statistical significance could not be established.

The data clearly showed *TaGCN2* to be involved in the regulation of gene expression in plants that were well nourished and also implicated *TaGCN2* in sulphur signalling. This was demonstrated dramatically in the analysis of asparagine synthetase (*AS1*) gene expression, which rose almost 10-fold in response to sulphur deprivation in wild-type plants but which was almost undetectable, with or without sulphur, in the transgenic lines. *AS1* gene expression has been shown to be induced by salinity and osmotic stress (Wang *et al.*, 2005) but has not previously been reported to increase in response to sulphur deprivation, although the fact that it does is not unexpected given the massive accumulation of asparagine seen in grain from sulphur-deprived wheat (Muttucumaru *et al.*, 2006; Granvogl *et al.*, 2007; Curtis *et al.*, 2009). *TaGCN2* overexpression also had profound effects on the expression of a gene used as a marker for sulphur deficiency, *sulphur deficiency-induced-1* (*SDI1*), and a gene encoding CGS. Expression of these genes was significantly reduced in the transgenic plants compared with controls when the plants were supplied with sulphur and *SDI1*, like *AS1*, also showed no induction in the transgenic lines in response to sulphur deficiency, whereas its expression increased significantly in controls.

The involvement of GCN2 or a related protein kinase in sulphur signalling has not been demonstrated so clearly in any organism before. However, phosphorylation of eIF2α, the substrate for GCN2, has been shown to be higher in liver cells of rats fed a diet deficient in sulphur-containing amino acids than in well-nourished rats (Sikalidis and Stipanuk, 2010). Fascinatingly, that study showed that asparagine synthetase gene expression was also increased.

The discovery of acrylamide in many popular foods (Tareke *et al.*, 2002) has stimulated great interest in the control of free amino acid and particularly free asparagine accumulation in grains, tubers and other crop products. Acrylamide forms as part of the Maillard reaction, a series of nonenzymatic reactions between reducing sugars and amino groups, principally those of amino acids. The Maillard reaction is an important one for the food industry because it produces the melanoidin compounds that give fried, roasted and baked products their colour and a host of volatiles that impart aroma and flavour. It is multistep, with amino groups participating in the first stage and the last, and is not one reaction but many. In the final stages, amino acids are deaminated and decarboxylated to give aldehydes (Strecker degradation) and the major route for acrylamide formation is a Strecker-type reaction involving asparagine (Mottram, 2007; Halford *et al.*, 2011). Asparagine concentration is the limiting factor for acrylamide formation in heated flour from wheat and rye grain (Muttucumaru *et al.*, 2006; Granvogl *et al.*, 2007; Curtis *et al.*, 2009, 2010). Aspara-

Table 2 List of genes that were analysed in transgenic wheat lines overexpressing *TaGCN2*

Abbreviation	Full name	Comment
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Reference gene
SDH	Succinate dehydrogenase	Reference gene
AAT	Aspartate amino transferase	Responsible for the conversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate
AK/HSDH	Aspartate kinase/homoserine dehydrogenase	Bifunctional: AK catalyses the phosphorylation of aspartate, first step in the synthesis of methionine, lysine and threonine. HSDH participates in glycine, serine and threonine metabolism and lysine synthesis
AlaAT	Alanine amino transferase	Responsible for the transfer of an amino group from alanine to α-ketoglutarate to give pyruvate and glutamate
ALS	Acetolactate synthase	Responsible for first step in synthesis of branched chain amino acids: valine, leucine and isoleucine
AS1	Asparagine synthetase	Responsible for the ATP-dependent transfer of amino group of glutamine to aspartate to generate glutamate and asparagine
CGS	Cystathionine gamma-synthase	Involved in methionine, cysteine, selenoamino acid and sulphur metabolism
DHDPS	Dihydrodipicolinate synthase	Responsible for first unique reaction of lysine synthesis
DHS2	3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase	Involved in early stages of aromatic amino acid biosynthesis
eIF2α	Translation initiation factor 2α	Substrate of GCN2
GCN2	General control nonderepressible	Overexpressed in transgenic lines
HDH	Histidinol dehydrogenase	Involved in histidine metabolism
NR	Nitrate reductase	Responsible for key step in incorporation of inorganic nitrogen into amino acids
PAT1	Phosphoribosylanthranilate transferase	Involved in tryptophan biosynthesis
PP2A	Protein phosphatase 2A	Dephosphorylates eIF2α
PSP	Phosphoserine phosphatase	Involved in glycine, serine and threonine metabolism
SDI1	Sulphur-deficiency-induced-1	Marker for sulphur deprivation; unknown function
14-3-3NR	14-3-3 protein	Involved in interaction with NR, regulating activity

gine accumulates to high concentrations in plants in response to a variety of environmental and biotic stimuli (Lea *et al.*, 2007; Curtis *et al.*, 2009, 2010); in wheat, sulphur deprivation has a particularly dramatic effect, causing increases of up to 30-fold in free asparagine concentration in the grain (Muttucumaru *et al.*, 2006; Granvogl *et al.*, 2007; Curtis *et al.*, 2009). A two-thirds reduction in free asparagine concentration in wheat grain and a mitigation of the effects of sulphur deficiency would be of great benefit to the food industry.

The expression of a similar suite of genes in a *gcn2* mutant of Arabidopsis showed little change with wild type (Zhang *et al.*, 2008). However, the Arabidopsis study did not include an overexpression experiment or use sulphur deprivation to perturb the system. Nor did it include an analysis of *AS1* or *SDI1* genes and it was these genes that differed most between the *TaGCN2* overexpressing lines and the controls. Wheat appears to be extremely sensitive to sulphur deprivation and to respond with dramatic changes in free amino acid accumulation. The wheat system may therefore simply be a better one for demonstrating the role of *TaGCN2* in regulating gene expression.

The transgenic plants may have been compensating for *TaGCN2* overexpression by increasing expression of eIF2α, the substrate for GCN2-type protein kinases, and of a protein phosphatase 2A, which reverses the action of GCN2. This may explain why there was no evidence of a negative effect on yield in the overexpressing lines. The fact that there were such profound effects on the expression of other genes despite this leads us to speculate that GCN2 regulates gene expression in plants through a different mechanism from that described in budding yeast. In that organism, eIF2α phosphorylation by GCN2 controls the translation of transcription factor, GCN4. However, no GCN4 homologue has been identified in plants, despite the extensive genome data that are now available (Halford, 2006). Animals, on the other hand, do have a GCN4 homologue, ATF4, but lack the ability to make many amino acids.

There are other differences between the regulatory system in yeast and the one that is being elucidated in plants. For example, overexpression of *TaGCN2* repressed the expression of genes encoding AS1, DHS and CGS, increased that of genes encoding AK/HSDH, PSP and DHDPS and had no consistent significant effect on genes encoding AAT, AlaAT, ALS, HDH and PAT. Obviously, this is not the same as the general amino acid control system of yeast, in which activation of GCN2 results in the translation of GCN4 and the promotion of expression of genes encoding enzymes in every amino acid biosynthetic pathway except cysteine (Natarajan *et al.*, 2001). The involvement of GCN2 in regulating genes in response to sulphur availability has also never been demonstrated in fungi. Clearly, while some of the components and mechanisms of the regulatory systems controlling protein synthesis and the expression of genes encoding enzymes of amino acid biosynthesis have been conserved as fungi and plants have diverged, others have changed substantially.

Experimental procedures

Isolation of wheat leaf RNA

Six-week-old wheat (*Triticum aestivum* cv. Cadenza) leaf material was snap-frozen in liquid nitrogen before being crushed to a fine powder using a chilled pestle and mortar. Total RNA was purified using the RNeasy Mini Kit (Qiagen Ltd, Crawley, UK) following the manufacturer's instructions. Alternatively, RNA

Table 3 Analysis of gene expression in *TaGCN2*-overexpressing lines 402 and 426 compared with wild-type wheat cv. Cadenza grown with sulphur supplied (S+) or withheld (S–). There were three biological replicates for each line by sulphur treatment combination. Genes encoding GAPDH and SDH were used as reference genes for all other genes. (A) *P*-values from the ANOVA for the result of the *F*-test on main effects and interactions between the line and sulphur treatment. The genes and *P*-values in boldface indicate that significant ($P < 0.05$) or marginal ($0.05 < P < 0.1$) differences were identified, warranting further investigation. Also shown are the value of residual variance (s^2) and the degrees of freedom (df). (B) Relevant means tables, on the $\log_2$[1/normalized relative quantity (NRQ)] scale, for comparison of overall line effects for genes showing no significant ($P > 0.05$) response to sulphur, using the least significant difference (LSD) (5%) values. Genes given in boldface show significant differences ($P < 0.05$) in expression between the control and both transgenic lines. Note that a low $\log_2$(1/NRQ) indicates high gene expression, whereas a high $\log_2$(1/NRQ) indicates low gene expression. (C) Relevant means tables, on the $\log_2$(1/NRQ) scale, for comparison of effect of line, sulphur, or line by sulphur interaction for genes showing a significant ($S < 0.05$) sulphur response, using the LSD (5%) values

Genes	Line	Sulphur	Line.Sulphur	s^2, residual df
A				
GAPDH	0.166	0.960	0.727	0.1261, 12
GAPDH2	0.131	0.772	0.952	0.1887, 12
SDH	0.166	0.960	0.727	0.1261, 12
AATx	0.006	0.926	0.948	0.1294, 12
AATy	0.080	0.862	0.489	0.2198, 12
AATz	0.237	0.967	0.518	0.08958, 12
AK/HSDH	0.131	0.292	0.084	0.1932, 12
AlaAT	0.397	0.167	0.215	0.2848, 12
ALS	0.030	0.381	0.790	0.7779, 12
AS1	<0.001	0.464	0.071	6.131, 12
CGS	0.026	0.645	0.207	0.1838, 12
DHDPS	0.012	0.607	0.837	0.3534, 12
DHS2	0.006	0.566	0.040	0.2601, 12
eIF2α	<0.001	0.503	0.640	0.09902, 12
GCN2	0.001	0.296	0.583	0.3432, 12
HDH	0.278	0.344	0.601	2.400, 12
NR	<0.001	0.517	0.155	0.3102, 12
PAT1	0.616	0.726	0.951	0.1285, 12
PP2A	0.001	0.532	0.502	0.1349, 12
PSP	0.076	0.138	0.071	0.1845, 12
SDI1	<0.001	0.434	0.055	2.256, 12
14-3-3	0.999	0.062	0.914	0.9885, 11

	Line					
Genes	Control	402	426	SED	df	LSD (5%)	Comment
B							
GCN2	5.64	4.23	4.20	0.338	12	0.737	Control significantly different ($P < 0.05$) from 402 and 426
AATx	1.27	0.888	0.441	0.208	12	0.453	Control significantly different ($P < 0.05$) from 426 but not 402
AATy	1.54	1.18	0.86	0.271	12	0.590	Control significantly different ($P < 0.05$) from 426 but not 402
ALS	−0.02	−0.94	−1.58	0.509	12	1.11	Control significantly different ($P < 0.05$) from 426 but not 402
CGS	−1.41	−0.62	−1.04	0.248	12	0.539	Control significantly different ($P < 0.05$) from 402 and 426
DHDPS	1.43	0.25	0.12	0.534	12	1.16	Control significantly different ($P < 0.05$) from 402 and 426
eIF2α	5.19	3.96	3.52	0.182	12	0.396	Control significantly different ($P < 0.05$) from 402 and 426
NR	7.21	5.60	5.97	0.322	12	0.701	Control significantly different ($P < 0.05$) from 402 and 426
PP2A	0.557	−0.103	−0.475	0.212	12	0.462	Control significantly different ($P < 0.05$) from 402 and 426

	Line						
Genes and S regime	Control	402	426	SED	df	LSD (5%)	Comment
C							
AK/HSDH							
S+	2.35	2.23	1.96	0.359	12	0.782	Control S+ significantly different ($P < 0.05$) from control S–. No S effect in 402 or 426

Table 3 Continued

Genes and S regime	Line			SED	df	LSD (5%)	Comment
	Control	402	426				
S–	1.44	2.55	1.88				
AS1							
S+	2.49	9.53	7.97	2.02	12	4.41	Control significantly different ($P < 0.05$) from 402 and 406 in S+ and S–.
S–	–2.55	9.89	10.0				Significant increase ($P < 0.05$) in expression in S– compared with S+ in control but not in 402 or 426
DHS2							
S+	0.08	–0.17	0.52	0.416	12	0.907	Control S+ significantly different ($P < 0.05$) from control S–. Control
S–	–0.77	0.51	1.13				significantly different ($P < 0.05$) from 402 and 426 in S–
PSP							
S+	2.93	2.04	1.75	0.351	12	0.764	Expression in control significantly higher ($P < 0.05$) in S– than in S+.
S–	1.96	1.74	2.06				Expression in control significantly different ($P < 0.05$) from 402 and 426 in S+ but not S–
SDI1							
S+	9.71	12.5	12.9	1.23	12	2.67	Control significantly different ($P < 0.05$) from 402 and 406 in S+ and
S–	6.41	13.5	13.5				S–. Significant ($P < 0.05$) increase in expression in control in S– compared with S+ but not in 402 or 426

SED, standard error of the difference.

was extracted from leaf material with Trizol reagent (Invitrogen Ltd, Paisley, UK). RNA was treated with DNase (Promega, Southampton, UK) to prevent DNA contamination. RNA quality was checked using a spectrophotometer and in some cases by agarose gel electrophoresis.

Isolation of RNA from grain

Up to 250 mg of frozen grain material was allowed to thaw momentarily, squashed to rupture the structure, then re-frozen in liquid nitrogen and ground to a fine powder. RNA was extracted from powdered grain tissue using the CTAB method (Chang *et al.*, 1993). RNA was further purified using the RNeasy MinElute clean-up column that included an on-column DNase treatment (Qiagen).

Molecular cloning of *TaGCN2*

The design of the antisense primer used to amplify a product from *TaGCN2* mRNA, GCCAATCAGCTCCAGATTGTAGGA, was based on a wheat EST matching the 3′ end of the Arabidopsis *AtGCN2* nucleotide sequence (Zhang *et al.*, 2003), which was identified using an *in silico* search of the WhETS database (Mitchell *et al.*, 2007). A similar search revealed a previously uncharacterized rice (*Oryza sativa*) *GCN2*-like nucleotide sequence (GenBank: XM473001) that was used to design a sense primer: ATGGGGCACAGCGCGAGGAAGAAGAA.

Complementary DNA was generated from the wheat RNA by reverse transcription using SuperScript III (Invitrogen). Amplification by PCR used Phusion High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland). Cycling conditions were as follows: 98 °C for 30 s; 40 cycles of 98 °C for 10 s, 50 °C to 70 °C gradient for 20 s and 72 °C for 3 min.; final hold at 72 °C for 10 min.

Polymerase chain reaction products were cloned and nucleotide sequences were determined using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Life Technologies, Carlsbad, CA). The reaction conditions were as follows: 96 °C for 1 min., followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Nucleotide sequence analysis was performed by Geneservice, Source Bioscience (Nottingham, UK) or MWG Biotech (Wolverhampton, UK).

Amplification of 3′ cDNA ends

The nucleotide sequence of the 3′ end of the *TaGCN2* transcript was determined by rapid amplification of the cDNA end (RACE) using a GeneRacer kit (Invitrogen), which incorporates Phusion High-fidelity DNA polymerase. Three primers were used: AGTCTGTTCAAAGGGTGGCGGTGG, GGTGGACTCTTAAACGAGC GCATGGA and ACCAATAACACAGGCCGAAG. The PCR conditions were as follows: 98 °C for 30 s; 40 cycles of 98 °C for 10 s, 65 °C for 15 s and 72 °C for 20 s; final extension at 72 °C for 10 min. An aliquot of the reaction product was analysed by agarose gel electrophoresis; another aliquot was then used as the template for nested PCR.

Production of transgenic wheat plants

The full-length *TaGCN2* open reading frame was spliced into a plasmid downstream of a rice actin gene promoter, which is constitutively active (McElroy *et al.*, 1990). The termination signal was from the *Agrobacterium tumefaciens* nopaline synthase gene (*nos*) (Jefferson, 1987). The plasmid was introduced into wheat by particle bombardment of scutella tissue. Plasmid pAHC20 (Christensen and Quail, 1996), which conveys resistance to the herbicide phosphinothricin (PPT), was used for cotransformation. Following selection, PCR was used to establish the presence of the transgene. Transgenic plants were self-fertilized, and PPT-resistant progeny that tested positive for the presence of the construct were selected. This was repeated to the T3 generation, and three independent, homozygous lines, 395, 402 and 426, were produced. Embryo isolation and bombardment and plant regeneration and selection were performed

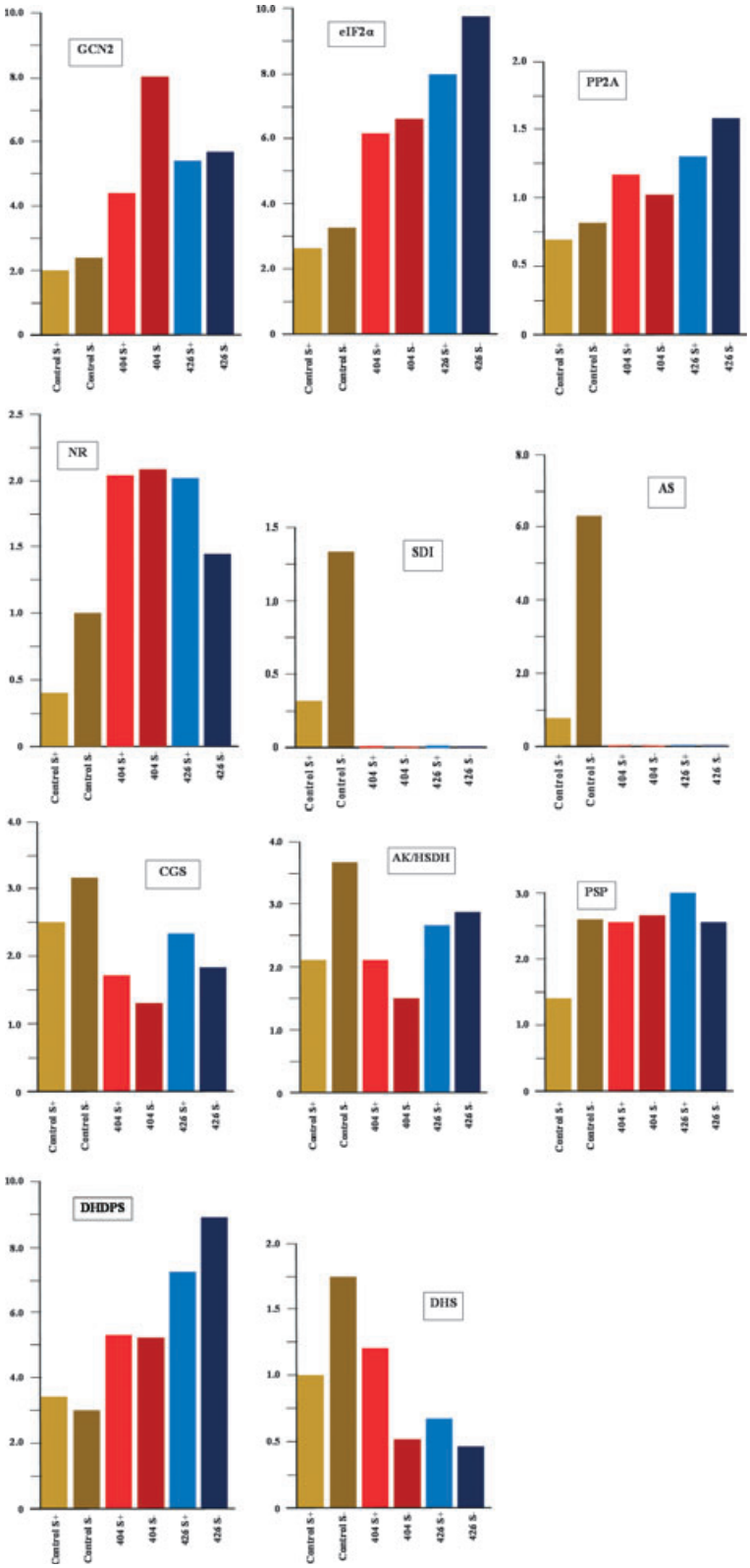

Figure 4 Expression [normalized relative quantities (NRQ)] values of a suite of genes (Table 3) in the leaves of transgenic wheat lines in which *TaGCN2* was overexpressed under the control of an actin gene promoter and in wild-type (cv. Cadenza) controls. The plants were grown with sulphur either supplied (S+) or withheld (S–). The analysis was carried out by quantitative real-time polymerase chain reaction using 3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase (SDH) as reference genes. Note that the graphs have different scales on the *y*-axis. In all cases shown, the levels of expression of the gene differed significantly ($P < 0.05$) between the transgenic and control lines in either S+ or S– conditions, or in both (Table 3).

within the Rothamsted Cereal Transformation Laboratory using the methods described by Sparks and Jones (2009). The presence of the transgene in individual plants was checked by PCR using genomic DNA as the template. The primers used were 5′-CAAGGACCACGCCGCGCAG, which anneals in exon 1, and 5′-GCTAAATCGGGTGTGAGGTGATTGTG, which anneals in exon 2. The product amplified from the endogenous gene therefore contained an approximately 0.8-kb intron that was not present in the transgene. Successive self-fertilization to the T_3 generation was carried out to achieve homozygosity.

Sulphur feeding

Transgenic and wild-type (cv Cadenza) wheat plants were grown in vermiculite in a glasshouse with a 16-h daylength (supplemental lighting was used as necessary) and a minimum temperature of 16 °C. Vermiculite does not retain nutrients, so once seed reserves were exhausted, the only nutrition available to the developing seedlings came from externally applied liquid feed solution. Feeding was started 3 weeks after potting and continued every 2 days until harvest. Distilled water was also supplied as required to prevent water stress. A completely randomized design was used for the pots in the glasshouse. Plants were supplied with either a medium containing a full nutrient complement of potassium, phosphate, calcium, magnesium, sodium, iron, nitrate (2 mM $Ca(NO_3)_2$ and 1.6 mM $Mg(NO_3)_2$) and sulphate ions (1.1 mM $MgSO_4$) (Muttucumaru *et al.*, 2006; Curtis *et al.*, 2009) or the same medium containing one-tenth the concentration of $MgSO_4$. RNA was prepared from flag leaves as described earlier.

Expression analyses by real-time quantitative PCR

First-strand cDNA synthesis was performed using SuperScript III (Invitrogen) to reverse-transcribe 1–2 μg DNase-treated RNA and was primed with an anchored dT_{20} primer in a final volume of 20 μL. The qPCR reaction mixture consisted of 10 μL SYBR Green JumpStart Taq ReadyMix (Sigma, Poole, UK), 5 μL diluted cDNA and 5 μL primers (125 nM final concentration). Samples were run in an ABI7500 real-time PCR system (Applied Biosystems), and the amplification conditions were 95 °C for 2 min, then 45 cycles of 95 °C for 15 s followed by 67 °C for 45 s. Primer nucleotide sequences are given in Table S1.

The efficiencies of the reactions were estimated using the LinReg PCR program (Ramakers *et al.*, 2003), and the *ct* (at threshold fluorescence) and efficiency values were then used to calculate the NRQ with respect to the reference genes, cyclophilin, SDH and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), for each sample/target gene combination.

$$NRQ = \frac{E_{target}^{-ct,target}}{\sqrt{E_{SDH}^{-ct,SDH} * E_{GAPDH}^{-ct,GAPDH}}}$$

where E_{target}, E_{SDH} and E_{GAPDH} are the estimated reaction efficiencies for a particular target gene and the two reference genes and where *ct,target*, *ct,SDH* and *ct,GAPDH* are the corresponding *ct* values.

Statistical analysis of the gene expression data from the sulphur feeding experiment data was performed using the GenStat® (2010, Thirteenth Edition; VSN International Ltd, Hemel Hempstead, UK) statistical system. There were three biological replicates (leaf tissue samples) for each line by sulphur treatment combination. Two genes, encoding GAPDH and SDH, were used as reference genes for all other genes. The stability of these genes across the line by sulphur treatment combinations was checked to confirm that they were suitable for this role. The NRQ for all genes were calculated. ANOVA was applied to the log (to base 2)-transformed inverse of the NRQ data. This transformation ensured homogeneity of variance across the line by sulphur treatment combinations and effectively provided values back on the *ct*-scale. Therefore, as for *ct* values, a low $\log_2(1/NRQ)$ indicated a high gene expression, whereas a high $\log_2(1/NRQ)$ indicated low gene expression. The analysis assessed the statistical significance of line and sulphur treatment main effects and the interaction between these two factors using the *F*-test. Following an *F*-test result indicating significant ($P < 0.05$) or marginal ($0.05 < P < 0.10$) differences, the least significant difference (LSD) at the 5% level of significance was used to separate pairs of means of interest in the appropriate table of means for each gene. Details of the genes analysed are given in Table 2. Primer sequences are given in Table S1.

Amino acid analyses

Free amino acid concentrations in mature grain of compost-grown plants were determined by gas chromatography-mass spectrometry (GC-MS) using methods described previously (Muttucumaru *et al.*, 2006; Curtis *et al.*, 2009). For each amino acid and the total, wheat lines were compared using ANOVA. Following an *F*-test result indicating significant ($P < 0.05$) overall differences between lines, specific comparisons of transgenic lines to controls were made using the SED in post-ANOVA *t*-tests based on the residual degrees of freedom (df). Analysis was performed using GenStat®.

Total nitrogen and carbon analysis

Measurements of total grain nitrogen and carbon were taken by the Analytical Unit of the Soil Science Department, Rothamsted Research, using the 'Dumas' digestion method and a LECO CNS 2000 Combustion Analyser (LECO Corporation, Saint Joseph, MI).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Table S1 Primers used for expression analyses. Gene details are given in Table 2. F and R refer to 'forward' and 'reverse' primers.

Table S2 Yield, 1000 grain weight, carbon and nitrogen content of transgenic wheat lines over-expressing *TaGCN2* compared with wild-type (cv. Cadenza) controls. The means over the control and transgenic lines are given in bold with SEs.

Data S1 *TaGCN2* nucleotide sequence data comprising the nucleotide sequence of the *TaGCN2* PCR product and wheat genome data (http://www.cerealsdb.uk.net/) contig 1723930 (together making up EMBL accession number FR839672), and additional wheat genome data overlapping with the 5 end of FR839672; derived amino acid sequence for *TaGCN2*; wheat eIF2α nucleotide and derived amino acid sequence.

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