RESEARCH PAPER

GCN2-dependent phosphorylation of eukaryotic translation initiation factor-2α in Arabidopsis

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Abstract

The yeast regulatory protein kinase, general control non-derepressible-2 (GCN2) plays a key role in general amino acid control. GCN2 phosphorylates the α subunit of the trimeric eukaryotic translation initiation factor-2 (eIF2), bringing about a decrease in the general rate of protein synthesis but an increase in the synthesis of GCN4, a transcription factor that promotes the expression of genes encoding enzymes for amino acid biosynthesis. The present study concerned the phosphorylation of Arabidopsis eIF2α (AteIF2α) by the Arabidopsis homologue of GCN2, AtGCN2, and the role of AtGCN2 in regulating genes encoding enzymes of amino acid biosynthesis and responding to virus infection. A null mutant for AtGCN2 called GT8359 was obtained and western analysis confirmed that it lacked AtGCN2 protein. GT8359 was more sensitive than wild-type Arabidopsis to herbicides that affect amino acid biosynthesis. Phosphorylation of AteIF2α occurred in response to herbicide treatment but only in wild-type Arabidopsis, not GT8359, showing it to be AtGCN2-dependent. Expression analysis of genes encoding key enzymes for amino acid biosynthesis and nitrate assimilation revealed little effect of loss of AtGCN2 function in GT8359 except that expression of a nitrate reductase gene, *NIA1*, was decreased. Analysis of wild-type and GT8359 plants infected with *Turnip yellow mosaic virus* or *Turnip crinkle virus* showed that AteIF2α was not phosphorylated.

Key words: Amino acid signalling, general control non-derepressible, metabolic regulation, phosphorylation, protein kinase, virus infection.

Introduction

The molecular cloning of an Arabidopsis (*Arabidopsis thaliana*) homologue of the key yeast (*Saccharomyces cerevisiae*) regulatory protein kinase, general control non-derepressible-2 (GCN2), was reported in 2003 (Zhang *et al.*, 2003). This supported the hypothesis that general amino acid control (Hinnebusch, 1992), the yeast signalling and response system through which the rate of protein and amino acid synthesis responds to amino acid levels, is conserved, at least in part, in plants (reviewed by Halford, 2006). In order to ensure that the distinction between the Arabidopsis and yeast homologues is clear, henceforth they will be referred to, respectively, as AtGCN2 and ScGCN2. Briefly, in yeast, amino acid starvation causes a build-up of uncharged tRNA (Wek *et al.*, 1989). This activates ScGCN2 by interacting directly with a regulatory domain in the ScGCN2 C-terminal region. The substrate for ScGCN2 is the α subunit of the trimeric eukaryotic translation initiation factor-2 (eIF2), which is phosphorylated by ScGCN2 at serine-52. eIF2 can bind either

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guanosine diphosphate (GDP) or guanosine triphosphate (GTP), but is able to bind Met-tRNA to the ribosome and transfer it to the 40S ribosomal subunit to initiate the synthesis of a protein only when bound to GTP. Following attachment of the [eIF2.GTP.Met-tRNA] complex to the 40S subunit, the GTP is hydrolysed to GDP and P_i and eIF2 is released as an inactive [eIF2.GDP] complex. Phosphorylation of eIF2α inhibits the recycling of bound GDP to GTP, thereby decreasing the rate of protein synthesis. Although there are some differences between the plant and fungal translation initiation complexes the three subunits that make up eIF2 are conserved (Browning, 1996, 2004).

As well as the global reduction in protein synthesis that results from eIF2α phosphorylation, there is a change in the expression of a large number of genes. This comes about through the action of a transcriptional activator, GCN4 (Hinnebusch, 1997), levels of which are controlled translationally. So, while there is a general decrease in protein synthesis, the synthesis of GCN4 actually increases, with translation initiating 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 others involved in a wide range of cellular processes (Natarajan, 2001). ScGCN2-dependent phosphorylation of eIF2α therefore controls amino acid biosynthesis gene expression at the level of transcription.

Screening of expressed sequence tag (EST) and genomic databases with the AtGCN2 sequence reveals the presence of similar genes and transcripts in rice, wheat, barley, potato, soybean, sugar beet, sugarcane, *Medicago*, cotton, poplar, onion, lotus, and *Zinnia* (Halford, 2006). Homologues of ScGCN2 have also been identified in *Drosophila melanogaster* (Santoyo *et al.*, 1997) and *Neurospora crassa* (Sattlegger *et al.*, 1998). There are two other eIF2α kinases that have similar catalytic domains to ScGCN2 but do not contain a GCN2-type regulatory domain and respond to different stimuli. These are the mammalian haem-regulated inhibitor (HRI) (Chen *et al.*, 1991; Mellor *et al.*, 1994*a*) and double-stranded RNA-dependent kinase (PKR) (Meurs *et al.*, 1990). HRI is activated in response to haem deficiency (Chen *et al.*, 1991; Mellor *et al.*, 1994*a*) and PKR to the presence of double-stranded RNAs after virus infection (Icely *et al.*, 1991; Meurs *et al.*, 1990; Mellor *et al.*, 1994*b*).

Previous studies had suggested that at least some elements of general amino acid control are present in plants. For example, blocking histidine biosynthesis in Arabidopsis with a specific inhibitor, IRL 1803, was shown to increase expression of eight genes involved not only in the synthesis of histidine but also the aromatic amino acids (tyrosine, tryptophan, and phenylalanine), lysine, and purines (Guyer *et al.*, 1995). Genes encoding

tryptophan biosynthesis pathway enzymes have also been shown to be induced by amino acid starvation caused by Glyphosate application and other treatments in Arabidopsis (Zhao *et al.*, 1998). However, the substrate for AtGCN2 remained to be confirmed as eIF2α and, perhaps surprisingly, no obvious candidate for a GCN4 homologue in Arabidopsis was identifiable (Halford *et al.*, 2004; Halford, 2006).

Another mystery that has remained unsolved for several years is the apparent plant PKR-like activity described in several studies conducted in the 1980s and 1990s (Hiddinga *et al.*, 1988; Chang *et al.*, 1999, 2000; Langland *et al.*, 1995, 1996). These studies described a protein kinase activity in wheat and tobacco that phosphorylated eIF2α and, like PKR in humans, was induced during virus infection. However, completion of the Arabidopsis genome nucleotide sequencing projects revealed that a single gene encoding AtGCN2 was the only apparent eIF2α kinase-encoding gene. Similarly, the rice genome was subsequently found to contain a single identifiable GCN2-type gene and, apparently, no other encoding an eIF2α kinase (Halford, 2006).

Here, experiments conducted to demonstrate the phosphorylation of eIF2α by AtGCN2 and to investigate the role of AtGCN2 in regulating genes encoding enzymes of amino acid biosynthesis and responding to virus infection are described.

Materials and methods

Plant growth and treatments

Seeds of Arabidopsis (*Arabidopsis thaliana*) accession Landsberg *erecta* (L*er*) and Genetrap line GT8359 were obtained from Cold Spring Harbour Laboratory, New York (http://genetrap.cshl.org). The Genetrap lines carry a transposable element insertion (*Ds*) in a L*er* background. Arabidopsis plants were either grown in sterilized nutrient medium plates for herbicide treatments or in non-sterilized soil for virus infection.

For herbicide treatment experiments, Arabidopsis seeds were surface-sterilized and sown on a piece of sterile filter paper (3MM) laid on the surface of B5 medium (Sigma G5893; Gamborg *et al.*, 1968) with 15 g l^{-1} sucrose (B5/S15) and solidified with 4 g l^{-1} Gelrite (Kelco Div., Merck & Co., Inc., San Diego, Calif.). In order to make 'side-by-side' comparisons, seeds of the mutant and wild type were sown on the same plates each on one half of the filter paper. After stratification for 3−5 d at 3−4 °C, plates were moved into a dedicated plant culture room to grow with a photoperiod of 16 h, a light intensity of approximately 120 μmol photons m^{-2} s^{-1}, and a temperature of 23 °C.

After growth for 10−14 d, seedlings were submerged in 100 ml of herbicide solution for 1 min. The herbicides solutions were 0.5 μM Chlorsulfuron (Sigma), 350 μM IRL 1803 (a gift from Dr Tim Hawkes), 150 μM Glyphosate (Sigma), 5 μM Acifluorfen (Sigma) or 40 μM Diuron (Sigma), in 0.01% (v/v) Silwet L-77 (Lehle Seeds). If amino acids were used to mitigate the effects of the herbicide they were added at a concentration of 0.5 mM each (leucine, isoleucine and valine for Chlorsulfuron, histidine for IRL 1803, and phenylalanine, tyrosine and tryptophan for Glyphosate).

After treatment, the seedlings were transferred onto fresh B5/S15 plates and incubated in the plant culture room for further growth.

For virus infection experiments, Arabidopsis seeds were sown in soil and seedlings were allowed to grow under a day/night regime of 10/14 h light/dark, 23/20 °C, in a controlled environment cabinet. After growth for 20 d, the plants were inoculated either with *Turnip yellow mosaic virus* (TYMV) or *Turnip crinkle virus (*TCV). Immediately after inoculation the plants were covered with a plastic lid and kept under the same conditions as before.

Antisera

Polyclonal antibodies for the detection of either AtGCN2 or AteIF2α were raised in rabbits using peptides as antigens. Antiserum against peptide KLRPYSKDMGYEDTD, a peptide present in the N-terminal region (from amino acid residue 65−79) of AtGCN2, was used to detect AtGCN2 protein, while antiserum against peptide IRRRMTPQPMKIRAD was used to detect AteIF2α. Peptide IRRRMTPQPMKIRAD is present in both isoforms of AteIF2α (amino acid residues 196−210). The two antisera were produced and affinity-purified by Eurogentec (Belgium). A commercially-available polyclonal antibody, phospho-eIF2α (S[51]) (Catalogue no. 9721 from Cell Signalling), which was raised in a rabbit against human phosphorylated eIF2α, was used for the detection of phosphorylated AteIF2α.

Plant protein extraction, SDS-PAGE, and western blotting

Total plant soluble proteins from *in vitro*-grown Arabidopsis seedlings or soil-grown Arabidopsis leaves were extracted in ice-cold extraction buffer containing 25 mM TRIS−HCl (pH 7.5), 75 mM NaCl, 5% (v/v) glycerol, 0.05% (v/v) Nonidet P-40, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT, 2% (w/v) insoluble PVP (Sigma P-6755), supplemented with 1× protease inhibitor cocktail VII (Catalogue no. 539138, CalBiochem) and protein phosphatase inhibitor mix of 50 mM sodium fluoride, 25 mM β-glycerophosphate, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate (Na_3VO_4). Insoluble cell debris was removed from the crude extracts by centrifugation for 15 min at 15 000 rpm and 4 °C. The cleared protein extracts were quantified by Bradford assay (Bradford, 1976), snap-frozen in liquid nitrogen and stored at −80 °C.

Equal amounts of proteins (10 μg) were size-separated on Invitrogen's NuPAGE Novex BIS-TRIS pre-cast gels (4−10%) in 1× NuPAGE MOPS SDS running buffer in a XCell SureLock Mini-Cell and the proteins were then transferred from the gel to a PVDF membrane using Invitrogen's XCell II blot module. The quality of protein separation for each sample was visually checked using 0.1% Coomassie Brilliant Blue staining and protein transfer onto the membrane was monitored using prestained protein markers (SeeBlue plus, Invitrogen).

For immunodetection of proteins, the blots were first 'blocked' by submerging them in blocking solution containing 2% Amersham's ECL advance blocking reagent in TBST buffer (20 mM TRIS−HCl, 137 mM NaCl, pH 7.6, 0.1% (v/v) Tween 20) for 1 h at room temperature with gentle shaking. The blots were then incubated with the first antibodies diluted in the blocking solution (1:5000) overnight at 4 °C, thoroughly washed with TBST buffer, and incubated for 1 h at room temperature with horseradish peroxidase-labelled secondary antibody (Amersham NA934) diluted in blocking solution (1:20 000). After plenty of washes, the blots were treated with Amersham's ECL advance solutions for chemiluminescence development. An image of the membrane was then produced on X-ray film.

RNA isolation, cDNA synthesis and real-time quantitative PCR (RT-QPCR)

Total RNA was isolated from frozen seedlings using Ambion's RNAqueous small-scale RNA isolation kit. RNA samples were checked by running on formaldehyde-containing denaturing agarose gels (1.2%) and quantified on a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies). In order to remove genomic DNA contamination, RNA samples were treated with DNase I using a Turbo DNA-free™ kit (Ambion). The first-strand cDNA was reverse-transcribed from 2.5 μg of DNase-treated RNA using random primers (Promega) and Superscript III reverse transcriptase (Invitrogen). Control reactions in which the reverse transcriptase step was omitted were included in all cDNA synthesis experiments to confirm the lack of genomic DNA contamination.

For gene expression analysis, real-time quantitative PCR was performed in 96-well micro-plates (Abgene) using an Applied Biosystems 7500 Real Time PCR system. Each reaction had a total volume of 10 μl, containing 5 μl Power SYBR® Green PCR master mix (Applied Biosystems), 1 μl 5-fold diluted cDNA, and 0.25 μM of each primer. The PCR reaction was carried out using the same parameters for all gene expression analyses. These were 95 °C for 5 min for initial denaturing followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR primers were designed using Applied Biosystems' Primer Express software v2.0 and are listed in Table 1. Seven reference gene candidates from a list proposed by Czechowski *et al.* (2005) were examined for their expression stability and gene At1G13320 encoding a protein phosphatase 2A subunit (PP2A) was chosen for use in all subsequent experiments (data not shown).

Two biological replicates were used for each of the 18 treatment combinations: two lines, wild-type and GT8359, by nine treatments (Control, Glyphosate, Glyphosate+FWY, IRL 1803, IRL 1803+H,

Table 1. *Nucleotide sequences (5′ to 3′) of primers used for real-time quantitative PCR analysis*

Annotation	AGI	Sense primer	Antisense primer
ALAAT1	At1g17290	ACAATTTCTACTGCAAACGCCTTC	AAAGCCAGAACCAGGGACAAC
DHS2	At4g33510	GGTCACGCACCATCACTTACAA	CTTGGGTCACAGTGAGTGTGGTAG
HDH	At5g63890	CAAAGACGCTGAGAAATGGGAG	CTCGCATAATCCCCAACACTCT
ALS	At3g48560	ATGTTGGTGGTGGTTGTTTGAAT	TCAACGTACTCGCAACAGGGA
DHDPS1	At3g60880	TGTCGTTTGGAGTGGAAATGATG	CATCAAACCCGGAACTAAATTGC
PSP	At1g18640	TCAAGAAGTGGAGGCAAAGCC	ACGTGCTTCGAGATCAGTAGCAC
AK/HSDH	At1g31230	TCTGATTGTTCGTGGACCTGG	GCAAGGCGAAGAATGTCACTG
PAT1	At5g17990	CAATGCGGATGTGCTAAGACG	CGGTTGCTAACCAGAAGAGCTG
AtGCN2	At3g59410	CGCAAAGCACTCGATGAGTTG	GATGTCCCAGAGCTATTTTCTTTGG
NIA1	At1g37130	TGGTCAACCCACGTGAGAAA	AACGTCGTGCGAGATCGAA
NIA2	At1g77760	CGGTTAGGAACCTCGCTTTG	TGGAAGTCTTTTCGACGAGTTG
PP2A	At1g13320	TAACGTGGCCAAAATGATGC	GTTCTCCACAACCGCTTGGT

Acifluorfen, Diuron, Chlorsulfuron, and Chlorsulfuron+ILV). For each biological replicate there were three technical replicates. Technical replicates were kept together on 96-well plates, but treatment combinations were spread across five plates, for each of the biological replicates separately. This was done in order to have as many genes under investigation as possible on any given plate complete with the control (reference) gene.

Data were processed using the 'window of linearity' method (Ramakers *et al.*, 2003) as implemented in the LinRegPCR™ computer package in order to extract the efficiency of each reaction. The plate-to-plate variation in the efficiencies was investigated using analysis of variance (ANOVA) and was found to be significant ($P < 0.05$), so the mean efficiency separately for the reference gene and target genes within plates was used to calculate normalized gene expression values

$$NE_{ijkl} = \bar{E}_{R\_i}^{Rct\_ijkl}/\bar{E}_{X\_i}^{Xct\_ijkl}$$

for the reference gene R and arbitrary target gene X on plate i ($i=1,\dots, 10$), line j ($j=1, 2$), treatment k ($k=1,\dots, 9$), and technical replicate pair l ($l=1,\dots, 3$); here $\bar{E}_{R\_i}$ and $\bar{E}_{X\_i}$ are the mean efficiencies for plates whilst Rct_ijkl and Xct_ijkl are the ct-values. A log transformation to base of the mean target gene efficiency for each plate was then applied to the inverse of normalized gene expressions to give values on the ct-scale (but having normalized) and account was taken for differential target gene efficiencies across plates by multiplying by the $\log_e$ of the mean target gene efficiency for each plate and dividing by $\log_e(2)$:

$$\log_{\bar{E}_{X\_i}}\left(\frac{1}{NE_{ijkl}}\right)\frac{\log_e(\bar{E}_{X\_i})}{\log_e(2)}$$

These transformed values were then free from heterogeneity of variance and so appropriate for statistical analysis. Due to the experimental design being unbalanced (i.e. with different treatment combinations occurring on different plates) the method of residual maximum likelihood (REML) (Patterson and Thompson, 1971) was applied to this transformed variable in order to assess the variability. In this analysis, statistical significance of the main effects of lines, treatments, and then their interaction were assessed using approximate F-tests, and the standard error of the difference (SED) between means was used to perform approximate t tests on the corresponding degrees of freedom (df). These analyses were performed using the GenStat® (2007) statistical system with reference to Payne *et al.* (2007).

Electron microscopy

Electron microscopy was performed by Jean Devonshire of the Rothamsted Centre for Bioimaging, UK. Plant tissue was prepared for examination under the electron microscope by 'leaf dip' in 2% phosphotungstic acid (PTA) negative stain and transfer to pre-prepared formvar/carbon coated Cu grids (200 mesh). The electron microscope used was a JEM2010 FasTEM (JEOL UK) with an Ultrascan 1000 camera (GATAN UK) for imaging.

Results

Immunodetection of AtGCN2 in Arabidopsis seedlings and production of a homozygous null line of mutant GT8359

Antiserum was raised to a peptide with the amino acid sequence KLRPYSKDMGYEDTD, which is present in the N-terminal region of Arabidopsis GCN2 (AtGCN2)

(Zhang *et al.*, 2003; GenBank accession number AJ459823). Western analysis of crude soluble protein extracts of Arabidopsis (ecotype Landsberg *erecta* (L*er*)) seedlings showed that this antiserum recognized a protein approximately of the size predicted for AtGCN2 (140 kDa) (Fig. 1A). Despite immunoaffinity purification the antiserum also recognized a number of smaller proteins, but the relatively large size of AtGCN2 meant that these were well separated from the putative AtGCN2 protein.

Mutant GT8359, a Genetrap line containing a *Ds* element insertion in the *AtGCN2* gene, was obtained from Cold Spring Harbor Laboratory (http://genetrap.cshl.org). The presence of the *Ds* element in the *AtGCN2* gene was confirmed by amplification of two fragments by polymerase chain reaction (PCR). The first was amplified using primers specific for the *AtGCN2*-Ds 3′ junction, and therefore was present in the homozygous mutant and heterozygote, but not in the wild type; the second was amplified using primers specific for a fragment of *AtGCN2* which is disrupted by insertion of the *Ds* element in GT8359, and therefore was present in the wild type and heterozygote, but not the homozygous null mutant. Subsequently, all seeds were grown on plates containing kanamycin.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that an mRNA molecule was still being transcribed from the gene in the homozygous line (not shown). However, western analysis showed very clearly that the AtGCN2 protein was not present in extracts
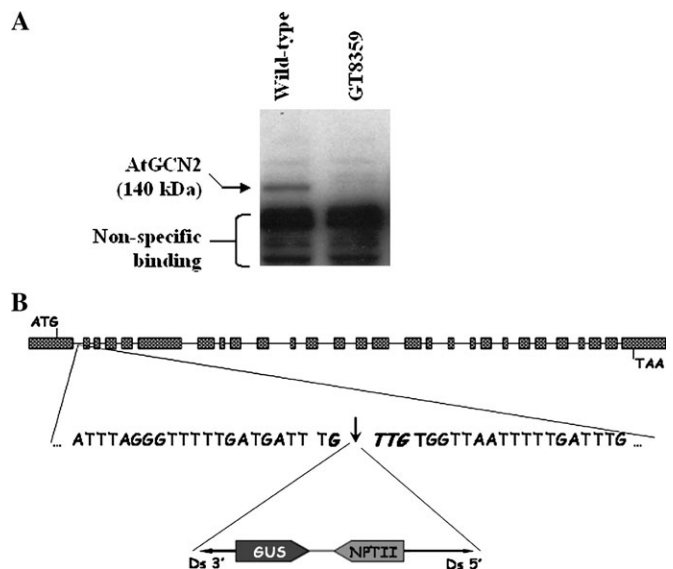
Fig. 1. (A) Western analysis showing immunodetection of AtGCN2 in wild-type Arabidopsis, ecotype Landsberg *erecta*, and the absence of AtGCN2 from mutant line GT8359. (B) Diagram showing the insertion site and orientation of the *Ds* element in the first intron of the *AtGCN2* gene in Genetrap mutant line GT8359 (see Zhang *et al.*, 2003 for full information on intron positions).
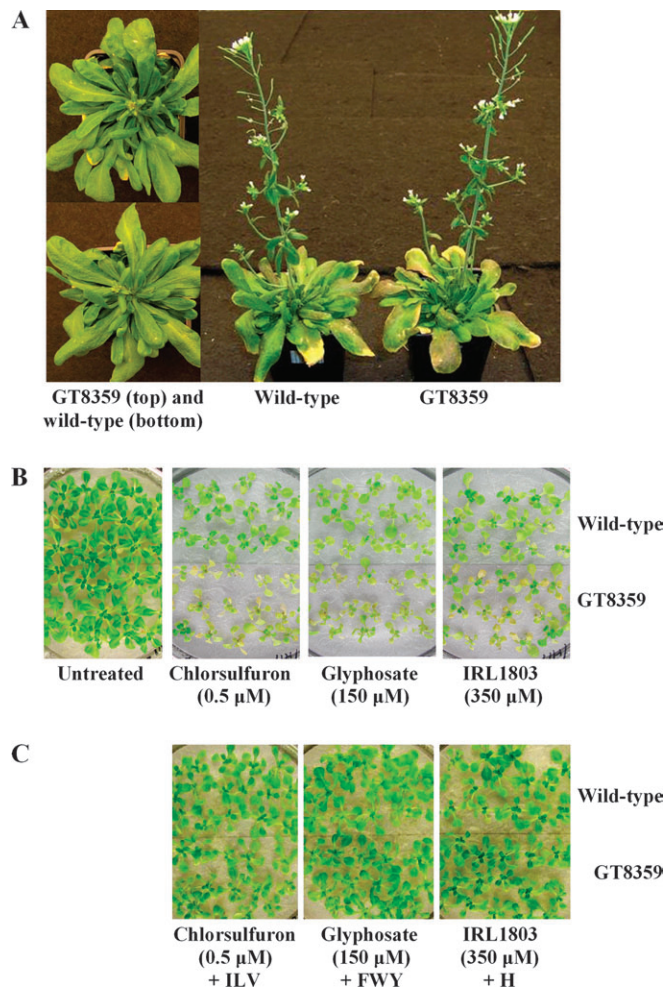
from homozygous null plants (Fig. 1A). DNA fragments containing both the *AtGCN2*-Ds3′ and *AtGCN2*-Ds5′ junctions were amplified by PCR and nucleotide sequence analysis confirmed that the *Ds* element was inserted in the first intron with an eight base-pair duplication at the insertion site (Fig. 1B).

Lack of AtGCN2 makes Arabidopsis more sensitive to herbicides that affect amino acid biosynthesis

Mutant GT8359 grew normally in soil (Fig. 2A) and in culture (Fig. 2B, far left panel). However, it did show an increased sensitivity to herbicides that affect amino acid

Fig. 2. (A) Wild-type Arabidopsis, ecotype Landsberg *erecta*, and gene trap mutant line GT8359 growing in soil. (B) Arabidopsis seedlings growing on agar plates. The plate on the left is a control while the other plates were treated with herbicide as indicated. In each case wild-type Arabidopsis, ecotype Landsberg *erecta,* was sown on the top half of the plate while Genetrap mutant line GT8359 was sown on the bottom half, as indicated. (C) As for (B), except that the seedlings were fed with the appropriate amino acids to compensate for the herbicide treatments; these were phenylalanine, tryptophan and tyrosine in the case of Glyphosate, histidine in the case of IRL 1803 and isoleucine, leucine and valine for Chlorsulfuron.

biosynthesis, namely Chlorsulfuron, Glyphosate, and IRL 1803 (Fig. 2B). Chlorsulfuron is a potent inhibitor of acetohydroxyacid synthase (AHAS) and causes depletion of valine, leucine, and isoleucine (Rhodes *et al.*, 1987). Glyphosate targets 5-enolpyruvoylshikimate 3-phosphate synthase (Padgette *et al.*, 1995), which catalyses the formation of 5-enolpyruvoylshikimate 3-phosphate from phosphoenolpyruvate and shikimate 3-phosphate. This reaction is the penultimate step in the shikimate pathway, which results in the formation of chorismate, which in turn is required for the synthesis of many aromatic plant metabolites including the amino acids phenylalanine, tyrosine and tryptophan. IRL 1803 is a competitive inhibitor of imidazoleglycerol phosphate dehydratase, an enzyme of histidine biosynthesis (Mori *et al.*, 1995).

Cultured wild-type and GT8359 seedlings were treated with the different herbicides at concentrations that were just high enough to be lethal for the wild type. In all three cases, a clear difference was seen between the wild type and the mutant, with the mutant bleaching and dying more rapidly (Fig. 2B). The effect of these herbicides could be mitigated almost entirely in both GT8359 and wild type by feeding the plants with the appropriate amino acids to compensate for the treatment; these were phenylalanine, tryptophan and tyrosine in the case of Glyphosate, histidine in the case of IRL 1803, and isoleucine, leucine and valine for Chlorsulfuron (Fig. 2C).
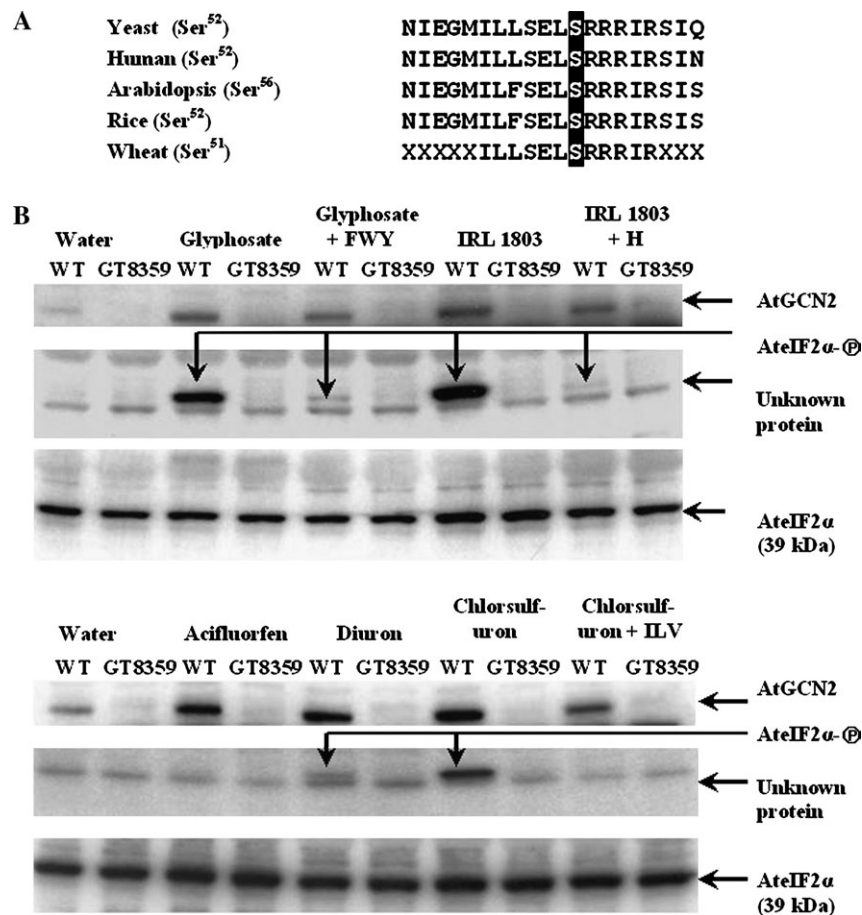
AtGCN2 is required for phosphorylation of eIF2α in response to treatment with herbicides that interfere with amino acid biosynthesis

The regulatory phosphorylation site in the N-terminal region of eIF2α is highly conserved in eukaryotes and an alignment of the amino acid residues around the target serine in budding yeast (*Saccharomyces cerevisiae*), human (*Homo sapiens*), Arabidopsis, rice (*Oryza sativa*), and wheat (*Triticum aestivum*) eIF2α is shown in Fig. 3A. Furthermore, ScGCN2 and human PKR have been shown to phosphorylate wheat eIF2α at this position (Chang *et al.*, 1999), while wheat eIF2α has been shown to complement its yeast counterpart but only if the putative target serine is present (Chang *et al.*, 2000). However, the apparent absence of PKR or GCN4 homologues in the Arabidopsis genome has led some to suggest that eIF2α phosphorylation might not be an important regulatory mechanism in plants (Browning, 2004).

Western analysis of protein samples from Arabidopsis seedlings treated with herbicides that inhibit amino acid biosynthesis and shown in Fig. 2B confirmed the presence of AtGCN2 in wild-type Arabidopsis and its absence in mutant line GT8359 (Fig. 3B). The level of AtGCN2 in the wild-type plants appeared to increase consistently in response to these herbicides, but also in response to two other herbicides, Acifluorfen and Diuron, that do not

A

Yeast (Ser[52])	NIEGMILLSELS**RRRIRSIQ**
Human (Ser[52])	NIEGMILLSELS**RRRIRSIN**
Arabidopsis (Ser[56])	NIEGMILFSELS**RRRIRSIS**
Rice (Ser[52])	NIEGMILFSELS**RRRIRSIS**
Wheat (Ser[51])	XXXXXILLSELS**RRRIRXXX**

B

Fig. 3. (A) Alignment of amino acids surrounding the phosphorylation site at the N-terminal end of eIF2α showing conservation of the sequence in plants, animals and fungi. All sequences come from the GENBANK database with the exception of that of wheat which was reported by Browning (Browning, 2004). The target serine is highlighted. (B) Western analyses showing immunodetection of AtGCN2, phosphorylated AteIF2α (AteIF2α-Ⓟ) or total AteIF2α in crude protein extracts from seedlings of Arabidopsis, ecotype Landsberg *erecta,* or gene trap mutant line GT8359, as indicated. The seedlings were treated with water, a herbicide or a herbicide plus the appropriate amino acids to mitigate the effect of the herbicide, as indicated. Note that the antisera reacted with other proteins as well; a strip of the western showing the reaction with a protein of the expected size is shown in each case.

affect amino acid biosynthesis (Acifluorfen is a protoporphyrinogen oxidase inhibitor while Diuron is an inhibitor of photosystem II).

Arabidopsis eIF2α (AteIF2α) was detected by western analysis using antiserum raised to peptide IRRRMTPQPMKIRAD; it was present in all of the extracts regardless of line or treatment. Commercially available antiserum specific for the phosphorylated form of eIF2α recognized a protein of the expected size (39 kDa) in wild-type plants treated with Glyphosate, IRL 1803 and Chlorsulfuron (Fig. 3B). This protein, the phosphorylated form of AteIF2α (AteIF2α-Ⓟ), was not detectable at all in line GT8359. Furthermore, it was undetectable or else present in greatly reduced amounts when the herbicide-treated wild-type seedlings were fed the appropriate amino acids to compensate for the treatment. On the other hand, AteIF2α-Ⓟ was not detectable at all in the Acifluorfen-treated seedlings and only just detectable in seedlings treated with Diuron.

As with the AtGCN2 antiserum, the AteIF2α-Ⓟ antiserum reacted with some other proteins, including one that was only slightly smaller than AteIF2αα-Ⓟ itself. It would be tempting to suggest that this protein was the unphosphorylated form of AteIF2α, but if it were one would expect the AteIF2α antiserum to show the presence of a doublet when AteIF2α-Ⓟ was present, which it did not. It is, therefore, concluded that this was an unrelated protein and it is labelled as an unknown protein in Fig. 3B.

These results indicate that AteIF2α is phosphorylated specifically in response to herbicides that affect amino acid biosynthesis and that this requires the presence of the protein kinase, AtGCN2.

Expression analysis of genes encoding key enzymes for amino acid biosynthesis and nitrate assimilation

The expression of a cohort of genes involved in amino acid biosynthesis was analysed in wild-type and GT8359 plants treated with the herbicides described above, using

real-time quantitative PCR (RT-QPCR). The genes were chosen in the main because they had been shown previously to respond to changes in amino acid metabolism or availability (Guyer *et al.*, 1995; Zhao *et al.*, 1998). There were two biological replicates, each with three technical replicates, for each treatment combination. The genes concerned were: dihydrodipicolinate synthase (*DHDPS1*; lysine biosynthesis), homoserine dehydrogenase/aspartate kinase (*HSDH/AK*; threonine biosynthesis), anthranilate phosphoribosyltransferase (*PAT1*; tryptophan biosynthesis), phosphoserine phosphatase (*PSP*; serine biosynthesis), acetolactate synthase (*ALS*; isoleucine, leucine and valine biosynthesis), dehydroshikimic acid synthase (*DHS2*; aromatic amino acid biosynthesis), histidinol dehydrogenase (*HDH*; histidine biosynthesis), alanine aminotransferase (*ALAAT1*; alanine biosynthesis), nitrate reductase (*NIA1* and *NIA2*; nitrate assimilation), and *AtGCN2* itself.

The method of residual maximum likelihood (REML) was used to analyse the transformed normalized expressions for each gene according to the design structure of treatment combinations (lines by treatments) arranged on 96-well RT-QPCR plates (see 'Materials and methods' section). Table 2A presents the mean ratios of normalized gene expressions for the wild type and the mutant with the control treatment, with the wild-type being taken as the reference and therefore always having a value of 1. There was a highly significant interaction ($P < 0.001$) between line and treatment for *ALS*, *HSDH/AK*, *PAT1*, *DHDPS1*, and *AtGCN2*. The interaction was also significant for *DHS2* ($P=0.003$), *HDH* ($P=0.022$), *ALAAT1* ($P=0.002$), and *NIA2* ($P=0.029$). Expression of the defunct *AtGCN2* gene was considerably up-regulated in GT8359 compared to the wild type for all treatments. This can be seen as a futile attempt by the plant to redress the loss of the AtGCN2 protein. In comparison, expression of *AtGCN2* was down-regulated in the wild type for all treatments except Diuron.

The most disparate regulation across the treatment combinations occurred for the *PAT1* gene, which was considerably up-regulated in both wild-type and GT8359 by treatment with Acifluorfen (the only gene, excepting *AtGCN2*, where there was any substantial up-regulation for this herbicide), Glyphosate and IRL 1803. In the case of Glyphosate and IRL 1803 it reduced again in recovery. There was also a substantial up-regulation of *DHDPS1* in both wild type and GT8359 after treatment with IRL 1803, while *NIA1*, *NIA2*, and *ALAAT1* were up-regulated by the Diuron treatment.

The interaction was not significant for *PSP* ($P=0.334$), but in this case the two main effects of line ($P=0.002$) and treatment ($P < 0.001$) were significant (Table 2B, C). Similarly, for *NIA1*, the interaction was not significant ($P=0.143$) but the two main effects were ($P < 0.001$). For these two genes, the effect of the treatments and the effect of lines were independent. For both, but to a greater extent

in *NIA1*, expression in GT8359 was largely down-regulated compared with wild type. In particular, for both genes, there were significant differences ($P < 0.05$) for all comparisons of herbicide with recovery treatments.

Considering recovery, the GT8359 and wild-type lines differed most in their response to the Chlorsulfuron recovery treatment. By contrast, the recovery treatment for Glyphosate generally brought the gene expressions closer together, with the expression reduced compared to control (excepting *PSP*), and a similar effect was seen for IRL 1803 plus histidine for *HSDH/AK*, *DHDPS1*, and *NIA1*, but with the expression being slightly up-regulated compared to control.

AteIF2α is not phosphorylated in response to infection by Turnip yellow mosaic virus or Turnip crinkle virus

Humans and other mammals have an eIF2α kinase, protein kinase R (PKR), that is activated by the binding of double-stranded RNA (dsRNA) produced in response to, amongst other things, viral infection (Meurs *et al.*, 1990). Human PKR has been shown to phosphorylate wheat eIF2α at serine-51 (Chang *et al.*, 1999) and a series of studies indicated the presence of a similar protein kinase in plants (Hiddinga *et al.*, 1988; Langland *et al.*, 1995, 1996). This protein kinase became known as plant-encoded dsRNA-dependent protein kinase (pPKR). It was something of a surprise, therefore, when the Arabidopsis genome sequencing project was concluded and no *PKR*-like gene was identifiable; indeed, the single-copy *AtGCN2* gene was the only identifiable eIF2α kinase gene (Zhang *et al.*, 2003).

In order to determine whether or not AteIF2α is phosphorylated in response to viral infection and if AtGCN2 is involved, wild-type Arabidopsis plants and plants of line GT8359 were infected with *Turnip yellow mosaic virus* (TYMV) or *Turnip crinkle virus* (TCV). These two unrelated, positive-strand RNA viruses are able to infect Arabidopsis and infection leads to the presence of dsRNA molecules as reproductive intermediates. The TCV-infected plants showed clear symptoms of slow growth and necrosis of the leaves (Fig. 4A), with no obvious difference between the wild-type and the mutant plants. The TYMV-infected plants also showed slow growth rate but other symptoms were not so clear (Fig. 4A). However, analysis of leaf material by transmission electron microscopy (TEM) confirmed that the plants were infected (Fig. 4B, virus particles indicated).

Western analysis was then performed using the antisera to AtGCN2, AteIF2α and AteIF2α-Ⓟ, using uninfected plants treated with IRL 1803 as a positive control. The herbicide-treated, wild-type plants showed an apparent increase in AtGCN2 and AteIF2α compared with un-treated, uninfected plants, and a very clear appearance of AteIF2α-Ⓟ (Fig. 4C). AteIF2α-Ⓟ was not detectable at

Table 2A. *The results of the analysis of data from RT-QPCR: mean ratios of normalized gene expression (NE) values, mean transformed NE values, in bold, for statistical analysis, and SEDs for comparisons of these means*

Note that two SEDs are given, the first for comparison of treatments which were located on the same 96-well plate and the second for comparison of treatments on different plates. Two biological replicates were used of each of the 18 treatment combinations: two lines: wild-type (WT) and GT8359 (M) by nine treatments: Control (cont), Glyphosate (Gly), Glyphosate+FWY (Gly+FWY), IRL 1803 (Irl), IRL 1803+H (Irl+H), Acifluorfen (Acif), Diuron (Diu), Chlorsulfuron (Chl), and Chlorsulfuron+ILV (Chl+ILV). Wild-type control is used as the reference (ref.). For each biological replicate there were three technical replicates.

Treatment/ Line	Gene																					
	ALS		DHS2		HDH		PSP[a]		HSDH/AK		PAT1		DHDPS1		ALAAT1		AtGCN2		NIA1[a]		NIA2	
1. WT cont (ref.)	1.000	**−3.289**	1.000	**−2.493**	1.000	**−1.639**	1.000	**−8.974**	1.000	**−0.502**	1.000	**−2.335**	1.000	**1.598**	1.000	**−3.442**	1.000	**−1.708**	1.000	**−1.793**	1.000	**−5.139**
2. GT8359 cont	1.036	**−3.226**	1.168	**−2.651**	1.045	**−1.659**	1.016	**−8.973**	1.016	**−0.528**	1.307	**−2.720**	0.940	**1.723**	1.167	**−3.664**	2.235	**−2.856**	0.889	**−1.623**	1.316	**−5.527**
3. WT Diu	1.282	**−3.510**	1.793	**−3.176**	1.376	**−2.027**	0.973	**−8.908**	1.070	**−0.602**	1.183	**−2.576**	1.273	**1.312**	2.024	**−4.435**	1.750	**−2.501**	3.824	**−3.527**	2.239	**−6.259**
4. GT8359 Diu	1.149	**−3.341**	1.389	**−2.888**	1.215	**−1.855**	0.854	**−8.726**	1.038	**−0.552**	1.670	**−3.069**	1.110	**1.496**	2.180	**−4.554**	5.624	**−4.179**	2.817	**−3.185**	2.124	**−6.158**
5. WT Acif	0.511	**−2.193**	0.672	**−1.793**	1.089	**−1.581**	0.307	**−7.132**	0.925	**−0.375**	2.026	**−3.239**	0.993	**1.781**	1.100	**−3.578**	0.528	**−0.751**	0.691	**−1.197**	0.599	**−4.288**
6. GT8359 Acif	0.601	**−2.394**	0.705	**−1.813**	1.134	**−1.747**	0.288	**−7.138**	0.919	**−0.374**	2.159	**−3.386**	1.096	**1.696**	0.969	**−3.389**	4.222	**−3.702**	0.589	**−1.030**	0.799	**−4.552**
7. WT Chl	0.968	**−2.619**	0.858	**−1.910**	0.811	**−1.158**	1.091	**−9.057**	0.704	**0.163**	1.127	**−2.430**	1.583	**1.099**	1.373	**−3.883**	0.736	**−1.271**	0.803	**−1.417**	1.099	**−5.064**
8. GT8359 Chl	0.767	**−2.383**	0.832	**−1.973**	0.758	**−0.972**	1.058	**−8.832**	0.702	**0.585**	1.683	**−3.742**	1.125	**1.550**	1.745	**−4.235**	9.884	**−5.007**	0.470	**−0.653**	1.324	**−5.375**
9. WT Chl+ILV	0.920	**−2.685**	0.902	**−2.066**	0.701	**−1.000**	1.196	**−9.202**	0.789	**−0.110**	0.843	**−2.018**	1.240	**1.354**	0.817	**−3.145**	0.952	**−1.633**	1.296	**−2.102**	0.876	**−4.649**
10. GT8359 Chl+ILV	0.927	**−2.685**	0.773	**−1.806**	0.692	**−1.001**	0.970	**−9.048**	0.624	**−0.064**	1.852	**−2.111**	1.139	**1.454**	0.964	**−3.360**	1.693	**−2.462**	1.338	**−2.058**	0.857	**−4.602**
11. WT Gly	0.544	**−2.281**	0.578	**−1.636**	0.836	**−1.345**	1.405	**−9.359**	0.476	**0.566**	2.800	**−3.809**	0.994	**1.648**	1.553	**−4.084**	0.780	**−1.415**	0.573	**−1.050**	0.527	**−4.263**
12. GT8359 Gly	0.694	**−2.523**	0.525	**−1.492**	0.858	**−1.359**	1.417	**−9.357**	0.388	**0.861**	3.581	**−4.146**	0.985	**1.677**	1.971	**−4.409**	6.851	**−4.446**	0.430	**−0.581**	0.551	**−4.280**
13. WT Gly+FWY	0.455	**−1.987**	0.529	**−1.477**	0.619	**−0.911**	1.644	**−9.618**	0.481	**0.551**	0.719	**−1.854**	0.702	**2.144**	0.671	**−2.852**	0.764	**−1.317**	0.907	**−1.566**	0.433	**−3.878**
14. GT8359 Gly+FWY	0.512	**−2.179**	0.554	**−1.568**	0.605	**−0.876**	1.702	**−9.601**	0.432	**0.715**	0.729	**−1.878**	0.688	**2.195**	0.814	**−3.071**	1.508	**−2.285**	0.831	**−1.485**	0.447	**−3.968**
15. WT Irl	0.566	**−2.375**	0.649	**−1.468**	1.087	**−1.700**	0.283	**−7.134**	0.742	**0.128**	1.691	**−3.068**	1.956	**0.695**	0.952	**−3.233**	0.568	**−0.867**	0.444	**−0.508**	0.979	**−4.639**
16. GT8359 Irl	0.624	**−2.515**	0.759	**−1.695**	1.235	**−1.886**	0.288	**−7.149**	0.724	**0.172**	3.041	**−3.937**	2.011	**0.632**	1.136	**−3.522**	5.718	**−4.226**	0.334	**−0.095**	0.948	**−4.617**
17. WT Irl+H	0.814	**−2.906**	0.724	**−1.753**	0.941	**−1.485**	0.418	**−7.693**	1.040	**−0.431**	1.333	**−2.636**	1.333	**1.257**	0.751	**−2.783**	0.679	**−1.147**	1.361	**−2.052**	0.824	**−4.274**
18. GT8359 Irl+H	0.932	**−3.070**	0.663	**−1.565**	1.036	**−1.602**	0.388	**−7.584**	1.150	**−0.536**	1.245	**−2.634**	1.333	**1.275**	0.740	**−2.823**	1.261	**−2.034**	1.552	**−1.961**	0.971	**−4.417**
SEDs (80 df.) (1)		**0.101**		**0.112**		**0.093**		**0.095**		**0.091**		**0.119**		**0.092**		**0.095**		**0.115**		**0.192**		**0.119**
SEDs (80 df.) (2)		**0.724**		**0.435**		**0.530**		**4.224**		**0.520**		**0.480**		**0.357**		**0.469**		**0.329**		**0.501**		**0.873**

[a] For these genes the interaction was not significant so refer to separate means tables (Tables 2B and C) for the main effects of line and treatment to make comparisons.

[b] (1) Between means 1, 2, 3, and 4; or 7, 8, 9, and 10; or 11, 12, 13, and 14; or 5 and 6; or 15, 16, 17. (2) All other comparisons.

Table 2B. *Mean transformed NE values and SEDs for comparisons for the main effect of line (PSP and NIA1)*

| PSP		NIA1	
WT	GT8359	WT	GT8359
−8.564	−8.490	−1.690	−1.408
SED (80 df.) = 0.029		SED (80 df.) = 0.062	

Table 2C. *Mean transformed NE values and SEDs for comparisons, for the main effect of treatment (PSP and NIA1)*

Treatment	PSP	NIA1
1. Control	−8.974	−1.708
2. Diuron	−8.817	−3.356
3. Aciflurofon	−7.135	−1.113
4. Chlorosulfuron	−8.944	−1.035
5. Chlorosulfuron+ILV	−9.125	−2.080
6. Glyphosate	−9.358	−0.816
7. Glyphosate+FWY	−9.609	−1.525
8. Irl 1803	−7.142	−0.302
9. Irl 1803+H	−7.638	−2.007
SEDs (on 80 df.)		
1 and 2; or 4 and 5; or 6 and 7; or 8 and 9.	0.064	0.133
All other comparisons	4.223	0.477

all in untreated, uninfected plants. As before, no AtGCN2 was detectable in the GT8359 line and while the level of AteIF2α increased in a similar fashion to wild type there was no detectable AteIF2α-Ⓟ. In contrast to the herbicide-treated plants, the levels of AtGCN2 and AteIF2α were not affected in the plants infected with the viruses, and virus infection did not cause AteIF2α to be phosphorylated sufficiently for AteIF2α-Ⓟ to be at all detectable. It was concluded that, in the case of Arabidopsis and these two viruses, at least, virus infection does not cause phosphorylation of AteIF2α by AtGCN2 or any other protein kinase.

Discussion

Phosphorylation of the highly conserved site in the N-terminal region of the α subunit of eukaryotic translation initiation factor-2 (eIF2α) is a key regulatory mechanism in a wide range of eukaryotes, bringing about a global reduction in protein synthesis and enabling the maintenance of cell homeostasis (Hinnebusch, 1992). In fungi it also results in a major change in gene expression through the action of a transcription factor, GCN4, the levels of which are controlled at the translational level and actually increase as general protein synthesis slows down (Hinnebusch, 1997). Genes under the control of GCN4 in yeast include many encoding enzymes of amino acid biosynthesis and one of the protein kinases that phosphor-
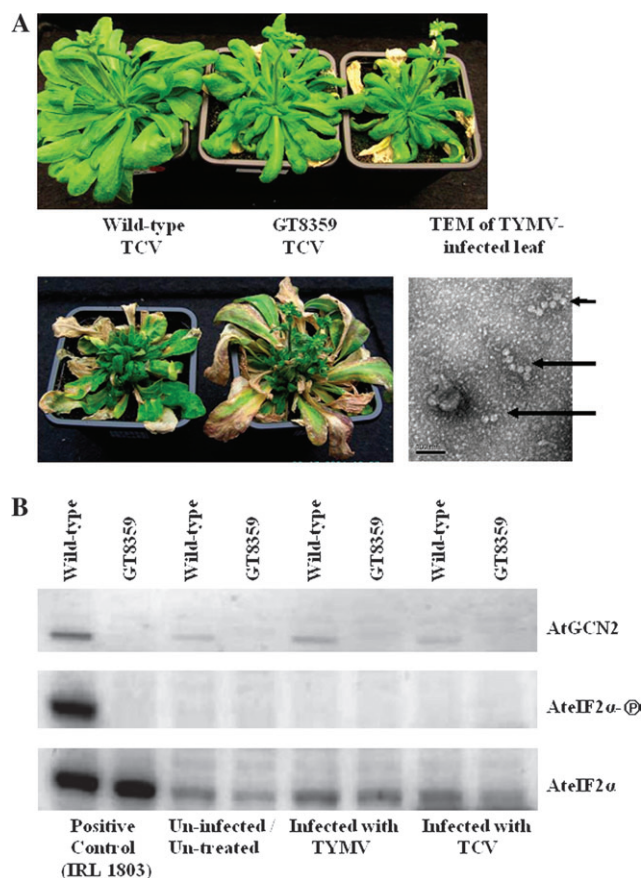
Fig. 4. (A) Arabidopsis, ecotype Landsberg *erecta,* and gene trap mutant line GT8359, uninfected, or infected with *Turnip yellow mosaic virus* (TYMV) or *Turnip crinkle virus* (TCV), as indicated. (B) Transmission electron microscope image confirming the presence of viral particles (arrowed) in the leaves of a TYMV-infected plant. The length of the bar corresponds to 100 nm. The image was prepared by Jean Devonshire of the Rothamsted Centre for Bioimaging. (C) Western analyses showing immunodetection of AtGCN2, phosphorylated AteIF2α (AteIF2α-Ⓟ) or total AteIF2α in crude protein extracts from leaves of Arabidopsis, ecotype Landsberg *erecta,* or gene trap mutant line GT8359, as indicated. The leaves had been inoculated with TYMV or TCV as indicated. A positive control of extracts from herbicide IRL-treated seedlings and a negative control of leaves inoculated with water are included.

ylate eIF2α, ScGCN2, is activated in response to low amino acid levels and the interaction of uncharged tRNA with its regulatory C-terminal domain.

In the present study, it has been shown that Arabidopsis eIF2α (AteIF2α) is phosphorylated at serine-52 in response to treatment with herbicides that interfere with amino acid biosynthesis, but not herbicides that affect other cellular processes. The effect of the herbicide treatment can be reversed by feeding with the appropriate amino acids. It has also been shown unequivocally that this phosphorylation is dependent upon AtGCN2, a protein kinase that is structurally and functionally related to ScGCN2 (Zhang *et al.*, 2003), because it does not occur in a mutant, GT8359, that lacks AtGCN2. Loss of AtGCN2 did not appear to affect plants growing under

normal conditions, but it did make them more sensitive to herbicides that interfere with amino acid biosynthesis.

The study provided little evidence for the involvement of AtGCN2 in the regulation of amino acid biosynthesis gene expression. While the expression of some of the genes that were analysed did change in response to the herbicide treatments, as has been shown in other studies, and this could be reversed by feeding with the appropriate amino acids, this occurred in GT8359 plants just as it did in wild-type plants; in other words it was not AtGCN2-dependent and did not require phosphorylation of AteIF2α. However, GT8359 did show a reduction in expression of a nitrate reductase gene, *NIA1*.

It is possible that AtGCN2-dependent phosphorylation of eIF2α is involved in the regulation of expression of amino acid biosynthesis genes in plants, but that other regulatory systems are able to compensate when AtGCN2 is not present. It is also possible that the experimental design did not allow for differences in the control of gene expression to become evident. On the other hand, we are reminded that the other key component of this system, the transcription factor GCN4, has not been identified in plants.

Another unanswered question regarding phosphorylation of eIF2α in plants concerned its involvement in the response to virus infection. Mammals have an eIF2α kinase that is activated by interaction with dsRNA molecules that are produced as a result of viral infection (Meurs *et al.*, 1990; Mellor *et al.*, 1994*b*) and there was considerable interest in the 1980s and 1990s in a plant protein kinase that appeared to have an equivalent function and became known as plant PKR (pPKR) (Crum *et al.*, 1988; Hiddinga *et al.*, 1988; Langland *et al.*, 1995, 1996; Chang *et al.*, 1999). It was something of a mystery, therefore, when it became clear that the Arabidopsis and, subsequently, rice genomes were found not to encode a PKR-like protein kinase. Given that AtGCN2 was the only eIF2α kinase that was identifiable in Arabidopsis (Halford *et al.*, 2004; Halford, 2006) we considered it possible that it was responsible for the PKR-like activity, in other words that PKR- and GCN2-like activities were consolidated in the single protein kinase. Alternatively, pPKR might exist but not be readily identifiable by sequence similarity with its mammalian counterpart. In fact, we could not show any phosphorylation of AteIF2α at all in Arabidopsis plants infected with either *Turnip crinkle virus* or *Turnip yellow mosaic virus*, regardless of the presence or absence of AtGCN2. It was concluded that it is unlikely that phosphorylation of eIF2α plays a part in the plant response to viral infection.

Acknowledgements

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