Molecular cloning of an Arabidopsis homologue of GCN2, a protein kinase involved in co-ordinated response to amino acid starvation

Article *in* Planta · September 2003

DOI: 10.1007/s00425-003-1025-4 · Source: PubMed

CITATIONS
88

READS
145

4 authors, including:

Matthew Paul
Rothamsted Research
133 PUBLICATIONS **8,997** CITATIONS

SEE PROFILE

Nigel Halford
Rothamsted Research
240 PUBLICATIONS **14,145** CITATIONS

SEE PROFILE

Planta (2003) 217: 668–675
DOI 10.1007/s00425-003-1025-4

ORIGINAL ARTICLE

Yuhua Zhang · J. Richard Dickinson
Matthew J. Paul · Nigel G. Halford

Molecular cloning of an arabidopsis homologue of GCN2, a protein kinase involved in co-ordinated response to amino acid starvation

Received: 14 December 2002 / Accepted: 24 February 2003 / Published online: 28 March 2003

Abstract DNA homologous to the yeast (*Saccharomyces cerevisiae*) protein kinase gene, *GCN2*, was amplified from arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] RNA and given the name AtGCN2. The AtGCN2 peptide sequence included adjacent protein kinase and histidyl tRNA synthetase-like domains and showed 45% sequence identity with the GCN2 peptide sequence in the protein kinase domain. AtGCN2 transcripts were detectable in RNA from roots, leaves, stems, buds, flowers, siliques and seedlings. GCN2 is required for yeast cells to respond to amino acid starvation. Expression of AtGCN2 in yeast *gcn2* mutants complemented the mutation, enabling growth in the presence of sulfometuron methyl, an inhibitor of branched-chain amino acid biosynthesis, and 3-aminotriazole, an inhibitor of histidine biosynthesis.

Keywords *Arabidopsis* · eIF2α kinase · Metabolic signalling · Phosphorylation · Yeast complementation

Abbreviations 3-AT: 3-aminotriazole · eIF2α: α subunit of eukaryotic translation initiation factor-2 · RACE: rapid amplification of cDNA ends · SM: sulfometuron methyl

The nucleotide sequence of AtGCN2 has been deposited in the EMBL database under accession number AJ459823

Y. Zhang · M. J. Paul · N. G. Halford (✉)
Department of Crop Performance and Improvement,
Rothamsted Research,
Harpenden,
Hertfordshire, AL5 2JQ, UK
E-mail: nigel.halford@bbsrc.ac.uk
Fax: +44-1582-763010

J. R. Dickinson
Cardiff School of Biosciences,
Cardiff University,
Cardiff, CF10 3TL, UK

Introduction

In yeast (*Saccharomyces cerevisiae*), amino acid starvation causes a general reduction in protein synthesis and initiates changes in expression of a huge number of genes in a process known as general amino acid control (Hinnebusch 1992). Fundamental to general amino acid control is the protein kinase, GCN2 (Wek et al. 1989). The substrate for GCN2 is the α subunit of eukaryotic translation initiation factor-2 (eIF2α), which is phosphorylated by GCN2 at serine-51 (Samuel 1993). eIF2 is a trimeric factor (subunits α, βand γ) that can bind either GDP or GTP. However, 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 and P_i and eIF2 is released as an inactive [eIF-2.GDP] complex. Phosphorylation of eIF2α inhibits the recycling of bound GDP to GTP, decreasing the rate of protein synthesis. The phosphorylation site at serine-51 is conserved in eIF2α homologues from a wide range of eukaryotes, including wheat, and yeast GCN2 will phosphorylate wheat eIF2α at this position (Chang et al. 1999, 2000).

GCN2-mediated phosphorylation of eIF2α under conditions of amino acid deprivation increases expression of amino acid biosynthesis genes through the action of a transcriptional activator, GCN4 (Hinnebusch 1997). Upregulation of GCN4 occurs at the translational level, translation initiating from a downstream initiation codon that is not used under normal conditions (Hinnebusch 1992, 1994). Micro-array analysis identified 539 yeast genes that are induced by amino acid starvation through the action of GCN4 (Natarajan et al. 2001). These included genes in every amino acid biosynthetic pathway except cysteine, genes encoding amino acid precursors, vitamin biosynthetic enzymes, peroxisomal components, mitochondrial carrier proteins, autophagy proteins and many genes encoding protein kinases and transcription factors.

GCN2 is believed to be activated through interaction with uncharged tRNA (Wek et al. 1989). The region of the protein responsible for this is a domain of approximately 400 amino acid residues that shows significant sequence similarity with histidyl-tRNA synthetases (Wek et al. 1989). The presence of adjacent kinase catalytic and His-tRNA synthetase-like domains is indicative of a GCN2-type protein kinase.

Homologues of GCN2 have been identified in *Drosophila melanogaster* (Santoyo et al. 1997) and *Neurospora crassa* (Sattlegger et al. 1998), and there are two other eIF2α kinases that have similar catalytic domains to GCN2 but do not contain a histidyl-tRNA synthetase-like domain and respond to different stimuli. These are the heme-regulated inhibitor (HRI) that has been cloned from rabbit and rat (Chen et al. 1991; Mellor et al. 1994a) and the double-stranded RNA-dependent kinase (PKR) that has been cloned from human (Meurs et al. 1990). HRI is activated in response to heme deficiency (Chen et al. 1991; Mellor et al. 1994a) and PKR to the presence of double-stranded RNAs after virus infection (Meurs et al. 1990; Icely et al. 1991; Mellor et al. 1994b). A PKR-like activity has been purified from wheat seedlings (Langland et al. 1996) and shown to phosphorylate wheat eIF2α specifically at serine-51 (Chang et al. 1999).

The link between amino acid signalling, nitrogen-use efficiency, crop yield and quality makes the elucidation of amino acid signalling pathways an important target for plant scientists. Here we describe the molecular cloning and characterisation of potentially one of the factors that is involved, an arabidopsis homologue of the yeast protein kinase, GCN2.

Materials and methods

Plant materials

Arabidopsis thaliana (L.) Heynh. (ecotype Columbia) plants from a seed stock held at Rothamsted Research were grown in soil in a growth chamber with a temperature/light setting of 20 °C/10 h.

Amplification of AtGCN2 DNA

Total RNA was isolated from leaf material of 4-week-old plants using an RNeasy Plant Mini Kit (Qiagen). First-strand complementary DNA (cDNA) was synthesized using SuperScript II (Life Technologies), using an oligonucleotide with the sequence 5′-CTTTAGCTCCAAACAGAGGGGTTTCTA as primer. This was then used as template for a polymerase chain reaction (PCR) using the same oligonucleotide and another, with the sequence 5′-TGAGGAAATGGGTCGCAGCAG, as primers. PCR was performed using *PfuTurbo* DNA polymerase (Stratagene). The PCR programme was 1 cycle of 98 °C for 1.5 min, 65 °C for 1 min, and 72 °C for 4 min followed by 30 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 4 min with a final extension of 10 min at 72 °C.

The PCR product was cloned into the pGEM-T Easy Vector (Promega). The nucleotide sequence of AtGCN2 was obtained by primer walking using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI Prism 377XL sequencing machine. Database searches were carried out with the BLAST version 2.0 program (Altschul et al. 1997) and pairwise protein sequence comparisons were made using the GAP program (Wisconsin Package version 10, Genetics Computer Group, Madison, WI, USA) with a gap creation penalty of 3.0 and a gap extension penalty of 0.1. Sequences were aligned using the PILEUP program with a gap creation penalty of 3.0 and a gap extension penalty of 0.1.

3′ and 5′ rapid amplification of cDNA ends (RACE)

The 3′and 5′ ends of the AtGCN2 transcript were amplified by RACE using a FirstChoice RLM-RACE kit (Ambion).

First-strand cDNA for 3′ RACE was performed using 1 μg total RNA as template and a 3′ RACE adapter (5′-CGAGCA CAGAATTAATACGACTCACTATAGGT$_{12}$VN). The cDNA was synthesised by MMLV reverse transcriptase at 42 °C for 1 h. Outer 3′ RACE PCR was performed using the Expand High Fidelity PCR system (Roche Molecular Biochemicals) using primers 5′-GCGAGCACAGAATTAATACGACT and 5′-AATGAAC CTCCCTGGTGCTGTTG. The PCR reaction was heated to 95 °C for 2 min, then cycled 35 times at 95 °C for 15 s, 65 °C for 30 s and 72 °C for 1 min, followed by 7 min at 72 °C. A nested PCR product was amplified using primers 5′-CGCGGATCCGAAT TAATACGACTCACTATAGG and 5′-CGCAAGCTTTCAA GAGGAGGTGGTGGTTTACTGG.

The template for 5′ RACE was prepared by treating 10 μg total RNA with calf intestinal phosphatase and tobacco acid pyrophosphatase. An adapter, 5′-GCUGAUGGCGAUGAAUGAA-CACUGCGUUUGCUGGCUUUGAUGAAA was then ligated to the RNA population using T4 RNA ligase. First-strand cDNA was synthesised from this template with MMLV reverse transcriptase at 42 °C for 1 h. First and nested PCR products were amplified as described for 3′ RACE using primers 5′-GCTGATGG CGATGAATGAACACTG and 5′-AAACTCTTGAGCAGC CTCCACCA for the first reaction and 5′-CGCGGATCCGA ACACTGCGTTTGCTGGCTTTGATG and 5′-ACTAAGCTTG CTGTTGTCAACCCTTGTTCTGGA for the nested reaction.

Expression analysis

Total RNA from arabidopsis root, leaf, stem, flower bud, flower, and young silique tissues was extracted using a small scale RNAqueous kit from Ambion. Total RNA was also extracted from 2-, 4-, 6-, 8-, and 12-day-old whole seedlings, which were grown on wet filter papers at 20 °C with 10 h of daylight.

For northern analysis, 10 μg of each RNA sample was separated through a 1% denaturing agarose gel. The RNA was then transferred onto an Osmonics Magnacharge Nylon membrane (Micron Separations Inc., Westborough, MA, USA) using standard procedures (Sambrook et al. 1989). Probe synthesis was carried out using the Prime-It II Random Primer Labelling kit (Stratagene). Hybridisation conditions were 50% (v/v) formamide, 5 × SSPE, 5 × Denhardt's solution, 0.5% (w/v) SDS, 100 μg ml^{-1} denatured herring sperm DNA, 4% (w/v) dextran sulphate at 42 °C for 16 h (Sambrook et al. 1989). Filters were washed in 0.2 × SSC, 0.1% (w/v) SDS for 30 min at 55°C. Hybridisation was visualised by autoradiography.

For RT–PCR analysis, first-strand cDNA was synthesised by SuperScriptII reverse transcriptase (Life Technologies) using a gene-specific oligonucleotide primer, 5′-CTTTAGCTCCAAACA GAGGGGTTTCTA. An aliquot (1 μl) of the reverse transcription reaction was used as template for PCR using oligonucleotide primers 5′-TCGTCCCTCTGCCACAG and 5′-CTTTAGCTC CAAACAGAGGGGTTTCTA, with one cycle at 95 °C for 3 min followed by 30 cycles of 95 °C for 15 s, 57 °C for 30 s and 72 °C for 1 min, then 72 °C for 10 min.

Yeast complementation

A 3,751-bp *Eco*RI fragment containing the full-length AtGCN2 PCR product was subcloned behind the *GAL1* promoter in the

*Eco*RI site of the low-copy yeast expression vector pYC2/CT (InVitrogen). Expression of the plant gene was terminated by the *CYC1* transcription termination signal. The plasmid containing the chimaeric gene was introduced into *gcn2*$^-$ yeast strains Y13642 (*Matα his3*Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YDR283c::kanMX4) and H1816 (*MAT*a *ura-3-52 leu2-3 leu2-112 trp1-Δ63* Δ*sui2* Δ*gcn2,* p1108 [*GCN4-lacZ TRP1*] at *trp1-Δ63*, *p1097* [*SUI2 LEU2*]) using the method of Gietz et al. (1995). Y13642 transformants were streaked on synthetic complete (SC) medium lacking uracil and grown for 2 days at 28–30 °C. H1816 transformants were selected on SC medium lacking uracil, leucine and tryptophan.

For complementation experiments, yeast transformants were grown in liquid SC medium lacking uracil overnight at 28–30 °C. They were then serially diluted with sterile distilled water to OD_{600} of 0.5, 5×10^{-2}, 5×10^{-3}, 5×10^{-4}, and 5×10^{-5}. An aliquot (5 µl) of each dilution was dropped onto plates containing glucose (non-inducing medium) or a combination of galactose (10% w/v) and raffinose (2% w/v) (inducing medium), with or without inhibitors. For Y13642, the medium was supplemented with leucine (380 mg/l), histidine (76 mg/l) and lysine (76 mg/l), and the inhibitor was sulfometuron methyl (SM) at 0.25 µg/ml. For H1816, the medium was supplemented with all of the amino acids except histidine, leucine, tryptophan and uracil, and the inhibitor was 3-amino-triazole (3-AT) at 10 mM. The agar plates were incubated for 4–7 days at 28–30 °C.

Results

Molecular cloning of a GCN2 homologue from arabidopsis

Total RNA was purified from arabidopsis seedlings and used as template in an RT–PCR reaction to amplify a *GCN2*-related PCR product. A single putative *GCN2*-related gene had been identified on arabidopsis chromosome 3 (accession number AL356014, protein id. CAB91611.1). The gene comprises 8,577 bp from the predicted translation start site to the end of the translation stop codon (77806–86382 in AL356014). Oligonucleotide primers for the PCR reaction were designed for the 5′- and 3′-ends of the coding region of the gene and a single product of 3,732 bp was produced. The PCR product, AtGCN2, was cloned and the nucleotide sequence of four independent clones was obtained. The sequence matched corresponding regions of the gene exactly, consistent with there being a single *GCN2*-related gene in arabidopsis. It was submitted to the EMBL database under accession number AJ459823.

The PCR product contained an open reading frame (ORF) of 3,723 nucleotides encoding a protein of 1,241 amino acid residues with a predicted molecular weight of 140,321 Da. Alignment of the nucleotide sequence with that of the gene showed that the coding sequence derived from 28 exons (Table 1). The predicted amino acid sequence of the AtGCN2 protein shows approximately 30% identity overall with that of yeast GCN2, rising to 45% in the catalytic domain (leucine-431 to arginine-719).

A schematic diagram of the AtGCN2 and GCN2 protein structures is shown in Fig. 1 and the amino acid sequences of the two proteins are aligned in Fig. 2. AtGCN2 contains both a kinase catalytic domain and a His tRNA synthetase-related domain. Sequence alignment

Table 1 Exon positions in AtGCN2. Locations refer to the nucleotide sequence of *Arabidopsis thaliana* chromosome III given in the EMBL databank entry AL356014. The locations of entire exons are given with the exception of exon 1, for which the first position given refers to the first nucleotide of the ATG translation start codon, and exon 28, for which the last position given refers to the end of the open reading frame

Exon	Location	Length	Exon	Location	Length
1	77806–77930	125	15	82649–82898	250
2	78278–78352	75	16	83202–83475	274
3	78459–78518	60	17	83547–83640	94
4	78601–78706	106	18	83941–84021	81
5	78799–78903	105	19	84163–84219	57
6	79055–79459	405	20	84433–84564	132
7	79735–79953	219	21	84658–84741	84
8	80041–80127	87	22	84849–84957	109
9	80212–80323	112	23	85024–85160	137
10	80675–80792	118	24	85374–85481	108
11	81185–81239	55	25	85717–85802	86
12	81419–81588	170	26	85874–85976	103
13	81905–82089	185	27	86070–86198	129
14	82388–82536	149	28	86272–86379	108
			Total		3723

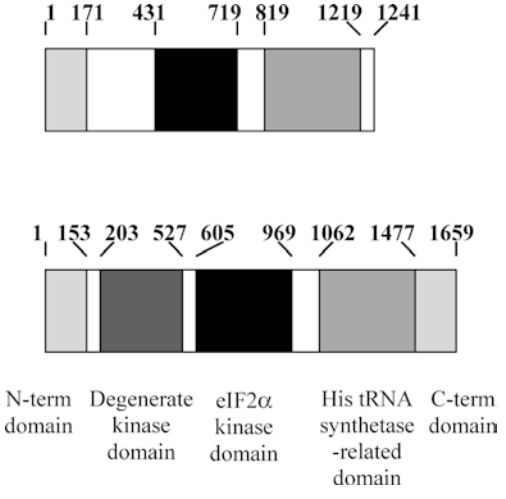

Fig. 1 The structures of *Arabidopsis thaliana* AtGCN2 (*top*) and yeast (*Saccharomyces cerevisiae*) GCN2 (*bottom*). The numbers shown indicate amino acid sequence positions

searches with the AtGCN2 kinase domain peptide sequence against SWISSPROT and TREMBL protein sequence databases matched it most closely with GCN2 and other eIF2α kinases. Similar searches with the His tRNA synthetase-related domain (isoleucine-819 to arginine-1219) matched it most closely with His tRNA synthetases from human and mouse (accession numbers AAH11807 and Q61035), with which it shares 26% amino acid sequence identity. This search also identified a peptide encoded by a short cDNA sequence from soybean (*Glycine max*), accession number BE347434. This 94-amino-acid peptide showed 73% amino acid sequence identity with the C-terminal region of AtGCN2 and is included in Fig. 2. The cDNA is almost certainly derived from the 3′ end of a soybean *GCN2* transcript.

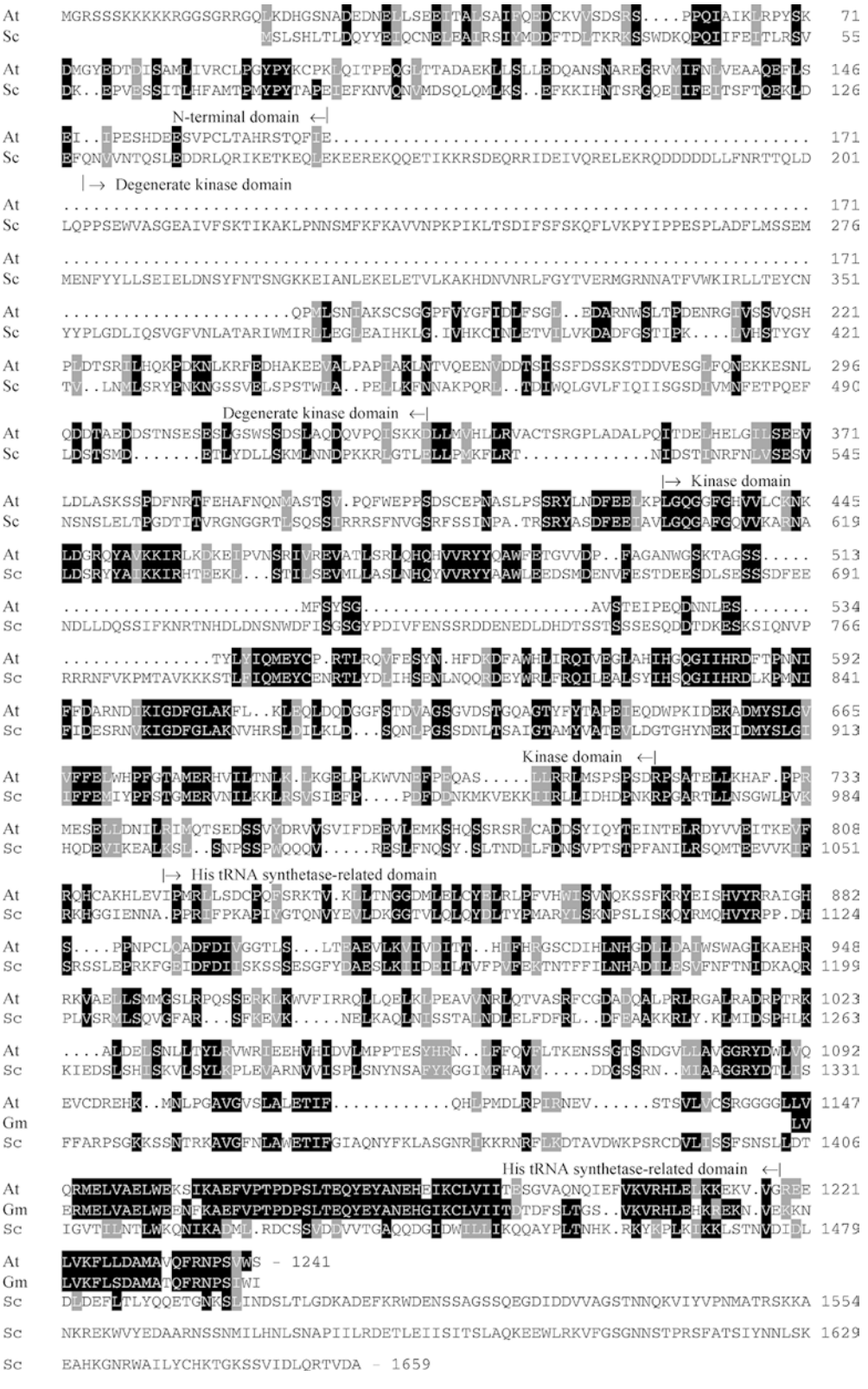

Fig. 2 Alignment of the peptide sequences of AtGCN2 (*At*) and yeast (*S. cerevisiae*) GCN2 (*Sc*) (accession number AAB62261). N-terminal, degenerate kinase, kinase and His tRNA synthetase domains are indicated. Matching amino acids at the same position are *highlighted in black* and similar amino acids at the same position are *highlighted in grey*. The C-terminal region of a putative soybean GCN2 homologue is also shown (Gm)

BLAST searches with the peptide sequence of the N-terminal domain of AtGCN2 matched it closely with the N-terminal domains of other eIF2α kinases, notably two that have been identified in mouse (accession numbers Q9QZ05 and Q9ESB7). For this reason, Fig. 2 was constructed from separate pairwise GAP alignments, one with the N-terminal domain and one with the rest of the protein. A single GAP alignment would not introduce a gap between the N-terminal domain and the rest of the AtGCN2 protein because the sequence identity

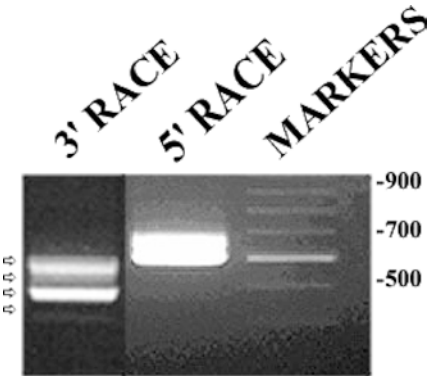

Fig. 3 Agarose gel electrophoresis of products of RACE experiments designed to amplify fragments at the 3′ and 5′ ends of AtGCN2 transcripts. Size markers (bp) are shown on the *right*. The positions of the four products (two major and two minor) of the 3′ RACE experiment are indicated with *arrows*

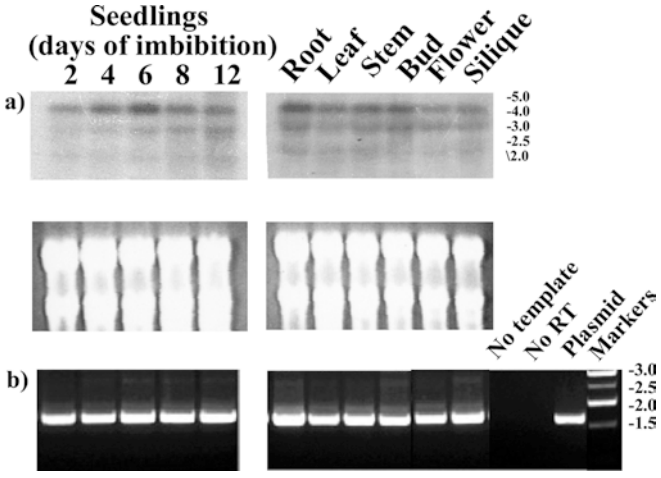

Fig. 4a, b AtGCN2 expression in various tissues of arabidopsis and in whole arabidopsis seedlings from 2 to 12 days after germination. **a** Northern analysis. Equal loadings of total RNA (10 μg) were separated in a 1% denaturing agarose gel and transferred onto a nylon membrane. RNA loadings were confirmed by ethidium bromide staining (*lower panel*). The blotted RNA was hybridised with an [α-^{32}P]dATP-labelled probe synthesized from the full-length AtGCN2 PCR product. Hybridisation was visualized by autoradiography (*top panel*). The positions of size markers are shown on the *right*. **b** RT–PCR analysis. Total RNA was used as template for RT–PCR reactions using AtGCN2-specific oligonucleotide primers. Negative controls shown are RT–PCR reaction using water template and PCR reaction using leaf RNA without first performing a reverse-transcription step. Positive control shown is the PCR product derived from plasmid DNA containing the cloned AtGCN2 PCR product. Size markers (bp) are shown on the *right*

between the two N-terminal domains was not sufficient to compensate for the gap penalty. In GCN2 and its homologues from *N. crassa* and *Drosophila melanogaster* there is a degenerate kinase domain between the N-terminal domain and the true kinase domain (Figs. 1, 2). The role of this domain is not known (it is incomplete and lacks some of the invariant amino acids present in true protein kinase catalytic domains and is therefore presumed to be catalytically inactive) and it is absent from AtGCN2. AtGCN2 and its homologue from soybean are also truncated at the C-terminal end compared with GCN2.

BLAST searches of the NCBI arabidopsis genome and protein databases (http://www.ncbi.nlm.nih.gov) did not identify any other GCN2-type protein kinase sequences from arabidopsis.

3′ and 5′ RACE

The ends of the AtGCN2 transcript were amplified by RACE (rapid amplification of cDNA ends). The 3′ RACE experiment resulted in the amplification of two major and two minor PCR products (Fig. 3). The nucleotide sequences of these products showed them to be 388, 458, 579, and 591 bp in length and identified four polyadenylation sites, 38, 105, 212, and 240 nucleotides downstream of the stop codon TAA. Apart from length, the nucleotide sequences were identical with each other and with the genomic sequence. Putative polyadenylation signals precede these polyadenylation sites at positions 9 (AATAAA), 93 (ATATAT), 199 (AATAT) and 223 (ATAAAT) nucleotides downstream of the stop codon.

The 5′ RACE experiment also identified multiple termini (Fig. 3). Two major bands were identified after separation of nested PCR products by agarose gel electrophoresis and the nucleotide sequences of these products revealed that the smaller band contained three products (613, 618 and 628 bp in length) and the larger band two (683 and 686 bp in length). Apart from length, these matched each other and the genomic sequence exactly and terminated at positions −236, −241, −251, −306, and −309 with respect to the translation start codon, ATG. There are no other ATG codons between these transcription start sites and the ATG identified as the translation start site. The genomic nucleotide sequence contains putative TATA boxes at −280 (TATAAAA) and −416 (TATAAATA) with respect to the ATG.

Expression analysis of AtGCN2

Northern analysis was performed using total RNA from root, leaf, stem, flower bud, flower and young silique tissues, and from whole seedlings 2, 4, 6, 8 and 12 days after germination. The RNA was hybridised with an AtGCN2 probe. A major band of just over 4,000 nucleotides was detected in all of the RNA samples (Fig. 4a). The predicted transcript size from the PCR and RACE experiments was 4,272 nucleotides, using the initiation and termination sites furthest up- and downstream of the coding region. The size of the major band seen in the Northern is in good agreement with this. The other initiation and termination sites that were identified in the RACE experiments would give slightly smaller

transcripts but it is unlikely that the different transcript sizes would be resolved on the northern.

Two smaller, fainter hybridisation bands, representing transcripts of approximately 3,000 and 2,000 nucleotides, were also visible. As we have stated above, a BLAST search of the arabidopsis genome did not identify other GCN2-type protein kinase genes in arabidopsis, but clearly the smaller hybridising bands indicate the presence of transcripts with sequences that are similar enough to AtGCN2 to cause hybridisation. It is possible that the regions of similarity are too short to have shown up in the BLAST search.

Another band of very high molecular weight was visible in some of the RNA samples (not shown in Fig. 4a), possibly indicating the presence of unprocessed RNA molecules.

A non-quantitative RT–PCR experiment was performed with the RNA samples to confirm the result of the Northern analysis showing the presence of AtGCN2 transcripts in all of the RNA samples. Plasmid DNA containing the AtGCN2 PCR product was used as a positive control. The PCR reaction was primed with oligonucleotides designed to amplify a 1,575-bp product from first-strand AtGCN2 cDNA. A negative control in which the reverse transcription step was omitted was performed to show that the PCR product did not derive from contaminating genomic DNA in the RNA sample, although a product amplified from the *AtGCN2* gene would be expected to be much larger (3,553 bp) anyway because of the presence of introns. The results are shown in Fig. 4b. PCR products of the expected size were amplified from all of the RNA samples whereas no product was produced without the reverse-transcription step. No smaller products were produced.

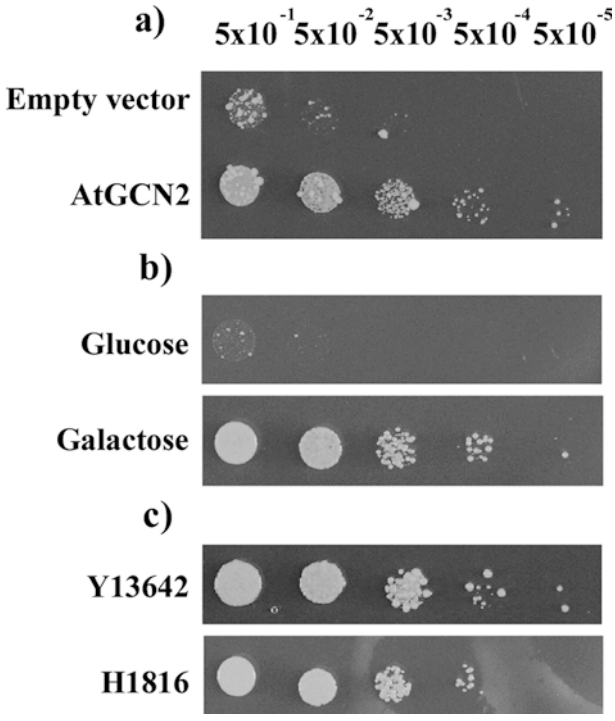

Fig. 5a–c Complementation of *gcn2* by AtGCN2. **a** Yeast *gcn2*$^-$ mutant strain Y13642 transformed with empty plasmid and plasmid for expression of AtGCN2 growing on glucose medium containing 0.25 mg/l SM, an inhibitor of branched-chain amino acid biosynthesis. **b** Yeast *gcn2*$^-$ mutant strain H1816 transformed with AtGCN2 expression plasmid growing on galactose (inducing) and glucose (non-inducing) media containing 10 mM 3-AT, an inhibitor of histidine biosynthesis. **c** Yeast strains Y13642 and H1816 transformed with AtGCN2 expression plasmid growing on galactose medium in the absence of any inhibitors. In each case, serial dilutions of cultures were spotted on to plates. The OD_{600} values of the suspensions are given across the top

Complementation of *gcn2* mutations by AtGCN2

In order to show functional conservation between AtGCN2 and yeast GCN2, the full-length AtGCN2 PCR product was subcloned behind the *GAL1* promoter in the low-copy-number yeast expression vector pYC2/CT. A low-copy-number plasmid was chosen because overexpression of eIF2α kinases on a multi-copy plasmid can cause slow growth (Chong et al. 1992).

In the first experiment, the plasmid containing the chimaeric gene was introduced into the *gcn2*$^-$ yeast strain, Y13642. Transformants were grown in liquid synthetic medium lacking uracil (to select for the presence of the plasmid) overnight at 28–30 °C. Serial dilutions were then made to adjust the OD_{600} to 0.5, 5×10^{-2}, 5×10^{-3}, 5×10^{-4} and 5×10^{-5} and an aliquot (5 µl) of each dilution was dropped onto plates. The medium on the plates contained either glucose (non-inducing) or galactose (inducing), and the inhibitor sulfometuron methyl (SM) at a concentration of 0.25 mg/l. SM is an inhibitor of branched-chain amino acid biosynthesis (Falco and Dumas 1985).

Transformants containing the AtGCN2 expression vector were able to grow in the presence of SM (Fig. 5a). However, it was not possible to show a clear difference between growth rates on the inducing and non-inducing media (the ideal control for the experiment). Indeed, if anything the yeast grew better with glucose as carbon source rather than galactose, suggesting that the *GAL1* promoter may be leaky in this yeast strain. Confirmation that AtGCN2 was enabling the yeast to grow in the presence of the inhibitor was obtained by comparing the growth rates of transformants containing the AtGCN2 expression plasmid with transformants containing an empty expression vector (Fig. 5a). The same result was obtained with glucose or galactose as carbon source, and Fig. 5a shows the result with glucose medium. The AtGCN2 gene enabled the yeast to grow much faster in the presence of the inhibitor, although growth was slightly slower than on medium without the inhibitor (Fig. 5c).

The complementation experiment was repeated in a different mutant strain, H1816, to allow the use of another inhibitor, 3-aminotriazole (3-AT). 3-AT is an inhibitor of the *HIS3* gene product, imidazoleglycerol-phosphate dehydratase, and thereby of histidine

biosynthesis. Growth on 3-AT medium requires an intact general amino acid control response (Hinnebusch and Fink 1983; Wolfner et al. 1975). 3-AT can not be used with strain Y13642 because Y13642 is *His*$^-$ and has to be supplied with histidine.

H1816 transformants containing the AtGCN2 expression plasmid grew well on galactose medium containing 10 mM 3-AT (Fig. 5b). In contrast to Y13642, H1816 transformants grew poorly on glucose medium containing the inhibitor, indicating that the *GAL1* promoter was not active in this strain in non-inducing conditions. This contrast between growth rates on inducing and non-inducing medium showed clearly that expression of AtGCN2 complemented the *gcn2* mutation. Furthermore, the transformants grew at similar rates on galactose medium whether the inhibitor was present or not (Fig. 5b, c).

Discussion

We have shown clearly the presence in arabidopsis of a gene, AtGCN2, that encodes a protein kinase that is structurally and functionally related to GCN2, a component of the regulatory system that is responsible for general amino acid control in budding yeast. AtGCN2 contains adjacent eIF2α kinase and His tRNA-synthetase-like domains, characteristic of GCN2-type protein kinases. It complemented the *gcn2* mutation well in two different yeast strains in the presence of two different inhibitors of amino acid biosynthesis.

There is evidence of co-ordinated regulation of genes encoding enzymes of amino acid biosynthesis in plants. For example, arabidopsis genes encoding tryptophan biosynthesis pathway enzymes have been shown to be induced by amino acid starvation caused by glyphosate and other treatments (Zhao et al. 1998). Furthermore, blocking histidine biosynthesis in arabidopsis with a specific inhibitor, IRL 1803, has been shown to increase the expression of eight genes involved in the synthesis of aromatic amino acids, histidine, lysine and purines (Guyer et al. 1995). However, this study found that starvation for aromatic or branched-chain amino acids did not initiate a general response. This is in contrast to general amino acid control in yeast, and the existence of general amino acid control in plants has not been accepted universally. The data that we present here are in agreement with the hypothesis that elements of general amino acid control are, indeed, conserved between yeast and plants.

The likely target for plant GCN2 would be serine-51 of eIF2α. This phosphorylation site is conserved in wheat eIF2α and has been shown to be phosphorylated by yeast GCN2 in vitro (Chang et al. 1999). Furthermore, wheat eIF2α has been shown to complement yeast mutants lacking eIF2α, enabling them to grow under conditions of histidine starvation imposed by 3-AT. The same yeast mutants expressing a mutant wheat eIF2α in which serine-51 was substituted with alanine could not grow under histidine starvation conditions. Nevertheless, further experiments will have to be undertaken to show that the mode of action of plant GCN2 is the same as that of GCN2 in yeast.

To date, the only plant protein kinase shown to phosphorylate eIF2α at serine-51 is a double-stranded RNA-dependent kinase, similar to human PKR (Meurs et al. 1990), that has been purified from wheat seedlings (Chang et al. 1999). As with its human homologue, this wheat protein kinase is activated in response to virus infection (Crum et al. 1988). The structure of plant PKR is not known but human and other animal PKRs do not contain a histidyl t-RNA synthetase-like domain, so the plant PKR activity is unlikely to correspond to AtGCN2.

Acknowledgement Rothamsted receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Chang L-Y, Yang WY, Browning K, Roth D (1999) Specific in vitro phosphorylation of plant eIF2α by eukaryotic eIF2α kinases. Plant Mol Biol 41:363–370

Chang L-Y, Yang WY, Roth D (2000) Functional complementation by wheat eIF2α in the yeast GCN2-mediated pathway. Biochem Biophys Res Commun 279:468–474

Chen J-J, Throop MS, Gehrke L, Kuo I, Pal JK, Brodsky M, London IM (1991) Cloning of the cDNA of the heme-regulated eukaryotic initiation factor-2 alpha (eIF2α) kinase of rabbit reticulocytes—homology to yeast GCN2 protein kinase and human double-stranded RNA-dependent eIF2α kinase. Proc Natl Acad Sci USA 88:7729–7733

Chong KL, Feng L, Schappert K, Meurs E, Donahue TF, Friessen JD, Hovanessian AG, Williams BRG (1992) Human P68 kinase exhibits growth suppression in yeast and homology to the translational regulator GCN2. EMBO J 11:1553–1562

Crum CJ, Hiddinga HJ, Roth DA (1988) Tobacco mosaic virus infection stimulates the phosphorylation of a plant protein associated with double-stranded RNA-dependent protein kinase activity. J Biol Chem 263:13440–13443

Falco SC, Dumas KS (1985) Genetic analysis of mutants of *Saccharomyces cerevisiae* resistant to the herbicide sulfometuron methyl. Genetics 109:21–35

Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/S-DNA/PEG procedure. Yeast 11:355–360

Guyer D, Patton D, Ward E (1995) Evidence for cross-pathway regulation of metabolic gene expression in plants. Proc Natl Acad Sci USA 92:4997–5000

Hinnebusch AG (1992) General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in *Saccharomyces cerevisiae*. In: Jones EW, Pringle JR, Broach JB (eds) Mol Cell Biol Yeast *Saccharomyces*, vol 2, Gene Expression. Cold Spring Harbor Laboratory Press, New York, pp 319–414

Hinnebusch AG (1994) Translational control of GCN4—an in vivo barometer of initiation factor activity. Trends Biochem Sci 19:409–414

Hinnebusch AG (1997) Translational regulation of yeast GCN4—a window on factors that control initiator-tRNA binding to the ribosome. J Biol Chem 272:21661–21664

Hinnebusch AG, Fink GR (1983) Positive regulation in the general amino acid control of *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 80:5374–5378

Icely PL, Gros P, Bergeron JJM, Devault A, Afar DEH, Bell JC (1991) TIK, a novel serine threonine kinase, is recognized by antibodies directed against phosphotyrosine. J Biol Chem 266:16073–16077

Langland JO, Langland L, Zeman C, Saha D, Roth DA (1996) Phosphorylation of plant eukaryotic initiation factor-2 by the plant encoded double-stranded RNA-dependent protein kinase, pPKR, and inhibition of protein synthesis in vitro. J Biol Chem 271:4539–4544

Mellor H, Flowers KM, Kimball SR, Jefferson LS (1994a) Cloning and characterization of cDNA encoding rat hemin-sensitive initiation factor-2 alpha (eIF2α) kinase—evidence for multi-tissue expression. J Biol Chem 269:10201–10204

Mellor H, Flowers KM, Kimball SR, Jefferson LS (1994b) Cloning and characterization of a cDNA encoding rat PKR, the double-stranded RNA-dependent eukaryotic initiation factor-II kinase. Biochim Biophys Acta 1219:693–696

Meurs E, Chong K, Galabru J, Thomas NSB, Kerr IM, Williams BRG, Hovanessian AG (1990) Molecular cloning and characterization of the human double stranded RNA-activated protein kinase induced by interferon. Cell 62:379–390

Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG, Marton MJ (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. Mol Cell Biol 21:4347–4368

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Samuel CE (1993) The eIF2α protein kinases, regulators of translation in eukaryotes from yeasts to humans. J Biol Chem 268:7603–7608

Santoyo J, Alcalde J, Mendez R, Pulido D, de Haro C (1997) Cloning and characterization of a cDNA encoding a protein synthesis initiation factor-2 alpha (eIF2α) kinase from *Drosophila melanogaster*—homology to yeast GCN2 protein kinase. J Biol Chem 272:12544–12550

Sattlegger E, Hinnebusch AG, Barthelmess IB (1998) cpc-3, the *Neurospora crassa* homologue of yeast GCN2, encodes a polypeptide with juxtaposed eIF2α kinase and histidyl-tRNA synthetase-related domains required for general amino acid control. J Biol Chem 273:20404–20416

Wek RC, Jackson BM, Hinnebusch AG (1989) Juxtaposition of domains homologous to protein kinases and histidyl transfer RNA synthetases in GCN2 protein suggests a mechanism for coupling GCN4 expression to amino acid availability. Proc Natl Acad Sci USA 86:4579–4583

Wolfner M, Yep D, Messenguy F, Fink GR (1975) Integration of amino acid biosynthesis into the cell cycle of *Saccharomyces cerevisiae*. J Mol Biol 96:273–290

Zhao J, Williams CC, Last RL (1998) Induction of *Arabidopsis* tryptophan pathway enzymes and camalexin by amino acid starvation, oxidative stress, and an abiotic elicitor. Plant Cell 10:359–370