

Complementation of *snf1*, a mutation affecting global regulation of carbon metabolism in yeast, by a plant protein kinase cDNA

(cereals/seeds/development/carbon partitioning)

ALISON ALDERSON*, PAOLO A. SABELLI[†], J. RICHARD DICKINSON[‡], DEBORAH COLE[‡],
MICHAEL RICHARDSON[§], MARTIN KREIS[¶], PETER R. SHEWRY[†], AND NIGEL G. HALFORD*^{||}

*Department of Biochemistry and Physiology, Agricultural and Food Research Council, Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Hertfordshire, AL5 2JQ, United Kingdom; [†]Agricultural and Food Research Council, Institute of Arable Crops Research, Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Bristol BS18 9AF, United Kingdom; [‡]School of Pure and Applied Biology, University of Wales, P.O. Box 915, Cardiff CF1 3TL, United Kingdom; [§]University of Durham, Department of Biological Sciences, Science Laboratories, South Road, Durham DH1 3LE, United Kingdom; and [¶]Universite de Paris-Sud, Biologie du Developpement des Plantes, F-91400 Orsay Cedex, France

Communicated by Ralph Riley, June 24, 1991

ABSTRACT A cDNA, cRKIN1, encoding a putative homologue of the yeast (*Saccharomyces cerevisiae*) *SNF1*-encoded protein-serine/threonine kinase, has been isolated from a library prepared from rye endosperm mRNA. Northern blot analysis demonstrated the presence of cRKIN1-related transcripts in developing endosperms but not in shoots, and Southern blot analysis showed the presence of a small gene family. *SNF1* plays a central role in carbon catabolite repression in yeast and expression of the RKIN1 sequence in yeast *snf1* mutants restored *SNF1* function. This suggests that the RKIN1 protein has a role in the control of carbon metabolism in endosperms of rye.

Protein kinases are known to play key roles in animals and yeasts in the control of cell division (1) and in cellular response to external stimuli and in metabolic regulation (2). For example, the role of insulin in controlling glycogen synthesis in mammals is mediated by an array of phosphorylation and dephosphorylation steps, the former catalyzed by protein kinases (3). Although it has been suggested that protein kinases play similar roles in plants (4), this remains to be demonstrated. However, recent studies have shown that protein kinases related to the *cdc2* cell cycle protein of fission yeast (5) and its homologues in budding yeast (6), human cells (7), and *Xenopus* (8) are also present in pea (9) and *Arabidopsis* (10). All protein kinases that have been characterized in detail contain a number of key residues and conserved regions in the catalytic domain (11) but can be divided into two classes: those that phosphorylate serine/threonine residues and those that phosphorylate tyrosine residues. In the present report, we present the nucleotide sequence of a cDNA, cRKIN1,** isolated from a rye endosperm cDNA library. The cRKIN1-encoded protein contains all the invariant residues and conserved domains characteristic of eukaryotic protein-serine/threonine kinases. It is particularly similar to the product of the *SNF1* gene of yeast (*Saccharomyces cerevisiae*) (12), a protein affecting global regulation of carbon metabolism, and the expression of cRKIN1 in yeast *snf1* mutants restores *SNF1* function.

MATERIALS AND METHODS

Construction of a cDNA Library from Rye Endosperm mRNA and Isolation of λ cRKIN1. Free and membrane-bound polysomes were prepared from rye endosperms 20 days after anthesis. Poly(A)⁺-rich RNA was purified by affinity chromatography using oligo(dT)-cellulose. cDNA was prepared

from this material using the Amersham cDNA synthesis kit and was cloned into the vector λ gt10. The resulting cDNA library contained 3×10^5 recombinants. λ cRKIN1 was isolated from this library in the course of an unrelated experiment.

DNA Sequencing. The cDNA insert from λ cRKIN1 was excised and ligated into pUC18 to produce the plasmid pcRKIN1. Sequential overlapping deletions of the insert were generated by linearization followed by digestion with exonuclease III. These were excised and cloned into M13mp18 for sequencing. The entire insert was sequenced in both directions and the sequences were analyzed using the programs of the University of Wisconsin Genetics Computer Group (13).

Southern and Northern Blot Analyses. A Southern blot of rye genomic DNA and a Northern blot of rye endosperm and shoot mRNA were probed with the full-length insert from pcRKIN1 using the methods described by Sabelli and Shewry (14). The blots were washed at high stringency (65°C in 0.5× standard saline citrate) before autoradiography.

Construction of Plasmid pDC1. The yeast expression plasmid pMA99 was kindly provided by S. M. Kingsman and A. J. Kingsman (University of Oxford, U.K.). It includes a *Bam*HI fragment containing the *Escherichia coli lacZ* gene downstream of the yeast phosphoglycerate kinase (*PGK1*) gene promoter. This fragment was removed and replaced with a *Stu*I-*Eco*RI fragment of cRKIN1 ligated to Pharmacia *Bam*HI 5'-phosphorylated linkers. This fragment contains 84 base pairs of the leader sequence, the whole coding region, and the 3' flanking region.

Yeast Strains. Yeast strain MCY1845 (*MAT α ade2 ura3 snf1 Δ 10*) kindly provided by M. Carlson (Columbia University, New York) was mated to strain DBY747 (*MAT α his3 leu2 ura3 trp1*) and the resulting diploid was sporulated to yield a variety of segregants including strain 30.1.6 (*MAT α his3 ura3 trp1 snf1 Δ 10*).

RESULTS AND DISCUSSION

A cDNA clone, pcRKIN1, was isolated from a rye endosperm cDNA library in the course of an unrelated experiment. The cDNA insert, consisting of 1809 base pairs plus a poly(A) tail, was completely sequenced in both directions (Fig. 1). It contains an open reading frame from the start of the sequence to a stop codon (TAG) at position 1616. The length of the transcript seen in Northern blot analysis (see

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

^{||}To whom reprint requests should be addressed.

**The sequence reported in this paper has been deposited in the GenBank data base (accession no. M74113).

1 AAACCTCTCTCGATTGGGTCGGAGGCCCTCCCTCGGCTCCACTGGGCGGGCGCTCTCGGCACTCCCTCCGTCGCGG
 1-H D G G E H S E A L K N Y
 76 GCGGTGATGACACCGGATTTGGCCAGTGAATAATGGATGGAGGGCGAACATTTGAAAGCATTTGAAATACTA
 15 Y L G K I L G V G T F A K V I I A E H K H T R R K
 151 CTATCTGGGTAAATATATTAGGTGAGGACATTTGCAAAAGATAAATTTGACAGAGCAATGACGATCAAGACACAA
 40 V A I E V L N R R O M R G A P E H E E K A K R E I
 226 AGTTGCTATAAAGTCTTGAACCGCGCTCAAATGCRAGCTCCAGAAATGGAGAGCAAGCAAGAGAAATCA
 65 I L R L F I D L I H P H I I R V Y E V I V T P R D
 301 GATATTGAGGTGTTGATTTGACTTAATTCACCCCTCATATCATCCGGGTTATGAGGTCATTGTGACCGAAAGA
 90 I F V V M E Y C Q N G D L L D Y I L E K R R L Q E
 376 TATTTTTGTGTGATGGAAATTTGCCAAATATGGTGAACCTATTGGACTACATTTTGAAAGACGGCGGTACAGGA
 115 D E A R R T F Q Q I I S A V E Y C H R N K V V H R
 451 AGACGAGCTCGTCAACCTTCAGCAGATTATATCTGCTGTTGAATCTGCCAGAAACAAGGTTGTTTCATCG
 140 D L K P E N L L L D S K Y N V K L A D F G L S N V
 526 TGATCTAAAGCCAGAAACCTGTACTTGAATTCATAATAATGTAAGCTTGGGTTAAGTAAVTG
 165 H H D G H F L K T S C G S L N Y A A P E V I S G R
 601 GATGATGATGACCAATTTTTCAGAGCTAGCTGGGAGTCTAAACTATGCTGCCAGAGGTCATCTCGGTA
 190 L Y A G P E I D V M S C G V I L Y A L L C G A V P
 676 ATTTGACCTGGACCTGAGATGATGTTTGGAGCTGTGGGTGATCTTATGCTCTCTTTGTGCTGCTGTTCC
 215 F D D D N I F N L P R Y L A V P P P D M H Q Q A K
 751 ATTTGATGATGACCAATTTCCCACTGTTCAAAGATAAGGGAGGTACTACATCTTCCAAATTTATTTATC
 240 D L V R D L I S R M L I V D P M K R I T I G E I R
 826 TGATCTTGAAGGATTTGATCTCAAGAAATGCTTATTTGATCCGATGAAGAGATCAACAATTTGTAATTCG
 265 K H S W F Q N R L P R Y L A V P P P D M H Q Q A K
 901 AAAACACTATGGTTTCAGAAATCGCTTCTCGCTAGCTGCTCCACAGATGATGTCGAGCAAGCCAA
 290 H I D E D T L R D V V K L G Y D K D H V C E S L C
 976 AATGATTGATGAAGATACACTTCGAGAGCTTGTCAAATCGGATGATGATAAAGATCATGTGTGTGAATCGCTGTG
 315 N R L Q N E E T V A Y Y L L L D N R F R A T S G Y
 1051 CAATAGCTGCAAAAAGAGAACTGTGTCATATTACTGCTTGGCAATCGGTTCCGGGCTACTAGTGCTCA
 340 L G A H Y Q Q P M E S A S P S T R S Y L F G S N D
 1126 TTTGGGGCTCACTATCAACCAAGTGAATGAGCAAGCCCAAGTACAGGAGTATCTTCCAGGAAGCAATGA
 365 S Q G S G L R P Y Y R C R V E R K M A L G L Q Q S R A
 1201 TTTCTAAGGAGCTGCTTGGCCATATTAACCGGTTGAAAGAAATGGCTTCTGGGCTCAGCAGTCTCGAAGC
 390 P P R A I M I E V L K A L K E L N V C W K R N G D
 1276 TCCCTCTGGTGCATATGATTTGAGGTTCTAAAGGCACTTAAGGAATTAATGCTGTGGAAAGAAAGTGGAGA
 415 C Y N H K C R W C P G P R V S D M L L D A N H S
 1351 CTGCTACAACTGAAATGCAGGTGGTCCCTGGGTTCTCGGGTCAGTGATATGTTGTAGATGCCAACACAG
 440 F V D D C A I K D N G D A N S R L P A V I K F E I
 1426 TTTTGTGATGACTGTGCCATCAAGGATAATGGCGATGCTAATAGTAGGCTACTGCTGTGATCAAGTTGAAAT
 465 Q L Y K T K D D K Y L L D M Q R V T G P Q L L F L
 1501 TCAGCTTTACAGCAAGGATGACAACTGCTGATGATGACAGAGTACTGCACTCAGCTCCTCTCTCTCT
 490 E F C A A F L T N L R V L stop
 1576 GGAATTTGTGGCGGCTCCCTTACCAACTTAGGTTCTATAGCTAGTGCATTTCTGGTGTGAGTGGTAAATAGC
 1651 AAACATAAGACTGCTCCGCTGAGTGTGTTCCAGCGCTCGCAGTGAATAAGTATGGTGTGTAATAACTAAAT
 1726 AGGCTGTGTGTTTTCATATGGTTTACCAGCTCCCTGACAGCGAACCGGTTGTTTGAAGTACTAGTATTGTG
 1801 TCAAGCACAAAAA

FIG. 1. Nucleotide and derived amino acid sequence of cRKIN1. Invariant residues characteristic of eukaryotic protein-serine/threonine kinases are indicated (*).

below) and sequences obtained for the 5' ends of genomic clones of homologous genes from barley and *Arabidopsis* (unpublished data) suggests that the cRKIN1 cDNA is complete or nearly complete and that the ATG sequence at position 110 is the translation start site. The open reading frame is, therefore, 1506 base pairs long and encodes a protein of 502 amino acid residues (Fig. 1) with a calculated molecular weight of 57,710. The protein is particularly rich in leucine (11.6 mol %) and lysine (7.2 mol %). It contains all the invariant or nearly invariant residues and all the conserved domains characteristic of eukaryotic protein-serine/threonine kinases (11). The invariant residues are Gly²¹, Gly²³, Val²⁸, Ala⁴¹, Lys⁴³, Glu⁶², Asp¹⁴⁰, Asn¹⁴⁵, Asp¹⁵⁸, Phe¹⁵⁹, Gly¹⁶⁰, Glu¹⁸⁴, Asp¹⁹⁷, Gly²⁰², and Arg²⁵⁷ (these are indicated in Fig. 1). The 11 conserved domains lie between Leu²⁰ and Arg²⁵⁷, with the core catalytic domain (11) consisting of residues Ala¹²⁷ to Tyr²⁰⁶. The sequence Asp¹⁴⁰-Leu-Lys-Pro-Glu-Asn is indicative of serine/threonine specificity, tyrosine kinases having a different consensus in this region.

Searches of the GenBank and EMBL nucleotide sequence data bases and the National Biomedical Research Foundation protein sequence data base (January 4, 1991) matched the RKIN1 amino acid sequence most closely with that of the *SNF1* gene product of the budding yeast (*S. cerevisiae*) (12). The *SNF1* gene product is a protein-serine/threonine kinase required for the derepression of several glucose-repressible genes, including *SUC2* (invertase). The RKIN1 and *SNF1* amino acid sequences are aligned in Fig. 2. The catalytic domains are most highly conserved but conserved regions

RKIN1 MDGGGEHSE 9
 SNF1 MSSNNNTNTPANANSHHHHHHHHHHHHGHGGSNSTLNNPKSSLADGA 50
 ALKNYYLGLKLVGVTFAKVI IAEHKHTRHKVAIKVLRNRQMRAPEMEKA 59
 HIGNYQIVKTLGEGSPGKVKLAYHTTTGQKVALKIINKKVLAKSDMQGRI 100
 KREIKILRLFIDLHPIH IIRVYEVIVTPKDFVVMVEYQNGDLDLYILEK 109
 EREISYLRL...LRHPHIIKLYDVIKSKDEIIMVIEYAGN.ELFDYIVQR 146
 RRLQDEARRTFQOIIISAVEYCHRNKVVHRDLKPENLLDLSKYNVKLADF 159
 DKMSEQEARRFQOIIISAVEYCHRNKIVHRDLKPENLLDHLNPKIADF 196
 GLSNVMHDGHFLKTS CGSLNYAAPEVISGKLYAGPEIDVWSCGVILYALL 209
 GLSNIMTDGNFLKTS CGSPNYAAPEVISGKLYAGPEVDVWSCGVILYVML 246
 CGAVPFDDDDNIPNLFKKIKGGTYILPIYLSDLVRDLISRMLIVDPMKRIT 259
 CRRLPFDDDESIPVLFKNISNGVYTLPLFSPGAAGLIRKMLIVNPLNRIS 296
 IGEIRKHSWFQNLPRYLAVPPDMMQAKMIDE..... 293
 IHEIMQDDWFKVDLPEYLL...PPDLKPHPEENENNDSKKDGGSSPDNDEI 344
 .DTLRDVV...KLGDKDHVCELSLNRNQ...NEETVAYYLLLDNRF... 333
 DDNLVNLISSTMGYKEDIYESLESSEDTAPAFNEIRDAYMLIKENKSLIK 394
RATSGYLGAHYQQP.....MESA 351
 DMKANKSVSDELDTFLSQSPPTFQQQSKSHQKSQVDHETAKQHARMASA 444
SFSTRSYLPGS.....NDSQSGSLR 371
 ITQRTYHQSPFMDQYKEEDSTVSIPLTSLPQIHRANHLAQGSPAASKIS 494
 PYY..RVERKRWALGQSRAPPRAIMIEVLKALKELNVCKWK..NGDCYN 417
 PLVTKSKTRWHFGI..RRSYPLDVMGEIYIALKNLGAEWAKPSEDLWT 543
 MKRCWCPGPPRVSDMLLDANHSFVDDCAIKDNGDANSRLPAVIFKEIQLY 467
 IKLRW.....KYDIGNKTNTNEKIPDLNKMVIQLF 573
 KTKDDKYLDM.....QRVTPQLLFLFCAAF 496
 QIETNNYLVDFKFDGWESSYGD DTTVSNISEDEMSTFSAYPFLHLTKLI 623
 TNLRVL-502
 MELAVNSQSN-633

FIG. 2. Derived amino acid sequence of the protein encoded by cRKIN1 aligned with that encoded by the *SNF1* gene of yeast. The alignment was made using the program GAP (13) with a gap weight of 3.0 and a gap length weight of 0.1.

and residues are present throughout the sequences. The GAP program (13) calculates a similarity of 66.9% between the RKIN1 and *SNF1* amino acid sequences. Of the 80 residues that constitute the core catalytic region of each protein, 69 are identical and 6 of the 11 substitutions are conservative.

Southern blot analysis of rye genomic DNA probed with the cRKIN1 cDNA insert showed several strongly hybridizing bands (Fig. 3A), indicating a small multigene family. The presence of other more weakly hybridizing bands showed that less closely related sequences were also present. Strongly hybridizing sequences are also present in *Arabidopsis*, barley, and wheat (unpublished data). Northern blot analysis showed that RKIN1-related transcripts of ≈1800 nucleotides in length were present in developing endosperms but not in shoots (Fig. 3B).

To determine the function of the RKIN1 protein, its ability to restore *SNF1* activity in yeast (*S. cerevisiae*) *snf1* mutants was tested. Strains of *S. cerevisiae* carrying *snf1* mutations fail to derepress many of the enzymes of oxidative metabolism. Consequently, they are unable to utilize substrates such as glycerol and ethanol. Yeast 30.1.6, carrying the *snf1* mutation, was transformed with plasmid pDC1 (Fig. 4A). pDC1 is maintained at low copy number (1-2 copies per cell) because of the presence of the CEN3 sequence, behaving like a tiny natural chromosome in terms of replication and partitioning. It contains the yeast phosphoglycerate kinase (*PGK1*) gene promoter up to nucleotide -2 (15), downstream of which a fragment containing the RKIN1 sequence from 84 base pairs upstream of the ATG to the 3' end has been

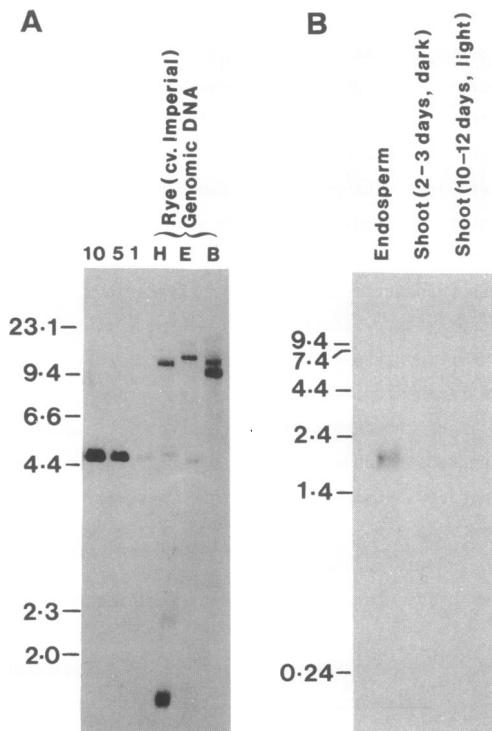


FIG. 3. (A) Southern blot analysis of genomic DNA from rye cv. Imperial, digested with restriction enzymes *Hind*III (H), *Eco*RI (E), and *Bam*HI (B), with linearized pcRKIN1 plasmid representing 10, 5, and 1 copies of the hybridizing sequence per haploid genome, as indicated. (B) Northern blot analysis of RNA from endosperms and shoots of rye cv. Imperial. The relative mobilities of size markers (in kilobases) are also shown. Both filters were probed with the full-length pcRKIN1 insert.

inserted. The resulting chimeric gene (Fig. 4B) contains all the yeast signals required for efficient initiation of transcrip-

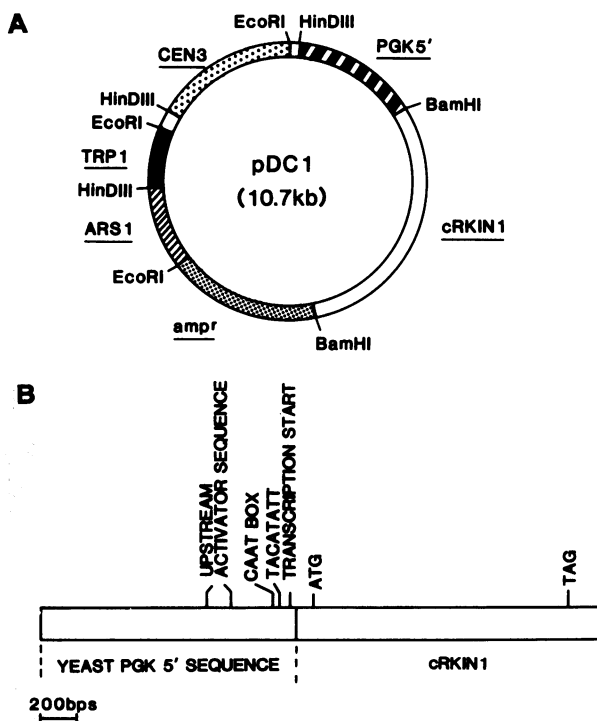


FIG. 4. (A) Plasmid pDC1 used for the expression of the RKIN1 protein in yeast. (B) The chimeric gene resulting from the ligation of the cRKIN1 sequence downstream of the *PGK1* gene promoter.

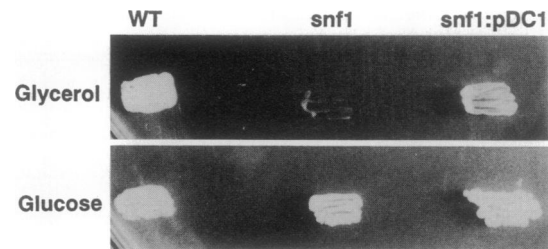


FIG. 5. Growth of yeast (*S. cerevisiae*) wild type (WT), *snf1* mutant strain 30.1.6 (*snf1*), and strain 30.1.6 transformed with plasmid pDC1 (*snf1:pDC1*) on glycerol and glucose media.

tion and uses the RKIN1 signals for the start and termination of translation. Transformants containing pDC1 were sought by plating-out on glycerol and ethanol minimal medium lacking tryptophan (the selectable marker in plasmid pDC1 is *TRP1*). *Bona fide* transformants grew on the glycerol and ethanol medium (Fig. 5). This indicates that the rye RKIN1 protein is able to perform the same function in yeast as the *SNF1* gene product.

SNF1 plays a central role in carbon catabolite repression in yeast (12). It was identified by screening for genes involved in the regulation of invertase gene expression and is required for the expression of several glucose-repressible genes in response to glucose deprivation. The *snf1* mutants are unable to utilize sucrose, melibiose, galactose, or nonfermentable carbon sources. *SNF1* controls *SUC2* (invertase) expression at the transcriptional level (16), *snf1* mutants failing to produce *SUC2* mRNA (17).

Although cereals are the major food crops in the world and starch is their major storage product (accounting for 70–80% of the grain dry weight), nothing is known of the factors controlling the partitioning of assimilated carbon between starch and other storage products (mainly oil and proteins). Similarly, the role of invertase in the developing endosperm is unclear. Doehlert and Felker (18) demonstrated that invertase activity was restricted to the basal and pedicel tissues in developing maize endosperms and consisted of soluble and cell wall-associated forms. They proposed that the location of the latter form is consistent with a role in phloem unloading, sucrose hydrolysis maintaining a concentration gradient between the phloem and apoplast. In contrast, Donovan *et al.* (19) demonstrated that sucrose was not metabolized during its movement along the wheat grain, suggesting that sucrose hydrolysis is not essential for phloem unloading.

Our isolation of the RKIN1 cDNA indicates that protein phosphorylation may play a regulatory role in carbohydrate metabolism in developing cereal endosperms, as in mammalian tissues. It also provides an opportunity to explore the control of carbohydrate metabolism and in particular the role of invertase *in vivo* in transgenic plants. Finally, our success in complementing the *snf1* mutation suggests that complementation of yeast mutations may prove to be a useful tool for isolating other genes that regulate plant metabolism.

We thank Jonathan Wingfield for advice on yeast genetic manipulation.

- Dunphy, W. G. & Newport, J. W. (1988) *Cell* 55, 925–928.
- Hunter, T. (1987) *Cell* 50, 823–829.
- Dent, P., Lavoigne, A., Nakielny, S., Caudwell, F. B., Watt, P. & Cohen, P. (1990) *Nature (London)* 348, 302–308.
- Ranjeva, R. & Boudet, A. M. (1987) *Annu. Rev. Plant Physiol.* 38, 73–93.
- Nurse, P. & Bissett, Y. (1981) *Nature (London)* 292, 558–560.
- Reed, S. I., Hadwiger, J. A. & Lorincz, A. T. (1985) *Proc. Natl. Acad. Sci. USA* 82, 4055–4059.
- Draetta, G., Brizuela, L., Potashkin, J. & Beach, D. (1987) *Cell* 50, 319–325.

8. Gautier, J., Norbury, C., Lohka, M., Nurse, P. & Maller, J. (1988) *J. Cell Biol.* **54**, 433–439.
9. Feiler, H. S. & Jacobs, T. W. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5397–5401.
10. John, P. C. L., Sek, F. J. & Lee, M. G. (1990) *The Plant Cell* **1**, 1185–1193.
11. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241**, 42–52.
12. Celenza, J. L. & Carlson, M. (1986) *Science* **233**, 1175–1180.
13. Devereux, J., Haerberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.
14. Sabelli, P. A. & Shewry, P. R. (1991) *Theor. Appl. Genet.*, in press.
15. Dobson, M. J., Tuite, M. F., Roberts, N. A., Kingsman, A. J. & Kingsman, S. M. (1982) *Nucleic Acids Res.* **10**, 2625–2636.
16. Sarokin, S. & Carlson, M. (1985) *Mol. Cell. Biol.* **5**, 2521–2526.
17. Carlson, M. & Botstein, D. (1982) *Cell* **28**, 145–154.
18. Doehlert, D. C. & Felker, F. C. (1987) *Physiol. Plant.* **70**, 51–57.
19. Donovan, G. R., Jenner, C. F., Lee, J. W. & Martin, P. (1983) *Aust. J. Plant Physiol.* **10**, 31–42.