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NPK1, a Tobacco Gene That Encodes a Protein with a Domain Homologous to Yeast BCK1, STE11, and Byr2 Protein Kinases

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We have isolated a cDNA (cNPK1) that encodes a predicted protein kinase of 690 amino acids from suspension cultures of tobacco cells. The deduced sequence is closely related to those of the protein kinases encoded by the *STE11* and *BCK1* genes of *Saccharomyces cerevisiae* and the *byr2* gene of *Schizosaccharomyces pombe*. STE11 and Byr2 function in the yeast mating pheromone response pathways, and BCK1 acts downstream of the yeast protein kinase C homolog encoded by the *PKC1* gene, which is essential for normal growth and division of yeast cells. Overexpression in yeast cells of a truncated form of cNPK1, which encodes only the putative catalytic domain, replaced the growth control functions of *BCK1* and *PKC1* but not the mating pheromone response function of *STE11*. Thus, the catalytic domain of NPK1 specifically activates the signal transduction pathway mediated by BCK1 in yeast. In tobacco cells in suspension culture, the *NPK1* gene is transcribed during logarithmic phase and early stationary phase but not during late stationary phase. In a tobacco plant, it is also transcribed in stems and roots but not in mature leaves, which rarely contain growing cells. The present results suggest that a signal transduction pathway mediated by this BCK1- and STE11-related protein kinase is also conserved in plants and that a function of NPK1 is controlled at least in part at a transcriptional level.

The growth of plant cells has several unique features. The most characteristic features of the growth processes are observed during cytokinesis and the elongation of plant cells, which are tightly coupled with the formation of new cell walls inside and outside the cells, respectively. The formation of new cell walls both inside and outside cells is thought to be controlled by specifically organized microtubules (13, 52). These observations suggest that division and growth of plant cells may involve mechanisms characteristic of plants as well as those that function generally in eukaryotic cells. However, an understanding of the molecular mechanisms that regulate these processes in plant cells is almost completely lacking, although plant homologs of yeast genes, such as *cdc2* (4, 10, 11, 16, 20, 21), the cyclin gene (18, 19), and *dis2* of *Schizosaccharomyces pombe* (39), which may be involved in the plant cell cycle, have recently been described.

Mechanisms that control the division and growth of yeast and animal cells are now being dissected at the molecular level, although our understanding is still far from complete. Among the factors that are required for cell division and growth, the protein kinase C (PKC) family is ubiquitous in both animals and yeasts and is thought to play a central role in the regulation of cellular functions, which include cell growth (22). Ten distinct subtypes of mammalian PKC have been reported to date (38). In addition, genes that encode putative PKCs have been isolated from yeasts: one homolog, designated *PKC1*, from *Saccharomyces cerevisiae* (33, 54) and two from the fission yeast *S. pombe* (48). Yeast cells depleted of the *PKC1* gene product show a cell lysis defect, although a medium with high osmolarity can prevent cells from lysing (32). Thus, PKC1 is essential for normal growth and division of yeast cells.

Although mammalian PKC induces the transcriptional activation of a wide array of genes, which include the proto-oncogene c-*fos* and the gene for collagenase (17, 36), the processes from activation of PKC to the induction of transcription remain obscure. In yeast cells, a class of dominant suppressor mutations which bypass the requirement for *PKC1* in cell division and growth has recently been isolated and the suppressor locus, *BCK1* (bypass of C kinase), has been characterized (31). The *BCK1* gene is predicted to encode a protein kinase with a putative catalytic domain that has 45% amino acid identity with that of the *STE11*-encoded protein kinase of *S. cerevisiae*. The latter protein is required for the response to mating pheromones of haploid yeast cells (34, 41). Deletion of *BCK1* results in a temperature-sensitive cell lysis defect, which is suppressed by osmotic stabilizers such as sorbitol (31). The *pkc1* deletion mutants also display a cell lysis defect, but at both low (25°C) and high (35°C) temperatures. On the basis of these observations, it has been proposed that *BCK1* might function downstream of *PKC1* within the same signal pathway. However, *PKC1* would regulate a bifurcated pathway in which *BCK1* functions on one branch (31).

The pathway steps that function downstream of STE11 and BCK1 in the respective signalling pathways activated by those protein kinases in budding yeast have been further investigated. Genetic and biochemical analyses indicate that STE11 acts upstream of the STE7, FUS3, and KSS1 protein kinases (2, 12, 45, 55). Recently it was revealed that STE7 directly activates FUS3, which belongs to a family of mitogen-activated protein (MAP) kinases, by phosphorylation, (9). *S. pombe* also has a mating pheromone response signal pathway, which involves Byr2 (a homolog of STE11), Byr1 (a homolog of STE7), and Spk1 (a homolog of MAP kinase) (34, 37). Similarly, the PKC1- and BCK1-activated signal pathway has been shown to contain two additional families

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of protein kinases that act downstream of BCK1, MKK1 and MKK2 (24) and MPK1 (30) (formerly SLT2 [49]), which are structurally related to STE7 (and also Byr1) and to FUS3 and KSS1 (and also Spk1), respectively. It has also been shown that activator kinases of MAP kinases (MAPKKs) from mice, rats, and *Xenopus* spp. share significant amino acid sequence identity with STE7, Byr1, and MKK1 and MKK2 (5, 27, 53). Thus, the signaling systems mediated by MAPKK (or MAPKK homologs such as STE7, MKK1 and MKK2, and Byr1) and MAP kinase (or its homologs) are conserved in a variety of eukaryotes and are probably ubiquitous. In addition to being isolated from these organisms, MAPKK homologs have been isolated from *Drosophila melanogaster* (34, 50) and *Ustilago maydis* (1). However, no protein kinases that exhibit structural similarity to STE11, BCK1, or Byr2 have been isolated from multicellular eukaryotes.

In the course of experiments in which we attempted to clone plant homologs of genes for PKC and cyclic AMP (cAMP)-dependent protein kinase, we have isolated a tobacco cDNA for a *BCK1*- and *STE11*-related gene, called cNPK1, which provides the first demonstration of a *BCK1*- and *STE11*-related gene from a multicellular organism. Overexpression of cNPK1 in yeast mutants was shown to complement the *bck1* deletion mutation but not the *ste11* mutation. Overexpression of a truncated form of cNPK1 also suppressed the growth defect associated with the *pkc1* deletion mutation. These observations suggest that a signal transduction pathway similar to that mediated by BCK1 or STE11 exists in plants.

MATERIALS AND METHODS

Plant materials. Tobacco plants were grown in a greenhouse. Suspension cultures of the BY-2 cell line of tobacco (26) were maintained in modified Linsmaier and Skoog medium (40) with 0.2 mg of 2,4-dichlorophenoxyacetic acid per liter and with weekly dilutions.

Yeast strains, growth conditions, and transformation. The *S. cerevisiae* strains used were DL251 (*MAT***a**/*MAT*α *leu2 ura3 trp1 his4 can-1*^{r} *bck1*Δ::*URA3*), KMY167-7A (*MAT*α *leu2 ura3 trp1 his3 tyr1 ste11*^{ts}), 15Dau (*MAT***a** *ade1 leu2 ura3 trp1 his2*), and SNY235 (*MAT***a** *leu2 ura3 his4 trp1 pkc1*Δ::*LEU2*). Yeast cultures were grown in YEP (1% Bacto yeast extract, 2% Bacto Peptone) supplemented with 2% glucose. SD medium (0.7% yeast nitrogen base without amino acids [Difco Laboratories, Detroit, Mich.] and 2% glucose), supplemented with the appropriate nutrients, was employed for selection of cells with plasmids. Yeast cells were transformed by the lithium acetate method (25). General genetic manipulations were carried out as described previously (14). Plates containing SD medium supplemented with the following appropriate requirements were used to monitor formation of diploids: 20 μg of leucine and 20 μg of uracil per ml for diploids formed with YEpGAP112 or its derivatives and 50 μg of tryptophan and 20 μg of uracil per ml for diploids formed with pSTE11.1 (3).

Preparation of RNA from plant cells. Total RNA was isolated as previously described (46) and further purified by precipitation in 0.8 M LiCl. Poly(A)$^+$ RNA was prepared by chromatography on oligo(dT)-cellulose (Pharmacia, Uppsala, Sweden).

Cloning of DNA fragments that encode putative protein kinases. First-strand cDNA was synthesized from poly(A)$^+$ RNA from BY-2 cells in mid-logarithmic phase by standard protocols with a cDNA synthesis kit from Amersham (Amersham, Buckinghamshire, England). A 50-ng sample of first-strand cDNA was used as a template for the polymerase chain reaction (PCR). The structure of the sense-oriented primer, which was designed on the basis of the amino acid sequence in subdomain I (GTGSFGRV) of the α-subunit of cAMP-dependent protein kinase from *S. cerevisiae* (47), was 5′-GGIACIGGI(T,A)CITT(T,C)GGI(C,A)GIGT-3′, and that of the antisense-oriented primer, which was designed on the basis of the sequence of subdomain VII [K(I,L)(A,T)DFG], which is conserved in most protein kinases (15), was 5′-CC(G,A)AA(G,A)TCIG(T,C)IA(T,G)(T,C)TT-3′. PCR was carried out as previously described (40), except that amplification was achieved during 30 cycles of 1 min of denaturation at 94°C, 2 min of annealing at 48°C, and 2 min of extension at 72°C. Amplified fragments of DNA of approximately 420 bp were treated with T4 DNA polymerase and ligated to the *Sma*I site in pUC18, and then the ligated DNA was used to transform *Escherichia coli* JM109. The DNA inserts of 12 plasmid clones were sequenced. Two clones had nucleotide sequences that potentially encoded protein kinases, but they were different from each other. These plasmid clones were named pUC-CA4 and pUC-CA7, but in the present study only pUC-CA7 was used in subsequent experiments.

cDNA cloning. First-strand cDNA was synthesized from poly(A)$^+$ RNA from BY-2 cells harvested in the mid-logarithmic phase by Moloney murine leukemia virus RNase reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.) under the conditions recommended by the manufacturer. Second-strand cDNA was also synthesized by the protocol recommended by Bethesda Research Laboratories. A 20-μl volume of the first-strand mixture was added to 140 μl of the second-strand mixture [21.5 mM Tris-HCl (pH 6.9)–104 mM KCl–5.3 mM $MgCl_2$–171 μM β-NAD–11.4 mM $(NH_4)_2SO_4$–0.21 mM each mixed deoxynucleoside triphosphate–4.3 mM dithiothreitol–15 U of *E. coli* DNA ligase (Takara Shuzo Co. Ltd., Kyoto, Japan)–40 U of *E. coli* DNA polymerase I (Takara Shuzo)–1.5 U of RNase H (Takara Shuzo)]. This reaction mixture was incubated at 16°C for 2 h, and then the double-stranded cDNA was blunted with T4 DNA polymerase. After the *Eco*RI-*Not*I-*Bam*HI adaptor (Takara Shuzo) had been ligated to the cDNA, the cDNA was inserted into the *Eco*RI site of λgt10 (Stratagene, La Jolla, Calif.). About 8×10^5 plaques were screened with the ^{32}P-labelled CA7 DNA fragment (see Fig. 1). The CA7 DNA fragment was isolated, for use as a probe, by digesting pUC-CA7 DNA with *Hin*cII, which can cleave the plasmid at the multiple cloning site of pUC18 and at a site near the opposite end of the CA7 sequence. A total of 13 λ-phage clones that gave positive signals were isolated. DNAs of the recombinant phages were purified and digested with *Not*I. The *Not*I fragments were subcloned into the *Not*I site of Bluescript SK$^-$ (Stratagene). Two plasmids (pNPK1-#6 and pNPK1-#13) were used for molecular analysis in the present study.

Plasmids. To generate the deletion mutant cNPK1Δ374 (see Fig. 2), pNPK1-#6 DNA was treated with *Eco*O109I, which can cleave the cDNA at nucleotide position 1166 (see Fig. 1), and then the linearized DNA was blunted with T4 DNA polymerase. The DNA segment corresponding to the kinase domain was obtained by digesting the linearized pNPK1-#6 DNA with *Xba*I, which can cleave within the multiple cloning site in Bluescript SK$^-$. This DNA fragment was reinserted between the *Spe*I site, which had been filled in by Klenow enzyme, and the *Xba*I site of Bluescript SK$^-$ to create a stop codon, in frame, immediately after the Thr-373 codon. The critical portion of the DNA of the

plasmid that had been constructed in this way was sequenced to confirm the presence of the anticipated termination codon. YEpGAP-NPK1 and YEpGAP-Δ374 were constructed by inserting the DNA fragment that contained cNPK1 or cNPK1Δ374 into the yeast expression vector YEpGAP112, which carries a cDNA cloning site between the promoter of the *TDH3* gene (the gene for glyceraldehyde 3-phosphate dehydrogenase) and the *TDH3* terminator, the origin of replication of the *S. cerevisiae* 2μm plasmid and *TRP1*. pRS314[*BCK1*] (31) includes the *BCK1* gene in the centromeric plasmid pRS314 (44). YEp352[*PKC1*] includes the *PKC1* gene in YEp352. pSTE11.1 (3) includes the *STE11* gene in YEp13.

Southern blot analysis and RNA blot analysis. RNA blot analysis was carried out as previously described (46) with the ^{32}P-labelled CA7 DNA fragment (see "cDNA cloning" above) used as the probe.

DNA sequencing. Nucleotide sequences of both strands of cNPK1 in pNPK1-#6 and pNPK1-#13 were determined by the dideoxy chain termination method (43).

RESULTS

Isolation of cDNAs that encode putative protein kinases of tobacco. We prepared cDNAs from mRNA that had been isolated from suspension cultures of the tobacco cell line BY-2, and then we amplified DNA fragments by PCR with synthetic oligonucleotide primers, the sequences of which had been deduced from consensus amino acid sequences for PKC or cAMP-dependent protein kinase (see Materials and Methods). On the basis of an analysis of nucleotide sequences of the PCR fragments which were cloned into plasmids, inserts in five plasmid clones were judged to encode portions of protein kinases because of their structural similarities to the conserved domains of members of the protein kinase family. However, PCR yielded no fragments with a reading frame that potentially encoded the expected protein kinases, such as PKC (unpublished observation). The DNA segment (CA7) in one plasmid clone, pUC-CA7, was predicted to encode an amino acid sequence that was homologous to that of the catalytic domains of the BCK1 and STE11 protein kinases of *S. cerevisiae* (31, 41). In the present study, we examined in detail a cDNA clone that was isolated from a cDNA library, prepared from poly(A)$^+$ mRNA from suspension cultures of tobacco cell line BY-2, by using the CA7 DNA fragment as a probe.

Nucleotide sequence of the cDNA and the deduced amino acid sequence. Two independently isolated plasmid clones (clones 6 and 13), containing cDNAs that corresponded to an almost-full-length (approximately 2,600-nucleotide) CA7-hybridizing mRNA (see Fig. 5C), were isolated, and their nucleotide sequences were determined. The nucleotide sequences of the two cDNA clones were identical, except that the cDNA in clone 6 was 20 nucleotides longer at the 5′ end than that in clone 13. Figure 1 represents the entire nucleotide sequence of the cDNA in clone 6. It is predicted to encode 690 amino acid residues if the coding region begins with the ATG codon near the 5′ end, which is located at nucleotide position 53 (Fig. 1). We designated the cDNA in clone 6 cNPK1 and the corresponding tobacco gene *NPK1*.

The N-terminal half (amino acid residues 78 to 327) of the predicted protein exhibits strong homology to the catalytic domains of members of the protein kinase family (15). The catalytic domain of NPK1 includes the amino acid sequences DIKGAN, GTPYWMAPE, and DIWSVG, which are similar to those of subdomain VIb (DLKXXN), subdomain VIII (GT/SXXY/FXAPE), and subdomain IX (DXWSXG), respectively, which are characteristic of the serine-threonine protein kinases (15). Therefore, it is likely that the *NPK1* gene encodes a protein kinase which is probably a member of the serine-threonine family.

A search for sequences with homology to the amino acid sequence of the predicted NPK1 protein was made by using the GenBank data base 71. As shown in Fig. 2A, significant amino acid sequence homology was found between the putative catalytic domain of NPK1 and the catalytic domains of the STE11 protein kinase (48% identical) and the BCK1 protein kinase (44% identical) of *S. cerevisiae* (31, 41) and the Byr2 protein kinase (47% identical) of *S. pombe* (51).

The predicted NPK1 protein has kinase-unrelated regions in the N-terminal region (amino acid residues 1 to 77) and the C-terminal half (amino acid residues 328 to 690) (Fig. 2B). The STE11, BCK1, and Byr2 proteins also have large kinase-unrelated regions in their N-terminal halves, which are thought to control their protein kinase activity. In contrast to the catalytic domains of these protein kinases, among which homology is found, the kinase-unrelated region of NPK1 exhibits no apparent homology to those of BCK1, STE11, and Byr2.

Functional analysis of the NPK1 gene with mutants of *S. cerevisiae*. To express cNPK1 in yeast cells, the DNA fragment containing the entire length of cNPK1 was joined to the constitutively active *TDH3* promoter in the yeast vector YEpGAP112 (see Materials and Methods). The same cDNA, without the nucleotide sequence that corresponds to the kinase-unrelated region (amino acid positions 374 to 690) (referred to as cNPK1Δ374 [Fig. 2B]), was also linked to this promoter. The resulting constructs were introduced into yeast strains bearing a *bck1* or a *pkc1* deletion (*bck1*Δ or *pkc1*Δ) or a temperature-sensitive *ste11* mutation.

The *bck1*Δ mutant cells carrying these constructs were examined for their ability to grow at 35°C in the presence and absence of sorbitol, since yeast cells defective in *BCK1* exhibit temperature-sensitive cell lysis that is prevented by high osmolarity. The mutant cannot grow at 35°C without osmotic support, but it can grow in the presence of 1 M sorbitol. Figure 3A shows that the *bck1*Δ mutant cells carrying either the full-length cNPK1 or cNPK1Δ374 were able to grow in the absence of sorbitol, although the extent of complementation by cNPK1Δ374 was greater than that by the full-length cNPK1. These results indicate that overexpression of either cDNA sequence complemented the *bck1*Δ mutation of the yeast.

We also examined whether overexpression of these NPK1 constructs could complement a *ste11* mutation. Figure 3B shows that neither the full-length cNPK1 nor cNPK1Δ374 was able to restore mating ability to a temperature-sensitive *ste11* mutant. Thus, overexpression of the cNPK1 constructs specifically complements the *bck1*Δ mutation.

Subsequently, we examined the effects of overexpressing cNPK1 and cNPK1Δ374 on the growth of *pkc1*Δ mutant cells in the presence and absence of sorbitol, because these cells also display a cell lysis defect. As shown in Fig. 4, a small fraction of the *pkc1*Δ mutant cells that carried cNPK1Δ374 grew in the absence of sorbitol, while no transformants with the full-length cNPK1 or the vector plasmid grew under the same conditions. These results indicate that the protein encoded by cNPK1Δ374 has the ability to suppress the growth defect of the *pkc1*Δ mutant, although such suppression is weak. In addition, the observation that only cNPK1Δ374 suppressed the *pkc1*Δ mutation, taken together with the fact that the extent of complementation of the *bck1*Δ

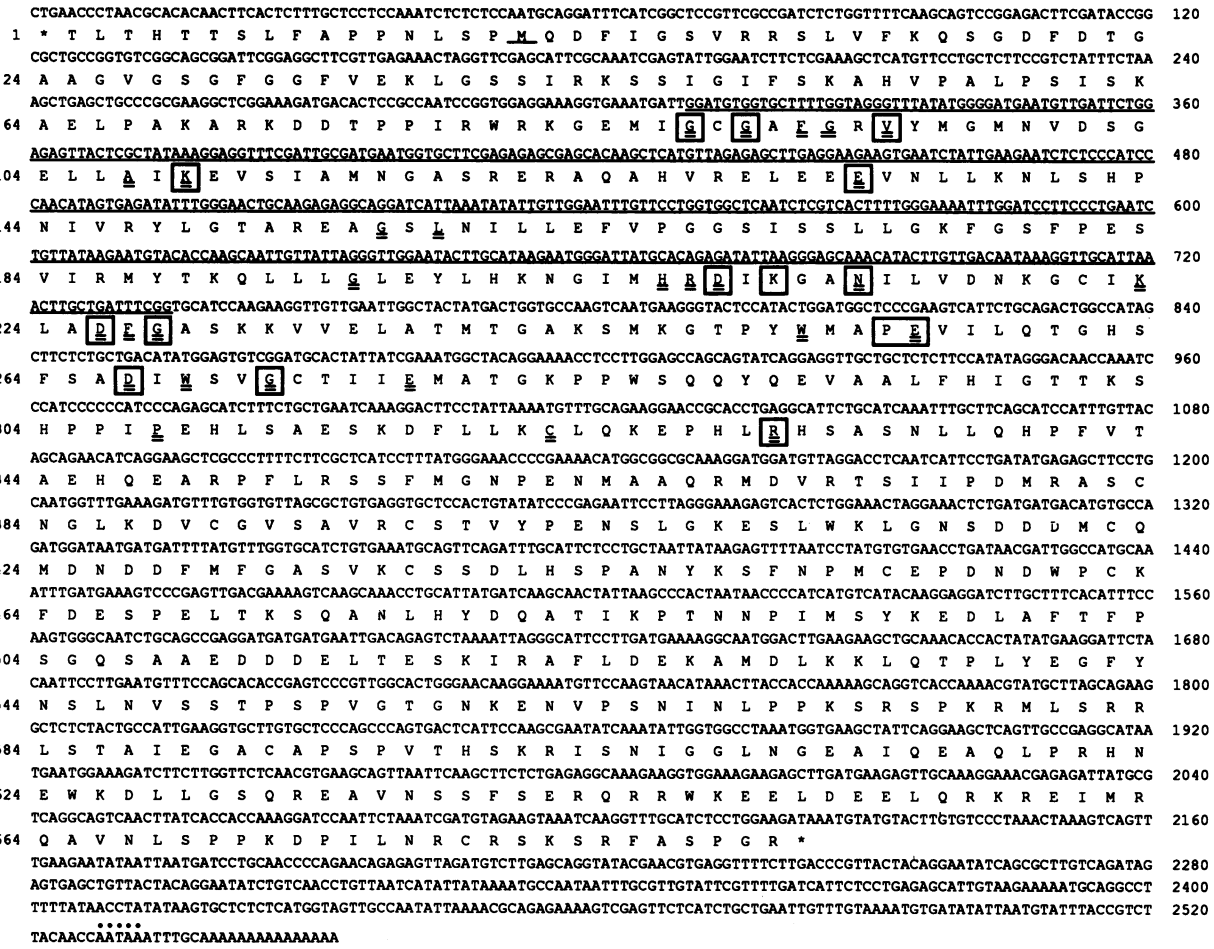

FIG. 1. Nucleotide sequence of *NPK1* cDNA (cNPK1) and the deduced amino acid sequence. Numbers on the right indicate nucleotide positions with respect to the first nucleotide of the sequence of cNPK1. Numbers on the left indicate positions of the amino acid residues with respect to the first methionine residue, which is underlined. The termination codon of the longest open reading frame is indicated by an asterisk. Dots indicate a nucleotide sequence similar to a polyadenylation signal near the 3′ end of cNPK1. The underlined nucleotide sequence indicates the region that corresponds to the DNA fragment obtained by PCR with the pUC-CA7 clone (see text). The CA7 fragment and cNPK1 have identical sequences. Boxes indicate amino acid residues conserved in 72 or more of the 75 known serine-threonine protein kinases (15). Double underlining indicates amino acid residues that are conserved in 39 or more of the 42 known tyrosine protein kinases.

mutation by cNPK1Δ374 was higher than that by the full-length cDNA, suggests that the predicted truncated protein encoded by cNPK1Δ374 is a deregulated form of the protein kinase. The C-terminal half of NPK1 may be involved in the regulation of protein kinase activity.

***NPK1* genes in *Nicotiana* species.** To estimate the number of copies of the *NPK1* gene in the tobacco genome, Southern blot analysis was carried out with genomic DNA from *Nicotiana tabacum*. Figure 5A shows that the labelled probe DNA hybridized with two bands from the *N. tabacum* genomic DNA that had been digested with restriction enzymes that do not cleave within the cNPK1 sequence which corresponds to the probe. Thus, it appears that *N. tabacum* contains two copies of *NPK1*-related genes. Genomic DNAs from *Nicotiana sylvestris* and *Nicotiana tomentosiformis* were also analyzed, since *N. tabacum* is an amphidiploid plant that contains two sets of genomes, one from *N. sylvestris* and the other from *N. tomentosiformis* (Fig. 5B). A single hybridization signal, which corresponded to one of the two signals from *N. tabacum*, was found for each of the two parental species. Therefore, the two genes in *N. tabacum* are probably derived from the two parental species.

Sites of expression of the *NPK1* gene. Poly(A)$^+$ RNA was purified from suspension cultures of BY-2 cells at various stages of growth: early logarithmic phase (1 day after the transfer of late-stationary-phase cells to fresh medium), mid-logarithmic phase (3 days), early stationary phase (5 days), and late stationary phase (7 days) (Fig. 6A). The poly(A)$^+$ RNA was then subjected to Northern (RNA) blot analysis. Figure 6B shows that *NPK1* expression is highest during logarithmic phase and decreases as cells enter stationary phase. No expression was detected in cells at late stationary phase. We also examined the expression of *NPK1* in mature leaves, stems, and roots of adult tobacco plants. Hybridization signals were detected with RNA from stems and roots but not with RNA from mature leaves (Fig. 6C).

DISCUSSION

We describe a tobacco gene, *NPK1*, which appears to encode a new protein kinase. Since Southern blot analysis showed that *Arabidopsis*, maize, and rice genes also have sequences that are homologous to the *NPK1* gene (unpub-

(A)

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NPK1   77  PIRWRKGEMIGCGAFGRVYMGMNVDSGELLAIKEVSIAMNGASRERAQAHVRELEE-
BCK1 1172  EFAWMKGEMIGKGSFGAVYLCLNVTTGEMMAVKQVEVPKYSSQNEAILSTVEALRS-
STE11 412  PKNWLKGACIGSGSFGSVYLGMNAHTGELMAVKQVEIKNNNIGVPTDNNKQANSDE{
Byr2   391  SIKWIRGALIGSGSFGQVYLGMNASSGELMAVKQVILDSVSESKDRHAKLLDALAG-
                      I                    II

NPK1   133  ---EVNLLKNLSHPNIVRYLGTAREAGSLNILLEFVPGGSISSLLGKFGSFPESVIR
BCK1  1228  ---EVSTLKDLDHLNIVQYLGFENKNNIYSLFLEYVAGGSVGSLIRMYGRFDEPLIK
STE11  504  36}EMNLLKELHHENIVTYYGASQEGGNLNIFLEYVPGGSVSSMLNNYGPFEESLIT
Byr2   447  ---EIALLQELSHEHIVQYLGSNLNSDHLNIFLEYVPGGSVAGLLTMYGSFEETLVK
               III          IV                              V

NPK1   187  MYTKQLLLGLEYLHKNGIMHRDIKGANILVDNKGCIKLADFGASKKVVEL-ATMTGA
BCK1  1282  HLTTQVLKGLAYLHSKGILHRDMKADNLLLDQDGICKISDFGISRKSKDIYSNSDM-
STE11  558  NFTRQILIGVAYLHKKNIIHRDIKGANILIDIKGCVKITDFGISKKLSPLNKKQNKR
Byr2   501  NFIKQTLKGLEYLHSRGIVHRDIKGANILVDNKGKIKISDFGISKKL-ELNSTSTKT
                             VI                  VII

NPK1   243  KSMK----GTPYWMAPEVILQTGHSFSA--DIWSVGCTIIEMATGKPPWSQQYQEV-
BCK1  1338  -TMR----GTVEWMAPEM-VDTKQGYSAKVDIWSLGCIVLEMFAGKRPWSN--LEVV
STE11  615  ASLQ----GSVEWMSPEVVKQTATT--AKADIWSTGCVVIEMFTGKHPFPDFSQ-M-
Byr2   557  GGARPSFQGSSFWMAPEVVKQTMHT--EKTDIWSLGCLVIEMLTSKHPYPNCDQ-M-
                        VIII              IX

NPK1   293  AALFHIGTTKSHPPIPEH----LSAESKDFLLKCLQKEPHLRHSASNLLQHPFVTAEHQ
BCK1  1387  AAMFKIGKSKSAPPIPEDTLPLISQIGRNFLDACFEINPEKRPTANELLSHPFSEVNET  (44%)
STE11  664  QAIFKIGTNTT-PEIPSW----ATSEGKNFLRKAFELDYQYRPSALELLQHPWLDAHII* (48%)
Byr2   610  QAIFRIGENIL-PEFPSNISSS----AIDFLEKTFAIDCNLRPTASELLSHPFVS*     (47%)
               X                                  XI
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(B)

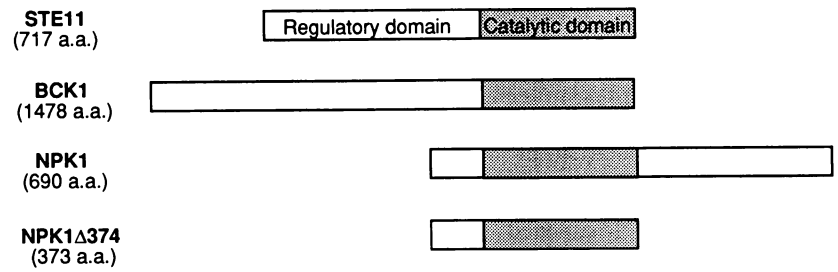

FIG. 2. (A) Comparison of amino acid sequences among putative catalytic domains: NPK1 of *N. tabacum* (Fig. 1), BCK1 of *S. cerevisiae* (31), STE11 of *S. cerevisiae* (41), and Byr2 of *S. pombe* (51). Numbers on the left indicate amino acid positions relative to the initiator methionine of predicted proteins. Sequences are aligned and dashes are introduced to give maximum matching. Residues identical to those in NPK1 are shaded. Percentages in parentheses on the right indicate homology for each predicted protein (percent identical residues). Roman numerals below the sequences indicate subdomains that are conserved in all the known members of the families of protein kinases. (B) Organization of domains of predicted STE11, BCK1, and NPK1 proteins. Numbers in parentheses are of amino acid residues (a.a.) in each protein. Shaded and open bars represent the putative catalytic domains and the kinase-unrelated domains which are thought to be regulatory domains, respectively. NPK1Δ374 is the truncated form of NPK1, in which the amino acid residues from positions 374 to 690 of the predicted NPK1 protein were deleted.

lished data), *NPK1*-related genes are probably present in many plant species.

The predicted NPK1 protein displays structural similarity within its putative catalytic domain to the protein kinases encoded by *BCK1* and *STE11* of *S. cerevisiae* and *byr2* of *S. pombe*. Overexpression of a C-terminal truncation of cNPK1 restored the ability of a yeast *bck1*Δ mutant to grow normally but neither complemented a *ste11* mutation (Fig. 3) nor activated transcription of a pheromone-inducible reporter gene (*fus1-lacZ*) (data not shown). We also examined the effect of overexpressing cNPK1 in a mutant lacking the *MPK1* gene, which has been shown recently to act downstream of *BCK1* in budding yeast (30). Overexpression of cNPK1 failed to suppress the *mpk1* mutation (unpublished data). These results indicate that the observed complementation is due to specific activation of the BCK1-mediated signaling pathway by the cNPK1 products but is not due to nonspecific phosphorylation by overexpression of the cNPK1 constructs in yeast cells. In addition, expression of cNPK1Δ374 suppressed the *pkc1*Δ mutation, but only weakly compared with the dominant *BCK1* mutants (31). Therefore, it is likely that plants could have a signalling pathway that is similar to the BCK1-mediated signaling system, although the presence of PKC in plants has not yet been demonstrated. Physiological evidence to indicate that enzymes which resemble PKC are involved in signal trans-

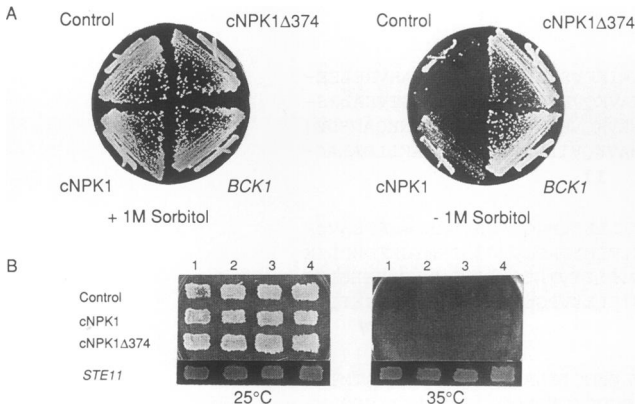

FIG. 3. (A) Complementation of the yeast *bck1Δ* mutation by cNPK1. *S. cerevisiae* DL251 (*bck1Δ/bck1Δ*) cells harboring only the vector plasmid YEpGAP112 (control), YEpGAP112-Δ374 containing cNPK1Δ374 (Fig. 2), YEpGAP112-NPK1 containing full-length cNPK1, or pRS314[*BCK1*] containing the *BCK1* gene were streaked on agar plates with (left) and without (right) 1 M sorbitol and grown at 35°C for 2 days. (B) Effects of expression of cNPK1 and cNPK1Δ374 in the *ste11* temperature-sensitive mutant. Patches of strain KMY167-7A, transformed with the plasmid that encoded the indicated cDNA (the control has no cDNA), were grown on selective medium plates at 25°C (left) or 35°C (right) and replicated on YEP-glucose plates seeded with a lawn of yeast strain 15Dau. Mating was allowed to proceed overnight at 25°C (left) or 35°C (right) before transfer onto synthetic glucose minimal medium plates, supplemented with the appropriate requirements, for monitoring formation of diploids. Four independent clones, indicated by numbers, were tested.

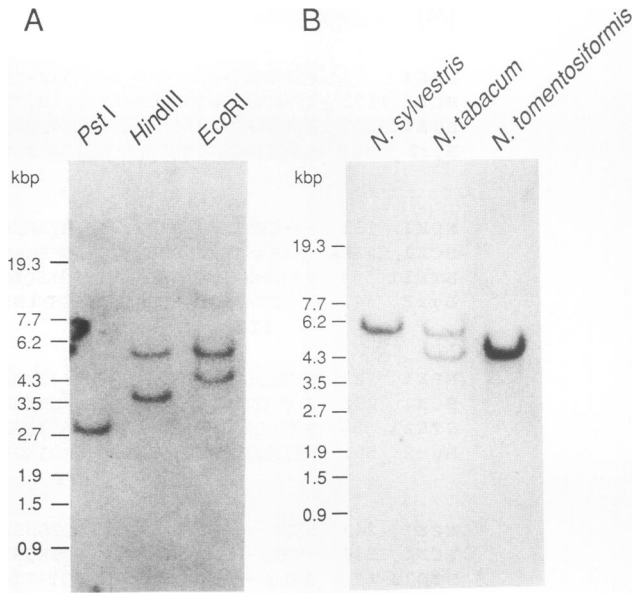

FIG. 5. Southern blot analysis of genomic DNAs from *Nicotiana* species. (A) Samples (10 μg each) of genomic DNA from *N. tabacum* were digested with *Pst*I, *Hin*dIII, or *Eco*RI. (B) Samples (10 μg each) of genomic DNA from each of the *Nicotiana* species indicated were digested with *Eco*RI. The digested DNAs were fractionated on a 0.7% agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labelled CA7 DNA (Fig. 1).

duction in the elicitor-induced synthesis of phytoalexin has been reported (28), and an enzyme with activity similar to that of PKC has been partially purified from *Amaranthus tricolor* (8).

Although NPK1 failed to suppress the *ste11* mutation, the data distinguishing which isotype NPK1 represents may be ambiguous. The data may reflect a different signal threshold for response in each of the STE11- and BCK1-mediated pathways: the cell lysis defect in the *bck1Δ* mutant may be suppressed by very low levels of activity of the pathway, whereas the mating response pathway may require a much higher level of activity. The observation that overexpression of *MKK1* or *MKK2* can suppress a deletion of *BCK1* (24) whereas overexpression of *STE7* fails to suppress a deletion of *STE11* (2) supports this idea.

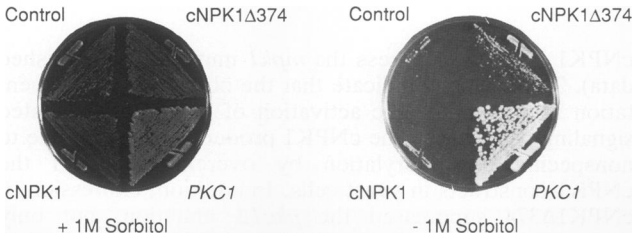

FIG. 4. Suppression of the *pkc1Δ* mutation in yeast by cNPK1Δ374. *S. cerevisiae* SNY235 (*pkc1Δ::LEU2*) cells harboring YEpGAP112 (control), YEpGAP112-NPK1, YEpGAP112-Δ374, or YEp352[*PKC1*] were streaked onto agar plates supplemented with 1 M sorbitol (left) or without 1 M sorbitol (right) and incubated at 25°C for 3 days.

The results of Northern blot analysis with mRNA from tobacco cells and organs (Fig. 6) are in agreement with the idea that the putative protein kinase NPK1 might be involved in the processes of the division and growth of tobacco cells. The *NPK1* transcript was specifically detected in suspension cultures of BY-2 cells in logarithmic phase and early stationary phase. It was also detected in stems and roots of mature tobacco plants but not in mature leaves, which rarely contain growing cells (Fig. 6C). The *NPK1* gene, however, was shown to be strongly expressed in developing leaves (shorter than 5 cm), which contain growing cells (unpublished observation). These results suggest that the *NPK1* gene is preferentially transcribed in growing cells of tobacco, though a correlation between the expression of *NPK1* and the growth of the cells remains to be proven at the cellular and molecular levels.

It is also intriguing to speculate about the substrate protein(s) for the putative NPK1 protein kinase in tobacco cells. As described in the introduction, recent studies with budding yeast have suggested that STE7, MKK1, and MKK2 function downstream of STE11 and BCK1 in the mating pheromone response pathway and in the PKC1-mediated pathway, respectively (2, 24, 45). STE7, MKK1, and MKK2 are homologs of vertebrate MAPKKs that function upstream of the yeast MAP kinase homologs FUS3, KSS1, and MPK1 in their respective signalling pathways (9, 12, 30). cDNAs that encode plant homologs of MAP kinase have recently been isolated from *Medicago sativa* (alfalfa) (7) and *Arabidopsis thaliana* (35), and protein kinase activity of the product from the *Arabidopsis* cDNA was shown to be activated by animal MAPKK (35). These observations allow us to speculate that plants also have a MAPKK homolog(s), which may be structurally similar to STE7, MKK1 and MKK2, and Byr1 and would be a candidate substrate of

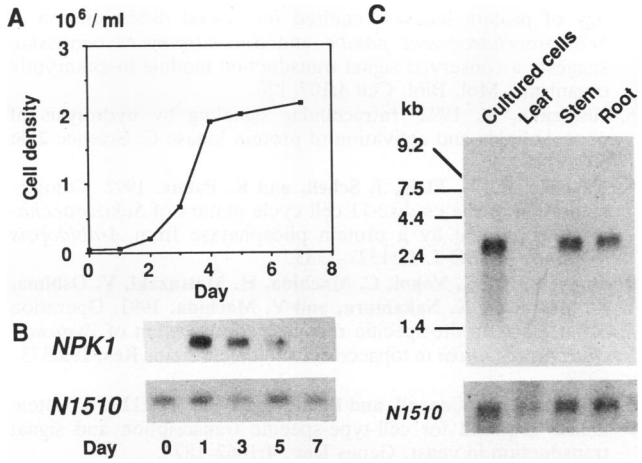

FIG. 6. Expression of the *NPK1*-related gene. (A) Growth curve for a suspension culture of BY-2 cells. Cell density was measured on the days indicated after the initial transfer of 2 ml of a 7-day-old culture to 95 ml of fresh medium. (B) Northern blot analysis of poly(A)$^+$ RNA from BY-2 cells harvested at various growth stages. Poly(A)$^+$ RNA was prepared from BY-2 cells immediately after transfer to fresh medium (day 0) or 1, 3, 5, or 7 days after transfer, as described in Materials and Methods. Samples (2 μg each) of poly(A)$^+$ RNA were fractionated on an agarose gel that contained formalin, transferred to a nylon membrane, and probed with ^{32}P-labelled CA7 DNA. After autoradiography, the CA7 probe was removed by washing, and then the membrane was rehybridized with the ^{32}P-labelled DNA fragment of the cDNA for the N1510 gene, which encodes an unknown protein but has been shown to be expressed in BY-2 cells at all stages of growth (our unpublished results). (C) Northern blot analysis of poly(A)$^+$ RNA from mature leaves, roots, and stems, carried out as described for panel B. Poly(A)$^+$ RNA from BY-2 cells on day 1 was analyzed in parallel. Numbers on the left indicate the sizes of marker RNA molecules.

NPK1. Recently, the cDNA for a MAPKK-related gene has been isolated from tobacco (unpublished data).

Several groups have reported that the Raf protein kinase of metazoans functions upstream of MAPKK (6, 23, 29, 50). Hence, Raf might have a role as an activator kinase of MAPKK (MAPKKK). STE11, BCK1, and Byr2, which exhibit no significant structural similarity to Raf, however, may serve as MAPKKKs in yeasts. Therefore, it is reasonable to speculate that MAPKKKs corresponding to these yeast kinases may exist in the signaling pathways of other eukaryotes. MAPKKKs other than Raf have been hypothesized previously to exist in animals (5, 23, 27, 42). The present study is the first demonstration of a protein kinase related to STE11, BCK1, and Byr2 in a multicellular organism. It should shed light on a new candidate MAPKKK in eukaryotes other than yeasts.

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ADDENDUM IN PROOF

After this article was submitted, Lange-Carter et al. reported a mouse STE11 homolog, MEKK, which can activate MAPKK by phosphorylation (C. A. Lange-Carter, C. M. Pleiman, A. M. Gardner, K. J. Blumer, and G. L. Johnson, Science **260**:315–319, 1993). The amino acid sequence of the kinase domain of MEKK is 45% identical to that of NPK1.

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