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RRB1 and *RRB2* Encode Maize Retinoblastoma-Related Proteins That Interact with a Plant D-Type Cyclin and Geminivirus Replication Protein

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Unlike mammalian and yeast cells, little is known about how plants regulate G_1 progression and entry into the S phase of the cell cycle. In mammalian cells, a key regulator of this process is the retinoblastoma tumor suppressor protein (RB). In contrast, G_1 control in *Saccharomyces cerevisiae* does not utilize an RB-like protein. We report here the cloning of cDNAs from two *Zea mays* genes, *RRB1* and *RRB2*, that encode RB-related proteins. Further, *RRB2* transcripts are alternatively spliced to yield two proteins with different C termini. At least one *RRB* gene is expressed in all the tissues examined, with the highest levels seen in the shoot apex. RRB1 is a 96-kDa nuclear protein that can physically interact with two mammalian DNA tumor virus oncoproteins, simian virus 40 large-T antigen and adenovirus E1A, and with a plant D-type cyclin. These associations are abolished by mutation of a conserved cysteine residue in RRB1 that is also essential for RB function. RRB1 binding potential is also sensitive to deletions in the conserved A and B domains, although differences exist in these effects compared to those of human RB. RRB1 can also bind to the AL1 protein from tomato golden mosaic virus (TGMV), a protein which is essential for TGMV DNA replication. These results suggest that G_1 regulation in plant cells is controlled by a mechanism which is much more similar to that found in mammalian cells than that in yeast.

Progression through the G_1 phase of the eukaryotic cell cycle is tightly regulated, allowing cells to integrate internal and external cues before initiating DNA replication and committing to a round of cell division. This process is governed by both positive- and negative-acting regulatory factors. Although substantial progress has been made in understanding the mechanisms that govern these events in yeast and mammals (reviewed in reference 61), relatively little is known about G_1 regulation in plants. The existence of cyclin-dependent kinases (Cdks) and their associated cyclin subunits in plants (reviewed in reference 14) suggests that at least some of the basic mechanisms which regulate the cell cycle have been conserved throughout eukaryotic evolution. However, identification of additional regulatory components of the plant cell cycle is clearly essential for understanding plant growth and development.

In the yeast *Saccharomyces cerevisiae*, progression through the G_1 phase is regulated by the Cdk Cdc28 (50), which in conjunction with G_1 cyclins activates the heterodimeric Swi4/Swi6 transcription factor (40), resulting in the transcriptional activation of genes necessary for G_1 progression and S-phase entry (12, 61). In mammalian cells, G_1 progression also depends upon a Cdk-cyclin-activated transcriptional control pathway. However, a major regulatory protein in this pathway, the retinoblastoma protein (RB), has not been found in yeast. RB is the 110-kDa product of the retinoblastoma susceptibility tumor suppressor gene and plays key roles in regulating both cell cycle progression through the G_1 phase and cellular differentiation (reviewed in references 7 and 69). RB appears to function through complex formation with a variety of proteins involved in G_1 progression (reviewed in reference 7). Of particular importance are the interactions of RB with three members of the E2F transcription factor family, which result in the inhibition of transcriptional activation by these factors (reviewed in reference 41). E2F sites are found in the promoters of several genes required for G_1 progression and S-phase entry (11), and the repression of these genes is thought to be a critical aspect of RB function.

RB activity is regulated by phosphorylation in a cell cycle-dependent manner (reviewed in reference 69). At or near the restriction point, RB becomes phosphorylated by Cdk4 and Cdk6 kinases in conjunction with D-type cyclins (16, 21, 38) and by the Cdk2-cyclin E complex (32). The cyclin D family can also physically interact with RB (16, 21, 38). Because all known RB-interacting proteins preferentially bind to the hypophosphorylated form of the protein (reviewed in reference 7), phosphorylation serves to functionally inactivate RB. For example, phosphorylation disrupts RB-E2F complexes, allowing transcription of E2F-activated genes and subsequent cell cycle progression.

The function of RB can also be abrogated by the binding of oncoproteins from several mammalian DNA tumor viruses, including the simian virus 40 (SV40) large-T antigen and the adenovirus E1A protein (6, 72). These proteins bind to RB via a conserved motif, LXCXE, which is also found in the D cyclins (16, 21, 23). Viral oncoprotein binding requires two large regions of RB, the A and B domains, which are conserved in the other two members of the mammalian RB family, p107 and p130 (22, 28). These domains are generally required for most associated proteins to bind pRb and are proposed to form a binding "pocket." The binding by viral oncoproteins is

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thought to mimic RB phosphorylation by releasing associated proteins, including E2F, and thus allowing cell cycle progression.

Recent evidence suggests that G_1 control in plants involves an RB-like protein. First, several plant D-type cyclin homologs, which are expressed during the G_1 phase and contain LXCXE motifs in their N termini, have been isolated (10, 62). Second, a DNA replication protein from a plant geminivirus binds to human RB (9) and p130 (70). While this paper was in preparation, partial cDNAs for a protein with homology to the A and B domains of human RB were also isolated from maize (25, 71). Here we show that maize has two genes, *RRB1* and *RRB2*, which encode RB-related proteins. A full-length cDNA from *RRB1* encodes a protein with a predicted molecular mass of 96 kDa. At least one of these genes is expressed in all tissues, with the highest levels in the shoot apex. The RRB1 protein can bind to both plant and mammalian LXCXE-containing proteins and to a geminivirus replication protein that has been shown to induce the expression of cellular proliferating cell nuclear antigen (PCNA) but does not contain an LXCXE motif. These findings indicate that despite the significant differences in cell growth and development between plants and animals, their mechanisms for regulation of the G_1 phase of the cell cycle may be strikingly similar.

MATERIALS AND METHODS

Plant materials. Maize genomic DNAs and RNAs were from the *Zea mays* inbred line B73. Maize vegetative-meristem and immature-ear cDNA libraries, constructed with a Stratagene Uni-ZAP XR kit, were gifts from Bruce Veit and Sarah Hake (USDA-UC Berkeley Plant Gene Expression Center).

Isolation of *RRB* cDNAs. Two oligonucleotides corresponding to nucleotides 64 to 93 and 189 to 219 of the 232-bp maize expressed sequence tag (EST) 6c02c02 (GenBank accession no. T18395) were constructed (60). PCR with these primers was performed with an aliquot of a maize vegetative-meristem cDNA library as previously described (1). A 155-bp amplified fragment homologous to the EST was gel purified and labelled with ^{32}P by random priming (Prime-It kit; Stratagene). This probe was used to screen maize vegetative-meristem and immature-ear cDNA libraries as previously described (3). Positive phage were plaque purified, and phage DNA was excised in vivo to recover pBluescript plasmid according to Stratagene's protocol.

RNA blot analysis. Total RNAs were isolated from various maize tissues (3). Samples of 15 μg were electrophoresed on an 0.8% agarose–formaldehyde gel, blotted, and hybridized (49) with a 2,065-bp radiolabelled *RRB1* probe corresponding to the region encoding the C-terminal 582 amino acids. After autoradiography, the filter was stripped and reprobed with a pea 18S nuclear rRNA gene probe (1).

Immunolocalization of RRB1. The myc-tagged RRB1 expression plasmid pA6M/RRB1 was constructed by digesting p28/ZmRb.2 with *Nde*I, blunting with Klenow fragment, and then digesting with *Xho*I. The resulting 2.1-kb fragment was ligated to *Eco*RV- and *Xho*I-cut pCANGlu2 (kindly provided by Ali Fattaey, ONYX Pharmaceuticals). The 2.2-kb NcoI-*Xba*I fragment from the resulting plasmid was cloned into pA6M (19a). This vector introduces an in-frame ATG and a five-myc epitope tag upstream of the cloned *RRB1* under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

Nicotiana tabacum (line XD) suspension cultures were grown as previously described (47), and protoplasts were prepared (33). After electroporation (47), cells were grown at 22°C in suspension medium (33) supplemented with 0.4% mannitol for 16 to 24 h. Cells were fixed with 3.7% (wt/vol) formaldehyde in fixation buffer containing 100 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.9), 10 mM EGTA, 5 mM $MgSO_4$, 10% dimethyl sulfoxide, and 100 mg of phenylmethyl sulfonyl fluoride per ml for 45 min at room temperature. Fixed cells were permeabilized with 0.5% Triton X-100 in fixation buffer for 5 min. All antibody and washing steps were performed in blocking buffer (100 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20, 5% [wt/vol] dry milk). myc-epitope-tagged proteins were detected with monoclonal antibody 9E10 (BAbCO) and goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Calbiochem). Cells were resuspended in Citifluor (Ted Pella, Inc.) prior to visualization. Digital images of FITC and DAPI (4′,6-diamidino-2-phenylindole) fluorescence and Normarski differential interference contrast were obtained by using a Zeiss Axiophot fluorescence microscope with a charge coupled device camera.

Yeast two-hybrid assays. Fusions with the Gal4 DNA-binding domain and the Gal4 activation domain were created in plasmids pAS1 and pACT (19), respectively. All sequences generated by PCR were verified by sequencing. A precursor to several of the deletion constructs, pZmRb.2, was constructed as follows. The *Hin*dIII-*Eco*RI fragment (encoding amino acids 285 to 866 of RRB1) from pZmRb.3.1.1, a partial *RRB1* clone, was ligated into pLITMUS28 (New England Biolabs) to create pZmRb.1. An in-frame *Nde*I site was introduced upstream of the coding sequence by PCR with the forward primer 5′-GCTGAGCTCCATAT GTTAAACTGCCCCCAATTTTTA-3′ and a reverse primer corresponding to nucleotides 1056 to 1035 of *RRB1*. The PCR product was digested with *Sac*I and *Hin*dIII and ligated to pZmRb.1 to create pZmRb.2.

The RRB1 deletion series was constructed in pAS1 as follows. 214C was constructed by ligating the 1.95-kb *Bcl*I fragment from the longest RRB1 clone, RRB1 (V.1.1.1), to pAS1 digested with *Bam*HI. 290C was constructed by ligating the 2.1-kb *Eco*RV-*Xho*I fragment from pZmRb.2 into pAS1. 413C was built by fusing the 1.7-kb *Bsp*HI-*Xho*I fragment from pZmRb.2 into pAS1 digested with *Nco*I and *Sal*I. To construct 290C/Δ563-586 and 290C/Δ483-586, *Spe*I sites were introduced at the 3′ end of the A domain and the 5′ end of the B domain by PCR to create pB/zRb-A and pB/zRb-B, respectively. These sites were positioned so as to be in frame with each other and the *Spe*I site in the spacer. The 920-bp *Sac*I-*Spe*I fragment from pZmRb.2 was ligated into pB/zRb-B to create pB/Δ563-586. The *Eco*RV-*Xho*I fragment from this plasmid was ligated to pAS1 to create 290C/Δ563-586. The *Spe*I-*Xho*I fragment from pB/zRb-B was fused to pB/zRb-A digested with the same enzymes, creating pB/Δ483-586. The *Eco*RV-*Xho*I fragment from this plasmid was ligated to pAS1, generating 290C/Δ483-586. 290-850 was built by ligating the *Eco*RV-*Bam*HI fragment from pZmRb.2 into pAS1. To construct 290-794, pZmRb.2 was first digested with *Sac*I and *Nsi*I and the resulting 1.55-kb fragment was fused to pLITMUS28 cut with *Sac*I and *Pst*I. The resultant plasmid was cut with *Eco*RV and *Xho*I and ligated into pAS1. 290-648 was built by first introducing the 1.1-kb *Eco*RI-*Cla*I fragment from pZmRb.2 into pBluescript KS and then ligating the *Eco*RV-*Sal*I fragment from the resultant plasmid into pAS1. Finally, the C653F mutation was introduced into the RRB1 coding sequence by PCR with the forward primer 5′-CACATCGATCAACTTA TCCTTTTCTGTC-3′ and a primer in the 3′ untranslated region which also introduces a new *Xho*I site. The PCR product was digested with *Cla*I and *Xho*I and ligated into pBluescript KS, generating pB/C653F. The *Eco*RI-*Cla*I fragment from pZmRb.2 was ligated to pB/C653F, creating pC653F.2, which was subsequently cut with *Eco*RV and *Xho*I and cloned into pAS1.

pAS1-cyclinD (46) and YIpPTG10 (19) were previously described. To construct pACT-δ3 and pACT-cyc1At, the coding regions of the *Arabidopsis* δ3 cyclin (62) and cyc1At cyclin (30) genes were amplified via PCR with the following primers: for δ3, 5′-CGGGATCCGGAAGGAGGAAGAAAGTAG-3′ and 5′-CCGGAATTCGATTATGGAGTGGCTACG-3′; for cyc1At, 5′-CGGG ATCCACACTAAGATGATGACTTCTCG-3′ and 5′-CCGGAATTCCTAAC TCCTAAGCAGATTCAG-3′. Amplified fragments were digested with *Eco*RI and *Bam*HI and cloned into pACT. The resulting plasmids were digested with *Xho*I, and the large fragments containing the pACT vector and the cyclin N-terminal coding regions were gel purified and self ligated to create pACT-δ3 and pACT-cyc1At. pACT-RRB1 was constructed by ligating the 2.2-kb *Eco*RI fragment from pZmRb.3.1.1 into pACT.

Yeast transformations were done with *S. cerevisiae* Y153 (19) as previously described (56), and β-galactosidase assays were performed by established protocols (19).

GST fusion constructs and in vitro binding assays. Glutathione *S*-transferase (GST)–δ3 and GST-cyc1At expression vectors were constructed by digesting pACT-δ3 and pACT-cyc1At with *Xma*I and *Eco*RI and ligating the insert with pGEX2TK cut with *Xma*I and *Eco*RI. The GST-T_{pep} expression plasmid was constructed by digesting pJ-T^{wt} (17a) with *Bam*HI and *Sal*I, filling the ends with Klenow fragment (36), and cloning the insert into pGEX2TK digested with *Sma*I. GST-E1A-12S- and GST-E1A-13S-expressing plasmids were gifts from Ali Fattaey (ONYX Pharmaceuticals).

^{35}S-labelled RRB1 protein was prepared by in vitro transcription with T7 RNA polymerase (2) and in vitro translation with [^{35}S]methionine (Amersham) by using a rabbit reticulocyte kit (Promega) according to the manufacturer's instructions. Binding assays were performed by washing purified GST fusion proteins bound to glutathione-Sepharose beads three times in phosphate-buffered saline and once in buffer A (50 mM HEPES [pH 7.7], 300 mM NaCl, 0.1% Nonidet P-40). Labelled RRB1 protein was added and incubated for 30 min at room temperature in buffer A supplemented with 10 mg of bovine serum albumin per ml. Beads were washed five times in buffer A without bovine serum albumin, boiled in sodium dodecyl sulfate (SDS) loading buffer, and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), followed by autoradiography.

Baculovirus expression and AL1-binding assays. Recombinant baculoviruses that express tomato golden mosaic virus (TGMV) AL1 protein and GST have been described previously (52). A baculovirus that expresses chloramphenicol acetyltransferase (CAT) was provided by Verne Luckow (Monsanto). The recombinant baculovirus for GST-RRB1 was constructed by digesting pZmRb.2 with *Nde*I, filling with Klenow fragment, and digesting with *Xho*I. The RRB1-containing *Nde*I-*Xho*I fragment was fused in frame to the 3′ end of the GST coding sequence in pNSB310 (52) previously digested with *Bam*HI, repaired with Klenow fragment, and recut with *Xho*I. This GST-RRB1 fusion was inserted as a *Sac*I fragment into pMON27025 downstream of the polyhedrin promoter, transferred to the bacmid vector bMON14242 by Tn7-mediated transposition, and transfected into *Spodoptera frugiperda* Sf9 cells as previously described (44).

Sf9 cells were infected with recombinant baculoviruses at a multiplicity of

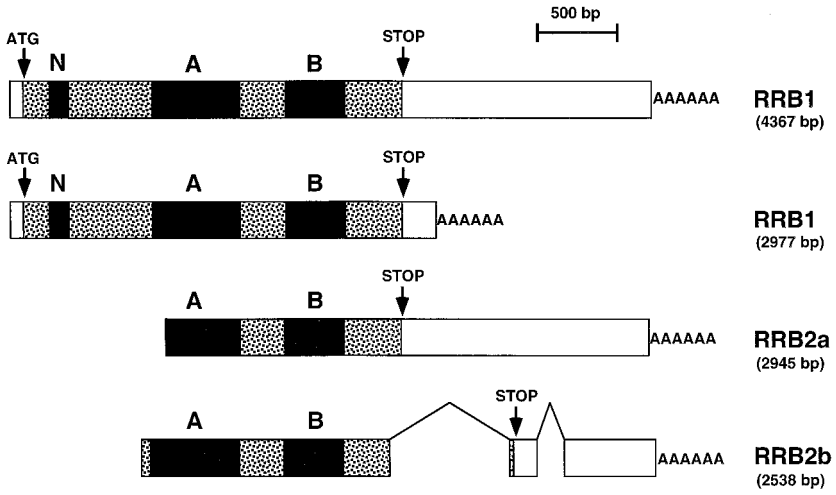

FIG. 1. Structures of maize *RRB* cDNAs. Shown are schematic diagrams of the two types of *RRB1* cDNAs and the *RRB2a* and *RRB2b* partial cDNAs, indicating alternative polyadenylation sites and alternative splicing. White boxes indicate noncoding regions, speckled boxes indicate coding regions with little or no homology to mammalian RB proteins, and black boxes indicate coding regions with high homology to the mammalian RB protein family. The lengths of cDNA clones are indicated.

infection of 5 and harvested at 72 h postinfection. Protein extracts were prepared and analyzed in GST-binding assays as described previously (58), except that 250 mM NaCl was included in the extraction buffer. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell), and analyzed by immunoblotting with an enhanced chemiluminescence detection system (Amersham). Primary antibodies were a mouse polyclonal anti-RRB1 serum and rabbit polyclonal anti-AL1 (27), anti-CAT (5′-3′ Inc.), and anti-GST (Upstate Biotechnology Inc.) sera.

To produce the anti-RRB1 serum, sequences that encode RRB1 amino acids 721 to 866 were fused to GST in pGEX-5X3 to create pGST/zRb-C. Protein was produced in *Escherichia coli* and purified by glutathione-affinity chromatography (2). Antisera were raised in mice injected subcutaneously with 50 μg of GST–zRb-C and given booster injections after 1 and 3 weeks, followed by induction of ascites by injection of T-180 sarcoma cells.

Nucleotide sequence accession number. The nucleotide sequences of *RRB1*, *RRB2a*, and *RRB2b* have been submitted to GenBank under accession numbers AF007793, AF007794, and AF007795, respectively.

RESULTS

The maize genome contains at least two genes encoding RB-related proteins. A partial maize cDNA that encodes a protein with homology to the B domain of the human RB protein family was previously identified in an EST sequencing project (60). This sequence was used to probe maize vegetative-meristem and immature-ear cDNA libraries for full-length clones. Two classes of cDNAs, corresponding to transcripts from two genes, *RRB1* and *RRB2* (for related to RB), were obtained (Fig. 1).

The two longest *RRB1* cDNAs were 4,367 and 2,977 bp long due to the use of alternate polyadenylation sites. *RRB1* transcripts contain a long open reading frame that encodes an 866-amino-acid (96-kDa) protein with homology to both the A and B domains of the mammalian RB protein family. 5′ rapid amplification of cDNA ends reverse transcription-PCR analysis of immature-ear RNA revealed several in-frame stop codons at the 5′ end of the *RRB1* mRNA (data not shown), indicating that the isolated cDNAs encode the full-length protein. We also isolated two partial cDNAs from the *RRB2* gene. These cDNAs are derived from alternatively spliced transcripts, with two regions of the *RRB2a* transcript spliced out of the *RRB2b* transcript. One of these splicing events occurs within the coding region, resulting in RRB2a and RRB2b proteins with different C termini.

The RRB1 and RRB2 proteins (Fig. 2A) are approximately 90% identical across the 611-amino-acid region for which RRB2 protein sequence is available. The RRB2 proteins are identical except at the C terminus, where RRB2b is 27 amino acids shorter and differs in its last 8 amino acids from RRB1 and RRB2a as a result of alternative splicing.

The maize RRB proteins have several regions of homology with the metazoan RB protein family. Most notable are the A and B domains, which make up the E1A- and T-antigen-binding pocket region (34, 35, 37) conserved among all metazoan RB protein family members (17). The sequences of the maize RRB proteins are approximately 35% identical to the human and *Drosophila* proteins in the A domain (Fig. 2B) and approximately 25% identical in the B domain (Fig. 2C). The maize proteins have a shorter B domain than do the human p107 and p130 proteins, similar to the human RB and *Drosophila* RBF proteins (Fig. 2C). The RRB B domains also contain a cysteine residue in a position equivalent to the critical Cys706 of human RB, which results in the loss of protein function when it is mutated (5, 39).

In addition to the A and B domains, there is a leucine-rich domain in the N-terminal region of mammalian and *Drosophila* RB-related proteins (17, 22, 28) which is also conserved in the maize RRB1 protein (Fig. 2D). Although the role of this sequence is unknown, its conservation among plants and animals strongly suggests that it is functionally significant.

There are a number of potential Cdk phosphorylation sites in the maize RRB proteins, with the highest concentration found in the C termini (Fig. 2A). A similar placement of Cdk phosphorylation sites is found in the mammalian RB, p107, and p130 proteins. The alternative splicing of *RRB2* removes the most C-terminal site from RRB2b, which could potentially alter regulation of the protein.

We believe that these genes represent most, if not all, of the immediate *RRB* gene family in maize for two reasons. First, extensive screening of maize cDNA libraries from several tissues yielded only *RRB1* and *RRB2* cDNAs. Second, Southern blot analysis of maize genomic DNA with a probe which hybridizes to both *RRB1* and *RRB2* under stringent conditions detected only one to three strongly hybridizing bands in several

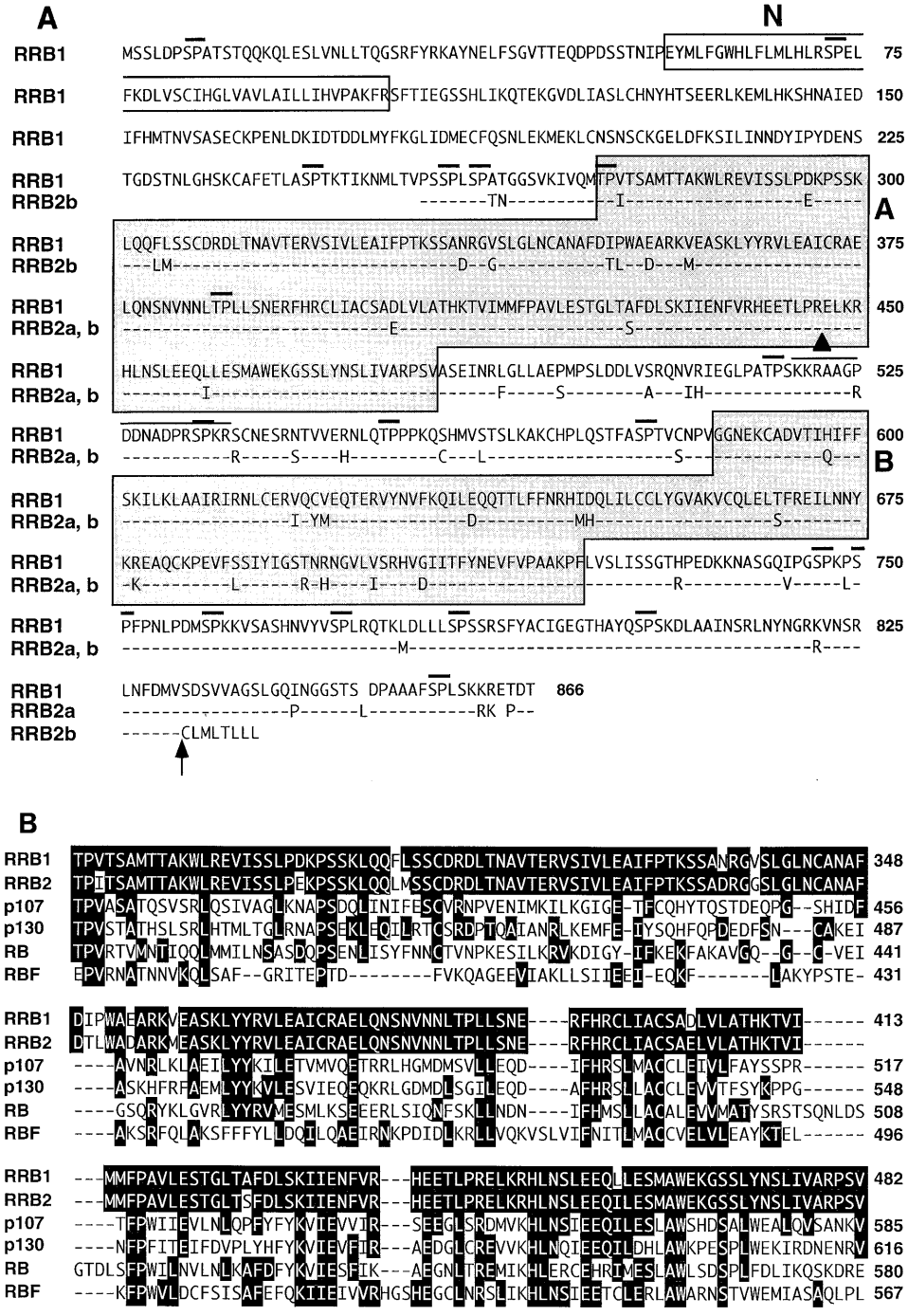

FIG. 2. Maize RRB proteins. (A) Complete amino acid sequence of the RRB1 protein and partial sequences of the RRB2a and RRB2b proteins. Dashes in the RRB2 sequences indicate amino acid identities with RRB1, and boxes indicate domains conserved between maize and human RB family members. The start of the RRB2a protein sequence is indicated by a black triangle, and an arrow designates the site in RRB2b that corresponds to the alternate splice junction in the mRNA. Potential Cdk phosphorylation sites are labelled by thick overlines, and a potential bipartite nuclear localization signal is marked by a thin overline. (B) Comparison of A domains of maize RRB1 and RRB2, human RB, p107, and p130, and *Drosophila* RBF proteins. Amino acid identities are boxed, and dashes indicate gaps in the sequences. (C) Comparison of the B domains of the proteins from panel B. An asterisk denotes the conserved cysteine residue corresponding to Cys706 of human RB (5, 39). (D) N-terminal homology domain.

digests (data not shown). PCR with maize genomic DNA amplified a sequence closely related to *RRB1* and *RRB2*, potentially encoding a third RRB protein (data not shown). However, cDNAs corresponding to this sequence were not isolated and we do not know whether this fragment represents another active *RRB* gene or a pseudogene.

***RRB* expression is ubiquitous and is highest in the shoot apex.** In mammals, the *RB* gene is expressed in all the tissues

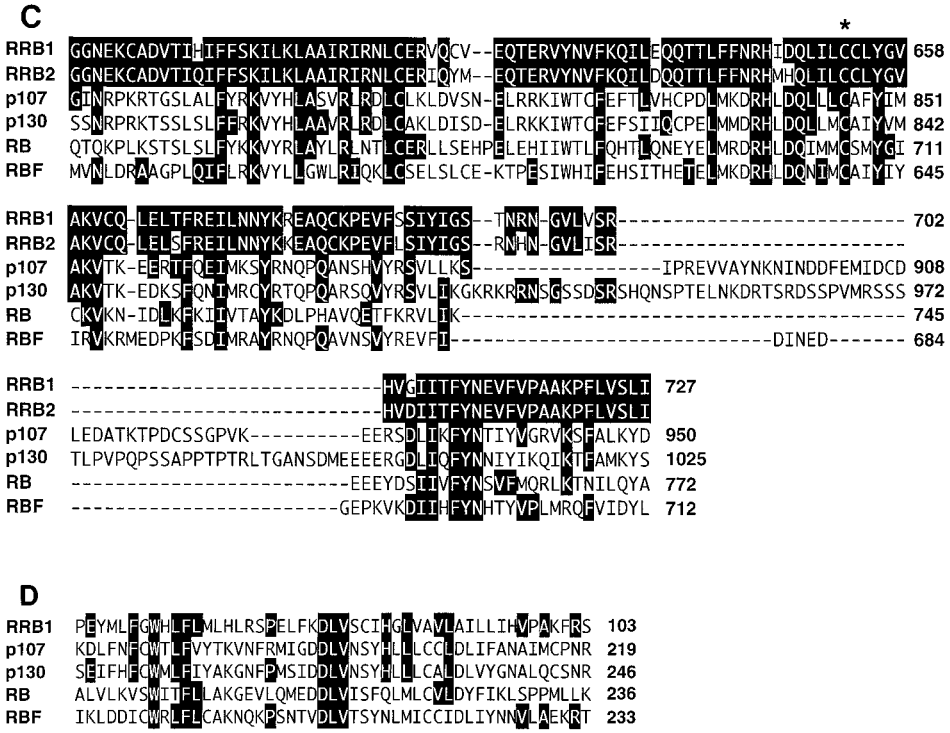

FIG. 2—*Continued.*

examined and is generally transcribed at high levels (4). To examine *RRB* expression in maize, a blot of total RNAs from various tissues was probed with an *RRB1* sequence that cross-hybridizes to both *RRB1* and *RRB2* transcripts (Fig. 3). In all the tissues examined, a transcript of approximately 3.2 kb was detected; a fainter, more diffuse band of 4.5 kb was also seen in some tissues. These transcript sizes correspond closely to the alternatively polyadenylated 4.4- and 3.0-kb *RRB1* cDNAs (Fig. 1). The highest level of *RRB* expression was seen in the shoot apex, in which cells undergo rapid division. Although the RNA blot analysis could not distinguish between *RRB1* and *RRB2* expression due to the extensive homology between the two transcripts, in an RNase protection assay mRNAs from the two genes were expressed at similar levels in the shoot apical meristem (data not shown). These results demonstrate that the maize *RRB* genes, like the mammalian *RB* gene, are ubiquitously expressed.

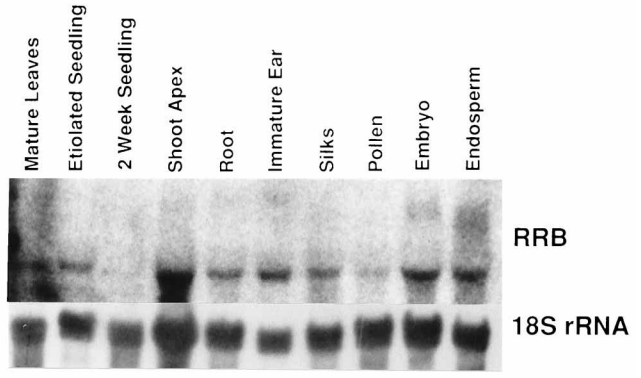

FIG. 3. Expression analysis of *RRB* mRNAs. Total RNAs (15 μg) from the indicated tissues were electrophoresed through an 0.8% agarose–formaldehyde gel, blotted onto nylon, and probed with a ^{32}P-labelled *RRB1* probe which hybridizes to both *RRB1* and *RRB2* transcripts. After autoradiography, the blot was stripped and reprobed with an 18S rRNA probe.

RRB1 is a nuclear protein in plant cells. To examine the subcellular localization of RRB1 in plant cells, a plasmid that expresses the C-terminal 581 amino acids of RRB1 fused to a myc epitope tag was constructed. This plasmid was electroporated into tobacco protoplasts, and the tagged RRB1 protein was localized by immunofluorescence with an anti-myc monoclonal antibody, 9E10. Like RB localization in mammalian cells, RRB1 was found exclusively in the nuclei of transfected protoplasts (Fig. 4A). Localization in the nucleus was confirmed by DAPI staining (Fig. 4B) and bright-field microscopy (Fig. 4C). This localization of RRB1 is possibly conferred by a consensus bipartite nuclear localization signal in the spacer region of the protein (Fig. 2A).

RRB1 can bind to mammalian DNA tumor virus oncoproteins and a plant D-type cyclin. The mammalian RB protein family binds to a large number of viral and cellular proteins (reviewed in references 7, 45, and 69), including DNA tumor virus oncoproteins (such as SV40 large-T antigen and adenovirus E1A), the E2F transcription factor family, and D-type cyclins. The ability of RB to bind to viral oncoproteins is conferred exclusively by the A and B pocket domains (34, 35, 37), although E2F and cyclin D binding also require sequences in the RB C-terminal region (31, 54). The conservation of the pocket domain in the RRB proteins suggests that they bind a set of proteins similar to those bound by RB. To test this prediction, the ability of RRB1 to bind various RB-associating proteins was tested by both yeast two-hybrid and in vitro binding assays.

For two-hybrid assays, a plasmid (214C) that expresses the maize RRB1 pocket–C-terminal region (amino acids 214 to

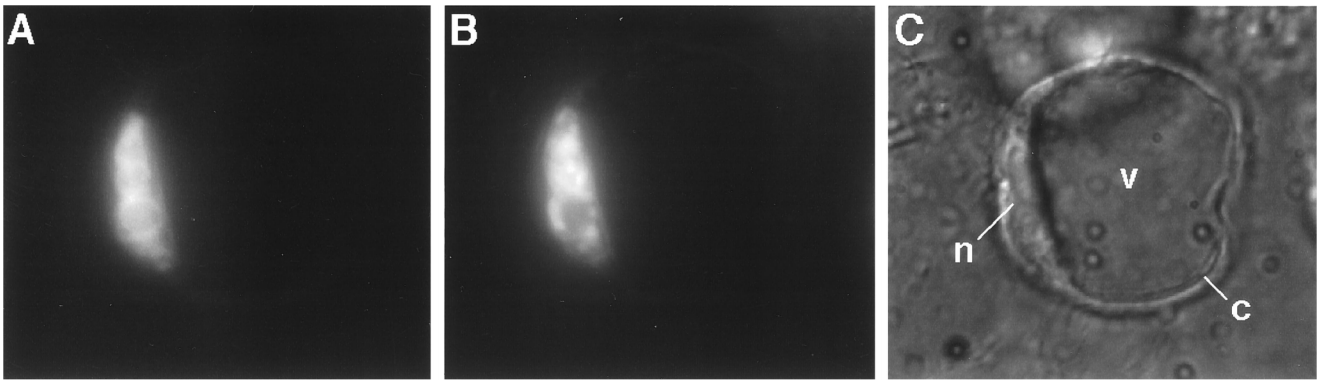

FIG. 4. RRB1 is nuclear localized in plant cells. Tobacco protoplasts were electroporated with a construct that expresses myc-epitope-tagged RRB1, and expression was analyzed after 24 h by immunoflourescence. (A) Tobacco protoplast transiently expressing myc-tagged RRB1 immunostained with anti-myc monoclonal antibody and FITC-conjugated secondary antibody. (B) DAPI staining of the same cell as in panel A. (C) Bright-field micrograph of panel A. In tobacco protoplasts, the vacuole (v) occupies most of the cell, with the cytoplasm restricted to a thin layer around the periphery of the vacuole and the region surrounding the nucleus (n). Magnification, ×900.

866) fused to the yeast Gal4 DNA-binding domain was constructed. 214C together with plasmids that express various proteins fused to the Gal4 activation domain were used to transform a yeast strain, Y153 (19), which harbors a *GAL1-lacZ* reporter gene. Transformants were assayed for β-galactosidase activity to detect any protein-protein interactions (Table 1). As a control, we also tested a plasmid which expresses a fusion of the human RB pocket and C-terminal regions to the Gal4 DNA-binding domain (19). Both RRB1 and RB bound to full-length SV40 large-T antigen, as well as to a 10-amino-acid peptide derived from large-T antigen which contains the LXCXE motif. This indicates that the sequences necessary for interacting with this critical binding motif have been conserved between the maize and human RB proteins. In contrast, RRB1 interaction with E2F-1 was only slightly above background in this assay (Table 1). In addition, a peptide corresponding to the RB-binding domain of E2F-1 (amino acids 409 to 426) (59) was also unable to bind RRB1. Thus, the precise sequences and/or structure for binding E2F-1 has not been conserved in RRB1. This is not unexpected, given that the C-terminal domain of RB is required for E2F binding (31, 54) and RRB1 has little sequence homology with RB in that region.

The abilities of RRB1 and RB to bind to plant G_1 and mitotic cyclins were also tested in the two-hybrid assay. The *Arabidopsis* δ3 cyclin is expressed at the G_1/S transition and is induced by the plant growth regulator cytokinin (62). This cyclin contains the canonical LXCXE RB-binding motif (16, 21). The N-terminal half of the δ3 cyclin, which contains the LXCXE motif, interacted strongly with both RRB1 and RB in this assay (Table 1). In contrast, full-length δ3 interacted very weakly, if at all, with either protein; this could be due to the improper folding of the fusion or the presence of an inhibitory domain. The *Arabidopsis* cyc1At cyclin is expressed predominantly in the G_2 phase of the cell cycle and lacks the LXCXE motif (30). This cyclin interacted weakly with RRB1 and not at all with human RB in this assay (Table 1). Together, these results indicate that the RB-related protein–D-type cyclin interaction is conserved between plants and mammals.

To confirm the results of the two-hybrid assay, we performed in vitro binding experiments. A ^{35}S-labelled RRB1 protein (amino acids 285 to 866) produced by in vitro transcription and translation was incubated with several GST fusion proteins bound to glutathione-Sepharose beads. After extensive washing, bound RRB1 was detected by SDS-PAGE, followed by autoradiography (Fig. 5). RRB1 bound strongly to the adenovirus 12S and 13S E1A proteins fused to GST, again demonstrating the ability of this maize protein to bind mammalian DNA tumor virus antigens. However, in contrast to the two-hybrid result, RRB1 did not bind to the 10-amino-acid T peptide fused to the C terminus of GST, possibly due to steric hindrance caused by GST. RRB1 bound strongly to the *Arabidopsis* δ3 cyclin, consistent with the two-hybrid results, al-

TABLE 1. RRB1 binding in the two-hybrid assay[a]

Activation domain fusion	β-Galactosidase activity	
	RRB1 (214–866)	Human RB (301–928)
Large-T antigen (1–273)	31.6	39.1
T peptide (105–115)	35.4	48.0
E2F-1 (284–437)	2.4	16.2
E2F-1 peptide (409–426)	1.8	11.9
Cyclin cyc1At (1–428)	10.7	<1
Cyclin cyc1At-N (1–114)	7.5	1.8
Cyclin δ3 (1–376)	3.1	8.8
Cyclin δ3-N (1–183)	20.2	44.8

[a] Y153 cells transformed with the indicated plasmids were assayed for β-galactosidase activity by chlorophenyl-red-β-D-galactopyranoside assay. Data are the averages of three independent transformants.

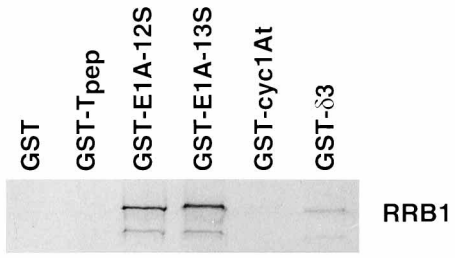

FIG. 5. In vitro binding of RRB1 protein to mammalian tumor virus antigens and plant cyclins. In vitro-translated ^{35}S-labelled RRB1 protein was incubated with the indicated GST fusion proteins bound to glutathione-Sepharose resin. Beads were washed extensively, and bound proteins were resolved by electrophoresis through an SDS–10% polyacrylamide gel, followed by autoradiography. T_{pep} corresponds to amino acids 105 to 115 of SV40 large-T antigen, E1A-12S and E1A-13S are the products of the adenovirus E1A gene, cyc1At is an *Arabidopsis* mitotic cyclin (30), and δ3 is an *Arabidopsis* D-like cyclin (62).

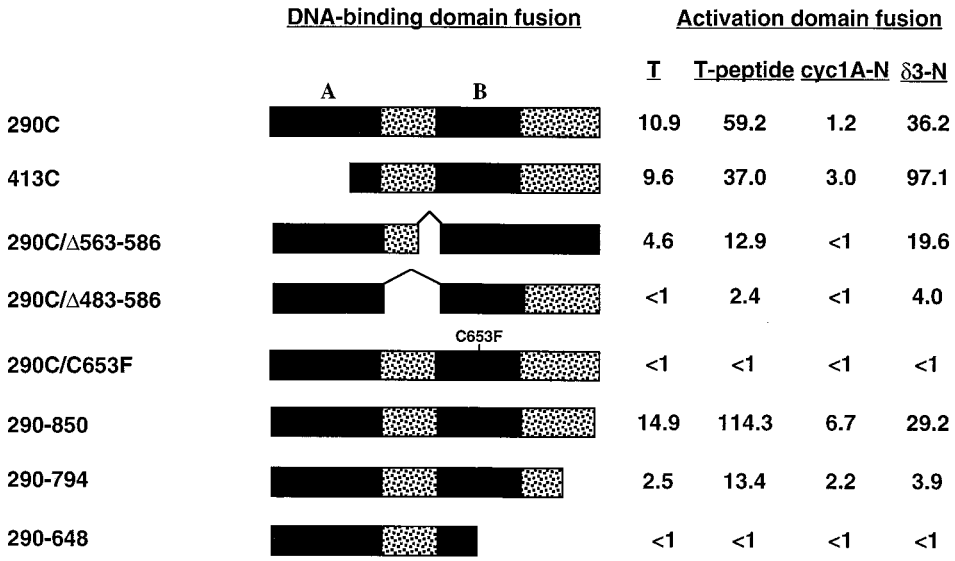

FIG. 6. Two-hybrid assay of binding abilities of RRB1 deletion mutants. Schematic drawings of the various deletion mutants of RRB1 are shown. Plasmids that express these mutants as Gal4 DNA-binding domain fusions were transformed pairwise into Y153 yeast cells along with plasmids that express Gal4 activation domain fusions of large-T antigen (T), T peptide, and cyclins cyc1At and δ3. β-Galactosidase activity was quantitated by a chlorophenyl-red-β-D-galactopyranoside assay (19). Three independent transformants were used in quantitation.

though it did not bind to the mitotic cyclin cyc1At. RRB1 did not bind to GST alone in this assay.

The A domain is less critical for binding large-T antigen and δ3 cyclin than is the B domain. To determine the regions of the RRB protein critical for binding large-T antigen and cyclins, a series of RRB1 deletions were constructed in the Gal4 DNA-binding domain vector. This deletion series was used to cotransform Y153 with plasmids that express the activation domain-tagged large-T antigen and cyclin fusions. Western analysis demonstrated that all deletion proteins were expressed at comparable levels (data not shown). Transformants were subsequently assayed for β-galactosidase activities (Fig. 6). A deletion mutant with the first 16 amino acids of the A domain removed, 290C, still bound to large T-antigen, T peptide, and the δ3 cyclin, although T binding was quantitatively reduced. 290C showed virtually no binding to cyc1At, indicating that the N terminus of the A domain is necessary for the low level of binding to this cyclin. N-terminal deletion of over half of the A domain (413C) did not significantly decrease the binding to large-T antigen or the δ3 cyclin compared to that of 290C; in fact, the binding to δ3 cyclin was enhanced. The ability to delete a large portion of the A domain and still retain binding is in sharp contrast to human RB, where an intact A domain is essential for virtually all protein interactions, including binding to large-T antigen (34, 35, 37).

Small internal deletions in the RB spacer region do not dramatically affect protein binding, although complete removal of this region blocks associated protein binding (34, 35). Similarly, deletion of 23 amino acids of the RRB1 spacer (290C/Δ563-586) resulted in a partial loss of binding to large-T antigen and the δ3 cyclin and removal of the entire spacer (290C/Δ483-586) completely blocked binding. Thus, the spacer is required for protein interaction.

A mutant with a C-terminal deletion of the last 16 amino acids of the protein (290-850) still retained the ability to bind to large-T antigen and the δ3 cyclin and also showed low-level cyc1At binding. In contrast, removal of the C-terminal 72 amino acids of RRB1 (290-794) greatly decreased the ability to bind to both large-T antigen and the δ3 cyclin, although some residual binding was observed. A deletion of the entire C-terminal domain and 74 amino acids of the B domain resulted in the loss of all binding activity. Thus, analogous to RB, RRB1 requires an intact B domain for protein association. Interestingly, the C-terminal domain is critical not only for binding the δ3 cyclin, as is this region of RB, but also for large-T-antigen interaction.

We also constructed a point mutant of RRB1 in which the cysteine at position 653 is changed to phenylalanine. This mutation is analogous to a naturally occurring mutation in human RB at position 706, which results in a total loss of protein function (5, 39). When this mutant was tested in the two-hybrid assay (Fig. 6), no binding was observed to any of the large-T-antigen or cyclin constructs, indicating that this conserved residue is also critical for maize RRB function.

RRB1 binds to a plant DNA virus replication protein. Geminiviruses are a group of plant viruses with single-stranded, circular DNA genomes that replicate in plant nuclei (reviewed in references 42 and 68). They encode only a few proteins necessary for replication (20, 27, 55, 63, 67) and are dependent upon the host cell DNA replication machinery. Recent experiments have shown that one geminivirus, TGMV, induces the accumulation of PCNA, a DNA polymerase accessory factor, in infected plant cells (48). Furthermore, the expression of one TGMV protein, AL1, is sufficient for the induction of PCNA expression in transgenic plants (48). This finding is reminiscent of the accumulation of mammalian cell replication factors in response to the expression of DNA tumor virus oncoproteins (51). Because the mammalian tumor virus oncoproteins exert their effects in part by binding to RB, we tested whether the geminivirus AL1 protein could interact with the maize RRB1 protein.

GST-RRB1 fusion protein and AL1 were coexpressed in insect cells by using a baculovirus expression system (Fig. 7, input, lane 1). Extracts from cotransfected cells were incubated with glutathione resin, and bound fractions were separated by SDS-PAGE and immunoblotted. Both proteins were detected in the bound fraction (Fig. 7, bound, lane 1), indicating that GST-RRB1 and AL1 form a complex. Two control experi-

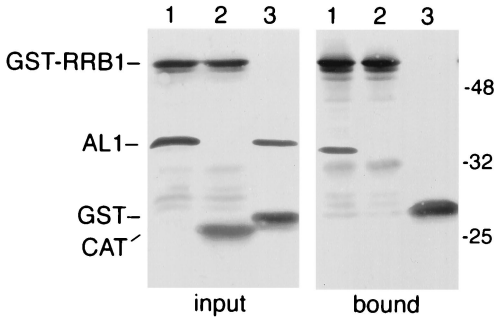

FIG. 7. RRB1 interacts with a plant geminivirus replication protein. Insect cells were coinfected with baculoviruses that express GST-RRB1 and the TGMV AL1 protein (lanes 1), GST-RRB1 and CAT (lanes 2), or GST and AL1 (lanes 3). Protein extracts from infected cells (input) were incubated with glutathione-Sepharose resin and eluted with glutathione (bound). Equivalent amounts of input and bound fractions were resolved on an SDS–16% polyacrylamide gel and analyzed by immunoblotting with antisera against RRB1, AL1, CAT, and GST. The identities of immunoreactive proteins are given on the left. The positions of prestained molecular mass (in kilodaltons) markers (New England Biolabs) are indicated on the right.

ments verified the specificity of this interaction. First, when extracts from insect cells coinfected with baculoviruses that express GST and AL1 (Fig. 7, input, lane 3) were incubated with glutathione resin, only GST was detected in the bound fraction (bound, lane 3), indicating AL1 does not bind to GST. Second, when extracts from cells that express both GST-RRB1 and CAT (Fig. 7, input, lane 2) were tested, only GST-RRB1 bound to glutathione resin (bound, lane 2). This indicates that GST-RRB1 does not aggregate nonspecifically with any coexpressed recombinant protein. Thus, RRB1 can specifically complex with the TGMV AL1 protein.

DISCUSSION

The genes and regulatory pathways involved in the control of G_1 progression in plants are largely unknown. The discovery of RB-like proteins in maize suggests that regulation of G_1 progression and S-phase entry during the plant cell cycle may be more closely related to the regulatory pathways in animal cells, where RB plays a central role, than to those in yeast, where no RB homologs have been found. Here we report the cloning of cDNAs from two maize genes, *RRB1* and *RRB2*, that encode proteins clearly related to the mammalian RB protein family. In addition, *RRB2* is alternatively spliced to yield two proteins that have different C termini. Unlike the sequence divergence seen in the mammalian RB, p107, and p130 proteins, however, the maize proteins have >90% amino acid identity. This similarity most likely reflects the pseudotetraploid origin of maize and suggests that the plant proteins are functionally more equivalent than are the three mammalian RB family members. While this paper was in preparation, two reports describing the cloning of partial *RRB1* cDNAs were published (25, 71). The C terminus of the RRB1 protein reported by Grafi et al. (25) differs from that reported here due to a frameshift caused by the absence of a G residue after position 1333 in their published sequence.

There are three regions conserved between RRB1 and the metazoan RB protein family, the A and B pocket domains and a short N-terminal domain. The arrangement of these regions, as well as the positioning of multiple potential Cdk phosphorylation sites throughout the protein, has also been conserved. The finding that a small region of the N-terminal domain is conserved among the maize and metazoan proteins is particularly intriguing, since the function of the N terminus is largely unknown. Although removal of the N-terminal domain from human RB results in a functional protein (24, 54), small deletions within this region abrogate the activity of the full-length protein (53). Binding studies have shown that the N terminus of human RB interacts with a nuclear matrix protein (18) and associates with a novel kinase activity (64). The RRB1 N-terminal domain (amino acids 1 to 290), however, was unable to interact with this mammalian nuclear matrix protein (17b). Construction of RRB mutants that lack either the N-terminal domain or the short conserved region will be needed to elucidate the functional roles of this region.

The A and B domains are essential for RB protein function in mammals, as all inactivating RB mutations isolated from tumors affect these domains (34, 35). The high degree of homology maintained in these domains throughout evolution further emphasizes their critical role and suggests that the maize proteins function similarly to their mammalian counterparts. The sequence homology also appears to confer similar biochemical properties to both plant and mammalian proteins. The A and B domains of RB were originally identified as regions essential for the binding of mammalian DNA tumor virus oncoproteins (34, 35, 37). These oncoproteins induce cellular DNA synthesis in part by binding to and thus inactivating RB (reviewed in reference 51). We have shown that RRB1 also has the ability to bind these oncoproteins, despite the significant sequence differences between the maize and human RB proteins. More importantly, the finding that certain geminiviruses encode proteins required for viral DNA replication that bind RRB1 strongly suggests that these plant pathogens use a similar mechanism to initiate DNA synthesis. Geminiviruses, like DNA tumor viruses, rely on their hosts to provide the DNA replication machinery necessary for their propagation. The ability of the TGMV AL1 protein to bind RRB1 suggests by analogy to the mammalian DNA tumor viruses, that this is an essential step in the induction of PCNA accumulation in differentiated plant cells that express AL1 (48). RRB1 also interacts with the equivalent C1 protein from wheat dwarf virus (WDV) (25, 71), possibly through an LXCXE motif in C1 (70). However, this motif is found only in a subset of C1 proteins from monocot-infecting geminiviruses and is absent in all AL1 and C1 proteins from dicot-infecting viruses, indicating that it is not generally required for geminivirus replication. It is presently unknown whether the WDV C1 protein has a role in the accumulation of host replication enzymes in infected cells. Xie et al. (71) reported that expression of RRB1 in cultured wheat cells under the control of the CaMV 35S promoter resulted in a reduction of WDV DNA replication. However, in similar experiments, we have found that RRB1 expression in tobacco protoplasts alters the activities of both the CaMV 35S and TGMV AL1 promoters (47a). Thus, RRB1 may have reduced viral replication in the wheat cell transfection assay by altering WDV C1 expression rather than through direct interaction with the C1 protein.

The maize RRB1 protein can also bind strongly to the *Arabidopsis* δ3 cyclin. The *Arabidopsis* δ3 cyclins are expressed in G_1 and S phases (62) and appear to be homologs of the human D cyclins that are essential for the inhibitory phosphorylation of RB during the G_1 phase (21, 38). The human D cyclins physically bind to RB (16, 21) and can also associate with RRB1 (17b). This conserved physical interaction, together with the presence of multiple potential Cdk phosphorylation sites in RRB1, argues that RRB1 is regulated by Cdk–D-type cyclin phosphorylation during G_1 progression. This idea is further supported by the finding that RRB1 is phosphorylated during maize endosperm development, a process characterized

by a G_1 transition into a repeated series of S phases (25). RRB1 can also bind to a mitotic cyclin, cyc1At, although at a much lower level than that of the δ3 cyclin. Although it is not known whether this weak interaction has functional relevance, it may indicate that the plant RB-like proteins also play a role in the entry into mitosis.

Despite the conservation in sequence and binding abilities, deletions in the pocket–C-terminal domain of the RRB1 protein differ somewhat in their binding properties compared to those of similar deletions in RB. Although the B domain is sensitive to deletion, a large portion of the A domain of RRB1 can be removed and still retain binding activity. The RRB1 C-terminal domain also appears to enhance large-T-antigen binding, a property not observed with RB. Thus, although the binding properties of RRB1 and RB are similar, structural differences between the two proteins obviously exist. These differences, together with the inability of RRB1 to bind human E2F-1, likely explain why the C-terminal 576 amino acids of RRB1 are unable to substitute functionally for RB in the flat cell growth suppression assay (16, 23a).

Like the mammalian *RB* gene (4), the maize *RRB* genes are ubiquitously expressed. Interestingly, the highest *RRB* mRNA levels were seen in the shoot apex, an organ with a large population of rapidly dividing cells. It is unclear why *RRB* mRNA levels are elevated in this tissue if the RRB proteins act as negative regulators of G_1 progression. It is possible that RRB proteins perform cell-cycle-regulatory roles other than negative regulation of G_1 progression and are thus required in higher amounts in cycling plant cells. In mammals, RB is also involved in inducing and maintaining a terminally differentiated state in at least some cell types (8, 26, 36, 43, 57). The presence of *RRB* mRNAs in organs with mostly differentiated, nondividing cells suggests that RRB also plays a role in the maintenance of the differentiated state in plant cells. However, plant cell differentiation is remarkably plastic, and unlike fully differentiated mammalian cells, plant cells can readily dedifferentiate and reenter the cell cycle (65, 66). Thus, if RRB does play a role in differentiation in plant cells, its exact function and regulation may be quite different.

While the loss of RB in mammalian cells appears to be a critical step in the development of many cancers (reviewed in reference 69), plants do not develop tumors outside of specialized responses to certain pathogens (reviewed in reference 15). Thus, in plant cells, the loss of an *RRB* gene may not have the same neoplastic consequence as it does in animal cells. This may simply be due to the multigenic nature of the *RRB* gene family (at least in maize). In addition, because overexpression of a G_2 cyclin in *Arabidopsis thaliana* increases the rate of cell division, G_2 phase rather than G_1 phase may be a rate-limiting step in certain plant cell cycles (14). Perhaps most importantly, plant development is surprisingly tolerant of changes in the control of cellular proliferation. In *Arabidopsis*, plants that overexpress a G_2 cyclin have longer roots with significantly more cells (13). However, these cells are incorporated into the normal body plan of the plant. Similarly, although transgenic tobacco plants that express a dominant negative mutant of cdc2 have significantly fewer cells than do wild-type plants, the wild-type plant size is maintained due to a compensatory increase in cell size (29). Because of this remarkable tolerance of plant development to changes in cell number, mutations of plant RRB may have entirely different consequences from those of mutations of RB in mammalian cells. The use of transgenic plants in overexpression and antisense experiments and transposon-tagged mutagenesis should help to elucidate the role(s) of the RRB proteins in regulating the cell cycle during plant development and cellular differentiation.

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R.A.A. and T.D. contributed equally to this work.

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REFERENCES

1. **Ach, R. A., and W. Gruissem.** 1994. A small nuclear GTP-binding protein from tomato suppresses a *Schizosaccharomyces pombe* cell-cycle mutant. Proc. Natl. Acad. Sci. USA **91:**5863–5867.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1993. Current protocols in molecular biology. John Wiley and Sons, New York, N.Y.
3. **Barkan, A., D. Miles, and W. C. Taylor.** 1986. Chloroplast gene expression in nuclear photosynthetic mutants of maize. EMBO J. **5:**1421–1427.
4. **Bernards, R., G. M. Schackleford, M. R. Gerber, J. M. Horowitz, S. H. Friend, M. Schartl, E. Bogenmann, J. M. Rappaport, T. McGee, T. Dryja, and R. A. Weinberg.** 1989. Structure and expression of the murine retinoblastoma gene and characterization of the encoded protein. Proc. Natl. Acad. Sci. USA **86:**6474–6478.
5. **Bignon, Y.-J., J.-Y. Shew, D. Rappolee, S. L. Naylor, E. Y.-H. P. Lee, J. Schnier, and W.-H. Lee.** 1990. A single cys706 to phe substitution in the retinoblastoma protein causes the loss of binding to SV40 T antigen. Cell Growth Differ. **1:**647–651.
6. **Chellappan, S., V. Kraus, B. Kroger, K. Munger, P. M. Howley, W. C. Phelps, and J. R. Nevins.** 1992. Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc. Natl. Acad. Sci. USA **89:**4549–4553.
7. **Chen, P.-L., D. J. Riley, and W.-H. Lee.** 1995. The retinoblastoma protein as a fundamental mediator of growth and differentiation signals. Crit. Rev. Eukaryotic Gene Expr. **5:**79–95.
8. **Clarke, A. R., E. R. Maandag, M. van Roon, N. M. T. van der Lugt, M. van der Valk, M. L. Hooper, A. Berns, and H. te Riele.** 1992. Requirement for a functional Rb-1 gene in murine development. Nature **359:**328–330.
9. **Collin, S., M. Fernandez-Lobato, P. S. Gooding, P. M. Mullineaux, and C. Fenoll.** 1996. The two nonstructural proteins from wheat dwarf virus involved in viral gene expression and replication are retinoblastoma-binding proteins. Virol. **219:**324–329.
10. **Dahl, M., I. Meskiene, L. Bogre, D. T. C. Ha, I. Swoboda, R. Hubmann, H. Hirt, and E. Heberle-Bors.** 1995. The D-type alfalfa cyclin gene cycMs4 complements G1 cyclin-deficient yeast and is induced in the G1 phase of the cell cycle. Plant Cell **7:**1847–1857.
11. **DeGregori, J., T. Kowalik, and J. R. Nevins.** 1995. Cellular targets for activation by the E2F-1 transcription factor include DNA synthesis- and G_1/S-regulatory genes. Mol. Cell. Biol. **15:**4215–4224.
12. **Dirick, L., T. Bohm, and K. Nasmyth.** 1995. Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*. EMBO J. **14:**4803–4813.
13. **Doerner, P., J.-E. Jorgensen, R. You, J. Steppuhn, and C. Lamb.** 1996. Control of root growth and development by cyclin expression. Nature **380:**520–523.
14. **Doerner, P. W.** 1994. Cell cycle regulation in plants. Plant Physiol. **106:**823–827.
15. **Doonan, J., and T. Hunt.** 1996. Why don't plants get cancer? Nature **380:** 481–482.
16. **Dowdy, S. F., P. W. Hinds, K. Louie, S. I. Reed, A. Arnold, and R. A. Weinberg.** 1993. Physical interaction of the retinoblastoma protein with human D cyclins. Cell **73:**499–511.
17. **Du, W., M. Vidal, J.-E. Xie, and N. Dyson.** 1996. RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in *Drosophila*. Genes Dev. **10:**1206–1218.

17a. **Durfee, T.** Unpublished data.

17b. **Durfee, T., and R. A. Ach.** Unpublished data.

18. **Durfee, T., M. A. Mancini, D. Jones, S. J. Elledge, and W.-H. Lee.** 1994. The amino-terminal region of the retinoblastoma gene product binds a novel nuclear matrix protein that co-localizes to centers for RNA processing. J. Cell Biol. **127:**609–622.
19. **Durfee, T., K. Becherer, P.-L. Chen, S.-H. Yeh, Y. Yang, A. E. Kilburn, W.-H. Lee, and S. J. Elledge.** 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev. **7:**555–569.

19a. **Durfee, T., and P. C. Zambryski.** Unpublished data.

20. **Elmer, J. S., L. Brand, G. Sunter, W. E. Gardiner, D. M. Bisaro, and S. G. Rogers.** 1988. Genetic analysis of tomato golden mosaic virus. II. Requirement for the product of the highly conserved AL1 coding sequence for replication. Nucleic Acids Res. **16:**7043–7060.
21. **Ewen, M. E., H. K. Sluss, C. J. Sherr, H. Matsushime, J.-Y. Kato, and D. M. Livingston.** 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell **73:**487–497.
22. **Ewen, M. E., Y. Xing, J. B. Lawrence, and D. M. Livingston.** 1991. Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell **66:**1155–1164.
23. **Figge, J., T. Webster, T. Smith, and E. Paucha.** 1988. Prediction of similar transforming regions in simian virus 40 large T, adenovirus E1A, and *myc* oncoproteins. J. Virol. **62:**1814–1818.

23a. **Goodrich, D. W., T. Durfee, R. A. Ach, P. C. Zambryski, and W. Gruissem.** Unpublished data.

24. **Goodrich, D. W., N.-P. Wang, Y.-W. Qian, E. Y.-H. P. Lee, and W.-H. Lee.** 1991. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell **67:**293–302.
25. **Grafi, G., R. J. Burnett, T. Helentjaris, B. A. Larkins, J. A. DeCaprio, W. R. Sellers, and W. G. Kaelin.** 1996. A maize cDNA encoding a member of the retinoblastoma protein family: involvement in endoreduplication. Proc. Natl. Acad. Sci. USA **93:**8962–8967.
26. **Gu, W., J. W. Schneider, G. Condorelli, S. Kaushal, V. Mahdavi, and B. Nadal-Ginard.** 1993. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell **72:**309–324.
27. **Hanley-Bowdoin, L., J. S. Elmer, and S. G. Rogers.** 1990. Expression of functional replication protein from tomato golden mosaic virus in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA **87:**1446–1450.
28. **Hannon, G. J., D. Demetrick, and D. Beach.** 1993. Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes Dev. **7:**2378–2391.
29. **Hemerly, A., J. de Almeida Engler, C. Bergounioux, M. van Montagu, G. Engler, D. Inze, and P. Ferreira.** 1995. Dominant negative mutants of the cdc2 kinase uncouple cell division from iterative plant development. EMBO J. **14:**3925–3936.
30. **Hemerly, A., C. Bergounioux, M. van Montagu, D. Inze, and P. Ferreira.** 1992. Genes regulating the plant cell cycle: isolation of a mitotic-like cyclin from *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **89:**3295–3299.
31. **Hiebert, S. W., S. P. Chellappan, J. M. Horowitz, and J. R. Nevins.** 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev. **6:**177–185.
32. **Hinds, P. W., S. Mittnacht, V. Dulic, A. Arnold, S. I. Reed, and R. A. Weinberg.** 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell **70:**993–1006.
33. **Howard, E. A., J. R. Zupan, V. Citovsky, and P. C. Zambryski.** 1992. The virD2 protein of A. tumefaciens contains a C-terminal bipartite nuclear localization signal: implications for nuclear uptake of DNA in plant cells. Cell **68:**109–118.
34. **Hu, Q., N. Dyson, and E. Harlow.** 1990. The regions of the retinoblastoma protein needed for binding to the adenovirus E1A or SV40 large T antigen are common sites for mutation. EMBO J. **9:**1147–1155.
35. **Huang, S., N.-P. Wang, B. Y. Tseng, W.-H. Lee, and E. H.-Y. P. Lee.** 1990. Two distinct and frequently mutated regions of the retinoblastoma protein are required for binding to SV40 T antigen. EMBO J. **9:**1815–1822.
36. **Jacks, T., A. Fazeli, E. M. Schmitt, R. T. Bronson, M. A. Goodell, and R. A. Weinberg.** 1992. Effects of an Rb mutation in the mouse. Nature **359:**295–300.
37. **Kaelin, W. G., M. E. Ewen, and D. M. Livingston.** 1990. Definition of the minimal simian virus 40 large T antigen- and adenovirus E1A-binding domain in the retinoblastoma gene product. Mol. Cell. Biol. **10:**3761–3769.
38. **Kato, J.-Y., H. Matsushime, S. W. Hiebert, M. E. Ewen, and C. J. Sherr.** 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. **7:**331–342.
39. **Kaye, F. J., R. A. Kratze, J. L. Gerster, and J. M. Horowitz.** 1990. A single amino acid substitution results in a retinoblastoma protein defective in phosphorylation and oncoprotein binding. Proc. Natl. Acad. Sci. USA **87:**6922–6926.
40. **Koch, C., A. Schleiffer, G. Ammerer, and K. Nasmyth.** 1996. Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at Start, whereas Clb/Cdc28 kinases displace it from the promoter in G2. Genes Dev. **10:**129–141.
41. **Lam, E. W.-F., and N. B. LaThangue.** 1994. DP and E2F proteins: coordinating transcription with cell cycle progression. Curr. Opin. Cell Biol. **6:**859–866.
42. **Lazarowitz, S.** 1992. Geminiviruses: genome structure and gene function. Crit. Rev. Plant Sci. **11:**327–349.
43. **Lee, E. Y.-H. P., C.-Y. Chang, N. Hu, Y.-C. J. Wang, C.-C. Lee, K. Herrup, W.-H. Lee, and A. Bradley.** 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoesis. Nature **359:**288–294.
44. **Luckow, V. A., S. C. Lee, G. F. Barry, and P. O. Olins.** 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. J. Virol. **67:**4566–4579.
45. **Ludlow, J. W., and G. R. Skuse.** 1995. Viral oncoprotein binding to pRB, p107, p130, and p300. Virus Res. **35:**113–121.
46. **Matsuoka, S., M. Edwards, C. Bai, S. Parker, P. Zhang, A. Baldini, J. W. Harper, and S. J. Elledge.** 1995. $p57^{KIP2}$, a structurally distinct member of the $p21^{CIP1}$ cdk-inhibitor family, is a candidate tumor suppressor gene. Genes Dev. **9:**650–662.
47. **McLean, B. G., J. Zupan, and P. C. Zambryski.** 1995. Tobacco mosaic virus movement protein associates with the cytoskeleton in tobacco cells. Plant Cell **7:**2101–2114.

47a. **Miller, A. B., and L. Hanley-Bowdoin.** Unpublished data.

48. **Nagar, S., T. J. Pedersen, K. M. Carrick, L. Hanley-Bowdoin, and D. Robertson.** 1995. A geminivirus induces expression of a host DNA synthesis protein in terminally differentiated plant cells. Plant Cell **7:**705–719.
49. **Narita, J. O., and W. Gruissem.** 1989. Tomato hydroxymethylglutaryl-CoA reductase is required early in fruit development but not during ripening. Plant Cell **1:**181–190.
50. **Nasmyth, K.** 1993. Control of the yeast cell cycle by the Cdc28 protein kinase. Curr. Opin. Cell Biol. **5:**166–179.
51. **Nevins, J. R.** 1992. E2F-A link between the RB tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
52. **Orozco, B. M., and L. Hanley-Bowdoin.** 1996. A DNA structure is required for geminivirus origin function. J. Virol. **70:**148–158.
53. **Qian, Y., C. Luckey, L. Horton, M. Esser, and D. J. Templeton.** 1992. Biological function of the retinoblastoma protein requires distinct domains for hyperphosphorylation and transcription factor binding. Mol. Cell. Biol. **12:**5363–5372.
54. **Qin, X.-Q., T. Chittenden, D. M. Livingston, and W. G. Kaelin, Jr.** 1992. Identification of a growth suppression domain within the retinoblastoma gene product. Genes Dev. **6:**953–964.
55. **Saunders, K., A. Lucy, and J. Stanley.** 1991. DNA forms of the geminivirus African cassava mosaic virus consistent with a rolling circle mechanism of replication. Nucleic Acids Res. **19:**2325–2330.
56. **Schiestl, R. H., and R. D. Geitz.** 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. **16:**339–346.
57. **Schneider, J. W., W. Gu, L. Zhu, V. Mahdavi, and B. Nadal-Ginard.** 1994. Reversal of terminal differentiation mediated by p107 in $RB^{-/-}$ mouse cells. Science **264:**1467–1471.
58. **Settlage, S. B., A. B. Miller, and L. Hanley-Bowdoin.** 1996. Interactions between geminivirus replication proteins. J. Virol. **70:**6790–6795.
59. **Shan, B., T. Durfee, and W.-H. Lee.** 1996. Disruption of RB/E2F-1 interaction by single point mutations in E2F-1 enhances S-phase entry and apoptosis. Proc. Natl. Acad. Sci. USA **93:**679–684.
60. **Shen, B., N. Carneiro, I. Torres-Jerez, B. Stevenson, T. McReery, T. Helentjaris, C. Baysdorfer, E. Almira, R. J. Ferl, J. E. Habben, and B. Larkins.** 1994. Partial sequencing and mapping of clones from two maize cDNA libraries. Plant Mol. Biol. **26:**1085–1101.
61. **Sherr, C. J.** 1994. G1 phase progression: cycling on cue. Cell **79:**551–555.
62. **Soni, R., J. P. Carmichael, Z. H. Shah, and J. A. H. Murray.** 1995. A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. Plant Cell **7:**85–103.
63. **Stenger, D. C., G. N. Revington, M. C. Stevensen, and D. M. Bisaro.** 1991. Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling-circle replication of a plant viral DNA. Proc. Natl. Acad. Sci. USA **88:**8029–8033.
64. **Sterner, J. M., Y. Murata, H. G. Kim, S. B. Kennett, D. J. Templeton, and J. M. Horowitz.** 1995. Detection of a novel cell cycle-regulated kinase activity that associates with the amino terminus of the retinoblastoma protein in the G2/M phases. J. Biol. Chem. **270:**9281–9288.
65. **Steward, F. C.** 1970. From cultured cells to whole plants: the induction and control of their growth and morphogenesis. Proc. R. Soc. Lond. Ser. B **175:**1–30.
66. **Steward, F. C., M. O. Mapes, A. E. Kent, and R. D. Holsten.** 1964. Growth and development of cultured plant cells. Science **143:**20–27.
67. **Sunter, G., M. D. Hartitz, S. G. Hurmuzdi, C. L. Brough, and D. M. Bisaro.** 1990. Genetic analysis of tomato golden mosaic virus: ORF AL2 is required for coat protein accumulation while ORF AL3 is necessary for efficient DNA replication. Virology **179:**69–77.
68. **Timmermans, M., O. Das, and J. Messing.** 1994. Geminiviruses and their uses as extrachromosomal replicons. Annu. Rev. Plant Physiol. Plant Mol. Biol. **45:**79–112.
69. **Weinberg, R. A.** 1995. The retinoblastoma protein and cell cycle control. Cell **81:**323–330.
70. **Xie, Q., P. Suarez-Lopez, and C. Gutierrez.** 1995. Identification and analysis of a retinoblastoma binding motif in the replication protein of a plant DNA virus: requirement for efficient viral DNA replication. EMBO J. **14:**4073–4082.
71. **Xie, Q., A. P. Sanz-Burgos, G. J. Hannon, and C. Gutierrez.** 1996. Plant cells contain a novel member of the retinoblastoma family of growth regulatory proteins. EMBO J. **15:**4900–4908.
72. **Zamanian, M., and N. B. LaThangue.** 1992. Adenovirus E1a prevents the retinoblastoma gene product from repressing the activity of a cellular transcription factor. EMBO J. **11:**2603–2610.