Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5863–5867, June 1994
Plant Biology

A small nuclear GTP-binding protein from tomato suppresses a *Schizosaccharomyces pombe* cell-cycle mutant

(Ran/*spi1*/TC4/plant cell cycle/*Lycopersicon esculentum*)

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Communicated by John Gerhart, March 10, 1994

ABSTRACT **Ran is a 25-kDa Ras-related nuclear GTP-binding protein which is very highly conserved in humans, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. Ran has been found to form a stable, noncovalent complex with the chromatin-associated protein RCC1, a negative regulator of mitosis. In *Sch. pombe*, a temperature-sensitive mutation in the RCC1 homolog encoded by the *pim1* gene causes premature induction of mitosis, and this mutation can be suppressed by overexpression of the Ran homolog encoded by *spi1*. We report here the cloning of three Ran cDNAs from tomato. The Ran protein is very highly conserved among plants, animals, and fungi. In tomato, Ran mRNA is expressed in all tissues examined, even those with little or no cell division, indicating that Ran in plants may have functions other than just control of mitosis. We have found that the tomato Ran protein can direct a β-glucuronidase reporter protein to the plant cell nucleus, confirming that Ran is a nuclear protein in plants. We show that the tomato Ran protein can suppress the *Sch. pombe pim1* mutation, indicating that the tomato Ran protein and the *Sch. pombe* spi1 protein are functionally homologous.**

The Ras-related protein Ran is a 25-kDa nuclear protein that was shown in mammalian cells to bind to the chromatin-associated protein RCC1 (1). RCC1 was originally isolated from a hamster cell line (2) where a temperature-sensitive RCC1 mutation causes either premature chromosome condensation, activation of $p34^{cdc2}$ kinase, and entry into mitosis if the temperature is shifted during the S or G_2 phase of the cell cycle or cell-cycle arrest if the temperature is shifted during G_1 (3, 4). RCC1 catalyzes the exchange of guanine nucleotides on the Ran protein (5), suggesting that RCC1 and Ran may constitute parts of a GTPase switch that is involved in the coupling of the completion of DNA replication to the onset of M phase (for reviews, see refs. 6–8). Like their mammalian counterparts, the RCC1 and Ran homologs of *Schizosaccharomyces pombe*, called pim1 and spi1, respectively, have also been shown to be involved in the coupling of DNA replication to mitosis (9). A temperature-sensitive mutation in the *pim1* gene causes premature chromosome condensation and entry into mitosis at the restrictive temperature. Overexpression of *spi1* can suppress this mutation, and pim1 and spi1 have been shown to physically interact *in vitro* (10). In *Saccharomyces cerevisiae* a temperature-sensitive mutation in the RCC1 homolog PRP20/SRM1 can be suppressed by overexpression of either of the two *S. cerevisiae* Ran homologs (11, 12). However, unlike RCC1 and pim1, PRP20/SRM1 was first discovered as a gene essential for a variety of nuclear functions including pheromone response, mRNA metabolism and export, nuclear structure, and plasmid and chromosome stability (13–17). Also, H1 kinase is not activated in *srm1* mutants (16), and disruption of the two Ran genes inhibits mRNA export (12), indicating that RCC1 and Ran play broad roles in nuclear structure and function in *S. cerevisiae*, which may differ from other organisms. However, findings that RCC1 is involved in mRNA export from mammalian nuclei (14) and in DNA replication in *Xenopus* oocyte extracts (18), that pim1 is involved in mRNA export in *Sch. pombe* (12), that Ran is involved in nuclear import of proteins in vertebrates (19), and that a mutant Ran protein can inhibit DNA replication in COS cells (20) indicate that RCC1 and Ran have broader roles than just the regulation of mitosis in other organisms as well (6).

We have started to investigate Ras-related GTP-binding proteins and their regulation during tomato fruit development in order to understand the regulatory pathways that operate to control division, growth, and differentiation of plant cells, which remain largely unelucidated. Tomato fruit is a uniquely valuable organ in which problems in plant cell growth and division can be studied, since cell division and expansion occur rapidly and in distinct developmental stages, and initiation and control of fruit development can be manipulated experimentally by application of exogenous hormones and growth inhibitors (reviewed in ref. 21). We describe here the isolation and characterization of Ran cDNAs from tomato.* We find that the Ran protein is remarkably conserved among plants, animals, and fungi, suggesting that this protein plays a fundamental role in cell division and nuclear function in all eukaryotes.

MATERIALS AND METHODS

Plant Materials. Tomato DNA and RNAs were from greenhouse-grown *Lycopersicon esculentum* cv. VFNT Cherry LA1221. The young-fruit cDNA library was made from mRNA from 3- to 8-mm fruit by using a Stratagene Uni-ZAP XR kit. The *Arabidopsis* cDNA library was made from mRNA from *Arabidopsis thaliana* var. Columbia leaves.

Degenerate PCR of Ran cDNAs. Two degenerate oligonucleotide primers were derived from aa 33–39 and 154–160 of the human Ran protein TC4 (22). Their sequences were 5′-GGGAATTCGGIGARTTYGAGAARAARTA-3′ and 5′-CGGGATCCGGYTTYTCGAARTTRTARTT-3′, where I is deoxyinosine. The PCR mixture contained 500 pmol of each primer, amplified phage library from tomato (1.2×10^7 plaque-forming units) or *Arabidopsis* (1.7×10^6 plaque-forming units), and 2.5 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus). Forty-four cycles were performed of 1 min at 94°C, 2 min at the annealing temperature, and 2 min at 70°C. The annealing temperature started at 52°C and was decreased 2°C every second cycle to a "touchdown" (23) at 38°C for the final 30 cycles. The final cycle was followed by a 7-min extension at 70°C.

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Abbreviation: GUS, β-glucuronidase.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L28713, L28714, and L28715).

cDNA Library Screening. Phage from a tomato young-fruit cDNA library were plated onto *Escherichia coli* XL1-Blue cells and were blotted and screened as described (24) with a random-primer ^{32}P-labeled PCR fragment (Stratagene Prime-It kit) containing RAN1 sequences. Positive phage were plaque purified and phage DNA was excised *in vivo* to recover pBluescript plasmid according to Stratagene's protocol.

Northern and Southern Blot Analysis. Total RNA was isolated from tomato tissues (24), and 2.5-μg samples were blotted and hybridized as described (25) with a random-primer ^{32}P-labeled RAN1 cDNA probe. After autoradiography, filters were stripped and reprobed with an *Xba* I fragment containing the pea nuclear 18S rRNA gene (26).

Southern analysis of tomato genomic DNA was performed as described (25).

Nuclear Localization. The plasmid pRTL-GUS/Nla (27), containing β-glucuronidase (GUS) coding sequence fused to coding sequence of the nuclear protein Nla of tobacco etch virus under control of the cauliflower mosaic virus 35S promoter, was digested with *Bam*HI/*Bgl* II to remove the Nla sequence. PCR was performed on the RAN2A cDNA clone, using the primers 5′-CGCGGATCCGCTTTAC-CAAACCAAC-3′ and 5′-CGCGGATCCGCTCTGAGC-TATGCC-3′ to produce a 698-bp fragment containing the RAN2A coding region. The PCR fragment was digested with *Bam*HI and inserted into the above vector to create an in-frame GUS–RAN2A fusion.

Growth and electroporation of *Nicotiana tabacum* suspension cultures (line XD) and GUS assays were as described (28). Two hours after initiation of the GUS assay, protoplasts were examined with a Zeiss Axiophot microscope with differential interference optics. Electroporation efficiency was ≈5%.

Yeast Transformation. RAN2A cDNA was inserted into the *Sch. pombe* expression vector pART3 (29) by cutting the excised pBluescript plasmids with *Bam*HI/*Xho* I, filling in with Klenow DNA polymerase, and ligating the cDNA insert into pART3 cut with *Nde* I and filled in with Klenow polymerase.

The *Sch. pombe pim1* strain SP1027 (*h⁻leu1-32 pim1-46*) (9) was transformed (30) and plated onto SD plates (31) at 23°C. Leu$^+$ transformants were restreaked and incubated at 23°C or 37°C.

RESULTS

Isolation of Tomato Ran cDNA Clones. We synthesized two degenerate oligonucleotides corresponding to aa 33–39 and 154–160 of the human Ran protein (22). PCR amplification using these two primers was performed with a tomato cDNA library constructed from mRNA from young tomato fruit, a rapidly dividing tissue. A DNA fragment of about 380 bp was seen upon amplification from this library, as well as from an *Arabidopsis* leaf cDNA library. Upon sequencing, both fragments were found to encode Ran homologs. The tomato PCR subclone was then used to screen 450,000 plaques from the

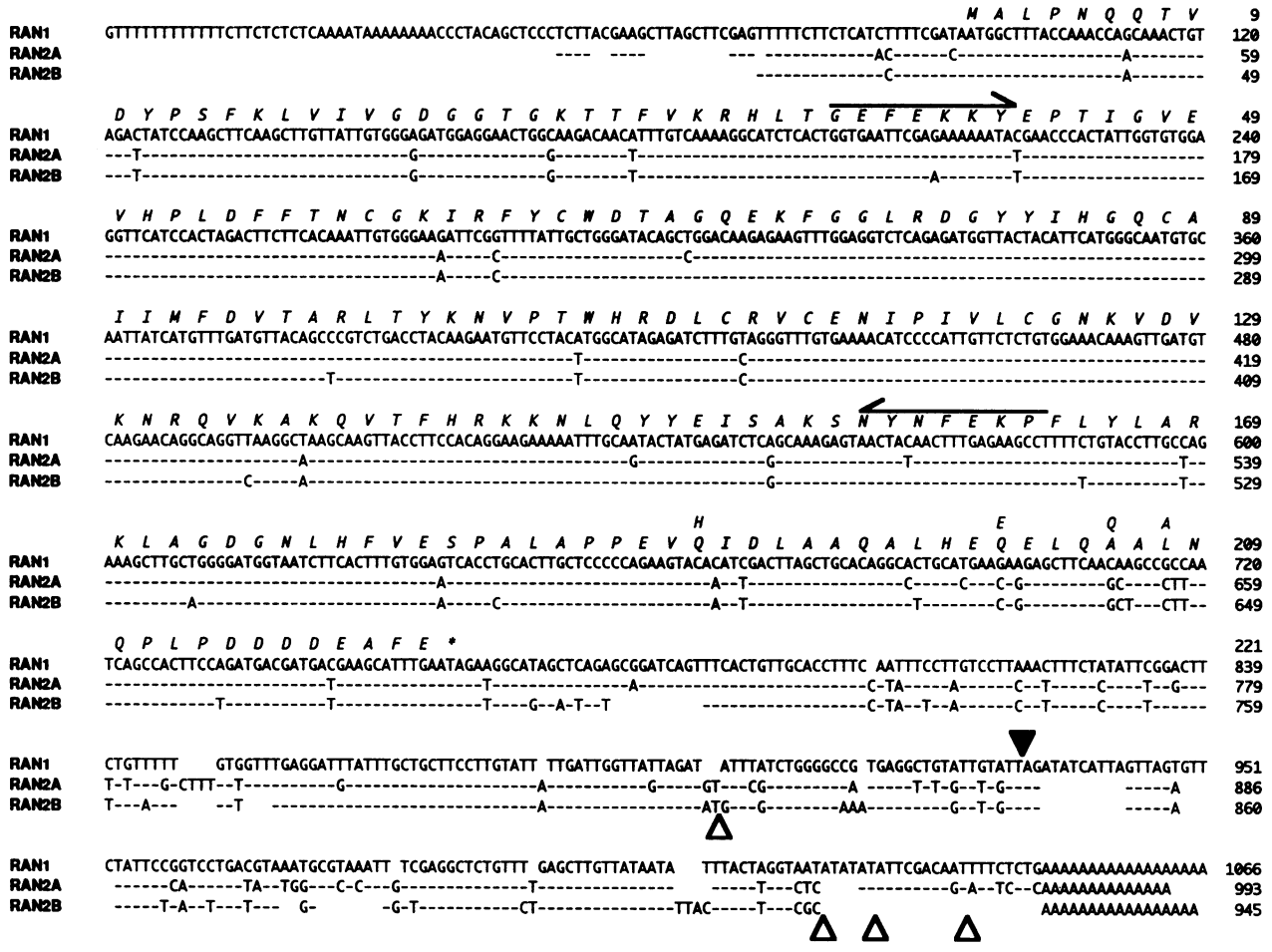

FIG. 1. Nucleotide sequence and conceptual translation of three tomato Ran cDNAs. Nucleotides in RAN2A and RAN2B cDNAs that are identical to RAN1 are denoted by dashes. Spaces indicate gaps in homology. Where the RAN1 protein sequence differs from RAN2A and RAN2B, the amino acid sequence for RAN1 is listed on top. The sequence of the longest cDNA of each type is shown. The filled-in triangle marks the alternate polyadenylylation site seen for RAN1, whereas the empty triangles mark the alternative polyadenylylation sites for the RAN2A cDNAs. Half-arrows note the regions corresponding to the two degenerate PCR primers.

young-fruit cDNA library, of which 150 hybridized to the Ran probe. Sequence analysis of 14 positive clones showed that they represented three different classes of Ran cDNAs, designated RAN1, RAN2A, and RAN2B. Of the 14 clones, 5 were RAN1, 8 were RAN2A, and 1 was RAN2B. Members of each class of cDNA had the same nucleotide sequence but differed in the amount of 5′ sequence and in the location of the polyadenylylation site.

The three classes of Ran cDNAs (Fig. 1) are ≈90% identical in nucleotide sequence, with the majority of the nucleotide differences in the 3′ untranslated region. All three cDNAs contain open reading frames encoding 221-aa, 25-kDa proteins with a high degree of homology to Ran proteins from mammals and fungi. The RAN2A and RAN2B cDNAs encode identical proteins, whereas RAN1 differs in four amino acids. The DNA sequence surrounding the initial ATG contains a good match to the plant translation initiation consensus sequence AACAATGGC (32). Several RAN1 and RAN2A cDNAs were isolated that differ in their polyadenylylation sites (Fig. 1). None of the 3′ untranslated regions contain an AATAAA polyadenylylation consensus sequence. The lack of such a sequence, along with the presence of multiple polyadenylylation sites, is common in plant genes (33, 34).

A comparison of the amino acid sequences of the two tomato Ran proteins with Ran homologs from *Sch. pombe, S. cerevisiae, Dictyostelium discoideum, Brugia malayi* (a parasitic filarial worm), and humans, along with the partial sequence of the *Arabidopsis* Ran protein we obtained by PCR, is shown in Fig. 2. The Ran amino acid sequence is highly conserved among the plant, fungi, and animal kingdoms. The most variable regions of the proteins are at the N and C termini, although the last 30 aa are generally very acidic and glutamine-rich. Unlike other members of the Ras superfamily of proteins, none of the Ran proteins have amino acid sequences at their C terminus that resemble the conserved amino acid motifs that serve as sites for prenylation (37). The Ran proteins share five regions of amino acid homology with the human Ras protein. Four of these regions are involved in guanine nucleotide binding (38), while the fifth, amino acids 42–50 of tomato Ran, corresponds to the effector domain of Ras, which interacts with the GTPase-activating protein GAP (38, 39).

To determine the approximate number of Ran genes in the tomato genome, a genomic Southern analysis was performed with a RAN2A cDNA probe (Fig. 3). The cDNA probe detected two strong and five faint bands in an *Eco*RI digest, six bands of varying intensity in a *Hin*dIII digest, seven bands in an *Xmn* I digest, and one strong band in a *Spe* I digest. Although the intron/exon structure of the Ran genes cannot be elucidated until genomic clones are isolated, we conclude that the Ran protein in tomato is encoded by a small multigene family of three to six closely related genes.

Expression of Ran mRNA in Tomato Tissues. To determine the expression levels of Ran mRNA in various tomato tissues, we performed an RNA blot analysis (Fig. 4). The probe used was the full-length RAN1 cDNA, which should hybridize with all three Ran transcripts. Identical results were obtained with a RAN2A probe (data not shown). In all tissues we detected a transcript of about 1 kb, which corresponds to the sizes of the Ran cDNAs that were isolated. The levels of Ran transcript were similar in total RNA from young leaves and all stages of fruit, lower in roots and light- or dark-grown cotyledons, and lower still in mature leaves. Interestingly, Ran mRNA levels stay relatively constant during fruit development, even though the amount of cell division decreases from a high level in 0.8 cm fruit to almost zero in red fruit (21). The presence of Ran mRNA in all tissues, including those where little cell division is occurring, suggests that Ran may play a more general role in plants than just cell cycle control. In all tissues Ran mRNA is relatively abundant and is present in light-grown cotyledons at ≈10% of the level of the mRNA for the small subunit of ribulose-1,5-bisphosphate carboxylase, a very highly expressed mRNA (data not shown).

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                                                                  52
Tomato 1    MALPNQQTVDYPSFKLVIVGDGGTGKTTFVKRHLTGEFEKKYEPT
Tomato 2    ------------------------------------------------
S. pombe    --    -PQNV-T----L----------------------IA-
S. cerev. 1 -SA- AANGEV-T----L----------------------IA-
S. cerev. 2 -SA-A-NNAEV-T----L----------------------IA-
Dictyostel. --EKE-I       ---L-----V------Q--------PR-I--
B. malayi   --    TGD-I-T----L-----------------D-----VA-
Human       --   A-GEPQVQ----L----------------------VA-
Arabidopsis                                          ----
Human Ras                    GAGGVGK                 YDPT
                                                     104
Tomato 1    IGVEVHPLDFFTNCGKIRFYCWDTAGQEKFGGLRDGYYIHGQCAI
Tomato 2    ------------------------------------------------
S. pombe    L-------H-H--F-E-C-NV--------L---------Q---G-
S. cerev. 1 --------S-Y--F-E-K-DV------------------NA----
S. cerev. 2 --------S-Y--F-E-K-DV------------------NA----
Dictyostel. L--S----I-Y--F---H-NV------------------Q-N---
B. malayi   L-------I-H--R-Q---NV------------------Q----F
Human       L-------V-H--R-P-K-NV------------------QA-----
Arabidopsis ------------------------------------------------
Human Ras   IEDSY                DTAGQE
                                                     156
Tomato 1    IMFDVTARLTYKNVPTWHRDLCRVCENIPIVLCGNKVDVKNRQVK
Tomato 2    ------------------------------------------------
S. pombe    ------S-I------H-W---V------------------E-K--
S. cerev. 1 ------S-I------N-----V------------------E-K--
S. cerev. 2 ------S-I------N-----V------------------E-K--
Dictyostel. ------S-IS-----N--S--T------------------D-K--
B. malayi   N-------V------N-----V------------------D-K--
Human       ------S-V------N-----V----------------I-D-K--
Arabidopsis ------------------------------------------------
Human Ras                                        NKCD
                                                     208
Tomato 1    AKQVTFHRKKNLQYYEISAKSNYNFEKPFLYLARKLAGDGNLHFV
Tomato 2    ------------------------------------------------
S. pombe    --AI----------D-------------W-----V-NP--E--
S. cerev. 1 --TI----------D-------------W-------NPQ-E--
S. cerev. 2 --TI----------D-------------W-------NPQ-E--
Dictyostel. PS-IV---RY--S--DV-----------VW-TS--L-NKAVTL-
B. malayi   --TI----------D-------------W-----L--P--E--
Human       --SIV---------D-------------W-----I--P--E--
Arabidopsis ---------------
Human Ras                ETSAK
                                                     221
Tomato 1    ESPALAPPEVHIDLAAQALHEEELQQAANQPLPDDDDEAFE
Tomato 2    ----------Q----------Q---A-L-------------
S. pombe    A---------QV-QQLL-QYQQ-MNE--AM----E-- -DL
S. cerev. 1 A---------QV-EQLMQQYQQ-ME--TAL----E-- -DL
S. cerev. 2 A---------QV-EQLMHQYQQ-MD--TAL----E-- -DL
Dictyostel. QQ-T-KL--TVL-SNLMS-Y-K-VAD--AL---E-V-  DL
B. malayi   AM--------QM-PTMV-QY-Q-IAA---AE-----E  DL
Human       AM--------VM-P-LA-QY-HD-EV-QTTA---E--  DL
```

FIG. 2. Compilation of Ran protein sequences. Amino acid sequences for eukaryotic Ran-like proteins (9, 11, 20, 35, 36) are shown with dashes indicating identity to the tomato RAN1 sequence. The *Arabidopsis* sequence was obtained by PCR from a leaf cDNA library (data not shown).

RAN2A Amino Acid Sequences Direct GUS into the Plant Cell Nucleus. With immunofluorescence, Ran has been shown to be a predominantly nuclear protein in mammalian tissue culture cells (1, 20) and in *S. cerevisiae* (11). To test whether the tomato protein is also localized in the nucleus, we constructed a translational fusion containing the RAN2A coding region placed at the C terminus of the GUS coding sequence, under the control of the cauliflower mosaic virus 35S promoter. When this construct, pRTL-GUS/RAN2A, was introduced into tobacco cell protoplasts by electroporation, GUS activity was predominantly localized in the nucleus in all cells examined (Fig. 5 *C* and *D*), although some blue staining was also

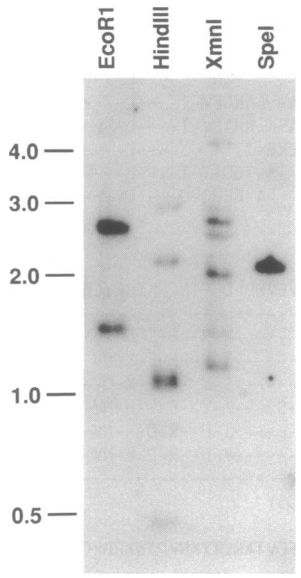

FIG. 3. Southern analysis of tomato genomic DNA. Genomic DNA (20 μg) was digested with *Eco*RI, *Hin*dIII, *Xmn* I, or *Spe* I and probed with labeled RAN2A cDNA. DNA marker sizes in kilobases are at left.

seen in the surrounding cytoplasm. In contrast, control experiments using the vector expressing GUS alone (pRTL-GUS) exhibited no nuclear localization in any cells (Fig. 5*A*). As a positive control, protoplasts were transfected with pRTL2-GUS/Nla, which expresses a fusion of GUS to the tobacco etch virus nuclear protein Nla (27). This resulted in nuclear localization of GUS activity (Fig. 5*B*), as previously shown (27, 28).

Suppression of the *Sch. pombe pim1* Mutation by Tomato RAN2A. Because the tomato Ran protein has a high level of sequence homology to the *Sch. pombe* Ran homolog spi1, we determined whether the two were functionally homologous as well. Since the *spi1* gene was originally isolated as a multicopy suppressor of the temperature-sensitive *pim1* mutation (9), we tested whether the tomato RAN2A cDNA could also suppress *pim1*. We inserted the RAN2A cDNA downstream of the alcohol dehydrogenase promoter in the pART3 expression vector (29), and then transformed the *pim1-46 leu1-32* strain SP1027 (9) with either the pART3-RAN2A construct, the pART3 vector alone, or the pART3

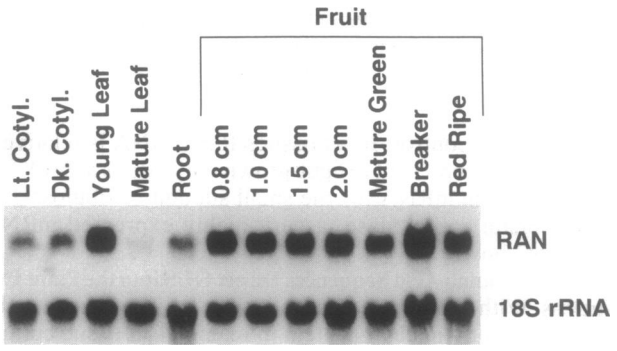

FIG. 4. Expression of Ran mRNA in tomato. Samples (2.5 μg) of total RNA were fractionated in an agarose/formaldehyde gel, transferred to nylon, and hybridized with a full-length ^{32}P-labeled RAN1 cDNA probe under conditions where all three Ran mRNAs should hybridize. RNAs are from the indicated tissues: Lt. Cotyl., 7-day-old cotyledons grown in 12 hr dark/12 hr light; Dk. Cotyl., 7-day-old cotyledons grown in constant darkness. As a control for loading and transfer, the blot was stripped and rehybridized with an 18S rRNA probe.

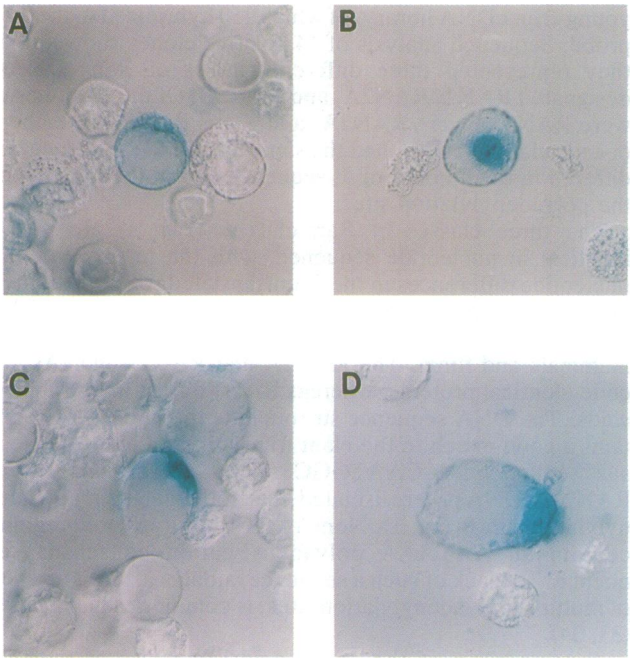

FIG. 5. Localization of GUS activity in tobacco protoplasts transiently transformed with GUS–RAN2A. *N. tabacum* protoplasts were transfected by electroporation with pRTL2-GUS (*A*), pRTL2-GUS/Nla (*B*), or pRTL2-GUS/RAN2A (*C* and *D*), incubated in medium for 24 hr, and assayed for GUS activity. Protoplasts were visualized with differential interference contrast optics at ×110 magnification. The protoplasts vary in diameter from 40 to 100 mm. The nuclei with one or two nuclei are clearly visible, as is the large vacuole (except in *B*).

vector containing the *Sch. pombe spi1* cDNA as a positive control (10).

Cells transformed with all three constructs grew at the permissive temperature on minimal plates (Fig. 6). At the restrictive temperature, cells containing the vector alone did not grow, whereas those containing the *spi1* or RAN2A cDNA did grow, demonstrating that both the *Sch. pombe spi1* cDNA and the tomato RAN2A cDNA can suppress the *pim1* mutation.

DISCUSSION

We show here that plants contain a homolog of the Ras-related GTP-binding protein Ran and that a Ran protein from tomato can suppress the *Sch. pombe pim1* mutation, as can the *Sch. pombe* Ran homolog spi1 (9). That a tomato Ran can suppress the *Sch. pombe pim1* mutation suggests that Ran plays a role in plants similar to spi1 in fission yeast—namely, as a regulator of the transition into M phase. However, Ran may also have other roles in plant cells. In mammalian cells, Ran may also regulate entry into S phase (20), and in *Xenopus* oocyte extracts RCC1 is necessary for the initiation of DNA replication, although the involvement of Ran has not been shown (18). The *S. cerevisiae*, *Sch. pombe*, and mammalian RCC1 homologs and the *S. cerevisiae* Ran homologs have all been shown to be involved in mRNA export (12, 14, 17), and the *S. cerevisiae* RCC1 homolog is involved in other nuclear functions as well (13, 15–17). Also, Ran has been implicated in nuclear protein import in vertebrates (19). Thus Ran and RCC1 have multiple roles in nuclear and chromatin organization, as well as in cell-cycle control, and these roles may differ in different organisms. In tomato, Ran mRNA is present in all plant tissues we examined, and the levels do not change during fruit development, even though the amount of cell division markedly decreases (21). In contrast, studies of cyclin expression in alfalfa (40) and cdc2 expression in

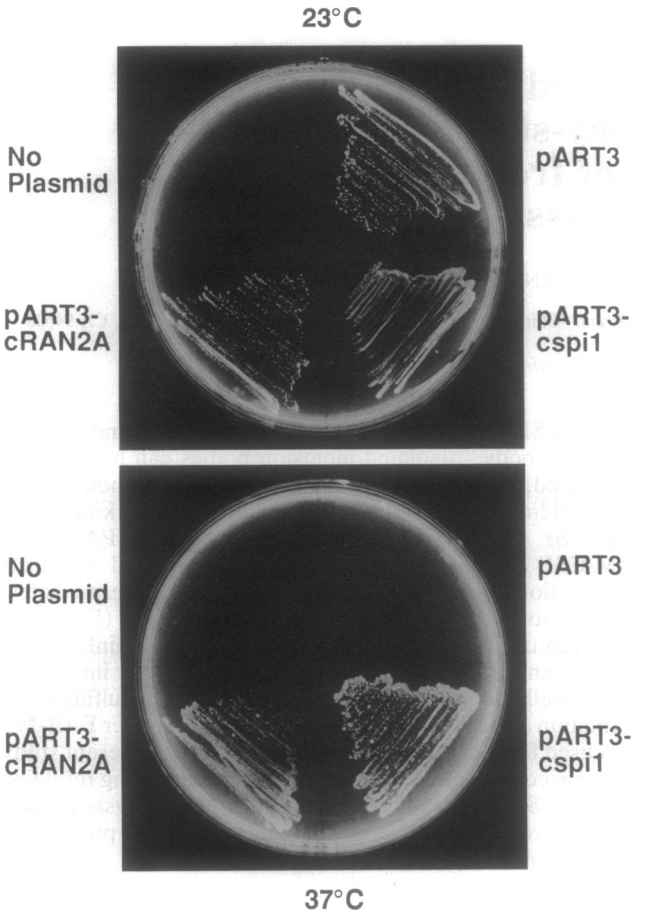

FIG. 6. Suppression of the *Sch. pombe pim1* mutation by tomato RAN2A. The *pim1* strain SP1027 without plasmid or transformed with pART3 expression vector alone, pART3-cspi (*Sch. pombe spi1* cDNA), or pART3-cRAN2A was grown on minimal plates at 23°C (permissive temperature) or 37°C (restrictive temperature).

Arabidopsis (41) found that mRNAs for these cell-cycle proteins are expressed at high levels in dividing tissues and at much lower or undetectable levels in nondividing tissues. Thus, the Ran mRNA expression pattern in tomato is consistent with the idea that Ran has other roles in plant cells besides regulation of the cell cycle.

Interestingly, Ran mRNA levels are lowest in mature leaves, a tissue in which many cells undergo endopolyploidization and polytenization during differentiation due to DNA replication without a subsequent mitosis (reviewed in ref. 42). If activated Ran inhibits the initiation of S phase in plant cells, as it may in mammalian cells (20), then lower levels of Ran in mature leaves may allow repeated DNA endoreplication. If Ran is involved in this process, it will be interesting to see how the plant cell cycle is regulated so as to allow the cells to repeatedly enter S phase without entering a subsequent M phase. The isolation of members of a plant Ran gene family is a first step toward the elucidation of their roles and regulation in the control of plant cell growth and division.

We thank Tomohiro Matsumoto and David Beach for the pART3 and pART3-cspi plasmids and the *Sch. pombe pim1* mutant strain. We thank George Prendergast for tips on degenerate PCR, David Lichtenfeld for technical advice, Jon Narita for help in screening the cDNA library, John Zupan for help with the nuclear localization studies, Ann Lorraine for help with the RNA blot analysis, and members of the Gruissem lab for helpful advice and comments. This work was supported by the National Science Foundation Center for Plant Development, by National Science Foundation Grant MCB 92-05713 to W.G., and by National Institutes of Health National Research Service Award GM14231 to R.A.A.

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