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Analysis of a *Nicotiana plumbaginifolia* cDNA encoding a novel small GTP-binding protein

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Small GTP-binding proteins belonging to the Ras superfamily have been found in evolutionarily divergent organisms. Here, we report the isolation and analysis of a cDNA encoding a putative small GTP-binding protein, designated Rhn1, from the plant, *Nicotiana plumbaginifolia*. The 21.8-kDa protein has 60% amino acid similarity with the mammalian Rab5 proteins. The Rhn1 protein is encoded by a small multigene family. Northern analysis shows the highest steady-state mRNA levels to be in roots and flowers. Furthermore, the Rhn1 protein has 80% amino acid similarity with an *Arabidopsis* small GTP-binding protein, designated Rha1.

GTP-binding protein; Protein secretion; *ras* gene expression; *Nicotiana plumbaginifolia*

1. INTRODUCTION

Sequence comparison of proteins belonging to the Ras superfamily revealed several regions of high homology. These regions are known to interact with GTP and to be involved in hydrolysis of the bound GTP to GDP [1,2].

One particular class of small GTP-binding proteins, belonging to the so-called Ypt or Rab subfamily, are thought to play a key role in vesicular-mediated protein transport (for review, see [3]). For example, protein transport studies in yeast indicate that the Ypt1 protein, which is localized in the Golgi apparatus, is essential for vesicular transport of proteins from the endoplasmic reticulum to the Golgi complex. In mammals, evidence is accumulating that each transport step between different compartments of the secretory pathway requires a specific GTP-binding protein. Specific small GTP-binding proteins have been found associated with the endoplasmic reticulum (Rab1), the post-endoplasmic reticulum and the pre-Golgi compartment (Rab2), different parts of the Golgi apparatus (Rab6), the plasma membrane (Rab5), early (Rab5, Rab4), and late endosomes (Rab7) [3].

In contrast to the extensive knowledge on GTP-binding proteins in yeast and mammals, virtually no information is available on such proteins in plants. Until now only four plant genes or cDNAs encoding small GTP-binding proteins have been described [4–7]. In this paper we describe the analysis of a cDNA encoding a novel small GTP-binding protein from *Nicotiana plumbaginifolia*, Rhn1.

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2. MATERIALS AND METHODS

2.1. *Oligodeoxynucleotide synthesis and labelling; construction and screening of the cDNA library*

The oligonucleotide used to screen the *N. plumbaginifolia* cell suspension cDNA library [8] contains deoxyinosines at ambiguous codon sites and has the sequence GAIATICTIGAIACIGCIGGICAI-GAIGAITAITCIGCITAG.

2.2. *Northern and Southern analysis*

Both DNA and RNA gel blot analyses were performed as described [9].

Flowering *N. plumbaginifolia* plants grown in greenhouse conditions (16 h light/8 h dark at 22°C and 70–80% humidity) were used as a source for nucleic acid preparation.

3. RESULTS AND DISCUSSION

3.1. *Isolation of the* rhn1 *cDNA clone and sequence analysis*

All Ras-related proteins identified until now share several highly conserved amino acid regions involved in GTP binding [1,10]. Therefore, we synthesized an oligonucleotide corresponding to the highly conserved 'DILDTAGQEEYSAM' peptide sequence, and subsequently used this oligonucleotide to screen a cDNA library of *N. plumbaginifolia*. The hybridization with 5×10^4 clones yielded one cDNA clone with an insert of 900 bp which will be further referred to as *rhn1*. The sequence of this cDNA clone is presented in Fig. 1. The sequence contains one continuous open reading frame. A comparison with members of other small GTP-binding proteins clearly indicates that the methionine at nu-

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1   ATGGCATCCAGTAGTCACAACAATCTCAATGCAAAACTCGTATTGTTAGGCGACATGGGAGCTGGT
      M  A  S  S  S  H  N  N  L  N  A  K  L  V  L  L  G  D  M  G  A  G    22
67  AAATCAAGCTTGGTCATACGATTCGTCAAGGGCCAGTTCCTTGAATTTCAAGAATCGACAATTGGG
      K  S  S  L  V  I  R  F  V  K  G  Q  F  L  E  F  Q  E  S  T  I  G    44
133 GCAGCTTTCTTTTCGTCTACGCTGGCAGTTAATAATGCTACAGTGAAGTTTGAGATATGGGATACT
      A  A  F  F  S  S  T  L  A  V  N  N  A  T  V  K  F  E  I  W  D  T    66
199 GCTGGACAGGAGAGGTACCATAGGTTAGCGCCTATGTATTATAGAGGAGCCGCTGCTGCTATTATT
      A  G  Q  E  R  Y  H  S  L  A  P  M  Y  Y  R  G  A  A  A  A  I  I    88
265 GTCTATGATATCACTAGCTCGGATTCATTTGCAAGGGCAAAAAAATGGGTGCAAGAATTGCAGAAG
      V  Y  D  I  T  S  S  D  S  F  A  R  A  K  K  W  V  Q  E  L  Q  K   110
331 CAAGGTAATCCTAACATGGTCATGGCTCTTGCTGGCAACAAGGCTGATCTAGAAGATAGAAGGAAG
      Q  G  N  P  N  M  V  M  A  L  A  G  N  K  A  D  L  E  D  R  R  K   132
397 GTTACTGCAGAAGAGGCACGCTTGTATGCTGAGGAAAATGGTCTCTTTTTCATGGAGACCTCTGCC
      V  T  A  E  E  A  R  L  Y  A  E  E  N  G  L  F  F  M  E  T  S  A   154
463 AAAnCTGCTGTGAATGTCAATGCTATTTTCTATGAAATAGCTAAACGGTTGCCTAGAGCTCAACCT
      K  T  A  V  N  V  N  A  I  F  Y  E  I  A  K  R  L  P  R  A  Q  P   176
529 GCTCAAAATCCAGCAGGAATGGTTCTTGTAGACAGAGCAGCAGAAGGGACCCGAGCTACATCATGC
      A  Q  N  P  A  G  M  V  L  V  D  R  A  A  E  G  T  R  A  T  S  C   198
595 TGTACTTAAAATTCACCATTGTTCATAAATTCATTAAACCTTTCCTAATCTTTGACTGTACTTCAC
      C  T  .                                                             200
661 TTGTTCTTCGTGTTAAACATGAAAGTCTGGAAATGGGAGTAGCATGGTTCAGTAGGAGATAGAGAG
727 CAAGCCGTTGTAAGGAAGCCCCTGAACCTGAAGTCATGCTCCTTGTTTCTTTAGTTTTTCTATTT
793 TGCATAGAATATGTAAGCTATGTACTTAGAAGCTCCCTATGTTAGAGGAATGTTTTGAATTTCCAG
859 CGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1. Complete DNA sequence of the cDNA clone, *rhn1*, from *N. plumbaginifolia*.

cleotide position 1–3 is the most probable start codon. Furthermore, the first amino acids are identical to the N-terminal amino acid sequence of an *Arabidopsis* small GTP-binding protein, Rha1, that we have recently characterized using the same strategy [6]. The overall percentage homology between Rhn1 and the Rha1 amino acid sequence is 79% with the C-terminal end of both sequences being the most diverged part. As such the *rhn1* cDNA insert encodes a protein of 200 amino acids with a calculated molecular weight of 21.8 kDa. Percentages of homology between Rhn1 and other members of the Ras superfamily range from 28 to 60% at the amino acid level. The highest homology was found with the Ypt(Rab) family and more specifically with the Rab5 protein, identified in dog and humans [11,12]. An alignment of Rhn1 with GTP-binding proteins belonging to the Ypt(Rab) family is presented in Fig. 2.

The fact that Rhn1 has a large overall homology with Rab5 and that the effector domain of Rhn1 (amino acids 37–49) and Rab5 are completely identical, indicates that Rhn1 could be the functional counterpart of Rab5 in plants. This would implicate a role for Rhn1 in endocytosis. However, the process of vesicle-mediated endocytosis in plants remains unclear. Despite observations that higher plants contain coated vesicles [13] and can internalize tracer molecules [14,15], the evidence for receptor- mediated endocytosis has been insufficient to substantiate the process.

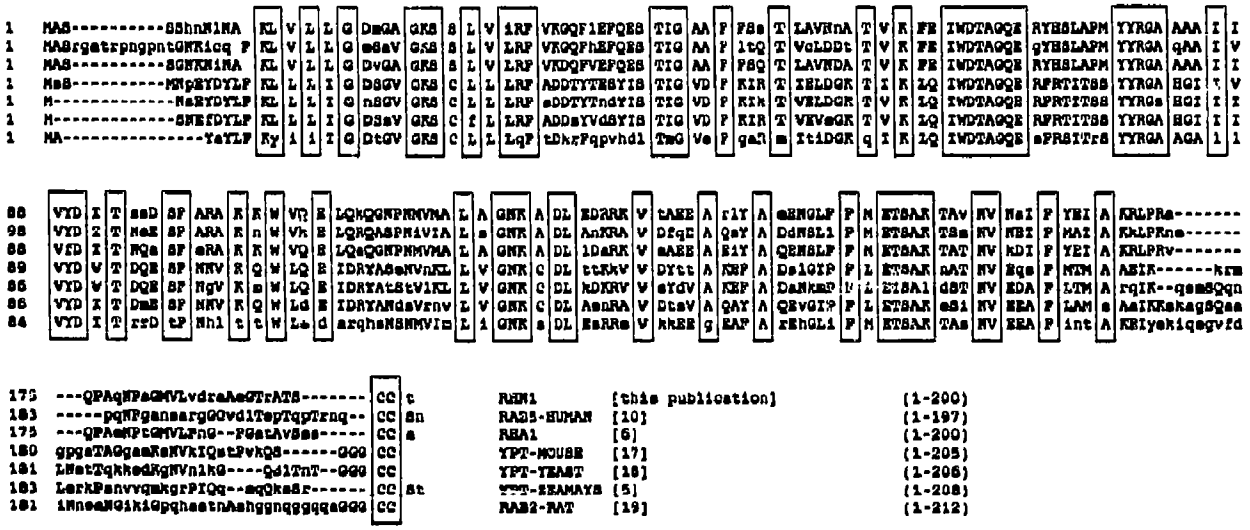

Fig. 2. Alignment of members of the Ypt(Rab) family. Conserved amino acids are boxed. Amino acids indicated with a capital are conserved in at least two sequences.

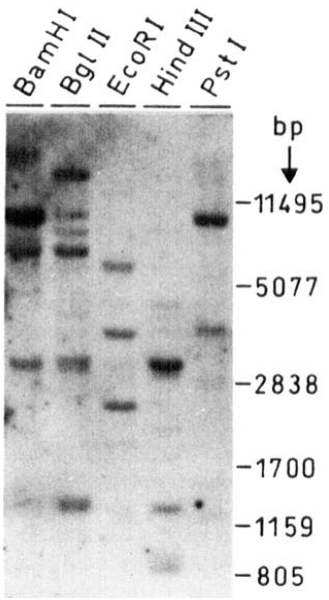

Fig. 3. Southern blot analysis of *N. plumbaginifolia* genomic DNA.

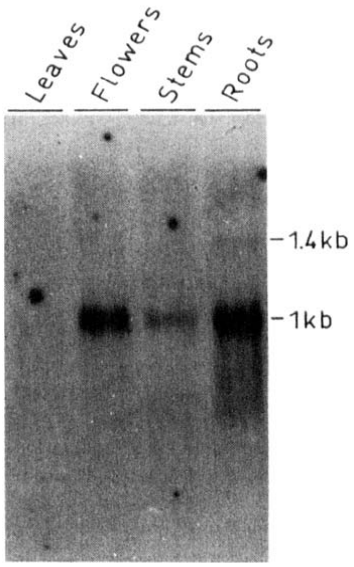

Fig. 4. Northern blot analysis of total RNA from leaves, inflorescences, stems and roots of *N. plumbaginifolia*.

3.2. *Southern analysis reveals that the* rhn1 *gene is part of a small multigene family*

To determine whether the *rhn1* gene is unique in the *N. plumbaginifolia* genome a *Hin*dIII/*Sst*I fragment containing the 5' end of the coding sequence (450 bp) was hybridized to different restriction digests of genomic DNA of *N. plumbaginifolia*. The result of this hybridization is presented in Fig. 3. Using the restriction enzymes *Hin*dIII, *Eco*RI, *Bgl*II and *Bam*HI, which do not cut within the 450-bp region, 4–6 hybridizing bands per digests could be observed. *Pst*I does cut within this 450-bp fragment and yields four hybridizing bands. These data clearly indicate that there are at least 3–4 genes for Rha1-homologous proteins present in *N. plumbaginifolia*.

3.3. *The* rhn1 *gene is differentially expressed in* N. plumbaginifolia

The levels of *rhn1* steady-state RNA in different organs of *N. plumbaginifolia* was determined by RNA gel blot hybridizations. As can be seen in Fig. 4 an abundant transcript of approximately 1 kb and a very weak band of higher molecular weight (1.4 kb) was detected in the RNA of roots and flowers. The transcript is about 8-fold and 15-fold less abundant in stem RNA and leaf RNA, respectively. At this stage we cannot rule out the possibility that highly related mRNAs might cross-hybridize with the same probe. The differential expression pattern of *rhn1* could reflect the relative abundance of specific cell types in which there is a need for the presence of Rhn1 protein. For example, the process of endocytosis, in which *rhn1* could play a role, has been demonstrated in root hairs [16] and this correlates well with the expression of the Rhn1 protein.

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