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Senescence-induced expression of a homologue of Δ9 desaturase in rose petals

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Abstract

cDNAs for senescence-inducible genes were isolated by differential hybridization from a cDNA library derived from mRNAs from the petals of rose flowers. The amino acid sequence deduced from these cDNAs exhibited significant homology to those of Δ9 acyl-lipid desaturases of cyanobacteria and of Δ9 acyl-CoA desaturases of a yeast and mammals. There was no amino-terminal sequence indicative of a leader peptide for targeting to the chloroplasts or to mitochondria. Northern blot analysis indicated that the transcripts of the cDNAs were expressed specifically in petals at late developmental stages and during senescence. It is proposed that a Δ9 desaturase in the senescing petals play an important role in the degradation of saturated fatty acids of membrane lipids.

Introduction

Fatty-acid desaturases are classified into three groups according to the form of the fatty acids with which they react [8]. Acyl-lipid desaturases, which are present in plants and cyanobacteria, desaturate fatty acids that are bound to glycerolipids [10, 13, 15, 16, 25, 27]; acyl-CoA desaturases in a yeast and mammals desaturate fatty acids that are esterified to coenzyme A (CoA) [5, 9, 22, 23]; and acyl-ACP desaturases in the chloroplasts of higher plants desaturate fatty acids that are esterified to acyl-carrier protein (ACP) [20]. These enzymes have been characterized in terms of both their biochemical features and their molecular biology. Each of the desaturases is specific to a particular site on the fatty acyl chain at which a double bond is introduced. The Δ9 acyl-lipid desaturases and the Δ9 acyl-CoA desaturases, which introduce a double bond at Δ9 positions, are similar to each other in their amino-acid sequences [15]. However, the Δ9 acyl-ACP desaturases are structurally unrelated to both the Δ9 acyl-lipid desaturases and the Δ9 acyl-CoA desaturases [15, 20].

The senescence of flowers is a well regulated process [1, 14]. In order to understand the molecular mechanism of senescence of flowers, we analyzed senescence-induced genes in petals of rose flowers and cloned cDNAs for these genes by differential hybridization. We identified three cDNA clones had encoded proteins homologous to the Δ9 acyl-lipid desaturases of cyanobacteria and the acyl-CoA desaturases of a yeast and

mammals. We report here the characterization of these cDNAs and discuss the possible roles of the corresponding enzymes in the degradation of membrane lipids during the senescence of rose petals.

Materials and methods

Plant materials

All the materials, except roots, were from *Rosa hybrida* cv. Kardinal that had been cultivated in a greenhouse in Van Wyke (Victoria, Australia). Roots in tissue culture were maintained under a photoperiod of 16 h of light (120 mmol m^{-2} s^{-1}; cool white fluorescent light) and 8 h of darkness at 23 ± 2 °C on solid medium that contained half of the standard concentration of the mineral salts of Murashige and Skoog's medium [7] supplemented with 0.1 mg/l indole-3-butyric acid, 0.05 mg/l α-naphthaleneacetic acid and 0.4% Gelrite (Merck, Rahway, NJ) (pH 5.7). Petals were harvested from flower buds at five different developmental stages (petals at stages 0 to 5) defined as follows (H, height; W, width, in cm): stage 1, closed and unpigmented buds (H = 1.0–1.2; W = 0.4–0.6); stage 2, closed and pigmenting buds (H = 1.2–1.5; W = 0.6–0.8); stage 3, closed buds with sepals that were beginning to open (H = 1.5–2.0; W = 0.8–1.0); stage 4, closed and heavily pigmented buds (H = 2.0–2.5; W = 1.0–1.5); and stage 5, buds that were starting to unfold (H = 2.5–3.0; W = 1.5–2.0). To obtain flowers at different stages of senescence, stems of ca. 20 cm with flowers were cut from plants when the outer petals started to open at stage 5 of development. The bottom halves of the excised stems were kept submerged in distilled water at room temperature. Petals were harvested from flowers 0, 1, 2, 3, 4, and 5 days after excision (day-0 petals, etc.).

Isolation of RNA and construction of a cDNA library

Total RNA was prepared from petals at each stage of development and senescence and from other floral and non-floral organs by the differential precipitation method with 2-butoxyethanol [6].

Poly(A)$^+$ RNA was purified from total RNA isolated from day-4 petals and cDNA was synthesized by the method of Brugliera *et al.* [3]. The cDNA was inserted into the *Eco* RI site of the phage vector λZAPII, and the resultant DNA was packaged into phage particles. The cDNA library of day-4 petals contained 300 000 pfu of recombinant clones.

Differential screening

Poly(A)$^+$ RNA was isolated from young leaves, petals at stage 2, day-0 petals and day-4 petals, and was used as the template for the synthesis of cDNA. The first strands of the resultant cDNAs were labeled with [α-^{32}P]dCTP to yield probes for plaque hybridization. Plaques derived from the cDNA library of day-4 petals were transferred onto Colony/Plaque Screen Membranes (Du Pont, Boston, MA). The membranes were then prehybridized at 42 °C in a solution that contained 50% formamide, 1 M NaCl, 10% dextran sulfate, 1% SDS and 100 mg/ml denatured salmon sperm DNA. The four sets of cDNA probes were used separately for hybridization, which was performed at 42 °C for 16 h in the same solution as that used for prehybridization. The membranes were washed at 65 °C for 2 h in 2× SSC that contained 1% SDS, and then at 65 °C for one hour in 0.2× SSC that contained 1% SDS. 239 phage clones preferentially hybridized to the probe cDNA that was specific for day-4 petals. They were suspended in SM buffer and dotted in ordered arrays onto plates of NZY medium (1% NZ amine, 0.5% yeast extract and 0.5% NaCl) with *Escherichia coli* XL1-Blue. The plates were incubated at 37 °C until plaques became visible. Duplicated plaques were probed separately with each of the four sets of cDNA probes described above. Prehybridization and hybridization were performed under the same conditions as used for the first screening.

Northern blot hybridization

The procedure for northern hybridization was essentially the same as described previously [3]. Each lane was loaded with 10 μg of RNA from floral or vegetative organs. Probes were labeled with [α-^{32}P]dCTP with a random primer DNA labeling kit (Takara, Kyoto, Japan).

Analysis of nucleotide sequences

Series of deleted cDNA sequences were prepared from the inserts of plasmid clones with the Erase-a-Base System (Promega, Madison, WI). Double-stranded plasmid DNAs were prepared with a Magic Miniprep Plasmid Purification System (Promega). Both strands of each cDNA were sequenced with an automated sequencer (model 373A; Applied Biosystems, Foster City, CA) or by the dideoxy chain-termination method [17] with Sequenase Enzyme (US Biochemical, Cleveland, OH). The alignment of amino acids was generated with a Gene Works Protein Analysis Program (IntelliGenetics, Mountain View, CA).

Treatment of flowers with ethylene

Excised stems with flowers that had been placed in distilled water were kept for 24, 48 or 72 h in an 850 ml chamber with a solution of 0.25 M Tris-HCl (pH 8.0) and 0.4% Ethrel (May and Baker Rural Pty., Sydney, NSW, Australia) which included 480 g/l ethephon, a precursor to ethylene, as the active constituent. Under these conditions, the concentration of ethylene in the chamber was ca. 650 ppm, as described previously [18]. As a control, excised stems were also incubated under the same conditions in the absence of ethephon. RNA was extracted from petals of both ethylene-treated and untreated flowers and used for northern blot analysis.

Results

Isolation of cDNAs for senescence-induced genes

The senescence of rose flowers usually becomes apparent 4–5 days after harvest [4]. Therefore, we used petals of day-4 post-harvest flowers for cloning of cDNAs for genes that are induced during senescence. The cDNA library of day-4 petals with ca. 100000 pfu was screened with the probes derived from the poly(A)$^+$ RNAs of young leaves, stage-2 petals, and day-4 petals. A total of 66 phage clones hybridized preferentially with the probe from the day-4 petals. The corresponding phagemids were recovered by *in vivo* excision. The resultant plasmid vector pBluescript was screened again with the three sets of probes. A total of 27 clones were obtained that hybridized with the day-4 petal probe but not with the stage-2 petal probe or the young-leaf probe. The cDNAs in these plasmids were designated RP (rose petal) cDNAs.

^{32}P-labeled probes were synthesized from the RP cDNAs and all 27 probes were individually used for northern blot analysis of total RNA extracted from petals at various stages of development and senescence, as well as from other floral and vegetative organs. The mRNA corre-

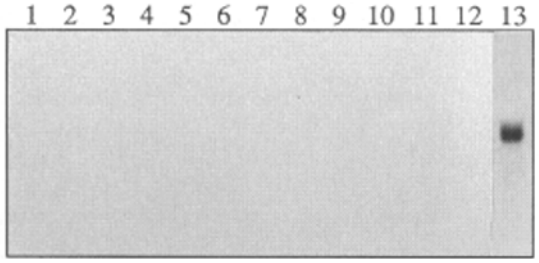

Fig. 1. Northern blot analysis of the organ-specific expression of the RP4 gene. Total RNA was extracted from roots in tissue culture (lane 1), young red leaves (lane 2), mature green leaves (lane 3), stems (lanes 4 & 5), sepals (lanes 6 & 7), ovaries (lanes 8 & 9), anthers (lanes 10 & 11), and petals (lanes 12 & 13). Plant materials, except petals, were sampled from flower stems on the day of harvest (lanes 2, 3, 4, 6, 8, 10) or from flowers 4 days after harvest (lanes 5, 7, 9, 11). Petals were sampled from buds at stage 2 (lane 12) or from flowers 4 days after harvest (lane 13). The [^{32}P]-labeled probe was derived from the entire sequence of the RP4 cDNA.

sponding to only one of the 27 RP cDNAs was expressed in the post-harvest petals and not in petals of developing buds or in any other organs, as shown in Fig. 1. The corresponding cDNA was designated RP4 cDNA.

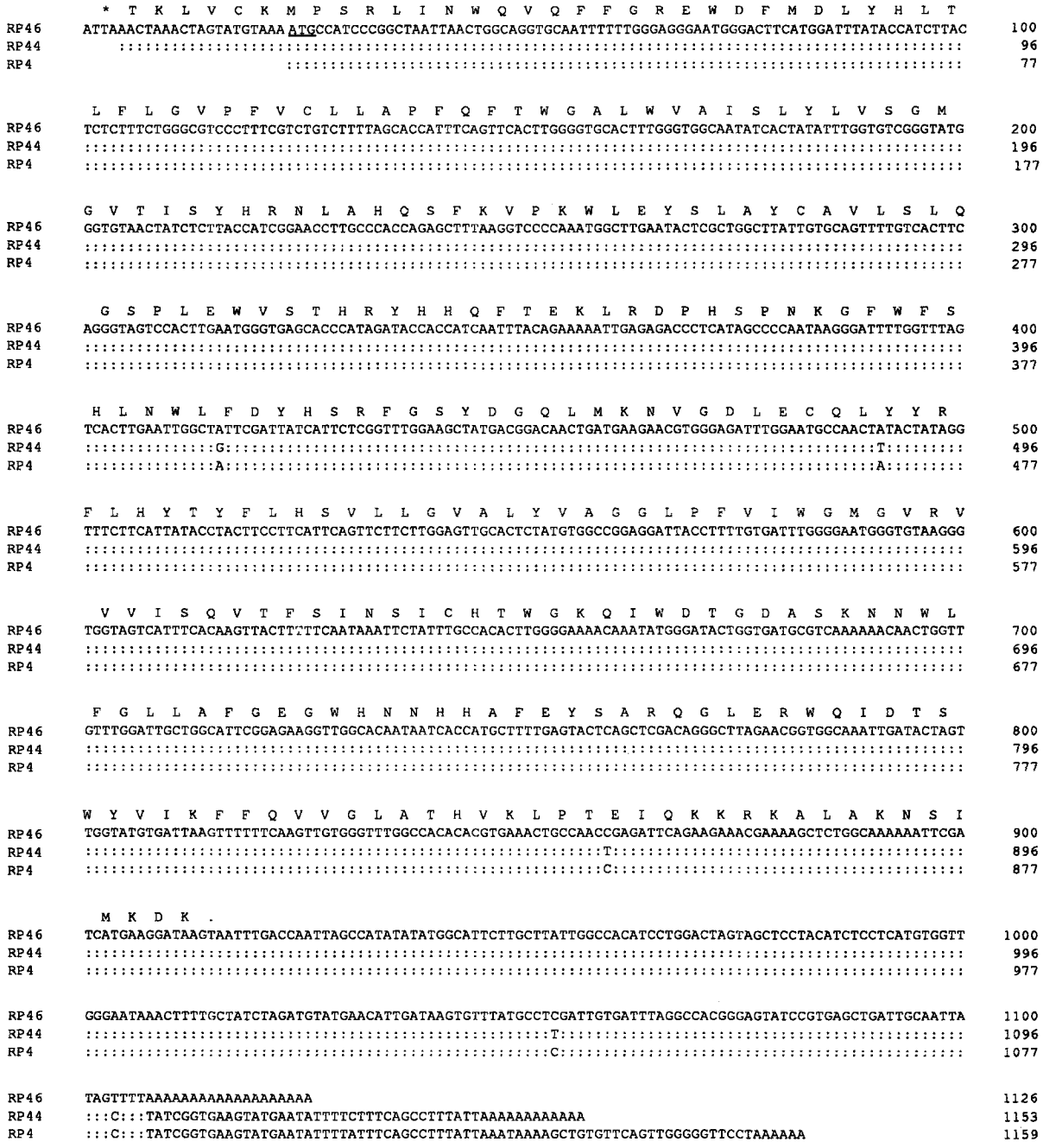

Fig. 2. Nucleotide and deduced amino acid sequences of RP4, RP44 and RP46 cDNAs. For RP4 and RP44, nucleotides identical to those of RP46 are shown by dots and only the nucleotides that differ among the three cDNAs are shown. The amino acid sequences deduced from the three cDNAs are identical. The initiation codon is indicated by underlining. Three nucleotides indicated by asterisks form a termination codon upstream of the open reading frame.

Isolation of cDNAs homologous to RP4

The cDNA library of day-4 petals was screened with the ^{32}P-labeled probe derived from the RP4 cDNA. Among ca. 300 000 pfu of recombinants, about 800 phage clones hybridized with this probe. This result suggests that genes for the RP4 cDNA or its homologous cDNA(s) were expressed at high levels in day-4 petals. Fifteen independent clones were arbitrarily selected, and partial nucleotide sequences of the cDNAs were determined. The cDNAs in the fifteen clones and RP4 were highly homologous to one another and differences were only found in the sequence of the polyadenylation site and the length of the 5′ region. Two of the cDNA clones, designated RP44 and RP46, were selected for further analysis.

Sequences of RP4, RP44 and RP46 cDNAs

The entire sequences of RP4, RP44 and RP46 cDNAs were determined (Fig. 2). All three cDNAs were ca. 1.2 kb in length and each contained an open-reading frame of 891 bp that corresponded to 297 amino acids. Apart from differences in their polyadenylation sites and in the lengths of their 5′ regions, there were three nucle-

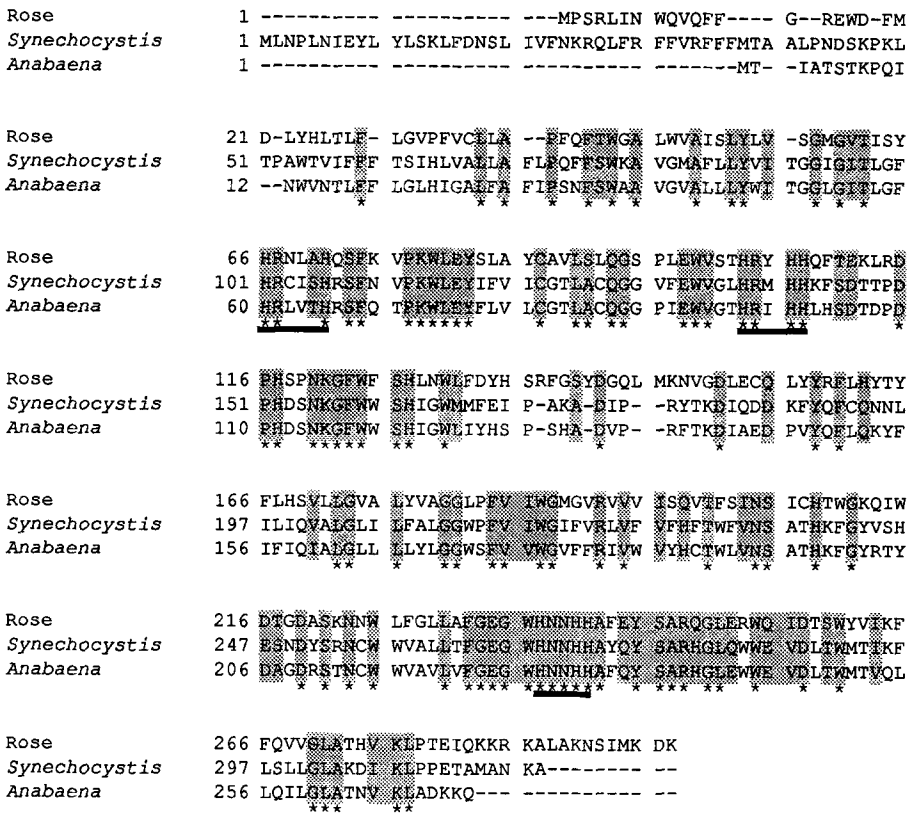

Fig. 3. Alignment of the product of RP4 cDNA with the amino acid sequences of Δ9 fatty acid desaturases. **A** (this page). Alignment with Δ9 acyl-lipid desaturases of cyanobacteria. **B** (next page). Alignment with Δ9 acyl-CoA desaturases of a yeast and two mammals. Rose, RP4 product; *Synechocystis*, Δ9 acyl-lipid desaturase of *Synechocystis* sp. PCC 6803 [15]; *Anabaena*, Δ9 acyl-lipid desaturase of *Anabaena variabilis* [15]; Mouse 1 and Mouse 2, stearoyl-CoA desaturases SCD1 [9] and SCD2 [5] of mouse; Rat, stearoyl-CoA desaturase of rat [23]; Yeast, yeast Δ9 desaturase [22]. The 5′ regions of acyl-CoA desaturases and part of the 3′ region of yeast acyl-CoA desaturases, indicated by dashed lines, are omitted. Conserved amino acid residues and conservative substitutions are shadowed and identical amino acid residues in all the aligned sequences are indicated by asterisks. Three clusters of histidine residues, H-X-X-X-X-H and H-X-X-H-H, are indicated by thick bars.

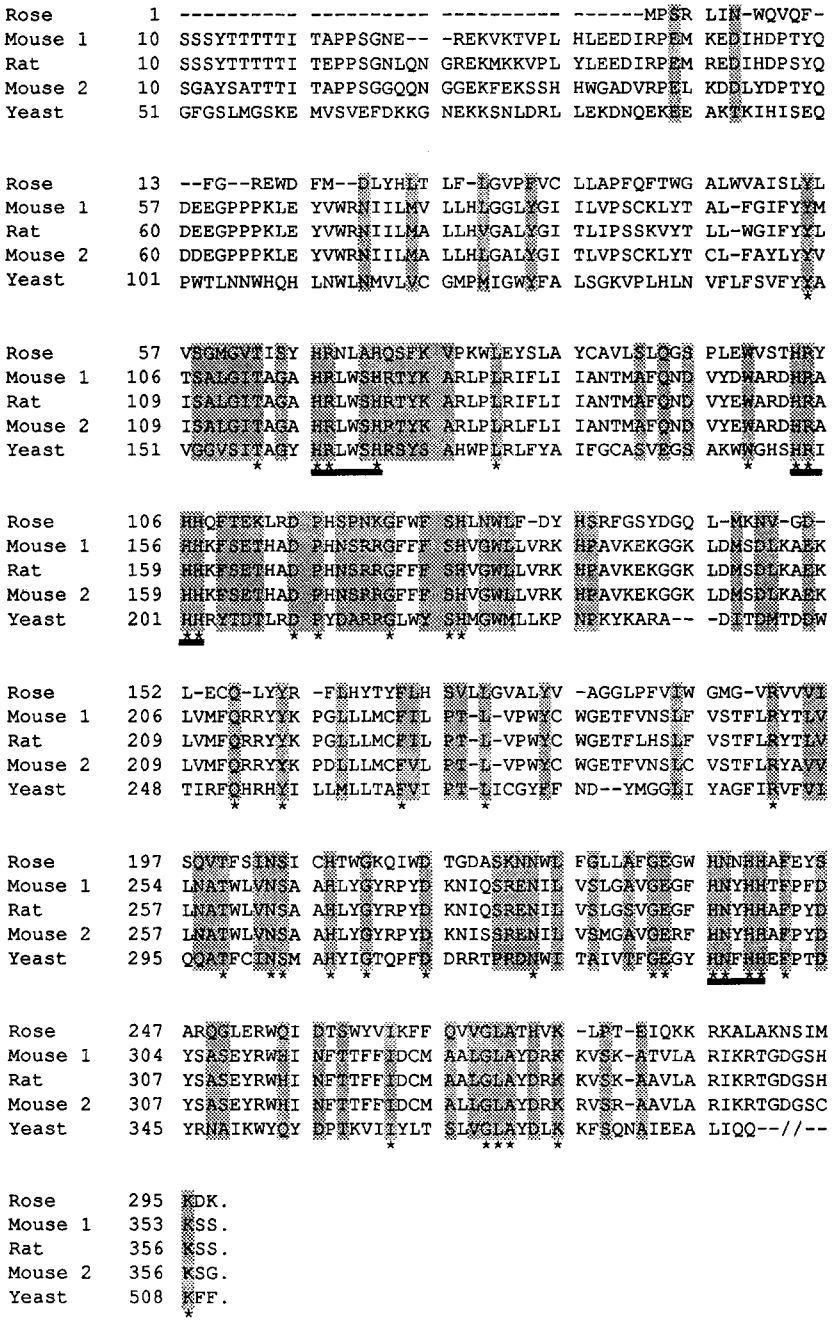

Fig. 3b.

otide differences among the open-reading frames of the three cDNAs. All the mismatches were located at the third position of a codon, with the result that no change in the encoded amino acid was indicated. The RP46 cDNA contained a stop codon (UAA) seven amino acids upstream of the putative first methionine codon in the reading frame (Fig. 2). This result, together with the size of an mRNA detected by northern blot analysis (Fig. 1), indicated that these three cDNAs con-

tained the entire coding regions of the corresponding genes.

When the amino acid sequence deduced from the cDNAs was compared with the sequences in GenBank and Swissprot data bases, we found that the amino acid sequence encoded by the RP4, RP44 and RP46 cDNAs was significantly homologous to the sequences of Δ9 acyl-lipid desaturases of cyanobacteria (Fig. 3A). The extent of the identity was 40% over the entire amino acid sequence. The amino acid sequence deduced from the RP4, RP44 and RP46 cDNAs was also similar to the sequences of Δ9 acyl-CoA desaturases of a yeast, mouse and rat (Fig. 3B) with the extent of identity being of 20–25%. In addition, the amino acid sequences contained three clusters of histidine residues, -H-X-X-X-X-H- (one cluster) and -H-X-X-H-H- (two clusters), which are

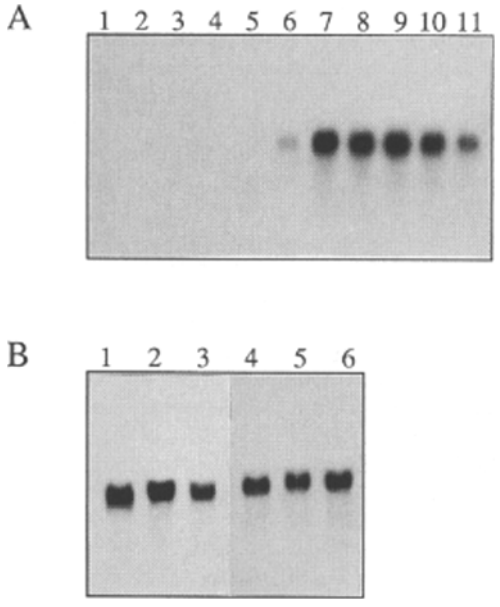

Fig. 4. Northern blot analysis of the expression of the RP4 gene in rose petals. A. RNA from petals at various stages of development, namely, stage 1 (lane 1), stage 2 (lane 2), stage 3 (lane 3), stage 4 (lane 4), and stage 5 (lane 5), on the day of harvest (lane 6), and at various times after harvest, namely, one day (lane 7), 2 days (lane 8), 3 days (lane 9), 4 days (lane 10), and 5 days (lane 11). B. RNA from petals of ethylene-treated flowers (lanes 1, 2 and 3) and non-treated flowers (lanes 4, 5 and 6). Flower stems were exposed to ethylene at ca. 650 ppm in a chamber for 1 day (lanes 1 and 4), 2 days (lanes 2 and 5) and 3 days (lanes 3 and 6).

well conserved in Δ9 acyl-lipid desaturases and Δ9 acyl-CoA desaturases and have been suggested to play an essential role at the catalytic site of the desaturases [15] (Fig. 3). Therefore, it is possible that the protein encoded by the three rose clones might be a Δ9 fatty-acid desaturase. The amino acid sequence deduced from the RP4, RP44 and RP46 cDNAs was, however, not similar to that of Δ9 acyl-ACP desaturases [20].

Expression of RP4

Figure 4A shows the results of northern blot analysis of the expression of the RP4 gene during senescence. The transcript was detected only in petals that were at the late developmental and senescent stages. Essentially the same results were obtained with petals from intact flowers that were still attached to plants (data not shown). The expression of the RP4 gene was unaffected by exposure to exogenous ethylene (Fig. 4B), which is known to induce a large number of senescence-related genes [12, 26].

Discussion

Differential screening of a cDNA library from rose petals allowed us to isolate three closely related cDNAs, namely, RP4, RP44, and RP46, which were expressed specifically in senescing petals. These three cDNAs encoded an identical protein of 297 amino acids. The deduced amino-acid sequence of this protein is highly homologous to those of Δ9 acyl-lipid desaturases of cyanobacteria and, to a lesser extent, of Δ9 acyl-CoA desaturases of a yeast and mammals. This is the first report of the isolation of cDNAs homologous to genes for Δ9 acyl-lipid and acyl-CoA desaturases from a higher plant.

In plant cells, desaturation of fatty acids is associated with the chloroplast membrane and the endoplasmic reticulum [21]. The deduced amino-acid sequence encoded by RP4, RP44 and RP46 (Fig. 2) lacks a leader peptide that is necessary for targeting of a protein to the chloroplast. We

also note that the gene that corresponds to RP4 was expressed specifically in mature petals and not in leaves which contain far more chloroplasts than petals. Therefore, it is likely that the putative desaturase is located in the endoplasmic reticulum. However, in the current scheme for the biosynthesis of lipids in higher plants [21], neither Δ9 acyl-lipid desaturation nor Δ9 acyl-CoA desaturation is involved in the main pathway of lipid synthesis on the endoplasmic reticulum.

The biological role of the temporal and petal-specific expression of the putative gene for a desaturase is unclear. However, Brown *et al.* [2] reported that, during senescence of carnation petals, microsomal phospholipids with polyunsaturated fatty acids were selectively degraded. Comparing the degradation of phospholipids of naturally aged membranes and membranes that had been aged *in vitro*, they obtained evidence that desaturases and retailoring enzymes have roles in generating polyunsaturated fatty acids that are more susceptible to catabolism that saturated fatty acids.

Methyl jasmonate might be involved in the biological role of the putative gene for a desaturase in the senescence of petals. Porat *et al.* [11] recently demonstrated that, in addition to its protective function in wounding and when there is a water deficit, methyl jasmonate accelerates senescence of flowers. In petunia and dendrobium, methyl jasmonate enhances senescence of flowers via promotion of the production of ethylene [11]. Methyl jasmonate is synthesized via the peroxidation of linolenic acid by lipoxygenase [19]. Therefore, senescing rose petals may require the synthesis of linolenic acid from less unsaturated fatty acids by senescence-specific desaturases. It is also worth noting here that a C18-unsaturated fatty acid was shown to accelerate senescence of leaves of *Ficus superba* [24]. If a similar phenomenon occurs in rose petals, the homologue of Δ9 desaturases should accelerate their senescence.

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