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Characterization of two cDNA clones for mRNAs expressed during ripening of melon (*Cucumis melo* L.) fruits

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Abstract

In vitro translation of mRNAs and polyacrylamide gel electrophoresis of proteins from melons revealed that several mRNAs increased in amount during ripening, indicating the existence of other ripening genes in addition to those cloned previously. To identify ripening-related genes we have screened a ripe melon cDNA library and isolated two novel cDNA clones (MEL2 and MEL7) encoding unidentified proteins. Southern analysis revealed that MEL2 and MEL7 are encoded by low-copy-number genes. The MEL2 cDNA clone is near full-length, corresponds to a 1600 nucleotide mRNA that accumulates during ripening and encodes a predicted protein rich in hydrophobic amino acids. The MEL7 cDNA clone is full-length, corresponds to a mRNA of 0.7 kb which accumulates during early ripening stages and is also present at low levels in other organs of the melon plant. The MEL7 predicted polypeptide is 17 kDa and shows significant homology with the major latex protein from opium-poppy. Wounding and ethylene treatment of unripe melon fruits 20 days after anthesis showed that MEL2 and MEL7 mRNAs are only induced by ethylene.

Introduction

Fruit ripening is a complex developmental process which has been extensively used as a model system to dissect genetically programmed organ differentiation. Studies with both non-climacteric and climacteric fruits such as apples, bananas, tomatoes, pears, avocados and mangos have provided evidence for differential gene expression during ripening [4]. Several enzymes showing altered activities during ripening have been reported and the respective genes have been cloned. The role of the ripening-related genes has been clarified in several cases but the function of many of them is still unknown [9].

Muskmelon (*Cucumis melo* L.) is an economically important fruit that has an associated climacteric rise in ethylene production during ripening [18, 29]. Studies in melon, as with other climacteric fruits, have shown that ripening is related to an increase in ethylene synthesis. A cDNA clone from melon with homology to the ACC oxidase (*Aco*1) from tomato, which catalyses the terminal step in ethylene biosynthesis, has been isolated and shown to increase during ripening [1]. The most notable physiological changes in fruit ripening are the softening of the mesocarp tissue, the accumulation of pigments, the development of the characteristic aroma and the sweet taste. Softening of the mesocarp is related to modifications of pectin and hemicellulosic polysacharides. In melon, these changes are believed to be caused mainly by β-galactosidases, whereas polygalacturonase is important in tomato, avocado and pears [8, 13, 25]. Other

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Z70521 (MEL2) and Z70522 (MEL7).

enzymes are also involved in cell-wall catabolism such as cellulase and xylanase [26]. The change of colour in ripe fruits is usually due to carotenoid or anthocyanin accumulation and chlorophyll degradation. This has been studied in detail in tomato which, like melon, synthesizes carotenoids during ripening. A cDNA clone with homology to tomato phytoene synthase, a key enzyme in the carotenoid pathway, has been isolated from melon and shown to be preferentially expressed during ripening [15]. Sweetness is a characteristic attribute of ripe muskmelon and it is also used in quality evaluation. Sugar level appears to be regulated by the balance of invertases and synthases present in the fruit tissue [12, 17]. Ripe fruit aroma is associated with a mixture of over fifty compounds, some of which include thioesters [33]. Production and release of aromatic volatiles are not well understood but they might be correlated with the presence of the plant hormone ethylene [32]. All these properties of the ripe fruit make it attractive to the consumer and their possible manipulation is scientifically and commercially interesting. There is, however, a need to identify additional genes involved in melon ripening.

In order to improve our knowledge of ripening in melon, we screened the original cDNA library [1] to isolate other clones for mRNAs showing differential expression during ripening in addition to ACC oxidase [1] and phytoene synthase [15]. Here we report the isolation and characterization of two novel cDNA clones, designated as MEL2 and MEL7, which encode mRNAs that increase in expression during ripening.

Materials and methods

Plant tissue

Melon seeds (*Cucumis melo* L. cv. Cantaloupe charentais) were provided by Tezier Breeding Institute, Velence, France and grown in a glasshouse in 5 l pots under 16 h of light. Freshly opened female flowers were hand-pollinated and tagged to identify fruit of known age. One fruit per plant was allowed to develop. Fruits were harvested after 15, 20, 25, 30, 35, 40 and 45 days after anthesis (daa). The ripening stage of fruit was also assessed by measuring the rate of ethylene evolution immediately after harvesting. The mesocarp tissue was separated from the seed cavity and epidermis, cut into small pieces, frozen in liquid nitrogen and stored at $-70\ ^{\circ}C$.

Ethylene measurements

Fruits were harvested and sealed in air-tight glass containers. They were incubated for 2 h at room temperature and 1 ml of gas, withdrawn from the container via a Suba seal, was used to quantify the external released ethylene, using a Pye Unicam PU4500 gas chromatograph [10].

Colour and texture measurements

The fruits were cut longitudinally and the colour measurements were taken with a Chroma meter (Minolta CR-200), by placing the probe on the fruit flesh 1.5 cm below the epidermis. For the texture measurements a cylindrical sample of fruit tissue 2 cm in length and 15 mm diameter was removed using a metallic cork-borer, starting from the epidermis inwards to the seed cavity. The cylinder was compressed with a 12 mm diameter probe against a metallic base. The required force was plotted against the deformation, till the sample collapsed, for each fruit, using a TA-XT2 Texture Analyser (Stable Micro Systems).

RNA extraction

Different methods were used for total RNA extraction according to the kind of tissue. Total RNA from fruit samples and ovaries was extracted using the method described by Smith *et al.* [30]. For leaf, stem, petal and seed material, total RNA was extracted according to the procedure of Wadsworth *et al.* [31] and for root samples the method described by Dean *et al.* [5] was used. Poly(A)$^+$ mRNA was isolated from fruit total RNA using the polyATract kit (Promega).

Differential screening

Replicate plaque lifts, about 30 000 pfu per 140 mm plate, were made of a dilution of the cDNA library [1]. Lifts were carried out with Hybond-N$^+$ (Amersham) membranes as described by the manufacturer. Replicate filters were hybridised to single strand cDNA probes generated from 0.5 μg poly(A)$^+$ RNA either from unripe or ripe fruit using Moloney Murine Reverse transcriptase (Stratagene) and [$\alpha-^{32}$P]dCTP (Amersham). Hybridization conditions were according to the Hybond-N$^+$ protocol (Amersham). Clones were isolated on the basis of their ability for preferential hybridization to probes. Primary isolates were put

through second and third round screens using similar probes until plaque-pure clones were isolated.

Northern blots

Northern blot analysis was carried out as described by John *et al.* [14]. The membranes were then exposed for autoradiography at −70 °C using intensifying screens. In addition to autoradiography, signal intensity on the membranes was quantified directly using an AMBIS 4000 radioanalytical imaging detector and analyzed using AMBIS QuantProbe version 4 software.

Genomic DNA extraction and Southern blots

Genomic DNA was extracted from young leaves according to the method of Bernatzky *et al.* [2]. Ca. 10 μg of genomic DNA was digested with restriction enzymes overnight and separated by electrophoresis on a 0.8% agarose gel. The DNA was transferred onto nylon membranes (GeneScreen Plus, DuPont) according to the manufacturer's instructions. The membranes were then hybridized with labelled probes (as described for the northern blots) at 42 °C and exposed on autoradiographic film at −70 °C.

Radiolabelled probes

DNA probes were synthesised according to the random priming method [27]. The plasmids (pMEL2 and pMEL7) were digested with *Eco*RI and *Xho*I to remove the cloned inserts which were then separated by agarose gel electrophoresis. The cDNA inserts from agarose gel were purified using GeneCleanII (Bio101) kit and used as templates for random prime labeling.

DNA sequence analysis

Sequencing was performed by the dideoxy chain termination method [28] using synthetic oligonucleotides as primers. The plasmid DNA for sequencing was isolated using Qiagen columns and sequenced with the Sequenase V.2.0 (UBS) and Taqtrack (Promega) sequencing kits. The DNA sequence data was analyzed using the University of Wisconsin Genetics Computer Group (GCG) [6] and DNA Strider programs [19].

In vitro *translation*

For *in vitro* translation 2 μg of poly(A)$^+$ mRNA from unripe (515 and 20 daa) and ripe (35 and 40 daa) fruit was used as template in the TNT-coupled wheat germ extract (Promega), labelled with ^{35}S-methionine (Amersham).

Wounding and ethylene treatment of unripe fruits

Unripe fruits (20 daa) were wounded by cutting into very small pieces using a scalpel blade and frozen in liquid nitrogen after 2 and 6 h. Control unwounded material from the same fruits was frozen immediately after harvesting. For ethylene treatment, unripe fruits were sealed for 48 h in 20 μl/l ethylene atmosphere inside air-tight glass containers. The containers were ventilated every ten hours to avoid low oxygen conditions, released and the ethylene concentration was restored.

Results

Ripening and fruit attributes

The change in melon flesh colour from green to characteristic orange started 30 days after anthesis (daa) (Fig. 1A) and was initially most obvious around the seed cavity. The change continued, spreading towards the epidermis till the final stage when the fruit reached a dark orange colour due to the accumulated pigments. The fruits also showed a dramatic decrease of firmness (force/deformation) between 30 and 40 daa (Fig. 1B). They started to soften 25 daa and became extremely soft and watery at 40 daa. The aroma of ripe melon was detectable 35 daa and increased till 45 daa. Ethylene from the ripening fruits was detectable at 35 daa which coincided with the time when the seeds were fully developed (Fig. 1C). It increased between 35 and 40 daa and then continued to increase at a slower rate till 45 daa. There was no ethylene detected from the green unripe fruits before 30 daa.

Changes in translatable mRNAs during ripening

The *in vitro* translation products of poly(A)+ mRNA from ripe and unripe fruit revealed proteins that changed in abundance. Proteins with molecular weights of 55, 51, 47, 43, 36, 31, 27, 20 and 17 kDa (Fig. 2) seemed to increase in the ripe fruit while there were some proteins that became undetectable as the fruit ripened. Similar changes were observed when the total proteins from different ripening stages were ana-

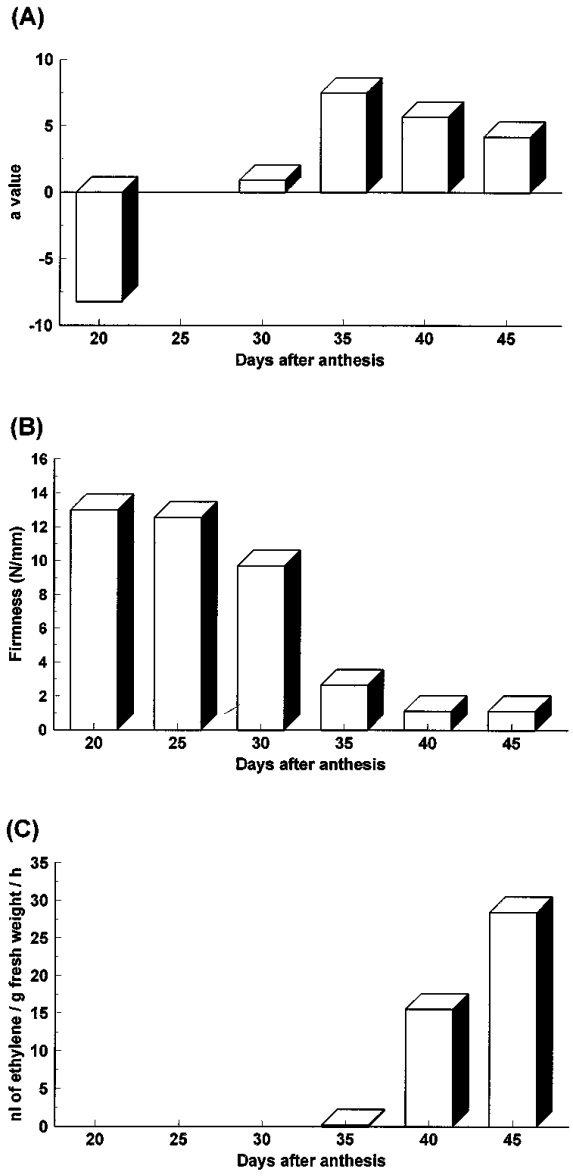

Figure 1. Changes in melon fruits during ripening. A. The change of colour during ripening. The a hue component from the colour measurements was used; negative values indicate green and positive values indicate red fruits. B. The firmness of the fruit flesh expressed in Newton (N) per mm of deformation. C. The release of ethylene from the detached fruits. All the data were plotted against the age of the fruits from anthesis.

lysed by SDS-PAGE (data not shown), although the molecular sizes of some proteins were different.

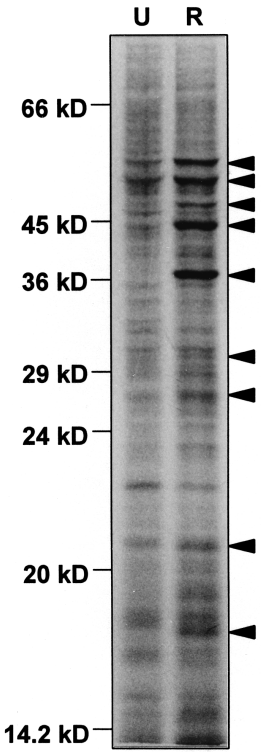

Figure 2. Changes in translatable mRNAs during ripening. *In vitro* translation products from unripe (U) and ripe (R) fruit poly(A)$^+$ mRNA were fractionated by SDS-PAGE. Proteins that increase in amount in the ripe fruit sample are indicated by arrowheads. The position of molecular weight markers are indicated on the left.

Isolation of MEL2 and MEL7 cDNA clones

Two novel cDNA clones showing differential expression were isolated from the melon ripe fruit cDNA library using single-stranded cDNA probes from unripe and ripe fruit poly(A)$^+$ mRNA, in addition to ACC oxidase and phytoene synthase. The MEL2 clone hybridized with a 1.6 kb transcript in ripening fruit RNA but the insert was 1512 bp, which indicates that it is not a full-length clone. It has an open reading frame (ORF) of 1370 nucleotides but lacks the initiation codon for the amino terminus. The 3′-untranslated sequence is 142 bases in length and contains the putative polyadenylation signal. The predicted protein has three potential glycosylation sites and one RNA-binding motif [7, 11]. Although the sequence is not complete the predicted protein has a high percentage (Leu 10.1%, Val 8.1%, Ile 6.2%, Ala 7.7%) of hydrophobic amino acids. Sequence analysis and hydropathy plot of the MEL2 predicted polypeptide did not reveal any signal peptide, although such a sequence if present might be

in the missing 5′ end of the clone. After sequence similarity search there was no significant homology with any of the known sequences in either nucleotide or protein databases.

The MEL7 cDNA insert is 686 bp in length with an ORF of 453 nucleotides. A 200 bp untranslated sequence is present at the 3′ end and has the putative polyadenylation signal at nucleotide position 646 to 651. Primer extension experiments showed that the MEL7 transcript is 14 bases longer than the cDNA (data not shown). The molecular mass of the predicted polypeptide is 17.3 kDa with 151 amino acids (Fig. 3A). There is no obvious signal peptide at the amino terminus of MEL7 and the hydropathy profile (Fig. 3B) shows no transmembrane regions in the polypeptide. There is one putative glycosylation site at position 33 to 35 of the amino acid sequence. The MEL7 polypeptide shows significant homology at the amino acid level with the major latex protein (33.5% identity and 61.6% similarity, Fig. 3A) isolated from opium-poppy (*Papaver somniferum*) [22] and the predicted polypeptide of the *Sn-1* gene (32.6% identity and 57.6% similarity) isolated from bell pepper (*Capsicum annuum*) [23]. All three polypeptides are similar in length and molecular weight.

Expression of the MEL2 and MEL7 mRNAs during fruit development and ripening and in other organs

Northern analysis using RNA from fruits and other organs of melon plants revealed that MEL2 mRNA accumulated only during ripening. It was less than 0.5% of maximum expression in unripe fruits before 30 daa and below the limit of detection in all other plant organs examined (Fig. 4A). The levels of MEL2 mRNA increased ca. 100-fold between 30 daa and 40 daa (Fig. 4A) and decreased 45 daa (ca. 40% of maximum), when the fruit became very soft and watery. The MEL7 mRNA was present in low levels during the early stages of ripening, increased from 25 daa to 40 daa (13-fold) and then declined at 45 daa (ca. 40% of maximum) (Fig. 4B). The MEL7 mRNA was expressed in very small amounts in various other plant organs investigated and was slightly higher in roots (0.6% of maximum), stems (0.65% of maximum) and ovaries (0.57% of maximum) as compared to seeds, leaves and petals. (Note the longer exposure times for part of the autoradiograph in Fig. 4B.) No MEL2 and MEL7 homologues were detectable when northern analysis of mRNA from tomato fruit using MEL2 and MEL7 as probes was performed under low stringency conditions of hybridization and washings (data not shown).

Expression of the MEL2 and MEL7 mRNAs after ethylene treatment and wounding

To examine the role of ethylene and wounding in the regulation of MEL2 and MEL7 genes, unripe fruits were incubated for 48 h in a high (20 μl/l) ethylene atmosphere and also wounded. Northern analysis of ethylene-treated and wounded fruits revealed that the minute amounts of MEL2 mRNA (undetectable on the autoradiograph but measureable by radioanalytical imaging) in the 20 daa control fruit were induced 27-fold after ethylene treatment. Wounding of the fruit tissue decreased the amount of MEL2 mRNA 7-fold, compared with the control, two hours after wounding and it became undetectable after six hours (Fig. 5A).

The MEL7 mRNA levels increased about 5-fold in response to ethylene when compared with the non-treated control samples. In wounded fruits there was more than an 80% decrease in MEL7 mRNA after two hours and ca. 95% decrease after six hours when compared with the control unwounded samples (Fig. 5B).

Genomic Southern analysis

Melon genomic DNA was digested with several restriction enzymes and hybridized with MEL2 and MEL7 radiolabelled probes for Southern analysis. Single digests of the genomic DNA generated hybridizing bands of high molecular weight. To overcome this problem, double digests were also used. The MEL2 probe hybridized to four *Eco*RV fragments of approximately 6.5, 4.9, 3.8 and 1.0 kb and five *Sal*I fragments of 9.0, 6.2, 4.3, 3.3 and 1.1 kb in size (Fig. 6A). In both lanes there were two classes of hybridizing signals. The 6.5 and 4.9 kb bands in the *Eco*RV digest and the 4.3, 3.3 and 1.1 kb bands in the *Sal*I digest gave very strong signal while the rest of the bands seemed to hybridize weakly. There is a single restriction site in the MEL2 cDNA sequence for the *Sal*I enzyme at position 650 of the MEL2 cDNA sequence but none for the *Eco*RV enzyme.

The MEL7 probe hybridized to a single 1.6 kb fragment when genomic DNA was digested with *Eco*RI and *Bam*HI restriction enzymes. Neither *Eco*RI nor *Bam*HI enzyme cuts the MEL7 cDNA insert. In the *Hin*dIII digest two fragments of 3.5 kb and 0.3 kb hybridized strongly with the MEL7 probe and two fragments with an approximate size of 1.0 and 0.7 kb hybridized very

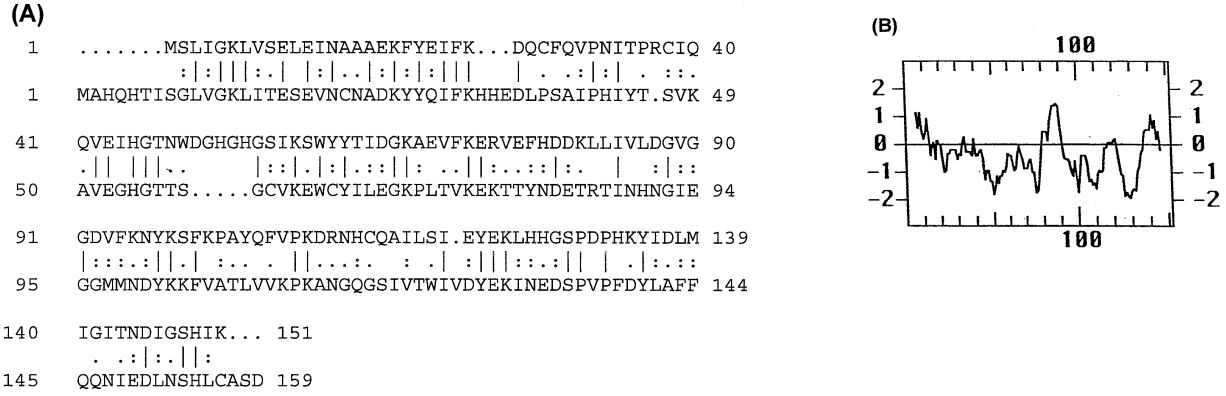

Figure 3. Comparison of MEL7 predicted polypeptide with the major latex protein and hydropathy plot. A. Homology of MEL7 polypeptide (upper sequence) to major latex protein of opium-poppy (bottom). A period indicates a weak similarity, a colon indicates a strong similarity and a vertical line indicates identity of the compared amino acids. Gaps (...) were introduced to both sequences to optimize the alignment. B. The hydropathy profile (window of 11 consecutive amino acids) was calculated according to Kyte and Doolitte [16] and plotted against the amino acid number.

weakly (Fig. 6B). The 0.3 kb fragment may be derived from the two internal *Hin*dIII restriction sites at positions 47 and 324 of the MEL7 cDNA sequence.

Discussion

To extend our understanding of the molecular basis of melon fruit ripening, we have used physiological and molecular approaches in this study. The period between 30 daa and 40 daa seemed to be the most active time in melon ripening although environmental factors such as temperature can affect the timing of ripening and the expression levels of some ripening-related genes. The ethylene measurements in this report agree with the previous data of ethylene production in melon cultivars [3, 20, 29].

In melon fruits an increase in the amounts of mRNAs encoding enzymes involved in the ethylene biosynthesis pathway, such as ACC oxidase [1], ACC synthase [21] and carotenoid production, including phytoene synthase [15], has been reported. Polyacrylamide gel electrophoresis of proteins synthesized *in vitro* using ripe and unripe melon fruit mRNA showed that several proteins increased in abundance during ripening. The 51 kDa and 36 kDa proteins may represent the ACC synthase and ACC oxidase respectively and the 43 kDa protein might represent the phytoene synthase, since the molecular weights of these proteins correspond to those established for these enzymes. The protein of 17 kDa that increased during ripening has the predicted size of the MEL7 polypeptide.

We have isolated two new ripening-related cDNAs designated as MEL2 and MEL7. The MEL2 mRNA was detected only in the ripe fruit and not in any other plant organ tested. Its expression peaked at 40 daa when the fruit was ripe and then decreased. The MEL7 mRNA was detected in various organs of the melon plant and in the early stages of ripening. It showed a significant increase in mRNA expression from 25 daa. The expression pattern of MEL7 mRNA during fruit ripening was similar to MEL2.

After 48 h in a high ethylene atmosphere MEL2 mRNA expression was induced and could be detected even in the 20 daa fruit. The MEL7 mRNA was also induced after 48 h of ethylene treatment. Wounding of the tissue resulted in a decrease of the MEL2 and MEL7 mRNAs which might mean that wounding affects their turnover rate or the transcription of the respective genes.

Southern analysis data showed that at least four genomic *Eco*RV fragments hybridized to the MEL2 cDNA probe, showing that there are more than one corresponding genes for MEL2. The differences in the hybridising signal in digestions with *Eco*RV and *Sal*I (Fig. 6A), might suggest that some genes have low homology at the nucleotide level. Since there is no internal *Eco*RV site in the MEL2 cDNA sequence, the detection of a 1.0 kb hybridizing band suggests that the restriction site might be located in an intron sequence. There was only one genomic fragment which hybridizing to the MEL7 cDNA probe, with approximate size

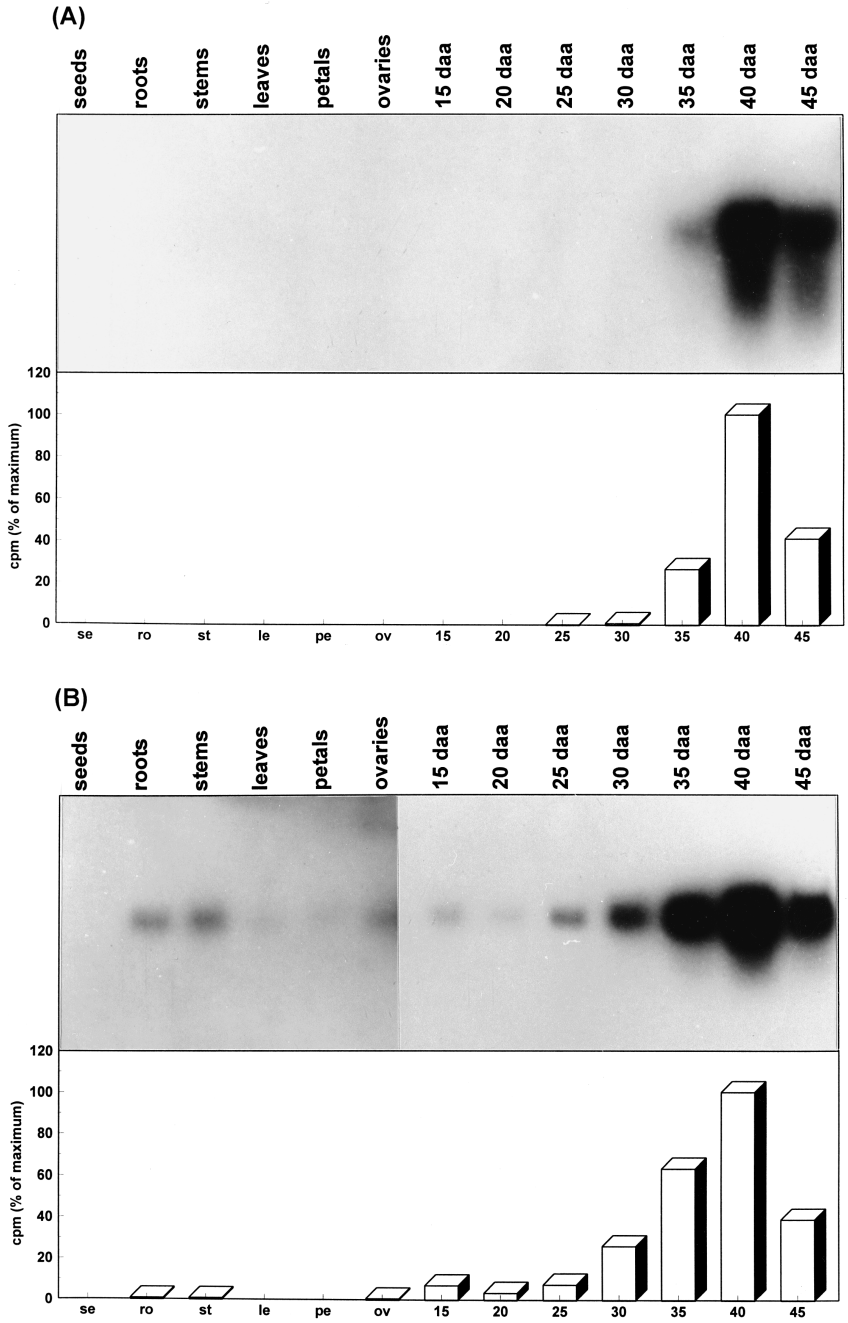

Figure 4. Expression of MEL2 and MEL7 mRNAs in melon organs and during fruit ripening. Total RNA from ripening stages and various organs of melon was electrophoresed in 1% agarose gels, blotted onto nylon membranes and hybridized with (A) MEL2 probe and (B) MEL7 probe. The MEL2 probed membrane was exposed for 12 h while the membrane probed with MEL7 was exposed for 18 h for the fruit samples and 8 days for the other organ samples. The bottom panels show the quantification of the hybridization of the membranes, expressed as a percentage of the maximum signal. Gels were checked for similar RNA loading in each lane by ethidium bromide staining (C).

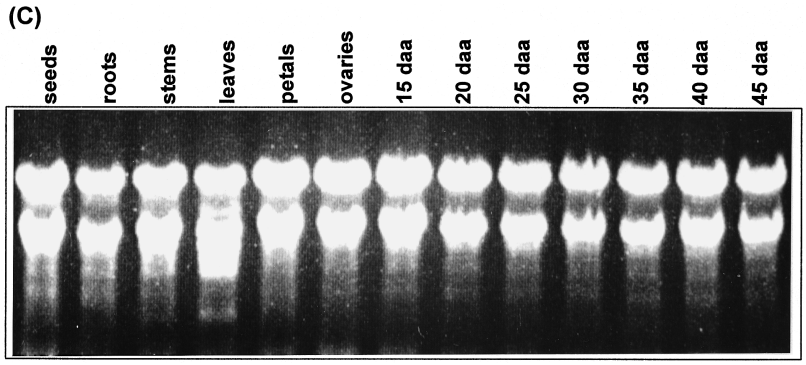

Figure 4. Continued.

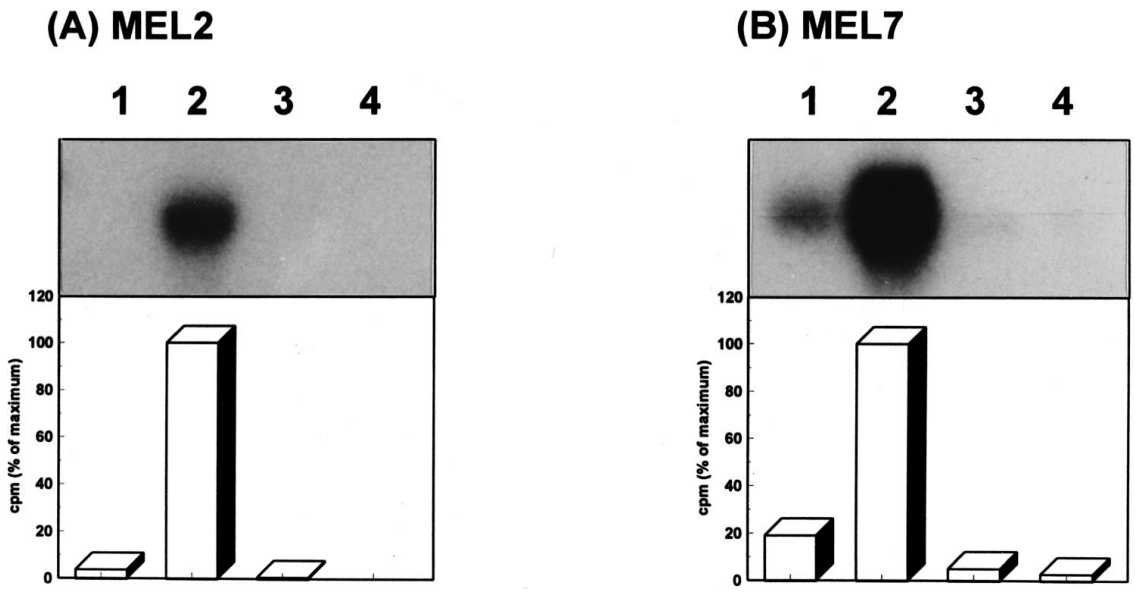

Figure 5. Expression of MEL2 and MEL7 mRNAs in unripe melon fruits after ethylene treatment and wounding. Unripe melon fruits (20 daa) were treated with ethylene (20 μl/l for 48 h) or wounded for two hours and six hours. The expression of MEL2 (A) and MEL7 (B) homologous mRNAs was determined using northern blot analysis. The RNA samples were: 1, control untreated and unwounded fruit, 2, ethylene-treated fruit; 3, wounded fruit after 2 h; 4, wounded fruit after 6 h. The accumulation of MEL2 and MEL7, determined by radioanalytical image detection of the northern blot membranes, is shown below the northern blot photographs. The results are expressed as a percentage of the maximum signal.

of 3.6 kb, when melon genomic DNA was digested with *Eco*RI and *Bam*HI enzymes. This result indicated that MEL7 might originate from a single or low-copy gene.

The RNA binding motif (RNP-CS1), the most conserved region identified as characteristic of RNA-binding proteins, was found in the MEL2 predicted polypeptide sequence. It is the first time that such a concensus sequence has been found in any ripening-related genes so far identified. Its presence indicated the possible involvement of this protein in the regulation of RNA turnover. It has been shown that nuclear-locating target signals function in plants but the absence of the N-terminal sequence makes it difficult to speculate about the location of the MEL2 protein and its possible regulatory role in transcription, pre-mRNA processing or translation of ripening-related gene(s) [7, 11]. However, this could be clarified by immunolocalization of MEL2 protein in the fruit cells.

The homology of the MEL7 predicted polypeptide to the major latex protein is interesting. This is the main protein of latex fluid in opium-poppy [22]. Latex is pro-

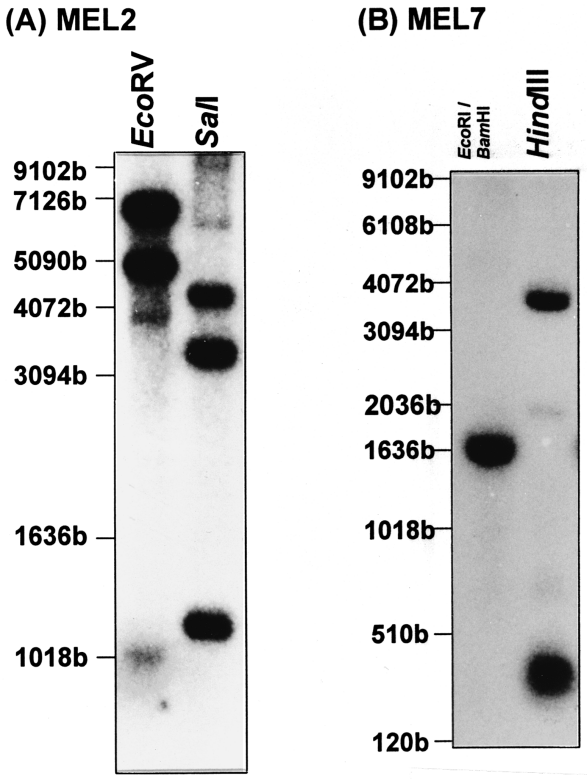

Figure 6. Southern analysis of MEL2 and MEL7 genes. Genomic DNA was isolated from melon leaves and digested with different restriction enzymes. The DNA was separated on a 0.8% agarose gel, transferred onto nylon membranes and probed with MEL2 (A) and MEL7 (B). Molecular weight markers are indicated on the left of each blot.

duced especially in differentiated cells called laticifer cells and its presence has been reported in many plant families. The detection of the MEL7 mRNA in higher levels in roots and stems than in other vegetative tissues coincides with the distribution of laticifer cells in plants. The dominant views about the function of latex are that it is involved in the sealing of wounds and the storage of secondary metabolites. It has been reported that ethylene can increase the production of latex and the activity of various enzymes in laticifer cells [24]. If the MEL7 protein proves to be the melon counterpart of the major latex protein then its role might be in the protection of the ripe fruit against infection and wounding. This view is supported by the fact that the *Sn-1* gene product was normally detected only in ripe bell pepper fruit but it could also accumulate in the green fruit 15 h after wounding [23]. It is known that fruits become more susceptible to infection and damage during ripening, probably because of the softening of the cell walls. It was assumed that the MEL7 expression would have been induced by wounding but our results showed that its mRNA levels dropped after wounding of the mesocarp tissue. This indicates that there is no general induction of this mRNA in response of wounding. It is possible, however, that the regulation and function of the protein vary in different cell and tissue types.

The accumulation pattern of mRNAs homologous to these MEL clones suggests their possible role in melon ripening, for example in RNA processing or turnover and wound sealing. Since the MEL2 mRNA is ripening-specific, the isolation of the MEL2 promoter could be very useful for genetic modification of melon. Future work to generate sense and antisense transgenic plants using these clones, and analysis of the fruit phenotype, may help us to clarify their role in melon ripening.

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