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Accumulation of specific mRNAs during almond fruit development

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Abstract

The mRNA accumulation of a number of different genes during different stages of almond fruit development has been studied. The probes used in the study correspond to almond cDNAs previously characterized in our laboratory. They include cDNAs encoding extensin, prunin (the main storage protein in almond seed), oleosin, α-tubulin and a cDNA clone (PA3BF11) not showing any similarity to known sequences but whose mRNA is very abundant in the almond seed. These probes have been used in RNA blot, tissue printing and in situ hybridization experiments. The tissue printing technique has proven to be a very useful tool for detecting gene expression in large organs such as whole almond fruits whereas in situ hybridization can be successfully applied in small fruits. It is shown that the distribution of specific mRNAs is highly controlled in the fruit. mRNAs related to storage proteins are abundant in seed tissues while mRNAs corresponding to genes related to cell division, such as extensin or α-tubulin, are accumulated in dividing cells mostly around the vascular tissues.

Keywords: cDNA; Gene expression; Tissue-printing; Fruit development; *Prunus amygdalus*

1. Introduction

Fruit development in angiosperms has been extensively studied in different plant model systems from a molecular point of view [1] although very few data are available in the *Rosaceae* family, an economically important group of plant species which includes fruit trees belonging to the genus *Prunus* such as peach, apricot, cherry, plum and almond. All these species share the same basic chromosome number ($x = 8$) and their DNA content is one of the smallest known: 0.54–0.67 pg of DNA/2C [2], which is approximately twice the size of the *Arabidopsis* genome [3]. There are in the literature some biochemical and physiological studies about almond seed and fruit development [4,5] but knowledge of the molecular mechanisms involved in these processes is still lacking.

Three main stages can be distinguished in almond fruit development: a first stage characterized by morphogenesis and tissue formation with a high rate of cellular divisions, comprising between fertilization and 80 days after flowering

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(DAF); a second stage which is characterized by the maturation and the accumulation of storage substances, where cellular expansion is found, and located between 80 and 170 DAF; a third and last stage in which loss of water and endocarp lignification occur from 170 DAF until fruit maturity. Among the species of *Prunus*, almond (*Prunus amygdalus*) is the only one where the seed is consumed and the species has an economical importance in the Mediterranean area: recently the first RFLP-based molecular map in *Prunus* was reported by our group [6]. As a first step towards the characterization of the structures which play a role in almond seed development we have studied the expression pattern of different genes that are important in specific cellular processes.

Tissue printing and in situ hybridization methodologies have been developed in this system and experiments have been carried out using cDNA probes previously characterized in almond. These probes encode extensin and α-tubulin [7,8] and they have been tested on fruit developmental stages where their expression had previously been detected by RNA blot. Other probes used in this study have been cDNAs coding for one of the main storage proteins (prunins) and an oleosin [9] which showed a seed-specific expression pattern when RNA blot experiments had previously been performed. The results obtained allow specific regions and periods to be defined where processes such as storage products accumulation or cell division occur.

2. Methods

2.1. Plant material

Almond (*Prunus amygdalus*, Batsch) fruit samples from the cultivar 'Texas' were collected at different stages of development from crop fields in Departament d'Arboricultura Mediterrània, IRTA (Reus) and were immediately frozen in liquid nitrogen and stored at −80°C until they were used.

2.2. RNA extraction and RNA blot analysis

RNA was extracted from different almond tissues using the methods described by Martin et al. [10] and Logemann et al. [11] depending on the tissue. Then 10 μg of total RNA were separated in an agarose formaldehyde gel and transferred to a nylon membrane (Nytran, Schleicher and Schuell) according to the method originally described by Lehrach et al. [12]. The membrane was hybridized with a random primed [^{32}P]dCTP-labelled cDNA probe and washed according to a protocol described by Church and Gilbert [13].

2.3. Tissue printing

The protocol used was originally described by Varner [14]. Almond fruits at different developmental stages, and stored frozen at −80°C, were thawed at room temperature and cut in sections with a double-edged razor blade. The sections were printed on a Zetaprobe nylon membrane (Biorad) for 15–30 s and after drying the membrane was exposed to UV light (50 mJ) to bind the RNA. Both sense and antisense riboprobes were synthesized with T7 and T3 RNA polymerase as described previously [15] using 0.5 μg of DNA and [^{35}S]rCTP (10 μCi/μl, 1000 Ci/mmol). The membrane was washed in SSC × 2, 1% SDS at 65°C for 16 h before prehybridization in hybridization solution (SSPE × 1.5, 1% SDS, Denhardt's × 5, denatured salmon sperm DNA 100 μg/ml, DTT 100 mM) at 68°C for 12 h. Hybridization was performed in hybridization solution with the riboprobe (10^7 counts/min per ml) at 68°C for 20 h. The membrane was washed three times in SSC × 2, 0.1% SDS at 42°C for 20 min and another wash in SSC × 2, 1% SDS was done at 65°C for the time necessary to remove any signal from the sense probe hybridization. The membrane was briefly rinsed in SSC × 2 at room temperature, air-dried and exposed to film (Kodak X-OMAT AR).

2.4. In situ hybridization

Thirty DAF almond immature fruits, stored at −80°C, were thawed, cut in cross- and longitudinal sections and fixed in ethanol/formaldehyde/acetic acid 80:3.5:5 for 1 week at 4°C. After this treatment, fruit sections were rinsed twice in 70%

ethanol and stored at 4°C. Tissue embedding in paraffin, sectioning, pretreatment of sections, hybridization and detection have previously been described [15]. The probes were labelled with digoxygenin following the manufacturer's recommendations (RNA colour kit for in situ hybridization, Amersham).

3. Results

3.1. Extensin mRNA accumulation in almond immature fruits

A cDNA encoding an almond extensin [7] has been used as a probe in a RNA blot experiment as shown in Fig. 1; 10 μg of total RNA from different seed and pericarp developmental stages, root and leaf, were fractionated in a formaldehyde denaturing agarose gel, transferred to a nylon membrane and hybridized with a 1.2 kb cDNA probe encoding the almond extensin. The expression of the extensin gene is detected in young seeds up to 60 DAF and no expression is found in later seed stages of development. The same result is found in the pericarp tissue with a high expression in 20 DAF and a faint detectable RNA band is observed at 60 DAF. The mRNA can also be detected in the root tissue, which was the source of the cDNA library from where the extensin cDNA was cloned [7].

In order to localize the tissues of the almond seed accumulating extensin mRNA, tissue printing hybridizations were performed on cross- and longitudinal sections of 60 DAF fruit. The printings were hybridized both with an antisense and a sense extensin riboprobe labelled with [^{35}S]rCTP. The results are shown in Figs. 2d and 2e for the antisense probe in a longitudinal and a cross-section, respectively. The extensin gene mRNA is mainly detected in the endocarp vascular bundles (vb), the testa layer (in) and in regions of the seed opposite to the embryo (in this developmental stage the seed content is mainly endosperm, the embryo not being developed yet). Tissue printing hybridizations in later stages of fruit development do not show any extensin mRNA accumulation (not shown). This result is consistent with the RNA blot results.

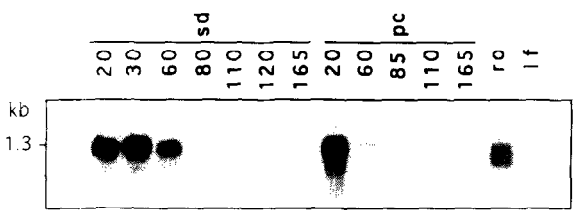


Fig. 1. RNA blot using the 1.2 kb almond extensin cDNA as a probe; 10 μg of total RNA from seed (sd) and pericarp (pc) in different stages of development, root (ro) and leaf (lf) were fractionated in a formaldehyde denaturing agarose gel and transferred to a nylon membrane which was hybridized with the 1.2 kb almond extensin cDNA probe. Numbers indicate days after flowering (DAF).

The in situ hybridizations using the extensin antisense digoxygenin-labelled riboprobe on 30 DAF fruits are shown in Figs. 3a to 3d. The mRNA accumulation is basically located in the pericarp and testa vascular systems (Figs. 3a and 3b); expression is detected with a higher magnification in a longitudinal section of a mesocarp vascular element (Fig. 3c), near the intersection between the pericarp and the seed. Extensin mRNA is mainly accumulated in the provascular cells contained in an endocarp vascular element (Fig. 3d).

3.2. Accumulation of α-tubulin mRNA in early almond fruits by in situ hybridization

A cDNA encoding an almond α-tubulin [8] has been used as a probe in in situ hybridization experiments on 30 DAF fruit sections. As has been previously reported by RNA blot analysis [8], this α-tubulin gene is expressed in young fruit tissue (20–30 DAF) and in later stages of seed development (100 DAF). The mRNA is also accumulated in roots. The antisense digoxygenin-labelled riboprobe used in the in situ experiments mainly hybridizes with the testa vascular elements and with some nucellar and testa cellular layers (Figs. 4a and 4b), tissues which are in a high growing rate in this stage of development.

3.3. Prunin storage protein mRNA accumulation in immature fruits

The pattern of expression of prunin genes which

has previously been described [9] indicates that it shows a seed-specific mRNA accumulation around 110 DAF, when the cotyledons are already developed. A tissue printing hybridization has been done on 110 DAF fruits with both antisense and sense ^{35}S-labelled riboprobes synthesized from the almond prunin Pru1 cDNA clone. The results obtained are shown in Fig. 2a for a cross-section and Fig. 2b for a longitudinal section, both hybridized with the antisense probe. In Fig. 2f the same sections are hybridized with a sense probe as a control. As can be seen in the figures, mRNA accumulation is limited to the cotyledonary tissue which has developed from the embryo; this result agrees with what had previously been obtained in the RNA blot experiments. The expression of storage protein genes in this developmental stage is very high as has been reported elsewhere [16].

3.4. Oleosin mRNA accumulation in almond immature fruits

As has been reported previously [9], the RNA blot hybridization experiments performed with an almond oleosin cDNA show a cotyledon-specific accumulation around 110 DAF with a still detectable expression around 165 DAF. Tissue prints of 110 DAF cross-sections of almond fruits hybridized with an antisense ^{35}S-labelled probe derived from the oleosin cDNA show a similar pattern of accumulation as the one obtained for the prunin gene (Fig. 2c).

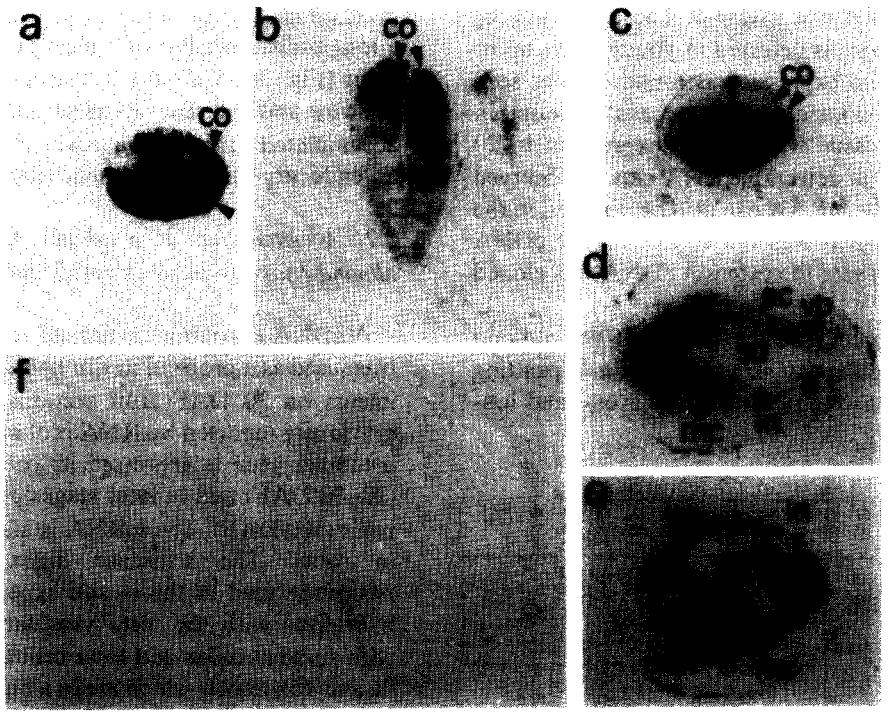
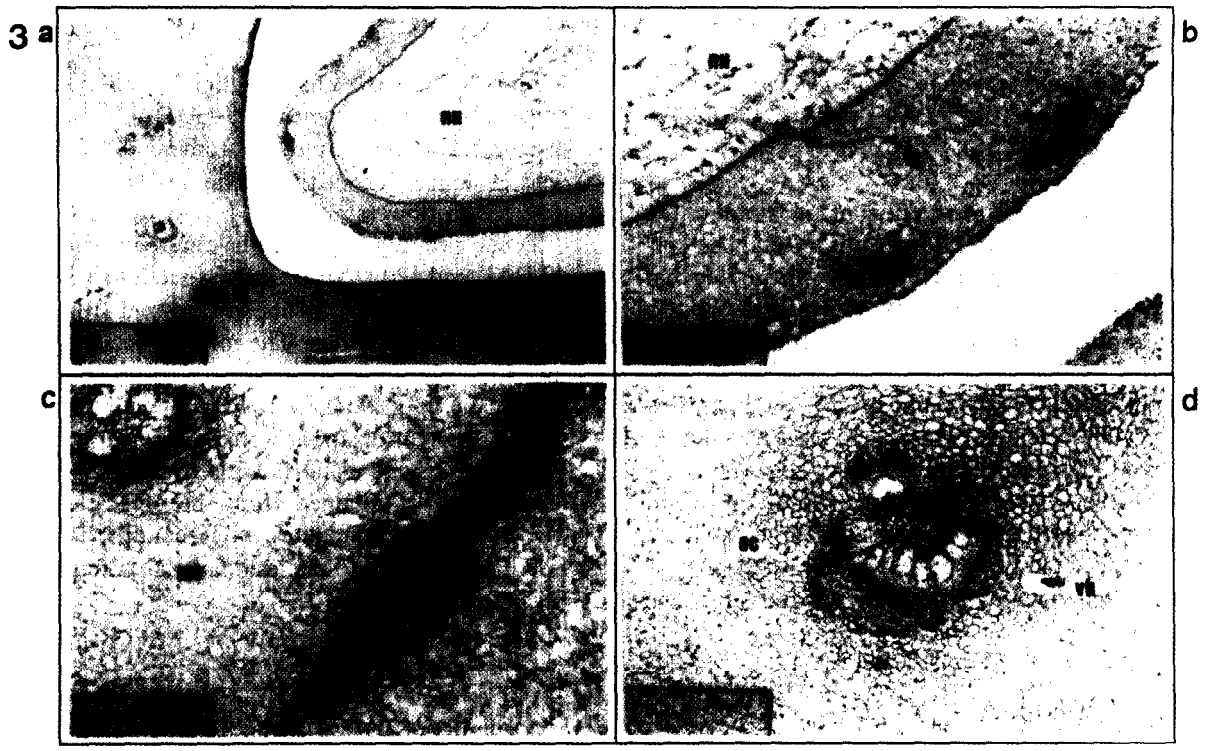


Fig. 2. Tissue printing hybridizations with different cDNA probes on almond fruit sections. (a), (b) Cross- and longitudinal sections of a 110 DAF almond fruit hybridized with an antisense RNA probe obtained from the almond prunin Pru1 cDNA clone. (c) Cross-section of a 110 DAF almond fruit hybridized with an antisense RNA probe obtained from the almond oleosin Ole1 cDNA clone. (d), (e) Longitudinal and cross-sections of a 60 DAF almond fruit hybridized with an antisense RNA probe obtained from the almond extensin cDNA clone. (f) Cross- and longitudinal sections of a 110 DAF almond fruit hybridized with a sense RNA probe obtained from the almond prunin Pru1 cDNA clone. co, cotyledon; ec, endocarp; in, integument; mc, mesocarp; sd, seed; vb, vascular bundle.



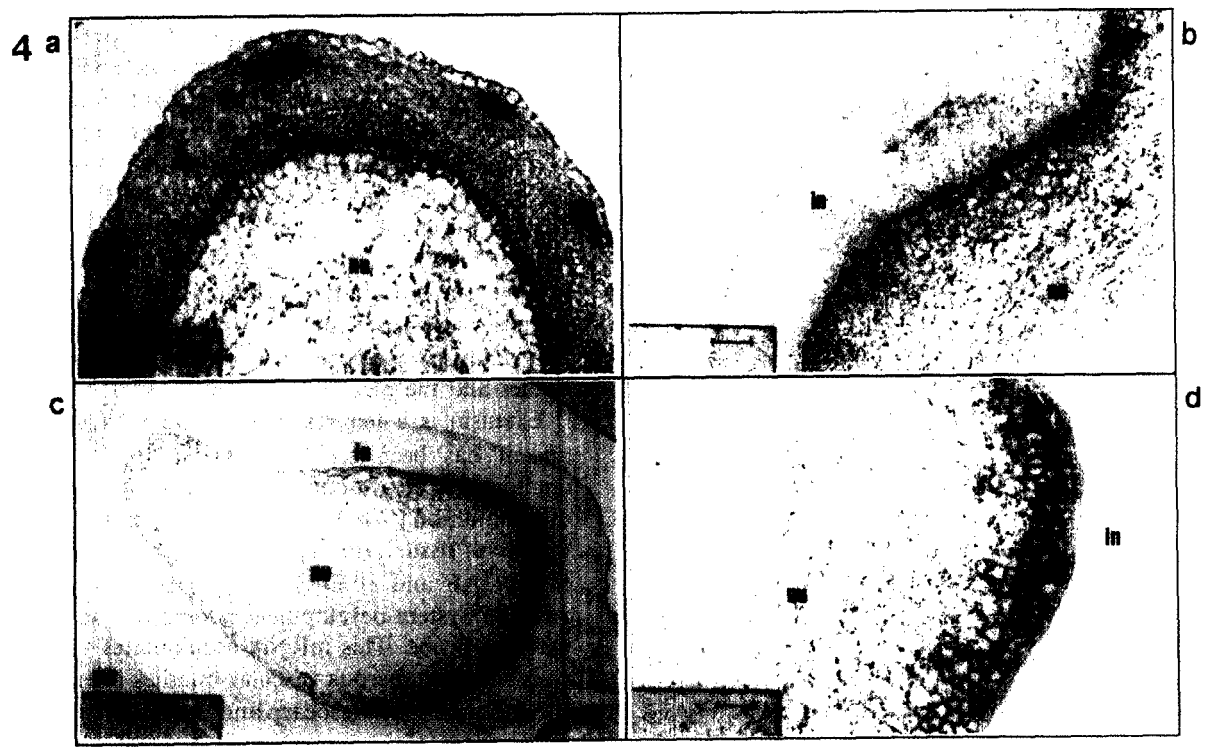


Fig. 3. In situ hybridizations with the digoxygenin-labelled extensin cDNA antisense riboprobe on 30-DAF almond fruit sections (a) to (d): ec, endocarp; in, integument; mc, mesocarp; nu, nucellar tissue; vb, vascular bundle.

Fig. 4. In situ hybridizations with the digoxygenin-labelled α-tubulin cDNA antisense riboprobe ((a), (b)) and cDNA clone PA3BF11 antisense riboprobe ((c), (d)) on 30-DAF almond fruit sections. in, integument; mc, mesocarp; nu, nucellar tissue.

3.5. mRNA accumulation of an unidentified gene corresponding to the cDNA clone PA3BF11 in almond immature fruits

Following the construction of an almond 110 DAF immature seed cDNA, library cDNAs corresponding to genes abundantly expressed at that period were characterized by screening the library with radioactively labelled cDNA from the same tissue. One of these clones, clone PA3BF11, corresponded to a highly abundant mRNA and it was further characterized. The 390 bp sequence obtained from the cDNA gave no significant similarity with sequences contained in the EMBL and Genbank databases, but we considered this clone to be interesting because of its expression pattern after RNA blot experiments (Figs. 5a and 5b). The mRNA accumulates at a very high level in the first stages of seed development (20–60 DAF) and it is also present in pericarp tissue from 60 to 110 DAF with a longer exposition. The mRNA is also detected in flowers.

Tissue printing experiments were performed in 30 DAF and 120 DAF fruit cross-sections with an antisense ^{35}S-labelled probe synthesized from the PA3BF11 cDNA clone. The mRNA accumulates in the nucellar tissue in 30-DAF sections (Fig. 5c) and in the cotyledons in 120-DAF sections (Fig. 5d). In situ hybridizations were performed with 30-DAF sections and using an antisense digoxygenin-labelled probe obtained from the cDNA clone. The mRNA accumulates preferentially in the nucellar cells close to the testa layer as is shown in Figs. 4c and 4d.

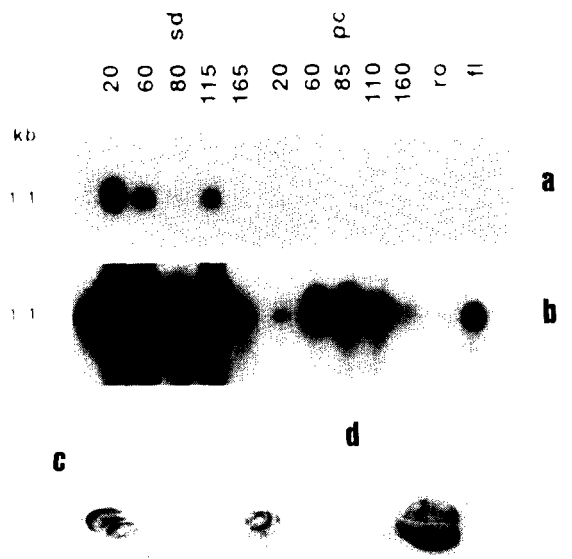


Fig. 5. (a), (b) RNA blot using the cDNA clone PA3BF11 as a probe (with a longer exposition in (b)); 10 μg of total RNA from seed (sd) and pericarp (pc) in different stages of development, root (ro) and flower (fl) were fractionated in a formaldehyde denaturing agarose gel and transferred to a nylon membrane which was hybridized with the cDNA clone PA3BF11 probe. Numbers indicate days after flowering (DAF). (c) Tissue printing from a cross- and a longitudinal section of a 30 DAF double-seeded almond fruit hybridized with an antisense RNA probe obtained from the cDNA clone PA3BF11. (d) Tissue printing from a cross-section of a 120 DAF almond fruit hybridized with an antisense RNA probe obtained from the cDNA clone PA3BF11.

4. Discussion

A number of cDNAs corresponding to genes expressed in almond seed have recently been described. In the present article results are presented showing that histochemical analysis methods such as tissue printing and in situ hybridization can be successfully applied to study the specific patterns of mRNA accumulation in this system. Extensin [7] is a marker of fruit vascular network formation, α-tubulin [8] of the development of new structures and organs where cell division is active and oleosin and prunin probes [9] allow detection of cells where deposition of storage nutrients in the cotyledon storage compartment (both the protein bodies and the oleosomes) occur.

Extensin is a marker of cell wall biosynthesis and it has been reported that its mRNA accumulates in provascular tissues, mainly xylem in differentiation [17]. As is shown in this paper, with the use of tissue printing and in situ hybridization in 60-DAF and 30-DAF fruits, respectively, the vascular system development is detected with the extensin probe. This mRNA accumulates specifically in the numerous vascular bundles present in the endocarp, the mesocarp and the testa. After 60 DAF no extensin expression is detectable which would correlate with the fact that all the vascular network needed in the fruit tissue is already present. Thus, the formation of vascular tissue in the almond fruit is detected with the use of the extensin probe as a molecular marker.

Another event that can be detected with the use of a molecular marker is cell division. α-Tubulin genes are preferentially expressed in dividing tissues [18] so the use of the almond α-tubulin cDNA as a probe during almond fruit development will detect those tissues and structures which are under a high rate of cellular division. As is shown in the in situ hybridizations performed on 30-DAF fruits, the α-tubulin mRNA accumulates in nucellar cells and in the testa and endocarp vascular systems of the fruit. The nucellar tissue is the source of nutrients for both the endosperm and the embryo development and supports a high rate of cellular divisions in the first stages of development until the growing of the endosperm, which absorbs the nucellar storage substances. Although not tested by in situ hybridization, the development of the embryo to form the cotyledons around 100 DAF correlates with the expression of the α-tubulin gene as has been shown by RNA blot [8]. Thus the formation of new tissues during the almond fruit development can be detected at a molecular level by the use of the α-tubulin gene as a probe in hybridization experiments.

On the other hand, the accumulation of storage molecules in seeds is one of the main processes occurring during fruit development. Once the fruit structures are already formed, the cotyledons store proteins, lipids and sugars to be used during seed germination. In almond the main family of storage proteins is the prunin family [9], which represents the majority of the protein fraction in the mature seed. The accumulation process of this gene mRNA can be detected by the use of the prunin cDNA probe, which shows a very fast mRNA accumulation pattern from 100 to 130 DAF in the cotyledons. Oleosin, the main protein present in the monolayer of the lipid storage organelles or oleosomes, may be a marker for lipid deposition and storage in the seed. The oleosin mRNA accumulation directly indicates the formation of the oleosomes and the lipid deposition from 100 up to 165 DAF. cDNA clone PA3BF11, although coding for an unidentified product, shows very high levels of expression in RNA blot experiments. The use of this clone in both tissue printing and in situ hybridization allows the detection of its mRNA in specific nucellar cells near the testa layer with a gradient of expression decreasing towards the centre of the nucellar tissue, in 30-DAF fruits. Its expression also appears to be preferential in cotyledons 110 DAF. These results show that other specific patterns of mRNA accumulation in the almond seed might be found by the use of other cDNA probes.

The application of in situ hybridization in almond fruit development is restricted to samples with a small size up to 30 DAF due to technical problems with the samples during their manipulation. The use of an alternative simple technique, such as tissue printing, allows the study of gene expression in the whole tissue with a high level of detail, the results obtained being complementary to those obtained by in situ hybridization.

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