Cloning and Characterization of cDNAs from Genes Differentially Expressed during the Strawberry Fruit Ripening Process by a MAST-PCR-SBDS Method

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A vast number of clones carrying cDNAs from genes differentially expressed along the strawberry (Fragaria × ananassa c.v. Chandler) fruit ripening process has been isolated by screening of a subtractive cDNA library. The library was constructed and screened using a powerful procedure that combines the differential screening technique with a Southern blot screening by means of the polymerase chain reaction (PCR-SBDS procedure). Several clones have been partially sequenced and characterized and main similarities with other known genes from higher plants are presented. These comparisons reveal putative functions of these genes in the strawberry fruit ripening process.

Although the molecular basis of the climacteric fruit ripening has been investigated in detail, few studies at a molecular level have been performed in a nonclimacteric fruit as strawberry. The ripening of this fruit is characterized by an initial auxin-regulated phase of growth and enlargement that is followed by a maturation phase during which the fruit acquires the ability to ripen (1–3). The ripening involves transition in color, flavor, aroma, and texture, resulting in fruit softening and liquefaction (1, 4–8). Many changes in mRNA populations during the strawberry ripening process have been reported (9). Strawberry cDNAs corresponding to genes whose expression is induced and/or repressed by auxin action have also been cloned and sequenced (10, 11), but no similarities to genes present in the data bases were found. Very recently, three fruit-specific cDNAs from strawberry have been cloned, two of which present similarities to the sequences of genes encoding chalcone synthase and annexins (12). So far, however, only a few genes have been isolated and studied, and the ripening process of this important nondimacteric fruit still remains poorly understood at the molecular level.

To elucidate the molecular mechanisms underlying the strawberry fruit ripening we have differentially screened a subtractive strawberry cDNA library generated by a modified magnet-assisted subtraction technique (MAST)2 (13), searching for cDNAs differentially expressed during this process. At present, the cloning of genes related to a specific biological process has been mainly achieved by three different approaches, namely, the conventional differential screening technique of cDNA libraries (14), the generation of subtractive expression libraries and their subsequent differential screening (13, 15–18), and, more recently, by means of a differential display method (12, 19, 20). Here, we present the successful cloning of cDNAs from a great variety of genes differentially expressed along the strawberry ripening process, using a reliable and easy procedure that combines a differential screening technique with a Southern blot screening of a subtractive library by means of the polymerase chain reaction (PCR). This method can be extended to isolate a vast number of cDNAs from genes differentially expressed.

2 Abbreviations used: MAST, magnet-assisted subtraction technique; DTT, dithiothreitol; DEPC, diethylpyrocarbonate; SBDS, Southern blot differential screening; LMW HSP, low molecular weight heat shock protein; MIP, membrane intrinsic protein; DHFR, dihydrofolate reductase; PRP, proline rich protein; GAST, gibberellins stimulated transcript.
along other physiological processes, increasing the probability of cloning cDNAs corresponding to genes weakly expressed. We also present the main identities found among some of these clones and others previously studied in higher plants.

**MATERIAL AND METHODS**

**Media, Strains, and Phages**

We have used the predigested λZapI/EcoRI/Clal cloning kit from Stratagene to generate the subtractive library. The Gigapack II Gold Packaging Extract from Stratagene was also used for packaging the λ phage. The Escherichia coli strain XL1-Blue MRPl, Δ(mra)183, Δ(mrcB-hsdSMR-mrr)173, endA1, supE44; thi-1, recA1, gyrA96, relA1, lac [F' proAB, lacIZΔM15, Tn10 (tet')], was used to plate and amplify the subtractive library. The E. coli SOLR strain, e14' (mra), Δ(mrcB-hsdSMR-mrr)171, sbcC, recB, Red, umuC::Tn5(kan'), uvrC, lac, gyrA96, relA1, thi-1, endA1, λ6', [F' proAB, lacIZΔM15]SU- (nonsense-preserving), and ExAssist helper phage (M13) were used for in vivo excision of the pBluescript SK(−) phagemid-derived clones from λZapI. Media and growing conditions are those described in the manufacturer's protocol and elsewhere (21).

**RNA Isolation**

Total RNA from different ripening stages of strawberry fruit (Fragaria × ananassa c.v. Chandler) and from roots, leaves, flowers, and stolons was isolated according to Manning (22), and the remaining carbohydrates were removed by passing total RNA through a cellulose column (23). Poly(A⁺)-RNA was obtained using an Oligo(dT) cellulose column (23).

The strawberry fruit stages used in this study were middle-size green fruit (G2-stage) and full-size red fruit (R-stage).

**Double-Stranded cDNA (ds-cDNA) Generation**

Double-stranded cDNA was obtained from poly(A⁺)-RNA, using the cDNA Synthesis System Plus Kit (Amersham) following the manufacturer’s instructions and essentially as follows: 5 μg of poly(A⁺)-RNA was used to synthesize the first-strand cDNA in a standard reaction mixture containing 10 μl of 5× first-strand synthesis reaction buffer (Amersham), 2.5 μl of sodium phosphate solution (Amersham), 2.5 μl of human placental ribonuclease inhibitor solution (20 units/μl, in 20 mM Hepes-KOH, pH 7.6, 50 mM KCl, 5 mM DTT, 50% (v/v) glycerol), 5 μl of deoxinucleoside triphosphate mixture (10 mM dATP, 10 mM dGTP, 10 mM dTTP, 5 mM dCTP), oligo(dT) (1.6 mg/ml), and distilled water (dH₂O) up to 49 μl. Two hundred units (1 μl) of M-MLV reverse transcriptase (Gibco-BRL) was added to the standard reaction and the mixture was incubated at 42°C for 1 h. Then, the reaction mixture was chilled on ice. To synthesize the second-strand cDNA, the following components were added to 50 μl of the first-strand cDNA reaction mixture: 93.5 μl of second strand synthesis reaction buffer (Amersham), 4 units (0.8 units/μl) of E. coli ribonuclease H, 115 units (3.5 units/μl) of E. coli DNA polymerase I, and dH₂O up to 248 μl. The mixture was incubated sequentially at 12°C for 1 h, at 22°C for 1 h, and 70°C for 10 min and then kept on ice. Afterward, 10 units (4 units/μl) of T4 DNA polymerase was added and the reaction was incubated at 37°C for 10 min. The reaction was finished by adding 10 μl of 0.25 M ethylenediamine tetraacetic acid (EDTA), and the double-stranded cDNA (ds-cDNA) was concentrated through a Centricon-100 unit. The quantity of cDNA was calculated spectrophotometrically. The yield of first- and second-strand cDNA synthesis was followed in separate reactions using [α-³²P]dCTP (data not shown).

Accordingly, poly(A⁺)-RNA from the red stage of fruit ripening (R-stage) was used to generate ds-cDNA for subtractive purposes (tracer cDNA) (Fig. 1, top right). Independently, poly(A⁺)-RNA from green (G2) and red (R) stages of fruit was used to generate ds-cDNA probes (G2-probe and R-probe, respectively) to perform differential screening of the subtractive cDNA library. These ds-cDNA probes were ligated to an adapter generated by mixing equimolecular amounts of two previously designed oligos with the sequences AATTCCGCAATTTATCTAGACC and GGCTCTAGATCCATG (with the 5'-end phosphorylated). The ligation was made in 50 μl of ligation mixture containing 100 pmol (2.5 μl) of adapter, 5 μl of 10× New England Biolabs (NEB) ligation buffer (500 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 100 mM DTT, 1 μM ATP, 25 μg/ml bovine serum albumin), 27.5 μl of sterile dH₂O, and 1 μl (30 Weiss units) of highly-concentrated T4 DNA ligase (NEB). The reaction was allowed to proceed at 12°C for 1 h. Then, an additional 30 Weiss units of T4 DNA ligase was added and the ligation mixture was incubated at 12°C for 16 h. Afterward, the mixture was incubated at 65°C for 10 min and then size fractionated using a G-50 spin column to remove the unligated adapters and oligos. After ligation, these ds-cDNA probes were PCR-amplified using the former oligo (21-mer) as a primer. The PCR reaction was carried out in a 100-μl reaction mixture containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.125 mM dNTPs, 50 pmol of the 21-mer primer, and 2.5 units of Taq polymerase (Pharmacia). To extend the 3'-recessed ends of the ligated adapter and thus to generate the primer binding site on the cDNAs, an initial step at 72°C during 2 min was carried out before the amplification steps. PCR conditions were 3 thermo-cycles, 1 min at 95°C, 2 min (with a ramping time of 1 min) at
FIG. 1. Scheme of the method used to generate the R-stage subtractive library. Details are given under the Materials and Methods section. Subtractive cDNA Library Construction. Tracer ds-cDNA (ds-cDNA synthesized from RNA isolated from the full-red stage (R-stage) of fruit ripening) was subject to two rounds of subtraction versus Driver ss-cDNA (ss-cDNA synthesized from RNA isolated from the G2-green stage (G2-stage) of fruit ripening. The product of subtraction was ligated to commercial EcoRI/NotI adapters and PCR-amplified so that only a yield of ds-cDNA (R-stage) differentially expressed was obtained. The amplified product was EcoRI digested and ligated to β-ZapI/EcoRI arms to produce a cDNA (R-stage) subtractive library.
48°C, and 10 min at 70°C; 22 step-cycles, 1 min at 95°C, 2 min at 52°C, and 10 min at 70°C; and 1 cycle, 10 min at 72°C. The PCR products were filtered through a microcon-100 to eliminate the primers before labeling. The probes were labeled to a specific activity of about 10^6 cpm/µg of DNA, using the oligolabelling kit (Pharmacia) and essentially as follows: about 100 ng (5 µl) of denatured ds-cDNA probe (either G2-probe or R-probe) was labeled in 50 µl of reaction mixture containing 10 µl of reagent mix (Pharmacia), 5 µl (50 µCi) of [α-32P]dCTP (3000 Ci/mmole), 29 µl of dH2O, and 1 µl (5 units/µl) of Klenow fragment. The reaction was allowed to proceed 37°C for 90 min. Finally, the unincorporated nucleotides were removed from the mixture by gel filtration using a NICK column (Pharmacia) before hybridization.

Subtractive cDNA Library Construction

To generate a cDNA subtractive library of the red stage versus green stage, a modified MAST method (13) was used according to the following procedure (Fig.1): a total amount of 2.8 mg (2.8 µg/µl in binding buffer) of G2-stage RNA was mixed with 14 mg (3.5 µg/µl in binding buffer) of oligo(dT)25 magnetic Dynabeads to capture poly(A+) mRNA. Thus, 10 individual samples, each containing 280 µg of total RNA (G2-stage) and 1.4 mg of magnetic beads, were made in 500 µl of binding buffer. The mixes were left to hybridize at room temperature for 15 min. Beads were collected using a magnetic support, combined in a single tube and then washed three times with 500 µl of buffer A (10 mM Tris–HCl, pH 7.6, 0.15 M LiCl, 1 mM EDTA). Beads were resuspended in 500 µl of cDNA synthesis solution (50 mM Tris–HCl, pH 7.6, 70 mM KCl, 10 mM MgCl2, 0.25 mM dNTPs, 10 mM DTT, 250 units of RNase inhibitor, and 15,000 units of M-MLV reverse transcriptase (Gibco BRL)) and split into 10 samples of 50 µl each. The cDNA synthesis reactions were allowed to continue overnight at 37°C in a hybridization oven with continuous rotation. Then, the beads were collected using a magnetic support, combined in a single tube, washed five times with 500 µl of diethylpyrocarbonate-treated water (H2O-DEPC) and boiled in H2O-DEPC (500 µl) for 5 min to denature mRNA–cDNA complexes. The beads were again collected and washed five times as above to elute the poly(A+)-RNA. This single-stranded cDNA (ss-cDNA) bound to the beads was used as driver cDNA (Fig. 1, top left).

Double-stranded cDNA from R-stage was generated as described in the section Double-Stranded cDNA Generation and used as tracer cDNA (Fig.1, top right). After boiling, 1 µg (in 200 µl of binding buffer) of this tracer cDNA was added to the immobilized driver cDNA (14 mg of beads). Hybridization was carried out at 65°C for 3 days in a hybridization oven with continuous rotation. Beads carrying hybrid ds-cDNAs (G2-stage:R-stage hybrids) were separated from the supernatant containing ss-cDNAs (R-stage) and annealed ds-cDNAs (preferentially expressed cDNAs from R-stage) using a magnetic support. Beads were washed with 200 µl of new prewarmed (65°C) binding buffer, and the supernatant was recovered and mixed with the previous one to get a volume of 400 µl. Then, this supernatant was subject to a second round of subtraction as follows: the collected beads were boiled with sterile water (200 µl) for 5 min, washed with the same volume of fresh sterile water (to remove the tracer ss-cDNA), and collected again with the magnetic support before using for further subtractions. Then, the 400 µl of supernatant was boiled and mixed again with the beads (driver ss-cDNA) to perform the subtraction. After this second round, no additional significant subtraction was observed. A yield of 46 ng of specific ds-cDNAs (R-stage) per microgram of input ds-cDNAs (R-stage) was obtained (4.6%). The subtracted library (500 ng in 14 µl dH2O) was ligated to commercial EcoRI/NotI adapter (Pharmacia) in 50 µl of ligation mixture containing 100 pmol (2.5 µl) of adapter, 5 µl of 10× NEB ligation buffer (500 mM Tris–HCl, pH 7.5, 100 mM MgCl2, 100 mM DTT, 1 mM ATP, 25 µg/ml bovine serum albumin), 27.5 µl of sterile dH2O, and 1 µl (30 Weiss units) of highly concentrated T4 DNA ligase (NEB). The reaction was allowed to proceed at 12°C for 1 h. Then, an additional 30 Weiss units of T4 DNA ligase was added and the ligation mix was incubated at 12°C for 16 h. Afterward, the mixture was incubated at 65°C for 10 min and then size fractionated with a 200-bp cut using a G-50 spin column. About 5 ng of adapter ligated specific ds-cDNA (R-stage) was PCR amplified using as a primer a complementary 21-mer of the lower strand of the EcoRI/NotI adapter with the sequence CTGCAGGAATTCCGGCCGCT. The PCR reaction was carried out under the same conditions as those described in the above section for the amplification of ds-cDNA probes.

The amplified cDNA was digested with EcoRI and ligated to λZapI using the λZapI/EcoRI/CIAF cloning kit (Stratagene). The ligation steps were controlled according to Caballero et al. (24) before packaging and plating.

Primary Differential Screening

The cDNA subtractive library was plated at a density of 2000–2500 pfu/150-mm plate (20 plates). Two replica filters (laid times of 2 and 4 min, respectively) (Amersham Hybond-N+) were produced from each plate according to Sambrook et al. (21). Each set of filters was prehybridized overnight at 65°C in 25 ml of prehybridization solution containing 5× SSC, 5× Denhardt’s, 0.1% (w/v) SDS, and 100 µg/ml of denatured salmon
sperm DNA (21). The two sets of filters were separately hybridized, overnight at 65°C, with the same amount (100 ng) and specific activity (10^6 cpm/μg DNA) of labeled probes (from G2-stage to one set of filters and from R-stage to the other set) in the prehybridization solutions.

Filters were washed (twice) at 65°C for 15 min, in 500 ml of 2× SSC, 0.1% SDS, and then exposed to X-ray film for 3 days at –80°C using intensifying screen. Positive clones were picked up and resuspended in 1.0 ml of standard SM buffer (21).

Secondary and Tertiary Southern Blot Differential Screening (PCR-SBDS)

Positive clones from the primary screening were subjected to a secondary screening (PCR-SBDS) according to the following conditions: 5 μl of each positive phage suspension was PCR amplified in a 100-μl reaction mixture as described above, except that 50 pmol (each) of T3 and T7 primer was used. PCR conditions were 1 cycle, 5 min at 95°C, 2 min at 50°C, and 2 min at 72°C; 35 cycles, 1 min at 94°C, 1 min at 50°C, and 2 min at 70°C; and 1 cycle, 1 min at 94°C, 1 min at 50°C, and 15 min at 72°C.

A duplicate set of samples, consisting of 10 μl of each PCR amplification mixture, was loaded and run on a 1.4% (w/v) agarose gel. The gel was alkaline blotted overnight to a Hybond-N+ membrane. Then, the membrane was divided in two identical halves. Each half was prehybridized and hybridized with labeled probes as described for the primary screening.

Phage suspensions that presented positive bands in the SBDS were plated at a lower density (about 10–15 pfu/plate). Five phage plaques were individually isolated and each one was PCR amplified as above. Ten microliters of each PCR product was loaded and run in a 1.4% (w/v) agarose gel. Pure phage suspensions whose inserts had the same size as the positive bands from 0.65 to 1.75 kb (Table 1 and Figs. 2 and 3).

DNA Sequencing and Computer Analysis

DNA was sequenced using an automatic sequencer and the Taq DyeDeoxy Terminator Cycle sequencing kit from Applied Biosystems, according to the manufacturer’s recommendations. Sequences were analyzed using programs from the UWGCG package (version 8.1, August 1995) (25). Sequences were compared with the GenBank (release 94.0, 4/96) and EMBL Nucleic Acid data bases (release 46.0, 3/96) and the PIR (release 48.0, 3/96) and SWISSPROT data bases (release 33.0, 3/96) using FASTA, TFASTA, BESTFIT, COMPARE, and DOTPLOT.

### RESULTS AND DISCUSSION

Using the procedure described above a subtractive cDNA library with a primary titer of 0.95 × 10⁶ pfu and a 91% of recombinant phages was obtained. We have used long elongation times in the PCR reaction because it has been demonstrated that they result in cDNA sequences longer than 500 bp, thus overcoming the problem of cDNA size heterogeneity (26). We have also found it necessary to fractionate the cDNA subtracted after the ligation of the EcoRI/NotI adapters, since a fair proportion of the molecules produced was less than 200 bp in length, which may represent the extent of cDNA degradation during the hybridization step (15). This fractionation has the additional benefit of removing the unligated adapters before the amplification, as the bottom strand of the adapter inhibits the PCR reaction by competing for the primer during the amplification reaction. Thus, a later partial characterization of these clones revealed insert sizes ranging from 0.65 to 1.75 kb (Table 1 and Figs. 2 and 3).

A great advantage of ligating the EcoRI/NotI adapters after the subtraction steps is that ss-cDNAs (R-stage) that could interfere in further steps are efficiently eliminated, as only reannealed ds-cDNAs (R-stage) differentially expressed can be used for ligation and, thus, for amplification.

After a first screening of only 50,000 pfu from this library, 116 positive clones, corresponding to genes putatively expressed in the R-stage, were isolated. Forty-five of them were excised to a plasmid and partially sequenced. Figure 2 shows the result of a secondary PCR-SBDS of some of the positive clones isolated from the primary differential screening. Several bands appear in some lanes as a result of the presence of different phages in one isolated phage suspension (Fig. 2a). Figure 2b shows the result of the hybridization of these bands with the two probes (left side for R-stage probe

<table>
<thead>
<tr>
<th>Clone</th>
<th>Identity</th>
<th>Insert size (kb)</th>
<th>%</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>njjs 1</td>
<td>Prunine</td>
<td>1.55–1.65</td>
<td>74.7</td>
<td>36</td>
</tr>
<tr>
<td>njjs 4</td>
<td>LMW HSPs</td>
<td>0.80–0.85</td>
<td>68.3</td>
<td>41</td>
</tr>
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<td>0.85–0.95</td>
<td>76.0</td>
<td>42</td>
</tr>
<tr>
<td>njjs 10</td>
<td>E4 protein</td>
<td>0.95–1.05</td>
<td>71.0</td>
<td>37</td>
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<tr>
<td>njjs 15</td>
<td>MIP protein</td>
<td>1.25–1.30</td>
<td>76.5</td>
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at a different intensity. The exact meaning of this difference in band intensity has to be established since the procedure has not been essentially designed for quantitative differences. However, the possibility of a change in the expression (increasing in the R-stage) of the corresponding gene may be considered as it has already been shown for several RNA populations that are involved in the strawberry ripening process (9). This possibility could be favored because of the design of the subtraction protocol. Thus, although conventional generation of subtractive cDNAs libraries recommends more than three cycles of subtraction using a large excess (at least 10- to 20-fold excess) of driver (15, 27), we only performed two rounds of subtraction. Perhaps this fact could avoid some genes whose expression is regulated quantitatively rather than qualitatively to be efficiently removed from the population as has already been described (15). The PCR product of lane 8 did not hybridize with the probes and can be considered as a false-positive clone eventually useful as an internal control.

So far, several methods have been used to clone and
characterize the genes differentially expressed along a particular biological process. Differential screening of cDNA libraries with labeled first-strand probes has been widely used. However, this technique can only detect clones representing about and above 0.1% of the total cellular mRNA (14). To detect low-abundance mRNAs, more sensitive subtractive hybridization techniques have been designed (28), although they are not routinely used. In addition, photobiotinylated cDNA is ordinarily used for the generation of cDNA subtractive libraries (17, 29, 30). However, photobiotinylated DNA is time consuming and requires pure nucleic acid as a substrate, since impurities are also labeled (29). So, it is preferable to incorporate biotin as Bio11-dUTP directly in the driver DNA during PCR (30). Another disadvantage is the need to remove all of the primers and unincorporated Bio11-dUTP, since the excess of Bio11-dUTP competes with the driver cDNA for the binding of avidin, which may eventually lower the efficiency of subtraction. Several procedures are available for selective removal of biotin-labeled nucleic acids, all of which exploit the high-affinity binding of avidin to biotin (18). However, most methods include relatively complex manipulations such as avidin coupled to cellulose columns (31), avidin-coated polystyrene beads combined with a filtration and centrifugation step, or several steps of phenol/chloroform extraction to eliminate streptavidin/biotin/nucleic acid complexes (17). Furthermore, the use of biotin to label driver cDNA is not easy. Thus, the amount of Bio11-dUTP must be empirically optimized depending on the GC content and the average size of DNA, as labeling at too high a density with biotin may hinder hybridization due to steric problems.

Other recent methods as the differential display (19) have also been applied with success to identify low-abundance and rare mRNAs differentially expressed in various cells or under altered conditions. Improvements of this method have also been published in the last 5 years (32–35), but this technique is not exempt of some technical problems as discussed very recently by others (12, 20, 32). In fact, recovering the positive from the amplified band on the gel is not technically simple, and, furthermore, coding regions of mRNAs are usually not cloned (12, 20). These problems are well overcome in our procedure as positives usually carry cDNA inserts representing the entire mRNA coding region and, once identified, these inserts are easily excised from the phage as plasmid derivatives. Moreover, the number of putative positive bands detected with the differential display method is usually not very high, and frequently only a small fraction (about 10 to 15%) of them corresponds to true differentially expressed genes (i.e., Ref. 12). In our procedure, 116 putative positive clones were isolated after the screening of only 50,000 pfu of the R-stage subtractive library. Of them, 45 clones were excised to plasmid derived and tested for their differential expression (data not shown). Thirty-one of them were positives (data not shown), thus representing a much higher percentage than that often obtained with the differential display method. Although other criticisms such as primer or sequence dependency can be also argued with regard to the differential display method, the technique has been proved, however, to be very efficient and, in fact, very valuable since large quantities of samples and conditions can be evaluated in a few days (19, 20). Nevertheless, our approach presents the advantage of its enrichment on the differentially expressed genes in a defined biological situation (fruit ripening process). Furthermore, the procedure that we describe here is easy and sensitive in nature and can be used as a reliable alternative to generate and screen a high-yield subtracted cDNA library, thus, avoiding all unwanted problems found so far with other techniques.

Very recently, a similar procedure for generating subtracted cDNA libraries has been applied to identify genes differentially expressed during spruce root infection by the fungal pathogen Pythium dimorphum (16). Our approach has several additional improvements. The screening of a subtractive cDNA library by a conventional differential methodology requires that each set of duplicate phage filter lifts contains exactly the same amount of DNA. This is difficult to achieve and usually produces a high background and many false positives. We have addressed this problem by using a combination of conventional primary differential screening with secondary and tertiary PCR-SBDS. Thus, the amount of insert cDNA PCR-amplified for every possible positive clone in each replica filter is exactly controlled, and bands rather than spots are visualized after hybridization, which drastically reduces the background and the number of false positives. The procedure also provides early information on the insert size, which is often a great help. Furthermore, in our hands, secondary PCR-SBDS may be sufficient to identify true positives, thus avoiding unnecessary hybridizations of the tertiary screening. Finally, many clones can be simultaneously analyzed in one filter by a single labeling and probing step (Figs. 2 and 3).

Table 1 shows the percentage of identity, at the nucleotide level of some strawberry clones, to known sequences in the data bases. These values are estimated from the sequence overlapping the insert. The role of each gene in the strawberry fruit ripening process has to be separately studied since they are apparently related to different metabolic processes. Thus, prunelike protein could be relevant on the achene matura-
(31, 37–41), although their specific role in this process is still unknown. The previous reports on Ca++-dependent signal transduction mediated by cyclophilins (42–45) make clone njjs 9 particularly interesting for further research. The reported involvement of MIP and DHFR proteins in source to sink transport (46, 47) and color development (48, 49) points to these genes as possible targets for a genetically mediated breeding program. Finally, two more assignments relate the strawberry clones to hormonally regulated genes. Thus, the 14-kDa PRP protein represents an extensin-like cell wall protein whose level responds to auxin and sucrose (6, 50), and the tomato GAST protein content is responsive to ABA and GA3 external application (51). The relevance of further research of these genes is emphasized by the absence of basic knowledge about the start and development of the nonclimacteric fruit ripening process.

In summary, we have been able to isolate a vast number of strawberry fruit cDNAs differentially expressed along the fruit ripening process by means of a sensitive, easy, low-cost, and reliable method. Although other recent techniques, such as the PCR differential display, are useful to clone differentially expressed genes during a biological process, our procedure has been shown to be also very powerful, overcoming some inconveniences of the differential display method. The great variety of independent clones isolated and their putative involvement in very different pathways confirm that strawberry fruit ripening is a very complex phenomenon which involves important changes in many metabolic pathways.

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