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DEVELOPMENT OF cDNA NORMALIZATION SYSTEM AND PRELIMINARY TRANSCRIPTION ANALYSIS OF KCS GENES IN APPLE TISSUES

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Abstract

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Wax production is an important aspect of apple (*Malus domestica* Borkh.) fruit development from both theoretical and practical point of views. The complex molecular mechanism that controls wax biosynthesis is still widely unknown but many studies focused on this topic. We aimed to develop further the experimental framework of these efforts with a description of an improved reference genes expression system. Results in the literature show that similarities exist among the expression of some housekeeping genes of different plant species. Based on these considerations and on gene expression data from *Arabidopsis thaliana*, some genes in apple were assigned for analysis. EST sequences of apple were used to design specific primers for RT-PCR experiments. Isolation of intact RNA from different apple tissues and performing RT-PCR reaction were also key point in obtaining expression patterns. To monitor DNA contamination of the RNA samples, specific primers were used that amplify intron-containing sequences from the cDNA. We found that actin primers can be used for the detection of intron containing genomic DNA, and tubulin primers are good internal controls in RT-PCR experiments. We were able to make a difference between tissue-specific and tissue-independent gene-expression, furthermore we found tissue specific differences between the expression patterns of candidate genes, that are potentially involved in wax-biosynthesis. Our results show that KCS1 and KCS4 are overexpressed in the skin tissue, this could mean that these genes have skin-specific expression in apple fruit.

wax, cuticle, RT-PCR, cDNA, gene expression, apple (*Malus domestica*)

ABBREVIATIONS: cDNA: complementary DNA; DAP: days after pollination; EST: Expressed Sequence Tag; KCS: 3-Ketoacyl-CoA synthase; gDNA: genomic DNA; RT-PCR: Reverse transcription-polymerase chain reaction; VLCFA: Very long chain fatty acid

The plant cuticle covers the surface of aerial parts of plants, as the outermost layer of the epidermis. The function of this layer is complex: it takes part in controlling non-stomatal transpiration, besides it is an active member of host-pathogen interactions, and it is also involved in plant development. Two constituents form its structures, which are cutin and waxes. The components of these differ significantly between species and even families. Waxes give up to sixty percent of the mass of the cuticle. This mixture consists of aliphatic C_{20} to C_{60} lipids, triterpenoids and flavonoids (SAMUELS *et al.*, 2008).

By studying wax biosynthesis in *Arabidopsis* twenty-two mutants were identified, these are called the Eceriferum mutants (cer). They have altered epicuticular wax patterns compared to the wild type (KORNNEEF *et al.*, 1989). Homologous mutations to Eceriferum were also found in other plants, e.g. in barley, where the locus is also termed Eceriferum, in maize and *Brassica napus* where the mutants are named glossy, referring to the leaves' glossy surface. Glossiness is caused by water assembling into droplets on the surface, due to low expressed levels of cuticular waxes (KUNST and SAMUELS, 2003).

The underlying molecular basis of wax biosynthesis in *Arabidopsis thaliana* L. (Heynch.) is described and understood to some details, although it is far from full description. In particular an elongase enzyme complex was found in this plant, which controls VLCFA biosynthesis. Interestingly the KCS genes, that take part in the control of VLCFA biosynthesis, show different tissue specific expression patterns shown by both molecular and histochemical tests. The varying expression levels of KCS isoforms in different tissues suggest specific roles for these enzymes (JOUBÉS *et al*., 2008).

The examination of the Eceriferum mutants also resulted in the discovery of the importance of the operation of the KCS gene family. Since the expression of these genes is essential for the production of VLCFAs, their inhibition results in the block of wax biosynthesis (JOUBÉS *et al*., 2008).

Concerning pathogen relations, a tomato line called dfd (delayed fruit deterioration) was described as a cutin-overexpressing cultivar. It has also been shown that it displays a higher level of water retention than the control cultivar, 'AilsaCraig'. This effect is possibly due to the altered composition and greater amount of cuticular components in dfd tomatoes (SALADIÉ *et al*., 2007).

In addition, many other post-synthetic modifications are present in plants concerning cuticular wax biosynthesis. Yephremov and Schreiber made a list of the candidate genes in wax biosynthesis, which suggests that the synthesized molecules by the elongase complexes are commonly modified, and therefore their proper constitution is crucial for the possibilities of later modifications (YEPHERMOV and SCREIBER, 2005).

In another practical perspective, in the case of apple fruits, waxes play important role in the customers' attitudes, a fresh, shiny apple can attract more attention.

Many different aspects support the examination of the genes in apple involved in wax-biosynthesis. The results of gene expression analysis could describe the genes involved in wax-biosynthesis, and thus could provide new tools for breeding new apple varieties.

In the work presented we tested and developed a method for RNA isolation from different apple tissues. We showed that some housekeeping genes could be used as good internal controls for gene expression. We also found alterations in the expression levels of some candidate genes, possibly involved in wax biosynthesis in apple fruit.

MATERIALS AND METHODS

Plant material In the experiments apple (*Malus domestica* "Gegesi Zöld") fruits were used. The plants were grown in the Experimental and Research Farm of the Corvinus University of Budapest, Soroksár. We harvested fruits at 90 DAP. Right after harvesting buds were packed into Eppendorf tubes, and dropped into liquid nitrogen, following storage at –80 °C. Pulp and the peel of fruits were separately sliced into Eppendorf-tubes, frozen in liquid nitrogen, and stored on –80 °C.

RNA extraction. We used the ethanol extraction protocol of Asif and co-workers (ASIF *et al*., 2006).

DNA extraction. We used the Qiagen DNeasy Kit for isolation of apple total DNA.

RT-PCR. For the reverse transcription we used the Fermentas First strand cDNA Synthesis Kit. We used oligo$(dT)_{18}$ as a reaction primer. In the PCR, we used the following protocol: denaturation on 95 °C for two and a half minutes. We used 32 cycles with the following cycling: 95 °C for 30 seconds, 55 °C for the annealing, for 30 seconds, 72 °C for one minute. After the last cycle we set 72 °C for 7 minutes, then we cooled the reaction down to 4 °C. The primers, which were used in the reactions are listed in Tab. I.

These primers were designed according to the apple EST sequences indicated, which are homologs to related *Arabidopsis thaliana* sequences. The accession numbers of these sequences are also included in Tab. I.

cDNA normalization For a proper comparison of the expression patterns of the housekeeping genes

I: *List of primers used in the PCR reactions*

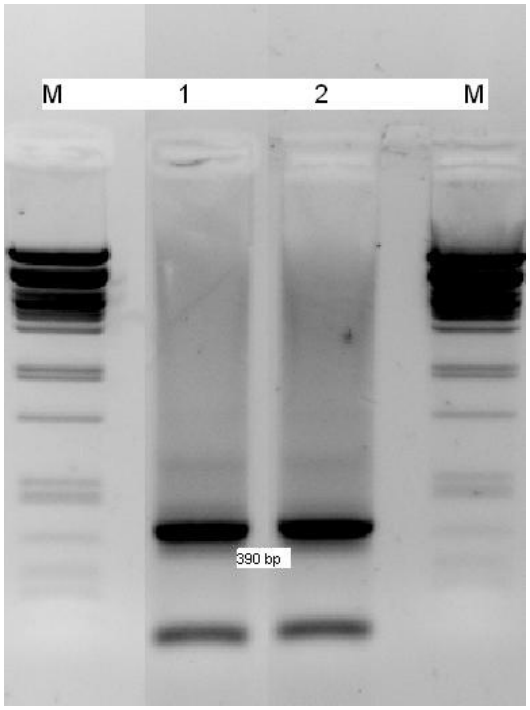
Gene name	Gene description	Sequence origin	Primer sequence	Amplicon length
ACT	Actin	GASIC *et al*., 2004	5'CTACAAAGTCATCGTCCAGACAT	≈1 000bp (cDNA)
			5'TGGGATGACATGGAGAAGATT	≈1 600 bp (gDNA)
TUA5	Tubulin 5	GO555180	5'GTGCGAAAACACGTAAAAACCA	390 bp
			5'CCATCAAGACCAAGAGGACAAT	
KCS1	3-ketoacyl-coA-synthase 1	GO562769	5'CGTCACTGTCCTCCATGATCGT	376 bp
			5'AGCGGGAAGACGACAAAGGCAC	
KCS2	3-ketoacyl-coA-synthase 2	DT001441	5'TTGTTCAATCCAACCCCATCTC	356 bp
			5'AGGGTGCTGATGATAAGTGCTT	
KCS4	3-ketoacyl-coA-synthase 4	EB127839	5'CGCTGAAATCGAACATCACGAC	348 bp
			5'AGGGTTTGGCAGATTGCGTTCG	
KCS4	3-ketoacyl-coA-synthase 4	CN880605	5'ACCCAGGCGGAGCAACCAATGT	364 bp
			5'ATTACGCCTGCTACCGCCCGCC	

and the genes involved in wax biosynthesis, the normalization of the cDNA levels and thereby the RNA levels is indispensable. The RNA levels were measured using a Shimadzu spectrophotometer, the calculation of the RNA concentration was achieved with the 1 O.D. at 260 nm = 40 mg/ml RNA formula. Next we calculated the concentrations of the RNA samples of the skin and flesh samples and we balanced their concentrations to each other (to 464 ng/µl). The RT-PCR reactions were done on the equalized RNA samples.

Agarose gel electrophoresis. The gel electrophoresis of the RT-PCR products were done using ethidium-bromide containing 1.2% agarose gel (Sigma).

RESULTS

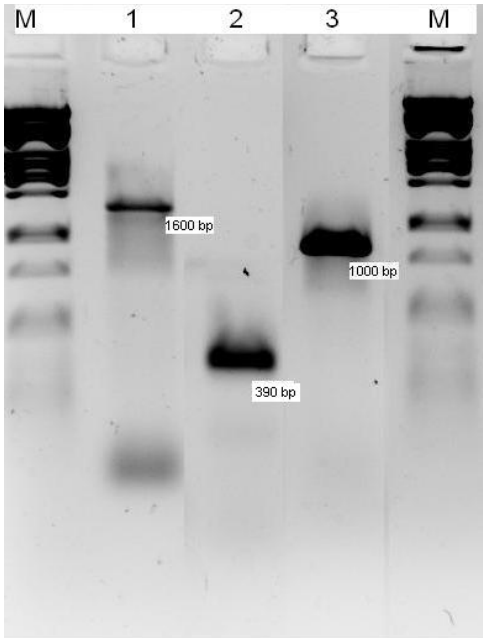
cDNA normalization. To test the accuracy of our normalisation, we run RT-PCR reactions on apple skin and flesh cDNAs, with the tubulin primers above. The products for skin (Lane 1) and pulp (Lane 2) are shown on Fig. 1.



1: *Agarose gel electrophoresis for the products of RT-PCR on fruit skin (Lane 1) and pulp cDNAs (Lane 2), using tubulin primers. (M = PstI digest of lambda phage DNA)*

The quantities of the products from these PCR reactions were equal, and they also had the same size.

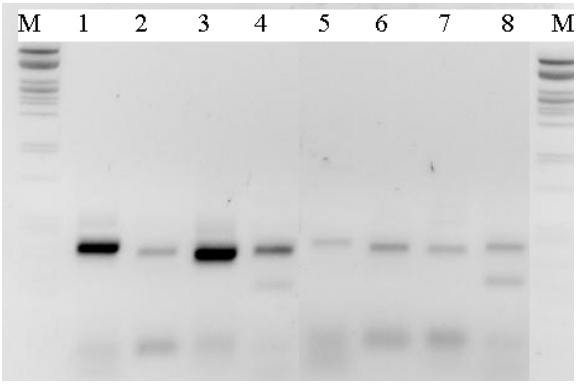
Actin primers can be used as gDNA controls Besides using internal controls for cDNA equalization we also needed a control primer that could be used for checking gDNA contamination in the cDNA sample. Here we found that an actin primer pair described in the literature could amplify a smaller product from cDNA and also a larger



2: *Agarose gel electrophoresis of PCR reactions on gDNA and apple peel cDNA, using actin and tubulin primers. The PCR products with actin primers from gDNA and cDNA differ greatly in their size (lane 1 and 3, respectively). The product of the tubulin specific RT-PCR reaction was run in lane 2.*

one from genomic DNA. This we used to monitor whether our cDNA samples contained genomic DNA contaminations. Fig. 2 shows agarose gel electrophoresis of the PCR products from apple gDNA with actin primers (lane 1) with the product size of ≈1 600 bp, cDNA with tubulin primer (lane 2, product size 390 bp), and cDNA with actin primers (lane 3), product size ≈1 000 bp.

RT-PCR products amplified with the primers specific to genes involved in wax-biosynthesis differ among tissues of fruit RNA was extracted from flesh and peel tissues of fruits, cDNAs were synthesized and the products amplified in RT-PCR reactions and run on an agarose gel (Fig. 3).



3: *Agarose gel electrophoresis of RT-PCR products from apple genes probably involved in wax-biosynthesis*

Products originating from fruit skin tissues are from lanes 1–4, those from flesh are in lanes 5–8. The primers used in this reaction are the following (in the same order, as in the gel): KCS1, KCS2, KCS4, KCS4/2. The two types of tissues show the same patterns, although they show large differences in intensity of the bands. KCS1 and KCS4, (lanes 1 and 3) show higher intensity than in the skin samples, while the same result cannot be observed in pulp. For the pulp it seems that KCS1, KCS2, KCS4 and KCS4/2 (lanes 5, 6, 7 and 8) show minimal expression. KCS4/2 amplifies two small-sized sequences, suggesting that these primers are not specific enough.

DISCUSSION

Actin primers as gDNA controls. Although actin genes are not advised to use as internal control in gene expression experiments, in this case we found that our actin primers was useful as controls of gDNA contamination. This result suggests that the sequence amplified by our primer contains an intron, which can be amplified from genomic DNA but cannot be found in cDNA.

Tubulin might be a good internal control. Our results show that after cDNA normalization the products amplified by tubulin primers had the same intensity. This suggests that this gene can be used in further tests, and it seems to be a good candidate for internal control in gene expression studies.

Wax-biosynthesis genes show tissue-specific expression. In the RT-PCR reaction the KCS genes tested showed varying expression levels in fruit skin and pulp tissues. This result is not surprising considering that these tissues have very different wax levels. The different expression patterns of these genes may mean that some of the genes may be involved in wax-biosynthesis in the epidermal tissue of apple fruit peel.

SUMMARY

We aimed at analyzing the expression levels of genes putatively involved in wax biosynthesis in different apple fruit tissues. We based our work on the use of proper internal controls. We describe a primer pair for an actin gene as an appropriate marker to monitor gDNA contamination of cDNA samples. We also managed to establish an internal control for cDNA equalization. A housekeeping tubulin gene was found as a good internal control, because it had stable expression levels in the tissues examined. The KCS genes putatively involved in wax biosynthesis showed variable expression levels in the fruit tissues. Furthermore they displayed different abundance relative to each other, which indicates that in the wax producing fruit epidermis the contribution of different KCS isoforms to this biochemical process may be widely different.

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