PhD Thesis

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MOLECULAR BIOLOGICAL CHARACTERIZATION OF EXOCARPS OF APPLE AND BELL PEPPER IN DIFFERENT DEVELOPMENTAL STAGES AND DURING STORAGE

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The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

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Introduction

The plant cuticle is an essential barrier from physiological, biochemical and plant protection points of view, its role is crucial in almost every species. One of the most important aspect of the plant properties provided by the cuticular layer is its regulative role in the water balance of the plant .

Literature review

As the outermost surface structure on the plant, the cuticle takes part in every process on the plant surface: regulation of water loss, transport of lipophilic substances, development of a protective layer against water, protection of the photosynthetic pigment system through attenuation of incoming radiation, boundary layer during morphogenesis, place of biotic interactions (Jenks and Ashworth, 1999, Riederer and Müller, 2006).

The fruit of bell pepper is usually chosen for the examination of its effect on water balance, since the exodermis of this fruit does not contain any stomata, and the only regulative structure on its surface is the cuticle. Differences in water loss through the cuticle have been described in different ripening stages (Díaz-Pérez *et al*., 2007). Major differences occur also between cultivars, that can be favourably modified through cold storage and packaging (Zsom, 2007). Alterations in the molecular components of the cuticle also significantly affect water loss, increases in

cuticular triterpenoids and sterols result in accelerated transpiration (Parsons et al., 2012).

The cuticle consists of three main parts, these are the matrix that is able to depolymerization, cutin, the polymer matrix that is unable to depolymerization, named cutan, and cuticular waxes, which can be divided into intra- and extracuticular waxes according to its disposition in the cuticle (Pollard *et al*., 2008). The examination of it can be done by microscopic techniques, and the quality and quantity of the components can be examined after their extraction from the surface with a solvent.

An overlap is present in the genes participating in the biosynthesis and modification of cutin and waxes, since both are derived from the fatty acid biosynthesis pathway (Pollard *et al*., 2008). Genes involved in the biosynthesis of cutin are divided into three larger subfamilies, these are the fatty acid oxidases in CYP86A gene family, the acyl-activator enzymes in LACS gene family and the acyltransferases in GPAT gene family. The *lacerata* mutation of the LCR gene resulted in organ fusions due to transposon insertion which points out the role of LCR in ω-hydroxilation of fatty acids and the importance of the biosynthesis of cutin during development. The gene encoding LACS2 has also been successfully identified in the above gene families, which encodes acyl-coenzyme-A-synthetase enzyme (Pollard *et al*., 2008). *Hothead* and *fiddlehead* mutants are also described in the context of prevention of postgenital organ fusions (Krolikowski *et al*., 2003, Pruitt *et al*., 2000). Mostly only assumptions are presented in the

case of intra- and extracellular transport mechanisms of the products synthetized, and the key enzymes involved in cuticular formation. The WBC11 gene with ABC transporter function takes part in cuticular wax secretion and participates in cutin layer development in presumed cooperation with the CER5 gene (Bird *et al.*, 2007, Bird, 2008).

Classification of enzymes involved in cuticular wax biosynthesis can be done from several points of view. According to function elongases, tioesterases, reductases and oxidases, decarbonylases and transacylases are known (Jenks and Ashworth, 1999). Biosynthesis of wax components is derived from the plastidial fatty acid biosynthesis, that elongates acetate by two carbons in several consecutive cycles. The synthetized fatty acid chain will be further elongated by the FAE (fatty acid elongase) enzyme complex residing in the endoplasmatic reticulum resulting in VLCFAs (very long chain fatty acid). Further modifications of VLCFAs can be done through two basic pathways: primary alcohols and wax esters are produced in the acyl-reduction pathway, while aldehydes, alkanes, secondary alcohols and ketons are produced in the decarbonylation pathway (Kunst and Samuels, 2003).

Objectives

Apple is one of the most substantial cultures in horticultural production in Hungary, hence we focused on it in our research. The fruits are usually sold after long storage periods, the shelf life is affected by postharvest water loss through the cuticle, that is

considered to be greatly affected by the composition and sturcture of the cuticle. We planned to determine molecular and plant physiological paramaters in our research. We wanted to predict the genes potentially involved in the development of cuticular structures *in silico*, and to examine the expression of these genes. These results could be used later in apple breeding programs. For this instance the set-up of a reliable control system as reference was necessary, that could be used for exact quantification in our gene expression studies. Additionally the examination of postharvest water loss was also planned, and moreover to determine microscopic, and putative ultrastructural alterations in the exocarp and the cuticle with confocal laser scanning microscopy.

Bell pepper can be also considered as one of the most prioritized vegetable cultures in Hungary, the plants are also sold as dry powder, hence water loss has direct economical importance. The examination of cuticular properties of the fruits is important since no stomata covers the fruits and the only barrier against water loss is the cuticle itself. We aimed to determine the postharvest water loss of the fruits in different cultivars and developmental stages, so the alterations of the culticle during development could also be identified. Proving the role of the cuticle can be done indirectly by the extraction of it, therefore our results would be compared against a group with extracted cuticle layer. We planed to determine the mass of the superficial wax layer both in bell pepper and in apple

fruits and to look for putative ultrastructural alterations in the cuticle of different apple cultivars with confocal laser scanning microscopy.

Materials and methods

Capsicum annuum L. ‘Titán’, ‘Hó’ and ‘Kárpia’ cultivars were the plant materials for our studies related to peppers, in the case of apples, the *Malus x domestica* Borkh. ’Gegesi zöld’, ‘Prima’ and ‘Florina’ cultivars were chosen. Studies with ‘Hó’ and ‘Titán’ cultivars were done with three different developmental stages: half-sized, immature, mature (in the sense of commercial definitions). The studies for water loss were carried out in two repeats: first time 26 ‘Titán’ and 24 ‘Hó’ fruits, in the second 45 fruits of each were divided according to treatment level and developmental stage. ‘Hó’ and ‘Kárpia’ peppers were also examined in two repeats, but only in mature stage.

To achieve the optimalization of experimental parameters for our studies with apples, ’Gegesi zöld’ cultivar was chosen. Total RNA was extracted from the leaves, fruit peel and pulp of the ‘Florina’ and ‘Prima’ cultivars in different developmental stages.

Water loss of peppers was determined through measuring their weight and surface area, in 100% ripe stage, in two repeats. The weights were measured for 10-10 days in the case of compairing ‘Titán’ -’Hó’, and for 11-13 days in the ‘Kárpia’ -’Hó’ case. The weight loss was assumed to be caused by peristomal water loss. Weights of the apples were similarly determined, stored under

laboratory circumstances, considering their surface area as a sphere. To characterize the daily water loss, Díaz-Pérez and co-workers introduced an indicator, named WLR (water loss rate), caluclated with $WLR = \frac{\Delta FW}{FW_0}$, and was supplemented by the total WLR, showing the total water loss during the whole study, calculated by $TotalWLR = \sum \overline{WLR_1, WLR_2 \dots WLR_n}$, where n is the last weighing day, and the water loss can be expressed as a percentage in both cases (Díaz-Pérez *et al.*, 2007).

To analyze the quantity of the components of the cuticle, the cuticles were dissolved by organic solvent in both apple and bell pepper studies.

One-way and two-way ANOVA were used to evaluate the cuticular thickness and the water loss parameters in pepper studies, linear regression was used for water loss studies with apples.

Viogene Plant Total RNA Mini Kit was used for RNA extraction. In order to extract higher quantities of electrophoretically detectable nucleic acid from apple tissues the ethanol extraction method published by Asif and co-workers in 2006 was used (Asif *et al.*, 2006). The genomic DNA contamination of the extracted total RNA was checked in a PCR with specific intron spanning primers.

cDNAs were created after DNase treatment, these were the templated for the latter RT-PCR and qPCR studies.

To design the primers for our apple specific PCRs, *Arabidopsis thaliana* L.(Heynch.) sequences related to cuticular wax biosynthesis appeared in publications from Gutierrez *et al.*, 2008,

Gasic *et al.*, 2004, Joubés *et al.*, 2008, Yephremov and Schreiber, 2005, and the Samuels *et al.*, 2008 were compared to the apple genome. Actin, EF1, EF2, EIF4-A, tubulin and ubiquitin genes were used as internal references, several KCS homologues for the description of the VLCFA biosynthesis. To describe the latter modifications and transport the following gene homologues were chosen: CER1, CER2, CER4, CER5, CER7, CER10, FDH, HTH, KCR1, LACS2, LCR, LTPG1, PAS2, WAX2, WBC11 and WIN1.

qPCR was done on balanced apple cDNA template in the volume of 0,75 µl in a total volume of 12,5 µl, with 0,4 µM specific primer, and in the presence of 2x ImmoMix and 20x EvaGreen with RotorGene 6000. To evaluate the results obtained in the qPCR, Rotor-Gene 6000 Series Sofware 1.7 was used, that can be used to draw the PCR-curves real-time.

Products obtained in RT-PCR were sequenced by BIOMI Kft., the results were then corrected using Chromas Lite Program and then compared against the original sequences.

For light microscopy sections were produced on -25 °C using a Leity Weitzlar cryostat. 1-2 cm^2 sections were cut, then fixed in Shandon Cryomatrix on the cryostat. 10 µm sections of the samples were cut then washed and stained by Sudan IV.

The same method was used for the fluorescent microscopy as described for the microscopic analysis, the fixed samples were stained with one of two different fluorescent dyes (Auramine O,

Calcofluor White) or both, as suggested by Buda and co-workers (Buda *et al.*, 2009).

Results and discussion

According to the results obtained for the control genes, tubulin and ubiquitin genes produced reliable and semiquantitatively equal expression in apple tissues in our RT-PCR studies. Excellent use can be declared for the screening of genomic DNA contamination in the case of the primer pair designed for the homologue of elongation factor 1 α.

The expression pattern of appple KCS-homologues specific to apple peel and less intense signal in leaf appeared in RT-PCR is similar to the results with thale cress studies published by Joubés and co-workers (2008). The differences in tissue specificity of the homologous KCS isoforms suggests the distinct function of them, that can be confirmed in later functional analyses.

CER1 is mentioned as a key enzyme in aldehyde-alkane conversion in thale cress, its function also has already been examined in wheat and rye. Results of the analysis of the fruit waxes of the cultivar 'Florina' has previously been published (Verardo *et al.*, 2003), and accumulation of C29 alkanes was found, that correlates with the exocarpial expression pattern of the putative CER1 gene.

Stem specific expression of the CER2 gene is shown in *Arabidopsis thaliana* (Joubés *et al*., 2011), the product of the gene

participates in the elongation of the fatty acids (Jenks *et al.*, 1995). The mainly peel- and pulp-specific expression pattern with no specificity for the cultivar of the CER2 homologue correlates with the mainly generative expression profile of CER2 gene on NCBI Unigene homepage.

CER5 participates in wax component transport in thale cress together with WBC11 (Bird, 2008). Interestingly CER 5 and WBC11 homologoues show equal expression sign according to tissue specificity and strength in the two years, that is explained by the heterodimer production shown by David Bird. The current model of the wax production shows the heterodimer production of the two gene, that can act in the cuticular wax transport to the cell wall.

The expression of the putative apple HTH gene appeared only in leaf tissue, which is slightly consistent with the expression pattern in thale cress, where stem and inflorescence showed the strongest signals, less intense signals were found in leaf, and even less intense sign in root and silique (Krolikowski *et al.*, 2003).

The most intense expression of the KCR1 homologue in apple in both years appeared in fruit tissues, while minor expression was detectable in the leaf. Therole of KCR1 in the FAE-complex was shown to be essential in *Arabidopsis thaliana*, its absence was lethal to the embryo (Beaudoin *et al.*, 2009). The expression patterns obtained in our studies can suggest that similarly to thale cress, the gene can have a similar essential function in apple, that is also

consistent with the results of a recent study on cherry (Alkio *et al.*, 2012).

The apple LCR homologue was found to have peel specificity in ’Prima’ in both years studied, in the case of the cultivar ‘Florina’, expression signal in the leaf also appeared. The gene has important role in cutin biosynthesis through the ω-hydroxylation of fatty acids (Wellesen *et al*., 2011), our results therefore imply active biosynthesis of cutin in ripe apple fruit skin.

The product of the LTPG1 gene was identified as a protein assisting the transport of wax components through the cell wall (DeBono *et al.*, 2009). The expression of an LTPG homologue was found to be intense in leaf and peel tissues, and weak or undetectable in the pulp, which can be related to the role of LTPG1 in the transfer of wax components.

Only minor differences were found between the postharvest water loss of cultivars ‘Florina’ and ‘Prima’ (winter and summer cultivars) during storage in room temperature, 60% relative humidity. Different storability of these cultivars is not supposed to be originated from the permeability of the cuticle but to be influenced by the intensity and the speed of the climacterium.

Interestingly the thicknesses of the cuticles were not proportional with the extractable superficial lipids. Differences in the two years wax coverage per surface area are explained by the year effect. Different environmental stresses can greatly affect the

cuticle, and the production of cuticular waxes (Shepherd and Griffiths, 2006).

Significant differences were found in the microscopic analysis of the cuticle, more intense Auramin O signal was detectable in the outermost layers of the cultivar ‘Florina’ with thinner cuticle, and the inner parts of cultivar ‘Prima’ with thicker cuticle. The ultrastructure of the cuticle was also presented by Buda and co-workers, the parts with more intense stain was shown as the intracuticular, the less intense parts were called the extracuticular region, which was explained by the altered loads of the different wax components (Buda *et al*., 2009). The two cultivars examined have different thickness and ultrastructure in their cuticle.

Relevant differences were found in the fruits of ‘Titán’ and ‘Hó’ bell pepper cultivars. ‘Titán’ peppers showed higher water loss rate than ‘Hó’ fruits according to WLR and total WLR values during storage. Additionally the solvent treated peppers lost 80-90% of their total weights during storage depending on developmental stage, while control peppers lost only 20-30%. Although the WLR values did not differ among the control peppers of the two cultivars, we found differences among developmental stages in the solvent treated groups: half-sized and immature peppers were distinguishable from mature peppers, that had higher water loss rates than immature ones.

Water loss analysis and comparison were carried our in the case of cultivars ‘Hó’ and ‘Kárpia’ based on their shelf life parameters (Zsom, 2007), and surprisingly although ‘Kárpia’ fruits had longer shelf life, they also lost more water. The water loss analyses of bell peppers were supplemented by our microscopic results, and the results obtained can be related to the differences in tissue structure.

Hypodermal collenchyma of 2-3 cell lines was detectable in the case of mature ‘Titán’ fruits and the covering cutin layer was thicker on ‘Titán’ fruits than on ‘Hó’ fruits. Although the cuticle was thicker in the case of ‘Titán’, the mass of cuticular waxes per surface area unit was higher in ‘Hó’ fruits. This can be a result of ultrastructural differences of the cuticle, with cuticular thickness mainly determined by the structure of the cutin layer. Our results confirm that no direct connection is present between the thickness of the cuticle and the water retention properties of the plant, as published also by other authors (Smith *et al.*, 2006). This is consistent with the qualitative alterations found in cuticular waxes of ‘Hó’ and ‘Titán’ friuts during ripening (unpublished results).

Dissolving surface waxes resulted in more wax from the fruits of cultivar ‘Hó’. The mass of surface waxes on fruits of ‘Kárpia’ was so little, that the results obtained from studies with the‘Titán’ peppers susceptible for high water loss rated showed even higher amounts. Summarizing these results confirms that the amount of cuticular waxes itself does not affect postharvest water loss rates.

Besides the cuticle, thickness of the periclinal cell wall, the number of hypodermal collenchyma, and the joint thickness of collenchyma and the exocarp were also found to be higher in the case of ‘Kárpia’ peppers. We assume that the better physical properties due to these tissue-specific traits, and not the low water permeability of the cuticle are the reasons of the better shelf life of ‘Kárpia’peppers.

New scientific results

- Reliable reference genes were found in the case of apple fruit tissues to facilitate gene expression studies with RT-PCR és RT-qPCR.
- To detect genomic DNA contaminationa PCR primer pair for an intron-containing sequence was introduced in apple.
- Genes putatively involved in cuticle biosynthesis with peel-specific expression were shown in apple
- Differences between the thickness and structure of the cuticles of ‘Prima’ and ‘Florina’ fruits were shown with microscopic techniques
- We characterized the water loss of ‘Hó’ and ‘Titán’ peppers in three developmental stages, and we found differences both among cultivars and developmental stages
- We described distinct wax loads and structures of the exocarp of the cultivars ‘Hó’ and ‘Kárpia’. Despite the better shelf life of ‘Kárpia’ peppers, higher water loss rates

were shown, so the putative directal relationship between shelf life and water loss was rejected.

Publications connected to the dissertation

Journal articles:

Albert Zs., Ivanics B., Molnár A., Miskó A., Tóth M., Papp I. (2013) Candidate genes of cuticle formation show characteristic expression in the fruit skin of apple. Plant Growth Regulation, 70:71–78

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