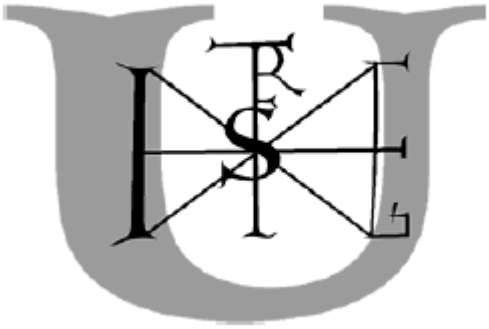
Szent István Egyetem
Gödöllő



Isolation of genes responsible for fruit development and ripening in cultivated strawberry

PhD thesis

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1. Background, aims

At the time when we started our research, out of the key genes involved in ethylene biosynthesis and signalling pathway, only the partial sequen ce of the gene encoding the ETR was known, this is why our primary aim was the isolation of genes and promoters of ACC-synthase, ACC-oxidase and CTR1.

Considering that in strawberry very few gene sequences are available, we used quantitative cDNA-AFLP which, next to the detection of gene exprassion pattern, also allows the detection of rare mRNA and the identification of novel genes involved in fruit development and ripening. Our aim was also to isolate and characterize full-length cDNAs which based on their putative function can be involved in the regulation of ripening, or they have already been associated with this process in other plant species. These are a Ring finger, bHLH and Hdzip genes, key genes taking part in signalling pathways (kinase), and a nitrilase-like protein involved in auxin biosynthesis.

2. Material and methods

The partial sequences of the genes encoding the ACC-synthase, ACC-oxidase and CTR1 were amplified by RT-PCR using degenerate primers. The full-length cDNAs were amplified based on the partial sequences with 5' and 3' RACE, which made possible the further amplification of the genomic clones of *ACS* and *ACO*, and the isolation of the promoters of *ACS* and *CTR1*. The genomic clones were amplified by PCR using primers designed for the UTR sequences of the genes, the promoters were isolated by TAIL-PCR. The structure of the promoters was analysed by means of bioinformatic tools.

In order to detect differential gene expression during ripening and to isolate novel genes involved in ripening we used cDNA-AFLP. The improved version of cDNA-AFLP used was developed in order to allow global, quantitative gene expression analysis. cDNA-AFLP, allows the detection of rare mRNAs, this way making it possible to identify novel genes involved in the process of fruit development and ripening. For the experiment we used strawberry fruit in green, white, pink and red stages of ripening (Fragaria x ananassa Duch. cv. 'Elsanta').

3. Results

This thesis aims the identification of novel genes involved in cultivated strawberry ripening and the better understanding of the ripening process. It embraces two main subjects worked out with different techniques.

The isolation of genes involved in ethylene biosynthesis and signalling pathway.

The 1902 bp full-length *FaACS* (AY661301) consists of 138 bp 5' and 288 bp 3' UTR surrounding the 1476 bp ORF. Based on similarities with sequences in the database, the strawberry *ACS* gene shows the highest homology both at nucleotide and protein level with an apple *MdACS-5* (AB034993) gene. The genomic clone is 2582 bp long, and similarly to other ACC synthase genes, it is interrupted by 3 introns. FaACS shares all the conservative amino acids and active site characteristic for ACC synthases, but its enzymatic activity remains to be proven. The expression pattern of FaACS was characterized by RT-PCR, in fruit it was present only in the green stage, and it is not accumulating during ripening, but it was shown to be present in ripe fruit infected by *Botrytis cinerea*. The promoter region of *FaACS* isolated by TAIL-PCR is 889 bp long. The presence of regulatory elements involved in biotic and abiotic stress- and auxin response was shown by means of bioinformatic tools.

The full-length *FaACO* (AY706156) is 1235 bp, with a 963 bp ORF, a 71 bp 5' UTR and 201 bp 3' UTR. On nucleotide level it shows the highest homology with an apple ACO gene (AB086888), on protein level with a peach ACO (*Prunus persica,* CAA54449). The genomic clone contains two introns apart from the majority of ACO genomic clones found in the databases, which contain 3 introns. The enzyme shows all the conservative amino acids required for ACO activity. The *FaACO* is ripening induced, but the transcript is present in all the tissue-types tested.

In the case of *FaCTR1* (AY538771) we could obtain the complete 2535 bp ORF and the 541 bp 3' UTR by RACE. The nucleotide sequence shows homology to CTR1 from apple (AY670703), on protein level the greatest homology is with a CTR1 from *Rosa hybrida* (AAK40361). Within its protein kinase domain FaCTR1 has a protein kinase ATP-binding signature (IGAGSFGTVH) as well as a serine/threonine protein kinase active site signature (IVHWDLKSPNLLV). The *FaCTR1* expression is constitutive during ripening, and in other tissues tested. The amplified upstream region of CTR1 is 1590 bp. Several enhancer elements, regulatory motives responsible for biotic and abiotic stress, circadian control were identified.

The isolation of novel genes involved in strawberry fruit ripening and the identification of different gene expression patterns during ripening with cDNA-AFLP

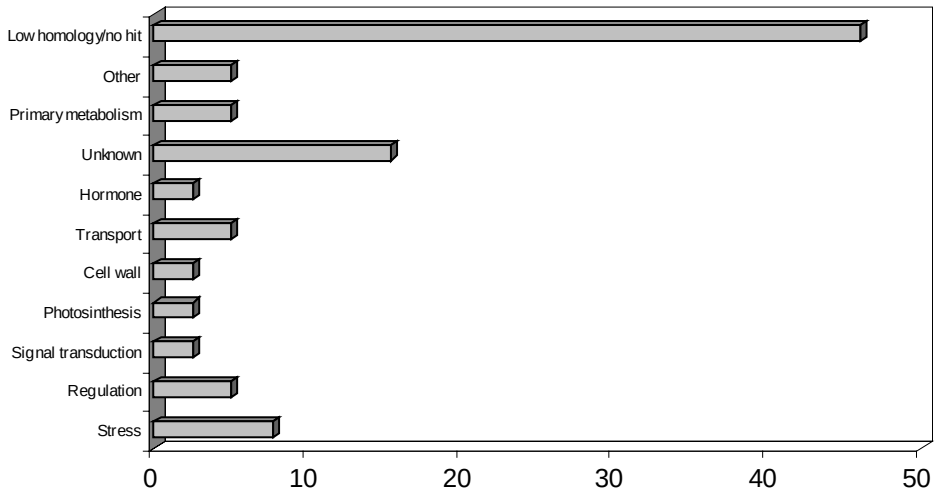
A total of 1403 cDNA-AFLP fragments were screened. After normalization of the AFLP-QuantarPro expression data and selection of differentially expressed genes based on the coefficient of variation (CV) > 1 criterion, 290 TDFs were found differentially regulated. After the hierarchical average linkage clustering of the 290 differentially expressed genes we were able to distinguish three major groups, the largest group is comprised of 120 achene specific TDFs, the rest of the transcripts belong to the green receptacle specific TDFs (86) and to the group of ripening induced transcripts (84 TDFs). 130 TDFs were isolated and sequenced based on their expression pattern and on suitability of the bands for isolation (size, sharpness). Each TDF was assigned to one of the functional categories on the basis of its BLAST search output. Most TDFs (34% in the ripe receptacle, 47% in the green receptacle and 50% in the achene tissue) did not show any homology to sequences with known functions (Fig. 1. A., B., C.), or their hits were below an E < e-0.002. All sequences obtained with hits of E < e-0.002 were submitted to NCBI GenBank. Among the genes identified only five showed homology with strawberry genes already submitted to the database, and other seven were similar to sequences isolated from species which belong to *Rosaceae* family, fact that shows that the public databases contain few information on strawberry genes.

As the TDFs are rather short (50-500 bp) and represent the 3' end of the cDNA we performed 5' RACE for 2 genes, and 5' and 3' RACE for 7 genes in order to get more precise sequence information (Table 1.). These fragments were selected based on their expression pattern (Fig. 2.), and also based on the function of their homologues with a potential role in fruit growth, development and ripening.

The 7 full-length cDNAs encode the following proteins (the strawberry gene names and GenBank accession number in brackets): a RING finger (*FaRingH2*, AY679613), a nitrilase like protein (*FaNit*, AY695666), a bHLH, homolog of SPATULA from *Arabidopsis* (*FaSpa*, AY679615), a Hdzip protein (*FaHd*, AY679614), two receptor-like kinase proteins (*FaRLK1*, AY940166, *FaRLK2*, AY679612), and a plasma membrane intrinsic protein (*FaPIP*, DQ022749).

It is possible that the functional analysis of the genes encoding the nitrilase-associated protein (which can be involved in the auxin biosynthesis), and the aquaporin (which is responsible for the growth of cells and fruit growth), and the functional analysis of the other genes can elucidate the non-hormonal regulation mechanism behind the process of the non-climacteric ripening.

A.



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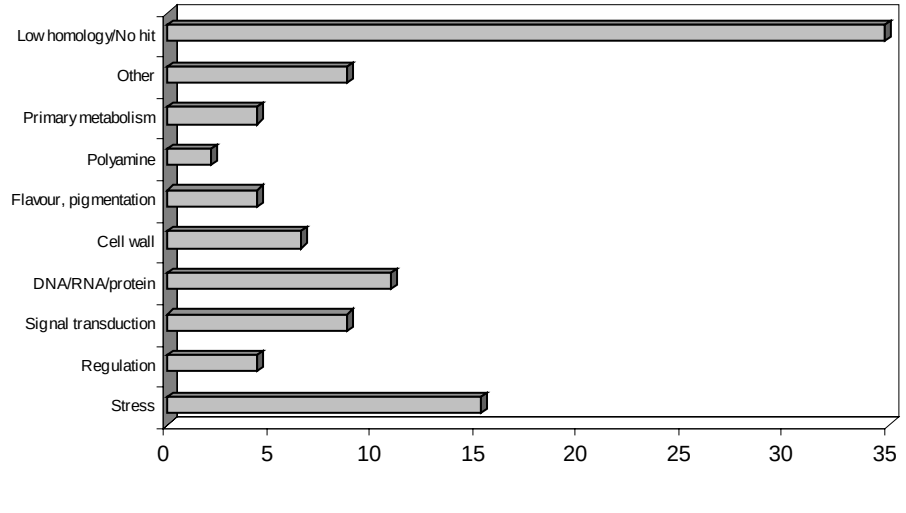

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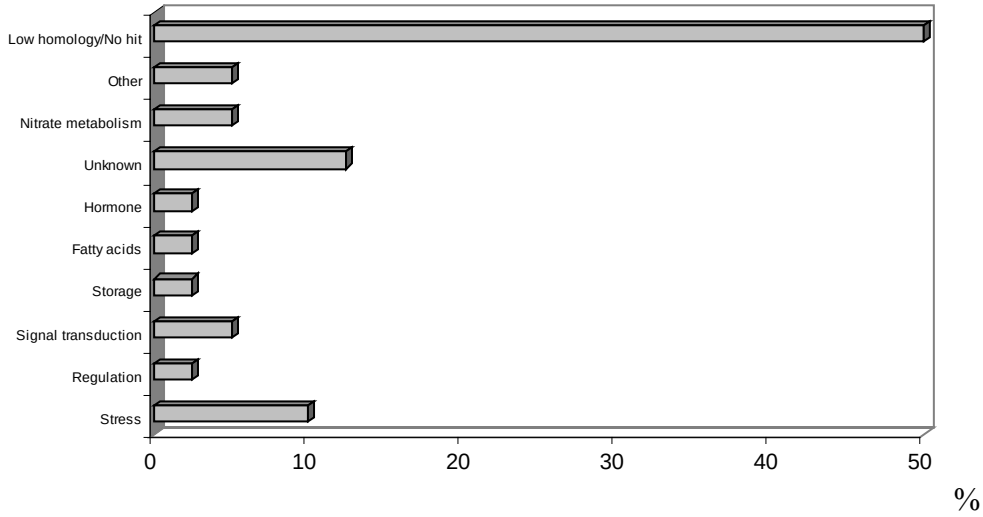

Fig. 1. Functional classification of TDFs differentially accumulated in strawberry fruit.

A: red receptacle, B: green receptacle, C: achene

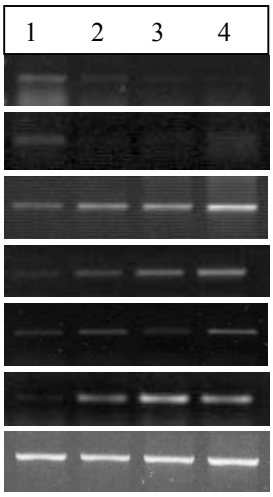

Fig. 2. Expression pattern of the ripening induced genes by semi-quantitative RT-PCR. Samples: fruits in 1: green, 2: white, 3: pink, 4: red ripening stage.

1. Table. The length of the initial and full length cDNA, their homologues and the E values before and after the RACE

cDNA-AFLP fragment	Length (bp) before and after RACE	Most similar homologue	E value before and after RACE (Blastx)
C11M32M003	400 → 737 bp	Putative nitrilase-associated protein	7e-14 → 2e-17 41/64 (64%) → 49/89 (55%)
C14M13M002	271 → 749 bp	RING finger protein	3e-26 → 2e-45 51/92 (55%) → 94/154 (61%)
C14M21M006	181 → 2400 bp	Receptor-like protein kinase	1e-12 → 0.0 33/49 (67%) → 379/698 (54%)
C24M33M004	240 → 1404 bp	bHLH protein SPATULA	0.005 → 3e-46 21/59 (35%) → 131/229 (57%)
C24M33M010	137 → 2077 bp	Receptor-like protein kinase	1e-05 → 0.0 26/41 (63%) → 395/593 (66%)
*C24M43M007	120 → 1277 bp	Plasma membrane intrinsic protein (aquaporin)	No hit → 8e-148 → 261/286 (91%)
C24M44M003	193 → 1188 bp	Putative HD-zip protein	0.011 → 5e-111 21/32 (65%) → 234/318 (73%)

*The only clone, where the full-length cDNA had different homology from the initial cDNA-AFLP fragment

3.1. New scientific results

1. The isolation of key genes involved in ethylene biosynthesis and signalling pathway in strawberry

We identified one member of ACC-synthase gene familiy, the *FaACS*. We described the structure and expression pattern of this gene. We isolated the full-lenght cDNA and the genomic clone. The promoter region of *FaACS* isolated by TAIL-PCR is 889 bp long and it was functionally analysed by means of bioinformatic tools.

We identified the member of ACC-oxidase, the *FaACO*. The full-length cDNA, and the genomic clone of the gene were amplified. The expression of *FaACO* is induced by ripening.

We isolated the strawberry *CTR1* gene, component of the ethylene signalling pathway. We identified the full-length cDNA. The *FaCTR1* expression is constitutive. We isolated a 1590 bp upstream region of *FaCTR1* by TAIL-PCR and we have done its *in silico* analysis. We submitted all of the sequences of the genes to the GenBank.

2. The characterization of strawberry ripening specific gene expression pattern, isolation of novel genes

We identified in total 72 genes from strawberry fruit and achene, these genes are expressed during different stages of ripening. We are also the first to report the expression pattern of these genes, analysed by quantitative cDNA-AFLP.

We have sequenced and submitted the sequences to the NCBI GenBank of 20 genes expressed in the green receptacle, 29 ripening induced genes, and 18 genes expressed in the achene.

Based on the transcripts identified by cDNA-AFLP we chose 7 clones, which are putatively involved in fruit development and ripening, we isolated their full-length cDNA, and considering their homologies, we determined their functions.

We defined the subcellular localization of FaRingH2 and FaHd using transient expression of GFP fusion proteins in rice protoplasts. The FaRingH2 was localized in cytoplasm, the FaHd in the nucleus.

4. Conclusions and suggestions

In strawberry, like in other plant species, the genes involved in ethylene biosynthesis and signalling pathway are present. The role of ACS identified by us, is probably similar to

the apple MdACS-5, as its expression was detected in young vegetative tissues and green fruit, and it is also inducible by elicitors. Contrary to ACS, the ripening induced pattern of the ACO suggests that the ethylene biosynthesis increases during strawberry ripening, too. The expression pattern of the *FaCTR1* gene, encoding the CTR1, the negative regulator of the ethylene signalling pathway, is constitutive, similarly to *CTR1* genes identified in other plants. The *in silico* analysis of the *ACS, ACO* and *CTR1* genes and their promoters isolated by us, adds many new information to the elucidation of the process of ethylene biosynthesis and signalling pathway in strawberry, but in order to determine the exact role of ethylene in strawberry it is necessary to isolate further members of the *ACC*-synthase and –oxidase gene families, to identify their expression pattern, enzyme activity and the functional analysis of their promoter.

Using cDNA-AFLP we could identify novel genes specific for strawberry ripening, and also ripening induced genes already described in the case of climacteric ripening. These represent the common elements of fruit ripening. It was possible to cluster tissue specific genes and genes specific for different ripening stages. Among the isolated clones there are present such regulatory elements which are characteristic both for climacteric and non-climacteric ripening. The functional analysis of these genes can elucidate the missing links in the regulation of non-climacteric ripening. The functional analysis of several genes mentioned in the thesis has already been started. In order to study the FaSpa function we overproduce the protein in tomato, as model plant, strawberry is also going to be transformed with sense and antisense expression vectors. The FaNit protein, which might be involved in the auxin biosynthesis, is overproduced in tobacco, but we also have considered investigating its enzyme activity.

The sequence information of the genes mentioned in this thesis it is a starting point in isolation of the promoters of differentially regulated genes, which will lead to the identification of the common regulatory elements found in the promoters of the genes expressed in different ripening stages, what will enable to determine a comprehensive transcriptional regulation of ripening of the strawberry fruit.

5. Publications

5.1. Publications related to the subject of the thesis

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