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Identification of ripening-related genes in strawberry fruit by cDNA-AFLP

Balogh A.[1], Koncz T.[1], Tisza V.[1], Kiss E.[1,2] & Heszky L.[1,2]

[1]Department of Genetics and Plant Breeding, Szent István University, Páter K. u. 1, H-2103 Gödöllő, Hungary e-mail kiss.erzsebet@mkk.szie.hu
[2]HAS-SIU Research Group for Molecular Plant Breeding, Gödöllő, Páter K. u. 1, H-2103, Hungary
Corresponding author: E. Kiss

Summary: An RNA fingerprinting study of strawberry receptacle and achene tissue was performed to identify candidate genes involved in fruit ripening. Quantitative cDNA-AFLP was used to detect differential gene expression in green, white, pink and red stages of fruit ripening. Based on hierarchical average linkage clustering the differentially expressed genes formed three major groups, genes expressed only in green receptacle, genes expressed mainly in white, pink and red receptacle, and in achene. 130 transcript-derived fragments (TDFs) were isolated and sequenced. Most TDFs did not show any homology to sequences with known functions, others were homologous to genes involved in oxidative stress response, signal transduction, regulation of development and cell-wall metabolism. Novel genes, so far not associated with strawberry ripening and ripening in general, were identified, such as genes encoding a bHLH protein, putative nitrilase-related protein, putative HD-zip protein. The differential pattern of gene expression draws the attention to the significance of ripening induced-or repressed promoters in strawberry fruit, whose isolation and characterization can be useful tool for functional genomics. For this purpose nine cDNA-AFLP fragments related either to ontogeny or senescing were completed with 5'UTR aiming at more precise annotation and future promoter isolation. Although tens of potentially important transcriptome changes were identified, the function of many ripening induced genes remain unknown.

Key words: cDNA-AFLP transcript profiling, *Fragaria x ananassa* Duch., fruit ripening

Introduction

Fruits can be divided into groups with contrasting ripening mechanisms. Climacteric fruits (tomato, apple, banana) show a burst of ethylene biosynthesis and increase in respiration during ripening, while non-climacteric fruits (strawberry, grape, orange) do not. Although this classification has clear boundaries, more and more studies report the involvement of ethylene in regulation of gene expression during non-climacteric ripening (*Balogh* et al., unpublished data, *Goldschmidt* et al., 1993, *El-Kereamy* et al., 2003, *Tesniere* et al., 2004). On the other side, recent studies describe the involvement of nonhormonal factors in the regulation of ripening both in climacteric and non-climacteric fruits. In a digital gene expression profiling the comparison of climacteric and non-climacteric ripening was done by using EST collections from ripening grape and tomato fruits. Among the homologous genes found in both species there were also three transcription factors present, including members of the MADS-box, zinc finger, and bZip transcription families (*Fei* et al., 2004). The same study reports the presence of 18 ripening induced and 14 ripening repressed transcription factors in tomato fruit, though their relationship with ripening remains elusive. Recently, in tomato two transcription factors have been functionally associated with ripening.

Two MADS-box genes (*LeMADS-RIN* and *LeMADS-MC*) are involved in ripening and sepal development, respectively, showing the contribution of nonhormonal (developmental) regulation of ripening (*Vrebalov* et al., 2002). The homolog of *LeMADS-RIN* was isolated also from strawberry (*Fv-MADS-9*). Fv-MADS-9 displays fruit-specific expression and clusters close to *LeMADS-RIN* in phylogenetic analysis (*Vrebalov* et al., 2002). *LeMADS-RIN* acts upstream to ethylene in climacteric fruit ripening and it might represent a common regulator of ripening in climacteric and non-climacteric fruit (*Vrebalov et al.*, 2002).

We used an improved version of cDNA-amplified fragment length polymorphism (cDNA-AFLP) technology (*Breyne* et al., 2003) to isolate genes associated with ripening of strawberry for understanding the processes underlying fruit maturation in this crop and non-climacteric ripening in general. 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. The improved version of cDNA-AFLP used was developed in order to allow global, quantitative gene expression analyis (*Breyne* et al., 2003). In the present study, we have sequenced 130 AFLP fragments in total, 70 transcript-derived fragments (TDFs) had homologues in the database, these were submitted to the GenBank and grouped functionally. No function could be assigned to 46% of the

TDFs, because they showed no or only poor sequence similarity to any database entry. Furthermore, the complete 5' end was isolated for nine cDNA-AFLP clones, potential candidates in key processes related to fruit development and ripening.

Materials and methods

Strawberry fruits (*Fragaria x ananassa* Duch. cv. Elsanta) were harvested from a commercial farm near Ghent (Belgium). Medium-size green, white, pink, and fully ripe red fruits were used for RNA isolation.

cDNA-AFLP experiment

Total RNA was extracted separately from the fruit flesh and achenes according to Salzman *et al.* (1999). First-strand cDNA synthesis was carried out starting from 10 µg of total RNA., and cDNA-AFLP analysis was conducted as described by *Breyne* et al. (2003). Restriction enzymes *Bst*YI and *Mse*I (New England Biolabs, Beverly, MA, USA) were used, and for preamplifications a *Mse*I primer without selective nucleotides was applied in pair with a *Bst*YI primer containing a C as a selective nucleotide (the primer sequences were: 5'-GACTGCGTAGTGATCC-3' for *Bst*YI and 5'-GATGAGTCCTGAGTAA-3' for *Mse*I). In the cDNA-AFLP reactions 48 primer combinations were tested, both primers contained two selective nucleotides. Amplification products were separated on 5% polyacrylamide gels using the SequiGen system (Bio-Rad, Hercules, CA, USA). Dried gels were exposed to Biomax films (Kodak) and scanned with a PhosphorImager 445 SI (Amersham Biosciences, Little Chalfont, UK).

Characterization of AFLP fragments

Based on the autoradiogram, the TDFs were excised from gels and hydrated in 100 µl water for 1 h before PCR amplification of 2 µl by using the same primers as in the selective amplification. Sequence information was obtained either by direct sequencing of the re-amplified product with the BstYI primer or after cloning the fragments in pGEM-T Easy (Promega, Madison, CA, USA). The reactions were analysed with an ABI Prism 310 Genetic Analyzer. Nucleotide and translated amino acid sequences were analysed for homology to known gene sequences using the BLASTN and BLASTX programs.

Amplification of complete 5' end of cDNAs by RACE

Total RNA was extracted from green and red receptacle tissues, and poly(A)$^+$ RNA was isolated with an Oligotex mRNA Mini Kit (QIAGEN, Germany). To determine the 5' end nucleotide sequence, RACE (rapid amplification of cDNA ends) was performed using a cDNA amplification kit (SMART^{TM} RACE cDNA amplification kit, BD Biosciences, Erembodegem, Belgium) according to the manufacturer's protocol. The primers used for amplification of the 5' end fragments are listed in *Table 1*. The sequences obtained from RACE reactions were analysed using the SeqMan program in the DNASTAR package.

Results and discussion

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 (*Eisen* et al., 1998) 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 belongs 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). 36% of the sequenced fragments were receptacle related and ripening specific, 30% were ripening repressed, showing higher expression in the green receptacle, and 34% of the TDFs were achene specific. 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, or their hits were below an E < e-0.002 (*Figure 1A,B,C*). All sequences obtained with hits of E < e-0.002 were submitted to GenBank, their accession numbers are listed in *Table 2, 3* and *4*. It is also notable that only five TDFs show homology with strawberry genes already submitted to GenBank, and other seven are similar with genes from species of the *Rosaceae* family, showing the absence of sequence information for strawberry in the database. In a next step of our experiment we intend to study the function of several genes described in the present work. As the TDSs are rather

Table 1. The sequence of primers used in 5' RACE reactions

TDF	5'-RACE primer sequences (5'→3')	TDF	5'-RACE primer sequences (5'→3')
C11M32M003	GTTTTGGCCATCTGCACGCATGTA	C24M14M007	AAGGCAGGTATTCAGCAAGGTGTA
C14M13M002	CTCGGATTCTGGTTGGAACTCACT	C24M33M010	AGGTGGATTGCTGGGTGCAGGAAGGT
C14M21M006	CTGGGAGAAAGAGAAGCCAAGACCAT	C24M43M007	AATGAGATTTGGGATGGTCCTGATC
C23M42M006	AGCTCCTCTCAAGCGAATGAGTAGCT	C24M44M003	TGCCACAGAACATGTTGCTCAAGC
C24M33M004	CTTGGATGAGTTTGGGAGCTGAAA		

short and represent the 3' end of the cDNA (*Breyne* et al., 2003), we performed 5' RACE on nine genes to get a more precise sequence information. These fragments were selected based on their expression pattern, and on the function of their homologues with a potential role in fruit growth, development and ripening. The length of the initial and complete 5' end fragments together with their homologues and the E values before and after the 5' RACE reactions are listed in *Table 5*. Although between the length of the initial and complete 5' end sequences there are significant differences, their homologues are the same, except in the case of the clone C24M43M007 where the initial 120 bp fragment showed No hit result after Blast search and for the complete 5' end fragment there is a strong match (E value = e-135). The results prove the reliability of the Blast hits listed in *Table 2, 3* and *4*. An extensive description of these genes is provided in a subsequent publication.

In the group of ripening induced transcripts all the genes expressed in white, pink and red stages are present, their expression show up-regulation during ripening. 47 TDFs were sequenced, no function could be assigned for 34% of the fragments because they showed no or only poor sequence

Table 2. Transcripts isolated from the ripe receptacle tissue, for which sequence homologies were identified

cDNA-AFLP fragment	GenBank accession No.	Putative identity, related sequence accession number	Putative function	E value
C11M33M001	AY679582	amino acid transport protein AAP1, T10100	Transport and carriers	5e-64
C11M33M011	AY679605	putative GTP-binding protein (DRG), BAC79856	Signal transduction	7e-13
C11M33M013	AY679583	T family 47, NP_565236	Prosthetic groups	3e-05
C11M33M014	AY679584	phospholipase PLDb2, AAG45488	Stress	7e-30
C11M34M005	AY679581	translation initiation factor AF499740	DNA/RNA/protein	3e-40
C12M11M011	DQ011163	late embryogenesis abundant protein PvLEA4-25, AAC49862	Stress	3e-12
C12M12M024	AY961593	U3 snoRNP-associated-like protein, At4g05410	DNA/RNA/protein	3e-13
C13M214M007	DQ011162	alcohol dehydrogenase, ripening-related, S39508	Flavour	5e-13
C14M21M006	AY940166	receptor-like protein kinase AAM62629	Signal transduction	e-108
C14M31M010	AF339024*	pectate lyase B, AF339024	Cell wall	2e-29
C14M33M006	AY679611	sedoheptulose-1,7-bisphosphatase precursor, AY188797	Primary metabolism	2e-49
C23M11M001	AY642687	phytochelatin synthetase, BAB10641	Stress	7e-65
C23M12M001	DQ012968	putative stress-responsive protein, AAT01418	Stress	4e-34
C23M21M001	AY679609	protein kinase 2, AAA34017	Signal transduction	7e-15
C23M21M011	DQ022748	putative endo-1,3;1,4-beta-D-glucanase, AAU10802	Cell wall	1e-15
C23M24M006	AY679593	18S ribosomal RNA gene, AF321262	DNA/RNA/protein	1e-27
C23M31M001	AY679594	putative integrase, AAD04177	DNA/RNA/protein	1e-38
C23M33M002	AY679608	flavonone-3-hydroxylase, AB074486	Flavonoid pathway	3e-43
C23M33M014	AY642689	putative splicing factor 3b, BAD10377	DNA/RNA/protein	7e-11
C23M42M006	AY679604	AAA-type ATPase family protein, NP_849842	Primary metabolism	e-119
C23M43M001	AY679602	wound-inducible P450 hydroxylase, AAG09208	Stress	2e-19
C23M43M008	AAW82451	putative glutathione S-transferase, Q03666	Stress	2e-30
C24M13M007	AY679595	quinone oxidoreductase homolog, T11672	Stress	4e-07
C24M14M001	AY679596	putative beta-coat protein, NP_913038	Transport and carriers	2e-46
C24M14M007	DQ074728	spermidine synthase, AB072915	Polyamine metabolism	e-147
C24M31M004	AAX09335	putative phosphatidylinositol glycan class S, NP_187374	Cell wall	2e-45
C24M33M004	AY679615	bHLH protein SPATULA, NP_568010	Regulation	2e-40
C24M33M008	AY171598*	UDP-glucosyl transferase AY171598	Carbohydrate metabolism	1e-47
C24M33M010	AY679612	protein kinase – related, NP_177209	Signal transduction	0.0
C24M44M001	AY679603	glycosyl transferase family 17 protein, NP_178963	Prosthetic groups	4e-42
C24M44M003	AY679614	putative HD-zip protein AAT39931	Regulation	e-111

*Strawberry genes already submitted to database

Table 3. Transcripts isolated from the green receptacle tissue, for which sequence homologies were identified

cDNA-AFLP fragment	GenBank accession No	Putative identity, related sequence accession number	Putative function	E value
C11M32M003	AY695666	nitrilase-associated protein AAG52493	Hormone metabolism	1e-17
C11M32M009	AY679616	cytochrome P450, putative NP_192969	Unknown	2e-14
C11M32M010	AY642688	probable mitochondrial carrier protein, T00435	Transport and carriers	1e-07
C11M33M010	AY946036	putative AAA-type ATPase AAO72381	Primary metabolism	5e-17
C11M34M012	AY679606	calreticulin 3, AY336743	Signal transduction	8e-20
C12M11M005	AY679601	Mal d1 homolog, AF020784	Unknown	8e-16
C12M11M006	AY946035	BEL1-related homeotic protein 30, AAN03627	Regulation	0.002
C12M12M008	AY679600	3-hydroxyisobutyrate dehydrogenase-like protein, AAM63893	DNA/RNA/protein	1e-28
C13M214M003	AAX23999	chlorophyll a/b-binding protein CP24 precursor, AAD27882	Photosinthesis	7e-75
C13M214M008	AJ278705 *	beta-galactosidase (beta-gal3) AJ278705	Cell-wall	2e-77
C14M12M015	AY679599	ribosomal protein S12, AF238068	Unknown	1e-05
C14M13M002	AY679613	C3HC4-type RING finger protein, NP_178507	Regulation	2e-26
C14M13M006	AY961594	unnamed protein BAB02057	Unknown	2e-07
C14M21M010	AY679598	polygalacturonase inhibitor-like protein, CAD56505	Stress	7e-08
C14M33M016	AY961595	NADH-ubiquinone oxidoreductase-related At5g52840	Primary metabolism	4e-04
C23M13M005	AY679607	cytochrome P450, AF386512	Unknown	4e-06
C23M32M007	DQ074727	hypothetical protein, BAD38514	Unknown	1e-04
C24M14M002	AY873806	S-adenosyl-L-methionine:carboxyl methyltransferase, NP_683307	Prosthetic groups	4e-08
C24M24M013	AY679610	ubiquitin-specific protease 26, NP_566922	Stress	1e-13
C24M32M014	AY679597	peroxidase (Prx2b), AF145348	Stress	6e-13
C24M43M007	DQ022749	plasma membrane intrinsic protein, BAD14371	Transport and carriers	e-100

*Strawberry genes already submitted to database

Table 4. Transcripts isolated from the achene, for which sequence homologies were identified

cDNA-AFLP fragment	GenBank accession No	Putative identity	Putative function	E value
C11M31M014	DQ074726	acid phosphatase type 5, NP_566587	Unknown	5e-10
C11M31M015	AAT46620	cytochrome P450, BAD06417	Unknown	2e-14
C11M32M003	AY695666	nitrilase-associated protein AAG52493	Hormone metabolism	1e-17
C11M34M001	AY912491	nitrite reductase AB061671	Nitrate metabolism	2e-75
C11M34M002	DQ022747	potassium channel, CAB62555	Unknown	5e-14
C13M13M002	AJ315844*	lpt46 gene for lipid transfer protein, AJ315844	Fatty acids	3e-06
C14M12M004	AY679585	heat shock protein, AAN74634	Stress	7e-17
C14M13M001	AY679586	48-kDa glycoprotein precursor, AAL86739	Unknown	1e-30
C14M13M002	AY679613	C3HC4-type RING finger, NP_178507	Regulation	1e-25
C14M21M003	DQ022746	calmodulin-binding protein, NP_565379	Signal transduction	8e-06
C14M31M001	AY679587	protein phosphatase 2C putative, At2g29380	Signal transduction	1e-10
C14M33M002	AY679588	putative nitrate transporter, BAB56042	Nitrate metabolism	5e-25
C14M33M003	AY679589	small heat shock protein P30222	Stress	1e-40
C23M12M004	AY633995	beta-amyrin synthase, BAB83088	Stress	2e-37
C23M21M002	AY679590	major storage protein, 1905429A	Storage	4e-23
C23M21M004	AY679591	1-cys peroxiredoxin, AAF12782	Stress	4e-25
C23M31M002	AY679592	UDP-glucuronosyltransferase, AAB99950	Carbohydrate metabolism	1e-04
C23M31M009	AY633994	cytochrome P450, CAA70575	Unknown	2e-16
C23M33M006	X15590*	18S ribosomal rRNA, X15590	Unknown	3e-08
C24M43M002	AY912490	putative auxin independent growth promoter, BAD69015	Unknown	6e-23

*Strawberry genes already submitted to database

Table 5. The length of the initial and complete 5' end fragments together with their homologues and the E values before and after the 5' RACE

cDNA-AFLP fragment	Length (bp) before and after 5'RACE	Most similar homologue before and after 5'RACE	E value before and after 5'RACE
C11M32M003	400 → 602	putative nitrilase-associated protein	7e-14 → 3e-17
C14M13M002	271 → 505	zinc finger (C3HC4-type RING finger) protein	3e-26 → 4e-44
C14M21M006	181 → 1430	receptor-like protein kinase	1e-12 → e-108
C23M42M006	346 → 1829	AAA-type ATPase family protein	1e-24 → e-119
C24M14M007	160 → 1212	spermidine synthase	0.015 → e-147
C24M33M004	240 → 1000	bHLH protein SPATULA	0.005 → 3e-46
C24M33M010	137 → 1800	putative protein kinase	0.007 → 0.0
C24M43M007	120 → 1151	plasma membrane intrinsic protein*	No hit → e-135
C24M44M003	193 → 1076	putative HD-zip protein	0.016 → e-111

*the only clone, where the complete 5' end had different homology from the initial cDNA-AFLP fragment

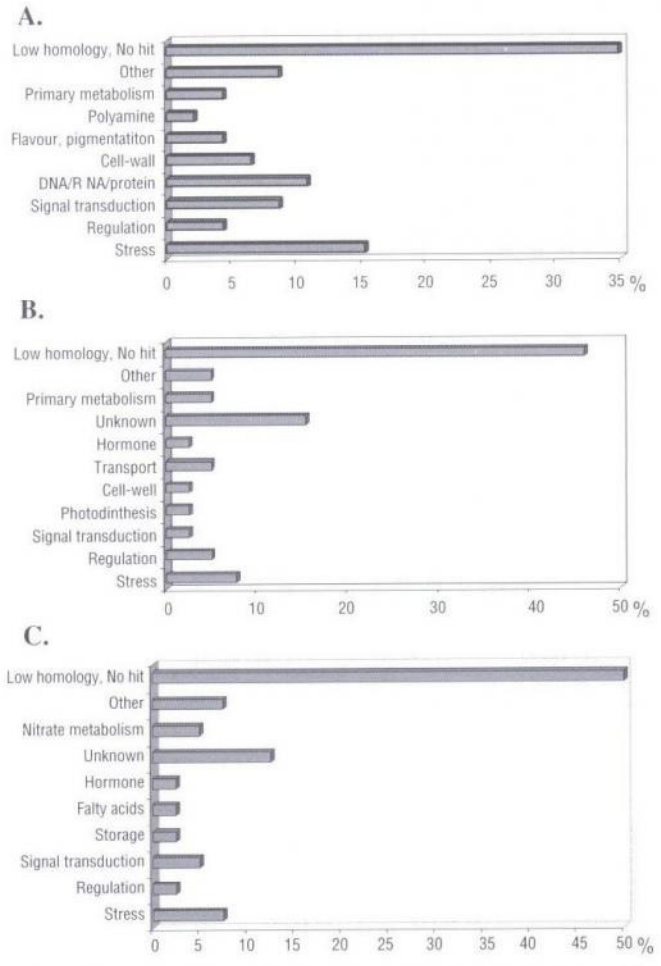

Figure 1. Functional classification of TDFs differentially accumulated in strawberry fruit. A: red receptacle, B: green receptacle, C: achene

similarity to any database entry (*Figure 1 A*). The major functional category of this group is represented by the genes involved in stress response. Increased free-radical-mediated peroxidative damage and loss of membrane integrity are characteristics of senescing plant tissues. Decline in free radical scavenging ability and the associated increase in oxidative stress may be prerequisites for mediating many of the physicochemical changes that facilitate ripening of the fruit of *Amelanchier alnifolia* Nutt. (*Rogiers* et al., 1998). The results reported by *Davies & Robinson* (2000) also show the accumulation of stress response proteins during grape berry ripening, another non-climacteric fruit. *Aharoni* et al. (2002) suggest that on the one hand active gene expression is induced to cope with oxidative stress conditions during ripening, on the other hand the strawberry ripening transcriptional program is an oxidative stress-induced process. In the present study we identified several putative oxidative-stress related genes. A strawberry homolog of the *TED2* gene (quinone oxidoreductase homolog) (C24M13M007) was already identified as ripening related and oxidative stress induced (*Aharoni* et al. 2002), in our study it was found that its expression is the highest in the pink stage, then decreasing in the ripe fruit. Two TDFs showing homology with a phytochelatin synthetase (C23M11M001) and putative glutathione S-transferase (C23M43M008) genes were also identified as ripening induced. Plants can produce Cys-rich peptides such as glutathione (GSH), phytochelatins (PCs), or metallothioneins (MTs) for detoxification or homeostasis of heavy metals (*Cobbett*, 2000). Many members of this group are associated with fruit ripening, e.g. in apple (*Reid & Ross*, 1997), banana (*Clendennen & May*, 1997), strawberry (*Aharoni & O'Connell*, 2002) and pineapple (*Moyle* et al., 2005). The TDF C11M33M014 encodes a phospholipase D which catalyses the hydrolysis of structural phospholipids to generate phosphatidic acid and a free head group. It is prevalent in plants where enhanced activity is associated with the response to: pathogens (*Van der Luit* et al., 2000), water stress (*Munnik* et al., 2000), oxidative stress; ethylene (*Fan* et al., 1997; Lee et al., 1998) and ABA (*Frank* et al., 2000). More traditionally, PLD was identified as a catabolic enzyme whose activity was associated with, germination, ripening and senescence. This clearly illustrates that phospholipase D exists as different enzymes, fulfilling different roles in plants. In our study phospholipase D is ripening specific, having the highest expression in the ripe fruit. Other stress-response related genes up regulated during ripening include a wound-inducible P450 hydroxylase (C23M43M001) and a TDF (C23M12M001) showing homology with a putative stress-responsive protein and cold acclimation protein (WCOR413-like protein from rice, AAG13395). The expression of the members of COR413 gene family is

correlated with the development of freezing tolerance in cereals and Arabidopsis. Several members of this gene family are also regulated by water stress, light, and abscisic acid (*Breton* et al., 2003). Other important functional groups are the ones comprising the transcripts involved in signal transduction (4 genes) and regulation (2 genes). In the receptacle tissue several members of the protein kinase family are represented with ripening induced expression pattern (C14M21M006, C23M21M001, C24M33M010). The clone C23M21M001 shares homology with protein kinase 2 from soybean (AAA34017) and with SAPK3 serine/threonine protein kinase from rice (BAD17999). The latter is the member of the sucrose nonfermenting1-related protein kinase2 (SnRK2) family and it was shown to be activated by hyperosmotic stress. Furthermore several members of SnRK2 family are also activated by ABA (*Kobayashi* et al., 2004). Together with a receptor-like protein kinase (C14M21M006) and a TDF encoding a protein kinase–related protein (C24M33M010) these genes could be part of the signal transduction pathway induced by ripening related stress.

In the regulation group two different strawberry receptacle-related cDNAs were identified showing homology to transcription factors. C24M33M004 is similar to basic helix-loop-helix (bHLH) protein SPATULA responsible for the development of carpel margin tissues in Arabidopsis (NP_568010) (*Heisler* et al., 2001). The cDNA fragment C24M44M003 shows the greatest identity to putative homeodomain-leucine zipper (HDZip) protein from *Solanum demissum* (AAT39931). HDZip proteins constitute a large family of transcription factors apparently unique to plants, the ones with known function play key roles in plant development (*Hanson* et al., 2002). The transcription factors identified here, might be involved in strawberry fruit development and ripening representing non-hormonal regulation of these processes. The group representing clones coding cell-wall related proteins, includes two TDFs (C14M31M010 and C23M21M011) encoding a pectate lyase and a putative endo-1,3,1,4-beta-D-glucanase respectively. Fruit ripening is usually associated with softening and cell wall disassembly, respectively involving the action of several enzyme families (*Brummell & Harpster*, 2001). Both genes mentioned above have already been associated with ripening-related cell-wall disassembly. It was shown that the pectate-lyase was expressed significantly higher in ripening-stage receptacle (*Benítez-Burraco* et al., 2003, *Aharoni & O'Connell*, 2002). Furthermore, the same authors showed that auxin treatment strongly repressed the expression of pectate lyase and endo-1,4-β-glucanase (*Aharoni* et al., 2002). Another ripening specific gene identified, might be associated with cell wall structure is putative phosphatidylinositol glycan (PIG) class S (C24M31M004) and is strongly upregulated in pink and red ripening stage. PIG gene is responsible for biosynthesis of the glycosylphosphatidylinositol (GPI) anchor. GPI-anchored proteins (GAPs) are involved in diverse physiological processes, such as root development, cell wall integrity and adhesion. In Arabidopsis the existence of 248 GAPs was predicted, among them cell wall related proteins also expressed during ripening, like several β-1,3 glucanases, a polygalacturonase and different pectate lyases (*Borner* et al., 2003). In the present experiment, phosphatidylinositol glycan could be responsible for the dynamic reorganization and modification of the cell-wall during strawberry fruit ripening.

Functional classification of ripening repressed genes expressed in the receptacle

Out of the 86 green receptacle specific TDFs 39 were sequenced. Almost half (46%) of the clones did not show any homology to sequences with known functions, another 6 fragments (18%) putatively encode proteins with unknown function (*Figure 1 B*). Based on their expression pattern the group of green receptacle associated TDFs are sharply differentiated from the group of ripening induced genes. This is in accordance with the different physiological and biochemical processes undergoing in the green and ripening receptacle. In this stage (middle green), under the effect of auxin, the fruit is growing mainly due to an increase in cell volume. We have identified two genes with key role in cell expansion. One of them (C13M214M008) shows homology with beta-galactosidase (Faßgal3), a cell wall hydrolase, mediating reversible wall loosening and thus allowing turgor-driven cell growth next to other cell wall modifying proteins. *Trainotti* et al. (2001) has already shown that the transcript amount of this gene appears to be very high in flowers and in young fruits (both small and large green ones), then it gradually decreases during fruit development and ripening and becomes almost undetectable in red fruits. The same author describes that in contrast with the expression pattern of *Faβgal3* the other two isoforms of beta-galactosidase (Faßgal1 and Faßgal2) are ripening induced, involved in cell wall disassembly. The other gene belongs to the group of transport and carriers, the complete 5' end sequence generated from the TDF C24M43M007 encodes a plasma membrane intrinsic protein (*PIP*) with high homology to *MdPIP1* (BAD14371) from apple. Together with Faßgal3, it is responsible for cell expansion during fruit growth by maintaining water homeostasis (*Hu* et al., 2003). The clone C11M32M003 shows similarity to a gene encoding a putative nitrilase-associated protein from Arabidopsis (AAG52493) representing the only member of the hormone metabolism group. Next to the sequence encoding a RING finger protein, it is one of the few transcripts present both in green receptacle and green achene tissue with the same expression pattern. Nitrilases (EC 3.5.5.1) hydrolyze nitriles (e.g. indole-3-acetonitril (IAN)) to the corresponding carboxylic acids (as indole-3-acetic acid (IAA)) and might have a potential role in biosynthesis of IAA, the most abundant natural auxin (*Park* et al., 2003). In strawberry high auxin levels are known to promote early fruit growth (*Nitsch*, 1950, *Perkins-Veazie*, 1995), and it was also shown that ripening is triggered by the cessation of auxin

supply to the receptacle from the achenes during achene maturation (*Archbold & Denni*, 1984; *Manning*, 1994, *Given* et al., 1998). Thus, the identification of a key enzyme in auxin biosynthesis could be helpful for further investigation of regulation and control of ripening by the hormone auxin. The group of stress induced transcripts present in the green receptacle tissue represents a lower percentage (7.7%) than in the ripening receptacle, the sequences account for a peroxidase (C24M32M014), a polygalacturonase inhibitor-like protein (C14M21M010) and a ubiquitin-specific protease 26 (C24M24M013). The signal transduction group is represented by a calreticulin 3 (C11M34M012). Calreticulin, a major Ca^{2+} -sequestering protein, has been implicated in a variety of cellular functions such as Ca^{2+} storage, signalling and chaperone activity within the cytoplasm and endoplasmic reticulum (*Sharma* et al., 2004). Distinct tissue-dependent expression patterns, stress-related regulation, and its modulation by phytohormones were observed for the isoform groups (*Borisjuk* et al., 1998, *Persson* et al., 2003). The TDFs representing the regulation group are C12M11M006 encoding a BEL1-related homeotic protein and C14M13M002 accounting for a zinc-finger protein (C3HC4-type RING finger). The clone encoding the Ring finger, is also expressed in the achene tissue, possibly representing an element of common regulatory mechanism present in both tissue types.

Functional classification of the achene specific genes

Based on the results obtained by the hierarchical average linkage clustering (*Eisen* et al., 1998) the achene specific TDFs represent one group sharply differentiated from the genes expressed in the receptacle. Including the achene in the cDNA-AFLP experiment, aimed to find not only common regulatory elements in the two tissue types but also to distinguish receptacle specific genes from the ones common for both tissue types. The fact that all the achene specific TDFs were clustered in one group shows the lack of significant fluctuations in gene expression pattern during achene maturation comparatively to the discrepancy noticed between the gene expression in green and ripe receptacle. 120 TDFs were achene specific, out of them 40 were sequenced. 50% of the TDFs represent fragments with low or no homology at all. The second biggest group includes 7 clones (12.5%), encoding proteins with unknown function. The group of stress and defence-related transcripts comprises 4 TDFs (10%) (*Figure 1 C*). This group of genes are responsible for acquisition of stress and desiccation tolerance during achene maturation. The *Fagopyrum esculentum* homologue (AAF12782) of the strawberry 1-cys peroxiredoxin (1-Cys Prx) (C23M21M004) was previously shown to be regulated in a seed-specific and temporal manner during seed development (*Lewis* et al., 2000). Peroxiredoxins are part of the antioxidant defence. They decompose reactive oxygen species (ROS) and lipid peroxides and tune ROS and peroxide levels in signalling events. Previous functional assays on 1-Cys Prx have revealed that it is a DNA-protecting enzyme, later a second role was identified in the maintenance of dormancy (*Stacy* et al., 1996). However, a rice 1-Cys Prx over-expressed in transgenic tobacco showed that the gene did not maintain dormancy in seeds but rather enhanced resistance against oxidative stress in transgenic plants, suggesting that antioxidant activity may be its primary function (*Lee* et al., 2000). A beta-amyrin synthase gene (C23M12M004) encodes the first committed step in the triterpenoid pathway leading to saponin biosynthesis. Saponins are secondary metabolites found in many major food crops, whose potent antifungal activity and presence at high concentrations in healthy plants, suggests that these molecules could act as barriers to fungal attack (*Papadopoulou* et al., 1999). The rest of the stress-related clones account for heat shock proteins (C14M33M003 and C14M12M004). A smaller group (5%) is involved in signal transduction and also 5% of the sequences account for proteins involved in nitrate metabolism. Among the few common genes found both in achene and receptacle tissues are the sequences encoding for a putative nitrilase, already described above, and a RING finger protein (C14M13M002). The transcript for zinc-finger protein is present in the green receptacle, and in all the maturation stages of the achene, where its expression is decreasing during the maturation of the achene. Ring zinc-finger proteins play an important role in the regulation of development in a variety of organisms. In plants only a few RING finger protein genes were characterized functionally. *BRH1* encodes a C-terminal RING-H2 finger protein, and it is down-regulated by brassinosteroids and can be induced by the pathogen elicitor chitin (*Molnár* et al., 2002). *RHA2*b is highly expressed in vascular tissues as well as in the transmitting tissue of the styles of flowers (*Lechner* et al., 2002); *RIE1* is involved in seed development (*Xu & Li*, 2003).

Conclusions

Combining cDNA-AFLP, RACE methods and sequencing enabled to identify novel genes expressed in strawberry ripening. From 130 transcripts only five were found in the GeneBank and annotated as *Fragaria x ananassa* genes, and other seven showed similarity to genes belonging to the *Rosaceae* family. Three major groups distinguished by hierarchical clustering from the achene-, green and red receptacle-related genes prove tissue-and developmental stage-specific regulation during maturation. Based on the complete 5' UTR of nine cDNA sequences related either to ontogeny or maturation the original homology changed only in one case, which actually meant a "no-hit ›hit" transition. This suggests that the sequence information based on sometimes very short fragments is reliable enough for identification of genes involved in the processes studied. Transcripts of many genes were described in both climacteric and non-climacteric (strawberry) fruits, including alcohol-dehydrogenase, phospholipase, flavonone-3-hydrxylase and spermidine synthase. These results can help

to understand more profoundly the common and/or peculiar mechanism of strawberry fruit ripening with that of non-climacteric and climacteric types. Achene and receptacle specific genes provide the basis for tissue specific promoter isolation and for further functional genomics research.

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