Identification, analysis and characterization of differential expressed genes involved in ripen fruits of blood and common cultivar of sweet orange

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Alla passione (per la ricerca),
all'amore (per i miei cari),
alla forza degli Angeli.

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SUMMARY

Italian and especially Sicilian *Citrus* are characterized by red pigmentation, due to the anthocyanin content. The amount of this secondary metabolism depend from different environmental and pedoclimatic conditions, as the light and the low temperature, and from genetic factors, as structural genes (involved in the biosynthesis of anthocyanins) and regulatory genes (controlling the activation of the pathway).

The aim of this work was the identification of differentially expressed genes between pigmented and common oranges (*Citrus sinensis* L. *Osbeck*) in flesh tissue at maturity time. We used Moro nucellare 58-8D-I, one of the cultivar with the highest anthocyanins content, and Cadenera, a common orange. The methodology of PCR Select SSH was chosen to enrich Moro cultivar of differential transcripts. Among the 1248 clones of the library, 260 are differential and 230 of them are over expressed in pigmented orange. 201 of the differential ESTs were submitted in the dbEST of GenBank database, to enrich informations on *C. sinensis*. Functional classification based on similarity analysis and references was divided into 10 categories. The frequency which transcripts are included into clones is function of the redundancy and it is a signal of the high expression level. The 260 clones correspond to 82 genes. We used the semi-quantitative RT-PCR to analyse the expression level of 10 genes, some of them were redundant, some other were chosen for their interesting function, as the regulatory genes. Three of these transcripts were validated also in Real time PCR, confirming previous differential data. Moreover 104 of the differential ESTs were spotted in a custom chip, together with ESTs deriving from a cDNA-AFLP library and from HarvEST *Citrus* database. It was hybridised using the samples of Moro and Cadenera at maturity time, that were used to the construction of the SSH library. It was confirmed the high expression level of genes involved in the anthocyanin biosynthesis as the redundant transcripts previously analysed. The 30,171 ESTs of various species of *Citrus* and intergeneric hybrids, deriving from different libraries spotted on the Affymetrix *Citrus* GeneChip, were hybridised with the same samples used for the SSH library and the custom chip. The screening of the *C. sinensis* database (94,127 ESTs) based on the differential data derived from various approaches, as the differential library and both of arrays, was used to isolate specific and unique transcripts of orange flesh tissue. The expression analysis performed with the reverse Northern, the semi-quantitative RT-PCR and the Real time PCR permitted to evidentiate that the only two flesh maturity specific transcripts are the dihydroflavonol 4-reductase (DFR) and UDP-glucose flavonoid glucosyl transferase (UFGT), enzymes involved in the anthocyanins pathway.

One of the most important enzymes involved in the pigmentation of oranges is the glutathione S-transferase (GST), that transports anthocyanins from cytoplasm to vacuole. Moreover six different classes of GSTs are known and they are classified according to their gene structure and function. The clustering/assembling analysis on the *C. sinensis* database, Blast and UniProt similarity, and *Arabidopsis* functional deduction were used to organize orange putative GSTs into the different classes using an *in silico* approaches. The expression analysis, performed through semi-quantitative RT-PCR on different orange tissues of Moro and Cadenera cultivars, confirmed the *in silico* deduction and a different expression pattern was evidentiated.

Experiments of homolog and heterolog transient transformation were conduced to investigate the regulatory function of *myc* and *myb*-like genes isolated in orange, deducing the involvement of these transcription factors on the anthocyanins pathway and the interaction between Myc and Myb-like transcription factor.

RIASSUNTO

Gli agrumi sono frutti di notevole interesse per le loro qualità organolettiche, le proprietà nutritive e terapeutiche. Tra i principali componenti (carboidrati, acidi organici, sostanze minerali, vitamine) godono di notevole importanza le antocianine.

Il primo riferimento di un'arancia pigmentata nell'area del Mediterraneo si trova nell'opera *Hesperides* del Ferrari (1646), che descrive un *Aurantium indicum* dalla polpa pigmentata introdotto in Italia da un missionario genovese che rientrava dalle Filippine. E' interessante inoltre constatare come, nonostante la coltivazione di arance bionde (Navel, Valencia) abbia seguito una crescita esponenziale rispetto al periodo in cui è stata descritta la prima arancia pigmentata, non si sono mai osservate mutazioni che producono antocianina dalle nuove selezioni di arancio biondo. Tra l'altro marcatori isoenzimatici e molecolari non rivelano polimorfismi tali da far ritenere l'origine dei due gruppi (arance bionde e pigmentate) legata a processi differenti di segregazione e ricombinazione. Sembrerebbe piuttosto che un evento mutazionale unico abbia separato i due gruppi, così come da successive mutazioni si sono differenziate tutte le cultivar bionde e pigmentate di arancio dolce.

In Italia, e in particolare, in Sicilia, le arance maggiormente sponsorizzate sono quelle rosse, Tarocco, Moro e Sanguinello (Zarbà, 1994). Più precisamente nella Sicilia Orientale e a Sud e Sud-Ovest del Monte Etna le arance rosse hanno trovato condizioni pedoclimatiche e ambientali ottimali, che consentono di manifestare al meglio le loro caratteristiche genetiche, quali il colore rosso intenso e un perfetto equilibrio tra zuccheri e acidità (Rapisarda et al., 1998). La produzione di antocianine è strettamente dipendente da fattori genetici (Honda et al., 2002) e nutritivi (Lancaster, 1992; Sparvoli et al., 1994). Uno dei fattori che maggiormente influenza la formazione di tale pigmento è la luce, anche se, in alcuni casi, ne accelera la degradazione (Maccarone et al., 1987). Tra l'altro la luce attiva la trasduzione del segnale e l'espressione genica coinvolta nella biosintesi (Mol et al., 1996). Inoltre, la pigmentazione variabile del frutto è dovuta anche alla posizione dello stesso nella pianta: solitamente una maggiore concentrazione di antocianine è stata riscontrata nei frutti esposti a nord (Rapisarda et al., 2001). Un altro fattore che influenza positivamente la biosintesi delle antocianine è costituito dalle basse temperature, in modo particolare l'escursione termica tra il giorno e la notte.

Nelle arance, il carattere "presenza di antocianina" è ad espressività variabile ed è anche probabile che l'accumulo di antocianine, che si diversifica in modo notevole nelle diverse cultivar, sia dipendente da condizioni e fattori di attivazione differenti. Questi pigmenti forniscono un importante contributo sensoriale, utile per la promozione dell'immagine e la tipizzazione del prodotto, ma esercitano un ruolo ancora più importante per le loro proprietà farmacologiche e antiossidanti. Le antocianine trovano impiego nella terapia oculistica, come principi attivi che favoriscono la rigenerazione della rodopsina, nella cura dell'ulcera e in angiologia per le proprietà epitelio-riparatrici e modulatrici della resistenza e della permeabilità capillare, e in tutte quelle condizioni fisiopatologiche caratterizzate da un eccesso di produzione di radicali liberi (Saija, 1994).

Le antocianine (dal Greco *antos*, fiore, e *kyanos*, blu), metaboliti secondari e flavonoidi, sono dei pigmenti naturali di colore rosso, porpora e blu, presenti soprattutto nelle cellule epidermiche della pianta, a livello dei vacuoli, in cui risiedono e colorano i tessuti (Rimari e Strommer, 1998). Negli organi riproduttori le antocianine attirano gli insetti per l'impollinazione e la dispersione dei semi (Holton e Cornish, 1995); nelle foglie autunnali prevengono il danno foto-ossidativo e la

senescenza fogliare (Field et al., 2001). Poco invece si conosce sull'accumulo e la funzione di tali pigmenti all'interno dei frutti. Nell'ambito dei frutti di agrume maturi le antocianine sono espresse esclusivamente nell'arancio rosso e nei suoi ibridi e soprattutto nella polpa e nella buccia; la presenza di antocianine nelle foglie e nei fiori di alcuni agrumi (limone, *Papeda* ecc.) si ha solo nella prima fase di crescita. In altri frutti di agrume (pompelmo, Vaniglia sanguigno, Cara Cara) il colore rosso non dipende dalle antocianine, bensì dal licopene.

La fisiologia e la base genetica della biosintesi delle antocianine sono state già ben studiate (Dooner et al., 1991) in: mais (*Zea mays*), bocca di leone (*Antirrhinum maijus*), petunia (*Petunia hybrida;* Mol et al., 1998; Holton e Cornish, 1995). Vari studi hanno portato alla conclusione che le antocianine sono sintetizzate quando intervengono: (1) geni strutturali, codificanti gli enzimi che catalizzano le reazioni della via metabolica; (2) geni regolatori, che codificano per fattori trascrizionali e controllano la loro espressione. I principali geni strutturali codificanti per enzimi coinvolti nella biosintesi dell'antocianina sono: *chs* (calcone sintasi), *dfr* (diidroflavonol 4-reduttasi), *ans* (antocianidina sintasi), *ufgt* (UDP-glucose:flavonoid 3-*O*-glucosiltransferasi) e *gst* (glutatione S-transferasi). È stato visto che il meccanismo di regolazione è direttamente controllato dalla combinazione di due diverse famiglie di fattori trascrizionali, omologhi alle proteine codificate dai proto-oncogeni dei vertebrati, *c-myb* (myeloblastosis; Mol et al., 1998) e *bHLH* (basic-Helix-Loop- Helix; Paz-Ares et al., 1987). L'interazione tra MYB e bHLH e il coinvolgimento delle proteine WD40 è il meccanismo chiave attraverso cui si sviluppa la regolazione della biosintesi delle antocianine e delle proantocianidine. La regolazione della biosintesi delle antocianine è stata particolarmente studiata in diverse piante, ma non esistono studi riguardo geni regolatori sulle arance.

Nonostante gli studi molecolari associati alla biosintesi delle antocianine siano stati compiuti estensivamente su mais, bocca di leone e petunia e, per quanto riguarda gli alberi da frutto, su vite (*Vitis vinifera* L.) e melo (*Malus communis*), poco si conosce sulla biosintesi delle antocianine nelle arance rosse. Lo Piero et al. (2005a) hanno isolato le sequenze parziali di cDNA di tre enzimi presenti nello stadio finale della biosintesi delle antocianine di *Citrus sinensis* (L.) Osbeck: DFR, ANS, UFGT. Cotroneo et al. (2006) hanno invece verificato il livello di espressione mediante Real time PCR e i risultati mostrano che i relativi trascritti sono espressi nelle arance rosse e sottoespressi nelle varietà bionde. Esiste inoltre una buona correlazione tra il livello di espressione dei geni biosintetici e il contenuto in antocianina. Tra l'altro l'accumulo di pigmento è strettamente correlato alla risposta di ogni genotipo alle condizioni ambientali.

L'obiettivo del lavoro sviluppato da questa tesi di dottorato ha riguardato l'identificazione dei geni differenzialmente espressi nella polpa di arancia pigmentata matura rispetto a quella di una cultivar bionda. La tesi è stata svolta seguendo diversi approcci investigativi. Considerate le limitate conoscenze che si hanno a proposito della variabilità genetica e di espressione della pigmentazione nelle arance, l'obiettivo prefissato ha riguardato la costruzione di una libreria sottrattiva a cDNA e quindi l'identificazione di trascritti differenziali presenti nella polpa di frutti maturi di una cultivar di arancio con un alto contenuto in antocianine (Moro nucellare 58-8D-1) rispetto a quella di una cultivar non pigmentata (Biondo cadenera). Per far ciò si è utilizzata una delle metodologie ritenuta più idonea (per moderata semplicità e per attendibilità dei risultati) per l'identificazione di trascritti differenziali, la PCR Select nella forma di Substractive Suppression Hybridization (SSH; Von Stein et al., 1997). La libreria è stata costituita partendo dall'RNA totale di polpa dei campioni in esame,

in maniera tale da arricchire in sequenze differenziali la cultivar pigmentata. La libreria consta di 1248 cloni, dei quali 260 sono differenziali e in particolare 230 sono sovra espressi in Moro e 30 in Cadenera. I 260 cloni corrispondono a 82 trascritti differenziali, di cui 30 sono risultati essere ridondanti (ovvero ritrovati più di una volta nella libreria) e 52 sono singoletti. L'analisi di similarità, effettuata mediante procedura di ricerca in banca dati (BlastN-nr; BlastX), supportata da riferimenti bibliografici, è stata indispensabile per dedurre la classificazione funzionale, suddivisa in 10 categorie. Si è visto che il 44% dei cloni differenziali era costituito dai geni che codificano per gli enzimi strutturali della biosintesi delle antocianine e dei carotenoidi. Sono stati isolati anche geni implicati nella biosintesi degli aromi. Una limitata percentuale ha interessato geni regolatori, geni coinvolti nella protezione del frutto nei confronti di attacchi patogeni e altri facenti parte dell'attività degli organelli cellulari. Rispettivamente l'8%, il 9% e l'11% invece ha riguardato geni implicati nel metabolismo primario, nell'organizzazione cellulare e meccanismi di trasduzione del segnale. Non sono mancati poi trascritti dalla funzione sconosciuta e altri che non sono stati fatti rientrare in una opportuna categoria funzionale. 201 delle sequenze differenziali di *C. sinensis* sono state sottomesse nella sessione dbEST della GenBank dell'NCBI.

Si è cercato poi di comprendere l'organizzazione dei vari cloni aventi la stessa similarità. Pertanto dopo l'allineamento multiplo ClustalW delle sequenze e ricostruzione delle varie porzioni, allineando le sequenze dei trascritti tra loro e con le sequenze a cui erano simili, è stato notato che 208 cloni erano ridondanti, vale a dire che le sequenze dei corrispondenti trascritti si erano inserite in più cloni e che codificavano in realtà per 30 geni. Questo dato di ridondanza è stato per noi motivo di ulteriore indagine analitica. Infatti i trascritti per l'analisi di espressione differenziale sono stati scelti tra quelli altamente ridondanti [(alcool acil transferasi (AAT), 10 idrossigeraniol ossidoreduttasi, fenilalanina ammonioliasi (PAL), citocromo b5, GST, valencene sintasi, pectinesterasi, putativo recettore S di chinasi (SRK)], perché si è avuto modo di ritenere che la ridondanza è direttamente e strettamente correlata con l'alto livello di espressione del trascritto. Ciò che ha consentito di fare questa deduzione è stato aver ritrovato ridondanti i geni codificanti gli enzimi implicati nella biosintesi delle antocianine. Inoltre è stato analizzato il livello di espressione dei geni regolatori *bHLH* e MADs box, al fine di comprendere meglio il loro coinvolgimento nella biosintesi delle antocianine. Nell'ambito dei geni codificanti gli enzimi strutturali del pathway delle antocianine [*pal*, *chs*, flavanone 3 idrossilasi (*f3h*), *dfr*, *ans*, *ufgt*, *gst*] è stato studiato il differente livello di espressione di PAL, GST e del citocromo b5 (substrato dei precursori della biosintesi delle antocianine). Lavori precedenti e già noti in bibliografia (Cotroneo et al., 2006; Lo Piero et al., 2005a, 2006) avevano analizzato mediante Real time PCR i livelli di espressione di CHS, DFR, ANS, UFGT e GST. L'analisi di RT-PCR semi-quantitativa ha mostrato un aumento di espressione di PAL e citocromo b5 nel corso della maturazione del Moro rispetto al Cadenera. I dati della GST, enzima coinvolto nella vacuolarizzazione delle antocianine (Marrs et al., 1995; Alfenito et al., 1998), sono stati ulteriormente validati con la Real time PCR, in cui si è evidenziato come durante la maturazione del frutto i livelli di trascrizione nel Moro aumentano al pari del contenuto di antocianine. Inoltre le due porzioni di GST, che sono state isolate attraverso la libreria, individuano la sequenza completa, come riscontrato in GenBank. Nonostante le sequenze di GST di Moro e Cadenera siano esattamente uguali, nell'arancio comune nessun trascritto sembra essere presente, confermando i dati forniti da Lo Piero et al. (2006). Questi dati confermano che le differenze tra Moro e Cadenera risiedono non tanto nella

differenza di sequenza dei geni biosintetici per le antocianine, ma nel loro livello di espressione, che è molto basso (quasi nullo) nella cultivar non pigmentata.

Tra tutti i trascritti facenti parte della categoria funzionale "Meccanismi di traduzione del segnale" un 'putativo recettore S di chinasi (SRK)' è risultato altamente ridondante all'interno della libreria. Il livello trascrizionale è stato analizzato sia attraverso RT-PCR semi-quantitativa sia in Real time PCR, e si è evidenziato un aumento del livello di espressione solo alla fine del periodo di maturazione del frutto e soprattutto nell'arancia pigmentata. Sebbene non si sia ancora a conoscenza della funzione specifica, anche per la mancanza di informazioni della sequenza completa dell'SRK, si è ipotizzato un possibile coinvolgimento nei meccanismi di regolazione delle antocianine, dal momento che la fosforilazione delle proteine attraverso Serina e Treonina chinasi sembra essere anche necessaria per attivare la regione C-terminale dei fattori trascrizionali Myc - Myb (Gu et al., 2002).

Il 7% dei trascritti differenziali appartiene alla categoria "Biosintesi degli aromi". L'RT-PCR semi-quantitativa eseguita sul valencene sintasi non ha mostrato alcuna differenza nel corso della maturazione tra Moro e Cadenera. Lo stesso vale per l'AAT e 10-idrossigeraniol ossidoreduttasi, anche se livelli di trascrizione leggermente più alti sono stati evidenziati nelle arance pigmentate.

Relativamente ai dati differenziali della pectinesterasi i risultati di Real time PCR (che hanno confermato quelli della RT-PCR semi-quantitativa) hanno mostrato una mancanza di espressione nel Cadenera, mentre un'evidente presenza di trascritto è stata riscontrata nel Moro, solo quando il frutto è altamente pigmentato e più specificatamente a maturità. Le pectinesterasi sono degli enzimi che demetilano le pectine, costituenti della parete cellulare del frutto (Castillejo et al., 2004), e conferiscono compattezza e turgidità. Infatti le arance pigmentate sono notevolmente più morbide e meno compatte delle corrispondenti arance bionde. Questo fenomeno costituisce un vantaggio per la facilità con cui le arance pigmentate si sbucciano, ma al tempo stesso ne rappresenta un inconveniente per la loro conservazione.

Il livello di espressione dei geni regolatori *bHLH* e MADs box, analizzato mediante RT-PCR semi-quantitativa, è risultato essere maggiore nel Cadenera più che nel Moro. Entrambi i geni appartengono a famiglie di fattori trascrizionali, coinvolti anche nella regolazione della biosintesi delle antocianine, ma non solo. Esperimenti di 5' e 3' RACE PCR e relativo disegno di primer interni hanno consentito di allungare e completare la sequenza di cDNA del bHLH, la cui cds è risultata essere lunga 1113 bp (EU240878). Le analisi di similarità in BlastX e dbEST di BlastN hanno mostrato alte similarità con sequenze implicate in meccanismi di attivazione dalle basse temperature, rispettivamente in *Tulipa gesneriana* e in *C. sinensis* e *C. clementine*. Tra l'altro una conferma del non coinvolgimento nel meccanismo di regolazione delle antocianine è stato dimostrato anche in seguito ad analisi filogenetica, nel cui albero erano stati inseriti gran parte dei fattori trascrizionali appartenenti alle famiglie MYB e MYC-like noti e dimostrati essere coinvolti nella regolazione delle antocianine in varie piante. Il bHLH da noi isolato è risultato segregare separatamente dai geni regolatori del pathway delle antocianine e, quando presente, si colloca vicino al GBOF-I di *T. gesneriana*.

I dati differenziali ottenuti attraverso la libreria sottrattiva hanno necessitato di un ulteriore sistema di validazione del livello di espressione, che dovrebbero confermare i dati ottenuti dalla Real time PCR. A tal proposito sono state utilizzate le potenzialità dei microarray. Tra le sequenze differenziali, 104 sono state spottate su un chip (costruito dalla Macrogen - Corea), insieme ad altre sequenze differenziali derivanti da altre fonti (129 da una libreria a cDNA-AFLP e 68 da HarvEST *Citrus*

database) per un totale di 302 sequenze; il chip è stato ibridato (CRIBI – Padova) con gli stessi campioni di polpa a maturità di Moro e Cadenera usati per la costruzione della libreria. Delle 104 sequenze SSH spottate sul chip, 35 sono risultate essere differenziali in seguito ad ibridazione. Tra questi sono stati evidenziati tutti i geni codificanti per gli enzimi strutturali della biosintesi delle antocianine; inoltre hanno risposto all'ibridazione anche, e non solo, quei trascritti che noi avevamo analizzato in RT-PCR semi-quantitativa perché ridondanti. I risultati ottenuti hanno mostrato che la ridondanza è manifestazione dell'alto livello di espressione genica; inoltre la ridondanza nella libreria e i dati di espressione differenziali, ottenuti attraverso gli array relativamente ai geni strutturali della biosintesi delle antocianine, hanno evidenziato come la principale differenza tra le cultivar pigmentate e quelle bionde risiede principalmente nei fattori implicati nella biosintesi di questo metabolita secondario. Questo avvalora la tesi secondo cui le due cultivar potrebbero essere considerate l'una la mutazione biologica dell'altra. Altro effetto non secondario ha riguardato la potenzialità della libreria sottrattiva a cDNA SSH, che si è dimostrata una tecnica molto sensibile nell'isolamento di sequenze differenziali, dato confermato anche dall'analisi array (infatti 35 delle 104 sequenze SSH sono risultate essere positive, contro 25 delle 129 isolate mediante libreria a cDNA-AFLP).

Inoltre si è eseguito un controllo sull'analisi del disegno dei 50mer spottati sul chip. Si è proceduto alla creazione di un database delle sequenze da analizzare e quindi si è eseguito un "Local Blast" tra le sequenze spottate sul chip e le sequenze dalle quali erano stati disegnati i 50mer. Questo approccio ha avuto lo scopo di essere sicuri che i 50mer fossero unici e specifici della sequenza originale sulla quale erano stati disegnati. In linea generale si è notato che il chip era stato costruito correttamente, anche se con alcune incongruenze. In alcuni casi è stata ritrovata la stessa sequenza di 50mer in due EST, delle quali una è la sequenza madre su cui era stata disegnata la porzione spottata, l'altra invece era una EST avente la stessa similarità ma in altro organismo. Viceversa sono state trovate alcune sequenze recanti più sonde, ma in uno di questi casi le due sonde e di conseguenza le due EST originali avevano similarità diverse. Si tratta di un caso di "artefatto da chimere", e meccanismo è possibile quando le sequenze di EST (utilizzate per la costruzione di un chip) derivano da una libreria sottrattiva a cDNA (Tomiuk e Hofmann, 2001). Questo tipo di dato deve essere tenuto in considerazione al momento della valutazione dei dati differenziali, perché in questi casi va verificata la stessa risposta all'ibridazione per la stessa sequenza spottata, nonostante sia localizzata su porzioni diverse. Se così non fosse si dovrebbe analizzare in dettaglio la struttura del gene.

Parallelamente è stato acquistato il GeneChip di *Citrus* dell'Affymetrix, il quale è costituito da 30,171 sequenze di cDNA rappresentanti 33,879 trascritti basati sulle sequenze EST di diverse librerie di varie specie di *Citrus* e di suoi ibridi. Il nostro interesse è stato rivolto all'analisi crociata dei dati differenziali ottenuti mediante l'analisi del chip mirato (costituito dalle sequenze isolate con la libreria sottrattiva e derivanti unicamente da polpa di arancio dolce a maturità) e dell'array Affymetrix (recante sequenze di origine differente), ma ibridati con gli stessi campioni di polpa di Moro e Cadenera a maturità. Avere a disposizione diversi dati differenziali derivanti da vari sistemi, quali la libreria sottrattiva SSH, il chip mirato e l'array Affymetrix, ci ha consentito di dedurre un sistema di analisi crociata, focalizzando l'attenzione sulla ricerca dei trascritti unici di polpa. L'iter analitico eseguito ha riguardato la costruzione di un database con tutte le sequenze EST di *C. sinensis* (94,127) presenti in banca dati fino al novembre 2006. Questa parte del lavoro è stata svolta in collaborazione con il gruppo della dott.ssa Maria Luisa Chiusano dell'Università

degli Studi di Napoli Federico II. Queste sequenze sono state sottoposte ad analisi di "clustering/assembling" (D'Agostino et al., 2005) al fine di organizzare le varie sequenze in *Tentative consensus* (TC) e singoletti. Tra tutti questi sono stati considerati i TC costituiti da almeno due EST, di cui almeno una di polpa. Dei 98 TC con almeno 2 EST, 70 sono risultati essere unici e specifici di polpa, di cui 43 derivavano dalla libreria sottrattiva, 18 dei quali sono risultati accesi in seguito all'ibridazione del chip mirato; 14 dei 70 TC invece hanno risposto positivamente all'ibridazione dell'array Affymetrix. La conferma differenziale di questi dati è stata fornita dai Reverse Northern (come conseguenza della costruzione della libreria sottrattiva), da RT-PCR semi-quantitativa e da Real time PCR. Dal punto di vista funzionale, le sequenze specifiche di polpa sono ricadute su varie categorie funzionali. Inoltre gli unici trascritti, che sono risultati essere isolati dagli approcci della libreria SSH e dai due array e la cui sovra espressione è stata confermata dagli approcci prima citati, sono risultati due enzimi della via biosintetica delle antocianine, vale a dire il DFR e l'UFGT. Questo dato è stato utile per supporre che nella polpa di arancio questi geni sono più specificatamente discriminanti e differenziali rispetto ad altri. Un altro dato per noi importante è stato aver constatato che dei 43 TC, le cui EST erano state isolate anche dalla libreria sottrattiva, 14 erano costituiti da EST isolate solo ed esclusivamente attraverso la libreria SSH. Questo vuol dire che la libreria differenziale costruita attraverso la metodologia della PCR select ha contribuito a fornire informazioni di sequenze non note prima in *C. sinensis.*

Non avere ancora a disposizione il genoma di *Citrus* ha limitato un po' le annotazioni delle sequenze isolate e la disponibilità di confrontarle con sequenze complete. Pertanto alcune sequenze derivanti dalla libreria SSH, che sono state trovate in banca dati in modo non completo in *C. sinensis* oppure complete ma in altre piante, sono state allungate utilizzando la tecnica della 5'-3' RACE (Rapid Amplification cDNA Ends) - PCR, al fine di ottenere informazioni (a livello strutturale) più dettagliate sulla regione codificante (cds). Attraverso la costruzione di primer interni (per colmare la porzione mancante di AAT) e in seguito all'applicazione della metodologia della 3' e 5' RACE PCR, sono state allungate le regioni codificanti di AAT, 10-idrossigeraniol ossidoreduttasi, citocromo b5, MADs e SRK e completato e sottomesso la cds di AAT (EU200366) e del bHLH (EU240878).

Tra tutti i trascritti isolati con la SSH, particolare attenzione è stata rivolta alla GST. Le GST costituiscono una famiglia genica, presente sia nel regno animale che vegetale. Nelle piante alcuni membri di questa famiglia svolgono ruoli nel metabolismo secondario, riconoscendo e trasportando sia substrati naturali sia xenobiotici (Sandermann, 1992). Inoltre le GST legano citotossine, come porfirine e droghe negli animali, e antocianine e ormoni nelle piante (Marrs, 1996; Mueller et al., 2000). Recenti studi hanno coinvolto le GST in processi di segnali indotti da stress (Loyall et al., 2000) e nella prevenzione dell'apoptosi (Kampranis et al., 2000). La compartimentalizzazione delle antocianine nel vacuolo è richiesta sia per limitare gli effetti mutageni e ossidativi degli intermedi del pathway (Ahmed et al., 1994; Rueff et al., 1995) sia per lo svolgimento delle principali funzioni biologiche. Non a caso le antocianine prodotte a carico dell'UFGT nel citoplasma, vengono legate al GSH e tramite la GST vengono dislocate a livello del vacuolo. Si distinguono sei diverse classi di GST (Edwards et al., 2000): Phi, Tau, Lambda, Theta, Zeta, MAPEG, a ognuna delle quali è assegnata una specifica funzione. Le 94,127 EST di *C. sinensis* (derivanti dal database) sono state sottoposte ad analisi di similarità contro banca dati UniProt, dalle quali sono state dedotte 370 ESTs putativamente codificanti per GST. La procedura di *clustering* e *assembling* (D'Agostino et al., 2005) ha dato modo

di dedurre 28 TC e 34 singoletti, che sono stati assegnati alle varie classi di GST sulla base della denominazione adottata per *Arabidopsis* secondo Edwards et al. (2000). L'impostazione preliminare bioinformatica è stata effettuata in collaborazione con il gruppo della dott.ssa Chiusano di Napoli. L'assegnazione delle varie classi è stata seguita dall'analisi e dall'identificazione della ORF più lunga, in maniera tale da distinguere una 5' UTR, la regione codificante (cds) e la 3' UTR. Quindi è stato analizzato il profilo di espressione sia sui pattern tessuto-specifici rispetto allo stadio di sviluppo e al tessuto (utilizzando l'approccio bioinformatico), sia dal punto di vista molecolare. Per quanto riguarda quest'ultimo approccio, l'analisi di RT-PCR semi-quantitativa è stata eseguita per verificare la deduzione e assegnazione effettuata *in silico* e per valutare il livello di espressione in diversi tessuti di arancio considerati: buccia, albedo, polpa, foglie giovani, foglie adulte e ovari sia nella cultivar pigmentata che in quella bionda. Da questa analisi è stato notato che la classe Phi sembra essere costituita da TC tessuto-specifici. In particolare la GST isolata in polpa in seguito alla costruzione della libreria sottrattiva, sia da deduzione *in silico* sia da analisi di espressione molecolare è risultata essere maggiormente espressa in polpa di arancio Moro, così come la Phi-29, che sembra essere specifica delle foglie giovani. Inoltre i dati sperimentali condotti sul livello di espressione, hanno mostrato una specificità in Cadenera del TC Tau-51. Questa parte del lavoro offre un interessante esempio di interdisciplinarietà e potenzialità che l'analisi bioinformatica e l'analisi biologico-molecolare possiedono, completandosi e supportandosi a vicenda.

L'ultimo filone considerato ha riguardato i geni regolatori, i quali rivestono grande importanza nel controllo di vari pathway e tra questi anche la biosintesi delle antocianine. Le analisi svolte in questa parte della ricerca sono state effettuate presso il laboratorio di Cathie Martin del JIC di Norwich (UK). Le informazioni a tal proposito in *C. sinensis* sono fino ai giorni nostri pressoché sconosciute. Si pensa infatti che, dal momento che l'unica differenza valida tra le arance pigmentate e quelle bionde riguarda il livello di espressione (piuttosto che le sequenze) dei geni strutturali codificanti gli enzimi della biosintesi delle antocianine, si è pensato di indagare il ruolo di alcuni geni regolatori di arancio dolce mediante esperimenti di trasformazione transiente sia di tipo omologo sia eterologo. E' noto che l'interazione tra i fattori trascrizionali appartenenti alle famiglie MYC e MYB-like (Grotewold et al., 2000) ha un maggiore effetto sull'attivazione della trascrizione e sul controllo della biosintesi delle antocianine rispetto all'azione dei suddetti fattori trascrizionali da soli. Esperimenti di trasformazione di protoplasti di foglie di *Nicothiana tabacum* hanno mostrato come il gene *myc2* (myc-like isolato da polpa di arancio Moro) se associato a un myb-like (*rosea*) di *Antirrhinum majus* attiva il promotore pDFR e ha un effetto maggiore rispetto all'azione dei corrispondenti geni da soli. Lo stesso dato è stato evidenziato utilizzando come promotore il pF3H. Inoltre la stessa analisi condotta con il *myb8* di arancio non ha mostrato dati di espressione interessanti, confermando il probabile non coinvolgimento di questo gene nei meccanismi di regolazione, come era stato già evidenziato in analisi di similarità e filogenesi. Esperimenti di trasformazione transiente effettuati con bombardamento e agroinfiltrazione sia omologa sia eterologa non hanno avuto alcun esito positivo e hanno inoltre mostrato e confermato la difficoltà esecutiva dovuta alla natura recalcitrante e fisiologica dei campioni di arancia.

In conclusione, dal punto di vista delle metodologie, questo lavoro di dottorato ha consentito di trarre le seguenti osservazioni:

- La libreria sottrattiva a cDNA mediante SSH si è rivelata un ottimo sistema di isolamento di sequenze differenziali. Inoltre, uno dei possibili svantaggi che questa metodologia offre, vale a dire la ridondanza, si è rivelata invece un buon criterio di analisi e discriminazione dell'alto livello di espressione.
- Si è inoltre avuto modo di utilizzare e confrontare diverse metodologie di analisi sul livello di espressione, quali la Real time PCR e due tipi di array, i quali, pur essendo costruiti in maniera diversa, sono stati utili nell'analisi crociata di dati differenziali.
- É stata sperimentata la stretta collaborazione tra il supporto bioinformatico e l'approccio sperimentale molecolare, dando modo di verificare praticamente delle supposizioni avanzate in una prima fase *in silico*.
- Esperimenti di trasformazione transiente di protoplasti su *N. tobacum* sono stati utili per studiare la funzionalità dei fattori trascrizionali. Inoltre esperimenti di trasformazione transiente quali bombardamento e agroinfiltrazione condotti anche su arancia hanno confermato la difficoltà nella realizzazione di tali analisi, soprattutto in funzione della difficile natura e fisiologia del frutto in questione.

Inoltre dal punto di vista concettuale:

- La libreria SSH ha dato modo di isolare e sottomettere alla dbEST della GenBank 201 sequenze nuove per *C. sinensis*.
- É stato confermato che una delle principali differenze tra le arance pigmentate e quelle bionde riguarda i geni implicati nella biosintesi delle antocianine. Questo ci ha dato modo di confermare la supposizione secondo cui le arance pigmentate si debbano considerare una cultivar sorta per mutazioni dalla cultivar non pigmentata. Tra l'altro sembrano essere particolarmente determinanti anche geni coinvolti nella composizione strutturale della parete dei frutti (non a caso l'importanza delle pectinesterasi) e della composizione aromatica. Non meno importanza rivestono trascritti implicati in altre categorie funzionali.
- Grazie all'utilizzo dei dati forniti dalla libreria sottrattiva, dal supporto dei due microarray e dai sistemi di analisi e validazione dei dati differenziali, si è estrapolato una prima analisi di identificazione di geni tessuto specifici di polpa di arancio, restringendo il campo a due geni codificanti per due enzimi strettamente coinvolti nella biosintesi delle antocianine, quali il DFR e l'UFGT.
- Relativamente all'importanza che le antocianine rivestono e in particolar modo alle GST (enzima implicato tra l'altro nella vacuolarizzazione di questi metaboliti secondari) e alla loro multifunzionalità, dal punto di vista sperimentale la relazione tra l'approccio bioinformatico e quello molecolare ci ha consentito di condurre delle analisi di espressione e dimostrare, tra l'altro, l'*up* regolazione in polpa della GST isolata attraverso la libreria sottrattiva. Inoltre questo approccio è stato interessante per determinare la specificità di altre GST nei vari tessuti e cultivar analizzate.
- Attraverso un'indagine rivolta all'analisi dei geni regolatori, si è dimostrato che esperimenti di trasformazione transiente di tipo eterologo mediante trasformazione di protoplasti hanno confermato la necessaria interazione tra le due classi di fattori trascrizionali Myc e Myb-like, per agire su specifici promotori, al fine di attivare il meccanismo di trascrizione e quindi di regolazione.

I principali ambiti che pensiamo di approfondire in futuro riguardano:

(1) l'analisi di quei trascritti che hanno in comune tutti i tessuti in cui si sa essere presente l'antocianina, in maniera tale da dedurre non solo un pathway biosintetico ma anche cercare di estrapolare una rete metabolica capace di relazionare tra loro i vari trascritti evidenziati. Inoltre pensiamo di focalizzare l'attenzione ai trascritti comuni a più tessuti, in maniera tale da dedurre una funzionalità di base.

(2) l'elaborazione dati degli array alla luce dei dati ottenuti dall'analisi del disegno delle *probe* del chip mirato e l'analisi dell'array Affymetrix, utilizzando un sistema che consente di monitorare, escludere e/o riammettere nella lista dei trascritti differenziali quelli che sono stati scartati, sulla base del protocollo di analisi considerato.

(3) l'analisi sulla tessuto specificità delle GST, monitorando tessuti e specie di *Citrus* caratterizzati dalla pigmentazione antocianica, in modo tale da circoscrivere l'analisi tra l'altro anche alla vacuolarizzazione delle antocianine. Questi dati dovranno essere supportati non solo dall'approccio bioinformatico, ma anche dall'analisi biochimica e da esperimenti di trasformazione transiente. Inoltre è nostra intenzione arricchire le conoscenze sull'organizzazione strutturale genica delle varie classi di GST.

(4) la messa a punto di un protocollo di trasformazione transiente utilizzando frutti di arancio, cercando di superare e risolvere gli inconvenienti e gli ostacoli di natura fisiologica di tali frutti, al fine di ottenere delle informazioni sulla specificità funzionale dei geni regolatori isolati in arancio, e gettare in questo le basi per una futura trasformazione stabile.

1. INTRODUCTION

1.1 The blood oranges

1.1.1 The origins

According to the historical notices, it seems that the first oranges in the Mediterranean area were blond ones. The first reference about a pigmented orange appears in the *Hesperides* of Ferrari (1646), in which he describes about an *Aurantium indicum* with a blood flesh, introducing in Italy through a missionary from Genova returning from Filippine. Even if there are many references about pigmented fruits in Chinese documents before that data, today we can't trace the origins of the Sicilian oranges ancestor.

Even if the growing of blond oranges (Navel, Valencia) had an exponential process in comparison to blood ones, there aren't mutations producing anthocyanins derived from new selections of common oranges. According to fingerprinting, isoenzimatic and molecular analysis, no segregation and ricombination gave rise to the two different oranges. Thus it seems that just one mutation event separated blood and common oranges and then other mutation events differentiated new selections.

1.1.2 The Sicilian oranges production

In Italy, and specially in Sicily, the most important and promote oranges are blood ones, such as Moro, Tarocco and Sanguinello (Zarbà, 1994). Blood oranges, specially grown in the East Sicily, have particular flavour and organoleptic characteristics (Fig. 1). In these areas the pedoclimatic conditions are suitable to obtain the red colour of the flesh and a perfect balance between sugar and acidity (Rapisarda et al., 1998). Mostly the range between night and day (sometimes almost 20°C) contributes to anthocyanins biosynthesis.

Fig. 1 The main production area of Sicilian oranges

1.2 The anthocyanins

1.2.1 General characteristics

Among all plant secondary compounds, anthocyanins have been investigated most extensively in the areas of chemistry, biochemistry and genetics.

The anthocyanins (from Greek *antos*, flower, and *kyanos*, blue) are natural red, purple and blue pigments, mostly present in plant epidermal cells, into the vacuoles, in which give colour to different tissues (Rimari and Strommer, 1998).

They are ubiquitous natural pigments, found in many plant species. In the reproductive organs and in the fruits, anthocyanins may attract pollination and help with seed dispersal, respectively (Holton and Cornish, 1995). During the autumn season, the red pigment in the leaves seems to prevent photo-oxidative damage, senescence (Field et al., 2001) and rapidly developing shoots of tropical trees. Several plants may mask themselves with anthocyanins to intercept UV irradiation (Holton and Cornish, 1995). They can act as antioxidants, phytoalexins or as antibacterial agents. Anthocyanins may be important factors along with other flavonoids in the resistance of plants to insects attack (Harborne, 1988). For example, cyanidin 3-glucoside was shown to protect cotton leaves against the tabacco budworm (Hedin et al., 1983).

The antioxidant activity of anthocyanins gives cause for a variety of medicinal usage: prevention of cancer, anti-inflammatory and anti-arteriosclerosis activities (Fauconneau et al., 1997; Nijveldt et al., 2001).

On *Citrus* mature fruits anthocyanins are exclusively expressed in blood oranges and its hybrids. In other *Citrus* fruits as grapefruit, blood Vaniglia, Cara Cara, the red colour depends by lycopene. Anthocyanins in rind, leaves and flowers of some *Citrus* (as limon, *Papeda*, etc) are evident only during the first developmental stage. In blood orange fruits anthocyanins production is strictly linked to genotype and environmental conditions. Moreover the expression levels change between flesh and rind. It causes the impossibility to ensure a constant amount of anthocyanins in *Citrus* fruits.

In horticultural crops, colour is an important consideration in consumer choice. In orange, in apple, grape, cherry, strawberry, and others fruits, there has been much interest in breeding varieties bearing fruits with altered colour, hues, patterns, or total anthocyanin content.

1.2.2 Orange fruits: clones and selections

Among blood oranges (Tarocco, Moro, Sanguinello) Tarocco is the most known and widespread (58%, against 20% and 22% of Moro and Sanguinello, respectively). It seems that it could be a mutation of Sanguinello and its origins are in the last century in Francofonte (Siracusa; Zarbà and Pulvirenti, 2006). The most important characteristics are the fruit size and the easy peeling. The last one could be a drawback, because it reduces its shelf life period. In the ’70 years, when genetic breeding of Tarocco started, the first selection diffuse in commercial plantings was the Tarocco nucellare 57-1E-1 (Fig. 2).

Fig. 2 Tarocco nucellare 57-1E-1 fruits

During the last 30 years, different selections were isolated and characterized. Nucellar or micrografted selections were used to ensure free virus propagation material (Reforgiato Recupero and Tribulato, 2000; Reforgiato Recupero and Russo,

2001, 2002). The request to have an high anthocyanins content in the flesh and in the rind and a prolonged maturation period was satisfacted by new different selections. A ripen fruit might have red colour in flesh and rind and a correct balance of sugar and acidity.

1.3 Chemical properties

The biosynthesis of the C15 flavonoid skeleton has been well-studied and reviewed (Heller and Forkmann, 1994; Stafford, 1990). Commonly, flavonoids are further modified by the addition of substituent groups (R1 and R2) such as methyl groups, aromatic acyl groups, and/or sugar moieties (Heller and Forkmann, 1994; Holton and Cornish, 1995).

The differences between individual anthocyanins are related to the number of hydroxyl groups, to the nature and the number of sugars attached to the molecule, the position of this attachment, and the nature and the number of aliphatic or aromatic acids attached to sugars in the molecule (Fig. 3).

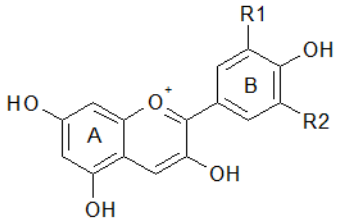

Fig. 3 Anthocyanin structure

Based on several reviews to date, it is estimated that more than 400 anthocyanins have been found in nature (Konga et al., 2003). The most common are listed in Tab 1. Only six anthocyanidins are common in higher plants: pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp). The glycosides of the three non-methylated anthocyanidins (Cy, Dp and Pg) are the most widespread in nature, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers. The distribution of the six most common anthocyanidins in the edible parts of plants is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%). So, the most widespread anthocyanin is the cyanidin 3-glucoside.

Name	Substitution pattern							Color
	3	5	6	7	3'	4'	5'	
Apigenidin	H	OH	H	OH	H	OH	H	Orange
Aurantinidin	OH	OH	OH	OH	H	OH	H	Orange
Capensinidin	OH	OMe	H	OH	OMe	OH	OMe	Bluish-red
Cyanidin	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	OH	OH	H	OH	OH	OH	OH	Bluish-red
Europinidin	OH	OMe	H	OH	OMe	OH	OH	Bluish-red
Hirsutidin	OH	OH	H	OMe	OMe	OH	OMe	Bluish-red
6-Hydroxycyanidin	OH	OH	OH	OH	OH	OH	H	Red
Luteolinidin	H	OH	H	OH	OH	OH	H	Orange
Malvidin	OH	OH	H	OH	OMe	OH	OMe	Bluish-red
5-Methylcyanidin	OH	OMe	H	OH	OH	OH	H	Orange-red
Pelargonidin	OH	OH	H	OH	H	OH	H	Orange
Peonidin	OH	OH	H	OH	OMe	OH	H	Orange-red
Petunidin	OH	OH	H	OH	OMe	OH	OH	Bluish-red
Pulchellidin	OH	OMe	H	OH	OH	OH	OH	Bluish-red
Rosinidin	OH	OH	H	OMe	OMe	OH	H	Red
Tricetinid	H	OH	H	OH	OH	OH	OH	Red

Tab. 1 The most common naturally occurring anthocianidins.

Among sweet oranges the blood ones distinguish firstly for the excellent sensorial properties, as a pleasant taste, an intense and characteristic fragrance, the balanced ratio sugar/acidity (Rapisarda, 2000). Pigmented oranges are mostly different for the rich burgundy brilliant red colour of the flesh and sometimes of the peel, due to anthocyanin pigments (Maccarone et al., 1983).

Pigmented oranges are different from common ones not only for anthocyanins, but also for higher ascorbic acid content, flavanones and hydroxicinnamic acids (Rapisarda et al., 2001a), substances with antioxidant properties.

Blood orange juice is an watery acidic solution (pH ~ 3.3), not clear because pectines, red colour, delicious sweet, acidic taste and characteristic fragrance (Maccarrone, 1997). Different substances, as glucoside flavanones (the most important is hesperidin; Trifirò et al., 1980) and vitamins C, A, B1, B2, PP, confer an important nutritive value to the juice.

The fragrance is constituted by a combination of volatile and semi-volatile compounds (alcohol, ester, terpen), and among them the limonene (originally present in the oil sacs of the rind) is the most important.

1.3.1 Blood oranges and antioxidant compounds

According biochemical compounds, blood oranges represent a rich source of bioactive substances, as vitamin C, polyphenol compounds, as hydroxicinnamic acids, flavanones and anthocyanins, as well as limonoids and corticoids. All of them act as antioxidants and free anti-radicals, because of their chemical nature, in particular the number and the position of the oxidrilic groups and the electron-donor substitutents.

1.3.1.1 Vitamin C

The request of *Citrus* in the human diet is mostly due to the ascorbic acid (vitamin C), variable in relation with the cultivar, the ripening time, the whether and other different agronomic factors (Di Giacomo and Calvarano, 1972; Maccarone, 1983; Rapisarda and Intelisano, 1996). Moreover, ascorbic acid is a strong antioxidant, that destroys oxygen free radicals (Gadjeva et al., 2005), takes part in cellular breathing and collagen synthesis and takes on iron permeability process (Cook and Monsen, 1977; Hazell and Johnson, 1987).

The ascorbic acid is one of the main nutrients in orange juice. Thus, some paper focused their attention on the evaluation of the ascorbic acid stability in red pigmented blood orange juice and its detrimental effect on colour stability. In blood orange juices vitamin C ranges between 40-80mg/100ml and in Tarocco cultivar it is about 70-80mg/100ml (Rapisarda and Intelisano, 1996). Moreover fruits storage for long period could decrease vitamin C content (Nagy, 1980). Infact after 65 days Tarocco and Moro registered a low decrement in ascorbic acid content, without compromising the antioxidant properties of the fruits.

Anthocyanin stability may also be influenced by other fruit components, especially the interaction of ascorbic acid with anthocyanins, and a subsequent mutual degradation has been reported in various fruit juice model systems including cranberry juice (Shrikhande and Francis, 1974; Starr and Francis, 1968), strawberry and blackcurrant products (Skrede et al., 1992). The interaction of ascorbic acid with anthocyanin pigments results in the degradation of both and a decrease in the colour and nutritional quality of the products (Markakis, 1982).

1.3.1.2 The hydroxicinnamic acids

The blood oranges contain esters and glucosides of hydroxicinnamic acids (caffeic, p-malic, ferulic and sinapic) in major quantity respect to common ones (Rapisarda et al., 1998). These substances (precursor of flavonoids and anthocyanins) act as antioxidants (Wang et al., 1997). The distribution of hydroxicinnamic acids is characteristic of blood and common cultivars, and it could be used to check the natural origin of blood orange juices against eventually addiction with blond orange juices (Arena et al., 1997).

1.3.1.3 The flavonoids

The flavonoids are a group of C-15 phenolic compounds, which comprise one of the most abundant groups of secondary metabolites in plants. Flavonoids serve a wide variety of roles: (i) UV protectants in leaves (Werner, 1998); an important cue in pollen development (Waser and Price, 1983) and in pollen germination (Mo et al., 1992; Vogt and Taylor, 1995; Vogt et al., 1995); (ii) phytoalexins giving resistance to pathogenic microbial attack; (iii) inducing nod genes of rhizobia for nodule formation of roots (Koes et al., 1994; Stafford, 1997); (iv) as defense agents against predation and pathogens (Dakora, 1995). It has been also well established that some flavonoids, such as anthocyanins, have an important role in the floral coloration and can serve as pollinator cues (Koes et al., 1994).

In *Citrus* the most important flavonoid classes (Fig. 4) are the flavanones and the anthocyanins (specific and unique of the pigmented oranges). In relation to the quantity, the most significant are hesperidin, narirutin and didimin (Tab. 2). Proteggente et al. (2003) saw that in blood oranges the hesperidin and the narirutin content is almost 2-3 times higher than blond oranges.

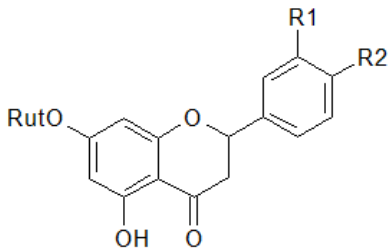

Fig. 4 Chemical structures of the flavonoid classes in oranges (Rut = Rutinosio)

	R_1	R_2
Hesperidin	OH	OCH_3
Narirutin	H	OH
Dimidin	H	OCH_3

Tab. 2 The most abundant flavonoid in oranges

Moreover *in vitro* and *in vivo* analysis demonstrated the anti-cancer and the anti-mutagen effects of flavonoids. For example, a decrement of almost 22% of colon cells tumor incidence on guinea pig following orange juice diet was demonstrated. Proteggente et al. (2003) indicated that glucuronide hesperidin protects fibroblast of the skin against oxidative stress induced by UVA ray. Finally, flavonoids own also therapeutical properties, as anti-inflammatory, anti-hypertensive, diuretic and analgesic effects.

1.3.1.4 The antocyanins and factors controlling

Tarocco, Moro and Sanguinello are cultivars of sweet oranges containing anthocyanins in mature fruits. These pigments accumulate mostly in the juice sacs of the flesh.

The anthocyanins are glicosilated in 3 and 5 position of A and B ring (Fig. 3). The sugars are usually glucose, galactose and ramnose. In the blood orange juices the most widespread anthocyanins are the cyanidin-3-glucoside and the cyaniding-3-(6''-malonyl)- β-glucoside (Maccarone et al., 1983; Maccarone et al., 1985; Maccarone et al., 1998). In Tab. 3 the most abundant and widespread anthocyanins are indicated.

Anthocyanidin	R_1	R_2
Cyanidin	OH	OH
Delphynidin	OH	OH
Pelargonydin	H	H
Peonydin	OCH_3	H
Petunydin	OCH_3	OH

Tab. 3 The most widespread antocyanins in blood oranges

The anthocyanins behave as pH indicators, because they have a red colour in acid environment (pH < 4). Moreover, the stability of anthocyanins at different pH and temperatures has been widely studied (Mazzaracchio et al., 2004).

In relation to the medical and pharmacological relevance, the anthocyanins are used in ophthalmology therapy, because the regeneration of rodopsin, in the ulcer treatment and in angiology for its epithelium-repair properties and capillary permeability, in all phisiopathological conditions characterized by an excess of free radicals production (Saija, 1994). Even the cyanidin is more efficient than the vitamin C as scavenger effect (Wang et al., 1997).

In the last years different Tarocco clones were tested to evaluate the anthocyanin content. It was seen that the red pigment in "Vitale", "Tringale", "Sciara", "Gallo" and "Rosso" depends by the genotype and increases during the ripening time. Anthocyanin biosynthesis in orange fruits is also depending by external factors:

1) the light, even if in some case it could be speed up its degradation (Maccarone et al., 1987).

2) the position of fruits in the tree. Rapisarda et al. (2001b) noted that the major anthocyanin concentration is in fruits facing north.

3) the temperature and mostly the range between day and night. This is the reason because East Sicily and South and West-South mountain Etna place (Catania, Enna, Siracusa) are suitable for growing blood oranges.

Anthocyanins thermal degradation has been studied for strawberry (Markakis et al., 1957), black raspberry (Daravingas and Cain, 1968), raspberry (Tanchev, 1972), Concord grape (Calvi and Francis, 1978), plum (Raynal and Moutounet, 1989) and sour cherry (Cemeroglu et al., 1994). Recently (Krifi et al., 2000), the degradation of anthocyanins in blood orange juice was investigated during storage at -18°C for few days and at 4°C in nitrogen for 12 months. However, there are no kinetic data for the degradation of blood orange anthocyanins. Kirca and Cemeroglu (2003) determined the kinetic parameters for blood orange anthocyanins in both juice and concentrates during heating and storage at various temperatures. Moreover Rapisarda et al. (2001b) reported how conservation at 8°C of Tarocco and Moro

fruits positively affects pigment content, mostly in the first cultivar, maybe for the activation of phenilpropanoid enzymes by low temperatures.

1.4 The anthocyanins and the nutraceutical value

In an endeavour to identify the active health-promoting ingredients, many researchers have focused on the properties of the flavonoids. Among them, the anthocyanins are of particular interest for the food colorant industry, because of their ability to impart vibrant colours to the product.

The anthocyanins were incorporated into the human diet many centuries ago. They were components of the traditional herbal medicines used by North American Indians, the Europeans and the Chinese, and they were habitually derived from dried leaves, fruits (berries), storage roots, or seeds.

They are important in nutrition because of their daily intake, which is estimated at around 200 mg in the USA (Kuhnau, 1976). Anthocyanin-rich mixtures and extracts (though not purified compounds) have been used historically to treat conditions as diverse as hypertension, pyrexia, liver disorders, dysentery and diarrhoea, urinary problems including kidney stones and urinary tract infections. They may reduce the risk of coronary heart disease (Renaud and de Lorgeril, 1992), the inflammatory insult (Youdim et al., 2002), they may modulate the immune response (Wang and Mazza, 2002) and exert anti-carcinogenic activities *in vitro* (Fimognari et al., 2004).

These actions might be mediated by their antioxidant activity (Tsuda et al., 1998; Mazza et al., 2002) owing to their particular chemical structure, which is characterized by an electron deficiency making them particularly sensitive to reactive oxygen species (Galvano et al., 2004). Although numerous studies have evaluated the absorption and the metabolism of anthocyanin glycosides (Miyazawa et al., 1999; Tsuda et al., 1999; Bub et al., 2001; Cao et al., 2001; Matsumoto et al., 2001; Felgines et al., 2002; Wu et al., 2002; Frank et al., 2003; McGhie et al., 2003; Nielsen et al., 2003; Bitsch et al., 2004; Cooney et al., 2004; Ichiyanagi et al., 2004, 2005; Kay et al., 2004; Talavera et al., 2006), only few reports are available on the absorption and metabolism of acylated anthocyanins (Suda et al., 2002; Harada et al., 2004).

About the use of *Citrus* extract, it was evaluated the absorption and metabolism of red orange juice anthocyanins in rats fed for 12 d with an anthocyanin enriched diet ('adapted rats'). Although numerous studies have focused on anthocyanin glycoside bio-availability (Miyazawa et al., 1999; Tsuda et al., 1999; Bub et al., 2001; Cao et al., 2001; Matsumoto et al., 2001; Felgines et al., 2002; Wu et al., 2002; Frank et al., 2003; McGhie et al., 2003; Nielsen et al., 2003; Bitsch et al., 2004; Cooney et al., 2004; Ichiyanagi et al., 2004, 2005; Kay et al., 2004; Talavera et al., 2006), there have been very few attempts to evaluate the absorption and metabolism of acylated anthocyanins.

1.4.1 The antioxidant properties

The excess production of free radicals and reactive oxygen species (ROS), such as singlet oxygen ($^{\cdot}O_2$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$), is thought to cause damage in cells. This damage may be involved in the aetiologies of diverse human diseases, such as atherosclerosis, ischemic injury, inflammation, cancer, aging, and neurodegenerative diseases (Parkinson's and Alzheimer's; Good et al., 1996; Gassen and Youdim, 1997; Halliwell and Gutteridge, 1999). ROS formed may cause cellular and subcellular damage by

lipid oxidation of membrane lipids, denaturing cellular proteins, breaking DNA strands and disrupting cellular functions (Maxwell, 1995). Oxidative damaged DNA bases, products of lipid oxidation, lipid radicals and antioxidant depletion have been found in UV irradiated skin (Fuchs et al., 1989; Jurkiewicz and Buettner, 1994; Hattori et al., 1997) and in the formation of apoptotic keratinocytes and depletion of Langerhans in the epidermis (McVean et al., 1999). Russo et al. (2002) showed the effect of anthocyanins for all the components present expressed by its capacity to protect DNA, to scavenge free radicals, and to inhibit xanthenes oxidase activity. Moreover, in relation to its antioxidant activity, this extract could have cosmetic benefits and may mitigate the consequences of skin photo aging. This natural compound could also prove useful to athletes, because the need for antioxidants is enhanced in individuals subjected to prolonged physical exercise. In fact, increased amounts of the products of free radical-mediated lipid oxidation and a depletion of antioxidant systems have been detected in muscle tissue following prolonged exercise (Sen, 1999).

Konga et al. (2003) showed that anthocyanins had strong antioxidative activity in a liposomal system and reduced the formation of malondialdehyde from UVB (320–290 nm) irradiation. The antioxidant activity in fruits and leaves from different cultivars of the thornless blackberry (*Rubus* sp.), red raspberry (*Rubus idaeus* L.), black raspberry (*Rubus occidentalis* L.) and strawberry (*Fragaria x ananassa* D.) was reported by Wang and Lin (2000). The results showed a linear correlation between the total phenolic content and ORAC activity for the fruits and leaves. For ripen berries, a linear relationship existed between ORAC values and the anthocyanin content. Horwitt (1933) observed that the urine of rabbits, that were fed with 500 mg of anthocyanin pigment from grapes, became highly pigmented. So small quantities of the grape anthocyanins or anthocyanidins were absorbed and passed through to the circulation.

There are a variety of methods to assess 'total' antioxidant activity (Huang et al., 2005; Prior and Cao, 1999; Prior et al., 2005) and they differ for the different chemistries and the sensitivity of the assays. A recent but widely accepted analysis, the ORAC (oxygen radical absorbent capacity) method (Cao et al., 1993; Cao and Prior, 1999; Cao et al., 1995; Ou et al., 2001; Prior et al., 2003) with subsequent development (Huang et al., 2002; Ou et al., 2001), was more sensitive than the iron based assay (FRAP) (Ou et al., 2002), 2,2- azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Leong and Shui, 2002; Miller and Rice-Evans, 1997) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams et al., 1995; Gil et al., 2002).

1.5 Factors controlling anthocyanins variability

The character '*presence of anthocyanins*' in oranges flesh and rind has a variable expression. Infact it's possible that the red pigment of both tissues could be regulated by different factors and situations.

Blood oranges growing is concentrated in East Sicily, mostly for pedoclimatic conditions, necessary to have a good anthocyanin expression and sugar/acidity ratio. Tarocco cultivar account for around 45% of Italian orange harvest with almost 50.000 hectars. New selections ensure orange availability from December to May.

1.5.1 The influence of genotype

In *Citrus* anthocyanins are expressed in young shoots and fruits and some floral tissues of lemon [*Citrus limon* (L.) Burm. f.], citron (*Citrus medica* L.), and *C. ichangensis* Swing. But among the mature fruits, they are expressed exclusively in

blood oranges and their hybrids. In pigmented cultivars the anthocyanin content is under genetic control. It is also highly variable in the rind and in the flesh. This leads to the conclusion that different factors occurrs to the anthocyanin biosynthesis.

Among pigmented oranges, the genotype hardly influeces the anthocyanin content. For example, Moro cultivar is the selection with a high anthocyanin content in flesh. Among Tarocco cultivars, TDV and TAPI selections have the most high content of the red pigment. But Cotroneo et al. (2006) showed that the hybrid OTA 9 ('Oroval' clementine X 'Tarocco' orange) has the most high content.

In relation with the presence of anthocyanin of the rind, the highest value are in Moro orange and in Tarocco Lempso.

1.5.2 The influence of rootstock

The main rootstock used to grow *Citrus* trees is the sour orange (*Citrus aurantium* L.). Rootstocks are important not only for tree performance and fruit quality, but also for Tristeza tolerance, a recently virus spraying unfortunately also in Sicily.

On the basis of trials carried out at experimental farm of Palazzelli (CRA-ISAGRU), trifoliate orange (*Poncirus trifoliata* Raf.) is the rootstock able to induce the highest content of anthocyanin and total soluble solids. The correlation between anthocyanin and total soluble solids was also confirmend in a trials with 20 rootstocks grafted on Tarocco TDV. Moreover in our laboratory (Cotroneo PS, personal communication) it was conduced a study about the importance of rootstock in relation with the influence on the anthocyanins content. It was compared three different rootstocks and sour oranges, used as control. One of them improves and increases anthocyanin content, compared with the control. But there isn't a strictly correlation between rootstock and ORAC value: maybe it is because ORAC amount depends not only by anthocyanins, but also by antioxidant factors in general, as vitamin C, hydroxicinnamic acid and flavonoids.

1.5.3 External factors

The anthocyanin accumulation depends by various stimulus: (i) light (activate gene expression involved in anthocyanin biosynthesis; Mol et al., 1996); (ii) temperature; (iii) nutritive (Lancaster, 1992), genetic (Honda et al., 2002) and physiological factors; (iv) pedoclimatic conditions. They are important not only in the activation of structural genes involved in the anthocyanin biosynthesis, but also in regulatory genes ones. The main differences between blood and common oranges seem to depend by transcription factors, acting in different steps of anthocyanins pathway.

1.5.3.1 The light

One of the most important environmental cue in anthocyanin synthesis is the light, which affects signal trasduction and gene expression involved in anthocyanin biosynthesis (Mol et al., 1996). Other factors, such as sugar addition (Tsukaya et al., 1991), phosphate limitation (Dixon and Paiva, 1995) and cold stress (Levya et al., 1995) can enhance anthocyanin accumulation induced by light. The effects of sugars have been studied especially in reproductive organs: for example in flowers the sugar levels increase during petal development.

The anthocyanin concentrations vary in relation with the cultural methods, the developmental stage of fruit, the position of fruit in the tree, or even the different sides of the same fruit (Saure, 1990; Ju et al., 1995b). In apple skin, sunlight is the most important external factor regulating anthocyanin synthesis (Saure, 1990;

Lancaster, 1992). It is believed that the bagging increases light sensitivity of fruit and stimulates anthocyanin synthesis when fruits are re-exposed to light after bag removal. However, the direct effect of fruit bagging is not to promote, but to inhibit anthocyanin synthesis (Ju et al., 1995a, 1995b, 1997). Therefore, it is possible to obtain fruits without anthocyanin accumulation in fruit peel if fruits were kept away from light during the total development period.

Moreover it was recently demonstrated that in the apple red skin of the Cripps' Red cultivar, several flavonoid genes required for anthocyanin synthesis were co-ordinately transcribed in response to light exposure (Takos et al., 2006).

1.5.3.2 The temperatures

The cold induction of pigmentation has been studied in flower development and related to the activation of the expression of anthocyanin biosynthetic genes, including *pal* (phenylalanine ammonia lyase), *chs* (chalcone synthase), *dfr* (dihydroflavonol 4-reductase) and *ans* (anthocyanidin synthase; Martin and Gerats, 1993). As regards tree fruits, studies on low temperature-induced anthocyanin accumulation have mostly been carried out on apple and grape skins (Cantos et al., 2000). It has been shown that post-harvest storage temperature can affect anthocyanin levels in small fruits, such as strawberries (Kalt et al., 1999), cranberries (Kalt et al., 1999), and several blueberry cultivars (Connor et al., 2002); in last one, Connor et al. (2002) showed that the anthocyanins contents of fully ripe blueberry fruits (100% degree of maturity) did not change significantly once they were subjected to a post-harvest low temperature exposure for periods ranging between 3 and 7 weeks; moreover depending on cultivar type, pigments accumulation might occur but only in fruits with other degrees of maturity (ranging between 50 and 75%).

However, the molecular basis of anthocyanins accumulation due to low temperature exposure, concerning the fruits edible portion, has not yet been established. Several cultivars of blood orange are characterized by the presence of anthocyanins in both the rind and the juice vesicles (Rapisarda and Giuffrida, 1992). It has shown that in the post-harvest phase, fruits can be stored at low temperatures for different long periods (Grierson and Ben-Yehoshua, 1986). The storage temperature, as well as the duration, turned out to be critical for orange fruits as prolonged storage, over 3 months, at 4°C negatively influences the sensory quality of blood orange juices, due to the increase of the malodorous substance vinylphenol (Fallico et al., 1996). Therefore, because the anthocyanins content of blood oranges represents a very high quality marker, it is desirable to find a convergence point, for temperature and storage duration, to obtain the maximum of anthocyanins accumulation and a high fruit's acceptability by consumers, trying also to limit the cost of too prolonged thermo controlled storage. Lo Piero et al. (2005b) studied the impact of a low temperature exposure (4°C) during a moderately long storage period (75 days) on Tarocco orange cultivar, the anthocyanins production and the expression of structural genes involved in their biosynthesis, such as *chs*, *dfr* and *ufgt* (UDP-glucose flavonoid glucosyl transferase; Lo Piero et al., 2005a). The maximum of anthocyanins accumulation has been gained after 75 days of storage, during which it has been shown that vinylphenols had not yet formed (Fallico_et al., 1996). Interestingly, orange fruits subjected to a brief low temperature exposure (45 days) still maintained higher levels of anthocyanins than those registered in control samples. Lo Piero et al. (2005b) also monitored the general pattern of total RNA content and it was seen that at 4°C the amount of total RNA was higher than in fruits stored at 25°C. Thus suggests that low temperature storage, at least in the first 30 days of storage, preserves processes linked to RNA biosynthesis, such as

transcription, and related to RNA function such as synthesis of proteins. Moreover the expression of PAL, CHS, DFR, and UFGT was strongly induced during low temperature exposure since levels of all transcripts increased at least 40-fold with respect to control samples. Maybe the increase of *pal* during cold storage could provide with substrates all of the enzymes located downstream in the pathway and it may represent the key enzyme for general control of stress induced plant response and therefore strictly controlled by environmental conditions. So the accumulation of anthocyanins due to cold exposure is related to the expression of their key biosynthetic enzymes in the edible portion of fruits.

As the low temperatures, also the humidity is an important factor. In fact the shortage or an excess of this parameter during irrigation decrease anthocyanin content in grape (Madero-Tamargo et al, 1979; Guilloux, 1981).

1.5.4 The influence of hormones

1.5.4.1 Gibberellins

The gibberellins (GAs) increase anthocyanin accumulation in the corolla of *Petunia* flowers by raising the transcription of flavonoid biosynthetic genes (Weiss et al., 1992). But gibberellins decrease the anthocyanin accumulation in carrot cell suspension cultures (Cheng et al., 1985; Hinderer et al., 1984; Ozeki and Komamine, 1986) and in radish seedlings (Jain and Guruprasad, 1989), even if the mechanism of GA inhibition has not been jet identified. When anthocyanin-producing carrot cells are cultured in presence of GA_3, a 3'-nucleotidase is rapidly synthesized, dephosphorylating the malonyl-CoA and producing an inhibitor of chalcone synthase (Ilan et al., 1994).

1.5.4.2 Abscissic acid

In grape berries anthocyanin accumulation is enhanced by ABA (Kataoka et al., 1982; Ban et al., 2003) and suppressed by synthetic auxins (Ban et al., 2003; Davies et al., 1997), high temperature and low light intensity. Recently, Ban et al. (2003) also reported that ABA treatment enhanced the mRNA accumulation of seven anthocyanin biosynthetic enzyme and that 2,4-dichlorophenoxyacetic acid suppressed them. Jeong et al. (2004) examined the effects of plant hormones, ABA and NAA (naphthaleneacetic acid) and shaded treatments on accumulation of the anthocyanin biosynthetic pathway genes and *VvmybA1* in berry skins, using the red wine grape Cabernet Sauvignon. As expected, the juice of the ABA-treated grape 4 weeks after veraison showed a higher concentration of soluble solids and lower acidity than that of the control, while the NAA-treated berries showed the opposite results.

1.5.4.3 Jasmonic acid

Jasmonic acid [JA; 3-oxo-2-(2-cis-pentenyl cyclopentane-1-acetic acid)] and its methyl ester (methyl jasmonate, MJ) are a class of oxylipins derived from lipoxygenase-dependent oxidation of fatty acid and have been found to occur naturally in a wide range of higher plants (Creelman and Mullet, 1997). In general, JA/MJ inhibits cell division, photosynthetic activities, flower bud formation, seed germination and embryogenesis (Koda, 1992; Sembdner and Parthier, 1993; Creelman and Mullet, 1997). In contrast, JA/MJ enhance the induction/ promotion of leaf senescence and petiole abscission, fruit ripening, chlorophyll degradation, carotenoid biosynthesis, tuber formation and protein synthesis (Yamane et al., 1981; Davies et al., 1986; Koda, 1992; Sembdner and Parthier, 1993; Creelman and Mullet, 1997). Moreover the MJ stimulates ethylene, ester, alcohol and acetic acid

production in pre-climacteric apple fruit and b-carotene synthesis (Saniewski and Czapski, 1983; Fan et al., 1997). For example ethylene caused an increase in anthocyanin contents by stimulating enzymes involved in the phenolic biosynthesis, in particular phenylalanine ammonia-lyase activity (Riov et al., 1969; Rigby et al., 1972). Pèrez et al. (1997) showed that the MJ stimulates the anthocyanin content during *in vitro* strawberry ripening. The effect of the MJ treatment on anthocyanin, total phenolic content and antioxidant capacity (as expressed as an ORAC value) in fruit of raspberry "Autumn Bliss" and "Jewel" were significant (Wang and Zheng, 2005). The increasing of the MJ concentration from 0.01 to 0.1 mM resulted in an increase in flavonoid content and antioxidant capacity.

Blando et al. (2005) reported the preliminary results on the establishment of an anthocyanin-producing callus system obtained from sour cherry leaf. They studied the growth of callus cultures and their anthocyanin production on various solid media in the dark or under light. Moreover the influence of JA on anthocyanin production in this cell system is also reported. Leaf explants cultured in the dark on A1M0 medium produced an abundant, friable and white callus and when these newly established cherry callus cultures were analyzed for anthocyanin content, they revealed an average anthocyanin content similar to that measured from the starting leaf tissue. The cell pigmentation was clearly stimulated also by the light. Anthocyanin accumulation, due to the light, did not have any dramatic effect on calli growth. So in cherry calli the induction of anthocyanin biosynthesis is triggered by light exposure rather than by media composition (Fig. 5).

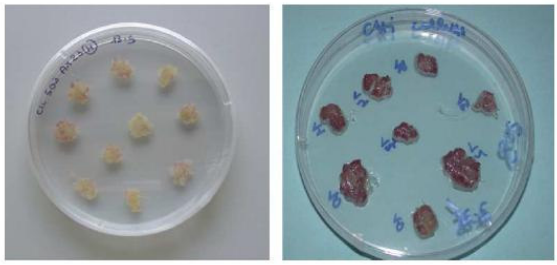

Fig. 5 Cherry callus colture in AIM0 media with the same concentration of the and different light exposure, after two (on the left) and seven (on the rigth) days (Blando et al., 2005).

1.6 The anthocyanins biosynthesis pathway

The anthocyanins physiology and genetic (Dooner et al., 1991) was studied in different monocotyledonous and dicotyledonous species (Holton and Cornish, 1995). Flower pigmentation is the most studied and there are at least 35 genes involved in flower colour of *Petunia hybrida* Vilm (Gerats and Martin, 1992; Wiering and De Vlaming, 1984). Moreover structural and regulatory genes were characterized and cloned in *Zea mays* L. (Radicella et al., 1991; Paz-Ares et al., 1986), *Antirrhinum majus* L., *Perilla frutescens* L. (Gong et al., 1997), *Arabidopsis thaliana* (L.) Heynh., etc. The anthocyanins biosynthesis in fruit trees is limited and focused mostly on *Malus sylvestris* (L.) Mill. *var. domestica* (Honda et al., 2002), *Vitis vinifera* L. and *V. labrusca* L. (Boss et al., 1996; Gollop et al., 2001; Kobayashi et al., 2001, 2002; Sparvoli et al., 1994), *Prunus persica* (L.) (Tsuda et al., 2004). Not so much is known about blood oranges anthocyanins. Different studies led to the conclusion that

anthocyanins are synthesised when two different kinds of genes take part: structural and regulatory genes. The first code for enzymes involved in the anthocyanins biosynthesis, the latter code for transcription factors (TFs) controlling the different expression of the structural genes (Dooner et al., 1991; Goodrich et al., 1992; Procissi et al., 1997; Quattrocchio et al., 1993). Thus, any significant genetic event in structural or regulatory genes could be important to the lost of the pigment. About regulatory mechanisms, tissues-specific regulation of structural genes is strictly correlated with combination of two different TF families: one is homolog to protein proto-oncogene of Vertebrate, c-MYB (myeloblastosis; Mol et al., 1998) and the other is the bHLH (basic-Helix-Loop- Helix) domain of MYC (Paz-Ares et al., 1987).

1.6.1 The structural genes

The anthocyanins, condensed tannins (CTs) and flavonols are synthesized via the flavonoid pathway, a branch of the phenylpropanoid ones. The flavonoids biosynthesis consists of a number of enzymatic steps, as illustrated in Fig 6.

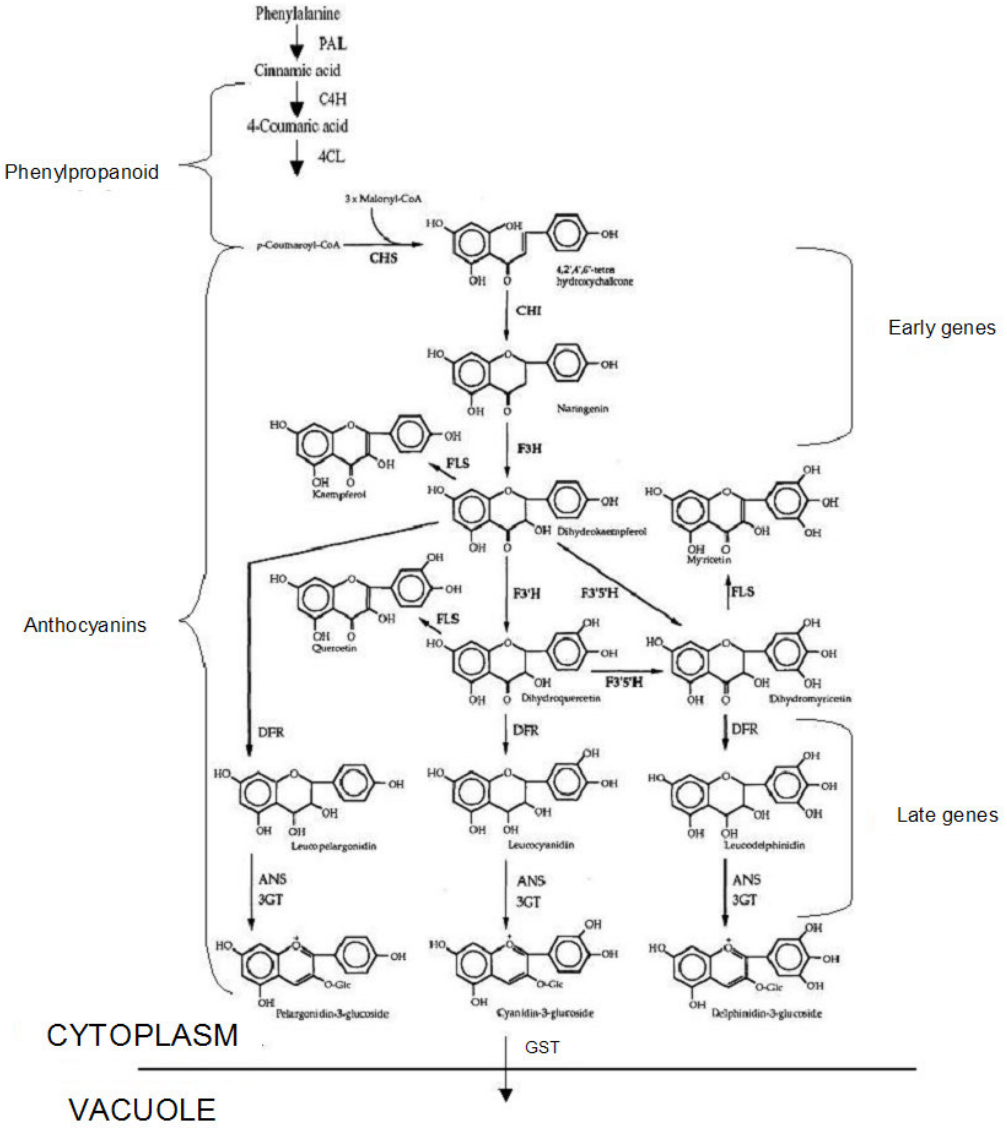

Fig. 6 Schema of anthocyanins biosynthesis

Anthocyanins are water-soluble compounds, accumulating in the vacuoles. Three major anthocyanin types contribute to flower colour: (1) pelargonidin-derived pigments are responsible for orange, pink or red colours; (2) cyanidin-derived pigments for red or magenta, and (3) delphinidin-derived pigments for purple or blue. Detailed biochemical and genetic analyses of anthocyanin production/accumulation (Holton and Cornish, 1995) have brought about the development of two main strategies for altering flower colour: introducing a foreign gene(s) to allow new branching in the anthocyanin biosynthetic pathway, and up/down-regulation of this pathway's native genes (Elomaa and Holton, 1994).

Nowadays, the anthocyanins biosynthesis has been almost completely elucidated and most of the structural genes encoding the enzymes responsible for each step have been isolated from different sources (Holton and Cornish, 1995). The general phenylpropanoid pathway is initiated by PAL, which catalyses the elimination of ammonia from L-phenylalanine to form *trans*-cinnammate; then, the metabolic pathway branches to give rise to a thousand of compounds, many of which are specific to particular plant species (Dixon and Palva, 1995). The first committed step for anthocyanins biosynthesis is catalysed by CHS, which condenses malonyl-CoA and 4-coumaroyl-CoA to form tetrahydroxy chalcone. This last compound is isomerized to flavanone naringenin, which is subsequently converted to the dihydroflavonol dihydrokaemferol by hydroxylation. The other two dihydroflavonols (dihydroquercetin and dihydromyricetin) are formed from dihydrokaemferol by further hydroxylation reactions. Then, anthocyanin 3-O-glycosides are synthesized from dihydroflavonols by the consecutive reactions catalyzed by DFR, ANS and UFGT (Dixon and Palva, 1995). Finally, GST is involved in the vacuolarization of anthocyanins, producted in the cytoplasm.

In *Citrus sinensis* [(L.) Osbeck] structural genes of *chs*, *chi* (chalcone isomerase), F_3OH (flavanone 3-hydroxylase), *dfr*, *ans* and *ufgt* were characterized and cloned (Lo Piero et al., 2005a; Moriguchi et al., 1999, 2001; Reforgiato et al., 2000). Cotroneo et al. (2006) verified the transcription expression level during ripening time through Real time PCR. They demonstrated that in common oranges (Valencia) the mRNA expression level is not absent but only down regulated. In Moro cultivar there is a strictly correlation between the transcription expression and the anthocyanin accumulation. The same genes were also compared in eleven different cultivars and selections harvest at the end of the winter, in which they could have the most high anthocyanin content (Cotroneo et al., 2006). Generally very high, high, medium or poorly pigmented samples synthesize respectively very high, high, medium or low mRNA target levels, in interdependent quantities, presumably co-ordinately regulated.

Structural genes involved in the anthocyanin biosynthesis of grape were cloned by Sparvoli et al. (1994). The mRNA of all structural genes, unless *Ufgt*, accumulates in early developmental stage and then it decreases until the veraison. But mRNA levels of all structural genes, included *ufgt*, increase in red grape cultivars (Boss et al., 1996; Kobayashi et al., 2001). So it seems that the *Ufgt* is the key enzyme involved in red skin grape (Jeong et al., 2004). Kobayashi et al. (2001, 2002) suggested that the phenotype of white grape (*Vitis vinifera* L.) cultivars probably is due to a *mybA* mutation, a regulatory gene that controls UFGT expression.

1.6.2. The glutatione S-transferase: the "last" enzyme

Anthocyanins are present in proportions and amounts that vary in relation with the plant species, organ, developmental stage and environmental growth conditions.

For example *Arabidopsis* contains flavonoid pigments in vegetative tissues and in seeds (Shirley et al., 1995).

Vacuoles offer a larger storage space than cell walls, which is important for flavonoids to reach concentrations great enough to function in the protection against predators and pathogens or as UV light sunscreens or attractants (Klein et al., 2000). Typically, the detoxification process for flavonoids requires three phases, in the last one of them (compartmentalization) the inactive water-soluble conjugates are exported from the cytosol by membrane-located transporters (Sandermann, 1992; Coleman et al., 1997; Rea, 1999; Klein et al., 2000). GSTs work also to protect cells by oxidative damage through quenching reaction with GSH. Even if it known that GST activity increases during oxidative damage, mechanisms involved in the protection against them is not clear jet (McGonigle et al., 2000).

In Fig. 7 the most common GST functions are summarized.

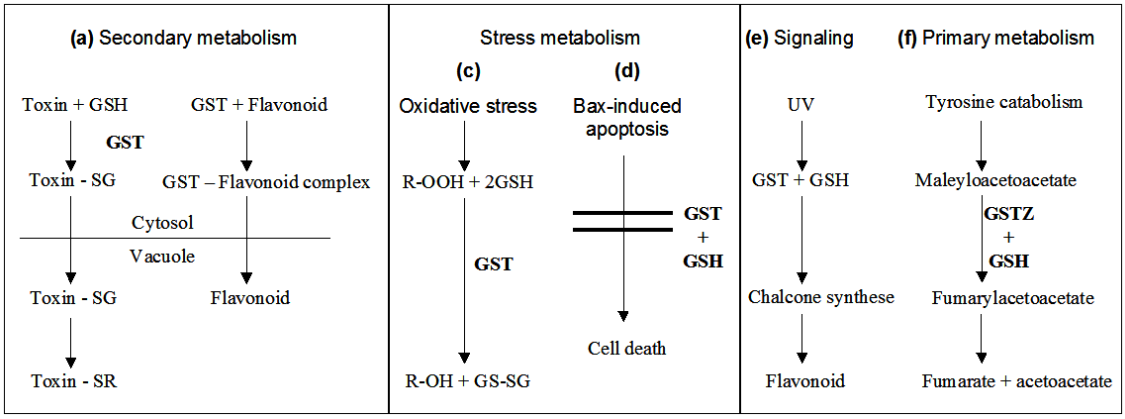

Fig. 7 Schema of GST function in planta. (a) In secondary metabolism, GST detoxify toxins through coniugation with GSH and then trasported into vacuols. (b) Phi and Tau enzymes for flavonoid trasport into vacuols. (c-e) GSTs on stress metabolism: (c) as glutathione peroxidase, to reduce citotixic DNA and lipidic ydroperoxid; (d) antioxidant value and protection against cellular death Bax induced; (e) in stress signal, GST induce CHS after UV exposition; (f) Zeta GST acts in primary metabolism as maleiloacetate isomerase (Dixon et al., 2002b).

The GST superfamily was firstly discovered in animals in the 1960s for their importance in the metabolism and the detoxification of drugs (Wilce et al., 1994). In plants it was firstly recognized in 1970 (cloning and sequencing in 1980s), when a GST activity from maize was shown to be responsible for conjugating the chloro-S-triazine atrazine with GSH, thereby protecting the crop from injury by this herbicide (Frear and Swanson, 1970; Edwards and Dixon, 2000). All plant GSTs were initially allocated to the most heterogeneous and widespread GST class known at that time, the Theta class, found in vertebrates, Drosophila, plants, and *Methylobacterium* (Mannervik and Danielson, 1988; Pemble and Taylor, 1992). Sheehan et al. (2001) indicated a possible phylogenetic deduction (Fig. 8).

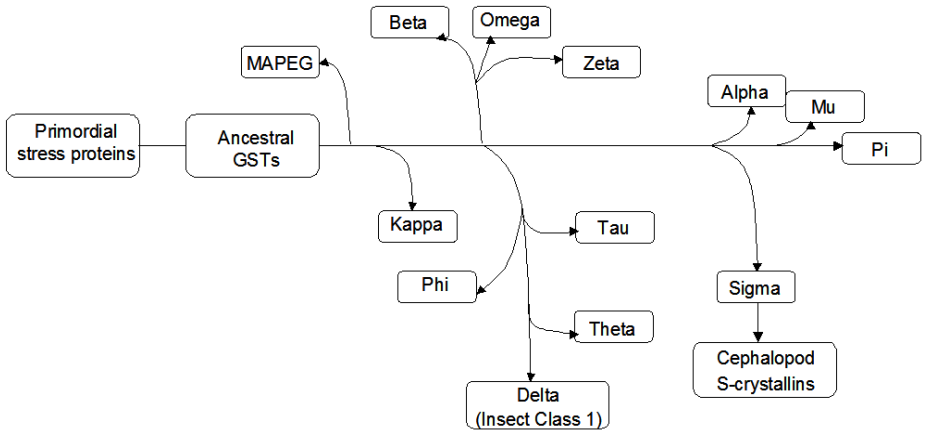

Fig. 8 Schematic diagram summarizing a plausible pattern of divergence of GST superfamily

The soluble GSTs (EC 2.5.1.18) are encoded by a large gene family in plants and they are involved in the detoxification of reactive electrophilic compounds by catalysing their conjugation to glutathione (GSH). The GSTs conjugate the glutathione tripeptide (g -Glu-Cys-Gly; GSH) to a broad variety of substrates. They are an ancient and ubiquitous gene family encoding ~ 25- to 29-kD proteins that form both homodimers and heterodimers *in vivo*. Stable anthocyanin pigmentation occurs when the molecules are transferred to the vacuole. It is necessary to limit the mutagenic and oxidative effects of synthesis of the intermediates pathway (Ahmed et al., 1994; Rueff et al., 1995) and for proper biological function of the final product. In maize, in the presence of a functional b*ronze2* (*bz2*) gene, anthocyanins accumulate exclusively within the vacuole. When *bz2* is missing, anthocyanins accumulate in the cytosol, conferring a tan-bronze phenotype from pigment oxidation (Marrs et al., 1995).

The GST gene families were firstly classified based on the exon/intron structure of the genes, on sequence similarity and on amino acid residue conservation. About this schema, three distinct classes of plant GSTs were initially indicated: type I, type II and type III GST genes. The *bz2* gene of maize encodes a type III GST (Marrs et al., 1995) and the *Petunia* gene *an9* encodes a type I GST. This classification schema has been underway refined (Frova, 2003) and six distinct classes have been characterized. Phi (type I) and Tau (type III) are GSTs plant specific and they are the most representative classes in terms of number of sequences per class.

1. Phi GSTs appear to function as defense or cellular protectant genes, expressing proteins in response to pathogen attack, wounding, senescence, and the resulting lipid peroxidation that accompanies these processes (Bartling et al., 1993; Dudler et al., 1991; Kim et al., 1994; Zhou and Goldsbrough, 1993). The genes are organized in three exons.
2. Tau GSTs were originally identified as inducible genes by a range of different treatments, particularly auxin, but also ethylene (Takahashi et al., 1989; Droog et al., 1993), pathogen infection (Taylor et al., 1990), heavy metals (Czarnecka et al., 1988; Hagen et al., 1988; Marrs and Walbot, 1997) and heat shock. The genes are organized in two exons.
3. Zeta (type II) and Theta GSTs are found in both animals and plants. The Zeta class GSTs are induced by ethylene and during senescence. Initially

discovered in carnation (*Dianthus caryophyllus* L.), they have later been found in several other plant species. Their genes have eight or nine introns and they are related to the mammalian and fungal Zeta class genes ((Meyer et al., 1991; Itzhaki and Woodson, 1993).

4. The Theta class includes few GSTs, that show similarity to the mammalian Theta enzymes. Their genes are usually made up of seven exons (Soranzo et al., 2004).
5. Dixon et al. (2002b) identified in *Arabidopsis thaliana* outlying members of the GST superfamily. These GST-like proteins are clustered into two distinct classes based on sequence similarity. Lambda genes are known to be co-induced with Phi and Tau GSTs in cereals following exposure to herbicide safeners, chemicals that increase tolerance to herbicides (Dixon et al., 2002b), while DHAR genes encode active glutathione-dependent dehydroascorbate reductases with members recently reported in *Arabidopsis*, rice and soybean (Dixon et al., 2002a). All the members of the two last classes adopted new catalytic functions though the substitution of an active serine site with a tyrosine.
6. A less numerous subfamily is represented by the microsomal GSTs, termed MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism; Jakobsson et al., 1999).

1.6.3 The involvement of regulatory genes

The control of flavonoids biosynthesis seems to be the key point for the regulation amongst the steps leading to the protein production. A separation of early biosynthetic genes (EBGs) from late biosynthetic genes (LBGs) is necessary. The point of division is at F3H or DFR, depending on whether there is a predominant co-production with anthocyanins of flavones or flavonols, respectively.

In seeds and vegetative tissues of *Zea mays* (maize) and leaves of *Perilla frutescens* the biosynthetic genes for anthocyanin production from CHS down to those involved in transport to the vacuole are regulated by common TFs (Dooner, 1983; Dooner et al., 1991; Saito and Yamazaki, 2002). In *V. vinifera* (grape) berries the UFGT is regulated separately from the other genes and seems to be the key step for triggering anthocyanin production during berry ripening (Boss et al., 1996; Kobayashi et al., 2002).

Two families of regulators, the bHLH and MYB proteins, are conserved in the regulation of the anthocyanin and CT pathways in all species analyzed to date (Koes et al., 2005). The bHLH (the conserved domain of the MYC TFs) may have overlapping regulatory targets (Zhang et al., 2003; Zimmermann et al., 2004), but the MYB proteins are the key components providing specificity for the subsets of genes activated. It is also necessary the involvement of WD40 proteins in assisting the process. Tab 4 summarizes the isolation of *myb*, *bHLH* and *WD40* genes, for which a role in regulation of anthocyanin or proanthocyanidin production has been confirmed through genetic mutants or transgenic studies.

Species	Metabolite	Protein type	Name	Reference
Antirrhinum majus	anthocyanins	MYB	ROSEA1	Martin et al., 2001
		MYB	ROSEA2	Martin et al., 2001
		MYB	VENOSA	Martin et al., 2001
		bHLH	DELILA	Goodrich et al., 1992
		bHLH	MUTABILIS	Martin et al., 2001
Arabidopsis thaliana	Anthocyanins and	MYB	PAP1 (AtMYB75)	Borevitz et al., 200o
	proanthocyanidins	MYB	PAP2 (AtMYB90)	Borevitz et al., 200o
		MYB	TT2	Nesi et al., 2001
		bHLH	TT8	Nesi et al., 2000
		WD40	TTG1	Walker et al., 1999
Fragaria x ananasa	anthocyanins	MYB	FaMYB1 (repressive?)	Aharoni et al., 2001
Gerbera hybrida	anthocyanins	bHLH	GMYC1	Elomaa et al., 1998
Prilla frutescens	anthocyanins	MYB	MYB-P1	Gong et al., 1999a
		bHLH	MYC-RP/GP	Gong et al., 1999b
		WD40	PFWD	Sompompailin et al., 2002
Petunia	anthocyanins	MYB	AN2, AN4	Quattrocchio et al., 1998, 1999
		bHLH	AN1, JAF13	Spelt et al., 2000
		WD40	AN11	De Vetten et al., 1997
Sorghum bicolor	phlobaphenes	MYB	Y	Chopra et al., 1999
Vitis sinifera	anthocyanins	MYB	MYBA	Kobayashi et al., 2002
Zea mays	anthocyanins and	MYB	C1	Cone et al., 1986; Paz-Ares et al., 1986
	phlobaphenes	MYB	P1	Cone et al., 1993
		MYB	P	Lechelt et al., 1989; Grotewold et al., 1991
		bHLH	B	Chandler et al., 1989
		bHLH	LC	Ludwing et al., 1989
		bHLH	R	Perrot and Cone 1989
		bHLH	SN	Tonelli et al., 1991
		bHLH	IN (repressive)	Burr et al., 1996

Tab. 4 The main transcription factors known in different plants (Davies and Schwinn, 2003)

The first plant system, for which the involvement of MYB and BHLH proteins was demonstrated, was *Zea mays*. The expression of one member of a *myb* (the *C1*/*Pl* family; Cone et al., 1986; Paz-Ares et al., 1986; Cone et al., 1993) and a *bHLH* gene families (the *R*/*B* family; Chandler et al., 1989; Ludwig et al., 1989; Perrot and Cone, 1989; Tonelli et al., 1991) is necessary and sufficient to induce the expression of most of the anthocyanin biosynthetic genes, from *chs* through to *bz2.*

In *A. majus*, *delila* (Goodrich et al., 1992) and *mutabilis* (Martin et al., 2001) encode the bHLH factors and the anthocyanin *myb* gene family consists of *rosea1*, *rosea2* and *venosa* (Martin et al., 2001). The *delila* gene is active in both petal lobes and tube, while *mutabilis* is active only in the lobes. The *rosea1* gives strong pigment production, *rosea2* weaker pigmentation and *venosa* gives pigmentation only in epidermal cells overlying veins, producing a striking venation pattern (Martin et al., 2001).

Several genes are also involved in controlling anthocyanin biosynthesis in floral tissues of *P. hybrida*. The *anthocyanin1* (*an1*) and *jaf13* genes encode bHLH factors, while *an2*, and probably *an4*, encode MYB proteins (Quattrocchio et al., 1998, 1999; Spelt et al., 2000, 2002). All these genes are required for the expression of the LBGs. JAF13 and AN1 diverge in their amino acid sequences, and they may also differ in function within the regulatory cascade. AN1 is structurally most similar to INTENSIFIER1 of maize. JAF13 is more similar to DELILA of snapdragon and R of maize, and *Jaf13* expression does not compensate for loss of *An1* activity in *Petunia* petals.

In *A. thaliana* TRANSPARENT TESTA 2 (TT2) up-regulates several proanthocyanidin biosynthetic genes in the seed coat, including *BANYULS* (Nesi et al., 2001), encoding the first committed step in flavan-3-ol production. TT2 requires

an interaction with a bHLH factor, encoded by TT8, and TT2 and TT8 are required for the transcription of the LBGs (Nesi et al., 2000). Mutant plants lacking activity of WD-repeat proteins in *Arabidopsis* (TRANSPARENT TESTA GLABROUS11) and *Petunia* (anthocyanin 11) lose the ability to produce anthocyanins (in *Petunia*; de Vetten et al., 1997) or anthocyanins, proanthocyanidins, seed mucilage and trichomes (in *Arabidopsis;* Walker et al., 1999; Western et al., 2001).

In *V. vinifera*, two *myb* genes, Vv*myb*A1 and Vv*myb*A2, which appear to regulate VvUFGT, contain mutations that segregate with white berries (Kobayashi et al., 2004; Walker et al., 2007). Since there were no differences in either the coding or the promoter sequences of *Ufgt*s between white cultivars and their red-skin sports, the phenotypic change from white to red was presumed to be the result of a mutation in a regulatory gene controlling the expression of *Ufgt* (Kobayashi et al., 2001). Recently, Kobayashi et al. (2002) reported that *VlmybA*s, putative regulatory genes isolated from Kyoho (*Vitis labruscana*: *Vitis labrusca* × *Vitis vinifera*), was involved in the regulation of anthocyanin biosynthesis and of the expression of *Ufgt*. Kobayashi et al. (2004) also reported that a retrotransposon-induced mutation in a homologue of *VlmybA*s, *VvmybA1* is associated with the loss of the pigmentation in white cultivars of *V. vinifera*.

In *Fragaria x ananassa* a MYB TF of anthocyanin and flavonol pathways has been identified, called FaMYB1, and based on the expression studies in tobacco, it is believed to repress these pathways (Aharoni et al., 2001).

In *C. sinensis* the regulation mechanism is not well identified. But two genomic sequences, showing structural similarities with proteins of the MYC-like (CsMYC2) and MYB-like (CsMYB8) transcription factors, were isolated from total RNA of flesh of 'Moro' orange and their implication in the regulation of anthocyanin's biosynthetic pathway was studied (Cultrone A., personal communication). We might hypothesize that purple pigment, in the flesh of blood oranges, is synthesized in response to a similar mechanism of interaction between the two classes of genes *myb* and *myc*-like. The regulation mechanism could be presumably different in the different tissues of the fruit, as well. Differences in the expression pattern may occur even between fruit rind and flesh.

1.7 Importance, potentiality and the reason of methods used

Among the different strategies to identify differential expression transcripts, as differential display (DD), representational difference analysis (RDA), serial analysis of gene expression (SAGE) and suppression and subtractive hybridization (SSH) (Liang and Pardee, 1992; Lisitsyn and Wigler, 1993; Velculescu et al., 1995; Watson and Margulies, 1993; Diatchenko et al., 1996), we decided to use the last one. In fact SSH methodology is a very accurate technique, that compare two RNA populations and allows to identify differentially expressed transcripts present/absent in one of them. This method is particularly well suited for the identification of target cDNAs that correspond to rare transcripts, which are typically the most difficult to obtain. Moreover the SSH library is a quite rapid technique, and not so much false positive are isolated. The drawback is the slow isolation and sequencing of all differential clones, the probability to find the same sequence into various clones and the necessity to validate the differential expression through other analysis, as Northern blot, Real time PCR and/or microarray, to be sure to eliminate the false positive (Cao et al., 2004). So we validated almost ten transcripts using semi-quantitative RT-PCR, then just three of them were confirmed using the Real time PCR. Moreover to confirm the results obtained through previous analysis, we chose two different kinds of

microarrays. The first is constituted by differential library cDNA (custom chip array); the other one is the Affymetrix cDNA *Citrus* GeneChip. Both of them were hybridised with the same samples used to the SSH library setting-up.

The SSH library allowed to isolate ESTs, most of them were completely new and we submitted 201 ESTs in the dbEST database. In some cases it was possible to obtain full length sequences, after clustering/assembling process. In other ones it was necessary to length and complete sequences that were never found and submitted in the GenBank database about *C. sinensis*. Thus we used 5' and 3' RACE-PCR to complete the partial sequences.

Moreover we focused our attention on the GST gene family, composed by six different classes with correspondent different functions. An *in silico* method and the molecular biology expression data on different orange tissues were performed to characterize the GST gene family in sweet orange. In this way we demonstrated the importance and the close correlaction between the bioinformatic and the experimental approaches.

The functional analysis about regulatory genes involved in the anthocyanin pathway was allow to understand better the role and the function of two *Citrus* transcription factors. This prospect has been tested by placement of *myb* and *bHLH* genes (Cs*myc2* and Cs*myb8,* gently provided by Cultrone A.) and *rosea* and *delila* from snapdragon (gently provided by Cathie Martin and Eugenio Butelli, from JIC of Norwich), under the control of the *CaMV 35S*. Different constructs were used for the protoplast transformation of tobacco leaves, agroinfiltration of orange fruits and the biolistic system of petunia white flowers and various orange. In fact transient gene expression systems were used for short-term studies of gene function (Barandiaran et al., 1998; Ferrer et al., 2000; Tian et al., 2004). They are rapid system and these techinques are well known for various plants, but not for woody trees such as *Citrus,* which are difficult to regenerate (Ghorbel et al., 1999) and whose fruits are obtained after a long juvenile phase. Moreover orange fruits have also a recalcitrant nature.

2. AIM OF WORK

The aim of this work was the identification of differential expressed transcripts between blood and common flesh orange fruits, mostly when they are in ripening time. Because nowadays is not so clear which characteristics are different between them, we wanted know if there's something different a part the anthocyanin content. We supposed that the construction of a cDNA library could be a good instrument to achieve our objective. Moreover the use of different expression analysis approaches (as the Real time PCR and the arrays) could be a well systhem to validate the expression level. The analysis on the transcription factors controlling the anthocyanin biosynthetic pathway has to be interesting to improve the knowledgments about differences between pigmented and common oranges.

3. MATERIAL AND METHODS

3.1 Plant material

Moro Nucellare 58-8D-I (cultivar highly pigmented) and Biondo Cadenera oranges (common cultivar) were harvest in the Palazzelli (Siracusa, the experimental farm of CRA-ISAGRU of Acireale, Catania) in eight different ripening periods: (I) at the beginning of October, when there's no pigmentation; (II) at the end of October; (III) at the end of November; (IV) at the beginning of December, when anthocyanin is just into some flesh sacs; (V) at the end of December; (VI) at the beginning of January, when Moro flesh is almost completely pigmented; (VII) at the end of January; (VIII) at the beginning of February, in which Moro in totally pigmented and ripe. Moro and Cadenera oranges harvested at ripening time (VIII sampling; Fig. 9) were used as tester and driver, respectively, for the cDNA library construction. The same samples were used to hybridized the custom chip and the Affymetrix array.

Flesh of I, IV, VI and VIII samplings (Fig. 10) were used for the expression analysis through semi-quantitative RT-PCR and Real time PCR.

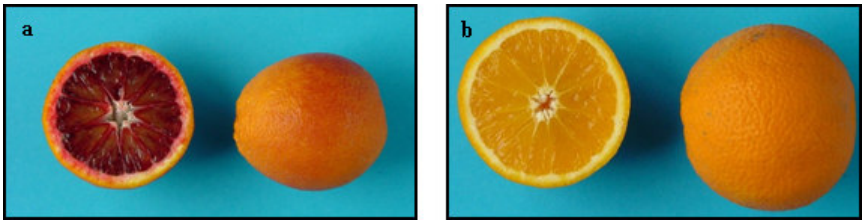

Fig. 9 Moro Nucellare 58-8D-I **(a)** and Biondo Cadenera **(b)** cultivars at maturity time, used as tester and driver, respectively, in the SSH library.

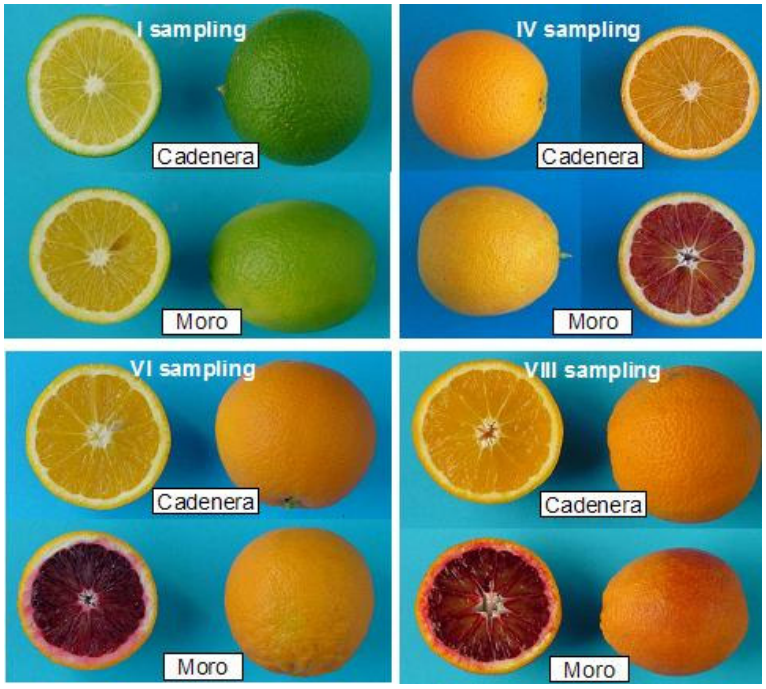

Fig. 10 Moro and Cadenera samples at different ripening time (I, IV, VI, VIII) used in the expression analysis data.

3.2 Total RNA extraction

All tissues were taken and frozen in liquid nitrogen. About the different compounds of tissues used, for their nature and developmental stage, different kind of protocols were used for total RNA extraction. It was obtained from 3 g of flesh using TRIzol® LS Reagent, according to the manufacturer's instructions (Invitrogen, Scotland UK). Total RNA of rind and adult leaves were extracted from 2 g of tissue using a modification of a standard extraction RNA protocol of Cl-Guanidine thiocyanate (Sambrook and Russel, 2001). The total RNA of the albedo (the spongy tissue) was extracted using Concert (Invitrogen, Scotland UK). The RNeasy plant mini kit (Qiagen) was used for the total RNA extraction from young leaves and ovaries. All the RNAs were treated with DNase (Promega) for 30 min at 37°C to remove the genomic DNA. The amount and the quality of the total RNA were estimated by spectrophotometer readings and by electrophoresis in agarose gel stained with ethidium bromide.

3.3 DNA extraction from leaves

Leaves were washed, frozen in liquid nitrogen and mill as pulverized substance. Almost 800µl of TES solution (0.2 M Tris, 1 mM EDTA, 1% SDS pH 8) were added to 100-200 mg of leaves, vortex and incubated 10 mins in ice. The same volume of phenol-chlorophorm-isoamylic acid was added, shaked and centrifuged 5 mins at 13.000 rpm. The same procedure was repeated with the surnatant and repeated again. Then the chlorophorm-isoamylic acid was added, shaked and spin 5 mins at 13.000 rpm. Almost 1/10 of 3M sodium acetate pH 5.2 and two volumes of Ethanol 100% were added to the surnatant. DNA was precipitated for at least 20 mins and then centrifuged 10 mins at 13.000 rpm. The pellet was washed two times with 70% ethanol and dry and resuspend in 100µl di TE (10 mM Tris, 1 mM EDTA pH 7.5) with RNase. The amount of DNA was analysed on agarose gel stained with ethidium bromide.

3.4 PCR Select

3.4.1 Construction of substractive cDNA library

The Suppression Subtractive Hybridization (SSH) cDNA library was constructed using the Clontech PCR-Select™ cDNA subtraction Kit (BD Biosciences Clontech, USA) according to manufacturer's instructions. The tester (Moro) and the driver (Cadenera) were retro transcribed from 100 ng of flesh total RNA, using the Super SMART™ PCR cDNA synthesis kit (BD Biosciences Clontech). Both cDNAs were digested with *Rsa*I restriction enzyme and then two different adaptors were ligated (Fig. 11). The first and the second amplification needed to isolate and to enrich differential expressed transcripts, respectively. The substracted cDNA (after Nested PCR) was used to generate the library.

3.4.2 Cloning in TA vector and colony PCR

The substracted cDNA was directly inserted into the pGEM T-Easy Vector (Promega) and transformed using *E. coli* DH5α Max Efficiency competent cells (Invitrogen). Then they were spreaded in LB and Ampicillin (100mg/ml) and growned on at 37°C. 1248 clones were picked randomly. Every clone was analysed through colony PCR and it was directly inoculated in 150 µl of Freezing broth media (Sambrook and Russel, 2001). Colony PCR mixture was of 20 µl final volume with 1x Platinum Taq DNA polymerase Buffer (Invitrogen), 0.1 mM dNTP mix, 2.5 mM $MgCl_2$,

0.4 µM of each Nested 1 and 2 primer, 1U Platinum DNA Taq Polymerase (Invitrogen). PCR program was about one cycle at 95℃ for 6 mins, 65℃ for 50 sec and 72℃ for 1.20 min, 35 cycles at 94℃ for 40 sec, 65℃ for 50 sec and 72℃ for 1.20 min. All the amplicons were analysed in 1% agarose gel in 1x TBE and stained with ethidium bromide.

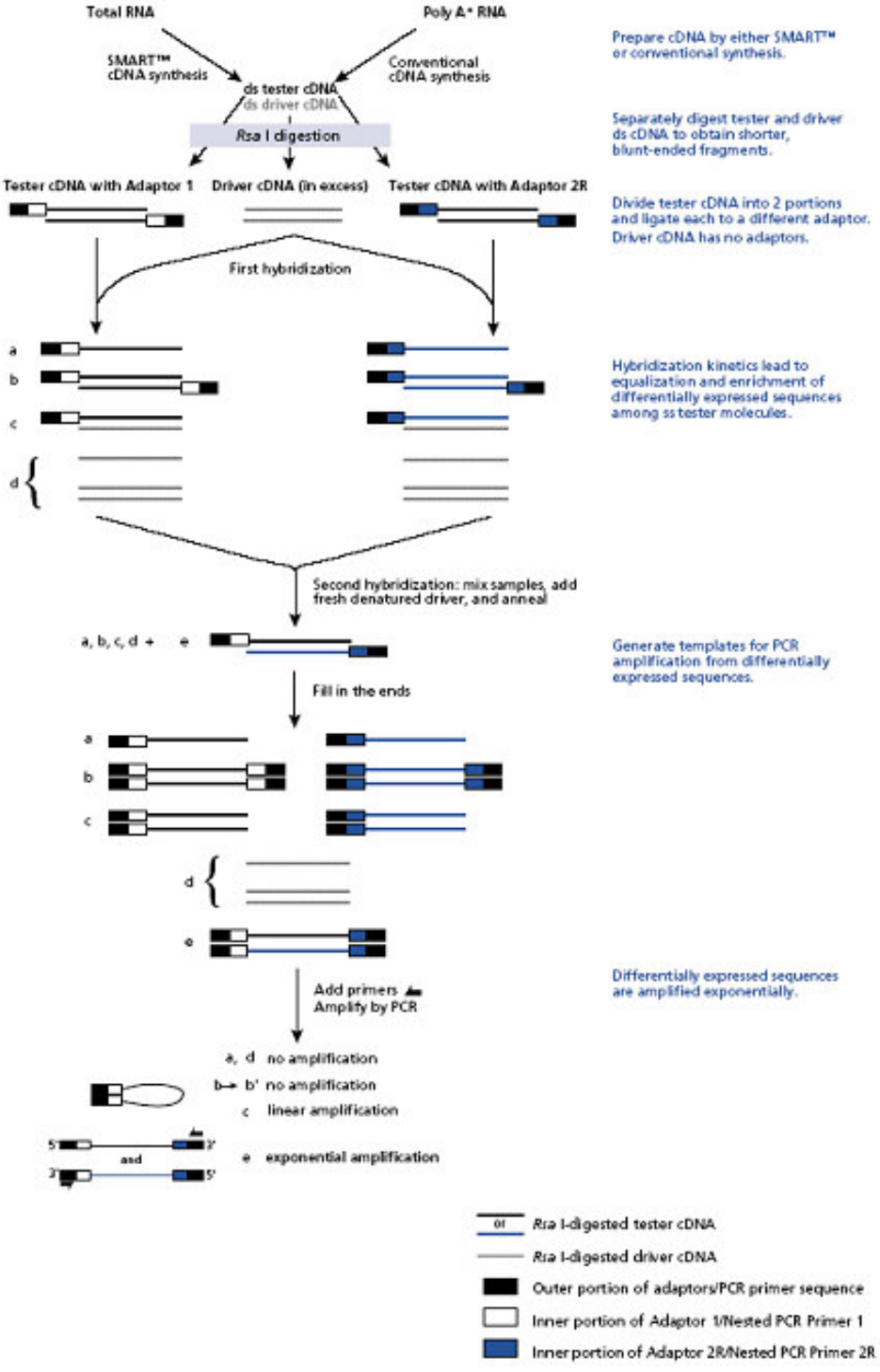

Fig. 11 Schema of PCR Select methodology.

3.4.3 Reverse Northern blot and screening of cDNA library

The differential clones were deduced after the reverse Northern blot hybridization (Fig. 12). The colony PCR DNAs were equilibrated with 1M NaOH, 10mM EDTA and they were denaturated for 10 mins at 100°C. Half volume was spotted on nylon membrane (Zeta-Probe® Blotting Membranes, Bio-Rad, USA) with 0.4M NaOH. The same procedure was conduced with the remained half volume. The membranes were washed in 2x SSC, dryed on air and fixed for 30 mins at 80°C.

About the cDNA probes, forward (tester) and reverse (driver) products derived from the Nested PCR were digested with *Rsa*I, to remove adaptors, and they were purified with YM30 microcoon filters (Millipore). Almost 100 ng of cDNA were labelled with ^{32}P dCTP. Probe preparation, pre-hybridization, hybridization and wash were conduced according to manufacture's instructions (BD Bioscience). Forward probe was used to hybridized a filter and reverse probe was used to hybridized the other one, and *vice versa*. Filters were analyzed with Typhoon 9210 Phosphorimager (Molecular Dynamics).

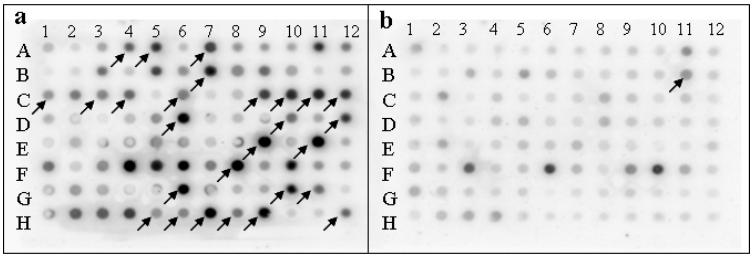

Fig. 12 Example of a reverse Northern blot filter. Arrows indicate some of the differential clones analysed, because overexpressed in Moro (**a.** tester-forward probe) or in Cadenera (**b.** driver-reverse probe) cultivars.

3.5 ESTs sequencing, similarity analysis and functional categories

The Reverse Northern analysis allowed us to evidentiate the over-expressed clones. Almost 5 µl of correspondent clones were inoculated in 5 ml of Louria Bertani medium and Ampicillin (100mg/ml) over night at 37°C at 200 rpm. The plasmidic DNAs were extracted with Wizard® Plus SV Miniprep-DNA Purification System kit (Promega) and they were digested with *EcoR*I. The clones with the insert were sequenced using the Applied Biosystems ABI PRISM 3100 DNA Sequencer (CRIBI DNA sequencing service, Padova University). The EST sequences were compared with the BlastN and BlastX non redundant (nr) database of the NCBI (Altschul et al., 1997). The BlastX E-value<0.01 was considered the limit of the acceptable similarity. 201 ESTs were submitted to the dbEST database of the NCBI.

The functional categories were achieved through bibliography references.

3.6 Differential expression analysis using the semi-quantitative RT-PCR and the Real time PCR

The first analysis used to validate the differential transcripts expression was the semi-quantitative RT-PCR. We chose the most redundant and functionally more interesting transcripts: PAL, cytochrome b5, GST, putative Ser receptor kinase (SRK), valencene synthase, alcohol acyl transferases (AAT), 10-hydroxigeraniol oxidoreductase, pectinesterase, bHLH, MADs box. The Elongation Factor 1 alpha (EF AY498567) was used as housekeeping gene.

The flesh RNAs were taken during four different ripening periods: I, IV, VI and VIII (as described in Plant Material). The first strand cDNA was synthesized from 250 ng of total RNA in a final volume of 25 µl, with 1x PCR Reaction mix, 0.2 µM of each primer (Tab. 5), 1U SuperScript III One-Step RT-PCR with Platinum Taq (end point; Invitrogen). The retro-transcription reaction was conduced for 30 mins at 50°C, following by the PCR with 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 120 sec and final extension at 72°C for 7 mins, in a GeneAmp PCR system 9700 (Applied Biosystems). 5 µl of each reaction was assayed at 20, 25, 30 and 35 cycles and visualized on 1.5% agarose gel in TBE 1x stained with ethidium bromide.

The Real time PCR was used to validate the expression level of three of transcripts analysed firstly in semi-quantitative RT-PCR: GST, SRK and pectinesterase. The EF 1 alpha was used as internal control.

Gene		Oligonuceotides		Ta (°C.)	Amplicon (bp)
Name	Acc. Number	Name	Sequence (5'-3')		
Glutathione S-transferase	EG358323	GST-61_fw GST-222_rv	ATCCTCTATTACTGGAACTTGCCCA TATGGTTCAGTTAAGGCAGCTTGC	60	162
Putative receptore Ser kinase	EG358294	SRK-255_fw SRK-400_rv	CTGCTCTATTTCGGTATGGTTGGA CTTTAGCGTTGCCAAGTTCTGAAC	60	146
Valencene syntase	EG358204	VS-79_fw VS-236_rv	CAAGGTCATGAGTTTGAGCAGAAGAG GGCTTCATCATCAACTCCTCGTTAA	60	158
Alcool acy transferase	EG358303	AAT-45_fw AAT-197_rv	GGATATTCCTCCAGGCTATTATGGC GCCGCAGACCTTATATACTCTTCGTT	60	153
10-hydroxigeraniol oxidoreductase	EG358189	HOR-343_fw HOR-492_rv	GAGGTCGGAAGCAAAGTGAGTAAAT CAGGTACTTGTTGGCATAGGTCATT	60	150
Pectinesterase	EG358218	PECT-19_fw PECT-168_rv	GTACTCGCGAACCGTTTACATCAAG ACTCTGTTAGCAGTTGAAGAACCCG	60	150
BHLH	EG358372	bHLH-25_fw bHLH-418_rv	TGACGACTACCGCTACTACTACTCGTATGG GGGCAGGTACATAGTTGATAA	60	390
MADs box	EG358383	MAD-309_fw MAD-463_rv	CAGGCCCGAGTCTGAATAATTACA TGATTGCGTGTTCCTAGTCACTGA	60	155
Cytochrome b5 DIF-F	EG358196	Citb5-328_fw Citb5-470_rv	GTAGTTGAAGAGCAACTCGTGAGCA CCGAGGTACTGAACGTTACAAGGTT	60	143
Phenylalanine ammonialyase	EG358295	PAL-22_fw PAL-180_rv	GCAGGTACGTTTTTTCATACGCTG CCTCCTCGAAAGCACCTATCTTTAAG	60	159
Elongation factor	AY498567	EF-161_fw EF-88_rv	CTGCTGGACGCTCTTGACAA TCCTGGAGTGGCAGACGAA	60	72

Tab. 5 Oligonucleotide sequences and characteristics used in semi-quantitative RT-PCR and in Real time PCR.

The RNA samples, used in the semi-quantitative RT-PCR, were used also in the Real time PCR. The total RNA was retro transcribed into cDNA using High-Capacity cDNA Archive kit (Applied Biosystems). The cDNA synthesis was conduced at 25°C for 10 mins and then at 37°C for 2 hours. The PCR reaction was done in 96 multiwells plate using ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Final volume of 25 µl consisted of 15 µl of Power SYBR Green mix (Applied Biosystems), 0.2 µM of each forward and reverse primer, 100 ng of cDNA. PCR program was 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec, 60°C for 1 min. Every sample was used in triplicate and two negative controls (without RNA sample and another without Taq) were also analysed. The relative quantification was used through the standard curve method.

3.7 Anthocyanin content

The anthocyanins were extracted from freeze-dried Moro flesh, using two times 40ml of 0.3% HCl in methanol and 20ml in the third extraction. The extracts were dried and resuspended in 10ml of water. Total anthocyanins were determinated through HPLC analysis, according to Rapisarda et al. (1994).

3.8 Phylogenetic analysis of the glutathione S-transferase gene family and bHLH transcription factor

ClustalW program (Thompson et al., 1994) was used to generate multiple alignments of amino acid sequences of GST and bHLH, isolated through the SSH library. Phylogenetic analysis was conduced using MEGA 3.1 version program (Kumar et al., 2004).

3.9 Lengthening of the uncomplete sequences through 5'-3' RACE PCR

The 5' and 3' RACE (Rapid Amplification of cDNA Ends) – PCR (Clontech) was performed to complete the partial cds of AAT, 10-hydroxigeraniol oxidoreductase, bHLH, cytochrome b5, SRK and MADS. There were not submission as the full length cds or no informations were about those sequences of *C. sinensis* in the GenBank database. The oligonucleotides used are indicated in Tab. 6. The annealing temperature and the amplification protocol were conduced according to the manufacture's instructions.

Name	Oligonucleotides	
	5' RACE-PCR (5'-3')	3' RACE-PCR (5'-3')
AAT	-	GGTCTGCGGGGGATCTTATGGTAAGCTACC
10-hydroxigeraniol oxidoreductase	-	ACCATTTACCCAATCGTCCCCGGACA
Cytochrome b5	TGATGGAGGCGGCAGGGAAGGATGCA	GTGCCACTCTTGGTTGCTGGTTCCTAC
SRK	TCAAAACTTTGGCTTCGGGCATCAACCA	TCTAGAGTATCTCCGAAGCCTCAAGTGA
bHLH	CACTTCTCTTGGATCCTTTGACTGAGC	GCAAGGAGGGGTCAAGCAACTGACAGCC
MADs box	ATCGGCGTGCCTTAGCGTCATCGTCCTT	-

Tab. 6 Primer sequences used to legthen the 5' and 3' ends

3.10 Flesh custom chip array construction

104 out the 201 differential ESTs (isolated through the SSH library), 129 differential ESTs (isolated through cDNA-AFLP library among Cadenera, Tarocco and Moro in ripening time and provided by Prof. Marocco of the University Cattolica of Piacenza) and 68 ESTs of Myc and Myb-like transcription factors (derived from *HarvEST Citrus* database) were spotted on a custom array by Macrogen (Korea) as 50mer probes. The EF 1 alpha was spotted as housekeeping gene control.

The array was hybridised with 1 µg/µl of Moro and Cadenera flesh at ripening time (the same samples used for the cDNA library construction). The hybridization and data analysis were provided by CRIBI of University of Padova.

3.11 Affymetrix GeneChip *Citrus* Array and elaboration of the data

The Affymetrix GeneChip *Citrus* array (available on February 2006) contains 30,171 probe sets representing 33,879 transcripts obtained from several *Citrus* species and *Citrus* hybrids (*C. aurantium*, *C. jambhiri*, *C. macrophylla*, *C. medica*, *C. paradisi* x *P trifoliata*, *C. resini*, *C. reticolata*, *C. sinensis*, *C. sinensis* x *P trifoliata*, *C. unshiu*, C. x paradisi, *P. trifoliate*) of different libraries (various tissues and developmental stages). The total RNA (1 µg/µl) of Moro and Cadenera flesh was

used to hybridised the Affymetrix chip. The hybridization and the elaboration data were conduced by Genopolis. The software GeneSpring GX 7.3 Expression Analysis Agilent Technologies was used for the elaboration differential data, considering differential gene expression in 3.0, 8.0 and 10.0 Foldchange.

3.12 Comparison of results from various methods used to validate the differential expression of the transcripts

94.127 orange EST sequences from different cDNA libraries were collected in a secondary EST database. The collection comprises also ESTs from the Moro and the Cadenera SSH cDNA library. All the sequences collected were used to feed a clustering/assembling procedure to obtain unique transcripts [tentative consensus sequences (TCs) and singletons]. Then, those TCs having a minimum of 2 ESTs and at least one of them from flesh were selected; this data-set was considered for the successive steps of the analysis. Some of the differential expression results were validated by semi-quantitative RT-PCR and/or by Real time PCR. Data from the Reverse Northern obtained from the SSH library were considered too.

A schematic procedure of the approach used is reported in Fig. 13.

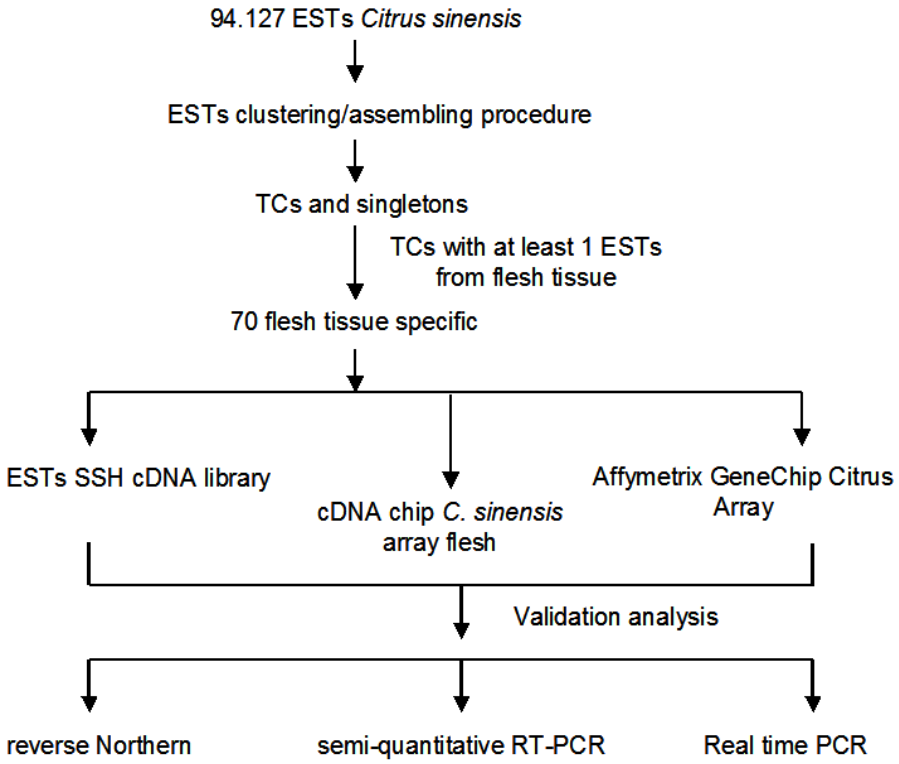

Fig. 13 Step by step procedure analysis

The characteristics considered were:

- the redundancy in the database and in the SSH library;
- the cluster ID;
- the number of ESTs in the TC;
- the similarity, considering the correspondent Best Hit and the Evalue obtained by BLAST based comparisons *versus* Protein database;
- the functional category, deduced considering also bibliography annotation;

- the expression data analysis according to (i) the sequence ID and the value of the hybridation of the custom chip; (ii) the sequence ID, the value expressed as 'ln' and the correspondent foldchange of the Affymetrix array; (iii) the validation data based on the semi-quantitative RT-PCR and Real time PCR; (iv) Reverse Northern blot results deduced following the setting up of the SSH cDNA library.

Comparison of results of differential expression analysis as derived from different kinds of sources was performed , focusing our attention on TCs with ESTs from flesh tissue.

3.13 Expressed sequence tag (EST) database search method for the identification of new members of gluthatione S-transferase superfamily in *C. sinensis*

The schematic view of the step-by-step procedure of the analysis is shown in Fig. 14. In succession, a brief description of each module of the analysis is discussed.

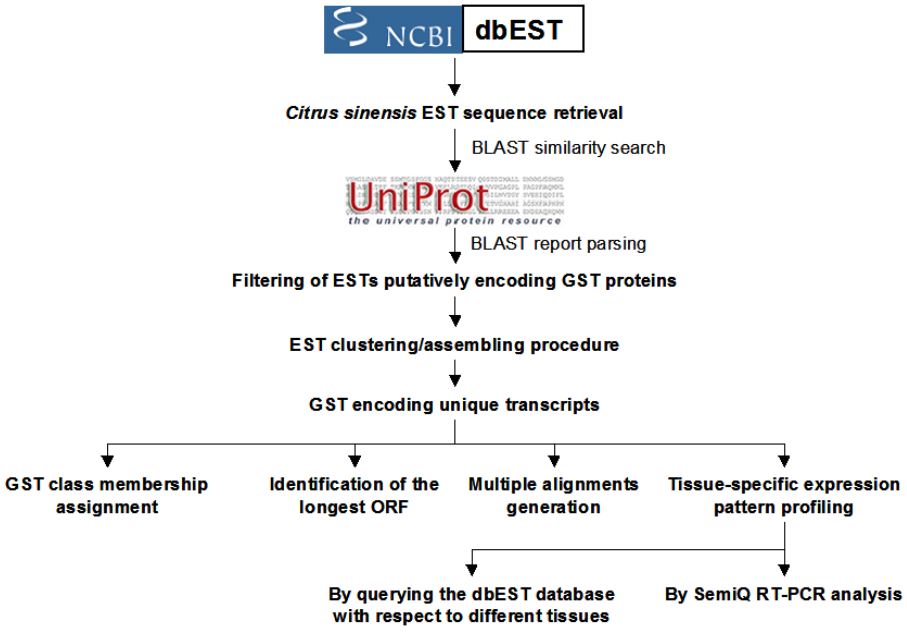

Fig. 14 Step-by-step procedure of the analysis using EST sequences as primary data source.

3.13.1 Identification of ESTs encoding putative GST proteins

Members of the GST gene family of *C. sinensis* were identified screening the EST collection retrieved from the dbEST divison of the GenBank repository (release 1st November 2006).

A preliminary functional annotation was based on BLASTX comparison of the 94.127 EST sequences against the UniProtKB/Swiss-Prot database. The NCBI BLAST formatted report file was parsed with an in-house Perl script in order to select orange ESTs that matched a GST protein as best hit. The original data-set was reduced to 370 putative GST encoding sequences. This collection was used to feed the clustering/assembling procedure. PaCE (default parameter) is the EST clustering software (Kalyanaraman et al., 2003): it was used in order to group ESTs putatively

derived from the same gene, while the assembly software CAP3 (Huang and Madan, 1999; with an overlapping window of 30 nucleotides and a minimum score of 95) was used in order to assemble into longer sequences i.e. tentative consensus sequences (TCs), the short ESTs which have been clustered in the same group.

3.13.2 Open Reading Frame finding

The EXPASY Translate tool (http://www.expasy.ch/tools/dna.html) was used to defined the longest Open Reading Frame (ORF) for each GST putative encoding transcript.

3.13.3 GST class assignment

Entrez query are carried out to retrieve all the *Arabidopsis thaliana* protein sequences belonging to the GST class Tau (resulting in 29 different sequences), class Phi (20 sequences), class Zeta (3 sequences), class Theta (2 sequences), class Lambda (6 sequences) and class MAPEG. All the protein sequences in each class were analysed by Block Maker (Henikoff and Henikoff, 1997), a tool for the identification of conserved blocks (i.e. segments corresponding to the most highly conserved regions of proteins) in a set of related sequences. Using COBBLER (COnsensus Biasing By Locally Embedding Residues; Henikoff and Henikoff, 1997) an embedded consensus sequence for each of the GST classes was generated. These COBBLER-embedded sequences were used as a reference to classify the putative *C. sinensis* GST sequences in order to determine their GST membership class.

3.13.4 Multiple alignments generation

The ClustalW program (Thompson et al., 1994) has been used to generate multiple alignments of nucleotide (mRNA) sequences for each GST class. Distance trees based on the alignment were generated using the program DNADist. The DNA distance matrix was used to identify subgroups of closely related sequences within each GST class. For this reason sequences with a distance lower or equal to 0.2 are considered closely related and they are indicated with the same colour (Fig. 15).

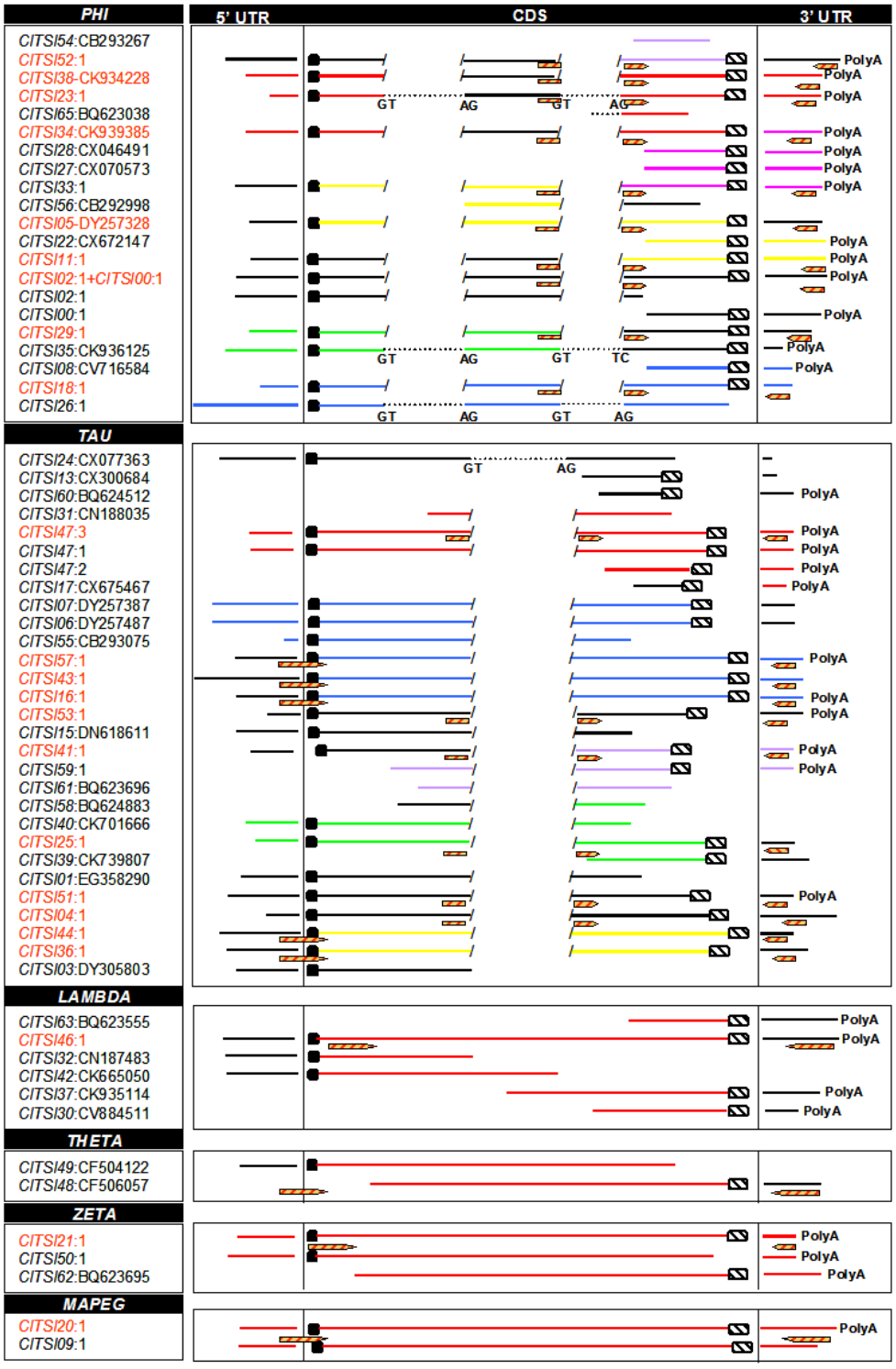

Fig. 15 Schematic view of the multiple alignments of the 62 GST transcripts into 6 different classes: Phi, Tau, Theta, Zeta, Lambda, MAPEG. In all the sequences the 5' and 3' UTR and the PolyA tail were indicated; initial triplet ATG and the stop codons are evidentiated with black and white and black rectangles; red and yellow thin rectangles are the positions of oligonucleotides. In Phi and Tau blocks interrupted lines are the introns location. In red are written the full length sequences.

The total RNA extraction of albedo, flavedo, flesh, young and adult leaves tissues and ovary is described in paragraph 3.2.

The oligonucleotide primers used to amplify target regions are shown in Tab. 7 where the sequences, the annealing temperatures and the sizes of the expected amplicon are reported.

Id sequence	Oligonucleotides characteristics			
	Forward (5'-3')	Reverse (5'-3')	Ta (°C)	Amplicon (bp)
CITSI52-Contig1	GGCCTTCCTTTCTTTGAATCCATTC	TTTTGATAAACCCATTGGGACAGTCGT	60	835
CITSI38-CK934228	GTACCTCAAATTGCAGCCTTTCGGA	TTTTCTCCCAAGGCCCCAAGCATTT	63	659
CITSI23-Contig1	ACGTTATACGGTAGAATCTCGAGCTATCA	TTTTGTCCTCCAAGGCCCCAAGCAT	63	610
CITSI34-CK939385	AGTACCTCAAATTGCAGCCTTTCGGT	TTTTGTCCTCCAAGGCCCCCAGCAT	64	655
CITSI33-Contig1	TTTATACGAGTCGCGAGCTATCATGAGGT	TTTTAAAGCTCCAACTCCAAACAT	60	658
CITSI05-DY257328	ATTTTATACGAGTCGCGAGCTATCATGAGG	ACCCCTTATCCAAGGAACATTTCCCA	64	565
CITSI11-Contig1	TGGGGATTTTACTCTATACGAATCGCGA	ATGGCGACAACAAGAAATCGCCGCA	63	640
CITSI29-Contig1	TCTGAAGATCCAGCCCTTTGGCCAA	TGGGAAATTATTAGACCATGCCA	60	732
CITSI18-Contig1	TCTTGCCAAGAATCCCTTCGGTCA	CATCAATGTAAAATCATCACGCAACCA	60	569
CITSI00-02	TCGAGGGCAATCATAAGGTACTACGCAGC	GATAACAGTAATGACAGCCAGCCGAA	63	539
DQ198153	ATGGTTGTTAAAGTGTATGGTTCAGTT	TTAATGAGCAAGACTCGCTAGTTTC	55	642
CITSI47-Contig3	TCGCCAAGCCCATTTGTGATGAGGGCA	ACGAGACAGGCTGCTGCCTAGTCCGA	66	866
CITSI57-Contig1	AGTAAGCTTCTGTAATAATGGCGGACGA	ACAATACCCTAAGATAACAGTCGGGGACA	64	879
CITSI43-Contig1	TCTGTCACAATGGCGGACGAAGTGGT	AGCAGGCAGCACGATTGCGCTGCT	68	732
CITSI16-Contig1	TCACTCGCCCTTAATTCTCAGTAAGGT	AGATTGACGCCACATAATATTCCCA	60	966
CITSI53-Contig1	TGCTGGGTTACTGGGCAAGCCCCT	ACCTTCATGCATGGGCAACCGCTGA	66	686
CITSI41-Contig1	GTTTACAGGGTGATTTGGGCTCTGA	ACCACTATGCTAGTCCCCCGAACT	63	757
CITSI25-Contig1	GTTCATCGACGAAAAGCTGTTGGCA	ACACAGAGAGAGAGCTAACCCAATCA	63	449
CITSI51-Contig1	TGGCCAAGCCCGTTTGTGTTTAGGGT	AGACTTTCCACACAACATCACACTAC	63	756
CITSI04-Contig1	AGACGTGGTCAAGCCCCTTTGGT	TGGAATGGGAAAAGGGCAAAAGGA	60	889
CITSI44-Contig1	GCAGAAGATTATGGCAACAAAAGTG	GAGCGTACAGAAAGGAGACACGTGCA	60	764
CITSI36-Contig1	GCGAAAATAATATGGCCAAAGAAGTGACGCT	GTCATTACAACACACCACAACACCACCT	64	765
Consensus CITSI48-49	TGGGTGGGCTAAAGAAAGGAAAATGAAGC	TGCGGAACATATAGGCAACATTGAAACCT	64	464
CITSI21-Contig1	ATGCTGAAACTGTATTCATACTGGAGGAGT	TGCTGCTTATTGAGGGTCAACAAAGGCTG	64	880
CITSI46-Contig1	CCTCCAAGATAGGCCCGCTTGGTAC	TCCAGCAACGTACACAAGCTCACATCGGCA	66	672
CITSI20-Contig1	CGACTCGACTATGGCGGATGCAAC	CTATGAGCTTATGCTTGCGCCATGCAGC	66	638

Tab. 7 Primers characteristics used in semi-quantitative RT-PCR to verify the different expression level of *Citrus* GST in different kind of tissues. ***Name*** refers to the GST family full length deduced after *in silico* analysis; ***Id sequence*** is the name of the cluster obtained after the assembling of GST ESTs and they could be contigs or singletons.

The primers (for location site see Fig. 15) designed aimed to specifically define the corresponding full length transcript. Therefore all the reverse oligonucleotides were designed in the 3' UTR region, near the PolyA tail. The forward primers of Phi class were chosen between the second and the third exon. About Tau class, the forward primers are located between the first and the second exon; when the similarity is lower than 0.2 (as deduced by distance matrix), as in some contigs of Tau class, in Theta and MAPEG classes, forward oligonucleotides were constructed between the 5' UTR end and the ATG initial codon. In Lambda and Zeta classes forward and reverse oligonucleotides were chosen in the internal region.

Semi-quantitative RT-PCR conditions and amplification program protocol is described in paragraph 3.6.

3.14 Construction of vectors with *delila, rosea, myc2* and *myb8* genes

3.14.1 Vectors used

The pJIT60 vector (with a double promoter 35S, a CaMV terminator and a Polylinker site; Fig. 16) was used to prepare the constructs with *rosea* and *delila* of *A. majus* and *myc2* and *myb8* of *C. sinensis* transcription factors. The pJIT166 vector (Fig. 16) was used as positive control of GUS activity for protoplast transformation.

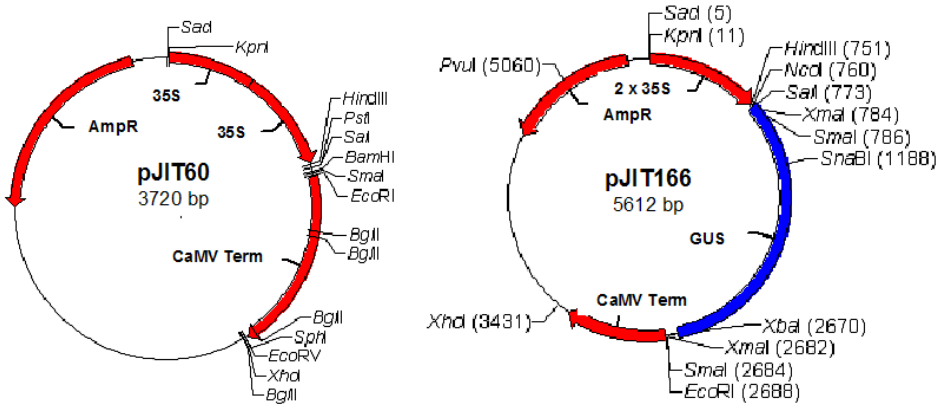

Fig. 16 pJIT60 and pJIT166 vectors maps. **AmpR** refers to Ampicilin resistant vector; **35S** and **2 x 35S** is the Cauliflower mosaic virus 35 promoter; **CaMV Term** is the terminator; **GUS** is the β-glucuronidase coding region.

The *delila* (M84913; myc-like) and *rosea1* (DQ275529; myb-like) genomic DNAs were kindly provided by Cathie Martin from JIC (Norwich) and they were used as positive control of the TFs involved in the regulation of the anthocyanin biosynthesis. The *myc2* (EF645810) and *myb8* (EF537874; myc and myb-like TFs, respectively) genomic DNAs were isolated and characterized by Antonella Cultrone (personal communication).

3.14.2 Bacterial transformation of different genes in Escherichia coli

DNAs were transformed using the DH5α competent cells, put 30 mins in ice, heat shocked 45 sec at 42°C and then 2 mins in ice again. Then 900 µl of SOC medium were added and incubated for 1 hour at 37°C at 300 rpm. 100 µl were spread in LB medium with Ampicillin (150µg/ml) plates and incubated over night at 37C. DNAs were inoculated in 10 ml of LB medium and Ampicillin and extracted with Mini prep Qiagen kit. The DNAs were inserted in the pJIT60 vector (Fig. 18) after digestions of 1µg of DNA with appropriate restriction enzymes. Different restriction enzymes were used also to verify the correct insertion and direction of the cassettes. The digestions were conduced at 37C for 1 hour and then visualized in 0.8% agarose gel in TBE 1x stained with ethidium bromide. The constructs were inoculated in 100 ml of LB medium and Ampicillin at 37°C on at 300 rpm. The plasmidic DNAs were extracted with Maxi prep Qiagen protocol, the correct insertion was verified after digestion and they were used in the transient transformation experiments.

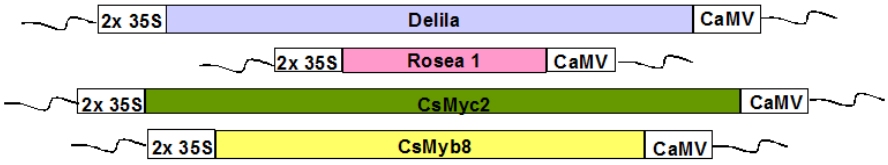

Fig. 18 Schema of cassettes inserted into pJIT60 vector used in protoplast transformation.

3.15 Northern blot analysis

Aliquots of 15 μg of total RNA of pigmented (Tarocco Meli) and common (Valencia) oranges flesh and rind were denaturated at 65°C for 5 minutes and then separated in a 1.2% agarose/formaldeyde gel electophoresis for 3h at 120V. The Northern blot analysis was conduced following the procedure described on Sambrook and Russel (2001).

The probe was obtained after digestion at 37°C for 1 hour of the plasmid pJIT60 with into the Cs*myc2* using appropriate restriction enzymes. The band fragment of the expected size was purified using the QIAquick Gel Extraction kit (Qiagen).

3.16 Transient transformation

3.16.1 Protoplast transformation of Tabacco leaves

Protoplasts preparation. Leaves are taken approximately 3-5 week old tissue-culture grown *Nicotiana Tabacum,* variety Samsun. The lower epidermis was peeled off using fine forceps by grasping the epidermis at the veins and pulling up towards the leaf base. Using a scalpel the peeled areas were cut away and floated peeled side down on CPW.9M solution pH 5.8 (Tab. 8), containing 0.2% cellulase and 0.05% macerozyme, in a petri dish. The dishes were incubated over night under low light conditions at 25°C.

CPW.9M	1000 X (G/20ml)	Use/500ml
KH_2PO_4	0,544	500 μl
KNO_3	2,020	500 μl
$MgSO_4$ $7H_2O$	4,92	500 μl
KI	0,0032	500 μl
$CuSO_4$ $5H_2O$	0,0005	500 μl
$CaCl_2$ $2H_2O$	-	0,74 gr
Mannitolo	-	45 gr

Tab. 8 Wash buffer compunds

Next morning dishes were gently shaker for approximately 60 mins to loosen protoplasts from the leaves. Protoplasts were gently sucked up with a 10 ml pipette an, filtered through 100 μm mesh and centrifuged at 1000 rpm for 5 mins to pellet. Then they were resuspended in approximately 25 ml of wash buffer (CPW.9M) and again with 10 ml of the same solution to wash protoplasts. Cells were counted using a haemocytometer and diluited (if necessary) to 0.5 x 10^6 ml. They were leaved under low light conditions for 90-120 mins. The DNAs for transformation were prepared in a total volume of 20 μl, as Tab. 9.

Sample	pDFR-GUS/ pF3H-GUS	*rosea*	*delila*	*myc2*	*myb8*	pJIT166
1	10 µg	/	/	/	/	/
2	10 µg	3 µg	4 µg	/	/	/
3	10 µg	3 µg	/	/	/	/
4	10 µg	3 µg	/	5,5 µg	/	/
5	10 µg	/	4 µg	/	/	/
6	10 µg	/	4 µg	/	3 µg	/
7	10 µg	/	/	5,5 µg	/	/
8	10 µg	/	/	/	3 µg	/
9	10 µg	/	/	5,5 µg	3 µg	/
10	/	/	/	/	/	10 µg
11	/	/	/	/	/	/

Tab. 9 Amount used and different mixture between of *A. majus* promoter pDFR-GUS or pF3H-GUS and *A. majus* and *C. sinensis* Myc and Myb-like transcription factors (*delila-myc2* and *rosea-myb8*). pJIT166 is a GUS vector used as positive control.

Protoplasts were centrifuged at 1000 rpm for 5 mins and resuspended in W5 pH 5.8 (Tab. 10) solution to a final concentration of 1 x 10^6 cells/ml and leaved for about 20 mins.

W5	100 X (g/200ml)	use/100ml
NaCl	180	1 ml
$CaCl_2$ $2H_2O$	-	1,84 gr
KCl	7.4	1 ml
Glucose	-	500 µl

Tab. 10 W5 buffer compound

Protoplasts were centrifuged at 500 rpm for 4 mins and resuspend in MaMg solution pH 5.6 (Tab. 11) to a final concentration of 1.66 x 10^6 cells/ml and use immediately.

MaMg	g/l	100ml
Mannitol	90	9 gr
$MgCl_2$ $6H_2O$	1.5	1 gr
MES buffer	0.5	100 mgr

Tab. 11 MaMg buffer compounds

Then 60 µl of protoplasts were added into tubes containing DNA. Contemporany 60 µl of PEG pH 8 (Tab. 12) was added into each tube. Mix gently and leaves 20 mins.

PEG.CMS	g/l	100ml
Mannitolo	73	7.3 gr
$Ca(NO_3)_2$ $4H_2O$	24	2.4 gr
PEG	300	30 gr

Tab. 12 PEG.CMS composition

Then 200 µl of 0.2M $CaCl_2$ were added to side of tubes, mixed gently and leaved 20 mins. After a centrifugation at 1000 rpm for 3 mins, samples were resuspend in 900 µl MSP.9M pH 5.8 (Tab. 13) with NAA 2mg/L and BAP 0,5 mg/L and everything was transferred to a 24 multiwell plate and incubate under light for approximately 40 hours.

MSP.9M	g/l
MS media	4.41
Saccarosio	30
Mannitolo	90

Tab. 13 MSP.9W solution compound

Harvesting. After 40 hours protoplasts were spinned for 2 mins at 1000 rpm. Supernatant was taken off and tubes were placed on ice. Then 200 µl CCLR buffer (Tab. 14) were added and tubes were leaved on ice for 30 mins, vortexing every 5 mins. The supernatant obtained after a spin in a coldroom for 3 mins at 13.000 rpm was transferred to microtiter plate.

CCLR	Final concentration
Phospahte buffer pH 7.8	100 mM
EDTA	1 mM
Triton-X-100	1%
Glicerolo	10%
B̃-Mercaptoethanol	7 mM

Tab. 14 CCLR solution compound

GUS assay. In a transparent microtiter plate were set up the reactions (25 µl extract and 225 µl MUG buffer - 22 mg/50 ml of CCLR) on ice. Then 50 µl were aliquoted in 3 black microtiter plates and they were closed and put at 37°C incubator. After 30, 60 and 90 mins each plate was removed, put on ice and 50 µl of 0.4M Na_2CO_3 were added to stop the reaction. Umbelliferone software was used to analysed data in a 1420 Multilaber Counter (Wallac).spectrofluorimeter.

Bredford assay for protein quantification. Protein standards ranging from 0.1 – 1.4 mg/ml using BSA standard were prepared and separated in the 96 well plate. To the blank wells, 5 µl of buffer was added. The unknown samples were prepared with an approximate concentration between 0.1 – 1.4 mg/ml. To each well being used, 250 µl of the Bradford Reagent (Sigma) were added and mixed on a shaker for approximately 30 seconds. Samples were incubated at room temperature for 5 to 45 minutes. Then the absorbance at 595 nm was measured. The statistic analysis were calculated considering the double measurement of the Multilaber Counter, the double analysis of GUS staining and the triplicate used for each sample.

3.16.2 Agroinfiltration of C. sinensis fruits

A single colony of different DNAs (*rosea*, *delila*, Cs*myc2*, Cs*myb8*) and of pBinl9:GUS HA in GV3101 *Agrobacterium* strain (positive GUS control) was inoculated in 100 ml of Louria Bertani medium containing appropriate antibiotic(s) and they were leaved over night at 28 C at 250-300 rpm. The cultures were pellet by

centrifugation at 6000 rpm for 20 mins and diluited to get a final OD_{600} of around 2.4 in MMA medium (MS salts, 10 mM MES pH 5.6, 20g/L sucrose, 200 μM acetosyringone). They were incubate at room temperature for 1 hour. Bacterial cultures were used to inject all the fruit with a sterile 1 ml hypodermic syringe.

For the hystochemical assay, the GUS assay buffer consisting of 1 mg/L X-Gluc, 0.5% Triton X-100, 20% methanol and 50 mM sodium phosphate buffer pH 7.0, according to Ahmad and Mirza (2005) and also 1mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide), 100 mM phosphate buffer pH 7.2, 0.1% Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 10 mM EDTA and 20% methanol (to reduce background), according to Spolaore et al. (2001), were used. After a vacuum treatment of at least 15 mins to facilitate the penetration of the drying solution, the immersed tissues were kept over night in the dark at 37°C.

3.16.3 Bombardment of orange fruits and Petunia white flowers

Orange rind, flesh and spongy tissues of Valencia orange (a common cultivar of sweet orange) were subjected to the bombardment. We used the rind of an entire orange; the spongy tissue of half an orange and some segments to shut flesh. The bombardment was carried out using a gun with pressurised helium. In order to monitorate and control the transformation efficiency, the control plasmid pJIT166 carrying the GUS gene under the CaMV 35S promoter was used in each bombardment experiment. Gold particles (10 mg) were coated with 10 μg of the positive plasmid control in a volume of 20 μl. 100 μl Xho buffer (150mM NaCl; 10mM Tris pH 8.0), 100 μl 0.1M spermidine, 100 μl 25% PEG M.W. 1300-1600 and 100 μl 2.5M $CaCl_2$ were added to the gold particles mixed with the plasmid while vortexing and sonicating. After an incubation of 10 mins, the pellet was spin on pulse and resuspended in 100% EtOH two times; finally 5 ml of 100% EtOH were added to the pellet in a sterile scintillation vial. A helium pressure among 100 and 400 psi was employed. After bombardment, *Citrus* tissues were kept at room temperature for 48h and then subjected to histochemical staining of GUS activity (GUS solution is constituted by 50mM sodium phosphate buffer pH 7, 0.5 mg/ml X-Glucuronide and 0.05% Triton X-100).

The same procedure was used for white petals of *P. hybrida* (cv Mitchell), with two mutations in two Myb TFs, *an2* and *an4* (Quattrocchio et al., 1993). The biolistic system was performed using the constructs described in paragraph 3.14.2. In these case no GUS staining was conduced, but it was simply observed the possible red purple spots as effect of regulation and activation of the anthocyanins biosynthesis.

The tissues were observed using an Zeiss stemi sv 6 model.

4. RESULTS AND DISCUSSION

4.1 Construction of a subtractive cDNA library and isolation of differential transcripts

The main object of this work was tthe isolation of the differentially expressed transcripts between a pigmented cultivar (Moro) and a common orange (Cadenera). The methodology used was the SSH cDNA library.

The subtractive cDNA library is comprised of 1248 clones. The hybridisation of reverse Northern filters through forward (tester) and reverse (driver) probes showed 260 differential transcripts: 230 were up-regulated in the Moro cultivar, and 30 were up-regulated in the Cadenera ones. The 260 differential clones corresponded to 82 differential genes, and 52 were singletons. In fact, gene redundancy is one of the drawbacks of the SSH methodology; however, this could be also an advantage because it considers possible signals from highly expressed transcripts. The ESTs length ranged from 250 to 750 bp. After clustering analysis and various alignments, 201 ESTs were submitted to the dbEST GenBank database.

The 208 redundant clones were assigned to 30 functional genes, and their putative functions were deduced from the literature. The different categories are shown in Fig. 18.

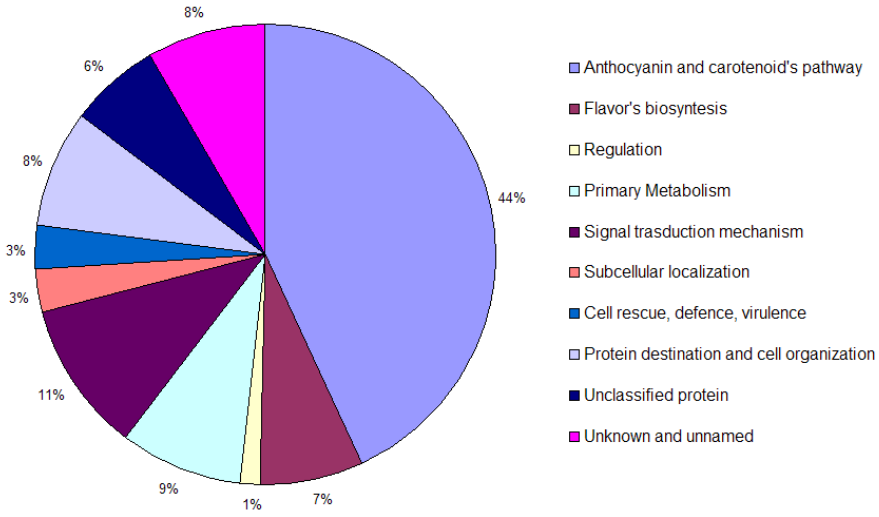

Fig. 18 Functional categories used to order and catalog differential clones

4.1.1 Anthocyanins and the carotenoid pathway

Forty-four percent (Fig. 18) of the differential clones are genes that encode enzymes implied in anthocyanin and carotenoid biosynthesis. We isolated six of the structural genes involved in anthocyanin biosynthesis (PAL, CHS, DFR, ANS, UFGT, GST), and all of them were up-regulated in the Moro cultivar, as revealed by reverse Northern analysis. The expression analysis of CHS, ANS and UFGT in the flesh of blood oranges was positively correlated with anthocyanin accumulation (Cotroneo et al., 2006). Lo Piero et al. (2005a) showed lower levels of DFR expression in common orange cultivars (Navel and Ovale) compared to those of blood oranges (Tarocco). Thus, DFR activity could be a key factor in the production of anthocyanins.

4.1.2 Regulatory genes and signal trasduction mechanisms

Just one percent (Fig. 18) of the differential clones encoded regulatory genes. We isolated two bHLH clones (EG358372) and one MADs box (EG358383). The bHLH transcription factors comprise one of the most widespread and common classes of regulatory genes. MADS box genes have been recently associated with flavonoid metabolism (Lalusin et al., 2006). They represent a large group of regulatory genes found in yeast, animals and plants (Ng and Yanofsky, 2001; Shore and Sharrocks, 1995; Theiben et al., 1995).

The category "Signal transduction mechanism" (11%; Fig. 18) included many clones that are up-regulated in both blood and common oranges (as reverse Northern showed). Among them, a transcript (EG358294) homologous to a putative serine receptor kinase (SRK) from rice (CAE05335.2) was isolated 25 times. We hypothesize that it may be involved in some regulatory mechanisms during fruit ripening. In fact, protein phosphorylation, which occurs via Ser/Thr kinases (and phosphatases), has a regulatory role in many signal transduction pathways (Buchanan et al., 2002; Gu et al., 2002).

4.1.3 Flavor biosynthesis and pathogen defense

Seven percent (Fig. 18) of the differential clones identified encoded for flavour biosynthetic enzymes. Using the SSH technique, we isolated cDNAs corresponding to proteins that were homologous to alcohol acyl transferases (EG358303), 10-hydroxigeraniol oxidoreductase (EG358189) and valencene synthase (EG358204). Based on reverse Northern analysis, these transcipts were up-regulated in blood oranges.

Three percent of the clones are implicated in various defense mechanisms (Fig. 18). Pathogenesis-related protein PR10A, acidic chitinase III, osmotin-like protein and Lea5 (EG358302, EG358203, EG358216 and EG358377, respectively) were isolated. With the exception of Lea5, the expression levels of these genes are higher in blood oranges than in the common cultivar, as indicated by reverse Northern analysis. The accumulation of PR (pathogenesis-related) proteins is one of the best characterized plant defense responses to pathogen attack (Park et al., 2003). Therefore, the presence of PR transcripts observed in our system is probably associated with defense-related responses or with undefined roles during orange ripening. Anthocyanins are also synthesized as a defense mechanism. This is evident in blood oranges when fruits are attacked by fungi; the fruit reacts by synthesizing a high concentration of anthocyanin.

4.1.4 Other functional categories

Almost 8% of all the differential clones (Fig. 18) belonged to the classes of "Protein destination" (i.e. DNAJ protein – EG358360-, aspartic proteinase – EG358343 -, Rad23 – EG358378-) and "Cell organization" (i.e. pectinesterase - EG358218 and POM30 - EG358259 -). On the basis of reverse Northern analysis, more of them are highly expressed in Moro than in the common orange fruit.

Other isolated cDNAs belong to the functional category of "Primary metabolism" (9%; Fig. 18) and "Subcellular localization" (3%; Fig. 18).

Finally, no matches were found in non-redundant databases for almost 8% of the SSH clones (Fig. 17), and we therefore classified them as having unknown and unnamed functions. Six percent of the remaining cDNAs matched ESTs with no assigned functions, and we called these "Unclassified".

4.2 Analysis of differential expression level through semi-quantitative RT-PCR and Real time PCR

Among different transcripts isolated using the SSH library, we have analyzed the differential expression level of 10 genes by semi-quantitative RT-PCR (Fig. 19). For the expression level analysis, we first chose the most redundant transcripts. Out of these, we then selected those that could give us relevant functional information about differences between blood and common oranges [PAL (isolated in 7 clones), cytochrome b5 (13 clones), GST (25 clones), pectinesterase (10 clones), valencene synthase (2 clones), alcohol acyl transferase (10 clones), 10-hydroxigeraniol oxidoreductase (6 clones) and putative SRK (25 clones)]. We also analyzed regulatory genes that are putatively involved in the anthocyanins mechanism control (bHLH and MADs box).

Among biosynthetic enzymes involved in flavor production, we analyzed the differential expression of AAT, 10-hydroxigeraiol oxidoreductase and valencene synthase. As demonstrated by semi-quantitative RT-PCR, the expression level of these transcripts remained almost the same during the ripening process in both cultivars, although higher levels of transcripts were detected in blood oranges (Fig. 19, 1-6). AAT belongs to a class of acyl-CoA-dependent acyltransferases; they play important roles in secondary metabolism in plants and fungi, including the biosynthesis of anthocyanins (Fujiwara et al., 1999; Suzuki et al., 2001, 2002, 2004). AAT activity is responsible for producing volatile esters, and it has been observed in plant tissues such as flowers and fruits (Ueda et al., 1997; Aharoni et al., 2000). The 10-hydroxigeraiol oxidoreductase is involved in the biosynthesis of geraniol, an acyclic monoterpene alcohol emitted from the flowers of many species, notably roses (Bayrak, 1994; Antonelli et al., 1997; Rao et al., 2000). The mixture of geranial and neral, also called citral, imparts a 'lemon' flavour. Valencene is an aromatic compound that increases during the last stages of ripening (Maccarone et al., 1998). *Citrus* fruit flavours and aromas are composed of complex combinations of soluble (mostly acids, sugars and flavonoids) and volatile compounds. The latter mostly consists of mono- and sesquiterpenes (the major components of citrus essential oils), which accumulate in specialized oil glands located in the flavedo (external part of the peel) and in oil bodies in the juice sacs (Sharon-Asa et al., 2003). The sequiterpenes valencene, alpha- and beta-sinesal, which are present in minor quantities in oranges, play an important role in the overall flavour and aroma of the fruit (Maccarone et al., 1998; Vora et al., 1983; Weiss, 1997).

As demonstrated by reverse Northern and semi-quantitative RT-PCR results (Fig. 19, 7-8), the putative SRK was slightly up-regulated in blood orange during the late phases of sampling. It might, therefore, regulate the accumulation of differential compounds in blood oranges. Real time quantitative PCR analysis (Fig. 20) confirmed semi-quantitative results. The SRK transcript increased continuously during maturation, even if the expression level was very low in the first and second samples. In Cadenera, all of the samples showed very low transcript levels. Results obtained with the clone EG358294 and the other kinases, that are identified in the present work, support a possible role for this class of proteins in ripening regulation and/or in the fruit pigmentation processes.

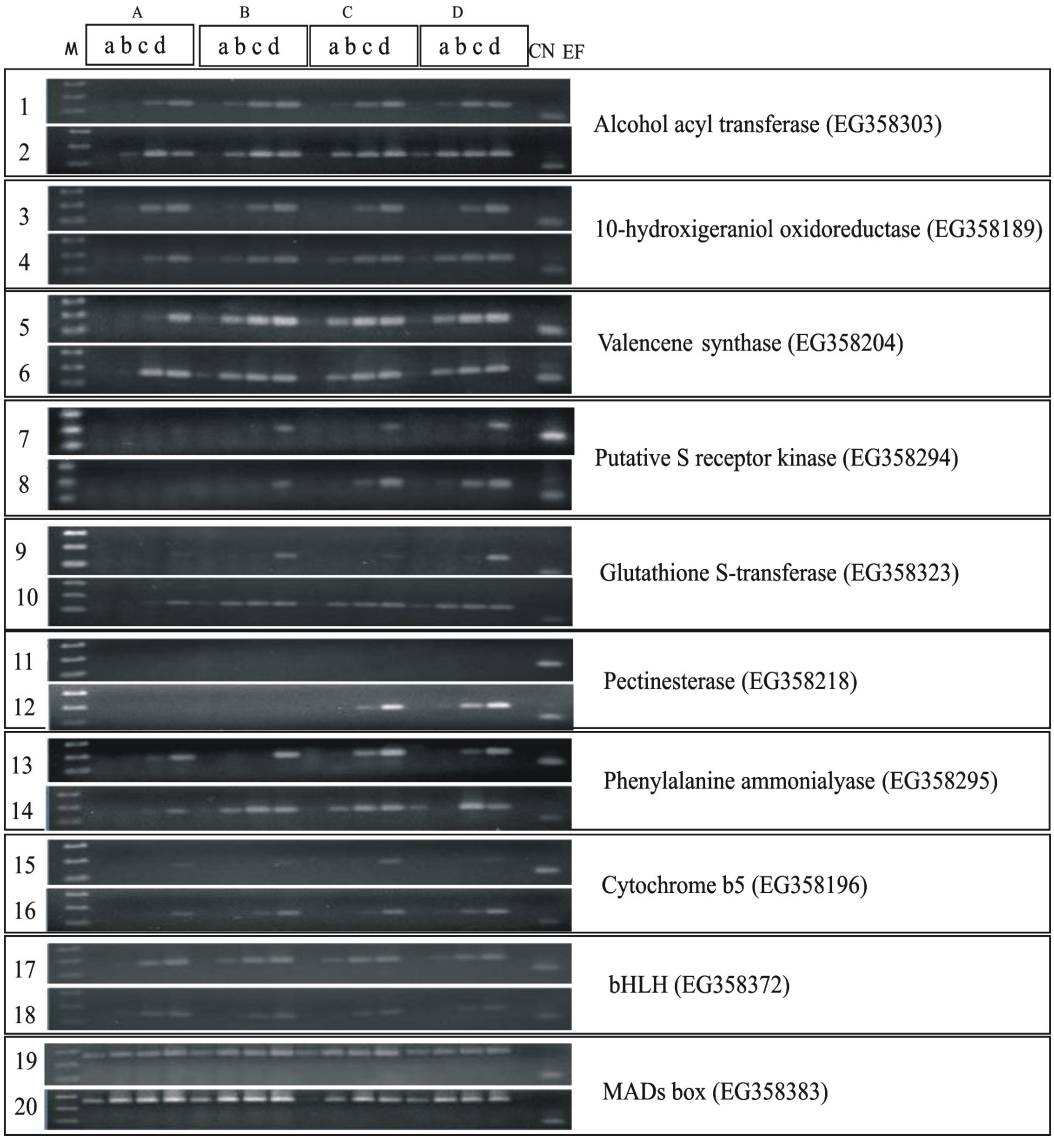

Fig. 19 Semi-quantitative RT-PCR data. **A**, **B**, **C**, **D** are I, IV, VI, VIII samples used (see Plant Material); **a**, **b**, **c**, **d** are the number of cycles used during the amplification to analyze the rise of transcription level (20, 25, 30 and 35 cycles, respectively); **1**, **3**, **5**, **7**, **9**, **11**, **13**, **15**, **17**, **19** are expression data of Cadenera, **2**, **4**, **6**, **8**, **10**, **12**, **14**, **16**, **18**, **20** are expression data of Moro.

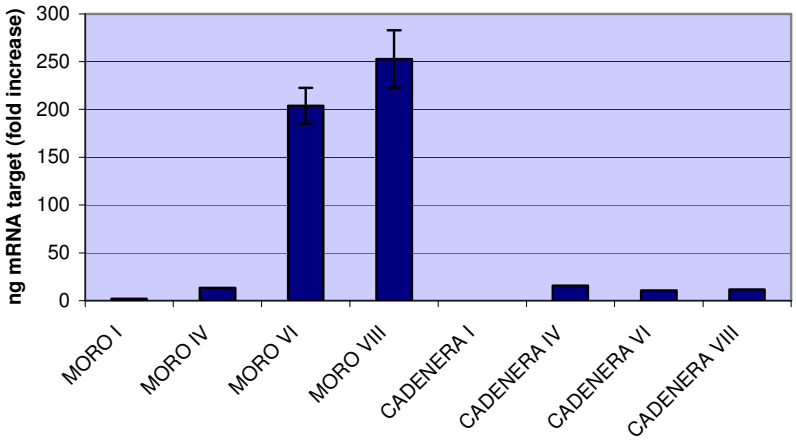

Fig. 20 Real time PCR expression level of putative Ser receptor kinase in Moro and Cadenera flesh during ripening.

Among the genes involved in the anthocyanin pathway, we analyzed PAL, cytochrome b5 and GST. Their expression was over-expressed at all time points in blood oranges compared to common fruits. PAL (Fig. 19, 9-10) is involved in the first committed step of phenylpropanoid metabolism, and its activity is essential for the synthesis of all flavonoids, including anthocyanins. In the common orange, the cytochrome b5 transcript was detected only during the last amplification cycle (Fig. 19, 11-12). Cytochrome b5 acts as a reductase and is essential for cytochrome P450 enzymes, which are involved in general phenylpropanoid metabolism (cinnamate 4-hydroxylase) and anthocyanin biosynthesis (flavonoid 3'-hydroxylase) (De Vetten et al., 1999). GST was isolated 25 times among the differentially regulated clones. The semi-quantitative RT-PCR results (Fig. 19, 13-14) showed that GST transcription was strongly enhanced in blood oranges compared to common oranges in all analyzed samples. In common oranges, no GST transcript was detectable at the first time point of sampling. These results were confirmed by Real time PCR (Fig. 21). Moreover, GST transcript levels increased at a constant rate in the Moro cultivar during the entire period of ripening time with the exception of the third sampling period. The transcript levels for GST at a given stage of fruit maturation were positively correlated with the accumulation of anthocyanin at the same stage (Fig. 22). Anthocyanins were totally absent in the first two samples, but they increased from 1.23 to 46.75 mg/100g. At stage VI of Moro maturation, the anthocyanins content remained almost constant, although the level of GST transcript was low. A discrepancy between the levels of transcripts (high) and pigment accumulation (low) at time point IV could reflect regulation at the level of protein functionality.

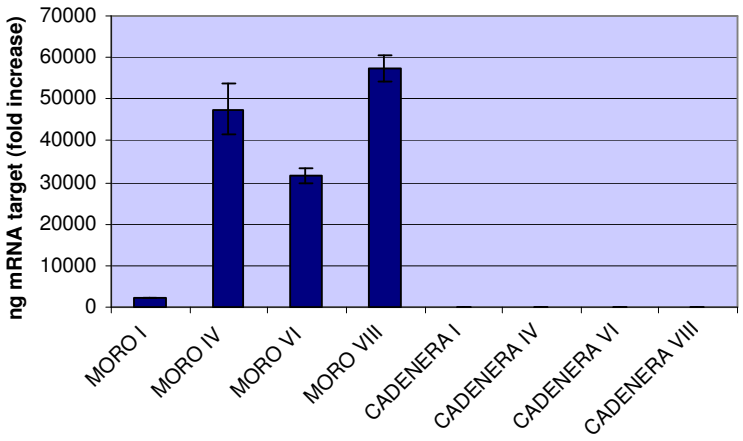

Fig. 21 Real time PCR expression level of GST in Moro and Cadenera, during stages of ripening.

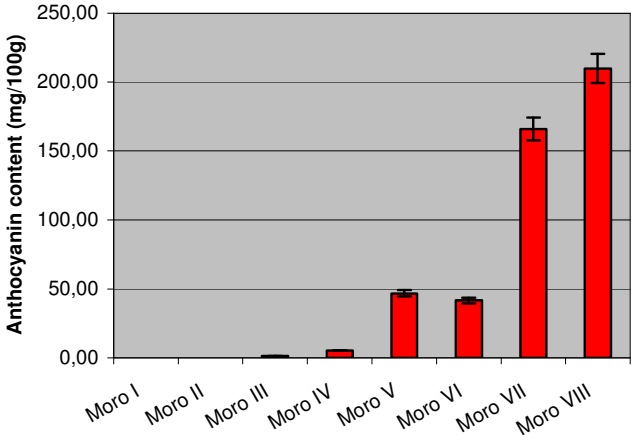

Fig. 22 Content of anthocyanin accumulated in Moro oranges in all eight samples during ripening.

Semi-quantitative RT-PCR showed that pectinesterase expression was completely absent in common orange fruit (Fig. 19, 15-16), while it became evident in ripe pigmented oranges. Real time data (Fig. 23) showed that the corresponding mRNA accumulated in Moro samples only during advanced ripening phases, whereas they were absent at the early phases of ripening and in all stages examined for the common orange Cadenera. Pectinesterases catalyze the demethylation of pectins, are the most abundant class of macromolecules within the cell wall matrix and are also abundant in the middle lamellae among primary cell walls. They regulate intercellular adhesion and are the major adhesive material in cells. Fruit cellular walls are usually highly enriched in pectins, often representing more than 50% of wall dry matter (Castillejo et al., 2004). During fruit softening, pectins typically undergo both solubilization and depolymerization, which contribute to wall loosening and disintegration (Fischer and Bennet, 1991). Pectins confer compactness and swollen consistency to fruit. According to these characteristics, our data were consistent with the observation that, at maturity, blood oranges are softer and less

swollen than common cultivars. This phenomenon can be advantageous because blood oranges are easy to peel, like mandarins, but it is also a drawback because it causes a shorter shelf-life.

Regarding regulatory genes, the expression analysis was performed on bHLH and MAD box transcription factors. The bHLH expression seemed to be higher in common oranges than in blood oranges. MAD box transcripts remained constant during the maturation process in the blood and in non-pigmented oranges, but the expression level was slightly higher in the common cultivar (Fig. 19, 17-20). This was in agreement with reverse Northern results.

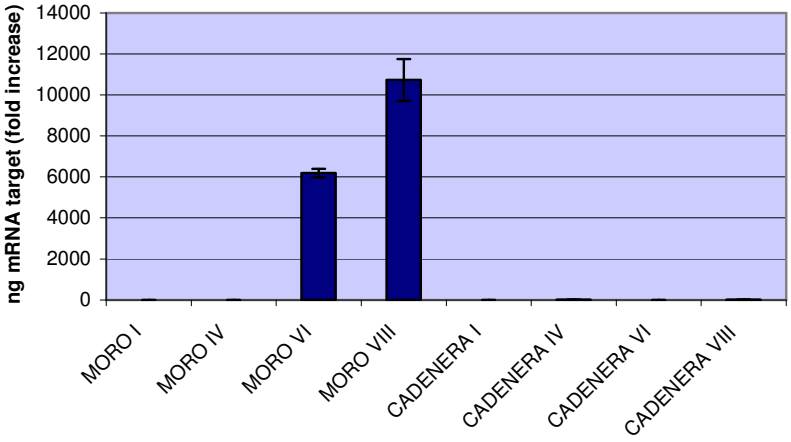

Fig. 23 Real time PCR expression level of pectinesterase in Moro and Cadenera during ripening..

4.3 Phylogenetic analysis

4.3.1 The GST gene family

Different types of plant GSTs and their various functions are known. In order to determine the class to which *C. sinensis* GST belongs, a multiple alignment of Cs-GST amino acid sequences (Fig. 24) against An9 (GST type III) from *P. hybrida,* ParB from *Nicotiana tobacum*, GmGST26A (GST type III) from soybean, type I maize (GSTI, GSTIII, GST IV) and Bz2 (GST type I) from *Zea mays* was performed. According to the conservation of amino acid residues (Fig. 24) and the phylogenetic tree (Fig. 25), we hypothesized that Cs-GST could be a type I (or Phi class) GST. Cs-GST was most closely related to the *Petunia* An9 and was also related to the type I maize genes GSTI, GSTIII and GSTIV. Based on the low sequence conservation among type I and type III GSTs, we propose that they diverged from a common ancestral gene before the evolution of An9 and B*z2* into specialized angiosperm GSTs, which are required for the efficient transport of anthocyanins to the vacuole.

```
Ph An9          -----MVVKVHGSAMAACPQRVMVCLIELGVDFELIHVDLDSLEQKKPEFLVLQP-FGQV 54
Cs-GST          -----MVVKVYGSVKAACPQRVLACLLEKGVEFEIVQVDLDEGEHKRPEFLLRQP-FGQV 54
Nt-ParB         -----MAIKVHGSPMSTATMRVAACLIEKELDFEFVPVDMASGEHKKHPYLSLNP-FGQV 54
Zm-GSTIII       ----MAPLKLYGMPLSPNVVRVATVLNEKGLDFEIVPVDLTTGAHKQPDFLALNP-FGQI 55
Zm-GSTI         --MAP--MKLYGAVMSWNLTRCATALEEAGSDYEIVPINFATAEHKSPEHLVRNP-FGQV 55
Zm-GST          --MATPAVKVYGWAISPFVSRALLALEEAGVDYELVPMSRQDGDHRRPEHLARNP-FGKV 57
Gm-HSP26A       MAATQEDVKLLGIVGSPFVCRVQIALKLKGVEYKFLEENLG---NKSDLLLKYNPVHKKV 57
Zm Bz2          --MTAGTMRVLGGEVSPFTARARLALDLRGVAYELLDEPLGP--KKSDRLLAANPVYGKI 56
                       ::: *   :    *    *      ::::        ::    *  :* . ::

Ph An9          PVIEDGD-FRLFESRAIIRYYAAKYEVKG-----SKLTGTTLEEKALVDQWLEVESNNYN 108
Cs-GST          PVIEDGD-FKLFESRAIIRYYAAKYANQG-----PNLLGNTLEEKALVDQWLEVEAHNFN 108
Nt-ParB         PAFEDGD-LKLFESRAITQYIAHVYADNG-----YQLILQDPKEMPSMSVWMEVEGQKFE 108
Zm-GSTIII       PALVDGD-EVLFESRAINRYIASKYASEG-----TDLLPATAS-AAKLEVWLEVESHHFH 108
Zm-GSTI         PALQDGD-LYLFESRAICKYAARKNKPE-------LLREGNLEEAAMVDVWIEVEANQYT 107
Zm-GSTIV        PVLEDGD-LTLFESRAIAPHVLPKHKPE-------LLGGGRLEQTAMVDVWLEVEAHQLS 109
Gm-HSP26A       PVFVHNE-QPIAESLVIVEYIDETWKNNP-------ILPSDPYQRALARFWSKFIDDKIV 109
Zm Bz2          PVLLLPDGRAICESAVIVQYIEDVAPESGGARAGSLLLPDDPYERAMHRFWTAFIDDKFW 116
                *.:   :   : ** .* .:       .        :        .    *  .  .:

Ph An9          DLVYNMVLQLLVFPKMGQTSDLTLVTKCANKLENVFDIYEQRLSKSKYLAG--EFFSLAD 166
Cs-GST          DLAFNLVLQLVILPRMGQRSDTALVHNLEQKLEAVLNIYEQRLSKSNYLAG--DSFTLAD 166
Nt-ParB         PPATKLTWELGIKPIIGMTTDDAAVKESEAQLSKVLDIYETQLAESKYLGG--DSFTLVD 166
Zm-GSTIII       PNASPLVFQLLVRPLLGGAPDAAVVEKHAEQLAKVLDVYEAHLARNKYLAG--DEFTLAD 166
Zm-GSTI         AALNPILFQVLISPMLGGTTDQKVVDENLEKLKKVLEVYEARLTKCKYLAG--DFLSLAD 165
Zm-GSTIV        PPAIAIVVECVFAPFLGREPNQAVVDENVEKLKKVLEVYEARLATCTYLAG--DFLSLAD 167
Gm-HSP26A       GAVSKSVFTVDEK------ERKKNVEETYEALQFLENELKDKK----FFGG--EEFGLVD 157
Zm Bz2          PALDAVSLAPTPG------ARAQAAEDTRAALSLLEEAFKDRSNGRAFFSGGDAAPGLLD 170
                                       . .    *  : :  : :     ::.*      * *

Ph An9          LSH------LPSLRFLMNEGGFSHLVTKPKCLHEWYLDISSRDSWKKVLDLMMKKISEIE 220
Cs-GST          LSH------LPALRYLMNEAGMAHLVTQPKHVNAWWDKISSRPAWKKLASLAH------- 213
Nt-ParB         LHH------IPNIYYLMS-SKVKEVFDSRPRVSAWCADILARPAWVKGLEKLQK------ 213
Zm-GSTIII       ANH------ALLPALTSARPPRPGCVAARPHVKAWWEAIAARPAFQKTVAAIPLPPPPSS 220
Zm-GSTI         LNH------VSVTLCLFA-TPYASVLDAYPHVKAWWSGLMERPSVQKVAALMKPSA---- 214
Zm-GSTIV        LSP------FTIMHCLMA-TEYAALVHALPHVSAWWQGLAARPAANKVAQFMPVGAGAPK 220
Gm-HSP26A       IAAVFIAFWIPIFQEIAGLQLFTS--EKFPILYKWSQEFLNHPFVHEVLPPRDPLFAYFK 215
Zm Bz2          LALGCFLPALRACERLHGLSLIDASATATPLLDGWSQRFAAHPAAKRVLPDTEKVVQFTR 230
                                          : *  :  :   .

Ph An9          AVSIPAKEEAKV 232
Cs-GST          ------------
Nt              ------------
III             SA---------- 222
GST             ------------
Zm-GSTIV        EQE--------- 223
Gm-HSP26A       ARYESLSASK-- 225
Zm Bz2          FLQAQFRVHVS- 241
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Fig. 24 ClustalW multiple alignments of amino acid sequences of Cs-GST and other plant GSTs involved in the vacuolarization of anthocyanins

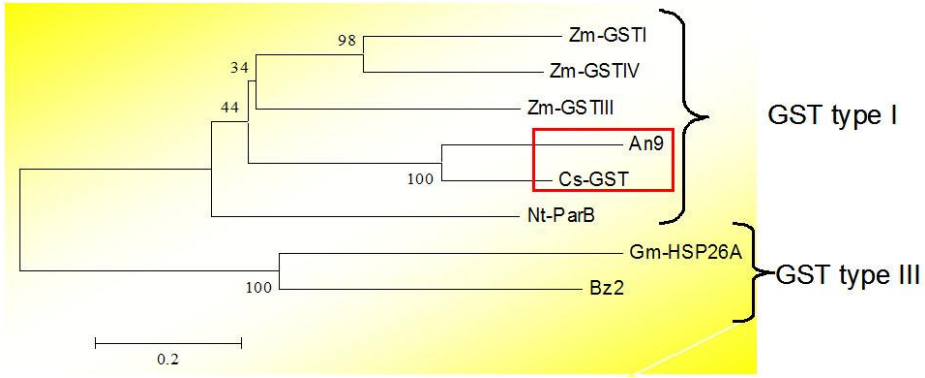

Fig. 25 Phylogenetic tree deduced from the alignment of some type I and III plant GSTs.

4.3.2 bHLH and MADs box transcription factors

Transcription factors are involved in controlling different pathways. To understand if *C. sinensis* bHLH (Cs-bHLH) might be involved in the anthocyanin pigmentation and explain and justify the high transcript expression level in Cadenera common oranges (as in semi-quantitative RT-PCR; Fig. 19, 17-18), a multiple alignment (Fig. 26) of Cs-bHLH amino acid sequences and other plant bHLHs involved in anthocyanin pigmentation was performed; other sequences included Lc and Sn from *Zea mays,* Delila from *Antirrhinum majus*, TT8 from *Arabidopsis thaliana*, JAF13 and An1 from *P. hybrida.* Lc expression, alone or in combination with c1 (a member of the c1 family that encodes MYB-type transcription activators), induces pigmentation and can activate the structural anthocyanin genes of dicots (Quattrocchio et al., 1998). The *delila* gene controls anthocyanin accumulation in the tube of *A. majus* flowers (Martin et al., 1991). JAF13 from *P. hybrida* is the ortholog of Delila and R (Quattrocchio et al., 1998). An1 regulates flower colour in *Petunia* (Quattrocchio et al., 1998) and activates the transcription of the structural anthocyanin gene *dihydroflavonol reductase* (*dfr*). TT8 regulates anthocyanin synthesis in *A. thaliana* seedlings (Shirley et al., 1995) and proanthocyanidin synthesis in siliques (Nesi et al., 2000). We also included GBOF-1 from *Tulipa gesneriana* [an auxin inducible G-box binding bHLH transcription expressed in low temperatures (Rietveld et al., 1999 unpublished data)] because a previous similarity search, using BLASTx, showed high similarity between the sequences of Cs-bHLH and GBOF-I.

Typically, a bHLH domain comprises a stretch of about 18 hydrophilic and basic amino acids at the N-terminal domain, followed by two regions of hydrophobic residues that are predicted to form amphypathic α–helices separated by an intervening loop (Murre et al., 1994). Amino acids at position His3-Glu7-Arg11 of *C. sinensis* sequence (H-E-R; Fig. 26) are important to link DNA. The DNA backbone is contacted by basic residues at positions R(8) and R(10), which are conserved in the majority of plant proteins. A leucine residue is present (and conserved) at position 21 of Cs-bHLH, which would be important for dimerization. However, as with In1 (intensifier) of *Z. mays* (a recessive mutation described by Fraser AC in 1924, that causes the derepression of anthocyanin biosynthesis, according to Burr et al., 1996), the Cs-bHLH sequence showed significant homology with a class of myc homologs involved in anthocyanin biosynthesis but lacks a leucine zipper domain on the C-terminal side of the HLH/ZIP domain (Jia et al., 1997).

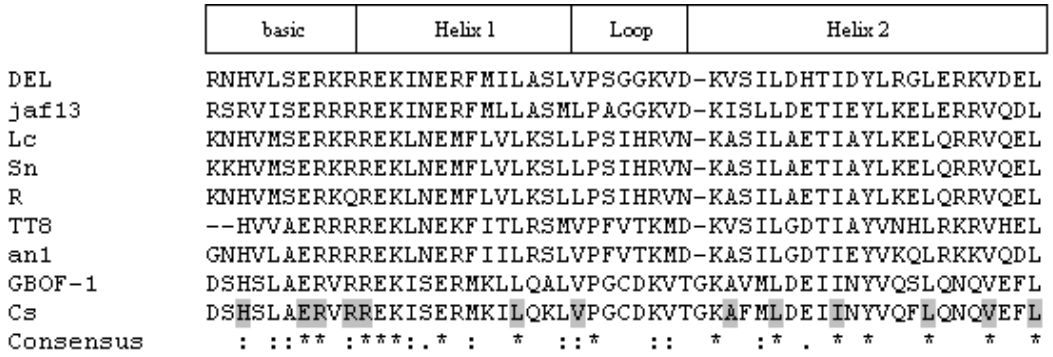

Fig. 26 ClustalW multiple alignment of the bHLH conserved domain involved in anthocyanins pathway regulation and Cs-bHLH and GBOF-1. Stars indicate conserved amino acids in all sequences; grey rectangles are the conserved amino acids specific for anthocyanin control.

Thus, according to the expression level obtained by semi-quantitative RT-PCR and phylogenetic analysis, the bHLH isolated through the SSH library could be not involved in orange anthocyanin pigmentation.

The expression level of the MAD box (Fig. 19, 19-20) did not suggest its involvement in anthocyanins biosynthesis. For this reason, we performed a multiple alignment (data not shown) of MADs from *C. sinensis* (Cs-MADS), isolated through SSH library, with TT16/ABS from *Arabidopsis*, FBP24 from *Petunia*, DEFH21 from *A. majus*, ZMM17 from *Z. mays,* GGM13 from *G. gnemon* (Nesi et al., 2002), SOC1 from *Malus*, MAD4 from *Malus* and *Vitis* (because a previous similarity search-based analysis BLASTx showed high similarity with these sequences), MADS10 from *Ipomea batatas*, DEFH28 from *A. majus* and MADS4 from *Betula pendula* (Lalusin et al., 2006). All of these plant MADS are involved in anthocyanin accumulation. The MADS box domain is composed mainly of four regions (Purugganan et al., 1995): the MADS box and the I, K and C regions (Shore and Sharrocks, 1995; Nesi et al., 2002). The analysis of the conserved domain and the typical amino acids of the MADS protein involved in anthocyanin pigmentation (data not shown) and its differential expression indicated that Cs-MADS box could not be involved in anthocyanin regulation. In fact, all functional amino acids were not conserved, and there was no differential expression between blood and common oranges during the ripening process.

4.4 Analysis of differential gene expression by microarrays

4.4.1 Custom chip

Differential transcripts data analysis. Two different kinds of microarrays were used to validate differential expression data of ESTs isolated through the SSH library. The custom array was constructed using 104 ESTs sequences isolated with the cDNA SSH library, 128 sequences isolated through a cDNA-AFLP analysis and 68 transcription factor sequences derived from the *HarvEST Citrus* database. The array was hybridised using total RNA from Moro and Cadenera ripe flesh. Among 302 ESTs, 67 are 'turned on' and differentially expressed (Fig. 27): 35 are derived from the SSH library, 25 are from the cDNA-AFLP library and 7 are from *HarvEST Citrus* database.

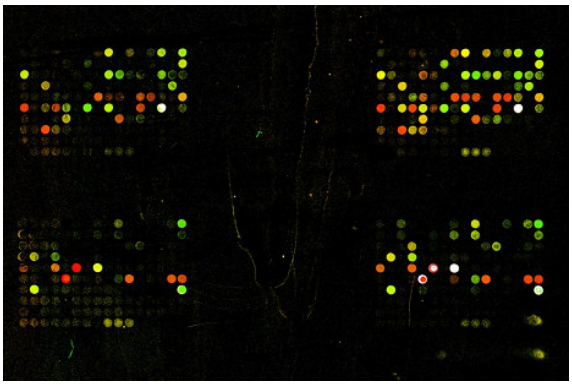

Fig. 27 Chip hybridazation.

Among the differential transcripts, 46 were up-regulated in pigmented oranges, and 21 were up-regulated in common ones. Schematic results are summarized in Tab. 14.

Sequence ID	Accession Number - Similarity	Library	UP	Functional category
Xcs.209	EG358308 GST	SSH	Moro	Anthocyanin biosynthesis
Xcs.216	UFGT	SSH	Moro	Anthocyanin biosynthesis
Xcs.260	EG358193 PAL	SSH	Moro	Anthocyanin biosynthesis
Xcs.241	EG358237 F3H	SSH	Moro	Anthocyanin biosynthesis
Xcs.229	EG358219 CHS	SSH	Moro	Anthocyanin biosynthesis
Xcs.232	EG358209 Cytochrome b5	SSH	Moro	Anthocyanin biosynthesis
Xcs.215	EG358256 CHS	SSH	Moro	Anthocyanin biosynthesis
Xcs.259	EG358296 PAL	SSH	Moro	Anthocyanin biosynthesis
Xcs.244	EG358290 GST Prabable	SSH	Moro	Anthocyanin biosynthesis
Xcs.206	EG358241 ANS	SSH	Moro	Anthocyanin biosynthesis
Xcs.234	EG358291 DFR	SSH	Moro	Anthocyanin biosynthesis
Xcs.017	EL492437 PAL	cDNA-AFLP	Moro	Anthocyanin biosynthesis
Xcs.145	EL492631 UFGT	cDNA-AFLP	Moro	Anthocyanin biosynthesis
Xcs.240	EG358237 F3H	SSH	Moro	Anthocyanin biosynthesis
Xcs.261	EG358227 PAL	SSH	Moro	Anthocyanin biosynthesis
Xcs.272	EG358317 Putative flavanone 3-hydroxylase	SSH	Moro	Anthocyanin biosynthesis
Xcs.270	EG358381 Putative cytochrome p450	SSH	Moro	Anthocyanin biosynthesis
Xcs.353	EG358199 Monoterpene glucosyl transferase	SSH	Moro	Carotenoid's biosynthesis
Xcs.213	EG358216 Osmotin-like	SSH	Moro	Defence
Xcs.199	EG358203 Acidic chitinase III	SSH	Moro	Defence
Xcs.201	EG358311 Al-induced protein	SSH	Moro	Defence
Xcs.161	EL492651 glutamate decarboxylase, putative	cDNA-AFLP	Cadenera	Defence
Xcs.048	EL492496 Lea14-A, late embryogenesis abundant protein	cDNA-AFLP	Cadenera	Defence
Xcs.120	EL492597 tyrosine aminotransferase	cDNA-AFLP	Cadenera	defense response to herbivores and pathogens
Xcs.203	EG358303 Alcohol acyl trasferase	SSH	Moro	Flavour's biosynthesis
Xcs.202	EG358236 Alcohol acyl trasferase	SSH	Moro	Flavour's biosynthesis
Xcs.279	EG358265 Sinapyl alcohol deydogenase	SSH	Moro	Flavour's biosynthesis
Xcs.210	EG358188 10-hydroxigeraniol oxidoreductase	SSH	Moro	Flavour's biosynthesis
Xcs.293	EG358205 Valencene synthese	SSH	Cadenera	Flavour's biosynthesis
Xcs.074	EL492535 pectinacetylesterase precursor	cDNA-AFLP	Moro	Fruit softening
Xcs.255	Nitropropane dioxygenase	SSH	Moro	Primary metabolism
Xcs.104	EL492574 glyceraldehyde-3-phosphate dehydrogenase	cDNA-AFLP	Moro	Primary metabolism
Xcs.037	EL492477 phosphoenolpyruvate carboxykinase (ATP)-like protein	cDNA-AFLP	Cadenera	Primary metabolism
Xcs.254	EG358366 NADH deidrogenase	SSH	Cadenera	Primary metabolism
Xcs.007	EL492421 acyltransferase 2	cDNA-AFLP	Moro	Primary metabolism (Fatty acid biosynthesis)
Xcs.098	EL492566 UDP-glucose 4-epimerase	cDNA-AFLP	Cadenera	regulation of cell wall carbohydrate biosynthesis
Xcs.393	transcription factor MYC7E _Zea mays_	HarvEST	Moro	Regulatory mechanism
Xcs.391	putative DNA_binding protein _Arabidopsis thaliana_	HarvEST	Moro	Regulatory mechanism
Xcs.195	EL492715 zinc finger (C3HC4-type RING finger) family protein	cDNA-AFLP	Moro	Regulatory mechanism
Xcs.392	myc_like protein _ Arabidopsis thaliana emb_CAB93714.1	HarvEST	Moro	Regulatory mechanism
Xcs.347	bHLH transcription factor GBOF_1 _Tulipa gesneriana_	HarvEST	Moro	Regulatory mechanism
Xcs.306	Myb family transcription factor _Arabidopsis thaliana_	HarvEST	Moro	Regulatory mechanism
Xcs.301	Myb_like protein _Nicotiana tabacum_	HarvEST	Moro	Regulatory mechanism
Xcs.398	basix helix_loop_helix (bHLH) family protein _Arabidopsis thaliana	HarvEST	Moro	Regulatory mechanism
Xcs.122	EL492599 purple acid phosphatase, putative	cDNA-AFLP	Cadenera	Response to oxidative stress
Xcs.149	C4690.SEQ annexin	cDNA-AFLP	Cadenera	secretion of polysaccharides
Xcs.212	OSJNBa0079M09.4	SSH	Moro	Signal trasduction mechanism
Xcs.257	EG358294 OSJNBa0079M09.4	SSH	Moro	Signal trasduction mechanism
Xcs.268	EG358365 Protein kinase C inibitor-like	SSH	Moro	Signal trasduction mechanism
Xcs.248	EG358276 Ipotetical Atg55230	SSH	Moro	Unclassified
Xcs.296	EG358231 Unknown	SSH	Moro	Unclassified
Xcs.290	EG358374 Unknown	SSH	Moro	Unclassified
Xcs.294	EG358320 Unknown	SSH	Moro	Unclassified
Xcs.344	diaminopimelate epimerase	SSH	Moro	Unclassified
Xcs.101	EL492569 HAC13 protein	cDNA-AFLP	Moro	Unclassified
Xcs.136	EL492616 AX110P -related protein	cDNA-AFLP	Cadenera	Unclassified
Xcs.119	EL492596 ubiquinol--cytochrome-c reductase-related protein	cDNA-AFLP	Cadenera	Unclassified
Xcs.075	C2660.SEQ translation elongation factor eEF-1 beta' chain, putative	cDNA-AFLP	Cadenera	Unclassified
Xcs.047	EL492492 snRNP Sm protein F-like	cDNA-AFLP	Cadenera	Unclassified
Xcs.174	EL492671 H+-ATPase catalytic subunit	cDNA-AFLP	Cadenera	Unclassified
Xcs.033	EL492465 auxin-regulated protein	cDNA-AFLP	Cadenera	Unclassified
Xcs.045	EL492489 expressed protein	cDNA-AFLP	Cadenera	Unclassified
Xcs.060	EL492515 expressed protein	cDNA-AFLP	Cadenera	Unclassified
Xcs.126	EL492603 protein F1N21.14	cDNA-AFLP	Cadenera	Unclassified
Xcs.173	EL492670 expressed protein	cDNA-AFLP	Cadenera	Unclassified
Xcs.125	EL492602 heat shock protein 22.0	cDNA-AFLP	Cadenera	Unclassified
Xcs.235	EG358279 Dicyanin	SSH	Cadenera	Unclassified

Tab. 14 Results of differential array data. ***Sequence ID*** is the codex corresponding to EST sequences; ***Accession number - Similarity*** refers to the GenBank Acc. numb. of submitted ESTs and the correspondent similarity to; ***Library*** is the original derivation of ESTs and in green rectangles are evidentiated SSH library ESTs; ***Up*** indicates over-expression in blood oranges (red) and common oranges (yellow); the ***Functional category*** is also shown.

The hybridisation revealed that enzymes involved in anthocyanins biosynthesis, such as PAL, putative F3H, cytochrome b5 (substrate for genes coding enzyme of anthocyanins biosynthesis), CHS, DFR, UFGT and GST were up-

regulated in blood oranges. CHS, DFR and UFGT were previously analyzed by Real time PCR (Lo Piero et al., 2005a, 2006; Cotroneo et al., 2006), where they showed higher expression levels in pigmented oranges than in common ones. Differential analysis with respect to GST expression was conduced through semi-quantitative RT-PCR and Real time PCR, and we observed a strong accumulation of the Moro transcript compared to Cadenera (Licciardello et al., 2007). Flavanone-3-hydroxylase (F3H) is one of the key enzymes acting at the flavanone branch point (Pelletier and Shirley, 1996). Its suppression, as opposed to that of CHS, does not block the production of the essential phytoalexins and isoflavonoids (Shirley, 1996). The suppression of F3H activity did not affect the downstream components of the anthocyanin biosynthetic pathway. In fact, the high efficiency of *f3h* suppression in terms of color modification could be due to it being a single-copy gene (Dedio et al., 1995). In this way, the higher expression of F3H in blood oranges could be due to a regulator gene that inactivates F3H or, perhaps, to the absence of transcripts in common oranges (as suggested by Zuker et al., 2003). According to the transcriptional regulation of F3H expression, the *delila* gene of snapdragons coordinately controls F3H expression with downstream 'anthocyanin-specific' genes such as *dfr* (Dooner et al., 1991; Martin et al., 1991); the *f3h* gene can be independently expressed, such as in *Petunia* (Quattrocchio et al., 1993), or co-ordinately controlled with the upstream genes for *chi* and *chs* by regulatory genes, such as *ttg* in *Arabidopsis* (Pelletier and Shirley, 1996).

Among transcripts that were 'turned on' in the hybridization with the Moro probe, we found some transcripts that we had previously analyzed by semi-quantitative RT-PCR, such as bHLH, 10-hydroxigeraniol oxidoreductase, AAT, putative SRK and valencene synthase. Array data confirmed the up-regulation of 10-hydroxigeraniol oxidoreductase, AAT and the putative SRK (also demonstrated by Real time PCR) in blood oranges. However, some array data contradict previous transcriptional analysis, as the bHLH transcription factor and the valencene synthase. In fact, semi-quantitative RT-PCR (Fig. 19, 17-18) demonstrated the over-expression of bHLH in common oranges, in contrast with the reverse Northern and microarray data. Moreover BlastN-BlastX similarity analysis and phylogenetic analysis (data not shown) of full length bHLH did not implicate this gene in anthocyanin control.

Functionally, genes involved in plant defense, such as the Al induced protein, acidic chitinase III, Lea 5 and dicyanin, are up-regulated in the Moro cultivar, as affirmed by reverse Northern. The remaining transcripts belong to other functional categories.

Analysis on the probe-set. The custom chip array was used to compare differential expression data through different methods. To ensure that the 50mer probe-set spotted on the chip was unique and did not create ambiguity, we created a database of all the original ESTs used for the custom chip. Then, we analyzed the 50mer ESTs through a local BlastN comparison of all of the probes versus all of the original ESTs.

We noticed different kinds of inconsistencies. First, the sequence Xcs.226 showed two regions corresponding to two different 50mer probes (Fig. 28). The first 50mer was specific for the Xcs.226 sequence (with a BlastX similarity to "Chloroplast small heat-shock protein class 1" EG358186), while the second probe also matched the Xcs.017 (encoding for phenylalanine-ammonia lyase, EL492437).

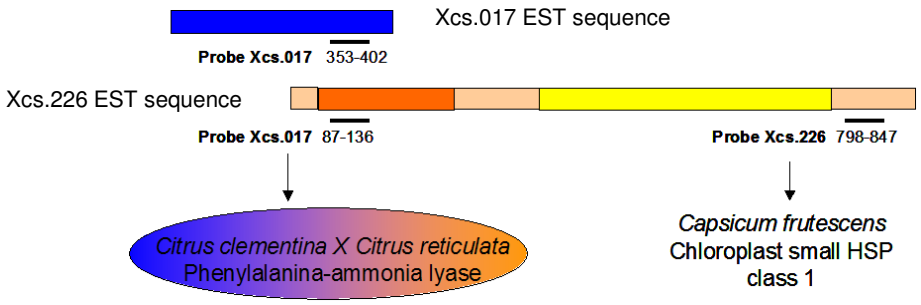

Fig. 28 Correlation between Xcs.017 50mer sequence and Xcs.017 and Xcs.226 EST sequences. Initial and final position of probes on sequences, probe species and similarity are indicated.

Xcs.017 and Xcs.226 ESTs sequences were similar to PAL only in the portion in which this second probe matches (blue and orange bars, respectively). In fact, the two original sequences were similar to two different proteins, PAL and HSP. We assumed that a chimeric artefact could be responsible for cDNA clones that contain sequences coming from more than one gene. Chimeric ESTs are more frequent than one might think, and this influences array design (Tomiuk and Hofmann, 2001).

Another problem was finding the same probe in different EST sequences (Fig. 29) in which only some of them (Xcs.017 and Xcs.260) were over-expressed in Moro cultivar.

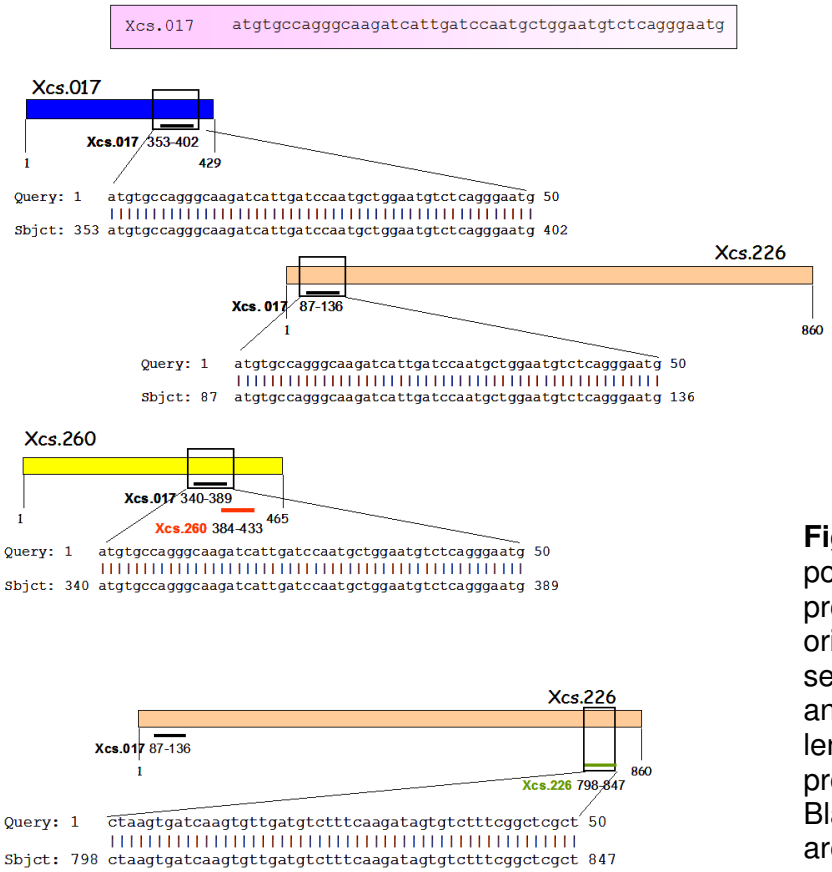

Fig. 29 Schema of the positions of the same probe in different original EST sequences. Position and corresponding length of ESTs and probes and the relative Blast2seq alignments are shown.

4.4.2 Differential transcripts as determined by an Affymetrix microarray

The Affymetrix GeneChip *Citrus* Array was hybridised using the same samples used to construct the cDNA library. The Affymetrix array was constituted by 30,171 ESTs sequences derived from different species of *Citrus* and hybrids. Differential data were obtained using GeneSpring GX 7.3 Expression Analysis software (Agilent Technologies). With respect to varying degrees of over-expression (10.0, 8.0 and 3.0-fold increase), we obtained 172, 214 and 721 differentially expressed ESTs, respectively.

4.5 Comparison of differential results of *C. sinensis* transcripts obtained using various approaches

4.5.1 Specific EST analysis of flesh

The *C. sinensis* database was built by considering 94,127 ESTs (at November 2006). The ESTs were from different libraries, tissues and developmental stages. All ESTs were assembled and organized into Tentative Consensus sequences (TCs). The first analysis focused on TCs that included at least two ESTs, which one of them had to derive from flesh tissue. This was useful for understanding which transcripts were specific for flesh. We identified 98 TCs and 70 out of them were specific for flesh. TCs generated from ESTs, derived only from flesh, are shown in Table 18 (cyan boxes). Forty-three out of the 98 TCs are assembled from clusters of ESTs that included sequences from the orange flesh SSH cDNA library (Tab. 18, green boxes). Out of these 43 transcripts, 23 were those used to design the 50mer oligonucleotide probes that were spotted on the custom chip array. Eighteen of them were up-regulated in either blood (Tab. 18, red boxes) or common oranges (Tab. 18, yellow boxes). Fourteen TCs have a match on the Affymetrix GeneChip *Citrus* Array. The hybridization results (red and yellow according to their up-regulation in Moro and Cadenera, respectively) and the fold-change from the Affymetrix array were also reported. “NULL” indicates no signal on the array (Tab. 15).

Genes encoding for some volatile substances are up-regulated in Moro, which suggests different expression of flavour compounds between pigmented and common oranges. Our results also showed differences for those genes involved in the ‘senescence and ripening fruit’. This may explain the softness and reduced turgidity of pigmented oranges with respect to common ones, especially when fruits are ripe. Some regulatory genes and kinases were isolated, as well as genes involved in the pathogen challenge response. Finally, genes with ‘no similarity’ and ‘unclassified’ genes were also detected but need further investigation to comprehend their role. As can be noted most of differential expression detected TCs, concerning the over expression in Moro orange, correspond to functional categories such as ‘Anthocyanin biosynthesis and transport’, ‘Flavour biosynthesis’ and ‘Stress and defense’ (Tab. 16, black rectangle). Specific TCs from flesh tissue isolated by SSH library, are also ‘on’ in the custom chip hybridisation. Some of them are also ‘on’ in the Affymetrix array and their over expression in the pigmented cultivar are also confirmed by validation experiments. Even if most of the genes involved in the ‘Anthocyanin’s pathway’ are reported, only DFR and UFGT were confirmed by all of the methodologies (black arrows in Tab. 15).

Moreover, 14 of the 43 contigs, including the ESTs isolated through the SSH library, were derived only from the SSH approach. This means that the SSH library introduced new sequences of *C. sinensis.*

Sample	SIMILARITY TO	FUNCTION	E value	EST			SSH	CUSTOM cDNA CHIP ARRAY	AFFYMETRIX CHIP		VALIDATION DATA		
				in contig	in flesh	in other tissues			Fold change	UP	Reverse Northern	SemiQ RT-PCR	Real time PCR
1	Anthocyanidin synthase	Anthocyanin's biosynthesis and transport	0	43	13	30	YES	Up in Moro		NULL	Up in Moro		Up in Moro
2	Phenylalanine ammonia-lyase 1 (EC 4.3.1.5)		0	7	4	3	YES	Up in Moro		NULL	Up in Moro	Up in Moro	
3	Dihydroflavonol-4-reductase		0	3	3	0	YES	Up in Moro	10	Up in Moro	Up in Moro		Up in Moro
4	UDP-glucose-flavonoid-3-O-glucosyl transferase		0	9	9	0	YES	Up in Moro	10	Up in Moro	Up in Moro		Up in Moro
5	Phenylalanine ammonia-lyase (EC 4.3.1.5)		0	4	2	2	YES	Up in Moro		-	Up in Moro	Up in Moro	
6	Cytochrome b5 DIF-F		3E-30	6	6	0	YES	Up in Moro		-	Up in Moro	Up in Moro	
7	Glutathione S-transferase (EC 2.5.1.18)		0	11	11	0	YES	Up in Moro		-	Up in Moro	Up in Moro	Up in Moro
8	Chalcone synthase 1 (EC 2.3.1.74)		4E-34	3	3	0	YES	Up in Moro		-	Up in Moro		Up in Moro
9	Chalcone synthase 1 (EC 2.3.1.74)		9E-35	3	3	0	YES	Up in Moro		-	Up in Moro		Up in Moro
10	Glutathione S-transferase (EC 2.5.1.18)		1E-29	11	11	0	YES	-		-	Up in Moro	Up in Moro	Up in Moro
11	Phloroglucinol O-methyltransferase	Flavour's biosynthesis	0	2	2	0	YES	NULL		NULL	Up in Cadenera		
12	10-hydroxygeraniol oxidoreductase		0	42	6	36	YES	Up in Moro		NULL	Up in Moro		
13	Alcohol acyl transferase		4E-33	2	2	0	YES	Up in Moro	10	Up in Moro	Up in Moro		
14	Alcohol acyl transferase		0	6	6	0	YES	Up in Moro		-	Up in Moro	Up in Moro	
15	Putative leucine aminopeptidase	Stress and defence	2,00E-39	5	5	0		-		NULL			
16	Hypothetical protein MAA21_50		1E-19	2	2	0		-		NULL			
17	Cysteine protease inhibitor		4,00E-25	2	2	0		-		NULL			
18	Polygalacturonase-inhibiting protein		0	2	2	0		-		NULL			
19	UDP-glucose:salicylic acid glucosyltransferase		0	2	2	0		-		Up in Moro			
20	Late embryogenesis abundant protein Lea5		0	310	35	275	YES	NULL		NULL	Up in Cadenera		
21	Pathogenesis-related protein PR10A		2E-36	29	2	27	YES	NULL		NULL	Up in Moro		
22	At4g15780		0	3	3	0	YES	NULL		NULL	Up in Moro		
23	Pyruvate kinase		0	23	4	19	YES	NULL	3	Up in Moro	Up in Moro		
24	Galactinol synthase	Stress and defence and sugar metabolism	0	2	2	0	YES	NULL		-	Up in Moro		
25	F6D8.22 protein (At1g52560) / Chloroplast HSP22	Stress tolerance	0	9	9	0		-		NULL			
26	Secretory peroxidase PX3	Senescence and cell wall ripening	0	3	3	0		-		NULL			
27	Extensin-like protein	Cell wall organization	3,00E-25	3	3	0		-		NULL			
28	Endo-1,3-1,4-beta-D-glucanase	Senescence and ripening fruit	0	7	2	5	YES	NULL		NULL	Up in Moro		
29	Pectinesterase-2 precursor (EC 3.1.1.11)		0	3	3	0	YES	-	10	Up in Cadenera	Up in Moro	Up in Moro	Up in Moro
30	Cytochrome P450	Primary and/or secondary metabolism	0	29	3	26	YES	NULL		NULL	Up in Cadenera		
31	Superoxide dismutase [Mn], mitochondrial precursor	Subcellular localization	0	38	13	25	YES	NULL		NULL	Up in Moro		
32	Pyruvate decarboxylase		0	27	6	21	YES	NULL		NULL	Up in Moro		
33	Aspartic proteinase		0	15	2	13	YES	NULL		NULL	Up in Moro		
34	Putative ATP citrate lyase a-subunit		0	10	3	7	YES	NULL		NULL	Up in Moro		
35	Putative terpene synthase		0	20	3	17	YES	Up in Cadenera		NULL	Up in Moro		
36	Putative NADH dehydrogenase 10.5K chain		2,00E-32	6	2	4	YES	Up in Cadenera		NULL	Up in Cadenera		
37	Putative 2-nitropropane dioxygenase		0	6	4	2	YES	Up in Moro		NULL	Up in Moro		
38	40S ribosomal protein S24		0	71	2	69	YES	NULL		NULL	Up in Cadenera		
39	Putative mitochondrial ATP synthase		0	21	3	18	YES	NULL		NULL	Up in Cadenera		
40	Anthranilate phosphoribosyltransferase, chloroplast precur		0	19	6	13	YES	NULL		NULL	Up in Moro		
41	ACT11D09.3	Defence pathogen attack and regulation mechanisms	0	2	2	0		-		NULL			
42	Wound induced protein-like (Fragment)	Defence pathogen attack and transcription factors	3E-09	2	2	0		-	3	Up in Cadenera			
43	Putative cinnamoyl-CoA reductase	Lignine regulation	0	2	2	0		-	3	Up in Moro			
44	(1-4)-beta-mannan endohydrolase precursor (EC 3.2.1.78)		0	2	2	0		-		-			
45	Auxin and ethylene responsive GH3-like protein	Auxin and sugar regulation	0	2	2	0		-		NULL			
46	Timing of CAB expression 1 protein	Circadian rithm regulation	8E-10	2	2	0		-		NULL			
47	F14O10.12 protein	Collagene structure	0	2	2	0		-		NULL			

Tab. 15 Each row presents an identified TC. TC details include the best Blast hit, the molecular function, the number of ESTs within a TC, the contribution of ESTs from flesh and the remaining tissues, which one were be isolated through the SSH library, the custom and Affymetrix chip array data and finally the validation of the differential expression from reverse Northern anaylsis, semi-quantitative RT-PCR and Real time PCR.

Sample	SIMILARITY TO	FUNCTION	E value	EST			SSH	CUSTOM cDNA CHIP ARRAY	AFFYMETRIX CHIP		VALIDATION DATA		
				in contig	in flesh	in other tissues			Fold change	UP	Reverse Northern	SemiQ RT-PCR	Real time PCR
47	F14O10.12 protein	Collagene structure	0	2	2	0		-		NULL			
48	Nuclear cap-binding protein	ABA Regulation	0	2	2	0		-		NULL			
49	Putative RING zinc finger protein-like protein	Regulation protein degradatin and Defence	7,00E-20	2	2	0		-		NULL			
50	Calcium-binding EF-hand family protein-like	Regulation to stress	3,00E-32	2	2	0		-		NULL			
51	Putative AT-hook protein 1	Regulatory genes to glucose	1,00E-23	2	2	0		-		NULL			
52	At1g49170 (Similar to serine/threonine kinase 9)	Signal transduction mechanism	7,00E-38	2	2	0		-		NULL			
53	T27D20.13 protein (Putative vesicle transfer ATPase)		1,00E-35	2	2	0		-		-			
54	Putative casein kinase	Signal trasduction mechanism and regulation mechanism	0	2	2	0		-		NULL			
55	MADS-box protein 4		0	37	8	29	YES	NULL		NULL	Up in Cadenera		
56	OSJNBa0079M09.4 protein		7E-35	13	11	2	YES	Up in Moro	10	Up in Moro	Up in Moro		
57	OSJNBa0079M09.4 protein		4E-37	2	2	0	YES	Up in Moro		-	Up in Moro	Up in Moro	Up in Moro
58	RING/C3HC4/PHD zinc finger-like protein	Transcription factors	1,00E-33	4	4	0		-		NULL			
59	WD-40 repeat protein		0	2	2	0		-		NULL			
60	F2D10.18	Unclassified	9E-37	116	5	111	YES	NULL		NULL	Up in Cadenera		
61	AT5g11200/F2I11_90 (AT5g11170/F2I11_60)		0	34	7	27	YES	NULL		NULL	Up in Cadenera		
62	Eukaryotic peptide chain release factor subunit 1-3		0	13	3	10	YES	NULL		NULL	Up in Moro		
63	AT5g06370/MHF15_11		0	11	4	7	YES	NULL		NULL	Up in Moro		
64	Hypothetical protein T10O8_50 (AT5g01340/T10O8_50)		0	5	2	3	YES	NULL		NULL	Up in Cadenera		
65	Ubiquitin-like protein		0.0008	2	2	0	YES	NULL		NULL	Up in Moro		
66	RRNA intron-encoded homing endonuclease B91		1E-20	23	2	21	YES	NULL		-	Up in Moro		
67	B1358B12.4 protein		4E-17	2	2	0	YES	Up in Moro	3	Up in Moro	Up in Cadenera		
68	F6N18.8		0	2	2	0		-		NULL			
69	Hypothetical protein		5,00E-20	2	2	0		-		NULL			
70	Hypothetical protein F5D21.24		2,00E-26	2	2	0		-		NULL			
71	Hypothetical protein At1g50510		0	2	2	0		-		NULL			
72	A_IG005I10.24 protein		1,00E-35	2	2	0		-		NULL			
73	Hypothetical protein P0030H07.11		0,00004	2	2	0		-		NULL			
74	Expressed protein		2E-07	2	2	0		-		NULL			
75	At5g24170		3E-19	2	2	0		-		NULL			
76	Putative ribosomal protein S17		2,00E-34	2	2	0		-		NULL			
77	Hypothetical protein MDF20.8		2,00E-38	2	2	0		-		NULL			
78	Similar to CGI-13 protein		0	2	2	0		-		NULL			
79	Hypothetical protein At5g52960/MNB8_2		0	2	2	0		-		NULL			
80	25.3 kDa vesicle transport protein		1,00E-31	2	2	0		-		NULL			
81	Putative Snf7-like protein (Fragment)		2,00E-39	2	2	0		-		NULL			
82	At5g04080		5E-13	2	2	0		-		NULL			
83	Hypothetical protein T10B6_10		3,00E-35	2	2	0		-	3	Up in Cadenera			
84	Hypothetical protein At2g46200		0	4	4	0		-	3	Up in Moro			
85	Expressed protein		0.0007	2	2	0		-	3	Up in Moro			
86	PGPD14 protein (RING finger family protein)		0	2	2	0		-	3	Up in Moro			
87	Expressed protein		1,00E-24	2	2	0		-		-			
88		No similarity		2	2	0		-		NULL			
89				2	2	0		-		NULL			
90				2	2	0		-		NULL			
91				2	2	0		-		NULL			
92				2	2	0		-		NULL			
93				2	2	0		-		NULL			
94				2	2	0		-		NULL			
95				2	2	0		-		NULL			
96				2	2	0		-		NULL			
97				2	2	0		-		NULL			
98				2	2	0		-		NULL			

(Continued Tab. 15)

The future aim of this work is to exploit the data to detect network of co-expressed genes from various analytical methods and set up a suitable strategy to compare different results from various approaches. Furthermore, our intention is to confirm differential gene expression, especially for those genes that are specific for flesh tissue, and to assign a biological function to these genes, thereby providing insight into the cellular mechanisms in which they are involved.

4.6 Ends extension of sequences of interest

Due to the absence (until this moment) of a *Citrus* Genome Project, public knowledge about *C. sinensis* full length sequences has been lacking. The SSH library allowed us to isolate ESTs. Some of these genes were previously isolated (through different methods) and known, while others were new, and no previous ESTs data existed. To focus on redundant and more interesting ESTs, the RACE-PCR method was used in order to complete some of the EST sequences.

The aromatic compounds AAT and 10-hydroxigeraniol oxidoreductase were chosen for their redundancy. The ClustalW alignment of different ESTs, compared to full length sequences from other plants, allowed us to better understand the regions of the each transcript that we isolated. The schema used is shown in Fig. 30.

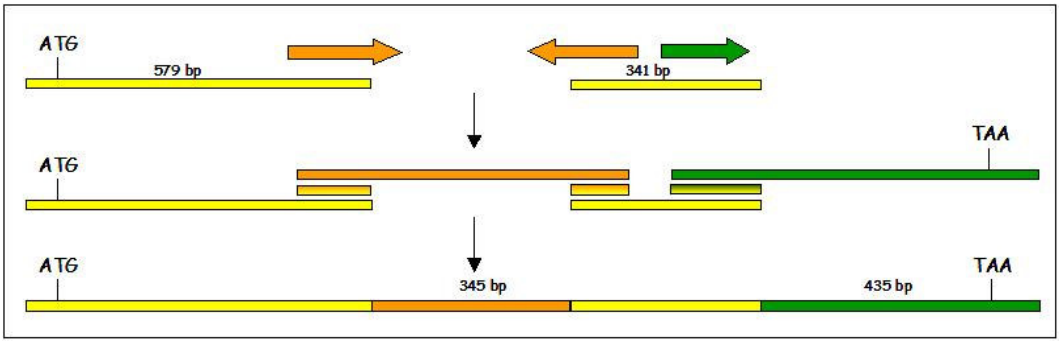

Fig. 30 3' RACE-PCR methodology was used to complete incomplete AAT sequences isolated through the cDNA SSH library. In yellow, the original ESTs; orange arrows show the localization of internal primers designed to fill up internal portion; green arrows are 3' RACE oligos.

The full length cDNA sequences were obtained after amplification using internal primers and 3' RACE PCR for AAT. Then, analysis using the Expasy tool (to deduce the transducted sequence) and oligos designed in the putative complete full length sequence confirmed the AAT complete cds sequence of 1371 bp (EU200366). Similarity analysis on BlastN and BlastX confirmed the putative function. According to 10-hydroxigeraniol oxidoreductase, the complete cds sequence was not yet obtained.

Among genes involved in the anthocyanin pathway and according to expression data (Fig. 19), cytochrome b5 is over-expressed in common oranges, although there is little data about the *C. sinensis* sequence. The isolated EST included the 3' final portion (compared to *P. hybrida* cytochrome b5 DQ352143 sequence). 5' RACE was conducted to complete the remaining 5' portion. Further analysis is necessary to confirm the 5' portion before submission to GenBank.

Afterwards our attention has been focused on regulatory genes. MAD box and bHLH EST sequences were a part of the internal portion of the correspondent submitted sequences of plants with a high similarity and both of them lacked of the ATG initial triplet and the final stop codon. It was necessary to complete the 5' and 3'

portions. With regard to the bHLH transcription factor, the full length cDNA sequence was 1113 bp (EU240878). According to the BlastN and BlastX similarity analysis and phylogenetic tree, we could confirm that *C. sinensis* bHLH does not play a role in the regulation of anthocyanin pathway by including it into a Myc-like TFs schema (thanks to Paul Barley, JIC Norwich, UK). The bHLH isolated through the SSH library and the complete cds sequence obtained after the RACE method suggested that it was involved in cold stress activation and not in anthocyanin control. According to MADs box, the complete cds sequence was not yet obtained.

Among the most redundant genes and apart from structural genes involved in the anthocyanin biosynthesis, there was also a transcipt similar to the SRK (in BlastX similarity analysis), which was isolated 25 times. However, there was no information about the function of this gene. Following the EST alignments and assembly, we deduced that is has two different internal portions. We are in process of filling the internal region and completing the 5' and 3' coding portion using the RACE PCR.

4.7 GST: tissue specificity analysis in *C. sinensis*

5.7.1 In silico individuation of the sweet orange GST family

Different members of the *C. sinensis* GST gene family were identified by comparing the 94,127 EST sequences, retrieved from the dbEST division of the GenBank repository, to the UniProtKB/Swiss-Prot database. The original dataset was reduced to 370 ESTs that putatively encoded GST proteins. The clustering/assembling procedure of this collection resulted in 62 putative distinct transcripts: 28 tentative consensus sequences (TCs) and 34 singletons (sESTs). The sequences were also compared with GenBank non-redundant nucleotide sequences to further confirm the data. 25 full-length GST transcripts and 37 partial transcripts were identified and classified in different classes typical of plant GSTs. In particular, 14 Tau class, 10 Phi class, 1 Lambda class, 1 Zeta class and 2 MAPEG class full length transcripts were identified. A Theta class sequence fragment was also identified. Once the putative full-length GST encoding transcripts were identified, tissue expression patterns of the putative full-length GST were inferred by querying the dbEST database with respect to different tissues/developmental stages.

The TCs CITSI00:1 and CITSI02:1, which corresponded to GST ESTs isolated through the subtractive cDNA library (Licciardello et al., 2007), were concatenated into the unique transcript sequence DQ198153 from GenBank.

Some of the deduced transcripts were full-length mRNAs, showing a complete Open Reading Frame (ORF). The remaining transcripts exhibited partial ORFs. Among these, those transcripts that included the start triplet ATG and lacked the stop codon were classified as '5' fragments'. On the other hand, those lacking the initiating ATG and presenting a termination codon were classified as '3' fragments'. Finally, transcripts that showed interspersed stop codons were classified as 'no good ORFs'. The list of all full length sequences is described in Tab. 16.

N° sequences	Name	Id sequences
1	Phi-1	CITSI52-Contig1
2	Phi-2	CITSI38-CK934228
3	Phi-3	CITSI23-Contig1
4	Phi-4	CITSI34-CK939385
5	Phi-5	CITSI33-Contig1
6	Phi-6	CITSI05-DY257328
7	Phi-7	CITSI11-Contig1
8	Phi-8	CITSI29-Contig1
9	Phi-9	CITSI18-Contig1
10	Phi-10	DQ198153 (CITSI00-02)
11	Tau-1	CITSI47-Contig3
12	Tau-2	CITSI57-Contig1
13	Tau-3	CITSI43-Contig1
14	Tau-4	CITSI16-Contig1
15	Tau-5	CITSI53-Contig1
16	Tau-6	CITSI41-Contig1
17	Tau-7	CITSI25-Contig1
18	Tau-8	CITSI51-Contig1
19	Tau-9	CITSI04-Contig1
20	Tau-10	CITSI44-Contig1
21	Tau-11	CITSI36-Contig1
22	Teta	consensus CITSI48-49
23	Zeta	CITSI21-Contig1
24	Lambda	CITSI46-Contig1
25	Mapeg	CITSI20-Contig1

Tab. 17 List of the full legth GST sequences.

The Phi class comprised nineteen sequences (Fig. 15), grouped as follows: ten transcripts were full-length, and nine were partial ORFs. Of these the sequence CITSI02-Contig1 was a '5' fragments', five were '3' fragments' (CITSI28-CX046491, CITSI27-CX070573, CITSI22-CX672147, CITSI00-Contig1, CITSI08-CV716584), CITSI54-CB293267 was a short fragment (110 bp length) and CITSI65- BQ623038 was a partial intron. In the sequences CITSI23-Contig1, CITSI26-Contig1 and CITSI35-CK936125, two putative transcripts still retaining introns were revealed. In the intron retaining sequences, the first and the second intron were present in the canonical 5'GT and the 3'AG splice sites, except for the second intron of CITSI35-CK936125 sequence whose 3' splice sites is TC. CITSI56-CB292998 is a short fragment with interspersed stop codons.

We assigned a total of twenty-nine sequences to the Tau class (Fig. 15). Fourteen out of twenty-nine were defined as full-length transcripts. Ten transcripts exhibited partial ORFs. Among these, the sequences CITSI55-CB293075, CITSI15-DN618611 and CITSI01-EG358290 were '5' fragments'. CITSI13-CX300684, CITSI60-BQ624512, CITSI47-Contig2 and CITSI17-CX675467 were classified as '3' fragments'. Furthermore, sequences CITSI31-CN188035, CITSI61-BQ623696 and CITSI58-BQ624883, which lacked both start and stop codons, were classified as 'fragments'. We considered CITSI24-CX077363 to be an intron-retaining sequence and the canonical 5' GT and the non-canonical 3' TC splice sites were identified at the limits of the region. CITSI59-Contig1, CITSI39-CK739807, CITSI03-DY305803 and CITSI40-CK701666 presented no ORFs of significant size.

Of the sequences in the Theta class (Fig. 15), CITSI49-CF504122 was a '5' fragments', while the CITSI48-CF506057 was a '3' fragments'.

The Zeta class consisted of 3 sequences (Fig. 15). The sequence CITSI21-Contig1 was full-length; CITSI50-Contig1 dids not represent a full-length sequence, because a few nucleotides were lacking, while CITSI62-BQ623695 was a '3' fragments'.

The Lambda class consisted of 6 sequences (Fig. 15). The sequence CITSI46-Contig1 was a full-length sequence, while the remaining 5 sequences exhibited partial ORFs. CITSI32-CN187483 and CITSI42-CK665050 were classified as '5' fragments'; CITSI63-BQ623555, CITSI37-CK935114 and CITSI30-CV884511 were classified as '3' fragments'.

Two sequences (CITSI20-Contig1 and CITSI09-Contig 1) corresponded to the MAPEG class and both of them were full-length.

4.7.2 Expression pattern of GSTs genes in different orange tissues and cultivars through semi-quantitative RT-PCR

Expression pattern analysis was performed on the 25 full length sequences using semi-quantitative RT-PCR. Both the different citrus tissues analyzed and the pigmented and common cultivars showed various expression patterns.

Semi-quantitative RT-PCR analysis of the Phi class (Fig. 31) showed the specificity of Phi-8 in young leaves of the pigmented oranges (red box in Fig. 31).

The over expression of the transcript Phi-10 in the flesh tissue was confirmed using oligonucleotides (DQ198153 in Tab. 7) to isolate the full length cds, from the ATG initial triplet to the stop codon, as used in a previous work (Lo Piero et al., 2006). In the Fig. 32 the compared expression pattern of the semi-quantitative RT-PCR performed on Moro and Cadenera tissues, using the two different oligonucleotide pairs, is showed. The Phi-10 amplification using the extreme primers showed a higher amplification on Moro flesh (Fig. 32, I); moreover a double band is evident in the pigmented young leaf (Fig. 32, II), of which the upper amplicon could be considered and intron retaining sequence, such as the only amplification band of Cadenera ovaries (Fig. 32, III).

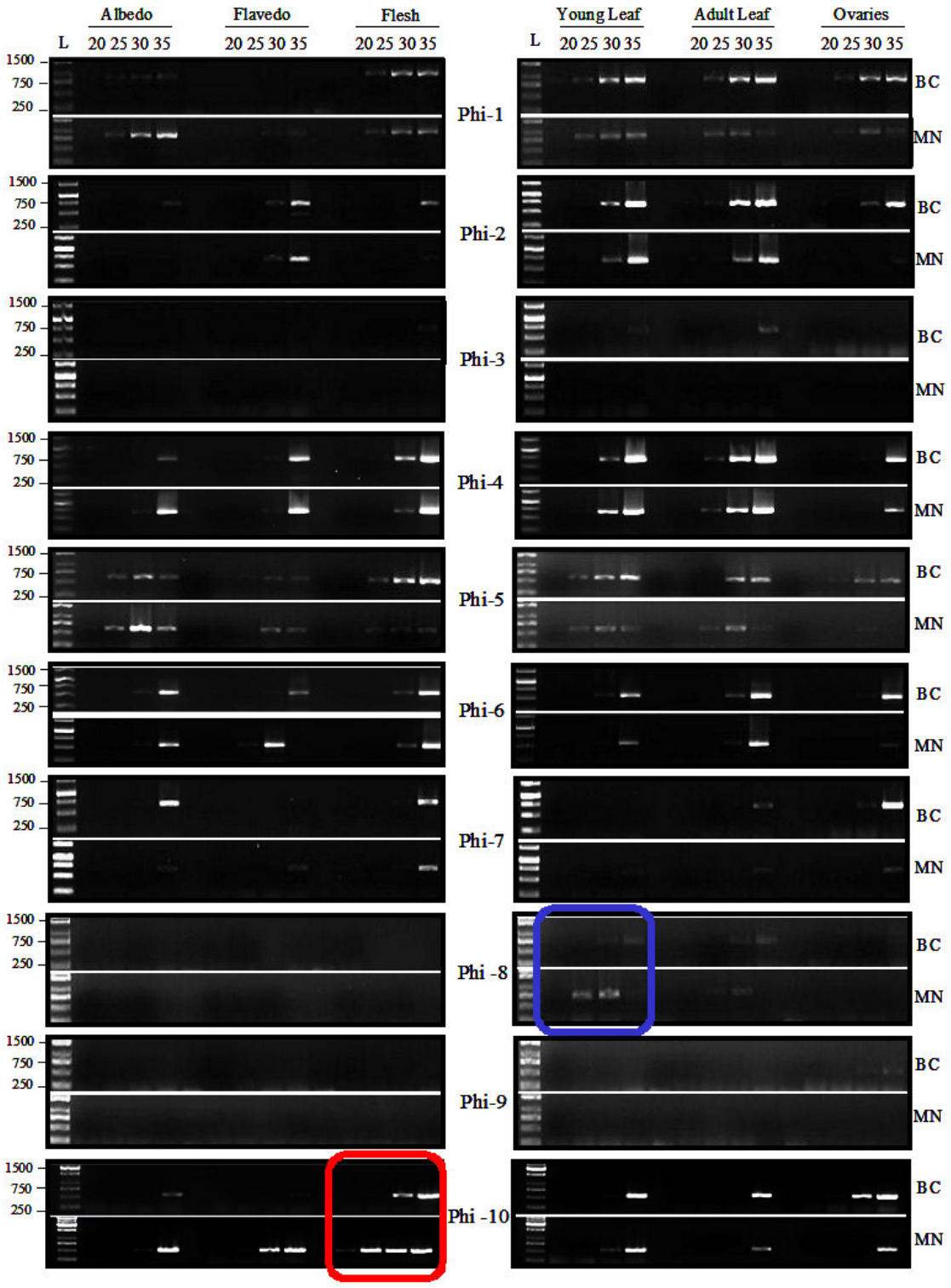

Fig. 31 Semi-quantitative RT-PCR data on different tissues and developmental stages of Phi class genes. **L** is 1Kb ladder; **20**, **25**, **30**, **35** are the amplification cycles at which transcripts were analyzed; **BC** is Biondo Cadenera; and **MN** is Moro Nucellare. The red rectangles indicate the specific tissue expression of Phi-8 in Moro young leaf and the higher expression level of Phi-10 in Moro flesh.

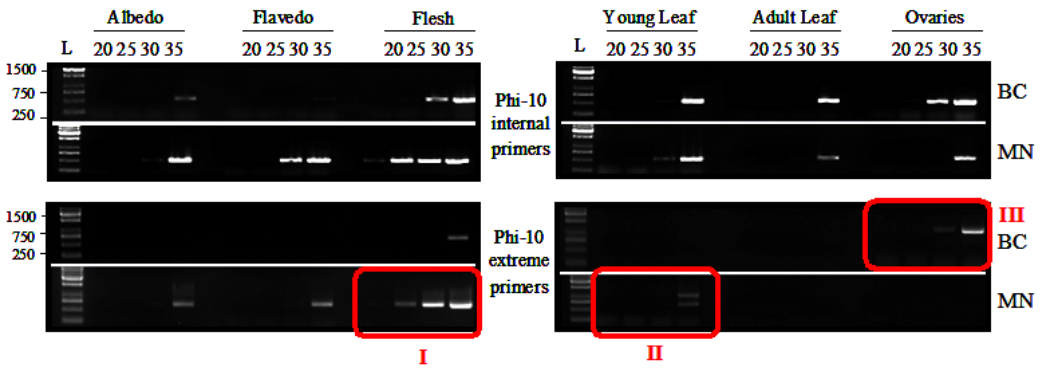

Fig. 32 Semi-quantitative RT-PCR results on Phi-10 using internal primers (to isolate an inner mature portion) and external primers (to isolate the entire cds). The red **I** rectangle indicates over-expression in Moro flesh tissue; the red **II** rectangle shows the double band in young Moro leaves; the red **III** rectangle points out the different lengths of the Cadenera ovaries amplicon.

Expression analysis on the Tau class (Fig. 33) revealed that Tau-8 is over-expressed in the different tissues of the Cadenera orange, with respect to pigmented ones (Fig. 33, red rectangle). Moreover, a longer amplification fragment in ovaries of the common cultivar was evident (Fig. 33, red arrows). This could be attributed to intron retention within the sequence or may be due to the different nature and function of ovaries (such as the fact that it is an organ and not a tissue, as in other examined samples) with respect to blood oranges.

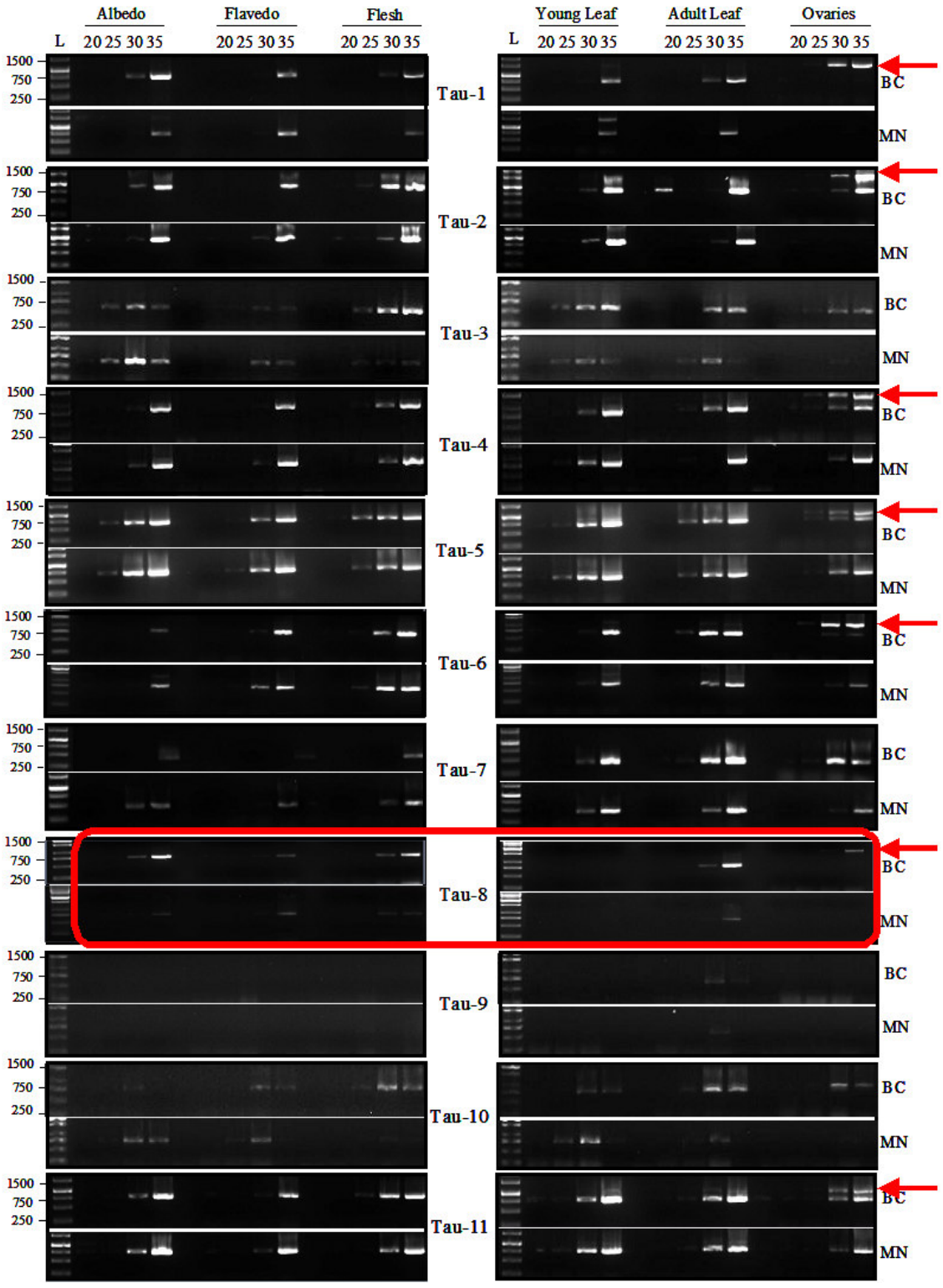

Fig. 33 Semi-quantitative RT-PCR data on different tissues and developmental stages of Tau class genes. Amplification cycles and cultivars are the same as in Fig. 31. The red rectangle indicates over-expression of Tau-8 in Cadenera cultivar; red arrows indicate longer amplicons in the ovaries of common orange fruits.

In the Theta and Zeta classes (Fig 34) multiple and double bands (respectively) were evident. The unspecific, not unique amplification in the Theta class was due to oligonucleotide construction in a non-specific region. In the Zeta class, double bands were probably caused by the non-specific experimental conditions.

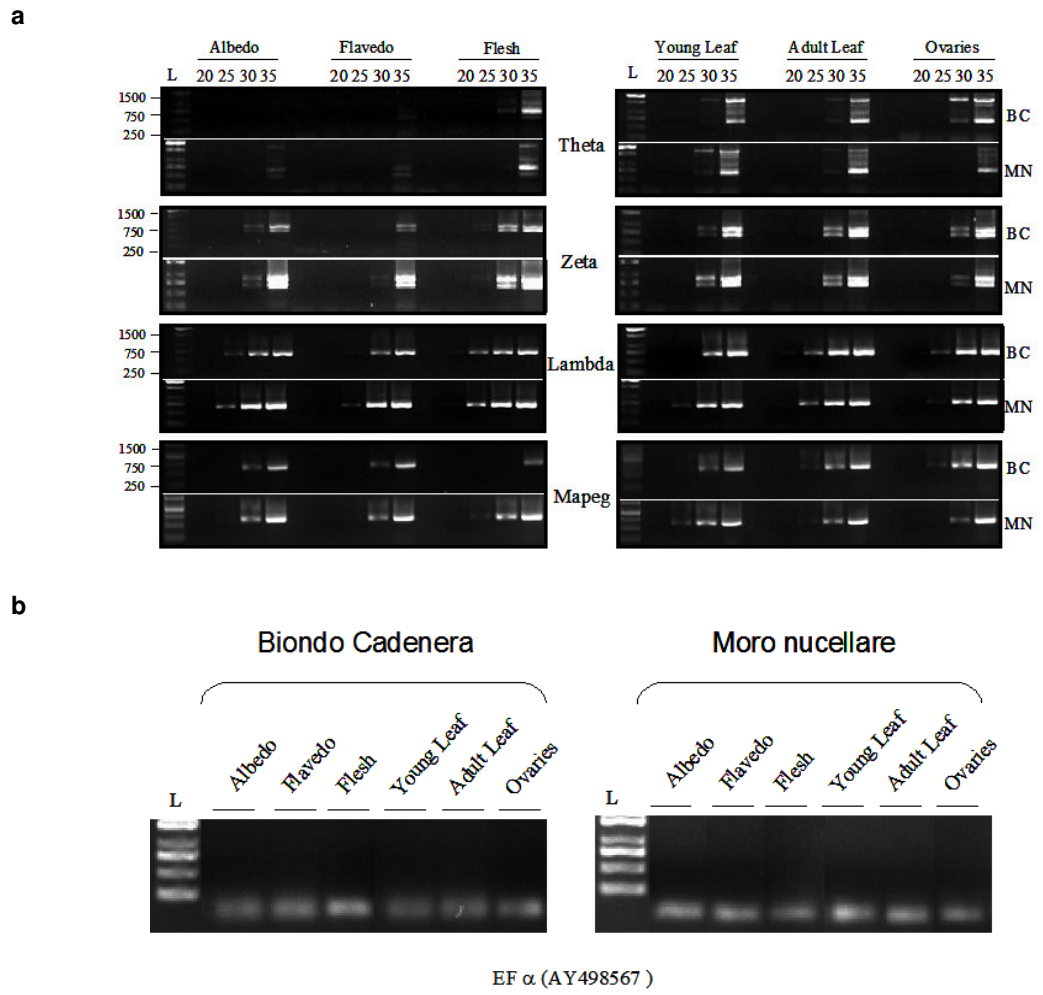

Fig. 34 Semi-quantitative RT-PCR data on different tissues and developmental stages of **(a)** Theta, Zeta, Lambda and MAPEG classes of genes and **(b)** housekeeping gene EF α.

4.8 Expression analysis determined by Northern blot of *C. sinensis myc2* transcription factor in the flesh and rind of pigmented and common oranges

Anthocyanin control is well-known in different plants, such as snapdragons, *Petunia*, maize, Arabidopsis. However, there is little information about woody trees, as well as *Citrus*. The only examples of transcription factors in *C. sinensis* are *myc2* (Cs*myc2*; EF645811) and *myb8* (Cs*myb8*; EF645810), which were isolated and characterized by Cultrone A. (personal communication). Similarity analysis, conduced in the BlastN and BlastX algorithms, and functional approaches have

shown that *myc2* was involved in anthocyanin control, but *myb8* appeared to be involved in a different pathway.

The expression analysis of Cs*myc2* was investigated using Northern blot. The total RNA of flesh and rind of Tarocco Meli and Valencia (a pigmented and a common cultivar, respectively) was analyzed and hybridised using a specific probe for orange *myc2* cDNA. Likely, the low amount of RNA or the low expression level of the transcripts is the limiting factor for understanding the expression level.

4.9 Transient transformation of transcription factors in *C. sinensis* orange fruits and heterologous analysis of *N. tabacum* leaves and *P. hybrida* flowers

Transient gene expression systems were used for short-term studies of gene function (Barandiaran et al., 1998; Ferrer et al., 2000; Tian et al., 2004). They have the advantage of being rapid, without the need of generating transformed cells, and are therefore commonly used for functional analysis of gene regulation (Tucker et al., 2002; Synek et al., 2004; Hoffmann et al., 2004; Luu et al., 2004; Chávez-Bárcenas et al., 2000). This is particularly valuable in the case of gene expression studies in fruits of woody trees such as *Citrus,* which are difficult to regenerate citrus trees (Ghorbel et al., 1999) and whose fruits are difficult to obtain due to a long juvenile phase.

4.9.1 Protoplast transformation

Protoplasts have often been used for transient expression analysis. Once set up, this method is reasonably fast and does not require expensive equipment. However, a number of problems can arise when trying to obtain protoplasts from ripe fleshly fruits, such as peaches, apples, pears and oranges (Spolaore et al., 2001). For this reason, protoplast transformation experiments on *N. tabacum* leaves using orange bHLH and Myb transcription factors were carried out by heterologous analysis.

The combinatorial protein-protein interaction specifities of the R2R3-MYB protein family and BHLH proteins seem to be necessary for regulatory activation (Grotewold et al., 2000; Hernandez et al., 2004; Zimmermann et al., 2004). Anthocyanin accumulation in maize is controlled by two classes of TFs acting synergistically: Myb and the bHLH domain (Ludwig and Wessler, 1990). Mol et al. (1998) showed that the myb-homologous *c1* or *pl* genes require a member of the bHLH-containing *r* or *b* gene family to activate transcription of anthocyanin biosynthetic genes. In *Petunia*, BHLH, WD40 and MYB proteins have been proposed to act together on late flavonoid metabolism (Mol et al., 1998).

Following these studies, protoplast transformation experiments were conducted using constructs with *bHLH* and *myb*-like TF genes separately and together to study the effect and efficiency of the interaction. The promoters of the flavonoid biosynthesis genes F3H and DFR were tested as potential targets for different combinations of orange and snapdragon Myb and bHLH TFs because of their different cis-acting elements. In this way, the effector and reporter plasmid constructs allowed quantification of both promoter activity and the effect of a TF by measuring β-glucuronidase (GUS) reporter activity (Fig. 35).

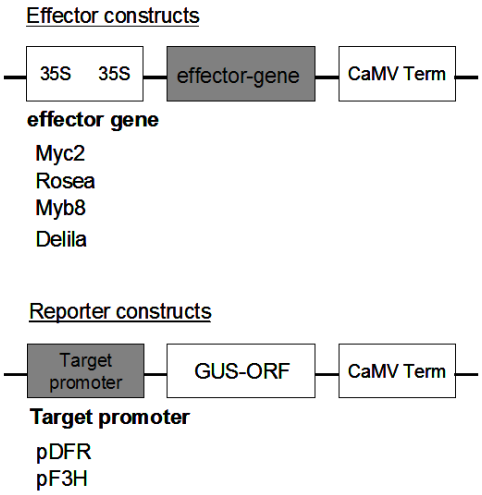

Fig. 35 Schema of the effector and reporter constructs used for transient analysis through protoplast transformation. The effector constructs are constituted by inserting the effector genes into the pJIT60 vector. The reporter constructs contain the target promoter fused to the GUS-ORF followed by a cauliflower mosaic virus terminator (CaMV Term)

The unknown function of the *myc2* gene isolated from *C. sinensis* was compared and associated with the well known involvement of *A. majus rosea* and *delila* (*myb* and *myc*-like genes, respectively) in anthocyanin control.

Fig. 33a shows the effect of TFs on the DFR promoter. The activation of Cs*myc2* with the *myb*-like *rosea* is due to both of them. The *rosea* gene alone has an activation effect of 10.549 (U/mg protein), and Cs*myc2* alone has an activation effect of 12.363 (U/mg protein); however, the interaction of both factors together produced an effect of 19.120 (U/mg protein). In contrast to Cs*myc2*, Cs*myb8* showed a different behavior. Only the combination of Cs*myc2 with* Cs*myb8* turned on the DFR promoter (10.053 U/mg protein); when Cs*myb8* was acting alone, no activation was evident. Although these are only preliminary results, they confirmed that *C. sinensis* Myb-like was not involved in anthocyanins regulation. The phylogenetic analysis also demonstrated no correlation of *myb8* with flavonoid control.

In a similar way, F3H promoter activation was also analyzed (Fig. 33b). The interaction of Cs*myc2* and *rosea* myb-like was stronger (63.964 U/mg protein) compared with their independent effects (*rosea*, 17.214 U/mg protein; Cs*myc2*, 12.158 U/mg protein). However, it seems that the individual TFs analyzed were unable to activate reporter expression.

This result supported the observation of different control mechanisms of anthocyanin biosynthesis by the effect of transcription factors on specific promoters. Moreover, the transient expression system using protoplasts of *N. tobacum* leaves demonstrated how orange Cs*myc2* activates anthocyanin regulation control when it was associated with a *myb*-like gene in a heterologous system.

However, the constructs used in this experiment were constituted by genomic sequences inserted into a pJIT60 vector. Further and more specific analyses had to be conducted using the correspondent ORF to ensure proper analysis of the transcription effect. In addition, these preliminary results confirmed that Cs*myb8* is not involved in anthocyanins regulation, which was suggested by phylogenetic analysis (data not shown).

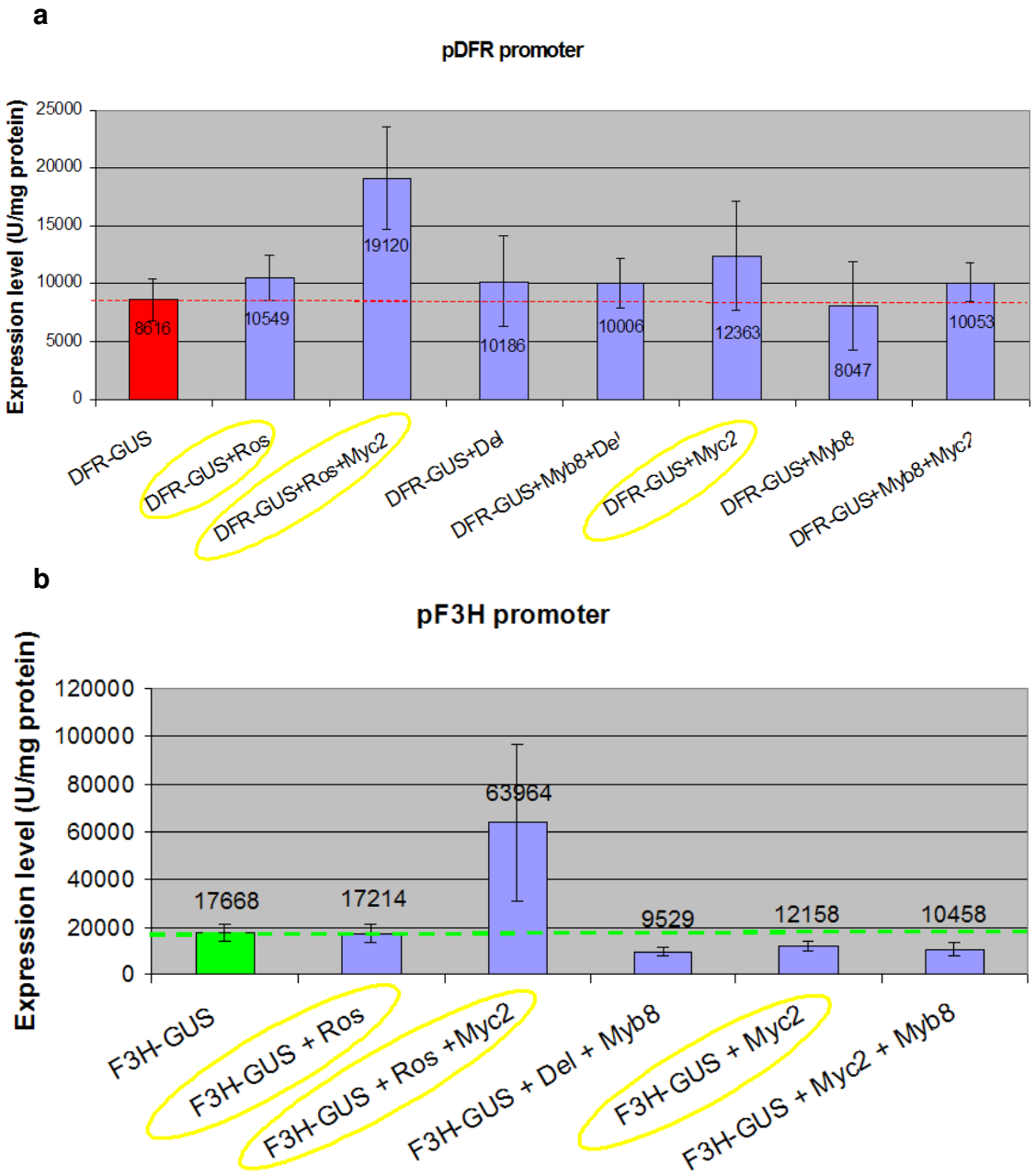

Fig. 36 Protoplast transformation on *Nicotiana tabacum* leaves using the promoter pDFR **(a)** and pF3H **(b)**. Red and green bars indicate the amount of the DFR and F3H promoter (respectively) alone as a function of the background transcription levels. Yellow circles indicate constructs with good expression results. Ros and Del are the *A: majus rosea* and *delila myb* and *myc*-like genes, respectively; Myb8 and Myc2 are *C: sinensis* Cs*myb8* and Cs*myc2* genes, respectively.

4.9.2 Bombardment

The biolistic method is a very common transient expression analysis system. However, in ripe fleshly fruits of recalcitrant and agronomic species, the cells change

their structure and the sugar that is present in vacuoles might cause problems for particle delivery transformation (Spolaore et al., 2001).

Our intention was to adjust the protocol for the bombardment of orange fruits. The first test was conducted in different orange tissues, such as the rind, spongy tissue and flesh, using a GUS binary vector as control. No red spots were evident after GUS staining in all tissues tested and with different pressure analysed. As is evident in Fig. 37, the red coloration of flesh was around cells and not within them (as expected). In an optimal situation, the gold particles linked with the construct would get through the cell wall, would transform cells with constructs used to product anthocyanins and coloring the internal cellular portion.

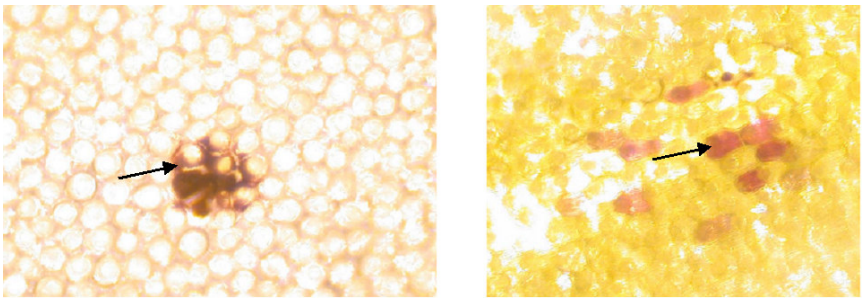

Fig. 37 Red pigmentation of orange flesh. Black arrows indicate the gold particles around cells (on the left) and the natural red pigmentation (on the right).

One of the reasons for the failed bombardment could be the recalcitrant nature of orange tissues. Differences in transformation efficiencies or transient expression may be associated with several anatomical and physiological changes that occur during ripening. High turgidity, pH variation and changes in osmolyte concentrations and types (Clements and Leland, 1962), which are characteristics of mature vesicles, have been reported to alter transformation efficiency and gene expression (Riva et al., 1998; Almeida and Huber, 1999; Hiei et al., 1994; Ishida et al., 1996; Mikolajczyk et al., 2000). Moreover, the developmental stage seems to be an important factor. In a previous paper (Ahmad and Mirza, 2005), transient GUS expression varied significantly in juice vesicles and seeds as the fruits matured.

A second test of the biolistic method was conducted on white petals of *P. hybrida* (cv Mitchell) that had two mutations in two Myb TFs, *an2* and *an4* (Quattrocchio et al., 1993) using the effector constructs (Fig. 32). A white background is the ideal condition to observe an eventual red spot pigmentation. No positive purple spots were observed after 48h of GUS staining.

4.9.3 Agroinfiltration

Among several techniques used for transient methods, *Agrobacterium* is the most widely used transformation tool (Wang and Fang, 1998; Broothaerts et al., 2005). In *C. sinensis,* agroinfiltration is complicated by the recalcitrant nature of orange tissues. The various drawbacks are connected with:

1. **The nature of the tissue.** This is linked to physiological characteristics (Shackel et al., 1991), osmolyte accumulation with maturity (Clements and Leland, 1962) and modulation of gene expression (Mikolajczyk et al., 2000). According to Ahmad and Mirza (2005), the mean percentage of transient GUS expression is highest in spongy tissue followed by the seed and rind. This might be explained by the preferential penetration of *Agrobacterium* in this

tissue (Spolaore et al., 2001). However, we observed that the albedo had a high level of blue background; even if the addition of methanol in the GUS staining solution reduced the background, the blue colour was too much and occluded the blue GUS signal. Moreover, the dense structure of fleshly fruits (such as the rind) did not allow any significant penetration of *Agrobacterium* through the epidermis. Even the use of vacuum infiltration with both slices of fruits and the entire fruit led to inconsistence data even if the injection was conducted with a sterile syringe to take advantage of the loose cell-to-cell contacts.

2. **The developmental stage.** Immature fruits are better than ripe ones. Highly significant ($P<0.01$) changes in the of the ripening is usually accompanied by changes in pH (Almeida and Huber, 1999), which has been reported to be an important factor in achieving efficient transformation of different plants by *A. tumefaciens* (Hiei et al., 1994; Ishida et al., 1996). The mature fruit juice vesicles do not show GUS expression. Higher turgidity, pH variation and changes in osmolytes (Clements and Leland, 1962) alter transformation efficiency and gene expression (Riva et al., 1998; Almeida and Huber, 1999; Hiei et al., 1994; Ishida et al., 1996; Mikolajczyk et al., 2000). The use of mature orange fruits probably negatively influenced the really difficult conditions of the agroinfiltration.
3. **The incubation time** for GUS staining. Ahmed et al. (1997) positively correlated transient GUS expression with the time elapsed after DNA uptake, with maximum activity at 48 h. Similar results have been reported for *Arabidopsis* (Abel and Theologis, 1994) and tomatoes (Stefanov et al., 1991). Different periods of incubation time were conduced without positive results.
4. **The reporter gene:** Spolaore et al. (2001) demonstrated how different results were obtained when different fleshly and recalcitrant fruits are transformed using the GUS or LUC reporter gene.

Our preliminary efforts, using the methodology reported in Spolaore et al. (2001) have been largely inconsistent.

5. CONCLUSION

The most important characteristic involved in the differentiation of Sicilian pigmented oranges is the presence of anthocyanins. The aim of this work was the identification of differentially expressed transcripts between blood and common oranges, using methodologies such as a subtractive SSH cDNA library, Real time PCR and two different kinds of microarrays. A characterization of the GST classes were investigated in different tissues of both blood and common oranges. Transient approaches, such as protoplast transformation, biolistic transfection and agroinfiltration, were conducted to evaluate functional potentiality of orange transcription factors in heterologous and homologous systems.

5.1 The differentially expressed genes

According to the methods used, the SSH cDNA library was an efficient and sensitive technique to isolate differential transcripts. In our experiments, the possibility and the drawback of isolating different portions of the same transcript (redundancy) could estimate the gene expression level. Moreover, the SSH library efficiently isolated new ESTs for sweet oranges, which were submitted to the dbEST of the GenBank database.

The 44% of the differentially expressed transcripts were genes involved in the anthocyanin pathway. In this way, the cDNA library was useful for deducing and confirming that one of the fundamental differences between blood and common oranges was differences in the enzymes in anthocyanins biosynthesis, supporting the idea that pigmented oranges are cultivars derived from a mutation of common oranges. In addition, many of the genes involved in red pigmentation were up-regulated in blood oranges, which is in agreement with the hypothesis that the absence of the red pigment in the common cultivar is not due to a lack of expression of individual biosynthetic genes (Cotroneo et al., 2006; Lo Piero et al, 2005a, 2006).

In addition, genes such as pectinesterase, which is involved in the structural composition of cell walls, could be used as a useful ‘parameter’ to distinguish ripe blood fruits from common oranges. Pectic substances are found above all in the albedo, flavedo and also in the pulp tissues and “cloudy” juices of citrus fruits (Safina, 1984). The pectinestarases catalyze the degradation of pectic polymers in plant cell walls; this depolymerization is generally associated with the process of fruit ripening (Wong, 1995). The pectinestarases are specific mostly for pigmented oranges. Ripe blood fruits have a high expression level on pectinesterase transcripts, and aromatic compounds may also be different and used to discriminate between blood and common oranges.

Expression level analysis was conduced using semi-quantitative RT-PCR (as a general screening method); Real time PCR and two different kinds of microarrays (a custom chip and the Affymetrix array) were used to validate differential transcription expression. Real time PCR and microarrays were used also as an integration system to connect differential gene expression data derived from various approaches. In this way, we narrowed down the investigation on specific and unique transcripts of orange flesh tissues. We can conclude that the only two specific transcripts refer to DFR and UFGT - enzymes directly involved in anthocyanins biosynthesis. The main differences between flesh and all the other analyzed tissues were probably involved in the anthocyanin content [it was mostly limited to late biosynthetic genes (LBGs)], in the flavour biosynthesis and in stress and defense

mechamisms. In addition, this approach was important to integrate *in silico* and molecular analysis.

5.2 The tissue-specificity of GSTs

A characterization of *C. sinensis* GST classes was performed on different tissues of pigmented and common oranges. The analysis, as with the *in silico* and experimental approaches, showed that mostly the Phi class and, in particular, an over-expression of ESTs isolated through the SSH library in Moro flesh tissue exhibited tissue specificity. The specificity and sequence information about intron retention and/or alternative splicing of double bands and of ovary tissues needs to be investigated.

5.3 Different transient approaches to investigate regulatory genes

If the main difference between pigmented and common oranges is closely connected to anthocyanins, it may be interesting to understand the involvement of two orange transcription factors, *myc2* and *myb8,* as regulatory genes involved in the activation of the anthocyanin pathway. Experiments to gain transient transformation on 'model' plants such as Nicothiana and *A. majus* were useful controls to ensure the proper outcome of the experiment. The same analysis on orange fruits confirmed real difficulties caused by the physiological, nature and restive conditions. In the meantime, an analysis on heterologous experiments regarding transient transformation confirmed the necessary interaction between Myc and Myb-like factors in the control of the anthocyanin pathway. Protoplast transformation experiments with the closely related bHLH (Myc2 of sweet orange) and Myb-like (Rosea of snapdragon) TFs have shown that, first, the same gene constructs may have markedly different effects according to the promoter used (pDFR or pF3H) and second, the activation effect was different if constructs were used together or independently. This variation may reflect differences in both the promoter sequences of the endogenous flavonoid biosynthetic genes and the presence or absence of interacting endogenous TFs. For the phenylpropanoid pathway, much is known about both the specific *cis*-elements that are involved in responses to environmental and developmental stimuli and the TFs involved, but little is known about regulation in citrus fruits.

5.4 Future aims

The results obtained through this work can be considered as a starting point for future analysis. In this way, our objects are detailed studies about using linked approaches among arrays and SSH library to isolate common genes among different tissues, and transcripts that are common in tissues with anthocyanin, and to investigate not only genes directly involved in the biosynthesis but also to link the pathways.

The microarray used could also be used to monitor different orange genotypes in various pedoclimatic conditions.

Our intention is also to analyze the data from the custom array, considering the connection between the design of the probe set and the hybridization data, to monitor, exclude or introduce a list of differential transcripts according to the analysis protocol used.

The GST tissue-specificity will be completed using tissues and *Citrus* species characterized by anthocyanin pigmentation, to confirm the isolation of the real GST Phi class contig involved in the vacuolarization of anthocyanins. These data will be

supported also by *in silico* analysis and biochemical and transient transformation approaches.

For transient transformation, our goal is to adjust a protocol for citrus agroinfiltration and bombardment, and to overcome the drawbacks of orange fruits. We wanted to use a homologous system to better understand the real involvement of regulatory genes on anthocyanin control. Furthermore, our results suggested that this method can also be used to develop an easy in-plant transformation system to produce transgenic *Citrus* plants. Before starting transient analysis, we compared the sequences of DFR and GST of pigmented and common oranges (the only one genes that are submitted in both cultivars) because the differences between blood and common oranges could be due to regulatory genes, but they were found to be identical. This confirmed that differences among biosynthetic anthocyanin genes are probably due to regulatory genes controlling differences in pigmentation.

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Paper

Identification of differentially expressed genes in the flesh of blood and common oranges*

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Abstract
The objective of this research was the identification of genes differentially expressed in blood oranges compared to common oranges and the identification of anthocyanin pathway genes that are up-regulated in flesh of blood oranges. A subtracted complementary DNA library of 1,248 clones was constructed using RNA from the flesh of a nucellar line (58-8D-1) of Moro (a blood orange) as tester and from Cadenera (a common orange) as driver. After screening by reverse Northern, a total of 230 clones were found to be up-regulated in blood orange, while 30 were up-regulated in the common blond one. Sequence analysis identified genes involved in the anthocyanin pathway including genes encoding biosynthetic enzymes like phenylalanine ammonialyase, chalcone synthase, dihydroflavonol-4-reductase, anthocyanidin synthase, UDP:glucose flavonoid 3-O-glucosyltransferase, glutathione S-transferase, and a regulatory gene encoding basic Helix-Loop-Helix protein, while others were related to primary metabolism, flavor biosynthesis, signal transduction mechanisms, and defense. Some sequences were classified as unknown and unnamed and some others were unclassified. Semiquantitative reverse transcription-polymerase chain reaction (PCR) and quantitative Real time PCR were used to confirm the differential expression patterns of selected candidate genes of different functional classes. Correlations between the expression of some genes and the processes involved in the ripening of blood oranges were identified.

Keywords
SSH-PCR Select, Anthocyanins, Differential expression, *Citrus sinensis* L., EST.

Introduction
Flavonoids represent a large group of secondary plant metabolites, of which, anthocyanins are the most ubiquitous class, and a wide range of colors results from their synthesis and accumulation. Anthocyanins are the red, purple, and blue pigments that accumulate in vacuoles, mainly in the epidermal cells, and color plant tissues. Their biosynthesis has been studied in detail and has provided considerable understanding of the regulation of gene expression in a great number of monocotyledonous and dicotyledonous plant species (Holton and Cornish 1995). Such studies have shown that these pigments are synthesized when two kinds of genes are expressed: structural genes, encoding the enzymes that catalyze the reactions of the metabolic pathway, and regulatory genes, which encode the

transcription factors controlling the expression of the structural genes. The accumulation of anthocyanins is influenced by environmental stimuli, such as light, temperature, and nutrition (Lancaster 1992; Sparvoli et al. 1994) as well as by genetic factors (Honda et al. 2002).

Some cultivars of sweet orange [*Citrus sinensis* (L.) Osbeck], which are known as 'blood oranges', accumulate anthocyanin pigments, while anthocyanin is not present in common orange, either in the rind or the flesh of the fruit. Blood orange cultivars are the mainstay of Italian orange production. The red color of fruit is an important factor influencing consumer appeal and marketability of both fruit and juices. In addition, the antioxidant activity of anthocyanins promotes health when they are consumed in the diet, including prevention of cancer, inflammation, and arteriosclerosis, conferring on plants that contain high levels, the character of 'functional foods' (Fauconneau et al. 1997; Nijveldt et al. 2001). Cyanidin 3-glucoside and an acylated anthocyanin, cyanidin 3-(4'-acetyl)-glucoside, constitute about 50 and 18%, respectively, of the total anthocyanins of blood oranges (Mazza and Miniati 1993). Among different anthocyanins tested, cyanidin 3-glucoside has the highest antioxidant value (Ding et al. 2006).

Although the molecular processes associated with anthocyanin biosynthesis have been studied extensively in maize, *Petunia, Antirrhinum*, and *Vitis* (Winkel-Shirley 2001), little is known about the biosynthetic pathways involved in anthocyanin biosynthesis in blood oranges. In previous work, partial complementary DNA (cDNA) clones encoding three enzymes active in the late steps of the anthocyanin biosynthetic pathway; dihydroflavonol-4-reductase (DFR), anthocyanidins synthase (ANS), and UDP:glucose flavonoid 3-O glucosyltransferase (UFGT), were isolated from *C. sinensis* (Lo Piero et al. 2005). The expression of these genes, assayed by Real timepolymerase chain reaction (PCR; Cotroneo et al. 2006), showed that higher levels of chalcone synthase (CHS), ANS, and UFGT messenger RNA (mRNA) transcripts accumulate in blood oranges compared to common cultivars, where the corresponding mRNAs are present at very low levels. Additionally, there was a strong correlation between gene expression levels and pigment content.

The presence of anthocyanin in the flesh and rind of blood oranges is rather variable and some selections show different anthocyanin contents in the rind and in the flesh of their fruit. Consequently, a major drawback in the marketing of blood oranges is the difficulty of producing fruit with a constant level of anthocyanins because the interaction of genotype with environmental conditions during fruit ripening and storage causes considerable variation in anthocyanin content (Cotroneo et al. 2006). This situation is particularly evident in the Tarocco accession, which is the most widely cultivated blood orange because of its large fruit size and broad ripening period. An understanding of the genes involved in anthocyanin biosynthesis and the regulation of their expression will allow for efficient management and breeding systems for improving both coloration and marketability.

In this paper a PCR-based, suppression subtractive hybridization (SSH) technique was used to identify genes that are up-regulated in blood orange flesh. The SSH procedure includes a normalization step, enriches for differentially expressed transcripts, and yields cDNA fragments that can be used directly for the construction of cDNA microarrays. We used a nucellar line (58-8D-1) of Moro (a blood orange) as tester and Cadenera (a common orange) as driver. A set of cDNAs corresponding to differentially accumulated mRNAs was isolated. The differential expression of some candidate genes was confirmed by semiquantitative and quantitative- Real time PCR at different time points of ripening. Analysis of the cDNA sequences and their

expression patterns during ripening allowed assignment of their possible functions in anthocyanin accumulation and in the ripening process of blood oranges.

Materials and methods

Plant material

Fruits of 58-8D-1, a nucellar line of Moro, the cultivar with highest content of anthocyanin, and Cadenera, a common orange, were harvested at Palazzelli (Lentini, SR, Italy), at eight different periods: I—at the beginning of October, when pigmentation is totally absent in Moro and total solublesolids/acidity ratio (S/A) is 3.9; II—at the end of October (S/A:4.7); III—at the end of November (S/A: 4.9); IV—at the beginning of December, when only some parts of the flesh of Moro oranges are weakly red (S/A: 5.9); V—at the end of December (S/A: 6.5); VI—at the beginning of January, when pigmentation in Moro flesh is clearly visible (S/A: 8.1); VII—at the end of January (S/A: 13.9); and VIII—at the beginning of February when pigmentation and ripening are complete (S/A: 16.4).
Moro and Cadenera fruits picked in period VIII (Fig. 1) were used as tester and driver, respectively, in the PCR-Select cDNA subtraction and cDNA library construction. Fruit collected at all the eight sampling periods were used to calculate the anthocyanin content.
Fruit sampled at periods I, IV, VI, and VIII were used to study the expression of genes by both semiquantitative reverse transcription (RT)-PCR and Real time PCR. Total RNA extraction Flesh was isolated from fruit and frozen in liquid nitrogen. Total RNA was extracted from 3g of flesh with TRIzol® LS Reagent according to the manufacturer's instructions (Invitrogen, Scotland UK) and treated with Dnase (Promega) for 30min at 37°C to remove genomic DNA. The amount and quality of the total RNA were estimated by spectrophotometer readings and by electrophoresis in formaldehyde-agarose gels stained with ethidium bromide.

PCR-select cDNA subtraction and cDNA library construction

PCR-select cDNA subtraction was performed using the Clontech PCR-Select™cDNA subtraction Kit (BD Biosciences Clontech, USA) according to the manufacturer protocol. The tester (Moro) and driver (Cadenera) cDNAs were reverse transcribed from 100ng of total RNA by using a Super SMART™PCR cDNA synthesis kit (BD Biosciences Clontech), digested with *Rsa*I, and then ligated to different adaptors. Hybridization and PCR amplifications were performed to enrich the differentially expressed sequences according to the manufacturer's instructions. The subtracted cDNAs were inserted directly into the T/A cloning vector pGEM-T Easy (Promega Corporation, USA) and transformed into *E. coli* DH5αMax Efficiency chemically competent cells (Invitrogen) to produce the subtracted cDNA library. Inserts were amplified by colony PCR; the PCR mixture (final volume 20µl) consisted of 1×Platinum Taq DNA polymerase Buffer (Invitrogen),0.1mM dNTP mix, 2.5mM MgCl2, 0.4µM of Nested 1 and 2 primers each, 1U Platinum DNA Taq Polymerase (Invitrogen). PCR parameters were: 1 cycle of 95°C for 6min, 65°C for 50s, and 72°C for 1.20min, 35 cycles of 94°C for 40s, 65°C for 50s, and 72°C for 1.20min. Each colony amplified by PCR was, in parallel, used to inoculate 150µl of Freezing broth-ampicillin (Sambrook and Russel 2001), and bacteria were allowed to grow overnight

at 37°C and finally stored at –80 °C. Two microliters of colony PCR amplification products were analyzed on 1% agarose gel in 1×TBE stained with ethidium bromide.

Dot blot and screening of cDNA library

The differential screening was carried out by reverse Northern analysis using Dot blot filters. DNA solutions obtained by colony PCR were equilibrated to 1M NaOH, 10mM ethylenediamine tetraacetic acid and denatured for 10min at 100°C. Half the final volume of denatured DNA was blotted onto nylon membranes (Zeta-Probe® Blotting Membranes, Bio-Rad, USA) with 0.4M of NaOH, according to the manufacturer's protocol. Membranes were washed in 2×SSC, dried in air, and fixed for 30min at 80°C. The same procedure was repeated for the remaining half volume. To prepare the cDNA probes, the products of forward (tester) and reverse (driver) subtraction were digested with *Rsa*I to remove the adapter sequences, purified on microcon YM30 filter devices (Millipore), and 100ng of cDNA was labeled with 32PdCTP. Probe preparation, prehybridization, hybridization, and washing conditions were performed according to the manufacturer's instructions (BD Biosciences). The labeled forward probes were hybridized to one membrane and the reverse probes to the duplicate. To ensure that the differences observed in the hybridization signals were ascribable to differential gene expression and not to the result of unequal loading of the filters, the membranes were stripped and filters, previously used for the hybridization with the forward probe, were used for hybridization with the reverse probe and vice versa. Exposures were analyzed with Typhoon 9210 Phosphorimager (Molecular Dynamics).

Expressed sequence tags sequencing and analysis

After analysis of the hybridization results, up-regulated expressed sequence tags (EST) clones were identified. Five microliters of the stored colonies in Freezing broth were used to inoculate 5ml of LB-ampicillin and were grown overnight at 37°C at 200rpm. Plasmid DNA was extracted with Wizard®Plus SV Miniprep-DNA Purification System (Promega) and sequenced by an Applied Biosystems ABI PRISM 3100 DNA Sequencer. After analysis of the nucleotide and of the derived amino acid sequences, ESTs were compared with those deposited in the non redundant public databases, using the BLASTN and BLASTX algorithms of the National Center for Biotechnology Information (Altschul et al. 1997). ESTs with BlastX E value $\leq$0.01 were deemed to have significant homology and were categorized according to their function. Analysis of gene expression by semiquantitative RT-PCR The expression patterns of transcripts putatively encoding for phenylalanine ammonio-lyase (PAL), cytochrome b5, glutathione S-transferase (GST), a putative S receptor kinase, valencene synthase, alcohol acyl transferase (AAT), 10-hydroxigeraniol oxidoreductase, pectinesterase, a basic Helix-Loop-Helix (bHLH) protein, and a MADs box protein and elongation factor 1 alpha (EF) were examined by semiquantitative reverse transcription polymerase chain reaction. We used SuperScript III One-Step RT-PCR with Platinum Taq (end point; Invitrogen) using samples harvested at four different periods (see above). The amplification of EF (AY498567) cDNA was used as an internal control. Oligonucleotide primers (Table 1) were designed with Primer Express 2.0 from the sequences of cDNAs isolated from the SSH library. For RT-PCR, firststr and cDNA was synthesized from 250ng of total RNA in a volume of 25 µl containing 1×PCR Reaction mix, 0.2µM of each gene-

specific amplification primer, 1U of SuperScript III One-Step RT-PCR with Platinum Taq. Reverse transcription was performed at 50°C for 30min, followed by PCR amplification: denaturation at 94°C for 6min followed by 35 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s, and extension at 72°C for 120s, with a final extension at 72°C for 7min, in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, 94404). Five microliters of each sample were analyzed at 20, 25, 30, and 35 cycles. The amplified DNA samples were separated on a 1.5% agarose gel in 1×TBE and stained with ethidium bromide.
Redundancy was used as a criterion to choose genes to be tested in semiquantitative RT-PCR and quantitative Real time PCR.

Real time PCR

Quantitative Real time PCR was used to confirm the differential expression of genes encoding glutathione S-transferase, putative S receptor kinase, and pectinesterase. Elongation factor 1 alpha (EF AY498567) was used as an internal control. Total RNA for qRT PCR analysis was extracted from samples harvested at four different time points (see above) and was retro-transcribed into cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems) according to the manufacture's instructions. cDNA synthesis was performed at 25°C for 10min and 37°C for 2h.
Polymerase chain reactions (PCRs) were performed in optical 96-well plates with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). For quantitative Real time, the PCR mixture (final volume 25µl) contained 15µl Power SYBR Green mix, 0.2µM of each gene-specific forward and reverse primers, 100ng of cDNA sample, using the protocol for the Power SYBR Green PCR Master Mix (Applied Biosystems). The following standard thermal profile was used for all PCRs: 50°C for 2min; 95°C for 10min; 40 cycles of 95°C for 15s, and 60°C for 1min. The analysis of each sample was replicated three times and a no-template negative control (H_2O control) was performed.
The analytic method used was the relative quantification standard curve method.

Anthocyanin content

Anthocyanins were extracted from lyoPhilized flesh of Moro oranges using 2 ×40ml of 0.3% HCl in methanol. In the third extraction, only 20ml of solution was used. The pooled extracts were dried by rotary evaporator and resuspended in 10ml of water. Total anthocyanins were determined by high-performance liquid chromatography (HPLC) according Rapisarda et al. (1994).

Comparative analysis of glutathione S-transferase, bHLH domain, and MADs box proteins

The program ClustalW (Thompson et al. 1994) has been used to generate multiple alignments of amino acid sequences for the following gene families: GST and bHLH.. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 3.1 (Kumar et al. 2004).

Results and discussion

Identification of cDNA fragments by PCR-select cDNA subtraction

SSH was used to isolate genes differentially expressed between blood and common oranges during fruit maturation (Fig. 1). A pool of genes putatively involved in ripening process was obtained after two rounds of subtraction and 1,248 colonies were picked randomly, in which the cDNA fragment sizes ranged from 250bp to 750bp. The results of filters hybridized with forward (tester) and reverse (driver) probes (Fig. 2) are reported in Table 3. All 260 clones were sequenced and the GenBank accession numbers, putative identities, species, scores, and *E* values of the best matching sequence found in the database are listed in Table 2.
cDNAs represented more than once are likely to be present more frequently in nonnormalized libraries as a result of their higher relative expression levels. Much of the redundancy results from the use of *Rsa*I-restricted cDNA fragments in the SSH procedure. Because of this restriction step, two or more different cloned cDNA fragments can represent a single transcript.
The 208 clones showing redundancy can be assigned to 30 functional genes (Table 3), and the putative functions as well as the number of PCR-select clones that could be assigned to each functional gene class for a selected sample of 13 functional genes are summarized in Table 4.

Isolation and expression of anthocyanin biosynthetic genes

A variety of genes were identified by SSH, some of which had already been deposited as full-length cDNAs in GenBank. They included structural genes of anthocyanin biosynthesis from *C. sinensis* [*CHS*-BAA81663.1, Flavanone 3-hydroxilase (*F3H*)-BAA36553.1, *DFR*-AAS00611.1, *ANS*-AAT02642.1, *UFGT*-AAS00612.1, *GST*-ABA42224.1] and 10-hydroxigeraniol oxidoreductase (AAQ55962.1) of *Chataranthus roseus* (Table 2).
The distribution of differentially expressed ESTs in functional classes is shown in Fig. 3.
More than 40% of the differentially expressed genes are related to genes involved in anthocyanin and carotenoid biosynthesis. We isolated six of the structural genes involved in anthocyanin biosynthesis (PAL, CHS, DFR, ANS, UFGT, GST), and all of them are upregulated in Moro cultivar, as revealed by reverse-Northern analysis (Table 2, *PAL*EG358333, *CHS*-EG358219, *DFR*-EG358289, *ANS*-EG358263, *UFGT*-EG358280 and *GST*EG358323). The expression of some structural genes (CHS, ANS and UFGT) in the flesh of blood orange had been previously shown to be positively correlated with anthocyanin accumulation (Cotroneo et al. 2006). Lo Piero et al. (2005, 2006) showed that there is much lower levels of DFR expression in common orange cultivars (Navel and Ovale) compared to those of the blood orange (Tarocco) suggesting that DFR enzyme activity could be a key factor determining the production of anthocyanins. Our data confirmed previous results (Cotroneo et al. 2006; Lo Piero et al. 2005, 2006) showing that many of the genes of the anthocyanin biosynthetic pathway are up-regulated in blood oranges, and they are in agreement with the hypothesis that the absence of pigment in the common cultivar is not due to lack of expression of individual biosynthetic genes. Genes involved in anthocyanin biosynthesis isolated in the present work include GST, PAL, Cytochrome b5, and F3H (Tables 2 and 4). GST was isolated 25 times among the differentially regulated

clones (Table 4). From the semiquantitative RT-PCR results, GST gene transcription was strongly enhanced in the blood orange in comparison to common orange in all the samples. Mammalian GST genes are divided into five groups (□, □, □, □, and □) based on sequence similarity, immunological cross-reactivity, and substrate specificity. All plant GSTs fall into the most ancient class, Theta. Droog et al. (1995) proposed three divisions of plant GSTs—type I, type II, and type III— based on combination of sequence conservation, immunological cross-reactivity, and intron/exon structure of the genes. To determine the class which *C. sinensis* GST (*Cs-GST*, isolated through SSH library) belongs to, we performed a multiple alignment of amino acid sequences (Fig. 4a) of *Cs-GST* against *An9* (GST type III) from *Petunia hybrida*, ParB from *Nicotiana tobacum*, *GmGST26A* (GST type III) from soybean, type I maize (*GSTI*, *GSTIII*, *GST IV*), and *Bz2* (GST type I) from *Zea mays.* According to the conservation of amino acid residues (see Fig. 4a) and to the phylogenetic tree (Fig. 4b), we hypothesize that *Cs-GST* falls within the class of type I GSTs. The *Cs-GST* is most closely related to the *Petunia An9* (more of 60% amino acid identity over the whole protein, data not shown), in contrast to *Bz2*, that is a type III GST and shows only the 18% amino acid of identity. *Cs-GST* is also related to the type I maize genes *GSTI*, *GSTIII*, and *GSTIV*. According to the low sequence conservation of type I and type III GSTs, we propose that they may have diverged from a common ancestral gene before *An9* and *Bz2* evolved into the specialized angiosperm GSTs that are required for the efficient transport of anthocyanins to the vacuole.

In common orange, no GST transcripts were detectable at the first time point of sampling (Fig. 5). Also, the results of quantitative Real time PCR performed on cDNA from 'Moro' and 'Cadenera' orange flesh samples during fruit maturation showed a significant difference in the expression of GST (Fig. 6a); in fact, in 'Moro' orange, GST transcript levels increased constantly during the entire period of maturation with the exception of the third sampling period. The transcript levels for GST at a given stage of fruit maturation positively correlated with the accumulation of anthocyanin at the same stage (Fig. 7); anthocyanins were totally absent in the first two samples, then increased from 1.23 to 46.75mg/100g. At stage VI, the levels of anthocyanins in 'Moro' remained almost constant, with reference to the previous one although the levels of GST transcript were low. A discrepancy between the levels of transcripts (high) and pigment accumulation (low) at time point IV could reflect regulation at the level of protein functionality. Studies performed on *Petunia* (Alfenito et al. 1998) and maize (Marrs et al. 1995), showed that GST is involved in vacuolar sequestration of anthocyanins; a similar role was observed in carnation, an Angiosperm plant phylogenetically distant from maize and *Petunia*, indicating that GST activity might represent a universal step in the anthocyanin pathway (Larsen et al. 2003). Stable anthocyanin pigmentation occurs when the molecules are transferred to the vacuole. In the presence of a functional *Bronze2* (*Bz2*) gene, which encodes for a GST protein, maize anthocyanins accumulate exclusively within the vacuole, while when *Bz2* is missing, anthocyanins accumulate in the cytosol, conferring a tan bronze phenotype derived from pigment oxidation (Marrs and Walbot 1997). Among the genes of anthocyanin biosynthesis, we also isolated genes encoding PAL and cytochrome b5 and their expression is up-regulated at all the time points in blood orange compared to the levels in common orange. For the cytochrome b5 gene, in the common orange, transcripts were detected only during the last amplification cycle (Fig. 5). PAL is the first committed step in phenylpropanoid metabolism and its activity is essential for the synthesis of all flavonoids including anthocyanins.

Cytochrome b5 acts as a reductase and its activity is essential for the Cytochrome P450 enzymes that function in general phenylpropanoid metabolism (Cinnamate 4-hydroxylase) and in anthocyanin biosynthesis (Flavonoid 3□-hydroxylase; De Vetten et al. 1999).

Genes encoding for signal transduction and regulatory

An additional objective of the SSH library construction was the isolation of regulatory genes that, by means of differential expression, might control differential biosynthetic gene expression. Two putative regulatory genes (1%; Fig. 3) were isolated: one encoding a bHLH protein (Table 2, EG358372) and one encoding a MADs box protein (Table 2, EG358383). To understand if *C. sinensis* bHLH (*Cs-bHLH*) was involved in the regulation of anthocyanin pigmentation and to explain the high expression level of *Cs-bHLH* cDNA in Cadenera blond orange obtained through semiquantitative RT-PCR (Fig. 5), we performed a multiple alignment of amino acid sequences of *Cs-bHLH* and other plant bHLHs involved in the anthocyanin pigmentation: *Lc* and *Sn* from *Zea mays*, *Delila* from *Antirrhinum majus*, *TT8* from *Arabidopsis thaliana*, *JAF13* and *An1* from *Petunia hybrida*, *GBOF-I* from *Tulipa gesneriana*.

Typically, a bHLH domain comprises a stretch of about 18 hydroPhilic and basic amino acids at the N terminal of the domain, followed by two regions of hydrophobic residues predicted to form amphypathic □-helices separated by an intervening loop (Murre et al. 1994). A high degree of sequence conservation was observed in those positions that are known to have important functions in DNA binding and protein dimerization. Within those bHLH proteins with proven ability to bind DNA, the amino acids at position His3-Glu7-Arg11 (H-E-R; Fig. 8) are the most critical (position of amino acids refers to *Cs-bHLH*). The DNA backbone is contacted by the basic residues at positions R(8) and R(10), which are conserved in the majority of plant proteins. A leucine residue is present (and conserved) at position 21 of *CsbHLH*, which emphasizes the likely importance of this residue in dimerization. However, as *In1* (intensifierl) of *Zea mays* (a recessive mutation described by Fraser AC in 1924, which cause the derepression of anthocyanin biosynthesis, according Burr et al. 1996), also the sequence of *Cs-bHLH* showed significant homology with a class of *myc* homologs involved in anthocyanin biosynthesis, but lacks a leucine zipper domain on the C-terminal side of the HLH/ZIP domain (Jia et al. 1997). So experimental results, reverse Northern analysis (Fig. 2; Table 2), and semiquantitative RT-PCR (Fig. 5) that revealed a higher expression level in common orange than in blood ones, and sequence analysis about the absence of the leucine zipper of C terminal (that falls in the longer sequence obtained from Cultrone A. through 3□RACE-PCR, personal communication; data not shown), induced us to suppose that the *Cs-bHLH* region, isolated through SSH, could not be involved in the anthocyanin regulation, but could be implicated in a different regulatory pathway. However, we propose to do further studies in N- and C-terminal region to understand better the role of the *Cs-bHLH*.

MADS-box genes represent a large group of regulatory genes found in yeast, animals, and plants (Ng and Yanofsky 2001; Shore and Sharrocks 1995; Theiben et al. 1995), in which they have been associated recently with flavonoid metabolism (Lalusin et al. 2006). The MADS box domain is the most highly conserved region of the protein (Purugganan et al. 1995), composed mainly of four regions: the MADS-box, the I, the K, and the C regions (Shore and Sharrocks 1995; Nesi et al. 2002). It

is found at the N terminus of the putative proteins; however, some plant proteins contain additional residues at the N terminal of the MADS-box protein. We performed a multiple alignment of MADS from *C. sinensis* (*Cs-MADS*), isolated through SSH library, and TT16/ABS from *Arabidopsis*, FBP24 from *Petunia*, DEFH21 from *A. majus*, ZMM17 from maize, and GGM13 from *G. gnemon* (Nesi et al. 2002), SOC1 from malus, MAD4 from malus and vitis (because a previous similarity search based analysis BLASTx shown an high similarity), MADS10 from *Ipomea batatas*, DEFH28 from *Antirrhinus majus*, and MADS4 from *Betula pendula* (Lalusin et al. 2006). All of these plant MADSs are involved in accumulation of anthocyanin.
MADs box protein transcripts accumulation remained constant during the maturation process in the blood and in common orange, but the expression level of the corresponding gene was slightly higher in the common cultivar (Fig. 5) and this is in agreement with reverse-Northern result (Fig. 2; Table 2). The analysis of the conserved domain and the typical amino acids of the MADS protein involved in anthocyanin pigmentation (data not shown) and the differential expression of the gene showed that *Cs-MADS* box is not involved in the anthocyanin regulation; in fact, any functional amino acids are conserved and there is no differential expression between blood and common orange during ripening process. The bHLH and the MADs box proteins belong to large families of transcription factors, therefore it cannot be excluded that these two genes isolated through SSH could not be involved in anthocyanin pathway regulation.
Other clones identified as highly expressed encoded different kinases (11%, Fig. 3), but these were up-regulated both in blood orange and in common one. One of these clones, homologous to a putative Serine receptor kinase from rice (EG358294 in Table 2; CAE05335.2 in Table 4) was isolated 25 times, supporting the view that it could be involved in regulatory mechanisms during fruit ripening. In fact, protein phosphorylation, which occurs via Ser/Thr kinases (and phosphatases), has a regulatory role in many signal trasduction pathways (Buchanan et al. 2002; Gu et al. 2002). According to reverse-Northern (Table 2) and semiquantitative RT-PCR (Fig. 5) results, the putative Ser receptor kinase was slightly up-regulated in blood orange during the late phases of sampling and might therefore operate in the regulation of accumulation of differential compounds in blood orange. Real time quantitative measurements of gene expression (Fig. 6b) confirmed our semiquantitative data that the putative S receptor kinase transcripts increased continuously during maturation although the expression levels were very low in the first and in the second samples. In 'Cadenera', all the samples showed very low levels of transcripts. Results obtained with the clone EG358294 and the other kinases identified in the present work support a possible role of this class of proteins in ripening regulation and/or fruit pigmentation processes.

Identification of genes for flavor biosynthesis

Seven percent of the differential genes identified encoded for flavor biosynthetic enzymes (Fig. 3). SSH allowed the isolation of cDNAs encoding proteins homologous to valencene synthase (EG358204, Table 2), which showed up-regulation in the blood orange after reverse-Northern analysis. However, using semiquantitative RT-PCR, no differences were observed in the expression level of this gene during the ripening process either in blood or common cultivars (Fig. 5). Other genes encoding biosynthetic enzymes involved in flavor production were also isolated by SSH: alcohol acyl transferase (AAT, EG358303 in Table 2) and 10-hydroxigeraiol

oxidoreductase (EG358189 in Table 2). According to semiquantitative RT-PCR results, the expression level of these genes remained almost the same during the ripening process in both cultivars although higher levels of transcripts were detected in blood oranges (Fig. 5). AAT belong to the class of acyl-CoA-dependent acyltransferases; they play important roles in the secondary metabolism in plants and fungi, including the biosynthesis of anthocyanins (Fujiwara et al. 1999; Suzuki et al. 2001; Suzuki et al. 2002; Suzuki et al. 2004). AAT activity is responsible for the production of volatile esters and was observed in plant tissues such as flowers and fruit (Ueda et al. 1997; Aharoni et al. 2000). 10-Hydroxigeraiol oxidoreductase is involved in the biosynthesis of geraniol, an acyclic monoterpene alcohol emitted from flowers of many species, notably roses (Bayrak 1994; Antonelli et al. 1997; Rao et al. 2000). The mixture of geranial and neral, also called citral, imparts a 'lemon' flavor.

Other gene classes differentially regulated

Genes implicated in defense, including those encoding the pathogenesis-related protein PR10A, acidic chitinase III, an osmotin-like protein, and Lea5 (EG358302, EG358203, EG358216, and EG358377, respectively, in Table 2) were also isolated in this work (3%; Fig. 3). With the exception of Lea5, the expression level of these genes was higher in blood orange than in the common cultivar, on the basis of reverse-Northern analysis (Table 2).

Almost 8% of all the differential clones (Fig. 3) belongs to the class of protein destination (Table 2; DNAJ protein -EG358360-, aspartic proteinase -EG358343-, Rad23 -EG358378-) and cell organization, like pectinesterase (EG358218 in Table 2) and POM30 (EG358259 in Table 2) encoding a porin protein. On the basis of reverse Northern (Table 2), most of them are more highly expressed in Moro than in common orange fruit. Semiquantitative RT-PCR showed that pectinesterase gene expression is completely absent in common orange fruit (Fig. 5), while its expression becomes evident in ripened blood oranges. Real time data for pectinesterase (Fig. 6c) showed that the corresponding mRNAs accumulated only in Moro samples in advanced ripening phase, whereas they were absent at the early phases of ripening, and in all the steps examined for the common orange 'Cadenera'. Pectin esterases catalyze the demethylation of pectins. Pectins are the most abundant class of macromolecule within the cell wall matrix and are also abundant in the middle lamellae among primary cell walls, where they functions in regulating intercellular adhesion, being the major adhesive material among cells. Fruit cell walls are usually highly enriched in pectins, often representing more than 50% of the wall dry matter (Castillejo et al. 2004). During fruit softening, pectins typically undergo solubilization and depolymerization that are thought to contribute to wall loosening and disintegration (Fischer and Bennet 1991). Pectins confer compactness and swollen consistency to fruit. According to these characteristics, our data are consistent with the observation that, at maturity, blood oranges are softer and less swollen than common cultivars; this phenomenon is an advantage because blood oranges are easy peeling like a mandarin, but this is a drawback during post-harvest causing shorter shelf life.

Other cDNAs isolated belonged to the functional category of primary metabolism (9%), genes of subcellular localization (3%), ribosomial proteins, mitochondrial ATP synthase, chloroplast small heat-shock protein, and chloroplast translational elongation factor (Table 2; Fig. 3).

Finally, for almost 8% of SSH clones (Fig. 3) no matches were found in the nonredundant databases, and we therefore classified them as being of unknown and

unnamed function. Six percent of the remaining cDNAs matched ESTs with no functions assigned and we called these 'unclassified'.
PCR-Select SSH allowed the isolation of genes involved in several different processes during orange fruit ripening; expression of some genes was directly linked to different phases of the ripening process, while for other genes more detailed experiments will need to be performed to obtain insights into their functions in orange fruit. Redundant and non-redundant cDNA clones were identified, and we have verified that redundancy is indicative of high levels of gene expression in the starting material. The study of the expression of all the genes identified in this work arrayed within a chip and analyzed within different genotypes and environmental conditions utilizing full-length coding sequences or domains specifically present in the members here identified will allow a better understanding of their individual roles during ripening.

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Fig. 1 a 'Moro' orange, used as tester, and **b** 'Cadenera' orange, used as driver

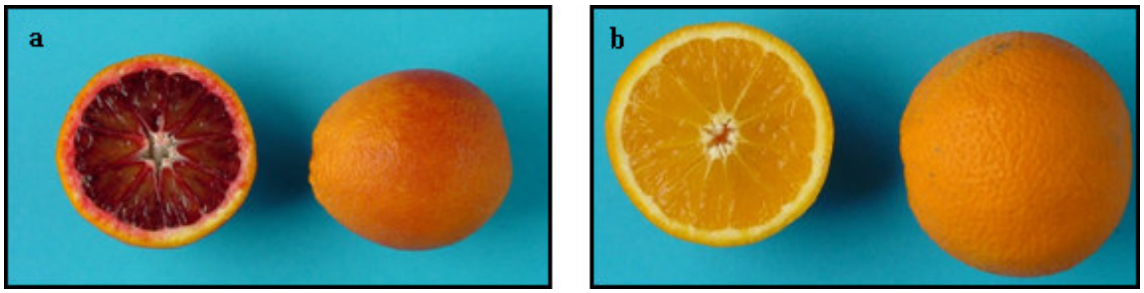

Fig. 2 Reverse-Northern analysis of dot blot array of 96 SSH clones showing putative up-regulated clones. **a** Hybridization with forward tester cDNA probe. **b** Hybridization with reverse driver cDNA probe. Clones selected for additional analyses are indicated by *arrows*

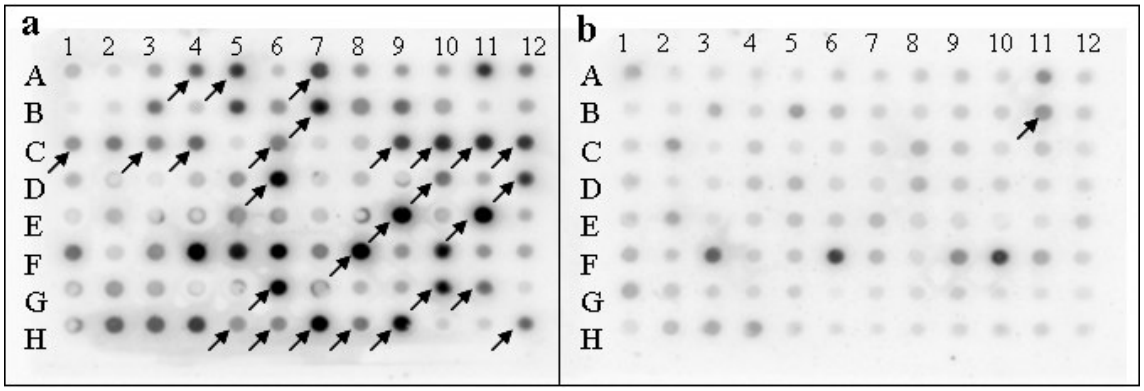

Fig. 3 Functional classification of ESTs identified by SSH

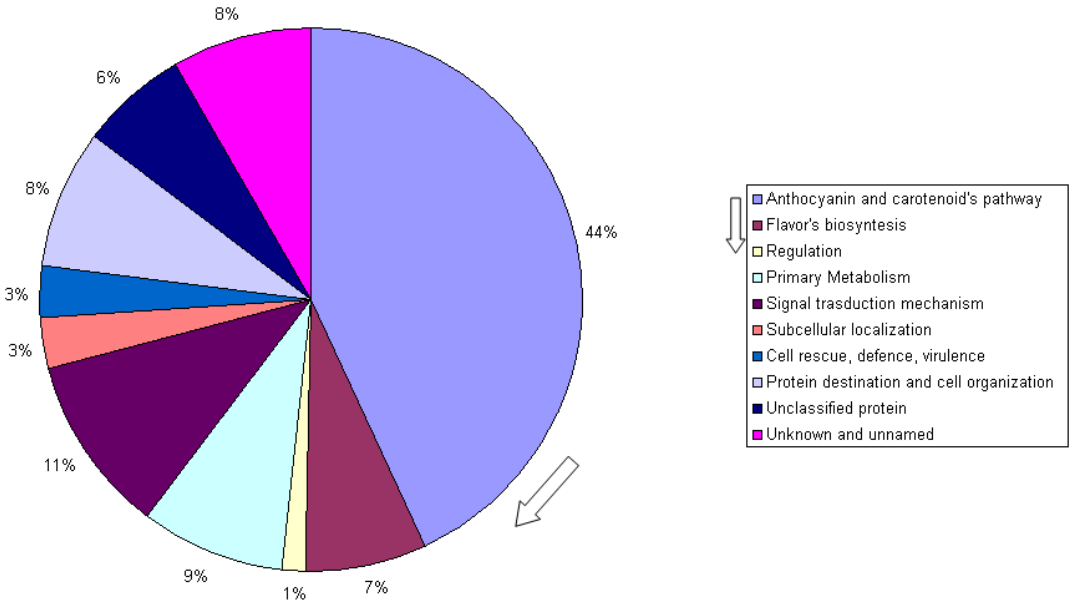

Fig. 4 Amino acid alignment and phylogenetic analysis of GSTs from different plants.
a

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Ph An9          -----MVVKVHGSAMAACPQRVMVCLIELGVDFELIHVDLDSLEQKKPEFLVLQP-FGQV 54
Cs-GST          -----MVVKVYGSVKAACPQRVLACLLEKGVEFEIVQVDLDEGEHKRPEFLLRQP-FGQV 54
Nt-ParB         -----MAIKVHGSPMSTATMRVAACLIEKELDFEFVPVDMASGEHKKHPYLSLNP-FGQV 54
Zm-GSTIII       ----MAPLKLYGMPLSPNVVRVATVLNEKGLDFEIVPVDLTTGAHKQPDFLALNP-FGQI 55
Zm-GSTI         --MAP--MKLYGAVMSWNLTRCATALEEAGSDYEIVPINFATAEHKSPEHLVRNP-FGQV 55
Zm-GST          --MATPAVKVYGWAISPFVSRALLALEEAGVDYELVPMSRQDGDHRRPEHLARNP-FGKV 57
Gm-HSP26A      MAATQEDVKLLGIVGSPFVCRVQIALKLKGVEYKFLEEDLG---NKSDLLLKYNPVHKKV 57
Zm Bz2          --MTAGTMRVLGGEVSPFTARARLALDLRGVAYELLDEPLGP--KKSDRLLAANPVYGKI 56
                       ::: *   :    *    *      ::::        ::    *  :* . ::

Ph An9          PVIEDGD-FRLFESRAIIRYYAAKYEVKG-----SKLTGTTLEEKALVDQWLEVESNNYN 108
Cs-GST          PVIEDGD-FKLFESRAIIRYYAAKYANQG-----PNLLGNTLEEKALVDQWLEVEAHNFN 108
Nt-ParB         PAFEDGD-LKLFESRAITQYIAHVYADNG-----YQLILQDPKKMPSMSVWMEVEGQKFE 108
Zm-GSTIII       PALVDGD-EVLFESRAINRYIASKYASEG-----TDLLPATAS-AAKLEVWLEVESHHFH 108
Zm-GSTI         PALQDGD-LYLFESRAICKYAARKNKPE-------LLREGNLEEAAMVDVWIEVEANQYT 107
Zm-GSTIV        PVLEDGD-LTLFESRAIARHVLRKHKPE-------LLGGGRLEQTAMVDVWLEVEAHQLS 109
Gm-HSP26A       PVFVHNE-QPIAESLVIVEYIDETWKNNP-------ILPSDPYQRALARFWSKFIDDKIV 109
Zm Bz2          PVLLLPDGRAICESAVIVQYIEDVARESGGAEAGSLLLPDDPYERAMHRFWTAFIDDKFW 116
                *.:   :   : ** .* .:       .        :        .   *  .  .:

Ph An9          DLVYNMVLQLLVFPKMGQTSDLTLVTKCANKLENVFDIYEQRLSKSKYLAG--EFFSLAD 166
Cs-GST          DLAFNLVLQLVILPRMGQRSDTALVHNLEQKLEAVLNIYEQRLSKSNYLAG--DSFTLAD 166
Nt-ParB         PPATKLTWELGIKPIIGMTTDDAAVKESEAQLSKVLDIYETQLAESKYLGG--DSFTLVD 166
Zm-GSTIII       PNASPLVFQLLVRPLLGGAPDAAVVEKHAEQLAKVLDVYEAHLARNKYLAG--DEFTLAD 166
Zm-GSTI         AALNPILFQVLISPMLGGTTDQKVVDENLEKLKKVLEVYEARLTKCKYLAG--DFLSLAD 165
Zm-GSTIV        PPAIAIVVECVFAPFLGREPNQAVVDENVEKLKKVLEVYEARLATCTYLAG--DFLSLAD 167
Gm-HSP26A       GAVSKSVFTVDEK------EREKNVEETYEALQFLENELKDKK----FFGG--EEFGLVD 157
Zm Bz2          PALDAVSLAPTPG------APAQAAEDTRAALSLLEEAFKDRSNGRAFFSGGDAAPGLLD 170
                                       . .    *  : :  : :     ::.*      * *

Ph An9          LSH------LPSLRFLMNEGGFSHLVTKRKCLHEWYLDISSRDSWKKVLDLMMKKISEIE 220
Cs-GST          LSH------LPALRYLMNEAGMAHLVTQRKHVNAWWDKISSRPAWKKLASLAH------- 213
Nt-ParB         LHH------IPNIYYLMS-SKVKEVFDSRPRVSAWCADILARPAWVKGLEKLQK------ 213
Zm-GSTIII       ANH------ALLPALTSARPPRPGCVAARPHVKAWWEAIAARPAFQKTVAAIPLPPPPSS 220
Zm-GSTI         LNH------VSVTLCLFA-TPYASVLDAYPHVKAWWSGLMERPSVQKVAALMKPSA---- 214
Zm-GSTIV        LSP------FTIMHCLMA-TEYAALVHALPHVSAWWQGLAARPAANKVAQFMPVGAGAPK 220
Gm-HSP26A       IAAVFIAFWIPIFQEIAGLQLFTS--EKFPILYKWSQEFLNHPFVHEVLPPRDPLFAYFK 215
Zm Bz2          LALGCFLPALRACERLHGLSLIDASATATPLLDGWSQRFAAHPAAKRVLPDTEKVVQFTR 230
                                          :  *   :  :    .

Ph An9          AVSIPAKEEAKV 232
Cs-GST          ------------
Nt              ------------
III             SA---------- 222
GST             ------------
Zm-GSTIV        EQE--------- 223
Gm-HSP26A       ARYESLSASK-- 225
Zm Bz2          FLQAQFRVHVS- 241
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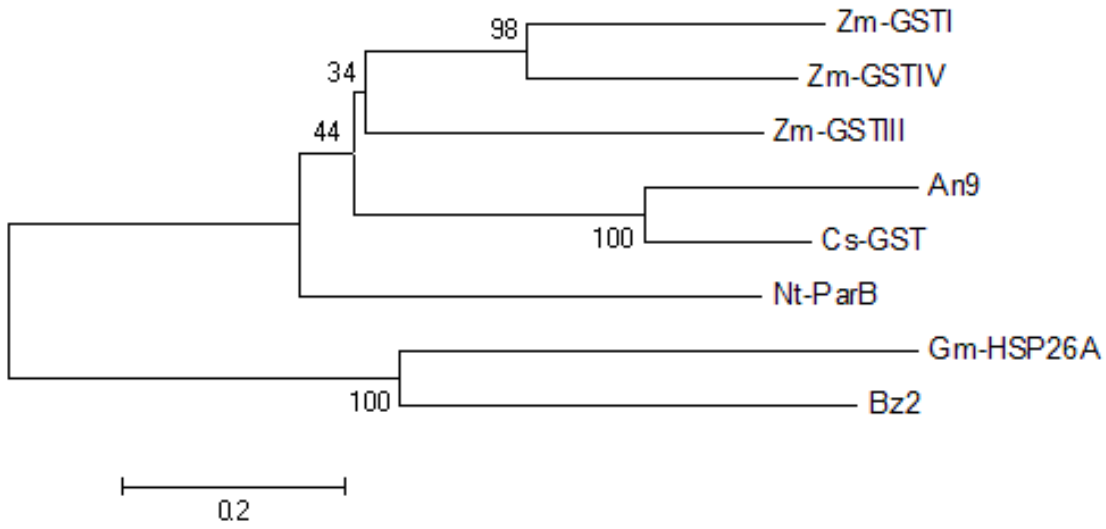
b



a Multiple alignment showing a comparison of Citrus GST (*Cs-GST*) with the type I and the type III GSTs. *An9* from Petunia; *ParB* from tobacco (Nt-ParB); GSTI and GSTIII from maize (Zm-GSTI and Zm-GSTIII); GSTIV from maize (Zm-GSTIV), are GSTs of the type I; *Bz2* from maize and *GST26A* from soybean (Gm-HSP26A) are GSTs of type III. In the bottom line in the alignment, asterisks indicate conserved amino acids, dashes indicate gaps in the alignments. Six of the 15 residues that are completely conserved correspond to the one reported in Alfenito et al. (1998).
b Phylogenetic tree derived from the sequences aligned in **a.** The length of the horizontal lines is proportional to the estimated genetic distance between the sequences. Bootstrap are indicated.

Fig. 5 Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) expression analysis of 10 selected SSH clones in common (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19) and blood (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20) oranges. M = Marker; a b c d = 20, 25, 30, 35 amplification cycles; A B C D = I, IV, VI, VIII sampling; CN = Negative Control; EF = Elongation Factor.

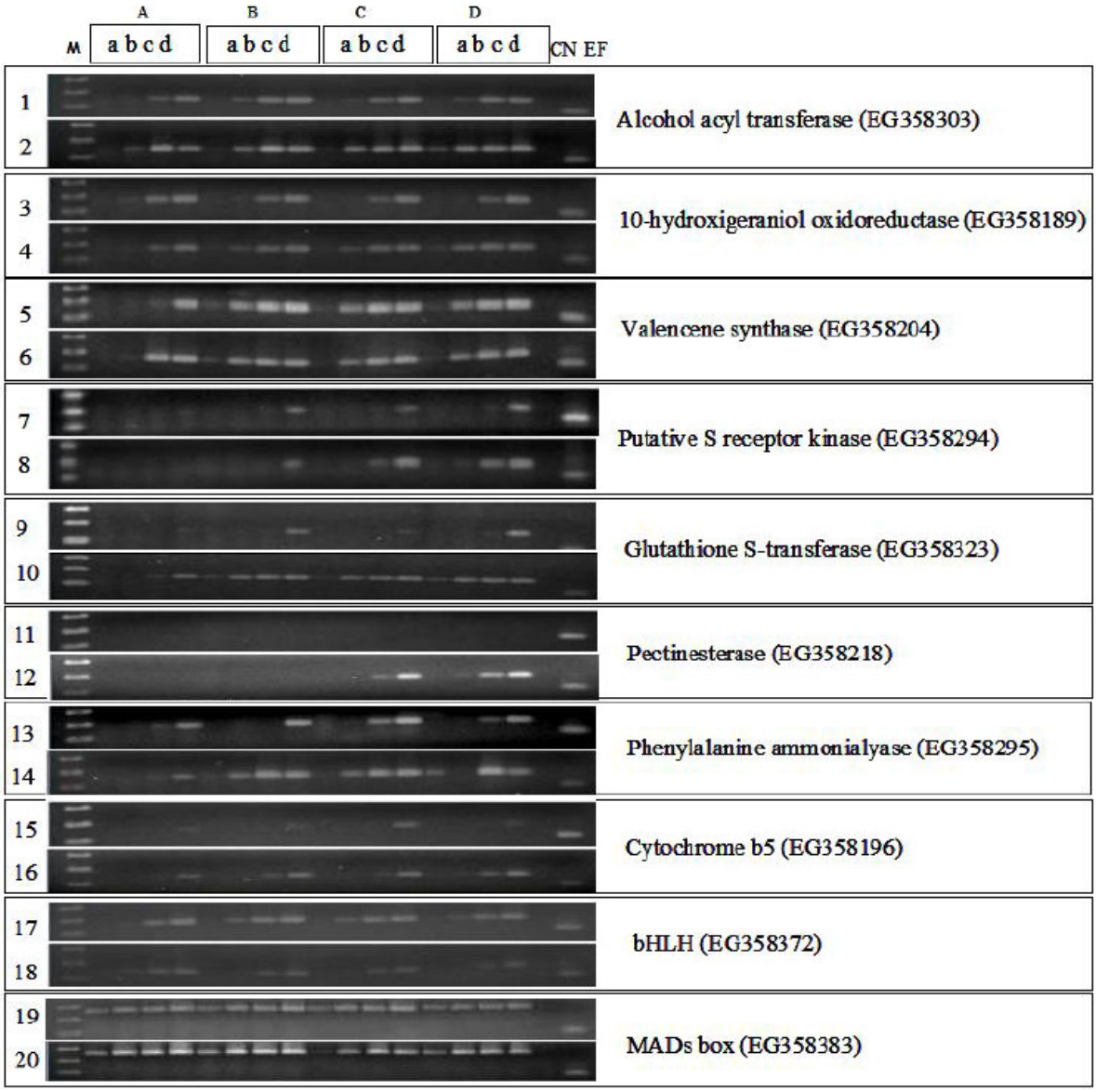


Fig. 6 Real time-PCR results of three selected SSH cDNA clones: **a** EG358323 (GST); **b** EG358294 (putative Ser receptor kinase); **c** EG358218 (Pectinesterase)

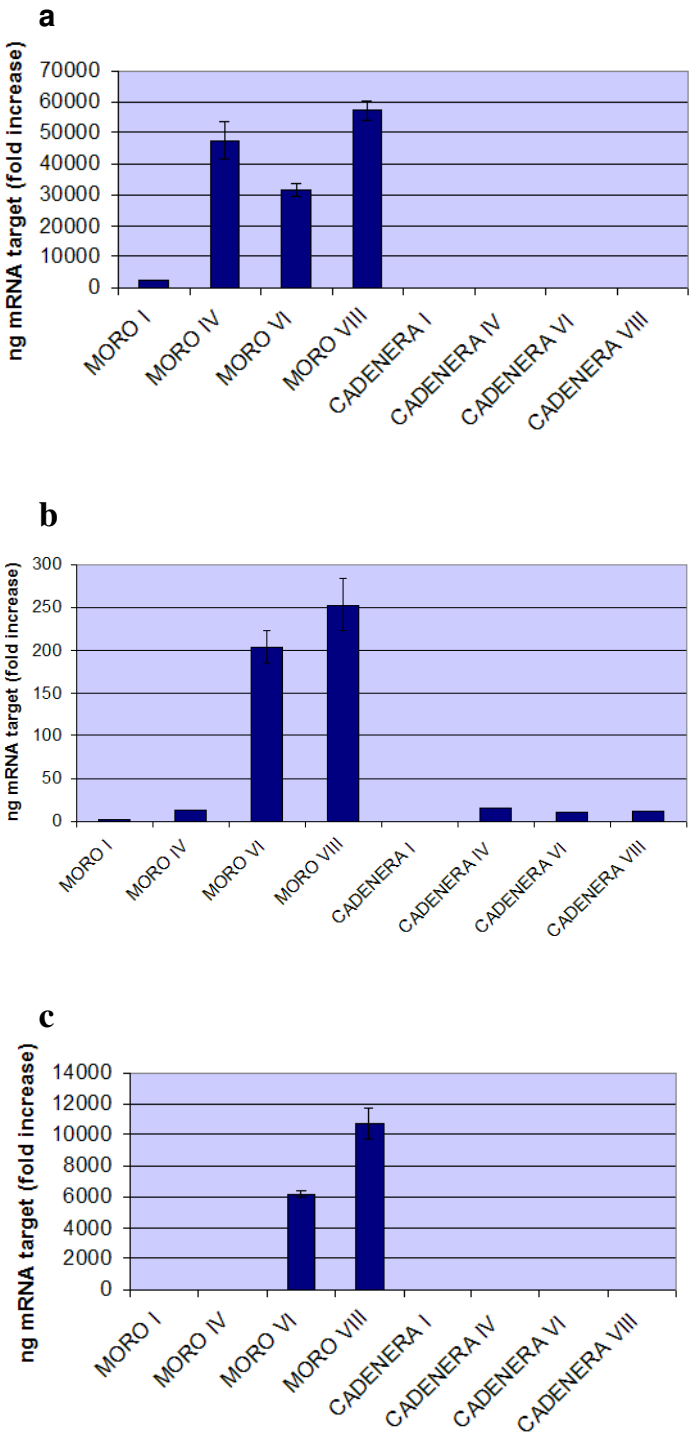


Fig. 7 Anthocyanin content in flesh of Moro oranges during ripening.

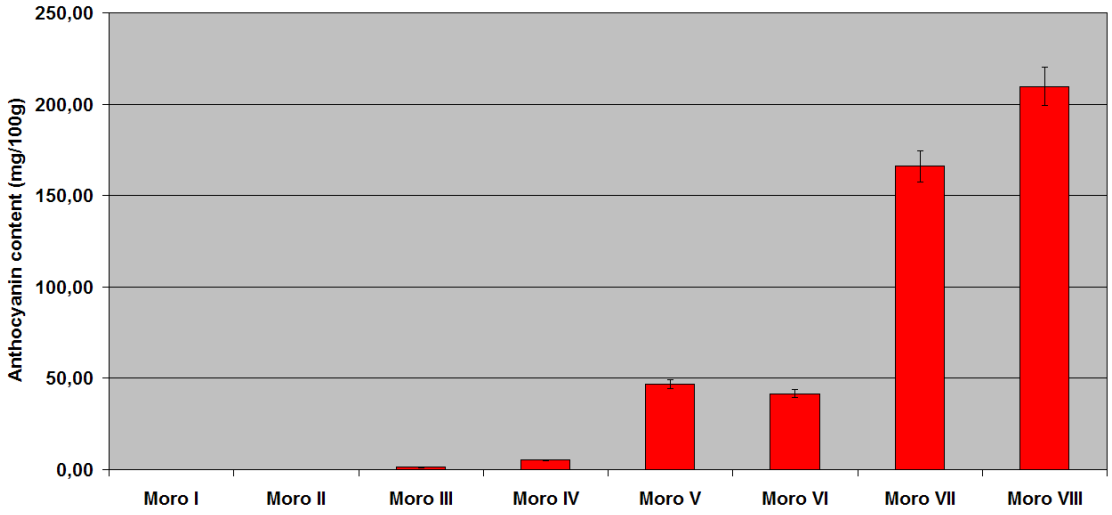


Fig. 8 Multiple alignment of Cs-bHLH and plant bHLHs involved in anthocyanin regulation. Amino acid sequence alignment of *Citrus sinensis* bHLH (Cs) with DELILA (from *Antirrhinum*; GenBank accession number M84913), Lc (from maize; GenBank accession number M26227), JAF13 (from petunia; GenBank accession number AF020545), Sn (from maize; GenBank accession number X60706), TT8 (from Arabidopsis; GenBank accession number NM_117050), An1 (from petunia; GenBank accession number AF260919) involved in plant anthocyanin pigmentation. Motif known to be conserved are shaded in *gray* (Heim et al. 2003)

	basic	Helix 1	Loop	Helix 2
DEL	RNHVLSERKRREKINERFMILASLVPSGGKVD-KVSILDHTIDYLRGLERKVDEL			
jaf13	RSRVISERRRREKINERFMLLASMLPAGGKVD-KISLLDETIEYLKELERRVQDL			
Lc	KNHVMSERKRREKLNEMFLVLKSLLPSIHRVN-KASILAETIAYLKELQRRVQEL			
Sn	KKHVMSERKRREKLNEMFLVLKSLLPSIHRVN-KASILAETIAYLKELQRRVQEL			
R	KNHVMSERKQREKLNEMFLVLKSLLPSIHRVN-KASILAETIAYLKELQRRVQEL			
TT8	--HVVAERRRREKLNEKFITLRSMVPFVTKMD-KVSILGDTIAYVNHLRKRVHEL			
an1	GNHVLAERRRREKLNERFIILRSLVPFVTKMD-KASILGDTIEYVKQLRKKVQDL			
GBOF-1	DSHSLAERVRREKISERMKLLQALVPGCDKVTGKAVMLDEIINYVQSLQNQVEFL			
Cs	DSHSLAERVRREKISERMKILQKLVPGCDKVTGKAFMLDEIINYVQFLQNQVEFL			
Consensus	: ::** :***:.* : * ::* :: * :* . * * * * *			

Table 1 Oligonucleotide primer sequences utilized for semiquantitative reverse transcription PCR and Real time analysis and expected fragment sizes of the amplification products

Gene		Primer		Temperature annealing (°C.)	Amplicon size (bp)
Name	Accession. number	Name	Sequence		
Glutathione S-transferase	EG358323	GST-61_fw GST-222_rv	ATCCTCTATTACTGGAACTTGCCCA TATGGTTCAGTTAAGGCAGCTTGC	60	162
Putative S receptor kinase	EG358294	SRK-255_fw SRK-400_rv	CTGCTCTATTTCGGTATGGTTGGA CTTTAGCGTTGCCAAGTTCTGAAC	60	146
Valencene synthase	EG358204	VS-79_fw VS-236_rv	CAAGGTCATGAGTTTGAGCAGAAGAG GGCTTCATCATCAACTCCTCGTTAA	60	158
Alcohol acyl transferase	EG358303	AAT-45_fw AAT-197_rv	GGATATTCCTCCAGGCTATTATGGC GCCGCAGACCTTATATACTCTTCGTT	60	153
10-hydroxigeraniol oxidoreductase	EG358189	HOR-343_fw HOR-492_rv	GAGGTCGGAAGCAAAGTGAGTAAAT CAGGTACTTGTTGGCATAGGTCATT	60	150
Pectinesterase	EG358218	PECT-19_fw PECT-168_rv	GTACTCGCGAACCGTTTACATCAAG ACTCTGTTAGCAGTTGAAGAACCCG	60	150
bHLH	EG358372	bHLH-25_fw bHLH-418_rv	TGACGACTACCGCTACTACTACTCGTATGG GGGCAGGTACATAGTTGATAA	60	390
MADs box	EG358383	MAD-309_fw MAD-463_rv	CAGGCCCGAGTCTGAATAATTACA TGATTGCGTGTTCCTAGTCACTGA	60	155
Cytochrome b5 DIF-F	EG358196	Citb5-328_fw Citb5-470_rv	GTAGTTGAAGAGCAACTCGTGAGCA CCGAGGTACTGAACGTTACAAGGTT	60	143
Phenylalanine ammoniolyase	EG358295	PAL-22_fw PAL-180_rv	GCAGGTACGTTTTTTCATACGCTG CCTCCTCGAAAGCACCTATCTTTAAG	60	159
Elongation factor	AY498567	EF-161_fw EF-88_rv	CTGCTGGACGCTCTTGACAA TCCTGGAGTGGCAGACGAA	60	72

Table 2 Functional categories of the cDNA identified after PCR-select differential

GenBank accession	Lengh (nt)	Highest homology (plant, GenBank accession no.)	Identity (%)	Score	E-value	Functional category [a]	Up-regulation
EG358344	999	Flavanone 3 hydroxylase (*C. sinensis*, dbj:BAA36553.1)	99	565	1,00E-160	1	Blood
EG358289	848	Dihydroflavonol-4-reductase (*C. sinensis*, gb: AAS00611.1)	98	530	1,00E-159	1	Blood
EG358333	1030	Phenylalanine ammoniolyase (*I. nil*, gb: AAG49585)	88	499	1,00E-147	1	Blood
EG358292	602	Phenylalanine ammoniolyase (*L. sativa*, gb:AAL55242.1)	94	381	1,00E-145	1	Blood
EG358263	779	Anthocyanidin synthase (*C. sinensis*, gb: AAT02642.1)	90	475	1,00E-134	1	Blood
EG358272	703	Succinyl-CoA-ligase beta subunit (*A. thaliana*, emb:CAA05024.1)	88	421	1,00E-117	8	Blood
EG358268	764	ATP citrate lyase a-subunity (*L. albus*, emb: CAC86995.1)	95	402	1,00E-112	8	Blood
EG358270	726	Putative peptide chain release factor subunit 1 protein (*O. sativa*, gb:AAX95517.1)	82	370	1,00E-102	10	Blood
EG358386	558	Piruvate decarboxylase 1 (*L. corniculatus*, gb: AAO72533.1)	89	357	1,00E-98	8	Blond
EG358280	633	UDP-glucose-flavonoid-3-O-glucosyl transferase (*C. sinensis*, gb: AAS00612.1)	93	337	1,00E-92	1	Blood
EG358356	546	T-protein precursor (emb: CAB16917.1)	82	322	6,00E-88	10	Blond
EG358274	669	Putative 2-nitropropane dioxigenase (*A. thaliana*, gb: AAL34288.1)	74	312	9,00E-87	8	Blood
EG358219	790	Chalcone synthase 1 (*C. sinensis*, dbj:BAA81663.1)	98	300	2,00E-81	1	Blood
EG358315	574	Manganase superoxide dismutase (*A. marina*, gb: AAN15216.1)	84	296	3,00E-80	8	Blood
EG358208	843	Phophoenol piruvate carboxialse (*A. thaliana*, ref: NP_850373.1)	83	265	6,00E-77	8	Blood
EG358314	578	MATE efflux family protein (*A. thaliana*, gb: AAU05531.1)	74	280	3,00E-75	9	Blood
EG358220	413	Phytoene synthase (*C. sinensis*, gb: AAO72533.1)	98	357	1,00E-74	1	Blood
EG358276	663	Ipotetical protein At1g55320 (*A. thaliana*, gb: AAX23787.1)	60	275	6,00E-74	10	Blood
EG358337	492	Leucoanthocyanidin dioxygenase-like protein (*A. thaliana*, gb:AAM61665.1)	75	264	2,00E-71	1	Blood
EG358200	462	Chloroplast translational elongation factor TU (*P. graveolens*, gb:AAK08141.1)	82	254	6,00E-68	7	Blood
EG358343	481	Aspartic proteinase (*T. cacao*, emb: CAC86004.1)	74	254	1,00E-67	8	Blood
EG358368	439	Unnamed protein product (*A. thaliana*, dbj: BAB08959.1)	79	245	4,00E-65	9	Blond
EG358362	517	Unknown protein (*A. thaliana*, gb: AAK93691.1)	71	245	5,00E-65	9	Blond
EG358204	450	Valencene synthase (*C. sinensis*, gb: AAQ04608.1)	97	241	7,00E-64	2	Blood
EG358275	669	Vacuolar sorting receptor, putative (*A. thaliana*, ref: NP_174375.1)	66	239	7,00E-63	7	Blood
EG358295	598	Phenylalanine ammoniolyase (Malus sp., emb: CAA48231.1)	78	235	9,00E-62	1	Blood
EG358303	583	Alcohol acyl transferase (*P. communis*, gb: AAS48090.1)	64	232	5,00E-61	2	Blood
EG358367	446	2-on-2-hemoglobin (*D. glomerata*, emb: CAD33536.1)	87	229	2,00E-60	8	Blond
EG358357	543	Caffeic acid o-methyltrasferase (*C. roseus*, gb: AAK20170.1)	59	229	4,00E-60	8	Blond
EG358323	550	Glutathione S-transferase (*C. sinensis*, gb: AAX81329.1)	98	285	7E-77	1	Blood
EG358186	860	Chloroplast small heat-shock protein class 1 (*C. frutiscens*, gb: AAQ19680.1)	68	221	2,00E-57	7	Blood
EG358189	467	10-hydroxygeraniol oxidoreductase (*C. roseus*, gb: AAQ55962.1)	77	221	3,00E-57	2	Blood
EG358273	703	Putative endol-1,3;1,4-beta-D-glucanase (*O. sativa*, gb: AAU10802.1)	53	218	2,00E-56	10	Blood
EG358373	389	Dead box helicase putative (*A. thaliana*, gb: AAN46806.1)	89	214	8,00E-56	3	Blond
EG358317	560	Putative flavanone 3-beta hydroxilase (*A. thaliana*, emb: CAB78172.1)	60	213	2,00E-55	1	Blood
EG358365	477	Protein kinase C inibitor-like protein (*A. thaliana*, gb: AAM63920.1)	86	212	4,00E-55	4	Blond
EG358213	436	Unnamed protein product (*O. sativa*, ref: NP_912885.1)	62	202	3,00E-52	9	Blond
EG358381	691	Putative cytochrome p450 protein (*A. thaliana*, gb: AAM20382.1)	50	200	4,00E-51	1	Blond

GenBank accession	Lengh (nt)	Highest homology (plant, GenBank accession no.)	Identity (%)	Score	E-value	Functional category [a]	Up-regulation
EG358218	416	Pectinesterase (*C. sinensis*, gb: AAB57669.1)	96	195	5,00E-50	6	Blood
EG358358	528	Calcium-binding EF and family protein (*A. thaliana*, gb: AAF80620.1)	56	195	8,00E-50	10	Blond
EG358325	541	Phenylalanine ammoniolyase (*R. idaeus*, dbj: BAA00887.1)	86	194	2,00E-49	1	Blood
EG358294	599	Putative S receptor kinase (*O. sativa*, emb:CAE05335.2)	58	174	2,00E-43	4	Blood
EG358203	456	Acidic Chitinase III (*N. Tabaccum*, emb: CAA77656.1)	73	172	3,00E-43	5	Blood
EG358278	661	Putative galactinol synthase (*A. thaliana*, gb: AAM61564.1)	81	173	4,00E-43	8	Blood
EG358379	235	Protein kinase CK2 regulatory subunity 2 (*N. tabaccum*, emb: CAD32500.1)	93	171	7,00E-43	4	Blond
EG358224	382	AnthranilateN-hydroxicinnamoil/benzoil transferase (*A. thaliana*, gb: AAM61636.1)	59	170	1,00E-42	8	Blood
EG358199	463	Monoterpene glucosyl trensferase (*E. perriniana*, dbj: BAD90935.1)	66	157	1,00E-38	1	Blood
EG358216	426	Osmotin-like (*T. cacao*, gb: AAV34889.1)	77	154	7,00E-38	5	Blood
EG358328	539	Expressed protein (*A. thaliana*, ref: NP_188866.1)	52	152	8,00E-37	10	Blood
EG358354	469	Putative S-receptor kinase (*O. sativa*, emb: CAE04682.1)	49	147	2,00E-35	4	Blood
EG358383	605	MADs-box protein 4 (*V. vinifera*, gb: AAM21344.1)	64	146	5,00E-35	3	Blond
EG358375	335	Putative mitochondrial ATP synthase (*S. demissum*, gb: AAT40531.1)	87	149	5,00E-35	7	Blond
EG358279	642	Dicyanin (*L. esculentum*, gb: AAF66242.1)	57	141	2,00E-33	10	Blood
EG358370	412	Steoroyl acyl carrier protein chromosome (*A. thaliana*, gb: AAD28287.1)	68	139	2,00E-33	10	Blond
EG358363	493	Putative NADH deidrogenase 10.5K Chain (*A. thaliana*, gb: AAM20072.1)	68	139	3,00E-33	10	Blond
EG358229	353	Adenylosuccinate syntethase (*A. thaliana*, emb: CAB41194.1)	85	134	9,00E-32	8	Blood
EG358196	464	Cytochrome b5 DIF-F (*P. hybrida*, gb: AAD10774.1)	57	133	2,00E-31	1	Blood
EG358311	1187	Al-induced protein (*G. hirsutum*, gb: AAQ74889.1)	73	111	8,00E-31	5	Blood
EG358378	412	RAD23 protein (*L. esculentum*, emb: CAB51544.1)	81	130	1,00E-30	6	Blond
EG358372	389	bHLH transcrition factor GBOF-1 (*T. gesneriana*, gb: AAD56411.1)	61	127	1,00E-29	3	Blond
EG358346	471	Chalcone synthase 1 (*C. sinensis*, dbj: BAA05641.1)	93	121	9,00E-28	1	Blood
EG358336	496	Tubulin alpha-chain (*O. sativa*, emb: CAD26891.1)	100	113	3,00E-25	10	Blood
EG358360	518	Putative DNAJ protein (*A. thaliana*, gb: AAQ54533.1)	73	108	9,00E-24	6	Blond
EG358259	254	Pom30 (*S. tuberosum*, emb: CAA63968.1)	86	105	5,00E-23	6	Blood
EG358265	182	Sinapyl alcohol dehydrogenase-like protein (*P.tremula* x *P.tremuloides*, gb: AAW45741.1)	80	102	3,00E-22	2	Blood
EG358302	584	Pathogenesis-related protein PR10A (*A. glutinosa*, emb: CAD33535.1)	49	98	2,00E-20	5	Blood
EG358231	353	Unknown (*S. ithaliaca*, gb: AAP93138.1)	44	87	1,00E-17	9	Blood
EG358258	262	O-diphenol-o-metiltrasferase (*C. annuum*, gb: AAC17455.1)	50	74	6,00E-14	8	Blood
EG358251	297	Pathogenesis-related protein PR10A (*D. glomerata*, emb: CAD33532.1)	57	74	1,00E-13	9	Blood
EG358364	488	Subtilisin inhibitor I (ASI-I) contain: Subtilisin inhibitor II (ASI-II) (gi: 124121)	43	63	5,00E-10	10	Blond
EG358377	252	Lea5 protein (*C. sinensis*, emb: CAA86851.1)	100	62	5,00E-10	5	Blond
EG358371	407	Cytochrome c reductase subunity (*S. tuberosum*, emb: CAA57768.1)	64	59	7,00E-09	8	Blond
EG358385	560	Putative cytokinin dehydrogenase (*O. sativa*, ref: XP_475155.1)	33	31	1,00E-04	8	Blond
EG358331	515	Unknown protein (*O. sativa*, gi: 50911783)	94	41	0,002	9	Blond

(continued Tab. 2)

For each clone the SSH identification name, the length of the homologous region, the putative function of the closest homolog and homology parameters of BlastX (identity, score, e value) and regulation as deduced from reverse-Northern analysis are reported.

[a]1. Anthocyanin and carotenoid's biosynthesis; 2. Flavor biosynthesis; 3. Regulation; 4. Signal transduction mechanism; 5. Cell rescue, defense, virulence; 6. Protein destination and cell organization; 7. Subcellular localization; 8. Primary metabolism, 9. Unknown and unnamed; 10. Unclassified proteins.

Table 3 Characteristics of the SSH library.

	Number	Percentage (%)
Total clones	1248	
Differential clones	260	
Up-regulated in blood	230	88,46
Up-regulated in blond	30	11,53
Differential genes	82	
Non-redundant	52 (52 clones)	63,41
Redundant	30 (208 clones)	36,58

Table 4 Assembled clusters of SSH cDNAs

BLAST Acc. No[a]	Putative identity	Organism	Number of hit[b]	Percentage[c]
CAB42793.1 AAG49585 AAL55242.1 CAA48231.1	Phenylalanine ammoniolyase	*Citrus sinensis* *Ipomea nil* *Lactuca sativa* Malus sp.	7	3.36
BAA81663.1 BAA05641.1	Chalcone synthase	*Citrus sinensis* *Camellia sinensis*	15	7.21
AAS00611.1	Dihydroflavonol 4-reductase	*Citrus sinensis*	4	1.92
AAT02642.1	Anthocyanidin synthase	*Citrus sinensis*	24	11.6
AAD10774.1	Cytochrome b5	*Petunia hybrda*	13	6.25
AAS00612.1	UDPglucose:flavonol 3-O-glucosyltransferase	*Citrus sinensis*	15	7.21
ABA42224.1	Glutathione S-transferase	*Citrus sinensis*	25	12.01
AAS48090.1	Alcohol acyl transferase	Pirus communis	10	4.80
AAQ55962.1	10-hydroxigeraniol oxidoreductase	*Catharanthus roseus*	6	2.88
AAD56411.1	bHLH	*Tulipa genseriana*	2	0.96
CAE05335.2	S receptor putative kinase	*Oryza sativa*	25	12.01
AAB57669.1	Pectinesterase	*Citrus sinensis*	10	4.80
AAQ04608.1	Valencene synthase	*Citrus sinensis*	2	0.96
	Other clones redundant not inserted in the table		50	24.03
	Sum of Redundant Clones		208	100

[a] GenBank accession number of the most similar sequence identified by BLASTX alignment.
[b] Number of clones assigned to the same putative functional class.
[c] Percentage of the number of hit on the total redundant clones

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