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Characterization of genes associated to induced resistance against *Penicillium expansum* in apple fruit treated with quercetin

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ABSTRACT

Penicillium expansum causes blue mould, a serious postharvest disease of apples, and is the main producer of the mycotoxin patulin. Since control by synthetic fungicides is no longer accepted by consumers, the demand for alternative means is pressing. In a recent study, the flavonoid quercetin, although scarcely effective in *in vitro* assays against *P. expansum* growth, significantly reduced blue mould rots on Golden Delicious apples, suggesting an enhancement of host disease resistance. To confirm or reject this hypothesis, genes differentially expressed in quercetin-treated Golden Delicious apples were identified by suppression subtractive hybridization (SSH) technique. A pool of 88 unique gene transcripts were obtained. Several sequences revealed high similarities with different classes of pathogenesis-related proteins (RNase-like PR10 and PR8), or with proteins expressed under stress conditions. Other transcripts had high similarity to genes of unknown function or genes coding for proteins having a role in pathogen recognition and in signalling pathways. SSH data were validated by analysing the expression of 14 genes by quantitative real time PCR (qPCR). Eleven genes proved to be up-regulated at a medium-high level in freshly harvested apples. Among these, 5 genes selected for temporal expression profiling revealed the existence of a combined effect, particularly at 24 h or 48 h, between wounding and phenolic treatment. These results provide evidence that quercetin induces resistance to *P. expansum* in apples, by acting on the transcription level of genes involved in several distinct metabolic processes.

Keywords: induced disease resistance; quercetin; *Penicillium expansum; Malus domestica*; quantitative real time PCR; suppression subtractive hybridization.

1. INTRODUCTION

Apples (*Malus domestica* Borkh) kept in cold storage are highly susceptible to fungal decay. Blue mould, caused by *Penicillium expansum* Link, is one of the most important postharvest rots and can account up to 50% of stored fruit losses (Mari et al., 2002). This disease is of economic concern not only to the fresh-fruit industry but also to the fruit-processing industry, since *P. expansum* is regarded as the major producer of patulin, a mycotoxin with severe acute and chronic effects on human health (Wouters and Speijers, 1996). Due to its solubility in water and stability in acidic media, this toxic metabolite can be transferred into fruit juices and purees, making them unmarketable (Acar et al., 1998). Indeed, the European Commission (2006) has laid down the maximum permitted patulin levels in apple-based products: 50 µg/kg for juices, 25 µg/kg for solid products and 10 µg/kg for baby food. When permitted, synthetic fungicides are the primary means to control postharvest diseases. However, the public's growing concern for human and environmental health risks associated with pesticide usage, the development of fungicide-resistant strains, and the lack of approval of some of the most effective fungicides have motivated the search for alternative approaches. Among these new control strategies, the use of plant or animal products with a fungicidal activity and the application of antagonistic microorganisms, either alone or as part of an integrated pest management policy, can be considered (Spadaro and Gullino, 2004).

Within plant products, phenolic compounds play a major role in the activation of resistance in plants (Nicholson and Hammerschmidt, 1992). Some of them occur constitutively and some are synthesized in response to biotic or abiotic stresses. Within phenolic compounds, particular interest is aroused by the flavonoids, which are potent dietary antioxidants present in several plant organs, including fruits. They are thought to improve human health and this effect seems to be related, at least partially, to their antioxidant effect (Nijveldt et al., 2001). Among flavonoids, quercetin was considered the most prominent

(Bock, 2003). It consists of 3 rings and 5 hydroxyl groups and occurs in food (i.e. apple, tea, onion, nuts, berries, cauliflower and cabbage) as the aglycone (attached to a sugar molecule) of many plant glycosides. Quercetin can scavenge superoxide and hydroxyl radicals and reduce lipid peroxidation. In addition, it has been reported that quercetin reduces the biosynthesis of heat shock proteins (Ishida et al., 2005). In a recent study the exogenous application of the flavonoid quercetin was proposed as alternative strategy to control blue mould and patulin accumulation in Golden Delicious apples (Sanzani et al., 2009a). Authors hypothesized that in some way quercetin might enhance natural host defence responses since, although at the tested concentration it didn't markedly reduced *P. expansum in vitro* growth, it controlled blue mould on apples. Interestingly, at the same concentration quercetin was able to reduce patulin accumulation both on apples and *in vitro*. Subsequent studies have demonstrated that quercetin reduces patulin production by acting on the transcript level of genes involved in its biosynthetic pathway (Sanzani et al., 2009b).

The overall objective of the present investigation was to attempt to elucidate at a molecular level the mechanisms by which quercetin induces resistance in treated apples. Among the numerous techniques that can be utilised to identify genes differentially expressed in response to a treatment, suppression subtractive hybridization (SSH) was chosen, since it can be performed in absence of sequence information and enables rare differentially expressed transcripts to be enriched by 1,000-5,000-fold (Diatchenko et al., 1996). Moreover, it yields cDNA fragments that can be used directly for sequencing and further analyses. Quantitative real time PCR (qPCR), i.e. one of the most powerful techniques for studying plant responses to biotic and/or abiotic factors (Schena et al., 2004), was utilised to validate SSH results and to study the temporal expression profile of 5 genes in quercetin treated apples.

2. MATERIALS AND METHODS

2.1. Chemicals

Quercetin (3,3′,4′,5,7-pentahydroxyflavone dihydrate) was purchased from Sigma (Sigma-Aldrich, Milan, Italy). Compound stock solution was prepared at a concentration of 5 g L^{-1} by dissolving a pure standard into a mixture of phosphate buffer (50 mM, pH 7.4) and NaOH (1 mol, pH 13) (9:1 v v^{-1}, pH 13).

2.2. In vivo *tests to evaluate induced resistance in apples*

Golden Delicious apples purchased from a local market in Valencia (Spain) were surface sterilized by immersion in 2 % hypochlorite for 2 min, washed with running tap water and air-dried. On each fruit, 4 equidistant wounds (3×3 mm) were made with a sterile nail-head along the equatorial axis and each wound was treated with 20 μL of quercetin stock solution. Non-treated wounded apples were used as a control. One hour after quercetin application or after 24, 48 and 72 h of incubation at 16 °C and high relative humidity (85-95 %), another identical series of wounds was made approximately 5 mm apart from the previous ones and inoculated with 10 μL of a 5×10^{7} conidia L^{-1} suspension of *P. expansum*. For each treatment and for each inoculation time, 12 apples were arranged in a randomized complete block design. Incidence of decay (percentage of infected wounds) and disease severity (diameter of the lesions, mm) were evaluated 4 d after pathogen inoculation.

2.3. Tissue sampling and RNA isolation

On the equatorial area of 8 surface sterilised Golden Delicious apples, 8 wounds were made and treated with quercetin as described above. An equal number of wounds were amended with the dissolving buffer and used as a control. After 24 and 48 h incubation, tissue cylinders (9×10 mm) were withdrawn around each wound with a cork borer and immediately

frozen in liquid nitrogen. Then, samples from each single thesis were pooled, grounded in liquid nitrogen and kept at -80 °C until RNA extraction.

Total RNA was isolated as described by Sánchez-Torres and González-Candelas (2003) with minor modifications. Two g of grounded apple tissue were homogenized with a tissue homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) for 2 min in presence of 10 mL of preheated (80°C) extraction buffer (200 mM Tris-HCl pH 8.0, 400 mM NaCl, 50 mM EDTA pH 8.0, 2 % sarcosyl, 1 % PVP-40, 1 % ß-mercaptoethanol) and 5 mL of Tris-equilibrated phenol. Samples were then incubated at 80 °C for 15 min and added with 5 mL of a mixture chloroform:isoamyl alcohol (24:1, v:v). After centrifugation for 20 min at 3000 ×g and 4 °C, the aqueous upper phase was extracted again with 10 mL of phenol:chlorophorm: isoamyl alcohol (25:24:1, v:v:v). The supernatant was collected, added with 1.5 volumes of ethanol, incubated on ice for 1.5 h and centrifuged for 15 min. The pellet was washed with 70 % cold ethanol, dissolved in 900 µL TESa buffer (10 mM Tris-HCl, 5 mM EDTA pH 8, 0.1 % sarcosyl), incubated at 65 °C for 10 min and centrifuged for 5 min. The supernatant was collected added with 0.3 volumes of 12 M LiCl and maintained overnight at -20 °C. After centrifugation for 45 min, the pellet was washed with 70 % cold ethanol and dissolved in 250 µL of 3 M NaOAc (pH 5.2). After a further centrifugation for 5 min, the pellet was rewashed with 70 % cold ethanol and dissolved in nuclease-free water (NFW). Finally, to completely eliminate polysaccharides, samples were brought to a saline concentration of 80 mM, in a final volume of 700 µL, by adding 3 M NaOAc (pH 6.0). Then they were added with 0.3 volumes of 2-butoxyethanol (2-BE) and incubated on ice for 30 min. After centrifugation for 10 min, the supernatant was added with 0.7 volumes of 2-BE, incubated at -20 ºC for 90 min and centrifuged for 20 min. The pellet was washed with 70 % cold ethanol, dissolved in NFW and stored at -80 °C until use. Total RNA integrity was checked on 1 % agarose gel and its quantity and purity was determined spectrophotometrically.

150

151 *2.4. Construction of a SSH cDNA library*

152 For each treatment and sampling time, RNAs from replicate extractions were pooled
153 together in equal amounts. Then, 75 µg of total RNA, from both quercetin-treated and non-
154 treated samples (Fig. 1), were used as starting material for the isolation of pure, intact polyA$^+$
155 mRNA with the Dynabeads® mRNA Purification™ Kit (Invitrogen, Barcelona, Spain). The
156 obtained mRNA was quantified using the RiboGreen® RNA Fluorimetric Assay (Invitrogen,
157 Barcelona, Spain).

158 SSH was performed with treated (tester) and non-treated (driver) samples (Fig. 1)
159 using the PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA), based on the
160 method developed by Diatchenko et al. (1996), following the manufacturer's instructions.
161 Double-stranded cDNA was produced from 2 µg of mRNA. In first-strand cDNA synthesis, 4
162 µL of mRNA and 1 µL of 10 µM cDNA Synthesis Primers (provided with the kit) were
163 combined. Samples were incubated at 70 °C for 2 min and then cooled on ice. Two µL of 5×
164 First-Strand Buffer, 1 µL of the dNTPs Mix (10 mM each), 1.5 µL of NFW, 1 µL of
165 SuperScript III Reverse Transcriptase (200 U) and 0.5 µL of 0.1 M DTT were added to each
166 reaction. Finally, tubes were incubated for 1.5 h at 42 °C. Second-strand cDNA synthesis was
167 performed according to the procedure described by the manufacturer.

168 The tester and the driver cDNA populations were digested with the restriction enzyme
169 RsaI (Gibco) to obtain short blunt-ended fragments. The tester pool was then divided into two
170 populations: the first was ligated to adaptor 1 and the second to adaptor 2R, provided with the
171 kit. Each tester pool was hybridized separately with excess driver cDNA, and finally mixed
172 together for a second subtractive hybridization. The fragments differentially expressed in the
173 tester were then amplified in two PCRs, according to the manufacturer's recommendations.

The resulting cDNA library was enriched in genes exclusively or more expressed in quercetin-treated apples as compared to non-treated ones.

The efficiency of subtraction was checked by running the subtracted sample on an agarose gel, side by side with the non subtracted one and with a control subtracted cDNA. A further evaluation was conducted by amplifying the constitutively expressed (housekeeping) gene elongation factor 1-α (EF 1-α) and 3 additional genes (chalcone synthase - CHS, class II chitinase - CHT 2, and phenylalanine ammonia lyase - PAL), whose expression proved not to be influenced by quercetin application (data not shown). PCR reactions were carried out in a 25 µL final volume. A 1:10 dilution of the second SSH PCR product was used as a template. The reactions contained 0.5 µL of dNTPs (10 mM), 1 µL of each primer (10 µM) (Table 1), 0.2 µL of EcoTaq (1 U, Ecogen, Barcelona, Spain), 0.75 µL of 50 mM $MgCl_2$ and 2.5 µL of 10× EcoTaq buffer. Amplification conditions consisted of 3 min of denaturation at 94 °C followed by 35 cycles of 30 s at 94 °C, 45 s at a temperature ranging from 54 and 58 °C (Table 1) and 1 min at 72 °C. Finally, amplification mixtures were maintained at 72 °C for 10 min. Aliquots (5 µL) of the amplification mixtures were withdrawn at regular cycle intervals of PCR reactions and analysed by electrophoresis on an agarose gel.

2.5. Cloning and differential screening

The subtracted sample was purified using the High Pure PCR Product Purification kit (Roche, Barcelona, Spain), ligated to the *pCR®II* cloning vector (Invitrogen) and utilised to transform *Escherichia coli* strain DH5α cells, according to standard protocols (Sambrook and Russel, 2001). One hundred fifty white colonies were randomly picked, grown in Luria-Bertani (LB) broth containing carbenicillin (100 mg L^{-1}), added with glycerol (20 %), and stored at -80 °C.

198 To check for the presence and size of individual inserts in the selected colonies, fragments
199 were amplified by colony PCR using Nested Primer 1 and 2R (Clontech kit) and the
200 corresponding PCR products were run on high-density agarose gels. Plasmids from positive
201 colonies were extracted with the GenElute^{TM} Plasmid Miniprep Kit (Sigma, St. Louis, MO,
202 USA) according to the manufacturer's recommendation.
203
204 *2.6. Sequencing and homology search on the databases*
205 All inserts were sequenced with primers T7 or Sp6, using external sequencing services
206 (IBMCP, UPV–CSIC, Valencia, Spain or PRIMM s.r.l., DNA Sequencing facility, Naples,
207 Italy). Raw sequences were retrieved, trimmed off vector and adaptor sequences and aligned
208 using the SEQtools program (Rasmussen, 2002). The resulting multiple sequence alignment
209 was manually corrected using the GENEDOC program (Nicholas et al., 1997).
210 To assign an identity to the contigs and singletons sequences, the nucleotide or their
211 derived amino acid sequences were compared with those deposited in the NCBI database
212 using the BLAST N or X algorithms. Moreover, the genetic database for the model higher
213 plant *Arabidopsis thaliana*, maintained by the Arabidopsis Information Resource (TAIR), was
214 utilized for identifying functions associated with many of the proteins that had significant
215 matches to our subtracted cDNA fragments.
216
217 *2.7. Data validation and expression profile analysis by qPCR*
218 For each trial 3 independent experiments were used as biological repetitions for RNA
219 extraction. Total RNA was extracted from tissue cylinders as reported above. For each thesis
220 and repetition, DNase RQ1-treated (Promega, Milan, Italy) RNA preparations were
221 subsequently converted to cDNA by AMV reverse transcriptase as recommended by the
222 manufacturer (Promega). PCR primers designed specifically to the nucleic acid sequence of

each SSH clone using the Primer 3 program (Rozen and Skaletsky, 2000) are reported in Table 2.

In order to calculate reaction efficiency (optimal range 90-110 %) and establish the most suitable template concentration, cDNAs synthesized from serial dilutions (from 1 ng to 2 µg) of total RNA were amplified. Standard curves and linear equations were determined by the iCycler associate software by plotting cycle threshold (Ct) values (y-axis) against logs of total RNA (x-axis). Amplifications were run in a 96 well-plates iCycler iQ thermal cycler (Biorad, Hercules, CA, USA) and quantification was performed with the iCycler iQTM associated software (Real time Detection System Software, version 3.0). Each reaction tube contained 10 µL of 2× iQ SYBR Green Supermix (Biorad), 0.5 µL of each primer (5 µM), 8 µL of NFW and 1 µL of cDNA. The following cycling conditions were used: 5 min at 95 °C, followed by 40 cycles of 20 s at 94 °C, 20 s at 60 °C and 20 s at 72 °C. At the end of each run, melt curves were analysed from 55 to 95 °C. No template and non reverse transcribed RNA controls were included in each run. Three technical replicates were utilised for each gene and the average ratio of these values was used to determine the fold change in transcript level.

The relative expression (RE) was calculated according to the ΔΔCt method (Livak et al., 2001) using the Eq. (1):

$$RE = 2^{(-\Delta\Delta Ct)} \quad (1)$$

where ΔCt = (average Ct of housekeeping gene - average Ct of target gene) and ΔΔCt = (average ΔCt of phenolic compound treated sample - average ΔCt of non treated sample). Data were transformed to $\log_2$ and levels of change were categorized as follows: “low” ≥ −1.0 to ≤1.0; “medium” ≥ −2.0 to <−1.0 or >1.0 to ≤2.0; “high” <−2.0 or >2.0 (Kim et al., 2008).

The real time PCR transcript quantification for validating SSH data was performed with 14 genes commonly involved in host defence response or highly represented in the subtracted

library and the *M. domestica* housekeeping gene EF1-α as an endogenous control (Table 2). The trial was repeated twice using long stored or freshly harvested Golden Delicious apples.

Among the 14 genes, 4 genes characterised by the highest up-regulation, plus a gene belonging to the most abundant contig, were selected for a time-course expression profile analysis at 4 distinct time points (1, 24, 48 and 72 h). Experiments were conducted as reported above, using freshly harvested apples that were quercetin-treated, non-treated or non-wounded, in order to discriminate among quercetin and wounding effects.

2.8. Statistical analysis

Data were subjected to ANOVA (one-way analysis of variance). Significant differences ($P \leq 0.05$) were identified by the General Linear Model (GLM) procedure with the Duncan's Multiple Range Test (DMRT). Percentage data of incidence of decay were subjected to arcsine-square-root transformation before ANOVA analysis. The data were processed using the statistical software package Statistics for Windows (StatSoft, Tulsa, OK, USA).

2.9. Reduction index calculation

The effect exerted by phenolics on the disease was expressed by a reduction index (RI, %) calculated according to the Eq. (2):

$$RI\ \% = [(A - B)/A] \times 100 \quad (2)$$

where A and B correspond to the mean percentage of infected wounds or mean lesion diameter measured in control apples (A, inoculated with *P. expansum*) and treated apples (B, quercetin-treated and inoculated with *P. expansum*).

3. RESULTS

3.1. In vivo *tests to evaluate induced resistance in apples*

On the whole, blue mould incidence and severity, as measured after 4 d of incubation on Golden Delicious apples treated with quercetin, were significantly diminished as compared to the non-treated wounded control. The highest disease reduction was obtained when the pathogen conidial suspension was applied 24 and 48 h after quercetin treatment (Fig. 2), being RIs 52-26 % and 57-37% for disease incidence and severity, respectively.

3.2. Differential screening and sequencing of SSH clones

The number of PCR cycles necessary to reach the plateau in the amplification of 4 tested genes was determined for subtracted and non-substracted cDNAs. In the non-subtracted library, the EF1-α-specific PCR product was recorded by the 14^{th} cycle, becoming saturated at 20–23 cycles (Fig. 3). However, in the subtracted library it required a higher number of amplification cycles, indicating that the elongation factor cDNA was partially depleted in the subtracted cDNA library. A higher subtraction efficiency was observed for the other 3 tested genes which were only amplified in the non-subtracted cDNA library (Fig. 3).

After cloning, PCR screening of 150 randomly picked white colonies enabled the identification of 125 colonies containing single inserts ranging in size from 116 to 945 bp. One hundred two high-quality sequences were obtained after the exclusion of poor quality sequences and trimming off vector and primers. This EST library corresponded to genes putatively differentially expressed in quercetin-treated apples, which were designated as AIR (Apple Induced Resistance) and deposited in GenBank (Table 3 and Table 4).

3.3. EST clustering and annotation

Eighty four EST sequences (82 %) showed significant homology to sequences with assigned putative identity in public databases, whereas 18 % showed no significant similarity

297 to any known gene. EST clustering according to the SEQtools protocol (Rasmussen, 2002)
298 revealed 9 clusters of redundant sequences (contigs) (Table 3) containing 23 clones and 79
299 single sequences (singletons) (Table 4), constituting 23 and 77 % of total clones, respectively.
300 Functional categorization using the MIPS FunCat analysis tool (vs *A. thaliana*
301 homologues) permitted to assign putative functions to about 80 % of the ESTs in the library
302 which were sorted into 21 primary functional categories (Fig. 4). Numerous clones were
303 classified into more than one category defined on the basis of different biochemical processes.
304 The largest set of genes was assigned to the category “metabolism”, whereas “protein with
305 binding function or cofactor requirement” and ”protein fate” formed the second and third
306 largest groups, respectively. Genes involved in cell rescue/defence/virulence accounted for 5
307 % of the library. Among genes coding for enzymes involved in protein fate, degradation (i.e.
308 cysteine protease) and modification (i.e. phosphorylation by protein kinase) were the 2 major
309 annotations; among proteins involved in cell rescue, defence and virulence processes,
310 oxidative stress response proteins (i.e. isoeugenol synthase) and cold shock stress response
311 proteins (i.e. Type II SK2 dehydrin) were included. Interestingly, within “interaction with the
312 environment” category, enzymes involved in the response to biotic stimulus (i.e.
313 γ-glutamylcysteine synthetase) were found. Furthermore, the “systemic interaction with the
314 environment” group comprised genes encoding enzymes involved in response to wounding
315 (12-oxophytodienoate reductase) and in systemic acquired resistance (E-4-hydroxy-3-
316 methylbut-2-enyl diphosphate synthase). Twenty percent of ESTs could not be allocated into
317 any functional category, since a function in *A. thaliana* has not been assigned to them yet or
318 they had no hit in public databases.
319
320 *3.4. Validation of SSH results by qPCR*

Quantification reactions were performed by using 400 ng RNA as starting material for cDNA synthesis. In stored apples, 10 out of 14 genes showed a positive relative expression and, among them, 4 were significantly up-regulated at a medium-high level (Table 2). In particular, a gene encoding a cinnamyl alcohol dehydrogenase (CAD) showed a 27-fold difference of expression in quercetin-treated apples as compared to the corresponding control. Other genes up-regulated at a medium-high level corresponded to a Clp protease proteolytic subunit 4 (CLPP4), a ribonuclease-like PR-10c protein (PR-10c) and an oxidoreductase belonging to 2OG-Fe(II) oxygenase family (OR-2OG). A different expression profile was assessed for freshly harvested apples (Table 2). All genes were up regulated in treated apples and 11 of the tested genes proved to be differentially expressed at a medium-high level. In particular, 7 showed at least a 2.1-fold induction in quercetin-treated apples and 4 showed at least a 4.2-fold difference in their RNA accumulation level. To the latter category belonged the genes encoding the CAD protein (31-fold), the CLPP4 protein, the OR-2OG protein and a 1-aminocyclopropane-1-carboxylate oxidase (ACC).

3.5. Time-course expression of differentially expressed ESTs

Expression profiles of the 4 most differentially expressed genes, plus a gene representative of the most abundant contig (Phenazine biosynthesis protein - PhzC/F), had a similar trend for “wounding effect” (Fig. 5A), “quercetin effect” (Fig. 5B) and “wounding+quercetin effect” (Fig. 5C), with gene expression being up-regulated 1 h after wounding/quercetin application and increasing over time. However, the quantitative nature of this trend was different. In particular, wounding caused a high level of up-regulation of ACC, CAD and OR-2OG, particularly at 48 h (13.13, 11.93 and 9.62-fold, respectively), whereas CLPP4 and PhzC/F expression was influenced at a medium-low level (Fig. 5A). Quercetin increased the expression of all examined genes. CAD, OR-2OG and PhzC/F had their

maximum RNA accumulation level at 24 h (29.47, 7.64 and 4.22-fold, respectively), whereas for ACC and CLPP4 it was later, at 48 h (Fig. 5B). Higher levels of relative expression were obtained for wounded apples treated with quercetin (combined effect). Tested genes showed a high differential expression, particularly at 24 h for OR-2OG and PhzC/F (41.90 and 4.45-fold, respectively) or at 48 h for CAD, ACC and CLPP4 (139.96, 171.24 and 8.49-fold, respectively) (Fig. 5C).

4. DISCUSSION

Induction of natural disease resistance in harvested horticultural crops using physical, biological and/or chemical elicitors has received increasing attention over recent years. In this study, Golden Delicious apples treated with the flavonoid quercetin in wounds spatially separated from the site of challenge with *P. expansum* exhibited resistance to blue mould, suggesting an induction of host defence response. As already reported by El Ghaouth et al. (2003), disease resistance in treated fruits was influenced by the time-lag between treatment application and pathogen inoculation. However, while in the present investigation the highest disease reduction was recorded when quercetin was applied 24-48 h before the pathogen, in the work by El Ghaouth et al. (2003), a significant reduction was obtained only when the yeast *Candida saitoana* was applied 48-72 h prior to inoculation with *Botrytis cinerea*.

Since a deeper understanding of the mode of action of induced resistance elicitors on harvested fruit is an important pre-requisite for their commercial application (Tian et al., 2006), a screening to identify the genes whose expression was up-regulated by the quercetin treatment was conducted. A cDNA library was generated by SSH, a technique that, by comparing two mRNA populations, allows the detection of differentially expressed transcripts highly or exclusively present in one population as compared to the other.

One-hundred two high quality sequences were generated by SSH analysis, cloning and sequencing: the majority (82 %) showed significant homology to sequences with putative assigned identity in GenBank database, whereas 18 % of them could not be annotated by similarity. These latter clones could represent some novel coding sequences that have not been previously isolated or contain insufficient coding sequences to be accurately assigned with an annotation based on homology. Twenty three ESTs were grouped in 9 clusters (contigs) of redundant sequences, whereas 79 sequences remained as single ESTs (singletons) with no significant homology to any other sequence in the data set.

The Mal d1 was the most represented protein group in the library and corresponded to 3 contigs and 6 singletons. These proteins are members of the pathogenesis-related proteins (PR proteins) family 10 (Gao et al., 2005). Among the 17 families of PRs which could play a role in restricting pathogen development and spread in the plant (Van Loon et al., 2006), the PR-10 family has a ribonuclease-like structure and is the only family consisting of cytoplasmic proteins. Walter et al. (1996) proposed that cytosolic ribonucleases, with the partial regulation of different ligands such as flavonoids, could be involved in the degradation of mRNAs present because of pathogen attack. PR-10c proteins were reported to be expressed in apple leaves in response to a challenge with *Venturia inaequalis* (Beuning et al., 2004) or in response to oxidative stress (Utriainen et al., 1998), with jasmonic acid (JA) functioning as the necessary systemic signal. The putative involvement of this pathway in the mode of action of quercetin could also be related to JA, because γ-glutamylcysteine synthetase, another gene known to be regulated by JA, was found in the library. Finally, Mal d1 homologue protein Bet V 1 allergen resulted to be up-regulate in apple leaves and roots exposed to water stress (Wisniewski et al., 2008).

The second most abundant cluster comprised ESTs coding a PhzC/F protein, which is involved in the biosynthesis of phenazine compounds that are nitrogen-containing

heterocyclic pigments. Almost all phenazines exhibit a broad-spectrum activity against various species of bacteria and fungi connected with their ability to undergo oxidation/reduction, thus causing the accumulation of toxic superoxide radicals in the target cells (Mavrodi et al., 1998).

The third most represented cluster gathered clones encoding the 26S proteasome regulatory non-ATPase particles subunit 12 (26S-RPN12). The proteasome is a large protein complex, whose main function is to degrade unneeded or damaged proteins into peptides. It consists of a proteolytic core that associates with a regulatory complex, composed of at least 15 different subunits (Dubiel et al., 1995). Subunit 12 is redundantly present in the library and, thus, probably highly synthesized in presence of the flavonoid quercetin. Lodish et al. (2004) reported that cellular stresses induce the expression of several proteins that identify misfolded or unfolded proteins and target them for proteasomal degradation. For instance, a 26S proteosome α subunit resulted associated to apple response to low temperature and water deficit (Wisniewski et al., 2008). The 26S proteasome degradation of Aux/IAA proteins, which function as transcriptional regulators of the various auxin responses and are present in the screened library, requires ubiquitin-ligating proteins (Kim et al., 1997). Among them, the F-box-containing proteins, also present in the library, might be found. These results seem to suggest the involvement of the ubiquitin/proteasome pathway in quercetin-induced resistance against *P. expansum*.

Library investigation by the MIP FunCat Analysis programme suggested that quercetin application led to the differential expression of several genes which have a function in the general plant defence system. As reported for other SSH libraries (Norelli et al., 2009; Wisniewski et al., 2008), one of the most represented functional category was ”metabolism” (14 %). In this group, proteins involved in lipid metabolism are highly present, such as the oxysterol-binding proteins (OBP), implicated in lipid transport and metabolism, vesicle

trafficking and cell signalling. The genes encoding these proteins have been reported to be up-regulated by oligogalacturonides (plant cell wall breakdown products generated by pectinase activities) in potato plants resistant to *Phytophthora infestans* (Avrova et al., 2004) and thus it could similarly act in promoting quercetin-treated apples resistance to *P. expansum*. Moreover, in the category metabolism a protein belonging to PR-8 family was ascribed. The PR-8 family is made of type III chitinases, commonly directed against fungal cell walls, that are robustly induced in apple, following pathogen attack (Norelli et al., 2009) or treatment with alternative control agents such as yeast antagonists (El Ghaouth et al., 2003), acibenzolar or salycilic acid (SA) (Maxson-Stein et al., 2002). SA is an important regulator of induced plant resistance to pathogens and it can be synthesized from phenylalanine via cinnamic and benzoic acids (Yalpani et al., 1993) or from chorismate via isochorismate (Shah, 2003). The presence of a CAD protein and a putative 4-coumarate-CoA ligase/4-coumaroyl-CoA synthase (4CL/S) in the subtracted library seemed to confirm the putative involvement of this metabolic pathway in quercetin-elicited defence response.

Equally interesting, although less represented, are ESTs related to protein fate and defence response. Particularly relevant was the presence in the 'protein fate' functional group of a protein kinase. *In vivo* studies have demonstrated that protein kinases and phosphatases are crucial for activation of early defence responses to several environmental stresses and plant–pathogen interactions (Romeis et al., 2000). Their activation appears to be regulated by an increase in the cytosolic Ca^{2+} concentration, which occurs within seconds after elicitation (Scheel, 1998). Quercetin treatment also led to the differential expression of a cystein protease, which function in many aspects of cellular regulation (Xu et al., 1999) and whose activity is influenced by ethylene production (Yang et al., 1994). Their involvement in quercetin mode of action might be corroborated by the presence in the library of an ACC synthase, a cytosolic enzyme that catalyzes the first committed step in ethylene biosynthesis

445 in higher plants. Among the transcripts included in the defence response category, we
446 observed clones corresponding to oxidative and cold shock stress response proteins. Indeed, it
447 is well documented that the plant defence against pathogens involves an oxidative burst and
448 that the reactive oxygen species play a major role in the outcome of plant-pathogen
449 interactions (Apel et al., 2004). These results are consistent with quercetin antioxidant
450 properties (Nijveldt et al., 2001). Moreover, the induction of cold shock stress response
451 proteins, such as Type II SK2 dehydrin, has been associated to the response of apples to fire
452 blight disease (Norelli et al., 2009) or of peaches to cold stress (Bassett et al., 2006).

453 Nine percent of the sequences could not be annotated by similarity, whereas 11 % of them
454 showed high similarity to known proteins or ESTs, although no function has yet been
455 assigned to them in *A. thaliana*. Interestingly, several of these latter fragments correspond to
456 genes involved in the pathogenic process, such as PR-10c or OR-2OG proteins, which are
457 involved in a large number of metabolic pathways, including the biosynthesis of flavonoids,
458 gibberellins and alkaloids (De Carolis and De Luca, 1994).

459 In the present study qPCR was utilised to validate SSH data, but also provided specific
460 information about quercetin-mediated resistance in apples. Relative expression assays showed
461 a higher RNA accumulation in quercetin-treated fruit for most of 14 tested apple genes.
462 However, a detectable expression was also found in control fruit, thus indicating that the
463 corresponding genes are constitutively transcribed in non-treated fruit and up-regulated upon
464 quercetin application. Indeed, often differences between susceptibility and resistance are
465 associated with differences in the timing and magnitude of gene expression changes rather
466 than with the expression of different sets of genes (van Loon et al., 2006). A stronger
467 differential expression was recorded when quercetin was applied on freshly harvested Golden
468 Delicious apples, thus highlighting the importance to apply quercetin or any other inducer of
469 resistance at the proper stage of maturity. Similarly, El Ghaouth et al. (2003) reported that the

induction of chitinase and β-1,3-glucanase by *C. saitoana* appeared to be dependent on fruit stage of ripeness. However, our results showed, at the same time, that quercetin induces the expression of defence-related genes even when applied on long stored apples, in which the natural responses to diseases are weakened due to the senescence progress (Droby et al., 1993). In particular, 86 % of the 14 analysed genes showed at least a 1.8-fold induction and this percentage is comparable with that recorded during the validation of other SSH libraries (Norelli et al., 2009), whose level of differentially expressed genes ranged from 10 % to 95 % and depended mainly on the starting biological material (Desai et al., 2000).

Furthermore, since for a more quantitative assessment of the actual contribution of quercetin and wounding to overall defence response, the expression of the genes of interest should be examined in greater detail (Bassett et al., 2006), the specific profile of the highest expressed genes (CAD, ACC, CLPP4 and OR-2OG), as well as of one of the most abundant contig forming gene (PhzC/F), was determined in freshly harvested apples. For these genes the wounding-induction effect and quercetin-induction effect were analyzed both singly and in combination at 1, 24, 48 and 72 h after treatment. In these experiments a higher up-regulation, as compared to the validation experiments, was observed. These results were probably due to the use in qPCR analyses of RNAs corresponding to each assessment time as standalone, whereas in the SSH data validation experiments cDNA synthesis starting material was made up of equal amounts of 24 and 48 h RNAs for both control and treated samples. However, the influence of the single fruit tissue composition should be also taken into account. For all genes the highest induction took place at 24-48 h after quercetin treatment. This finding further support the initial choice to use 24-48 h RNAs for the library construction and it is in accordance with the higher reduction of disease incidence and severity achieved during *in vivo* tests at 24 and 48 h. It has, indeed, been reported that stress-inducible genes reach their maximum expression at 24 h (Rabbani et al., 2003) or 48 h (Strizhov et al., 1997)

from treatment, depending on the gene and the suffered stress. Moreover, it has to be underlined that the tested genes seemed to be involved in the early response to stresses, since they showed a considerable induction level just 1 h after wounding and/or quercetin application. A similar behaviour has been described for stress-related genes in salt-treated *Arabidopsis* seedlings (Strizhov et al., 1997), salt-stressed rice (Kawasaki et al., 2001) and *Erwinia amylovora* challenged apples (Norelli et al., 2009). The well-timed response might be the key to explain quercetin activity since it has been reported that in compatible plant-fungus interactions resistance mechanisms may be activated too slowly to be effective or may be suppressed by the invading pathogen (Van Loon et al., 2006). CAD, ACC, CLPP4, OR-2OG and PhzC/F expression proved to be influenced by both wounding and quercetin application. Likewise Bassett et al. (2006) reported that a number of genes induced in peaches by low temperatures are also induced by other stresses. However, while 1 h after wounding the response is already triggered almost to its maximum level, in presence of quercetin a further increase is seen also up to 48 h. Therefore, quercetin application seems to enhance the natural defence responses triggered by wounding, as confirmed by the strongest gene up-regulation caused by wounding+quercetin application, and by the highest quercetin efficacy recorded during *in vivo* tests at 24 and 48 h. Similarly, Mur et al. (1996) reported that the expression of defence genes after wounding could be enhanced by treating tobacco plants with exogenous SA. However, it has to be observed that the quercetin 'potentiation' of wounding effect concerned mainly CAD, ACC, and OR-2OG genes. This finding is of particular interest since it seems to suggest that some apple responses are specific to quercetin application. Similarly, it has been reported that, although wounding and pathogen attack induce common defence strategies, the expression of some gene is exclusive to pathogen attack (Mur et al., 1996).

5. CONCLUSIONS

It can be concluded the application of quercetin on Golden Delicious apples induces resistance to *P. expansum.* The defence response appears to be correlated with the up-regulation of a substantial number of transcripts encoding proteins with a role in the adaptation process to various stresses. This molecular response seems to sum up to apple natural response to wounding, enhancing its protective effect. Although a causal connection has not yet been established, as a consequence, the fruit might have a better chance for a successful defence against the pathogen.

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REFERENCES

Acar, J., Gökmen, V., Taydas, E.E., 1998. The effects of processing technology on the patulin content of juice during commercial apple juice concentrate production. Z Lebensm Unters Forsch A 207, 328–331.

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55, 373–399.

Avrova, A.O., Taleb, N., Rokka, V.M., Heilbronn, J., Campbell, E., Hein, I., Gilroy, E.M., Cardle, L., Bradshaw, J.E., Stewart, H.E., Fakim, Y.J., Loake, G., Birch. P.R.J., 2004. Potato oxysterol binding protein and cathepsin B are rapidly up-regulated in independent defence pathways that distinguish R gene-mediated and field resistances to *Phytophthora infestans*. Mol. Plant Pathol. 5, 45-56.

Bassett, C.L., Wisniewski, M.E., Artlip, T.S., Norelli, J.L., Renaut, J., Farrell, R.E. Jr. 2006. Global analysis of genes regulated by low temperature and photoperiod in peach bark. J. Amer. Soc. Hort. Sci. 131(4), 551-563.

545 Beuning, L.L., Bowen, J.H., Persson, H.A., Barraclough, D., Bulley, S., MacRae, E.A., 2004.
546 Characterisation of Mal d 1-related genes in *Malus*. Plant Mol. Biol. 55, 369–388.
547 BocK, K.W., 2003. Vertebrate UDP-glucuronosyltransferases: functional and evolutionary
548 aspects. Biochem. Pharmacol. 66, 691–696.
549 De Carolis, E. and De Luca, V., 1994. 2-Oxoglutarate-dependent dioxygenase and related
550 enzymes: Biochemical characterization. Phytochem. 36(5), 1093-1107.
551 Desai, S., Hill, J., Trelogan, S., Diatchenko, L., Siebert, P.D., 2000. Identification of
552 differentially expressed genes by suppression substractive hybridization. In: Hunt, S.,
553 Livesey, F. (Eds), Functional genomics. Oxford University Press, Oxford, pp. 81–112.
554 Diatchenko, L., Yun-Fai, C.L., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B.,
555 Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D., Siebert, P.D., 1996.
556 Suppression subtractive hybridization: A method for generating differentially regulated or
557 tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. USA. 9(12), 6025-6030.
558 Droby, S., Chalutz, E., Horev, B., Cohen, L., Gaba, V., Wilson, C.L., Wisniewski, M., 1993.
559 Factors affecting UV-induced resistance in grapefruit against the green mould decay
560 caused by *Penicillium digitatum*. Plant Pathol. 42, 418-424.
561 Dubiel, W., Ferrell, K., Dumdey, R., Standera, S., Prehn, S., Rechsteiner, M., 1995.
562 Molecular cloning and expression of subunit 12: a non-MCP and non-ATPase subunit of
563 the 26 S protease. FEBS Lett. 363, 97-100.
564 El Ghaouth, A., Wilson, C.L., Wisniewski, M., 2003. Control of postharvest decay of apple
565 fruit with *Candida saitoana* and induction of defense responses. Phytopathol. 93, 344-
566 348.
567 European Commission, 2006. Commission Regulation (EC) No. 1881/2006 setting maximum
568 levels for certain contaminants in foodstuffs. Off. J. Eur. Union L 364, 5-24.

569 Gao, Z.S., van de Weg, W.E., Schaart, J.G., Schouten, H.J. Tran, D.H., Kodde, L.P., van der
570 Meer, I.M.A., van der Geest, H.M., Kodde, J., Breiteneder, H., Hoffmann-Sommergruber,
571 K., Bosch, D., Gilissen, L.J.W.J., 2005. Genomic cloning and linkage mapping of the *Mal*
572 *d 1* (PR-10) gene family in apple (*Malus domestica*). Theor. Appl. Gen. 111, 171-183.
573 Ishida, T., Naito, E., Mutoh, J., Takeda, S., Ishii, Y., Yamada, H., 2005. The plant flavonoid,
574 quercetin, reduces some forms of dioxin toxicity by mechanisms distinct from aryl
575 hydrocarbon receptor activation, heat shock protein induction and quenching oxidative
576 stress. J. Health Science 51, 410-417.
577 Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D.,
578 Bohnert, H.J., 2001. Gene expression profiles during the initial phase of salt stress in rice.
579 The Plant Cell. 13, 889–905.
580 Kim, J.K., Harter, K., Theologis, A., 1997. Protein–protein interactions among the Aux/IAA
581 proteins. Proc. Natl. Acad. Sci. USA. 94, 11786–11791.
582 Kim, J.H., Yu, J., Mahoney, N., Chan, K.L., Molyneux, R.J., Varga, J., Bhatnagar, D.,
583 Cleveland, T.E., Nierman, W.C., Campbell, B.C., 2008. Elucidation of the functional
584 genomics of antioxidant-based inhibition of aflatoxin biosynthesis. Int. J.Food Microbiol.
585 122, 49-60.
586 Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-
587 Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods. 25, 402-408.
588 Lodish, H., Berk, A., Matsudaira, P., Kaiser, C.A., Krieger, M., Scott, M.P., Zipursky, S.L.,
589 Darnell, J., 2004. Molecular Cell Biology (5th ed.). W.H. Freeman and Company, New
590 York.
591 Mari, M., Leoni, O., Iori, R., Cembali, T., 2002. Antifungal vapour-phase activity of allyl-
592 isothiocyanate against *Penicillium expansum* on pears. Plant Pathol. 51, 231–236.

593 Mavrodi, D.V., Ksenzenko, V.N., Bonsall, R.F. Cook, R.J., Boronin, A.M., Thomashow, L.S.,
594 1998. Seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas*
595 *fluorescens* 2-79. J. Bacteriol. 180, 2541–2548.
596 Maxson-Stein, K., He, S.Y., Hammerschmidt, R., Jones A.L., 2002. Effect of Treating Apple
597 Trees with Acibenzolar-S-Methyl on Fire Blight and Expression of Pathogenesis-Related
598 Protein Genes. Plant Dis. 86(7), 785-790.
599 Mur, L.A.J., Naylor, G., Warner, S.A.J., Sugars, J.M., White, R.F., Draper, J., 1996. Salicylic
600 acid potentiates defence gene expression in tissue exhibiting acquired resistance to
601 pathogen attack. The Plant J. 9(4), 559 – 571.
602 Nicholas, K.B., Nicholas, H.B. Jr, Deerfield, D.W., 1997. GeneDoc: analysis and
603 visualization of genetic variation. EMBNEW News 4, 14.
604 Nicholson, R.L., Hammerschmidt, R., 1992. Phenolic Compounds and Their Role in Disease
605 Resistance. Ann. Rev. Phytopathol. 30, 369-389
606 Nijveldt, R. J., van Nood, E., van Hoorn, D.E.C., Boelens, P.G., van Norren, K., van
607 Leeuwen, P.A.M., 2001. Flavonoids: a review of probable mechanisms of action and
608 potential applications. Am. J. Clin. Nutr. 74, 418 – 425.
609 Norelli, J.L., Farrell, Jr., R.E., Bassett, C.L., Baldo, A.M., Lalli, D.A., Aldwinckle, H.S.,
610 Wisniewski, M.E., 2009. Rapid transcriptional response of apple to fire blight disease
611 revealed by cDNA suppression subtractive hybridization analysis. Tree Gen. Genom. 5,
612 27 – 40.
613 Rabbani, M.A., Maruyama, K., Abe, H., Khan, M.A., Katsura, K., Ito, Y., Yoshiwara, K.,
614 Seki, M., Shinozaki, K., Yamaguchi-Shinozak, K., 2003. Monitoring expression profiles
615 of rice genes under cold, drought, and high-salinity stresses and abscisic acid application
616 using cDNA microarray and RNA gel-blot analyses. Plant Physiol. 133, 1755–1767.

617 Rasmussen, S.W., 2002. SEQtools, a software package for analysis of nucleotide and protein
618 sequences. Source code available at http://www.seqtools.dk.
619 Rozen, S., Skaletsky, H.J., 2000. Primer3 on the WWW for general users and for biologist
620 programmers. In: Krawetz, S., Misener, S., (Eds.), Bioinformatics Methods and Protocols:
621 Methods in Molecular Biology. Humana Press, Totowa (NJ), pp 365-386. Source code
622 available at http://primer3.sourceforge.net/.
623 Romeis, T., Piedras, P., Jones J.D.G., 2000. Resistance Gene-Dependent Activation of a
624 Calcium-Dependent Protein Kinase in the Plant Defense Response. The Plant Cell. 12,
625 803–815.
626 Sambrook, J., Russell, D.W., 2001. Molecular cloning: a laboratory manual. (3th ed.). Cold
627 Spring Harbor Laboratory Press, USA.
628 Sánchez-Torres, P., González-Candelas, L., 2003. Isolation and characterization of genes
629 differentially expressed during the interaction between apple fruit and *Penicillium*
630 *expansum*. Mol. Plant Pathol. 4(6), 447–457.
631 Sanzani, S.M., De Girolamo, A., Schena, L., Solfrizzo, M., Ippolito, A., Visconti, A., 2009a.
632 Control of *Penicillium expansum* and patulin accumulation on apples by quercetin and
633 umbelliferone. Eur. Food Res. Technol. 228(3), 381–389.
634 Sanzani, S.M., Schena, L., Nigro, F., De Girolamo, A., Ippolito, A., 2009b. Effect of
635 quercetin and umbelliferone on the transcript level of *Penicillium expansum* genes
636 involved in patulin biosynthesis. Eur. J. Plant Pathol. 125(2), 223-233.
637 Scheel, D., 1998. Resistance response physiology and signal transduction. Curr. Opin. Plant
638 Biol. 1, 305–310.
639 Schena, L., Nigro, F., Ippolito, A., Gallitelli, D., 2004. Real-time quantitative PCR: a new
640 technology to detect and study phytopathogenic and antagonistic fungi. Eur. J. Plant
641 Pathol. 110, 893-908.

642 Shah, J., 2003. The salicylic acid loop in plant defense. Curr. Opin. Plant Biol.
643 6(4), 365-371.
644 Spadaro, D., Gullino, M.L., 2004. State of the art and future prospects of the biological
645 control of postharvest fruit diseases. Int. J. Food Microbiol. 91, 185– 194.
646 Strizhov, N., Abrahám, E., Okrész, L., Blickling, S., Zilberstein, A., Schell, J., Koncz C.,
647 Szabados, L., 1997. Differential expression of two P5CS genes controlling proline
648 accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2
649 in *Arabidopsis*. The Plant J. 12(3), 557–569.
650 Tian, S., Wan, Y., Qin, G., Xu, Y., 2006. Induction of defense responses against Alternaria rot
651 by different elicitors in harvested pear fruit. Appl. Microbiol. Biotechnol. 70, 729 – 734.
652 Utriainen, M., Kokko, H., Auriola, S., Sarrazin, O., Kärenlampi, S., 1998. PR-10 protein is
653 induced by copper stress in roots and leaves of a Cu/Zn tolerant clone of birch, Betula
654 pendula. Plant, Cell and Env. 21(8), 821-828.
655 van Loon L.C., Rep, M., Pieterse, C.M.J., 2006. Significance of inducible defence-related
656 proteins in infected plants. Annu. Rev. Phytopathol. 44, 135–162.
657 Walter, M.H., Liu, J.W., Grand, C., Lamb, C.J., Hess, D., 1990. Bean pathogenesis-related
658 (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new
659 class of conserved PR proteins including pollen allergens. Mol. Gen. Genet. MGG. 222(2-
660 3), 353-360.
661 Wisniewski, M., Bassett, C., Norelli, J., Macarisin, D., Artlip, T., Gasic, K., Korban, S., 2008.
662 Expressed sequence tag analysis of the response of apple (Malus x domestica 'Royal
663 Gala') to low temperature and water deficit. Physiol. Plant. 133, 298 – 317.
664 Wouters, M.F.A., Speijers, G.J.A., 1996. Patulin Food Additives Series 35 Toxicological
665 Evaluation of certain food additives and contaminants (Geneva: World Health

Organization) 35, 377–402. Home page http://www.inchem.org/documents/jecfa/ jecmono /v35je16.htm

Xu, F.X., Chye, M.L., 1999. Expression of cysteine proteinase during developmental events associated with programmed cell death in brinjal. Plant J. 17(3), 321–327.

Yalpani, N., Enyedi, A.J., León, J., Raskin, I., 1994. Ultraviolet light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. Planta. 193(3), 372-376.

Yang, S.F., Hoffmann, N.E., 1984. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35, 155–189.

677 **Figures caption**

678 **Fig. 1.** Flowchart of the experimental design to obtain a cDNA-library enriched in genes
679 differentially expressed in apples in response to quercetin application.

680

681 **Fig. 2.** Incidence of decay (infected wounds, %) and disease severity (lesion diameter, mm)
682 on Golden Delicious apples non-treated or treated with quercetin (100 µg/wound), 24 or 48 h
683 before *Penicillium expansum* inoculation in other but close wounds, and incubated at 16°C for
684 4 days. Bars represent the mean of 48 wounds ± standard error of mean (SEM). Bars with
685 different letter in the same group are significantly different ($P \leq 0.05$).

686

687 **Fig. 3.** Subtraction efficiency of 4 constitutively expressed genes. Elongation factor 1-α (EF1-
688 α), chalcone synthase (CHS), chitinase II (CHT2) and phenylalanine ammonia lyase (PAL).
689 Aliquots were taken from PCR reaction mixtures, containing the subtracted and non
690 subtracted sample as template, at regular intervals of amplification cycles (numbers on the
691 figure) and run on agarose gels.

692

693 **Fig. 4.** A pie chart showing genes analysis according to MIPS FunCat, i.e. the number of
694 genes assigned to each functional category and their corresponding fraction. The categories
695 were the following: (A) organ differentiation; (B) protein with binding factor or cofactor
696 requirement; (C) regulation of metabolism and protein function; (D) metabolism; (E) organ
697 localization; (F) cellular transport/transport facilities and routes; (G) protein synthesis; (H)
698 development (systemic); (I) interaction with the environment; (J) systemic interaction with
699 the environment; (K) biogenesis of cellular compounds; (L) cell rescue/defence/virulence;
700 (M) energy; (N) cellular communication/signal transduction mechanism; (O) protein fate; (P)

storage protein; (Q) subcellular localization; (R) cell cycle/DNA processing; (S) unclassified; (T) transcription; (U) without identity.

Fig. 5. Relative expression kinetics of 5 selected apple gene as influenced by wounding, quercetin application or their combination effect. Graph (A) represents gene expression in wounded apples as compared to non-wounded control; graph (B) represents gene expression in wounded quercetin-treated apples as compared to non-treated wounded control; graph (C) represents gene expression in wounded quercetin-treated apples as compared to non-wounded control. Bars represent data from three independent experiments ± standard error of mean (SEM). * indicates significant differences between treated and non treated samples according to Ct values ($P \leq 0.05$).

Table 1. Specific primer sequences, expected PCR product sizes (bp) and optimal annealing temperatures (°C) for non differentially expressed genes utilised for subtraction efficiency analysis.

Name	*Sequence (5'- 3')*	*Gene*	*Accession no.*	*Annealing T (°C)*	*Amplicon (bp)*
EF1F	*GATCTCAAGCGTGGTTTCGT*	*Elongation factor 1-α*	*U80268*	*54*	*250*
EF3R	*CCATACCTGCATCTCCGTTC*				
CHS F	*ACCCACTTGGTCTTTTGCAC*	*Chalcone synthase*	*AB074485*	*58*	*392*
CHS R	*GTCGATAGCCCCATCACTGT*				
CHT2 F	*GATGGGAAAGTGCACCAGAT*	*Class II chitinase*	*AF494396*	*56*	*227*
CHT2 R	*AACCGGGTCTGTAGCCACTA*				
PAL F	*GGCATTTGGAGGAGAATTGA*	*Phenylalanine ammonia lyase*	*AF494403*	*56*	*219*
PAL R	*TCAACAAGCACTTGCCTCAG*				

Table 2. Selected genes utilised to validate SSH results by qPCR. Specific primers, amplicon sizes (bp) and relative expression (RE) for stored (SA) and fresh (FA) apples are reported. Data are expressed in $\log_2$ form and represent the average relative expression of three independent experiments ± standard error of mean (SEM).

Annotation	Acronym	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon (bp)	RE ($\log_2$) ± SEM	
					SA	FA
Phenazine biosynthesis PhzC/PhzF protein	PhzC/F	CTGCTGCGAGGAAAGGCTGT	GGTTCTTTCCCCATAAAATTGAGTC	122	-0.6 ± 0.3	1.2 ± 0.2*
Cysteine protease	CLPP4	GGGCCGTCGTTTAGTGGTG	CCTCTACTCCAAGTAAACGTGCAA	144	1,8 ± 0.5*	2.0 ± 0.2*
26S proteasome regulatory particle non-ATPase subunit 12	26S-RP12	ACGGAGCGATTTCGATACCTG	CTTGACAGCATTAGGTGTGTCTTG	110	0.3 ± 0.2	1.3 ± 0.1*
Major allergen Mal d 1.03G	MdG	GAAGCCAGTTTTCGAAGGGAAA	TTGGAGCACCAGGATGCCTA	139	0.3 ± 0.1	1.0 ± 0.1*
Ribonuclease-like PR-10c	PR10c	TCATCATGGGTGTCTGCACATT	ATTGCCTGGGGTGCAATCTT	121	1.1 ± 0.3*	1.2 ± 0.1*
Pathogenesis-related protein 8	PR8	AGGGGCTTGGTTGTTTCCAA	TCATGCAAGTCCTCCCAAGC	147	-0.2 ± 0.1	1.5 ± 0.2*
Calcium binding EF hand family protein	CaBP	TGCACTCCACTCCACTAAGAAACA	TGACTGTCCTGCCATTCCTCA	129	0.5 ± 0.1	0.6 ± 0.2
Oxysterol-binding protein	OBP	GAATGGGAGCGAGCAGAGGT	TGAGGATTTGGCTGCTGCTT	162	1.0 ± 0.2*	1.4 ± 0.3*
4-coumarate-CoA ligase/ 4-coumaroyl-CoA synthase	4CL/S	GTGTCCCCAAGTCAGCATCG	CAAGCCTTCCCGATAAAGCAA	124	-0.2 ± 0.0	0.7 ± 0.1
Cinnamyl alcohol dehydrogenase	CAD	CGGGCGAGAAAGATGTGATG	TTCATGTCCGGGAACCAATG	116	4.6 ± 0.8*	5.0 ± 0.2*
Serine carboxypeptidase S10 family protein	SC-S10	ACAGGAGGCAATTCGTGGTG	AAGATTGAGCTTCGACGGATGA	143	0.4 ± 0.1	0.9 ± 0.0
ACC oxidase	ACC	CCGGCAGTGCTTGAGAAGAA	TGTTCTCGGACGGCTCTCAG	182	0.5 ± 0.3	3.0 ± 0.8*
Oxidoreductase, 2OG-Fe(II) Oxygenase family protein	OR-2OG	TGAGGAGGCCCGGAAATCTA	TGCAGCAAGAAACGAAACGAA	125	2.1 ± 0.2*	2.2 ± 0.2*
WAX2; catalytic	WAX	CACAAGAAATCGCCGGATCA	TGATGGCACCAAACAGCAGA	127	0.8 ± 0.4	1.2 ± 0.1*
Elongation factor 1 alpha	EF-1α	CAAGCCCATGGTTGTGGAGA	CACCGCTGGGATCCTTCTTC	125	0	0

* indicates For each gene significant differences between treated and non treated samples according to Ct values ($P \leq 0.05$).

Table 3. Contig-forming ESTs identified by SSH in quercetin-treated Golden Delicious apples.

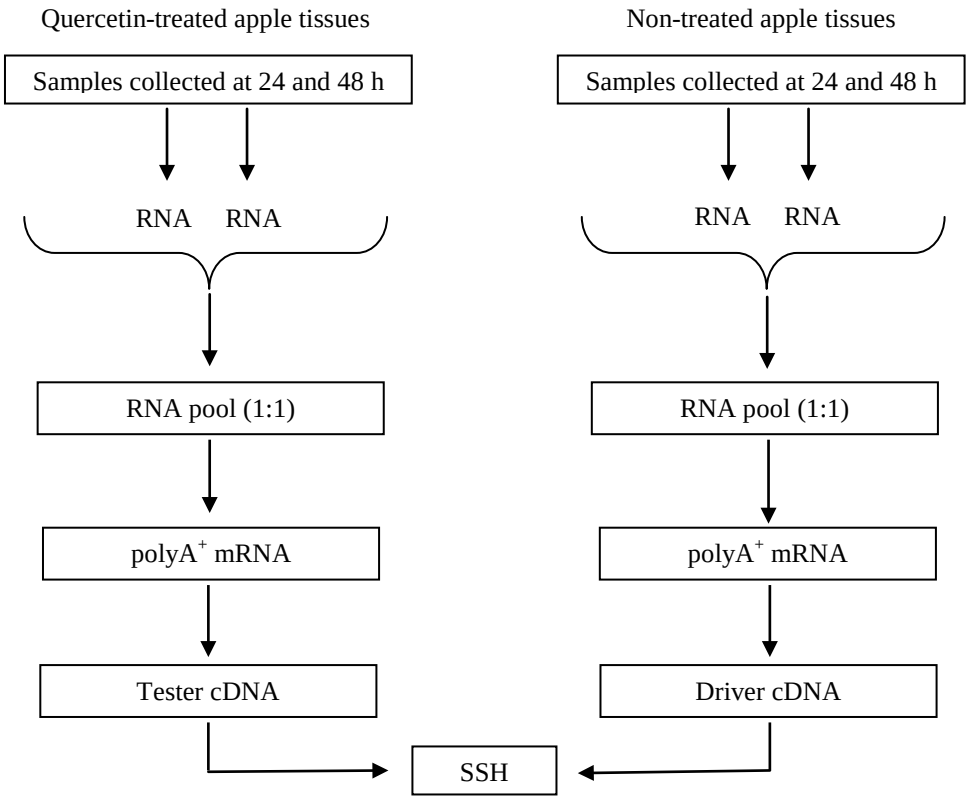
Contig no.	Clone	GenBank accession no.	Size (bp)	Accession no. of matching sequence	Best e-value (BlastX/N)	Sequence origin	Protein similarità
1	AIR050	GR882730	222	ABE77454.1	1e -37	*Medicago truncatula*	Phenazine biosynthesis PhzC/PhzF protein
	AIR062	GR882742	222	ABE77454.1	1e -37	*Medicago truncatula*	Phenazine biosynthesis PhzC/PhzF protein
	AIR072	GR882752	222	ABE77454.1	1e -37	*Medicago truncatula*	Phenazine biosynthesis PhzC/PhzF protein
	AIR082	GR882759	222	ABE77454.1	1e -37	*Medicago truncatula*	Phenazine biosynthesis PhzC/PhzF protein
2	AIR048	GR882728	257	ABI31652.1	8.00e-33	*Camellia sinensis*	26S proteasome regulatory particle non-ATPase subunit 12
	AIR055	GR882735	257	ABI31652.1	8.00e-33	*Camellia sinensis*	26S proteasome regulatory particle non-ATPase subunit 12
	AIR077	GR882755	257	ABI31652.1	8.00e-33	*Camellia sinensis*	26S proteasome regulatory particle non-ATPase subunit 12
3	AIR028	GR882711	320	AAX18320.1	2.00e-12	*Malus domestica*	Major allergen Mal d 1.03E
	AIR098	GR882773	323	AAK13029.1	4.00e-09	*Malus domestica*	Major allergen Mal d 1.03E
	AIR099	GR882774	293	AAX18320.1	4.00e-13	*Malus domestica*	Major allergen Mal d 1.03E
4	AIR008	GR882693	273				No hit
	AIR011	GR882696	273				No hit
	AIR026	GR882709	273				No hit
5	AIR001	GR882686	329	AAX18324.1	7.00e-12	*Malus domestica*	Major allergen Mal d 1.03G
	AIR004	GR882689	459	AAX18324.1	9.00e-34	*Malus domestica*	Major allergen Mal d 1.03G
6	AIR019	GR882703	190				No hit
	AIR021	GR882705	190				No hit
7	AIR049	GR882729	572	AAD26552.1	2.00e-42	*Malus domestica*	Major allergen mal d 1
	AIR068	GR882748	444	AAD26552.1	5.00e-48	*Malus domestica*	Major allergen mal d 1
8	AIR066	GR882746	557	CAB61744.1	6.00e-33	*Cicer arietinum*	Hypothetical protein
	AIR070	GR882750	557	CAB61744.1	6.00e-33	*Cicer arietinum*	Hypothetical protein
9	AIR080	GR882757	424	CAB85633.1	7.00e-24	*Vitis vinifera*	Putative ripening-related protein
	AIR096	GR882771	424	CAB85633.1	7.00e-24	*Vitis vinifera*	Putative ripening-related protein

Table 4. Singleton-forming ESTs identified by SSH from quercetin-treated Golden Delicious apples.

Clone no. [a]	GenBank accession no.	Size (bp)	Accession no. of matching sequence	Best e-value (BlastX/N)	Sequence origin	Protein similarità
AIR002	GR882687	246	AY742306.1	1.00e-106	*Malus domestica*	Calcium-binding EF hand family protein
AIR003	GR882688	800	Q40280	4.00e-80	*Malus domestica*	Major allergen Mal d 1 (AP15)
AIR005	GR882690	344	AB094988.2	4.00e-07	*Prunus persica*	UDP-glucose:flavonoid 3-O-glucosyltransferase
AIR007	GR882692	424	BAD25771.1	4.00e-08	*Medicago truncatula*	F-box protein-like
AIR009	GR882694	814	ABE89192.1	2.00e-83	*Medicago truncatula*	Protein kinase
AIR010	GR882695	664	BAD14371.1	4.00e-104	*Malus domestica*	Plasma membrane intrinsic protein
AIR012	GR882697	220	AAB97142.1	2.00e-37	*Prunus armeniaca*	Cysteine protease
AIR014	GR882699	334	NP_566725.2	1.00e-46	*Arabidopsis thaliana*	Dehydration-responsive protein-related
AIR015	GR882700	315	AAM20701.1	5.00e-35	*Arabidopsis thaliana*	Chloroplast inner envelope protein
AIR016	GR882701	449	AAS00046.1	2.00e-36	*Malus domestica*	Mal d 1-like
AIR018	GR882702	205	ABE83112.1	1.00e-28	*Medicago truncatula*	Peptidase aspartic, catalytic; UBA-like
AIR020	GR882704	590	AAX20990.1	8.00e-68	*Malus domestica*	Mal d 1.06C05
AIR022	GR882706	525	CAA10129.1	8.00e-35	*Cicer arietinum*	hypothetical protein
AIR023	GR882707	357	ABA46790.1	9.00e-26	*Solanum tuberosum*	60S ribosomal protein L13a-like protein
AIR024	GR882708	225	ABO31359.1	1.00e-122	*Malus domestica*	Starch branching enzyme II-2
AIR027	GR882710	713	P22778	9.00e-58	*Ipomoea batatas*	ATP synthase delta chain, mitochondrial precursor
AIR029	GR882712	220	AAY27752.1	7.00e-44	*Hevea brasiliensis*	12-oxophytodienoate reductase
AIR030	GR882713	324	YP_398330.1	7.00e-42	*Lactuca sativa*	Photosystem I assembly protein Ycf3
AIR031	GR882714	180	ABB85235.1	e-19	*Glycine max*	Malonyltransferase
AIR033	GR882716	313	NP_001031110.1	7.00e-44	*Arabidopsis thaliana*	Zinc ion binding
AIR035	GR882718	261	ABA93724.1	9.00e-35	*Oryza sativa*	Protein transport protein Sec24-like CEF, putative
AIR037	GR882719	654	YP_740685.1	3.00e-19	*Nandina domestica*	Translational initiation factor 1
AIR038	GR882720	286	BAD27390.1	3.00e-26	*Zinnia elegans*	Gamma-glutamylcysteine synthetase
AIR041	GR882721	254	AAK13029.1	1.00e-29	*Malus domestica*	Ribonuclease-like PR-10c
AIR042	GR882722	286	ABE87918.1	5.00e-33	*Medicago truncatula*	Cupin region
AIR043	GR882723	238	AAL66292.1	2.00e-05	*Glycine max*	Serine acetyltransferase
AIR044	GR882724	366	CAA74054.1	5.00e-39	*Arabidopsis thaliana*	Transcription factor
AIR045	GR882725	318	AAX18314.1	6.00e-41	*Malus domestica*	Major allergen Mal d 1.03B
AIR046	GR882726	302	P16148	2.00e-16	*Lupinus polyphyllus*	Protein PPLZ12
AIR051	GR882731	304	P49299	6.00e-69	*Cucurbita maxima*	Citrate synthase, glyoxysomal precursor
AIR052	GR882732	287	ABE87751.2	3.00e-49	*Cucurbita maxima*	Ras GTPase; Ras small GTPase, Rab type
AIR053	GR882733	334	AAZ83586.1	1.00e-08	*Cucurbita maxima*	Type II SK2 dehydrin
AIR054	GR882734	433	CAC84712.1	8.00e-27	*Cucurbita maxima*	aux/IAA protein
AIR056	GR882736	544	NP_200588.2	1.00e-33	*Cucurbita maxima*	WAX2; catalytic
AIR057	GR882737	465	ABA03057.1	2.00e-41	*Cucurbita maxima*	ACC oxidase
AIR058	GR882738	175	BAB33421.1	4.00e-25	*Cucurbita maxima*	Putative senescence-associated protein

AIR059	GR882739	371	AAF26091.1	4.00e-11	*Cucurbita maxima*	Low temperature and salt responsive protein
AIR061	GR882741	369	P62302	9.00e-63	*Glycine max*	40S ribosomal protein S13
AIR063	GR882743	381	CAN61534.1	7.00e-51	*Vitis vinifera*	Hypothetical protein
AIR064	GR882744	254	ABC47924.1	2.00e-27	*Malus domestica*	Pathogenesis-related protein 8
AIR065	GR882745	404	ABO84639.1	2.00e-30	*Medicago truncatula*	CHCH
AIR067	GR882747	945	ABD17322.1	5.00e-36	*Petunia hybrida*	Isoeugenol synthase 1
AIR068	GR882748	444	AAD26552.1	5.00e-48	*Malus domestica*	Major allergen mal d 1
AIR071	GR882751	205	BAC06946.1	8.00e-22	*Oryza sativa*	Putative GTP binding protein
AIR074	GR882753	202	BAD08451.1	7.00e-05	*Oryza sativa*	Glucose-6-phosphate isomerase
AIR076	GR882754	383	AAR25799.1	2.00e-05	*Solanum tuberosum*	Oxysterol-binding protein
AIR078	GR882756	884	NP_179464.1	5.00e-17	*Arabidopsis thaliana*	Small nuclear ribonucleoprotein E, putative
AIR081	GR882758	122	AF318061.1	4.00e-35	*Peach asteroid spot virus*	Putative coat protein
AIR085	GR882761	405	EAY86632.1	3.00e-22	*Oryza sativa*	Hypothetical protein
AIR086	GR882762	298	AAZ32845.1	8.00e-18	*Medicago truncatula*	Serine carboxypeptidase S10 family protein
AIR088	GR882763	195	BAF44098.1	1.00e-15	*Pyrus bretschneideri*	1-aminocyclopropane-1-carboxylate oxidase
AIR090	GR882765	432	O24058	4.00e-27	*Malus domestica*	Metallothionein-like protein type 2
AIR091	GR882766	345	CAA98170.1	5.00e-59	*Lotus japonicus*	GTP-binding protein RAB7C
AIR092	GR882767	290	NP_175688.1	9.00e-08	*Arabidopsis thaliana*	Oxidoreductase, 2OG-Fe(II) oxygenase family protein
AIR094	GR882769	175	AAU09444.1	2.00e-28	*Fragaria ananassa*	UDP-glucose glucosyltransferase
AIR097	GR882772	116	AAD38148.1	3.00e-58	*Prunus armeniaca*	Beta-amylase
AIR101	GR882775	118	AB067683.1	6.00e-22	*Pyrus communis*	Pectin methylesterase 3
AIR103	GR882776	440	CAB61744.1	2.00e-10	*Cicer arietinum*	Hypothetical protein
AIR106	GR882779	161	AAB84202.2	2.00e-10	*Kosteletzkya virginica*	Plasma membrane proton ATPase
AIR107	GR882780	362	BAF42040.1	6.00e-68	*Pyrus communis*	Pectin methylesterase 3
AIR108	GR882781	144	NM_117310.2	1.00e-07	*Arabidopsis thaliana*	Stress-inducible protein, putative
AIR110	GR882782	230	NP_192425.1	4.00e-12	*Arabidopsis thaliana*	4-coumarate-CoA ligase, putative / 4-coumaroyl-CoA synthase, putative
AIR111	GR882783	254	ABG75916.2	3.00e-34	*Stevia rebaudiana*	(E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase
AIR117	GR882784	234	ABE91065.2	5.00e-08	*Medicago truncatula*	Hypothetical protein MtrDRAFT_AC146564g30v2
AIR121	GR882785	489	O24059	1.00e-19	*Malus domestica*	Metallothionein-like protein type 3
AIR123	GR882786	301	AAK28509.1	3.00e-36	*Fragaria ananasa*	Cinnamyl alcohol dehydrogenase
AIR127	GR882787	229	NP_568644.1	3.00e-17	*Arabidopsis thaliana*	CLPP4 (Clp protease proteolytic subunit 4)

[a] No matching sequences were found in the NCBI database for the following EST clones: AIR006 (GR882691, 168bp), AIR013 (GR882698, 240 bp), AIR032 (GR882715, 154 bp), AIR034 (GR882717, 309bp), AIR047 (GR882727, 323bp), AIR060 (GR882740, 263bp), AIR069 (GR882749, 384bp), AIR084 (GR882760, 228bp), AIR089 (GR882764, 228bp), AIR093 (GR882768, 143bp), AIR095 (GR882770, 305bp), AIR104 (GR882777, 134bp), AIR105 (GR882778, 162bp).


Quercetin-treated apple tissues
Non-treated apple tissues
Samples collected at 24 and 48 h
Samples collected at 24 and 48 h
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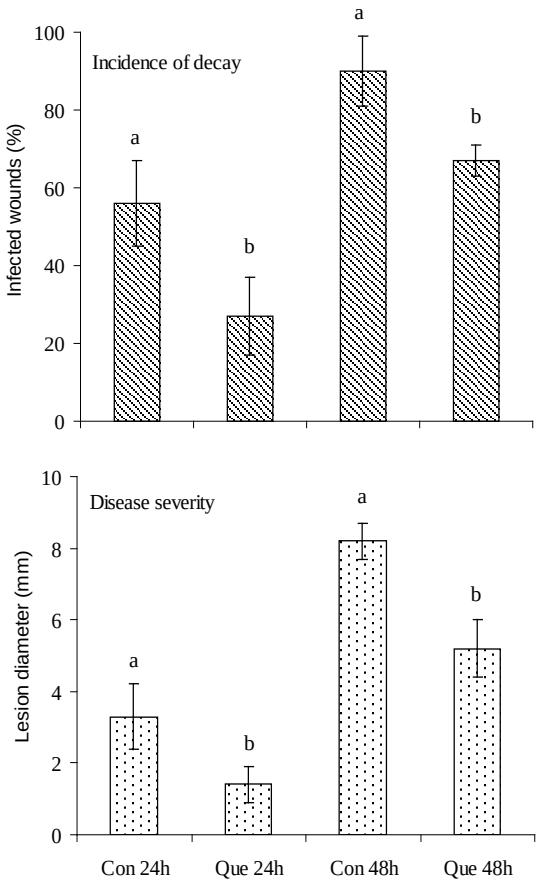
RNA RNA
RNA pool (1:1)
RNA pool (1:1)
polyA+ mRNA
polyA+ mRNA
Tester cDNA
Driver cDNA
SSH

Fig. 1.


Incidence of decay
Infected wounds (%)
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80
60
40
20
0
a
b
a
b
Disease severity
Lesion diameter (mm)
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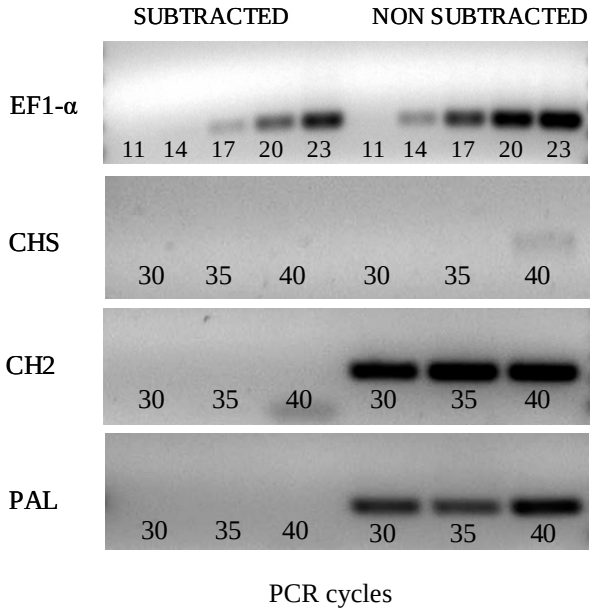
8
6
4
2
0
a
b
a
b
Con 24h
Que 24h
Con 48h
Que 48h

Fig. 2.


SUBTRACTED
NON SUBTRACTED
EF1-α
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CHS
30 35 40 30 35 40
CH2
30 35 40 30 35 40
PAL
30 35 40 30 35 40
PCR cycles

Fig. 3.

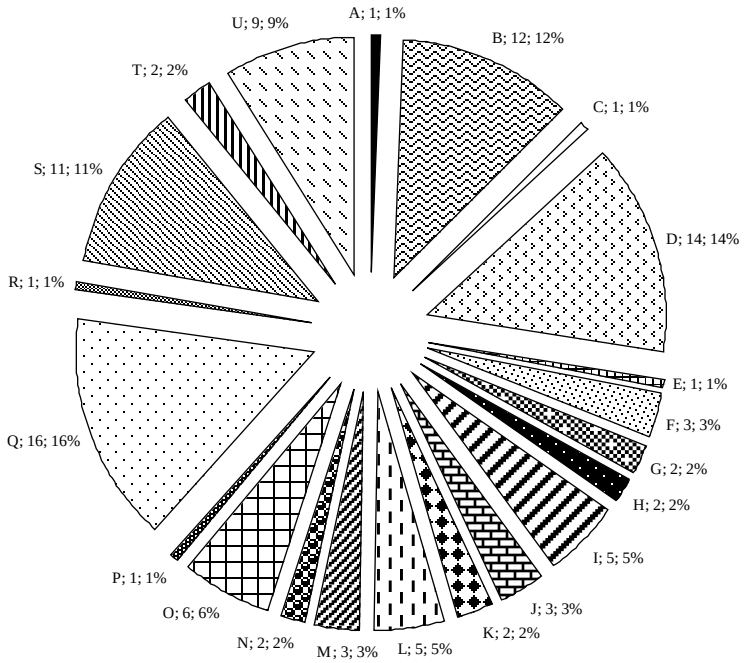
A; 1; 1%
B; 12; 12%
C; 1; 1%
D; 14; 14%
E; 1; 1%
F; 3; 3%
G; 2; 2%
H; 2; 2%
I; 5; 5%
J; 3; 3%
K; 2; 2%
L; 5; 5%
M; 3; 3%
N; 2; 2%
O; 6; 6%
P; 1; 1%
Q; 16; 16%
R; 1; 1%
S; 11; 11%
T; 2; 2%
U; 9; 9%

Fig. 4.

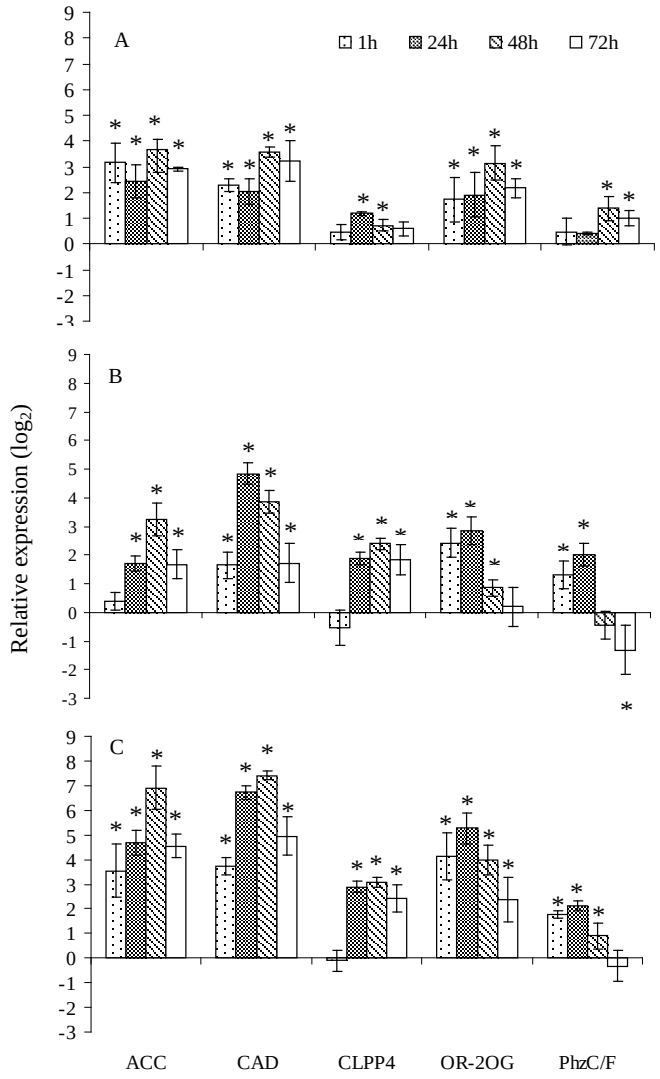
A
B
C
1h
24h
48h
72h
Relative expression (log2)
ACC
CAD
CLPP4
OR-2OG
PhzC/F

Fig. 5.