The use of qRT-PCR approach for comparative phenylpropanoid gene expression studies in two *Rubus* spp.

Utilizarea metodologiei qRT-PCR in studii de analiza comparativa a expresiei genelor implicate in metabolismul fenilpropanilor la doua specii din genul *Rubus*

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Abstract. *Rubus spp. is economically-important crops worldwide and represents an invaluable source of healthy-related compounds, being used traditionally for therapeutic purposes. In berries, development of fruit quality traits is critically dependent on flavonoids-derived metabolites. Moreover, these compounds are often involved in resistance against biotic and abiotic stress. Better knowledge and understanding of the flavonoid biosynthetic pathway and its regulation is crucial for identifying strategies for enhancement product accumulation and quality, in specific environments. In the present study, a qRT-CR approach was performed in order to assess the accumulation in the transcript of several genes (pal1, pal2, chs, 4cl1, 4cl2, 4cl3), involved in flavonoid biosynthesis pathway. Comparative transcriptional profiling was carried out in raspberry (Ruvi cv.) and blackberry (Lochness cv.) plants grown under controlled, greenhouse conditions. The determination of the relative expression levels of pal1 and chs genes showed that in raspberry, these transcripts were 3- and 150-fold, respectively more abundant than in blackberry. Similar, the accumulation of the three gene transcripts encoding 4-coumarate: CoA ligase (4cl1, 4cl2 and 4cl3) was higher in raspberry than in blackberry. The importance of qRT-PCR in studies of secondary metabolism in raspberry and blackberry is discussed.*

Key words: *rubus, phenylpropanoids, gene expression, real-time quantitative PCR*

Rezumat. *Genul Rubus cuprinde specii cu importanta economica recunoscuta, care produc si acumuleaza compusi cu valoare terapeutica. La aceste specii calitatea fructelor este strict dependenta de acumularea flavonoizilor. In plus,acesti compusi sunt adesea implicati in mecanismele de rezistenta la stresul biotic si abiotic. Pentru o mai buna cunoastere si intelegere a caii de sinteza a flavonoizilor este necesara identificarea strategiilor de stimulare a acumularii produsilor si calitatii in conditii specifice de mediu. In studiul de fata, metoda qRT-CR a fost aplicata in vederea determinarii acumularii produsilor de transcriptie ai unor gene implicate in calea de biosinteza a (pal1, pal2, chs, 4cl1, 4cl2, 4cl3). Analiza comparativa a profilului transcriptomic a fost realizata pe plante de zmeur (Ruvi cv.) si mur (Lochness cv.) crescute in conditii controlate de sera. Determinarea nivelelor relative de expresie ale genelor pal1 si chs a aratat ca la zmeur, acumularea acestor produsi de transcriptie este de 3 si 150 ori mai abundenta decat la mur. Nivele mai crescute de expresie la zmeur decat la mur, au fost detectate si in cazul celor trei gene ce codifica 4 cumarat- CoA ligaza (4cl1, 4cl2 si 4cl3). Importanta tehnicii qRT-PCR in studiul metabolismului secundar la specii de zmeur si mur este discutata.*

Cuvinte cheie: *rubus, fenilpropani, expresie genica, analiza cantitativa qRT-PCR.*

INTRODUCTION

Phenylpropanoids occur widely in plants and represent a major group of secondary metabolites. *Rubus* spp. is a very rich source of these healthy-related compounds with biological and pharmacological properties (Rao A.V., and Snyder D.M., 2010). Phenolics play an important role in plant growth and development and are also involved in resistance against environmental stresses (Treutter D, 2006). In berries, phenylpropanoids-derived metabolites are significant determinants for plant quality (Weisshaar, B., and Jenkins G.I., 1998, Kumar and Elis, 2003a). Moreover, the bioactive content of fruits varies among different genotypes (Pantelidis et al., 2007). To gain insights of the phenylpropanoids biosynthetic pathway and its regulation, the changes in the transcript level of several key genes involved in their biosynthesis was assessed by a qRT-CR approach. For this purpose, raspberry (Ruvi cv.) and blackberry (Lochness cv.) plants were grown under controlled, greenhouse environment, and gene expression in their leaf tissues was investigated. Total RNA was isolated from harvested samples and subjected to reverse transcription and real-time quantitative PCR. Comparative transcriptomic analysis was performed using relative quantification method, the most adequate approach to investigate physiological changes in gene expression level (Pfaffl M.W., 2004). To eliminate non-biological variation, optimization and accurate normalization of qRT-PCR reaction was required. The importance of qRT-PCR method for assessing expression of target genes and ultimately for gathering significant information of their role in the metabolites biosynthesis and accumulation is also discussed.

MATERIAL AND METHOD

Plant tissues preparation. Plant leaves were collected from three biological replicates ground to a fine powder in liquid nitrogen and stored at -80^{o}C to preserve full-length RNA.

RNA isolation and quantification. Aliquots of 100 mg grounded plant material were subjected to total RNA extraction and purification according to previously described protocol (Salzman et al., 1999), or by using Spectrum Plant Total RNA kit. RNA quality was electrophoretically verified by ethidium bromide staining of RNA samples in 1% agaroze gel, by spectrophotometer analysis at 230, 260 and 280 nm and by Agilent Bioanalyzer analysis using an RNA 6000 Nano kit. Total RNA samples were digested with RQ1 DNaseI (Promega) to remove any trace of contaminating genomic DNA.

cDNA synthesis and RT-PCR. Two µg of purified DNAse-treated RNA was reverse transcribed with SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's protocols. The resulted first-strand cDNA was diluted to a final volume of 100ng, and target cDNAs were amplified using gene-specific primers (Table1). The oligonucleotides were designed from the transcribed region of *Rubus idaeus* specific - target genes using Primer Express 1.5 software (Applied Biosystems, Darmstadt, DE).

qRT-PCR analysis. Quantitative real-time PCR analysis was performed on the Rotor-Gene 6000 (Corbette) using MyTaq^{TM}RedMix (Bioline), gene specific primers at a final concentration of 0.25uM and 2 ul of the cDNA as template. The temperature cycle

used comprised 40 cycles at 95°C for 15 sec and 60°C for 1 min. To monitor PCR specificity a dissociation curve was performed. For the relative quantification of transcript levels, a modification of the comparative threshold cycle method was used. Relative transcript levels of the gene of interest (X) were calculated as a ratio to the histone H3 gene transcripts (U), as $(1 + E^{)-\Delta Ct}$, where ΔCt was calculated as $(Ct_X – Ct_U)$. PCR efficiency (E) for each amplicon was calculated employing the linear regression method (Ramakers et al. 2003). All real-time qPCR reactions for relative quantification were performed as triplicates.

RESULTS AND DISCUSSIONS

In Rubus spp., the accumulation of metabolites, essential for plant growth and development, requires integrated expression of genes encoding enzymes in the phenylpropanoid pathway, such as phenylalanine ammonia-lyase (PAL), 4-coumarate CoA ligase (4CL), and chalcone synthase (CHS). The employed real-time quantitative PCR (qRT-PCR) method has become a very powerful tool for the quantification of gene expression, for its high sensitivity and reliability (Pfaffl M.W., 2004.). The reliability and efficiency of gene expression evaluation is affected by several critical factors including RNA and cDNA quality and PCR optimization. Moreover, a successful qRT-PCR assay strongly depends on the accurate transcript normalization using an appropriate reference gene.

Assessment of RNA quality. *Rubus* plants contain many secondary metabolites that can interfere with RNA preparation and its use in downstream PCR-based applications. Therefore, different protocols for RNA extraction and purification from difficult tissues were employed in order to obtain excellent quality RNA, highly suitable for qRT-PCR approach. Sigma's Spectrum Plant total RNA kit provided the most simple, rapid and efficient procedure for total RNA extraction. Total RNA isolated samples were quantified by spectrophotometry, (A260/280>1.8 and A260/A230>1.95) and agarose gel electrophoresis by ethidium bromide staining (data not shown). Moreover, RNA integrity and quality was verified using Agilent 2100 Bioanalyzer. This analysis showed clear and well defined 28S and 18S peaks, low noise between the peaks and minimal to moderate low molecular weight contamination and good RNA integrity numbers, RIN>7, for all investigated samples.

Evaluation of primer specificity. The primer specificity of target genes was monitored by agarose gel electrophoresis and dissociation curve analysis (Fig.1). Our data confirmed that for each investigated gene, only a unique PCR product of the expected size and melting temperature was obtained (see table 1). Moreover, no primer-dimers and non-specific products formations were detected.

Table 1

Table 1: Primers used for qRT-PCR assay

Target genes	Gene-specific primers		Amplicon size(bp)
Phenylalanine ammonia-lyase1 (pal1)	pal1F	5'-TCGACAATGCCAGGATCGA-3'	79
	pal1R	5'-CAACGGATAAGACCTGCATTCC-3'	

Phenylalanine ammonia-lyase2 (pal2)	pal2F	5'-ACCTCTTCCGATCTGCTAGCC-3'	70
	pal2R	5'-CGAAGTGGAATGGAATGACACA-3'	
4-coumarate:coA ligase1 (4cl1)	4cl1F	5'-TGCTCGTCACCCATCCTAACA-3'	89
	4cl1R	5'-TCACGACAAATGCAACCGG-3'	
4-coumarate:coA ligase2 (4cl2)	4cl2F	5'-CGGCTACTTTCCCAAATCGATA-3'	85
	4cl2R	5'-TCACCCCGGCCATTATAGAA-3'	
4-coumarate:coA ligase3 (4cl3)	4cl3F	5'-TCCGCAAAAAGATGATGCTG-3'	70
	4cl3R	5'-GCTCATTGCCGCCATTAGAT-3'	
chalcone synthase (chs)	chsF	5'-TCACAGTGTGGCAGCTTCAAC-3'	62
	chsR	5'-ACTGATCAAGGAGATCACCCAA-3'	
histoneH3 (his)	hisF	5'-TTCCAGAGCCATGCAGTTTTG-3'	93
	hisR	5'-TGGCATGAATGGCACAGAGA-3'	
Actin (act)	actF	5'-ATTGCAGACCGTATGAGCAAAG3'	62
	actR	5'-GGTGCCACAACCTTGATCTTC-3'	

Furthermore, the PCR conditions were optimized with respect to *Taq* DNA polymerase, primers concentrations, various annealing temperature, $MgCl_2$ and dNTP concentrations.

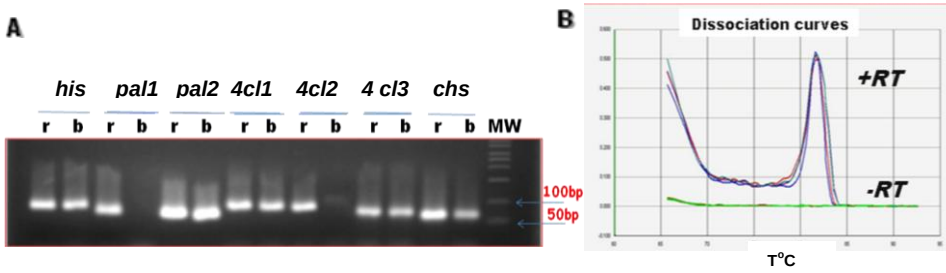

Fig.1. Validation of primer specificity of target genes**. A**. Visualization of RT-PCR specific products of the expected length on a 4% (w/v) agarose gel. **B.** Confirmation of primers specificity by qRT-PCR analysis using specific primers for the reference gene histone H3. No amplicon was detected within 40 cycles without RT to confirm absence of genomic DNA. Similar results were obtained for all investigated genes (data not-shown).

Validation of the reference gene. Prior to qRT-PCR analysis of target gene expression it is essential to identify and validates the reference gene, to accurately compare mRNA transcript among different samples. For this purpose, two potential reference genes, actin and histone H3, were chosen for a preliminary qRT-PCR assay. The frequently used reference gene in plant, actin, was the least stable (data not-shown). Therefore, histone H3 gene was selected as reference gene based on its performance data such as earlier Ct values, no primer-dimers and non-specific products artifacts, clean melt curves in all replicates and stable expression across all investigated samples.

Comparative transcriptional analysis of key genes involved in phenylpropanoid metabolism. Transcriptional profiling was performed using relative quantification of target gene transcripts in comparison to the previously selected reference gene. The expression levels of the control gene, was used as internal standards to normalize small variations in cDNA template amounts. The relative transcript levels of the gene of interest were calculated as a ratio to the histone H3 gene transcripts (Fig.2).

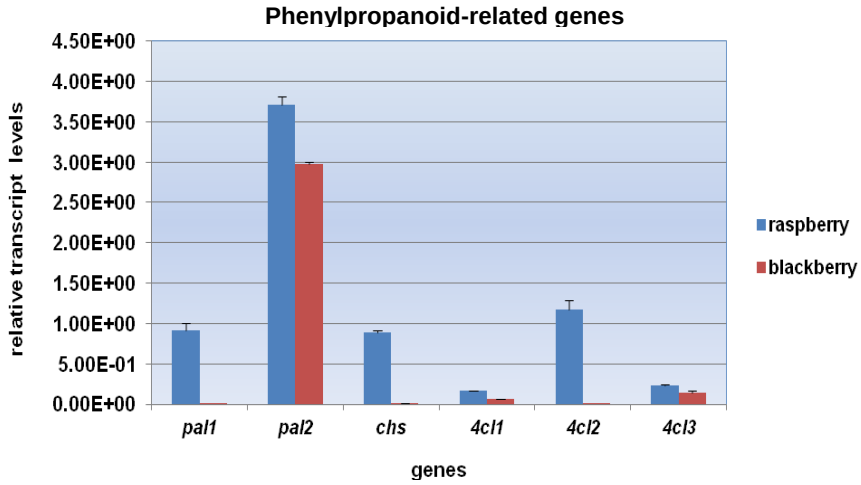

Fig. 2. Accumulation of key gene transcripts involved in phenylpropanoid pathways in two *Rubus spp*. Total RNA was isolated from leaves, reverse transcribed to cDNA, and subjected to real-time quantitative PCR. Transcript levels in the different samples were normalized to those of the reference gene, histone H3. Relative mRNA level was calculated with respect to the level of histone H3 transcripts. Bars show means +SD (n = 3).

Although expression of both *pal1* and *chs* genes was detected in examined tissues, determination of the relative expression levels of the two transcripts showed that in raspberry, *pal1* and *chs* transcripts were 3- and 150-fold, respectively more abundant than those in blackberry, while *pal2* was detected only in raspberry. Similar, the transcript accumulation of the three genes encoding 4-coumarate: CoA ligase (*4cl1*, *4cl2* and *4cl3*) in raspberry was higher than the expression levels of genes in blackberry. Previously published data showed a differential pattern of expression for the investigated genes in various vegetative tissues as well as during fruit development which suggest their involvement in the formation of various phenylpropanoid-derived metabolites (Kumar and Ellis, 2001, 2003a, b). Furthermore, we are going to investigate whether the changes in gene expression due to various stress condition or genotypes are also reflected on the metabolite level. Transcriptional profiling data obtained by the extremely sensitive and reliable qRT-PCR approach, corroborated with further metabolic results will facilitate a better understanding of plant growth and development and will contribute to the optimization of productivity in specific environments.

CONCLUSIONS

1. *RNA samples with identical high quality were investigated using a transcript profiling assay and processed with the same efficiency in every step of the analysis.*
2. *Dissociation curve analysis and agarose gel electrophoresis of the RT-PCR products amplified using gene-specific primers, confirmed reaction specificity.*
3. *To assess gene expression in Rubus plants, histone H3 housekeeping gene has been validated as reference gene for qRT-PCR normalization.*
4. *Relative quantification approach revealed that several key genes involved in phenylpropanoid metabolism are differentially expressed in the two investigated cultivars, their transcript levels being more abundant in raspberry (Ruvi cv.) than in blackberry plants (Lochness cv.)*
5. *Furthermore, a combined transcriptomic and metabolomic approach will allow the direct estimation of the plant nutritional and physiological state in various environmental conditions.*

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