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

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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 level of several genes (PAL, 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 PAL and CHS genes showed that in raspberry, these transcripts were 3- and 150-fold, respectively more abundant than in blackberry. Similar, the accumulation level of the three genes encoding 4-coumarate: CoA ligase (4CL1, 4CL2 and 4 CL3) in raspberry was higher than in blackberry. The importance of qRT-PCR in studies of secondary metabolism in raspberry and blackberry is discussed.

Introduction

Phenylpropanoids occur widely in plants and represent a major group of secondary metabolites. These compounds exhibit biological and pharmacological properties, including antioxidative and antimicrobial activities, and have been used for the treatment of cardiovascular diseases and cancer prevention (Wang, 2011, 2003; Treutter, 2006;). They are also involved in resistance against environmental stresses. 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, gene expression in the leaf tissues collected from raspberry and blackberry plants (Ruvi cv and Lochness cv, respectively), grown under controlled, greenhouse environment, was investigated. Samples were harvested and total RNA was isolated and subjected to reverse transcription and real-time quantitative polymerase chain reaction (qRT-PCR) analysis. The real-time quantitative RT-PCR analysis has become a very powerful tool for the quantification of gene expression, widely used for its high sensitivity and reliability. Comparative gene expression analysis was carried out using relative quantification of target gene transcripts in comparison to a reference gene transcript the most adequate approach to investigate physiological changes in gene expression level (Pfaffl, 2001; Udvardi 2008). To eliminate non-biological variation, optimization and accurate normalization of qRT-PCR reaction was required.

Furthermore, we are going to investigate whether the changes in gene expression due to various stress condition is also reflected on the metabolite level. Transcriptional analysis correlated with metabolic data in response to stress will contribute to the optimization of productivity in specific environments.

Materials and methods

Plant tissues preparation. The investigated *Rubus* plants (Ruvi and Lochness cultivars) were grown under controlled, greenhouse environment. Plant leafs were collected from three biological replicates ground to a fine powder in liquid nitrogen and stored at -80oC 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 protocols (Brusslan and Tobin, 1992; 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 Labchip kit.

cDNA synthesis and RT-PCR. Two µg of purified RNA was reverse transcribed with either SuperScript II Reverse Transcriptase kit (Invitrogen) or iScriptTM (Bioline) 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 designed from the transcribed region of each gene specific for *Rubus idaeus* spp., using Primer Express 1.5 software (Applied Biosystems, Darmstadt, DE).

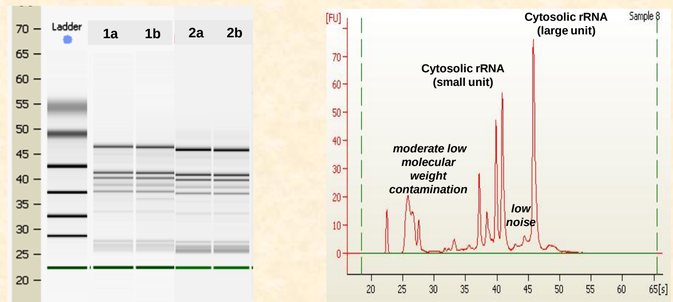
qRT-PCR analysis Quantitative real-time PCR analysis was performed on the Rotor Gene 6000 (Corbette) using SYBR Green-My Taq TM Red Mix (Bioline), gene specific primers at a final concentration of 0.25uM and 2 ul of the cDNA as template. The temperature cycle used comprised 40 cycles at 95°C for 15 sec and 60°C for 1 min. A dissociation curve was also performed, (95°C for 15 sec, 60°C for 1 min and 95°C for 10 sec), to monitor PCR specificity. For the relative quantification of gene expression, 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)-ΔCt, where Δ Ct was calculated as (CtX–CtU). PCR efficiency (E) for each amplicon was calculated employing the linear regression method on the Log (Fluorescence) per cycle number data, using the LinRegPCR software (Ramakers et al. 2003). All real-time qPCR reactions for relative quantification were performed as triplicates.

Results

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, several protocol for RNA extraction and purification from difficult tissues (see materials and methods) were employed in order to obtained excellent quality RNA, highly suitable for qRT-PCR approach. Sigma's Spectrum Plant total RNA kit provided the most rapid and efficient procedure for total RNA extraction.

Total RNA isolated samples were quantified by spectrophotometry and agarose gel electrophoresis by ethidium bromide staining. Moreover, RNA integrity was verified using Agilent 2100 Bioanalyzer. Prior qRT-PCR analysis total RNA samples were digested with DNaseI to remove any trace of contaminating genomic DNA.



RNA quality for investigated samples:

•A260/280 > 1.78 and A260/A230 > 1.95

•RNA integrity number, RIN>7

Figure 1. *Agilent Bioanalyzer analysis of total RNA sample purified by Sigma's Spectrum Plant Total RNA Kit from berries leaves. There are clear and well defined 28S and 18S peaks, low noise between the peaks and minimal to moderate low molecular weight contamination.*

Confirmation of primer specificity

RT-PCR analysis was performed in DNAse-treated RNA samples using gene specific-primers, to confirm absence of genomic DNA. Furthermore the PCR conditions were optimized with regard to *Taq* DNA polymerase, primers concentrations, various annealing temperature, MgCl2 and dNTP concentrations.

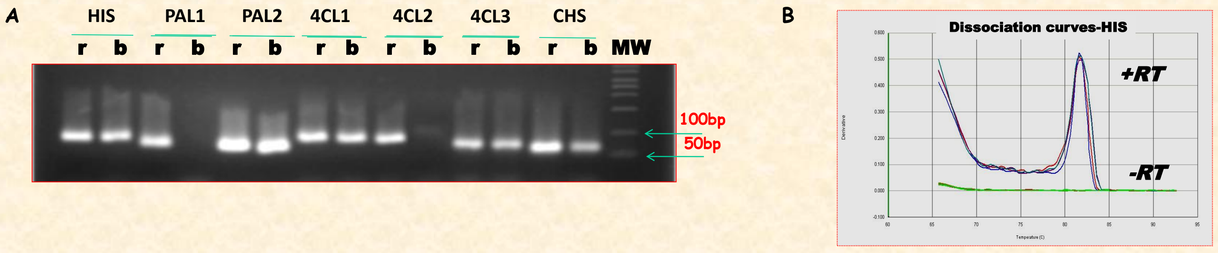


Figure 2: The primer specificity and formation of primer-dimmers monitored by dissociation curve analysis and agarose gel electrophoresis. A. Confirmation of amplicon size (see table 1) and primer specificity of target genes on a 4% (w/v) agarose gel electrophoresis showing specific RT-PCR products of the expected size for each gene investigated. **B.** Validation of primer specificity by qRT-PCR analysis using specific primers for the housekeeping gene histone H3. No amplicon was detected within 40 cycles without RT. Similar results were obtained for all investigated genes (data non-shown).

Validation of the reference gene

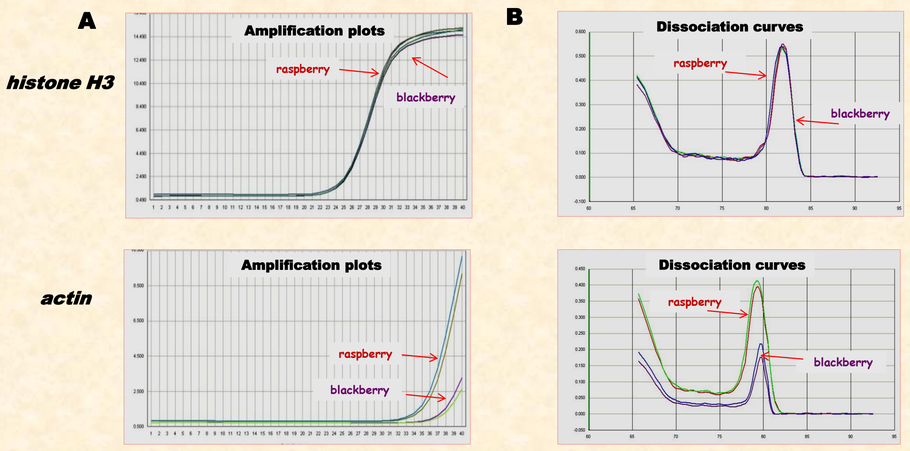


Figure 3. Quantitative RT-PCR of berries RNA samples isolated from berries leaves on Rotor Gene 6000 Corbett using gene-specific primer for two housekeeping genes; actin and histone H3 A. Amplification curves (fluorescence vs. cycle number) correspond to 200ng of input pure total RNA. **B. Dissociation curves** for the qRT-PCR products were generated using the following primers set: Upper Panel: HIS F and HIS R; Lower panel, ACT F; ACT R.

Prior qRT-PCR analysis of target genes expression it is essential to identify and validates the appropriate reference gene to accurately compare mRNA transcript across different samples.

Histone H3 gene was selected as reference gene based on its performance data:

- *Earlier Ct values*
- *No primer-dimmers and non-specific products artifacts*
- *Clean melt curves in all replicates*
- *Stable expressed across all investigated samples*

Comparative transcriptional analysis of key genes involved in phenylpropanoid metabolism

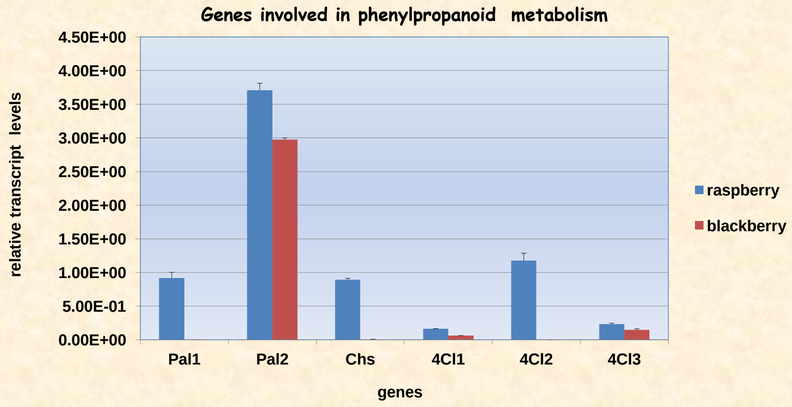


Figure 4. Accumulation of key gene transcripts involved in phenylpropanoid pathways in two *Rubus spp*. Total RNA was isolated from leaves, reverse transcribed to cDNA, and subjected to real-time quantitative PCR. Transcript levels in the different samples were normalized to those of the reference gene, histone H3. Relative mRNA level was calculated with respect to the level of histone H3 transcripts. Bars show means +SD (n = 3)

Table 1: Primers used for qRT-PCR assay

Key target genes	Gene-specific primers		Amplicon size (bp)	Accesion no.
Phenylalanine ammonia-lyase 1 (pal1)	pal1F	5'-TCGACAATGCCAGGATCGA-3'	79	AF237954.1
	pal1R	5'-CAACGGATAAGACCTGCATTCC-3'		
Phenylalanine ammonia-lyase 2 (pal2)	pal2F	5'-ACCTCTTCCGATCTGCTAGCC-3'	70	AF237955.1
	pal2R	5'-CGAAGTGGAATGGAATGACACA-3'		
4-coumarate:coA ligase 1 (4cl1)	4cl1F	5'-TGCTCGTCACCCATCCTAACA-3'	89	AF239687.1
	4cl1R	5'-TCACGACAAATGCAACCGG-3'		
4-coumarate:coA ligase 2 (4cl2)	4cl2F	5'-CGGCTACTTTCCCAAATCGATA-3'	85	AF239686.1
	4cl2R	5'-TCACCCCGGCCATTATAGAA-3'		
4-coumarate:coA ligase 3 (4cl3)	4cl3F	5'-TCCGCAAAAAGATGATGCTG-3'	70	AF239685.1
	4cl3R	5'-GCTCATTGCCGCCATTAGAT-3'		
chalcone-synthase 6 (chs)	chsF	5'-TCACAGTGTGGCAGCTTCAAC-3'	62	AF400567.1
	chsR	5'-ACTGATCAAGGAGATCACCCAA-3'		
histone H3 (his)	hisF	5'-TTCCAGAGCCATGCAGTTTTG-3'	93	AF304365.1
	hiSR	5'-TGGCATGAATGGCACAGAGA-3'		
Actin(act)	act F	5'-ATTGCAGACCGTATGAGCAAAG3'	62	GQ339772.1
	act R	5'-GGTGCCACAACCTTGATCTTC-3'		

For the relative quantification of gene expression, a modification of the comparative threshold cycle method was used. The relative transcript levels of the gene of interest was calculated as a ratio to the histone gene transcripts. The expression levels of this reference gene, was used as internal standards to normalize small differences in cDNA template amounts. Although expression of both PAL1 phenylalanine ammonia-lyase and CHS chalcone synthase 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 accumulation level of the three genes encoding 4-coumarate: CoA ligase (4CL1, 4CL2 and 4CL3) in raspberry was higher than the expression levels of genes displayed in blackberry.

References

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Conclusions

- *RNA samples being analyzed in any transcript profiling analysis must be of equivalent high quality and should be processed with the same efficiency in every critical step of the analysis.*
- *The primer specificity and primer-dimmers formation was determined by dissociation curve analysis and agarose gel electrophoresis of the RT-PCR product amplified using gene-specific primers.*
- *Following a validation experiment performed for the identification of reference gene, histone H3 housekeeping gene was selected for the normalization of qRT-PCR assay .*
- *Relative quantification approach (ΔCt method) revealed that the 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.)*
- *Furthermore, the changes in transcript profiling, will be correlated with the metabolite profile and will allow the direct estimation of the plant nutritional and physiological state in various environmental conditions.*