Effect of Reaction Parameters in the Real-Time PCR Detection of Grape Chitinase

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Abstract
Grapevine is a well-known and popular cultural plant that is grown on thousands of hectares worldwide. It possesses a lot of nutritional benefits but, on the other side, some of the people may suffer an allergic reaction when eating grapes or even in the case of consumption of the processed forms. Although grape allergy is not a common allergic reaction, the prevalence of this type of allergy is increasing mainly in southern European countries. One of the main proteins that has the potential to cause grapes allergy is chitinase. Here, a real-time PCR protocol was optimized to analyse the expression of chitinase in the mature grape berries. Different parameter such as analytic kit, primer annealing temperatures or cDNA dilution effects were tested. The final optimized protocol was proved for the chitinase unique isoform amplification by post melting analysis and testing of the protocol was performed for selected grape varieties.

Keywords: real-time PCR, grapevine, optimization

1. Introduction

Vitis vinifera ssp. *sativa* is one of the oldest and most important fruit crops in the world. Its evolution history is about 65 million years old. Nowadays, the cultivation of grapevine is widespread and is the most commonly grown from vine species with the portion of 98% from world's vineyards [1].
The grapevine is known for the presence of a large number of different health beneficiary bioactive compounds which represent secondary metabolites such as flavonoids, linoleic acid, pigments, anthocyanins, procyanidins or vitamin E. High concentration of procyanidins has been confirmed in grape seeds [2].

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On the other side, allergens are present in grapes, too, and became an object of different allergologic studies quite long ago when the first occurrence of grape allergy was reported [3]. Despite the fact that the occurrence of this type of allergy is not so common when compared to other food allergies, allergic reactions to various grape varieties or to various grape products such as vine were described in the last twenty years. Allergens such as 30 kDa molecular endochitinase 4A, lipid transfer protein (LTP) and 24 kDa taumatin-like protein (TLP) have been identified in grapes [4].
Endochitinase 4A is considered as the major allergen in young wines and wine called Fragolino. For many people, several allergic reactions have been observed after the consumption of grapes and also after drinking two kinds of red wine - young wine and Fragolino wine. The cause of the allergic reaction to these two types of wine may be related to the wine making process itself. In the production of red

wines, the polymerization of polyphenols causes the precipitation of residual proteins, which can be filtered off after the wine matures. The amount of chitinases present in the grapes represents 50% of the soluble proteins and is capable of persisting terribly during the wine making process, then aggregating and forming a cloud in the wine [5].
The formation of protein haze is an unwanted effect during the wine production, especially in the case of the wines produced from the white grape varieties. The amount of all proteins found in grape juice or wine ranges from 10 to 500 mg/L. Despite this low concentration, they cause problems in the wine making process. These include chitinases and thaumatin-like proteins. Several studies have confirmed that chitinases are more thermally unstable and more subject to aggregation in wines compared to chitinases [6].
The aim of the presented study was to analyse the effect of the different chemistry and reaction parameters of real-time based method of amplicon specifity and detection of the expression of chitinaze in mature grape.

2. Materials and methods

Biological material and its processing

Mature grapes of *Vitis vinifera*, L. Dornfelder variety from winery Sabo, Vrbové were used for the total RNA extraction. It was performed by ISOLATE II RNA Plant Kit, BIOLINE® following the manufacture´s instruction. Quality and quantity of extracted RNA were analysed using Nanodrop Nanophotometer™ (Implen). Reverse transcription and cDNA preparation were performed from 70 ng of extracted RNA by Tetro cDNA Synthesis Kit, BIOLINE®, using the mix of oligo-dT and random hexamer primers 1/1. Final testing of protocol was performed for varieties – Dornfelder, Cabernet, Alibernet and Frankovka.

Primer design and real-time PCR conditions

Two sets of primers were designed for testing both, chitinase and actin. Primer 3 software (primer3.ut.ee) was used for primer design based on the sequences with NCBI accession codes DQ267094 and AY847627 (table 1).
Different real-time PCR parameters were tested in the optimization analysis: primer annealing temperature (54°C - 64°C); primer concentration (300 – 500 nmol); dilution of cDNA (non-diluted vs. 1/10 diluted) and type of analytical kit (Luminaris Color HiGreen qPCR Master Mix™ - ThermoFisher Scientific; 5 x HOT FIREPol® EvaGreen® qPCR Mix - Solis BioDyne). The starting time and temperature profiles were in individual analytical kits as the manufacturer recommends and the reactions were performed in eight separated samples with the same content.

Table 1. Sequences of primers used in the study.

Actin/F1	5´GTGGCACCACCTGAGAGAA3´
Actin/R1	5´CATCTGCTGGAAGGTGCTGA3´
Chitinase/F1	5´AAGCTGGGGGTAGAGTTTGC3´
Chitinase/R1	5´GTGGAACTGCACTGGCTTTG3´
Actin/F2	5´TGGAAGCTGCGGGAATTCAT3´
Actin/R2	5´CGACCCACCACTAAGCACAA3´
Chitinase/F2	5´AGGGGTAGTTCCTCAGGTCC3´
Chitinase/R2	5´TGGTTGTTGCAGTCCAGGTT3´

Amplicon visualization

Upon completion of PCR and some of the real-time PCR reactions, 1.5% agarose gel electrophoresis was performed to check the amplicons and their specificity. Ultra low range Ladder (ThermoFisher Scientific) was used as the length marker. Gene BOX transilluminator was used to visualize and documented the amplified PCR products.
Melting analysis of amplicons was used further in the real-time PCR reaction within the temperature range 70°C - 90°C with the fluorescence reading time of 5 seconds. High resolution melt analysis was used to prove the specificity of amplicons in final analyse.

3. Results and discussion

The first step in setting up of appropriate PCR conditions was initiated by testing the functionality of the designed primer pairs for both, metabolic gene actin and the gene of interest chitinase. Verifying the functionality of designed primer pairs in the experiment is considered an important step in optimizing of real-time PCR conditions to ensure that we have selected appropriate and reliable primer pairs in the experiment. The standard PCR reaction was used

for this purpose using the MyTaq™ Red Mix protocol and 1/10 of cDNA in the reactions. The temperature gradient for primer annealing was set at the range of 54 °C – 64 °C. Visualization of amplified fragments confirms the functionality of all of the designed primer pairs for the actin and chitinase genes (figure 1) where the estimated amplicon length around 100 bp was detected. In the case of primer pair for actin F1+R1, small portion of dimers were present in the gel.

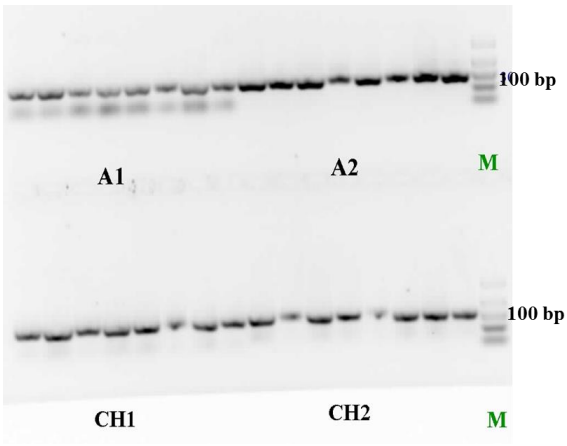

Figure 1. Amplicon checking for actin (A1, A2) and chitinase (CH1, CH2) in the analysis of designed primer pairs functionality. M – ladder.

The first optimization analysis was performed by Luminaris Color HiGreen qPCR Master Mix ™ (ThermoFisher Scientific) and the primer annealing temperature gradient was tested. In the case of primer pair actin F1/R1, no amplification products were obtained for the temperatures in the range of 54°C - 57°C. At the temperatures where the PCR product amplification occurred, the plateau effect appeared but was not in the sufficient amount and a fluorescence was very weak, ranging around only 200 relative fluorescence units (figure 2). Compared to primer pair actin F2/R2, the lowest Ct (34.87) was reached at the same annealing temperature as the primer at F1/R1 - 58.0 °C, but the plateau effect was not recorded in the reaction at all.

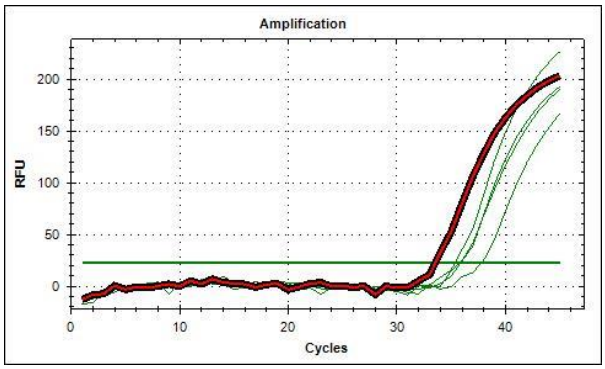

Figure 2. Amplification profile of actin primer pair F1/R1 when Luminaris Color HiGreen qPCR Master Mix ™ was used in the reaction.

Product amplification using primer pair chitinase F1/R1 was performed over the entire range of tested annealing temperatures, but with very different fluorescence growth patterns, and also without the presence of a sufficient plateau effect. The lowest Ct (32.68) was reached at 60.0°C. Compared to primer pair chitinase F2/R2, amplicon formation has taken place over the entire range of temperatures tested, but a non-standard of fluorescence growth curves was obtained (figure 3). The lowest Ct (14.59) was reached at 64 ° C.

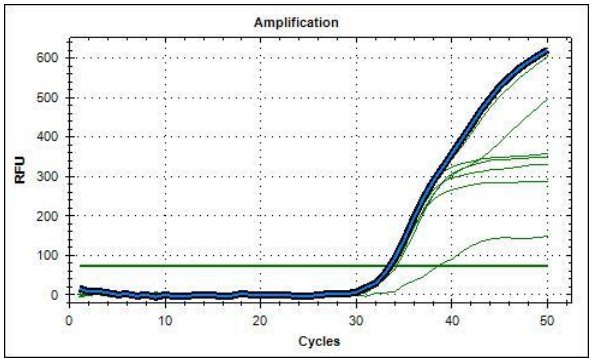

Figure 3. Amplification profile of chitinase primer pair F1/R1 when Luminaris Color HiGreen qPCR Master Mix ™ was used in the reaction.

Second optimization analysis was performed by 5x HOT FIREPol® EvaGreen® qPCR Mix - Solis BioDyne and the primer annealing temperature gradient was tested, too. Product amplification of both primer pairs for actin was performed in the whole range of tested annealing temperatures. The lowest Ct (34.31) was achieved at 56.0 °C with the highest fluorescence increase value of 633 relative fluorescence unit (figure 4).

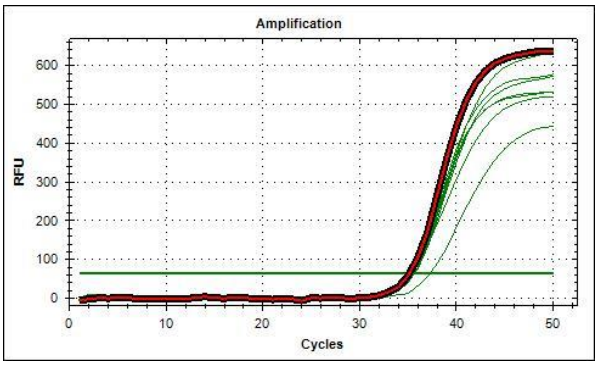

Figure 4. Amplification profile of actin primer pair F1/R1 when 5x HOT FIREPol® EvaGreen® qPCR Mix - Solis was used in the reaction.

Compared to primer pair chitinase F1/R1, the lowest Ct (32.25) was reached at 63.5°C for the primer pair chitinase F2/R2 with a lower fluorescence value of 566 relative fluorescence unit. The highest relative fluorescence unit - 588 value for primer pair chitinase F1/R1 was reached at 60.4 ° C. Compared to the previous protocol where the optimization analysis was performed, the plateau phase began to manifest itself sufficiently, using this type of chemistry.

Further step in chitinase real-time PCR amplification was tested for the effect of the cDNA dilution. The concentration and dilution of the cDNA template is one of the very important optimization steps that can significantly influence the results of the real-time PCR experiment. The degree of dilution of the cDNA template varies depending on the nature of the experiment. For dilution of the template cDNA, it is recommended to use either ultra-pure nuclease-free water or a buffer composed of Tris and EDTA. At the low cDNA concentration in the reaction, it is recommended to dilute the cDNA samples with nuclease-free water because of the possible inactivation of the DNA polymerase by the presence of EDTA in the PCR reaction using Tris and EDTA buffer [7]. Dilution of the cDNA sample also dilutes the concentration of possible contaminants present that inhibit the correct course of PCR analysis [8]. Using undiluted cDNA template in real-time PCR analysis, there is a higher risk of amplification of unwanted, non-specific products [9].

Using the 5x HOT FIREPol® EvaGreen® qPCR Mix protocol and cDNA dilution 1/10, both product amplification (actin and chitinase gene) was obtained when both primer pairs actin F1/R1 as well as chitinase F1/R1 where used, but with different fluorescent intensity of obtained amplicon (table 2).

Table 2. Comparison of amplification characteristics for cDNA dilution in this study.

F1/R1 primer pair	Undiluted cDNA	
	lowest Ct	*temperature (°C)*
actin	34.31	56.0
chitinase	32.25	63.5
	highest RFU	*temperature (°C)*
actin	633	56.0
chitinase	588	60.4
	Diluted cDNA (1/10)	
	lowest Ct	*temperature (°C)*
actin	30.27	64.0
chitinase	32.49	63.5
	highest RFU	*temperature (°C)*
actin	645	54.7
chitinase	383	61.4

RFU – relative fluorescence unit

Dissociate curves of amplified chitinase products calculated during the melting procedure showed a single melting peak with melting temperature (Tm) of 80.5 °C, indicating specific product (figure 5).

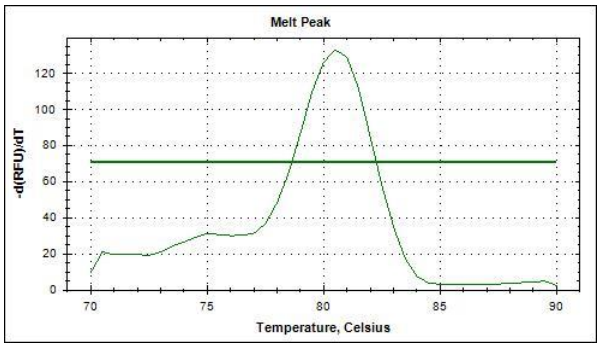

Figure 5. Melting temperature of analyzed chitinase amplicon as visualized by dissociation curve.

Final analysis was performed at the time and temperature profile as follows: 95°C 1 min; 30x (95°C 15 s, 60°C 15 s, 72°C 15 s) with the cDNA extracted from grape varieties Dornfelder, Cabernet, Alibernet and Frankovka. Selected protocol was proved as an efficient one for chitinase expression analysis, when the RNA normalization was performed for 70 ng of extracted total RNA (figure 6).

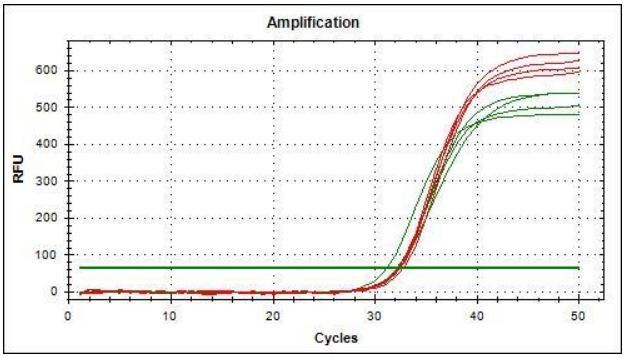

Figure 6. Amplification profile of actin (red profile) and chitinase (green profile) in the optimized PCR protocol.

Real-time PCR analyse is comprised of several steps that are necessary before the amplification of the cDNA. Typical steps include the sampling strategy and the extraction of the RNA; the process of reverse transcription and converting the mRNA it into cDNA; and the amplification and expression analysis. All of these steps are important to be optimized [10].

It is known that many molecular techniques, characterized by their advantages and disadvantages, are currently used in studies of gene expression [11]. In our experiment, chitinase expression reaction optimization parameters were analyzed by reverse transcription of RNA to cDNA and subsequent real-time PCR, which is widely used to detect food allergens [12]. RNA isolation was the first important step in our analysis because of the mature vine fruit contains a high concentration of water and secondary metabolites. Several plant species, including fruit, are known to be rich in primary and secondary metabolites - polysaccharides and phenol compounds whose presence affects the overall recovery and quality of the extracted RNA [13]. Also, mature grape berries include aqueous plant tissues characterized by the presence of a large number of phenolic substances that are capable of affecting the yield of RNA from the tissue. The difficulty of isolating RNA from aqueous plant tissues and the different recovery and purity of the isolated RNA by various commercially available isolation kits that use different types of chemicals was confirmed previously [14].

4. Conclusions

Chitinase is one of allergen in mature grape berries, but its importance is quite high, because of its persistence during the technological processing of wine. Preparation of protocol of rapid analysis of expression differences of chitinase in the *Vitis vinifera* L. varieties will help to analyse its natural variability in the future.

Acknowledgements

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