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A THAUMATIN-LIKE GENOMIC SEQUENCE IDENTIFICATION IN *Vitis vinifera* L., STORMY WINES AND MUSTS BASED ON DIRECT PCR

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ABSTRACT

Direct polymerase chain reaction method was use to amplify a thaumatin-like sequence of *Vitis vinifera* L. in grapes as well as in stormy wines and musts. Thaumatin-like proteins (TLPs) of *Vitis vinifera* possess beside its function in abiotic and biotic stress response another one – they are able to cause protein haze in wine unless removed prior to bottling. Direct PCR is an approach where omission of DNA extraction is typical prior the amplification of the target site of plant genome. Crude extract or small pieces of plant tissues are used in the analysis directly without steps of extraction and purification of gDNA. The biological material that was used in analysis was collected during August – October 2017 in local stores and winery Sabo and comprises from cultivars Iršai, Muškát, Savignon Blanc, Svätovavrinecké, Dornfelder and Pálava. Direct PCR was performed by a cutted piece of grape tissue and a dilution buffer was use in 1:2 for stormy wine or must, respectively. Direct amplification of thaumatin-like protein sequence of *Vitis vinifera* was performed along with the control reactions with the primers for conserved region of plant chloroplast. Possitive amplification of thaumatin-like allergen sequence resulted in 570 bp amplicon. The most abundant amplicons were amplified in stormy wines, followed by musts and the amplicons from grapes were weaker when comparing them to others. The amplicon specificity checking of obtained PCR product of thaumatin-like allergen was performed by restriction cleavage by Psi I and resulted in restriction amplicons of the 80 bp, 81 bp, 94 bp and 315 bp in length. Confirmation of the amplicon specificity by restriction cleavage support the potential of direct PCR to become a reproducible method that will be fully applicable in routine analysis of not only plant genomes in the future, but it was demonstrated, that it works in liquids, too.

Keywords: direct PCR; *Vitis vinifera* L.; thaumatin-like sequence; stormy wine; must

INTRODUCTION

Thaumatin protein was firstly described in fruits of *Thaumatococcus danielii* Benth. **(Van der Wel and Loeve, 1972)**. Thaumatin comprises from a typical osmotin-like protein domain and thaumatin-like protein family **(Wang et al., 2011)**.

Thaumatin-like proteins are reported as a very diversified in their function **(Liu et al., 2010)** from the stress related responses to drought, cold or salt **(D´Angeli and Altamura, 2007; Husaini and Abdin, 2008; Parkhi et al., 2009)** or the resistance to different pathogens **(Garcia-Casado et al., 2000; Chu and Ng, 2003; Ho et al., 2007)**. Some authors have demonstated the engagement of thaumatin-like proteins of *Vitis vinifera* L. in the pathogen resistance response to *E. ampelina* and *E. necator* **(Jayasankar et al., 2000; Yan et al. 2017)**.

Thaumatin-like proteins (TLPs) of *Vitis vinifera* L. possess beside its function in plant-pathogen interactions another one – they are able to cause protein haze in wine unless removed prior to bottling **(Marangon et al., 2014)**. This is the most important instability that is not caused by microorganisms especially for white wine production, as the pathogen-related proteins has the potential to aggregate to form a visible haze **(Ferreira et al., 2001; Waters et al., 2005)**.

Here, a direct polymerase chain reaction method was use to amplify a thaumatin-like sequence of *Vitis vinifera* L. in grapes as well as in different liquid products. Direct PCR is an approach where omitting of DNA extraction is typical prior the amplification of the target site of plant genome. Crude extract or small pieces of plant tissues are used in the analysis directly without steps of extraction and purification of gDNA **(Chum et al., 2012)**. If none DNA extraction step is used in the PCR workflow, the benefits of utilization of samples without loss, saving time and reducing the cost of analysis are typical.

Direct PCR can facilitate routine genotyping widely in the future, but to date, its application is not typically used in plant genomes analysis. This is caused by a specific composition of plant cells where much more PCR contaminats exist **(Bošeľová et al., 2016; Bošeľová, Žiarovská, 2016; Žiarovská et al., 2016, 2017).** Direct

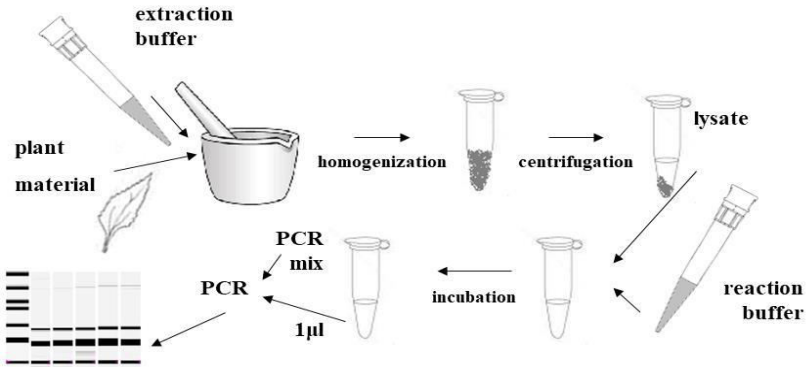

Figure 1 Workflow of direct PCR **(Žiarovská et al., 2016).**

PCR was used in plants firstly to amplify the *Nit1* gene of *Arabidopsis thaliana*, L. **(Young et al., 2007)**. The combination of commercial extraction buffer and in laboratory prepared specific PCR buffer was used to amplify the target (Figure 1).

The aim of the study was to analyse the possibility of amplification of thaumatin-like sequence of *Vitis vinifera* L. in grape and liquid products directly without DNA extraction and to optimize this for routine analysis.

Scientific hypothesis

The concentration of PCR inhibitors in stormy wines and musts can be overcome by direct PCR and a routine amplification protocol can be set-up for the purpose of thaumatin-like allergen DNA screening.

Statisic analysis

Testing of the hypothese based on the reported chemical content **(Aubert and Chalot, 2018)** of *Vitis vinifera* L. is primary the qualitative analysis of obtained (or not) amplicons by resolution through an agarose gel. Statistical evaluation of the results was applied for data of obtained amount of amplicons where the ezANOVA software for Windows (http://www.cabiatl.com/mricro/ezanova/) was used. The analyses of the amount of amplified PCR products were performed by the interpolation of density of pixels to the marker of knowing amounts using the software SynGeneGeneTools 4.01.04. Measurements of triplicate samples were subjected to the multifactorial analysis of variance and pairwise comparisons with Tukey HSD with the level of significance associated to the statistical test 0.05. The null hypothesis was tested that the difference exists among the amounts of obtained PCR amplicons depending on the type of used biological material.

MATERIAL AND METHODOLOGY

Biological material

The biological material that was used in analysis was collected during August – October 2017 in local stores and winery Sabo (Vrbové, Slovak Republic) (Table 1).

Primer design

BLAST alignment **(Zhang et al., 2000)** of the thaumatin-like protein, (NCBI accession code AF227324.1) was done by BLASTtn against *Vitis vinifera* (taxid: 29760) nucleotide sequences in the NCBI database to check the specifity or existing nucleotide differences. Primer design was performed in Primer-BLAST **(Ye et al., 2012)**.

Table 1 Codes of samples used in the study.

Code of sample	*Vitis vinifera* variety	Type of sample
I	Iršai	grape
M	Muškát	grape
SB	Savignon Blanc	stormy wine
SV	Svätovavrinecké	stormy wine
D	Dornfelder	must
P	Pálava	must

Direct PCR

Direct PCR was performed by Phire® Plant Direct PCR Kit (Thermo Scientific). A 0.35 mm cutter was used to obtain a piece of grape tissue and a dillution buffer was use in 1:2 with stormy wine or must, respectively. All the cutted pieces as well as dillutions were prepared in technical triplicates.

The following thermal and time profile was used: 98 °C for 5 min; 39 cycles of : 98 °C for 10 sec; 55 °C for 10 sec; 72 °C for 30 sec with final 72 °C for 5 minutes. All the amplification reactions were performed in C1000 thermocycler (BioRad).

Product specificity verification

Nucleotide sequence of *Vitis vinifera* thaumatin-like protein was uploaded into the NEBcutter v 2.0 **(Vincze et al., 2003)** and the specific restriction enzyme was selected to verify the PCR amplicons. The amplified direct PCR product was inspected for the specificity using the Psi I restriction endonuclease that cleaves in total four restriction sites.

RESULTS AND DISCUSSION

Thaumatin-like allergen of *Vitis vinifera* L. is defined well on both, protein as well as genomic sequence level. To date, a total of twenty-eight sequence accessions are available in the NCBI nucleotide database, from which ten are predicted (Table 2).

Table 2 Names and accessions of known thaumatin-likesequences.

Thaumatin-likesequences in NCBI
Vitis vinifera mRNA for putative thaumatin-likeprotein, partial 612 bp linear mRNA; Accession code: AJ237998.1
Vitis vinifera mRNA for thaumatin-like protein 918 bp linear mRNA; Accession code: AJ237999.1
Vitis vinifera thaumatin-like protein (TL3), mRNA 678 bp linear mRNA; Accession code: NM_001281202.1
Vitis vinifera thaumatin-like protein VVTL1 mRNA, complete cds 954 bplinear mRNA; Accession code: AF003007.1
Vitis vinifera cultivar Regent thaumatin-like protein mRNA, complete cds 678 bplinear mRNA; Accession code: DQ406688.1
Vitis vinifera thaumatin-like protein (Tl3) mRNA, complete cds 915 bp linear mRNA; Accession code: AF532965.1
Vitis vinifera cultivar Riesling thaumatin-like protein mRNA, complete cds 678 bp linear mRNA; Accession code: DQ406687.1
Vitis vinifera thaumatin-like protein gene, complete cds 2,777 bp linear DNA; Accession code: AF227324.1
Predicted Thaumatin-like sequences in NCBI
Vitis vinifera thaumatin-like protein 1 (LOC100265945), mRNA 862 bplinear mRNA; Accession code: XM_002265816.3
Vitis vinifera thaumatin-like protein (LOC100261232), mRNA 981 bp linear mRNA; Accession code: XM_003634158.3
Vitis vinifera thaumatin-likeprotein 1 (LOC100246972), mRNA 1,939 bp linear mRNA; Accession code: XM_010664644.2
Vitis vinifera thaumatin-like protein (LOC100242321), mRNA 2,096 bp linear mRNA; Accession code: XM_010662681.2
Vitis vinifera thaumatin-like protein (LOC100253199), mRNA 1,534 bp linear mRNA; Accessioncode: XM_002273235.4
Vitis vinifera thaumatin-like protein (LOC100265026), mRNA 945 bplinear mRNA; Accession code: XM_002282957.3
Vitis vinifera thaumatin-like protein (LOC100259841), mRNA 903 bp linear mRNA; Accession code: XM_002282994.3
Vitis vinifera thaumatin-like protein (LOC100254732), mRNA 772 bp linear mRNA; Accessioncode: XM_002283006.4
Vitis vinifera thaumatin-like protein (LOC100242737), mRNA 987 bp linear mRNA; Accession code: XM_002282952.4
Vitis vinifera thaumatin-like protein (LOC100244461), mRNA 905 bp linear mRNA; Accession code: XM_010662912.2
Vitis vinifera thaumatin-like protein 1b (LOC100248638), mRNA 890 bp linear mRNA; Accession code: XM_002265565.3
Vitis vinifera thaumatin-like protein (LOC100854994), mRNA 1,176 bp linear mRNA; Accessioncode: XM_003632623.3
Vitis vinifera thaumatin-like protein 1b (LOC100247111), mRNA 1,207 bp linear mRNA; Accessioncode: XM_002265769.3
Vitis vinifera thaumatin-like protein 1b (LOC100264253), mRNA 4,490 bp linear mRNA; Accession code: XM_002265889.4
Vitis vinifera thaumatin-like protein 1b (LOC100265907), mRNA 1,840 bp linear mRNA; Accession code: XM_002277512.4
Vitis vinifera thaumatin-like protein 1b (LOC100232855), mRNA 1,317 bp linear mRNA; Accession code: XM_002277426.3
Vitis vinifera thaumatin-like protein 1b (LOC100257373), mRNA 1,677 bp linear mRNA; Accession code: XM_002274101.4
Vitis vinifera thaumatin-like protein 1b (LOC100242003), mRNA 1,537 bp linear mRNA; Accession code: XM_010666529.2
Vitis vinifera thaumatin-like protein (LOC109124045), mRNA 351 bp linear mRNA; Accession code: XM_019225556.1
Vitis vinifera thaumatin-like protein (LOC100247919), mRNA 1,143 bp linear mRNA; Accession code: XM_002278043.3

Note: Thaumatin-like DNA sequence is written in bold in the table.

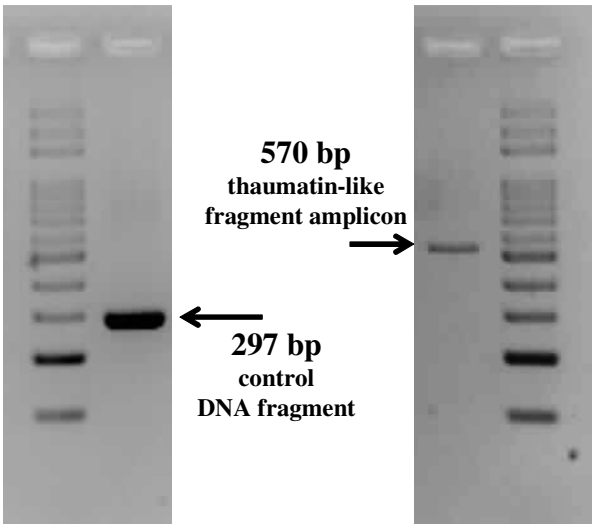

Figure 2 Control and amplified thaumatin-like amplicons lenght checking in agarose gel electrophoresis.

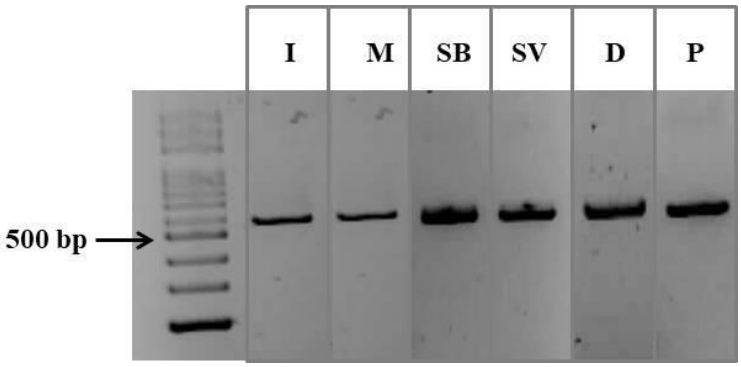

Figure 3 Amplicons of thaumatin-like allergen sequences resulted in direct PCR.

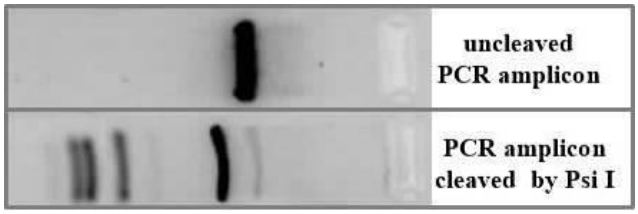

Figure 4 Restriction cleavage of amplified sequence of thaumatin-like allergen of *Vitis vinifera*, L.

Direct amplification of thaumatin-like protein sequence of *Vitis vinifera* was performed along with the control reactions with the primers for conserved region of plant chloroplast. In all the samples, control reaction was positive with the amplicon of 297 bp (Figure 2). This shows the suitability of Phire® Plant Direct PCR Kit for working with liquid products such as stromy wines and musts. Positive amplification of thaumatin-like allergen sequence resulted in amplicon of the length of 570bp (Figure 2).

Subsequent direct PCR was performed when using all the three types of samples – grapes, stormy wines and musts. In all the cases, an amplicon of appropriate length was obtained (Figure 3). The most abundant amplicons were amplified in stormy wines, followed by musts and the amplicons from grapes were the weaker when comparing them to others. The amplicon abundancy was statistical relevant among the types of the biological material, not between different varieties for individual types of biological material except of the must samples (Table 2).

Actually, amplifications of DNA regions of different, not only plant species, is well established method. Molecular and DNA analysis became a standard part of the research in many areas and these analysis are performed in a wide range of different approaches ranged from DNA markers based analysis up to the specific analysis of plant allergens detection or their expression (**Medo et al., 2015; Žiarovská et al., 2015; Oslovičová et al., 2014; Revák et al., 2014; Trebichalský et al., 2013; Židek et al., 2012; Milella et al., 2011**).

Table 2 ANOVA analysis of theyield of ampliconsobtained by direct PCR.

Descriptive details/	Types of samples and varieties					
	grape Iršai	grape Muškát	stormy wine Savignon Blanc	stormy wine Svätovavrinecké	must Dornfelder	must Pálava
Mean	101.75	90.57	179.82	151.26	190.34	191.55
StDev	1.59	1.9	3.73	1.48	1.86	1.71
SE	0.92	1.1	2.16	0.85	1.07	0.99
Var	2.53	3.62	13.95	2.18	3.45	2.92
CI 95%	2.75	2.75	2.75	2.75	2.75	2.75
N	3	3	3	3	3	3
Skew	1.393	-0.314	-1.466	-0.443	0.266	1.708
zSkew	0.985	-0.222	-1.037	-0.313	0.188	1.208

PAIRWISE COMPARISONS
[grape_Iršai]vs[grape_2] t(4) = 7.83 p <0.0014
[grape_1] vs [stormy wine_1] t(4) = 33.30 p <0.0001
[grape_1] vs [stormy wine_2] t(4) =39.49 p <0.0001
[grape_1] vs [must_1] t(4) = 62.71 p <0.0001
[grape_1] vs [must_2] t(4) = 66.58 p <0.0001
[grape_2] vs [stormy wine_1] t(4) = 36.88 p <0.0001
[grape_2] vs [stormy wine_2] t(4) = 43.65 p <0.0001
[grape_2] vs [must_1] t(4) = 64.98 p <0.0001
[grape_2] vs [must_2] t(4) = 68.38 p <0.0001
[stormy wine_1] vs [stormy wine_2] t(4) = 12.32 p <0.0002
[stormy wine_1] vs [must_1] t(4) = 4.37 p <0.0120
[stormy wine_1] vs [must_2] t(4) = 4.95 p <0.0078
[stormy wine_2] vs [must_1] t(4) = 28.53 p <0.0001
[stormy wine_2] vs [must_2] t(4) = 30.91 p <0.0001
[must_1] vs [must_2] t(4) = 0.83 p <0.4530

Note: Codes of *Vitisvinifera* L. varieties in pairwisecoparison are as follows: grape 1 – Iršai, grape 2 – Muškát, stromy wine 1 – Savignon Blanc, stromy wine 2 – Svätovavrinecké, must 1 – Dornfelder, must 2 – Pálava.

Direct PCR method was applied here to amplify a thaumatin-like allergen sequence of *Vitis vinifera* L. This method was succesfully used by **Belstedt et al. (2010)** previously to amplify fragments of plant genomes of thirty-two different plant families. The authors have used the extraction and PCR buffer to one amplicon analysis to be fully function in the species, that possess a wide range of PCR contaminants – *Coffea arabica* L.; *Thymus vulgaris* L. *Olea europea*, L. or *Lauru snobilis* L. PCR amplification in their study was carried out using standard universal primers of the chloroplast-encoded trn L-F locus with a positive amplification in all of the tested plant species except of two – *Cyatheade albata* (G. Forst.) Swartz and *Carpobrotus* sp. **Bošeľová and Žiarovská (2016)** reported direct PCR approach applicable in the marker based analysis of plants, too. They have used the direct PCR protocol to analyse the PBA polymorphism of *Hedera helix*, L.

The amplicon specificity checking of obtained PCR product of thaumatin-like allergen was performed by restriction cleavage by Psi I (Figure 4). In total, four restriction sites are predicted in the amplicon by NebCutterv 2.0 **(Vincze et al., 2003)** that resulted in restriction amplicons of the 80 bp, 81 bp, 94 bp and 315 bp in length.

The wine production is one of the most profitable agricultural activities and a wide diversity of *Vitis vinifera* L. cultivars are involved in the production of wine **(Briciu et al., 2010)**. This need to verify different DNA based techniques to characterize and authentify grapevine genepool. DNA of *Vitis vinifera* was reported previously as to be extracted from different parts of the plant and many studies were aimed to the methods of extracting DNA from grapevine products such as grape juice, musts or wines of different stages of the processing **(Faria et al. 2000; Siret et al. 2002; Baleiras-Couto and Eiras-Dias 2006; Faria et al. 2008; Drábek et al. 2008; Işçi et al., 2014)**, but in this studies it is concluded, that an efficient DNA extraction and subsequent PCR analysis from must and wines is not fully reproducible an still difficult. **Garcia-Beneytez et al. (2002)** suppose these difficulties because of specific processes of wine production. These together resulted completely removing of grapevine DNA. On the other hand, extraction of DNA from must is difficult thank to the presence of high levels of polyphenols and polysaccharides **(Briciu et al., 2010)**.

Confirmation of the amplicon specificity by restriction cleavage support the potential of direct PCR to become a reproducible method that will be fully applicable in routine analysis of not only plant genomes in the future, but it was demonstrated, that it works in liquids, too. The development a proving the efficient and reliable methods for tracing *Vitis vinifera* cultivars and its products is an

important aim as demand grows for origin product and sources of knowledge.

CONCLUSION

Direct PCR can be used well in *Vitis vinifera* L. genomic sequences amplification. Direct PCR was proved here to work on all the three types of tested samples – grapes, stormy wines and musts. In all the cases of qualitative analyse of the results an amplicon of thaumatin-like genomic sequence was obtained with the appropriate length of 570 bp. The most abundant amplicons were amplified in stormy wines, followed by musts and the amplicons from grapes were the weaker when comparing them to others. When regarding the quantitative analyse of results, grape and stormy wine amplicons abundance obtained by direct PCR is statistical relevant for different varieties with the p <0.05 and for must the p value was 0.453. Direct PCR provides a reliable method for rapid screening of allergen genomic sequences and can be utilized in liquid products, too.

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