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COMPARATIVE ANALYSIS OF ALLERGENIC CHITINASE EXPRESSION IN GRAPE VARIETIES

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Abstract

Vitis vinifera L. is a well-known cultural plant, which is grown on thousands of hectares throughout the world. However, it has the potential to cause allergic reactions. Even allergy to grapes is not a common allergic reaction, the prevalence of this type of allergy is growing, particularly in southern Europe countries. Here, we focused on the differences in the chitinase expression in selected varieties of grape vine.

Matured grapes of following varieties were analysed: Sauvignon blanc, Riesling white, Pesecká leánka, Alibernet, Pinot blanc, Valteliner blanc, Cabernet, Irsay Oliver, Müller Thurgau, Dornfelder, Limberger, Riesling blanc, and Pálava. Two step real-time PCR were performed to analyse the expression of chitinase allergen. The chitinase expression was measured via delta delta Ct method and all samples were cross-compared.

In the group of blue grape varieties, the lowest average Ct value was obtained for Cabernet variety with the value of 30.18 and in the group of white grape varieties, the lowest average Ct value was obtained for Pesecká leánka variety with the value of 32.34. The resulting values of the chitinase expression are in three different groups: a 2 - 20 fold changes in the chitinase expression, a 20 - 60 fold changes and two extreme values > 60 fold changes in the chitinase expression. In every comparison, Sauvignon blanc variety showed an increased chitinase expression, and in contrary, Riesling white variety showed a decrease of the chitinase expression in cross-comparisons. Expression fold change less than 2 times was in cross-comparisons among all of the analysed varieties obtained for Alibernet and Pinot Blanc.

The obtained results confirmed the existence of a naturally high variability occurring in the chitinase expression within different varieties of one specie.

***Key words**: Expression, Grape varieties, Chitinase, Relative quantification.*

1. Introduction

Vitis vinifera ssp. sativa is one of the oldest and a very popular fruit in the world. Its cultivation history began as early as 65 million years ago. The cultivation of grape is widespread worldwide and is the most commonly grown from *Vitis* specie [1]. Currently, the availability of *Vitis vinifera* annotated genome allows the identification of the expression of many genes, what contribute s to the efficiency of genomic and proteomic studies of grape [2].

Questions connected to food allergy in humans and more particular allergic reactions to various fruits, including grapes, is actually an increasingly discussed topic. Plant tissues that are consumed by humans contain many of diverse proteins that may have the potential to cause allergic reactions. They are referred as plant food allergens, which are classified

intoa specific groups according to their structure and functional properties of the proteins. Most food plant allergens belong to several protein families and superfamilies [3]. The first case of grapes allergy was reported [4]. Despite the fact that the incidence of this type of allergy is not frequent when compared to other food allergens, allergic reactions to different grape vine varieties or grape products such as grape vine have been described over the last two decades. In grapes, different allergens were identified such as endochitinase, lipid transfer protein (LTP) and taumatin-like protein (TLP) [5]. In wines made from both blue and white grape varieties, several studies have confirmed the presence of proteins, especially in the Mediterranean region, which are often discussed as potential allergens. In the wine production process, proteins are exposed to several conditions such as e.g. high alcohol content and low pH, which could ultimately alter the protein's structure and thus affect its allergenic potential. When investigating the structural stability of LTPs during the wine making process, no structural changes were found, suggesting that this protein may continue to be allergenic in wines [6].

Endochitinase 4A is refered to be the major allergen in young wines and Fragolino wines. Many allergic reactions have been observed in people after the consumption of grapes and also after the consumption of two types 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 has matured. The amount of chitinases present in the grapes represents 50% of the soluble proteins and are able to persist in the grapes during the wine making process, subsequently aggregating and creating haze in the wine [7].

The formation of protein haze is considered to be an undesirable effect during wine production, particularly in wines prepared from white varieties of grape vine. The amount of all proteins found in grape juice or wine is in the range of 10 - 500 mg/L. Despite this low concentration, they cause problems in the technological process of wine production. These include chitinases and TLP. Several studies have confirmed that chitinases are more thermally unstable and more subject to aggregate formation in wines compared to TLP [8]. During the ripening process, the concentration of sugars is significantly increased, the thickness of the grape skin is reduced, making the grapes more susceptible to fungal attack. Higher expression of chitinase in grapes has been confirmed during the maturation phase, which may be considered as a possible defense response of the plant against fungal attack [9].

Several studies that have examined chitinase expression in grapevine have confirmed increased expression of chitinase type IV in the maturation phase of vine fruit. This activity is attributed to the possible defense of the plant against fungal attack as well as abiotic stress. Chitinase type IV has been detected in grape juice from several grape varieties as well as in wines processed from it [10].

The aim of the study was to quantify the expression of chitinase in selected varieties of grape vine (*Vitis vinifera*) harvested from a vineyard in Vrbové belonging to the Sabo Winery, which is part of the Small Carpathian wine region of Slovakia.

2. Materials and Methods

2.1 Plant material, RNA extraction and preparation of cDNA

Matured grapes of following varieties were obtained in the vineyard in Vrbové (Slovak Republic; Small Carpathian Wine Region of Slovakia): Sauvignon blanc, Riesling white, Pesecká leánka, Alibernet, Pinot blanc, Valteliner blanc, Cabernet, Irsay Oliver, Müller Thurgau, Dornfelder, Limberger, Riesling blanc and Pálava. The grapes were collected in closable sterile paper bags. Until the subsequent processing, sampled grapes were stored in a deep-freezer at - 80 ^{0}C for 1 month.

Total RNA was extracted by Isolate II RNA Plant Kit (BIOLINE®). The quality and quantity of extracted RNA was measured by NanoPhotometer® (Implen). Two step real-time PCR were performed to analyse the expression of chitinase allergen. A total of 10 ng of RNA was used to prepare cDNA according the manufacturer's instructions of cDNA Tetro cDNA Synthesis Kit (BIOLINE®) when combine both, oligo-dT and hexamer random primers.

2.2 Real-time PCR analysis and data processing

Chitinase transcripts were amplified by 5 x HOT FIREPol® EvaGreen® qPCR Mix (Solis BioDyne) using the 300 nM of each primer and 1 µL of 1/10 diluted cDNA. Primers for the chitinase specific amplification were designed by Primer3web version 4.0.0, [22], based on the *Vitis vinifera* chitinase class I mRNA complete cds sequence stored in NCBI database under the accession number DQ267094. Actin was used as the housekeeping gene and the primers designed for its amplification were based on the nucleotide sequence of actin mRNA of Riesling vine variety stored in the NCBI under acession AY847627.1.

Thermal and time profile of PCR reactions were as follows: 95 ^{0}C 15 min; 40 x (95 ^{0}C 30 sec; 60 ^{0}C 30 sec; 72 ^{0}C 30 sec.) and final polymerization 95 ^{0}C 10 sec.

Amplified products were checked by melt point analysis.

The chitinase expression was measured via delta delta Ct method [23], and all samples were cross-compared.

3. Results and Discussion

A number of vegetables and fruits, including grapes, are described as minor allergenic foods. Nevertheless, many researches in the last two decades have described cases of manifesting allergic reactions after consumption of grapes, grape juice and wine [5]. Chitinase, whose relative expression has been detected in 13 varieties of grape vine in this work, is reported to be the main potential allergen expressed to an increased extent during the ripening of grape vine fruit, according to several studies. In addition to chitinase, other proteins with potential allergenicity and, in some cases, possible cross-reactivity have been gradually discovered in mature grape vine berries [11]. Together with chitinase, they are among the known PR proteins.

The analysis of the data of expression ratios in selected grape vine varieties, 3 general groups were found be different in the relative expression of chitinase. Several studies evaluating relative gene expression of RT-qPCR analysis find values achieving less than a 2-fold change in gene expression as statistically insignificant when comparing expression profiles of multiple samples with each other [12].

When comparing varietal differences in gene expression of grapevine chitinase, most often was a 2 - 20 fold change. However, a change in expression ratio of less than 2-fold was most often detected when comparing varieties Alibernet and Pinot Blanc to all of others. These two grape varieties show only a minimal change in chitinase expression compared to the others except of the Sauvignon Blanc variety. Based on these results, it can be assumed that the Alibernet and Pinot Blanc varieties have approximately the same gene expression of chitinase in mature grapes when compared to the other varieties. Compared to the Sauvignon blanc variety, which in any case had an increased expression of chitinase over the other varieties, the varieties Alibernet and Pinot Blanc showed the same decreased chitinase expression - 2.9-fold. Similarly, when comparing the Alibernet and Pinot Blanc varieties with the Riesling variety, that showed a reduced chitinase expression for each combination of varieties, the Alibernet and Pinot Blanc varieties achieved almost the same increase in chitinase expression compared to the Rhine Riesling variety - 40-fold higher. A minimal change in the expression profile of chitinase when compared to all of other analysed was obtained for the Riesling and Sauvignon blanc varieties. The Riesling variety showed a differently reduced chitinase expression with the varieties compared, with the highest difference compared to Alibernet, Pinot Blanc, Sauvignon blanc - more than 40-fold. In contrast, the Sauvignon blanc variety showed a differently increased expression of chitinase over the other varieties compared, in two cases an increase of more than 100-fold. Such a high change in chitinase expression was not observed in other varietal comparisons (Table 1).

Chitinase activity has been observed and confirmed in different *Vitis vinifera* L tissues, such as in leaves, roots

Table 1. Relative quantification of expression fold changes of chitinase in analysed *Vitis vinifera* L., grape varieties

	Pese. leánka	Ries. white	Alibe.	Rulan. biele	Valte. blanc	Cabe r.	Müller Thurg.	Irsay Oliver	Limbe.	Dorn felder	Ries. blanc	Sauvi. blanc	Pálava
Pesecká leánka		2.3	x	18.9	2.4	x	3.2	7.1	x	5	x	52.6	x
Riesling white	2.3		40	43.5	5.5	x	7.1	15.9	2.6	11	4.4	117	57
Alibernet	x	40		x	x	x	x	x	x	x	x	2.9	x
Rulandské biele	18.8	x	43.5		x	x	x	x	x	x	x	2.9	x
Valteliner blanc	2.4	5.5	x	x		x	x	2.9	x	2	x	21.3	x
Cabernet	x	x	x	x	x		7.1	16.6	2.6	10.5	4.2	111	x
Müller Thurgau	3.2	7.1	x	x	x	7.14		2.2	x	x	x	16.6	x
Irsay Oliver	7.1	15.9	x	x	2.9	16.6	2.2		x	x	x	7.7	x
Limberger	x	2.6	x	x	x	2.6	x	x		4.2	x	43.5	x
Dorn felder	5	11	x	x	2	10.5	x	x	4.2		x	11	x
Riesling blanc	x	4.4	x	x	x	4.2	x	x	x	x		33.3	x
Sauvignon blanc	52.6	117	2.9	2.9	21.3	111	16.6	7.7	43.5	11	33.3		57.4
Pálava	x	2.1	x	x	x	x	x	x	x	x	x	57.4	

Legend: - decreased fold change of chitinase expression - increased fold change of chitinase expression x - fold change of chitinase expression less than 2-times.

and grapes. However, the highest specific activity of chitinase has been detected in grapes, where it exceeds about 10 times the chitinase activity existing in grape leaves [13]. Both the many PR-proteins present in the mature grapes and the enzyme chitinase exist in several different isoforms having chitinolytic activity. A study of chitinase isoforms present in wine produced from the variety Manzoni Bianco confirmed the presence of several isoforms of the chitinase enzyme, which may contribute to the formation of undesirable haze proteins in wine production [14].

A recent study analyzed defense mechanisms of grapevine under abiotic stress factors in drought conditions, confirmed the decreased or overexpressed genes encoding various proteins, including chitinase [15]. Increased accumulation of secondary metabolites and phytohormones, synthesized by various metabolic pathways were found mainly in mature grapes and may be related to chitinase activity, has also been confirmed under drought stress.

The study confirmed 130-fold and 80-fold higher chitinase activity in the mature fruits of *Vitis vinifera* of Cabernet Sauvignon and Chardonnay varieties compared to the muscadine grape of *Vitis rotundifolia* [16]. The presence of allergizing proteins was compared in grape juice of Sauvignon blanc and Chardonnay and found an increased content of chitinase (mg/L) in grape juice prepared from Sauvignon blanc [17]. Also in [18], the presence of PR proteins, including chitinase, in mature berries compared to grape peel, grape pulp and grape seeds was relatively quantified in Sauvignon blanc. They found that PR proteins TLP and chitinase are present in high concentrations in grape peel and pulp, while their presence was not detected at all in grape seeds. Also, the concentration of chitinase and TLP in the flesh was much higher than in the berry skin. This finding contributes to the fact that PR proteins such as TLP allergens and chitinase are able to persist in grape juice and wine and have the potential to cause allergic reactions.

Not only in grapevine but also in other fruits, the expression of various proteins with the potential to cause allergic reactions was confirmed during the ripening stage. The expression of Fra and allergen was analyzed during the ripening of strawberries that were treated with exogenous auxin [19]. The study found that auxin may affect the gene expression of Fra and the allergen, but probably does not participate in its direct regulation. More likely, the gene expression of Fra and allergen in strawberries is induced by metabolic processes and by the action of abiotic and biotic stress factors. Another study analyzed the expression of allergen Fra and 1 belonging to PR proteins at each stage of strawberry ripening [20]. At the same time, the weight and anthocyanins content of strawberries was measured in the analysis to assess the potential correlation with Fra and 1 allergen. It was found that the expression level of Fra and 1 gene was highest in the early ripening stage of strawberry and about 70 times. As the Fra a 1 allergen is a PR protein, the result of the study may indicate the possible effect of oxidative stress on strawberries in the early stages of ripening. It has also been confirmed that genes encoding Fra and allergen are overexpressed in response to stress conditions. Possible causes of overexpression of Fra genes are elements related to the response to stress conditions - the so-called. cis elements interacting with the Fra a gene promoter region. Thus the different gene expression of chitinase in selected varieties of grape vine could be influenced by the activity of cis-regulatory elements in the promoter region of the gene encoding the enzyme chitinase and transcriptional factors [21].

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4. Conclusions

- Different types of food allergies are actually clinical relevant and many of them are connected to specific parts of continets are minor. Our knowledge about natural genomic and transcriptomic variability of allergens in the raw foodstuff such as fruits or vegetables is still limited.

- Quantitative real-time PCR was used in the study to characterize varietal differencies in chitinase expression among thirteen matured grape varieties. The resulting values of the chitinase expression are in three different groups: a 2 - 20 fold changes in the chitinase expression, a 20 - 60 fold changes and two extreme values > 60 fold changes in the chitinase expression.

- In every comparison, Sauvignon blanc variety showed an increased chitinase expression, and in contrary, Riesling white variety showed a decrease of the chitinase expression in cross-comparisons.

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