Expression analysis of gibberellin metabolism genes and metabolites during berry development in table grape

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Abstract

 Berry size is a highly desired trait in table grapes. However, the processes of berry development and growth are still not well understood, even when gibberellins are clear candidates to have a crucial role in the regulation of berry size. Looking for clues about the participation of gibberellins in determining berry size, in this work we used segregants of a 'Ruby Seedless' × 'Sultanina' population with contrasting phenotypes for this trait, searching for transcriptional and metabolic information. We found three GA-oxidases genes (VvGA20ox2, VvGA3ox4 and VvGA2ox2) that showed differential expression during berry development. We also found that, depending on the stage of development, the bioactive metabolites GA_1 and GA_4 exhibit different levels.

Keywords: berry development, gibberellin oxidases, gibberellin metabolites

INTRODUCTION

Berry size is one of the most important traits for table grape producers, because of the better prices obtained for large berries. The expression of this trait depends on the genetic background, on environmental conditions and on hormonal control in which the gibberellins are thought to play a crucial role. Gibberellins are phytohormones that regulate several development processes, including berry development. In fact, spraying berries with gibberellic acid (GA_3) of seedless cultivars produces a notorious increase in size (Weaver, 1958; Pérez et al., 2000). Furthermore, the application of GA_3 to seeded genotypes also induces a certain degree of seed abortion. The success of exogenous GA treatment has broadened its use in the commercial production of table grapes.

Regarding table grapes, little is known about the factors that control final berry size, but it is known that the metabolic pathway of GAs is highly conserved in plants. Gibberellins metabolism is controlled by seven genes, each having several isoforms. The first four genes of the pathway regulate the production of GA precursors until the formation of GA_{12}, the first proper GA produced and the source of all other types of GAs. The last three genes are GA dioxidases (GA20ox, GA3ox and GA2ox), which are in charge of the conversion of GAs from C-20 to C-19 compounds, 3β-hydroxylation to obtain bioactive GAs and finally inactivation of hormones through 2β-hydroxylation, respectively.

The aim of this work was to search for genes and metabolites involved in gibberellin metabolism in three developmental stages of berry development (from flowering to 6-8 mm berry size) in table grapes, looking for the different expression of these genes depending on the distinct phenotypes of berry size.

MATERIALS AND METHODS

Plant material

Samples from 12 siblings of a population of 'Ruby Seedless' × 'Sultanina' were

collected in three different stages of berry development (50% flowering, berry-setting and 6-8 mm). The samples were selected according to berry size and seed content, combining extreme phenotypes: large berries/large seeds (LB/LS), large berries/seedless (LB/SS), small berries/ large seeds (SB/LS) and small berries/seedless (SB/SS). All samples were collected in triplicate, frozen in liquid nitrogen and stored at -80°C.

RNA extraction

RNA was extracted using a hot borate method adapted from Wan and Wilkins (1994). The method consisted of grinding the berry samples using liquid nitrogen. Then, 3 g of this powder was put in a 50 mL OakRidge tube (Nalgene) containing a buffer of 0.2 M Na borate decahydrate, 30 mM EGTA, 1% (w/v) SDS, 1% deoxicholate, 1% Nonidet P-40 (Igepal), and 2% (w/v) PVP. The extraction continued with the following steps:

1) Day one: samples were incubated for 60 min at 42°C; 0.08 volumes of KCl 2M were added to the mix and incubated on ice for 30 min. Then, samples were centrifuged at 12,000g for 20 min and supernatants were collected on a new OakRidge tube; one volume of LiCl 4M was added to the supernatant and the mixes were incubated overnight at 4°C.

2) Day two: Samples were centrifuged at 20,000g for 40 min and the supernatant was discarded; the pellet was resuspended with 500 μL of DEPC Water plus 50 μL of sodium acetate. The solution was transferred to 2-mL eppendorf tubes and 500 μL of chloroform/isoamyl alcohol was added before being centrifuged for 5 min at 14,000 rpm. The supernatant was recovered into another 2-mL eppendorf tube. This step was repeated and one volume of isopropanol was added to the tube and incubated on ice for 60 min. Later, the solution was centrifuged at 14,000 rpm for 40 min and the solution was discarded, leaving only the pellet. Then, 400 μL of ethanol was used for careful washing of the pellet. Another centrifugation at 14,000 rpm for 5 min was performed and the ethanol was discarded. Finally, the pellet was resuspended in 50 μL of DEPC water.

RNA-seq and bioinformatic analysis

RNA-seq was performed on RNA samples using an Illumina platform. Then, sequence assembly and database construction was performed at Centro de Modelamiento Matemático (CMM), Facultad de Ingeniería, Universidad de Chile.

qPCR

Real-Time PCR was performed using StepOne® equipment (Applied Biosystems). Three genes were selected for further studies based on the RNA-seq data: VvGA20ox2, VvGA3ox4 and VvGA2ox2.

Identification and quantification of GA metabolites in berries

Endogenous GA levels were searched and quantified in all samples from the three developmental stages previously mentioned. Extraction of GA derivatives was performed at Fondazione E. Mach following a protocol adapted from Chiwocha et al. (2003), Hirano et al. (2007) and Kojima et al. (2009). This protocol required only 0.1 g of starting material.

The eight gibberellins analysed were: GA_1, GA_3, GA_4, GA_8, GA_{20}, GA_{29}, GA_{34} and GA_{51} using a Xevo-MS (Waters) arrangement.

RESULTS AND DISCUSSION

Candidate genes selection

Using previously available information on GA oxidase genes for *Arabidopsis thaliana* and *Oryza sativa* (Yamaguchi, 2008), we searched for orthologues in the *Vitis vinifera* reference genome PN40024 (Jaillon et al., 2007). We found 12 GA-oxidases (Table 1). Then, using RNA-seq data from transcript expression experiments, we selected three genes for further validation by qPCR: VvGA20ox2, VvGA3ox4 and VvGA2ox2.

Table 1. GA-oxidase genes found in the PN40024 reference genome.

Vitis orthologue	Genoscope code	Chromosome
GA2ox1 *V. vinifera*	GSVIVT01000687001	19
GA2ox2 *V. vinifera*	GSVIVT01012628001	10
GA2ox3 *V. vinifera*	GSVIVT01021468001	10
GA2ox4 *V. vinifera*	GSVIVT01028169001	10
GA3ox1 *V. vinifera*	GSVIVT01017173001	9
GA3ox2 *V. vinifera*	GSVIVT01035796001	4
GA3ox3 *V. vinifera*	GSVIVT01017178001	9
GA3ox4 *V. vinifera*	GSVIVG01026415001	2
GA20ox1 *V. vinifera*	GSVIVT01027572001	15
GA20ox2 *V. vinifera*	GSVIVT01018453001	16
GA20ox3 *V. vinifera*	GSVIVT01018453001	16
GA20ox4 *V. vinifera*	GSVIVT01026453001	16

Twelve GA-oxidases genes found in PN40024 genome. The respective Genoscope number and chromosome location is indicated.

Differential expression by qPCR

- VvGA20ox2: GA20-oxidases are in charge of producing precursors of bioactive gibberellins. Differential expression was only observed in the 50% flowering and fruit-setting stages. Interestingly, higher expression levels were found for the SB/SS (small berries seedless) phenotype. The two phenotypes with large berries (LB/LS and LB/SS) had similar expression levels at 50% flowering, but at fruit-setting, higher expression in LB/SS segregants was observed (Figure 1).
- VvGA3ox4: The transcription level for this gene was higher at the 50% flowering and 6-8 mm stages in comparison to the fruit-setting stage. In both stages, there was a clear difference between seedless segregants with large berries compared to those harbouring small berries.
- VvGA2ox2: Expression levels were higher in the 50% flowering and 6-8 mm stages. There was no clear difference in gene expression level between the different phenotypes.
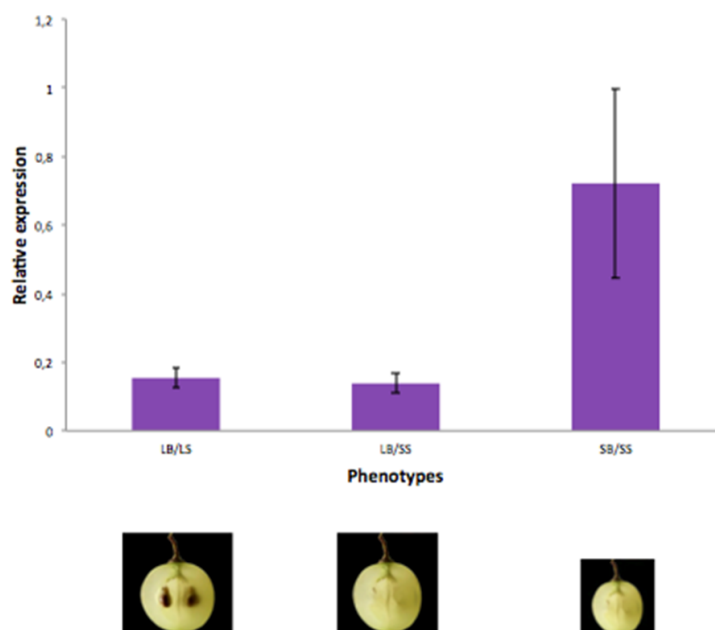

Figure 1. Relative expression of Vv-GA20ox2 gene at the 50% flowering stage. Phenotype according to berry size and seed content is shown.

Endogenous GAs quantification

The search for endogenous GAs revealed the presence of important levels for two bioactive GAs, GA_1 and GA_4, and their inactivation metabolites, GA_8 and GA_{34}, respectively. The related pair GA_1 plus GA_8 was predominant in the 50% flowering stage in phenotypes with large berries (data not shown). For GA_4 (Figure 2) and GA_{34}, contents were higher at the 6-8 mm stage, indicating the presence of both bioactive GAs at different stages during berry development. Also, this could indicate a possible correlation between bioactive GA levels and the sigmoidal growth curve observed for berry development; however, further metabolite studies are needed to corroborate this assumption.
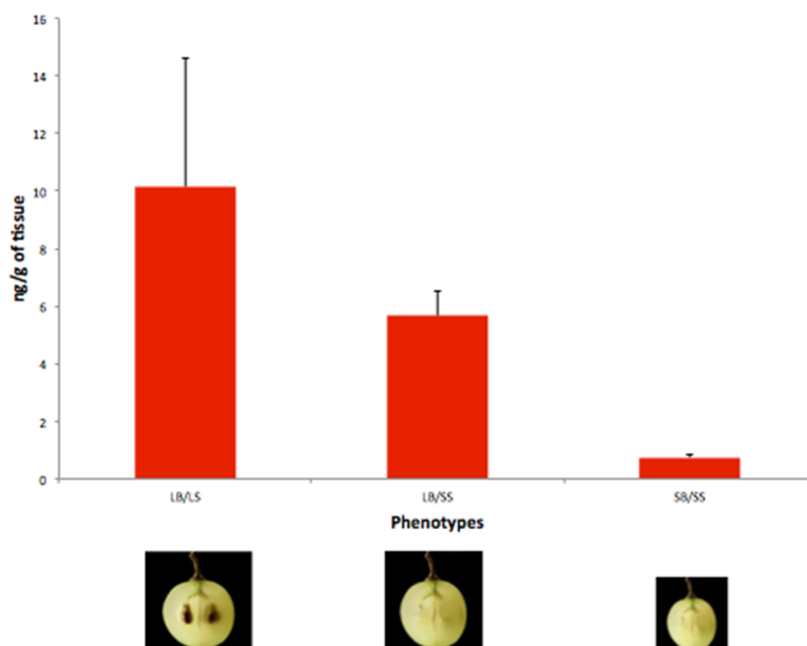

Figure 2. Contents of GA_4 metabolite in berries of segregants representing different phenotypes, at 6-8 mm developmental stage. Half berry is shown in each case.

CONCLUSION

Metabolite analyses revealed the presence of the two main plant bioactive gibberellins (GA_1 and GA_4) and their corresponding inactivated forms (GA_8 and GA_{34}).

GA_1 and GA_8 were observed mainly at the earliest phenological stage (50% flowering) considered in this work, as has been previously described. Unexpectedly, the pair GA_4 plus GA_{34} was predominant later during berry development, at the 6-8 mm stage. The confirmation of these results would require a more thorough evaluation considering additional samples and genotypes.

Results obtained in RNAseq were key to identifying genes of the GA pathway that were expressed at different levels during berry development. These genes were selected for further characterisation by qPCR. Of the three genes analysed, only two had differential expression between phenotypes (VvGA3ox4 and VvGA20ox2).

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