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Expression of two indole-3-acetic acid (IAA)-amido synthetase (GH3) genes during fruit development of raspberry (*Rubus idaeus* Heritage)

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ABSTRACT

The conjugation of indole-3-acetic acid (IAA) to amino acids by indole-3-acetic acid (IAA)-amido synthetases (GH3) is an important part of auxin level regulation. However, the auxin conjugation during development of soft fruits such as raspberry is poorly understood. In this study, indole-3-acetic acid (IAA)-amido synthetases in raspberry, designated as RiGH3 (RiGH3.1, RiGH3.5 transcripts) were evaluated during fruit development of raspberry cultivar *Rubus idaeus* Heritage, and under IAA treatment. The results showed that before to the onset of ripening the fruit size, weight and the expression of IAA-amido synthetase RiGH3.1 transcript levels increased. Then when the fruits attain full development, fruit firmness and titratable acidity decreased, in the contrast to ethylene production and total soluble solids content increasing. However, the RiGH3.5 transcript was found to be expressed primarily in flowers. When compared to untreated control fruit, fruit treated with 1 mM of IAA at white stage, showed an increase of RiGH3.1 transcript during *in-vitro* assay (10 °C by 18 h). However, no significant change in the levels of RiGH3.5 was observed during IAA treatment. Multiple alignments of the full-length predicted RiGH3.1 protein sequences revealed a high sequence homology with proteins deduced sequences described for other fruit of *Rosaceae* species. The RiGH3.1 deduced sequence showed the presence of binding motives for IAA and aspartic acid, and indicate that the isolated sequence have the typical motives of GH3.1 protein family. These findings give new insights into the possible role of RiGH3.1 transcripts, and the IAA conjugation (in maintaining the low concentration of free IAA) during raspberry fruit ripening.

1. Introduction

Raspberries are high value fruit crops due to their unique flavor, attractive color, high cost of production, and multifaceted health benefits (rich source of minerals and vitamins, dietary fiber and various antioxidants) (Pritts, 2003; Wang et al., 2009; Graham and Simpson, 2018). However, raspberries are soft fruit with a rapid ripening rate, leading to short postharvest shelf life. It has been reported that fruit ripening in raspberry is characterized by a progressive decrease in fruit firmness, associated to cell-wall modification and over-expression of softening related genes; starting from the large green to over ripe stages of ripening (Stewart et al., 2007; Vicente et al., 2007; Fuentes et al., 2015; Simpson et al., 2017; Graham and Simpson, 2018). Raspberry is categorized as a non-climacteric fruit (Pritts, 2003), although ethylene production and respiratory peaks have been detected in receptacle of white fruit stage until fruits attain full maturity (Blanpied, 1972; Fuentes et al., 2015).

Hormones such as auxins plays a critical role in fruit development

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and ripening, and indole-3-acetic acid (IAA) is described as an important regulator of fruit development (Cherian et al., 2014). In plants, two major pathways for IAA biosynthesis have been proposed: the tryptophan Trp-independent and Trp-dependent pathways (Mano and Nemoto, 2012; Yue et al., 2014; Rosquete et al., 2012). It has been reported that the over-expression of auxin-synthesis related gene (DefH9-iaaM) in strawberry and raspberry plants resulted significant increases in the number and size of fruits (Mezzetti et al., 2004). Among the three species tested, *F. vesca*, *F.*x *ananassa* and *R. idaeus* cv. 'Ruby', the transgenic plants showed an increased number of flowers per inflorescence and an increased number of inflorescences per plant (Mezzetti et al., 2004), suggesting an important role for IAA as regulator of raspberry fruit development.

The free-IAA is the biologically active form of the hormone, with amino acid conjugation leading to inactivation. Therefore, the conjugation of IAA to amino acids by indole-3-acetic acid (IAA)-amido synthetases (GH3) is an important step in auxin homeostasis (Peat et al., 2012). Conjugation of either alanine or leucine to IAA leads to an inactive but readily hydrolyzed storage form. However, the conjugation of IAA with either aspartate or glutamate leads to hormone degradation. It has been reported that the IAA-Trp conjugate has an anti-auxin activity role in plant growth, and also act as an important regulator during storage, and degradation, or during inhibition of auxin signaling pathways (Westfall et al., 2013).

The role of GH3 genes in fleshy fruit development has been poorly understood. A few years ago, Böttcher et al. (2011a,b) reported the presence of these genes in grapes (*Vitis vinifera*), and observed that the VvGH3.1 enzyme is involved in the process of berry maturation, and their transcript increases under exogenous-auxin treatment. The VvGH3.1 recombinant protein showed greater affinity for conjugation with aspartic acid as substrate (Böttcher et al., 2010). The combination of IAA with aspartic acid by GH3.1 enzyme action could reduce the average levels of free IAA, and was regulated by abscisic acid (ABA) (Böttcher et al., 2010).

Despite our understanding of the basic physiological and biochemical aspects of fruit ripening in non-climacteric fruit like raspberry, the molecular mechanisms involved in the raspberry fruit development and ripening and their influence on quality parameters is not fully understood, In this study, we attempt to elucidate the role of IAA and their conjugation by RiGH3, during the berry development of raspberry.

2. Materials and methods

2.1. Plant material and auxin treatments

Raspberry (*Rubus idaeus* L.) Heritage fruits were collected from commercial orchards that are located in Chimbarongo (34°41′45.54S; 71°10′01.71W; 333 masl), Chile. The raspberry plants used in the present study were of 3 years old and raised in soil with sandy loam texture. During the development of the orchard, the fertilization doses used were of 76, 38, 76, 19, 19, 1 kg ha^{-1} $year^{-1}$ for the elements nitrogen, phosphorus, potassium, magnesium, sulfur, calcium and 1.9 kg ha^{-1} $year^{-1}$ for the element boron, respectively (Hirzel, 2013). Flowers (F), and fruits bound to the receptacle and with peduncle were harvested and sorted by size and colour as: small green (SG), medium green (MG), large green (LG), white (W), pink (P), red (R) and overripe fruit (OR) (Vicente et al., 2007; Fuentes et al., 2015; Kayal et al., 2017). Immediately after harvest, half of the collected fruits were frozen in liquid nitrogen for RNA isolation and the other half was used to determine the quality and physiological parameters during development, and under auxin treatments. The fruit size and weight of twenty fruits from each developmental and ripening stage were measure using a caliper and analytical balance, respectively.

In-vitro auxin treatments were performed using W stage fruit according to the method of Figueroa et al. (2009). Briefly, fruits were divided into three groups; the first group was submerged for three minutes in 1 mM indole-3-acetic acid (IAA) dissolved in 0,06 M citric acid buffer (pH 4.5) containing 0,074 M di-sodium phosphate (Na2HPO4), 5 mM dithiothreitol (DTT) and 2% (v/v) dimethyl sulfoxide (DMSO), the second group was submerged for three minutes in 1 mM indole-3-propionic acid (IPA) dissolved in 0,06 M citric acid buffer (pH 4.5) containing 0,074 M di-sodium phosphate (Na2HPO4), 5 mM dithiothreitol (DTT) and 2% (v/v) dimethyl sulfoxide (DMSO), and a third group of control fruits were submerged for three minutes in a buffer solution containing 0,06 M citric acid buffer (pH 4.5) containing 0,074 M di-sodium phosphate (Na2HPO4), 5 mM dithiothreitol (DTT) and 2% (v/v) dimethyl sulfoxide (DMSO). All fruits were kept in a growth chamber at 10 °C throughout the experiment. From each group, five replicates (each one contains 10 fruits with average weight of 2.4 g per fruit) were sampled at 0, 18 and 36 h of treatment for determination of firmness and ethylene production and RNA extraction.

2.2. Quality and physiological measurements

The fruit firmness was measured using the Firm Tech II equipment (BioWorks Inc., Wamego, KS, USA) and expressed as Newton (N) (Muñoz-Robredo et al., 2013). A total of 8 g of fruit tissue (drupelets) from each replicate was homogenized in a mortar, and the juice was analyzed for total soluble solids (TSS) using a refractometer (ATAGO, Tokyo, Japan), expressed as the percentage of sugar per 100 g of fresh weight (FW) of fruit, and the titratable acidity (TA) was expressed as percentage of citric acid per 100 g of FW of fruit FW.

For respiratory production and ethylene determination, five independent replicates of 20 intact fruits binding to the receptacle (approximately 40 g) for each stage were introduced into close tight chambers (500 mL) and were incubated at 20 °C for 1 h. Further, 1 mL of the gas sample was quantified for ethylene in a gas chromatograph (Shimadzu 8A, Tokyo, Japan) equipped with a flame ionization detector (Muñoz-Robredo et al., 2013), and the results were expressed as μL ethylene kg^{-1} h^{-1}. For respiratory determination, the needle of a CO_2 detector (MAP Head space Gas Analyzer, Bridge Analyzers, USA) was introduced into the same chambers, and the CO2 concentrations were recorded. The respiration rates were expressed as mg CO_2 kg^{-1} h^{-1}. Five replicates per stage and 10 fruits of each replicate were used for all quality and physiological assessments analyzed during development and auxin *in-vitro* assay, and all results were expressed as the mean ± standard error (SE).

2.3. Isolation of RiGH3-related transcripts

The complete sequences of RiGH3.1 and RiGH3.5 were obtained from *R. idaeus* cv. Heritage transcriptome database available from the Genome Database for *Rosaceae* species (https://www.rosaceae.org/blast) (Jung et al., 2004, 2008, 2014).

2.4. Phylogenetic analysis

The sequence alignments were performed using the BioEdit (Hall, 1999) tool, and the phylogenetic trees were constructed using the Neighbor-Joining method of MEGA 6.06 (Tamura et al., 2011). The percentages of replicate trees in which the associated proteins clustered together in the bootstrap test (10,000 trials) are shown next to the branches. The sequences used and their GenBank accession numbers are as follows; *R. idaeus* L., RiGH3.1 (MK105830), *Fragaria vesca*, GH3.1 (XP_004287982.1); *Malus domestica*, MdGH3.1 (XP_008338396.1); *Prunus mume*, PmGH3.1 (XP_008238544.1); *Pyrus x bretschneideri*, PbGH3.1 (XP_009367779.1); *Vitis vinifera*, VvGH3.1, (XP_002283886.1); *Arabidopsis thaliana*, AtGH3.1 (NP_179101.1); *R. idaeus* L., RiGH3.5 (MK105831); *Fragaria vesca*, FvGH3.5 (XP_004304120.1); *Prunus mume*, PmGH3.5 (XP_008230245.1); *Malus domestica,* MdGH3.5 (XP_008372082.1); *Pyrus x bretschneideri*, PbGH3.5 (XP_009357080.1); *Vitis vinifera*,VvGH3.5 (XP_002272560.1).

2.5. Quantitative real-time PCR (qPCR) expression analysis

The total RNA was isolated from fruit of each ripening stage and also from drupelets of each auxin treatment, using the RNAqueous® Kit (Ambion, ThermoFisher Scientific Inc. Waltham, MA, USA). The first-strand cDNA was generated using a Revert Aid First-Strand cDNA Synthesis kit (Fermentas, Thermo Fisher Scientific Inc.). Five individual extractions for each sampling were performed. Specific forward and reverse primers were designed using primer premier tools (PREMIER Biosoft, Corina Way, Palo Alto, CA, USA) with high stringency. The following primers were used as forward and reverse primers for RiGH3.1; and RiGH3.5 transcripts respectively. RiGH3.1-F 5′-TTGCTG ACCTGGAAGTGGGAA-3′, and RiGH3.1-R 5′-GAAACCCGTGACTTGGA GGAT-3′; RIGH3.5-F 5′-TCGGCTTCTACCCTCACTCA-3′ and RiGH3.5-R 5′-AAATCTTCCCTCCTTCCCTTG-3′. The expression of RiGH3.1 and, RiGH3.5 genes were analyzed using qPCR (LightCycler® 96 Real-Time PCR System, Roche Diagnostics, Mannheim, Germany) according to Fuentes et al. (2015). The results were normalized against a reference gene Ri18S (GenBank, KP125886) by using the forward and reverse primers as: Ri18S-F, 5′- CTACCTATTGTAAGGAATGGTGCCT-3′ and Ri18S-R, 5′- TTCTGCATCCGAGATATCAAGTAGT-3′, respectively.

2.6. Statistical analysis

The data were analyzed using R Statistical Software (R Core Team, 2018). The growth and weight variables, ethylene production and firmness value obtained during development correlated by means of Pearson's correlation matrix, and then the trend was graphically represented by a scatter plot with a smooth fitting between variables. An analysis of variance was performed for levels of RiGH3.1 and RiGh3.5 transcripts, firmness and ethylene data obtained from auxin *in-vitro* assay, and significant differences from IAA- to control and IPA-treated fruits were grouped by time of treatment and determined at $P \leq 0.05$ (*) and $P \leq 0.01$ (**) (ANOVA test).

3. Results

3.1. Quality parameters and ethylene production during raspberry fruit development

A sharp increase of fruit weight and less pronounced increase of size was observed from small green (SG) to the white (W) stages during raspberry fruit development. Then, the weight increase was less pronounced at onset of ripening, *i.e.* W stage (Fig. 1A). The relation between both variables shows a potential growth until 1.75 cm, and afterwards a less pronounced increment in fruit growth (after the fruits attained 2 g of weight) (in Data in Brief, Monsalve et al., 2018). Firmness is an important quality determinant that changes significantly during raspberry ripening. A constant decrease of firmness and titratable acidity (TA), and increase of total soluble solids (TSS) from LG to ripe (R) stages was observed (Fig. 1B). However, no significant changes was observed for TA or TSS between medium green (MG) and large green (LG) stages (data no showed). All these results indicate that fruit development was carried out from SG to LG stage and that ripening beginning from stage W, giving way to quality changes such as decrease in fruit firmness and acidity an increase of TSS. On the other hand, the ethylene production were found very low in the LG fruit, (the levels reached below 0.99 $\mu L\,kg^{-1}\,h^{-1}$) (Fig. 1C), and then increased from W stage to over ripe (OR) stage. The ethylene and fruit firmness showed a negative correlation (in Data in Brief, Monsalve et al., 2018), indicating that, ethylene could play a role in fruit ripening and that could further determine the quality of the raspberry fruit.

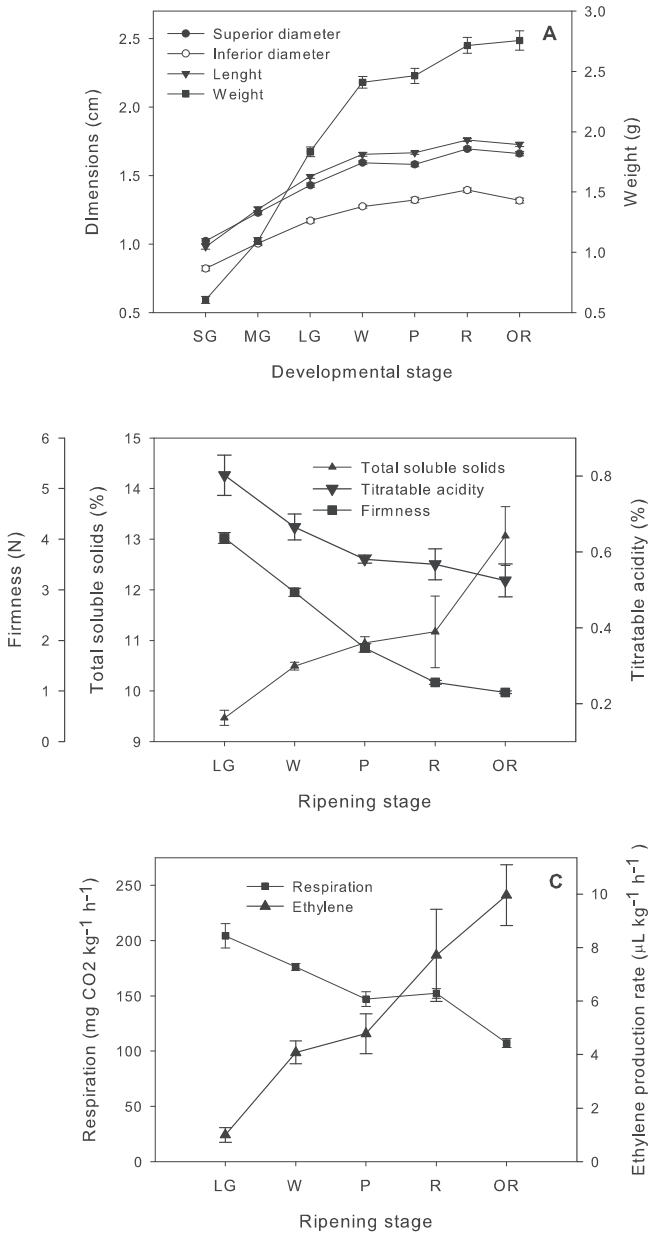


Fig. 1. Quality parameters during different raspberry development and ripening. Total soluble solids (%), titratable acidity (%), berry dimensions (cm), weight (g) and firmness (N) ethylene production ($\mu L\,kg^{-1}\,h^{-1}$) and respiration ($mg\ CO_2\,kg^{-1}\,h^{-1}$) were determined for different stages. Development stages are shown as small green (SG), medium green (HG) and large green (LG), and ripening stages are shown as white (W), pink (P), red (R) and overripe fruit (OR). Weight and dimension data represented as the means ± SE from 20 fruit of all different stages, Firmness, ethylene and respiration data represented as the means ± SE from 5 replicates of ripening stages (each one contains 10 fruits). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2. Identification of Indole-3-acetic acid-amido synthetase (GH3) transcripts in raspberry

Multiple alignments of the full-length predicted RiGH3.1 protein sequences (599 residues) revealed a high sequence homology to other GH3.1 genome sequence of *Rosaceae* species described, particularly with wild strawberry (*F. vesca*) (FvGH3.1 (92.4% sequence identity). It has shown 89.1% similarity with apple (*M. domestica*) (MdGH3.1) 89.1% with plum (*P. mume*) (PmGH3.1), 88.1% with pear (*P. bretschneideri*) (PbGH3.1), and 83.8% sequence identity with isolated VvGH3.1

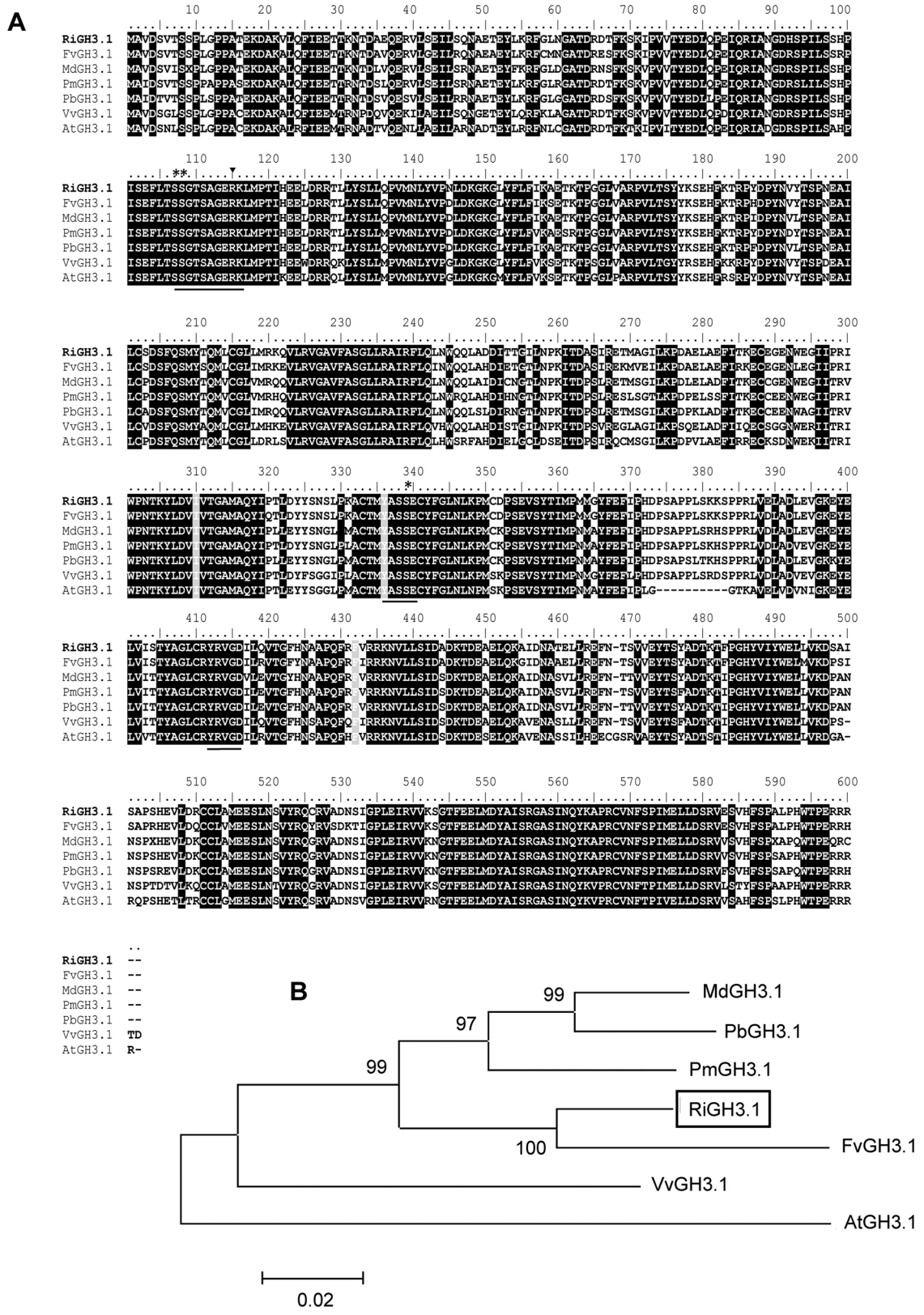


Fig. 2. (A) Alignment of the deduced RiGH3.1 sequence with other GH3.1 genome sequences from *Rosaceae* and *Arabidopsis* species. Gaps are indicated by dashes; letters with black background are identical amino acids; letters with gray background are residues associated with the formation of hydrophobic pocket that allows the adenine ring binding; the black line showed the binding motifs for ATP/AMP; black asterisk indicated residues associated with the union of IAA; black triangle showed the Arginine residue (R^{115}) associated to substrate affinity (Asp). Sequences were aligned using Bioedit Sequence Alignment Editor v7.0. At the end of alignment, the percentage of identity with RiGH3.1 is shown. (B) Phylogenic analysis of RiGH3.1. The phylogenic tree was built using MEGA software (version 6). The RiGH3.1 is indicated in black box. Numbers on branches indicate bootstrap values (as percentage).

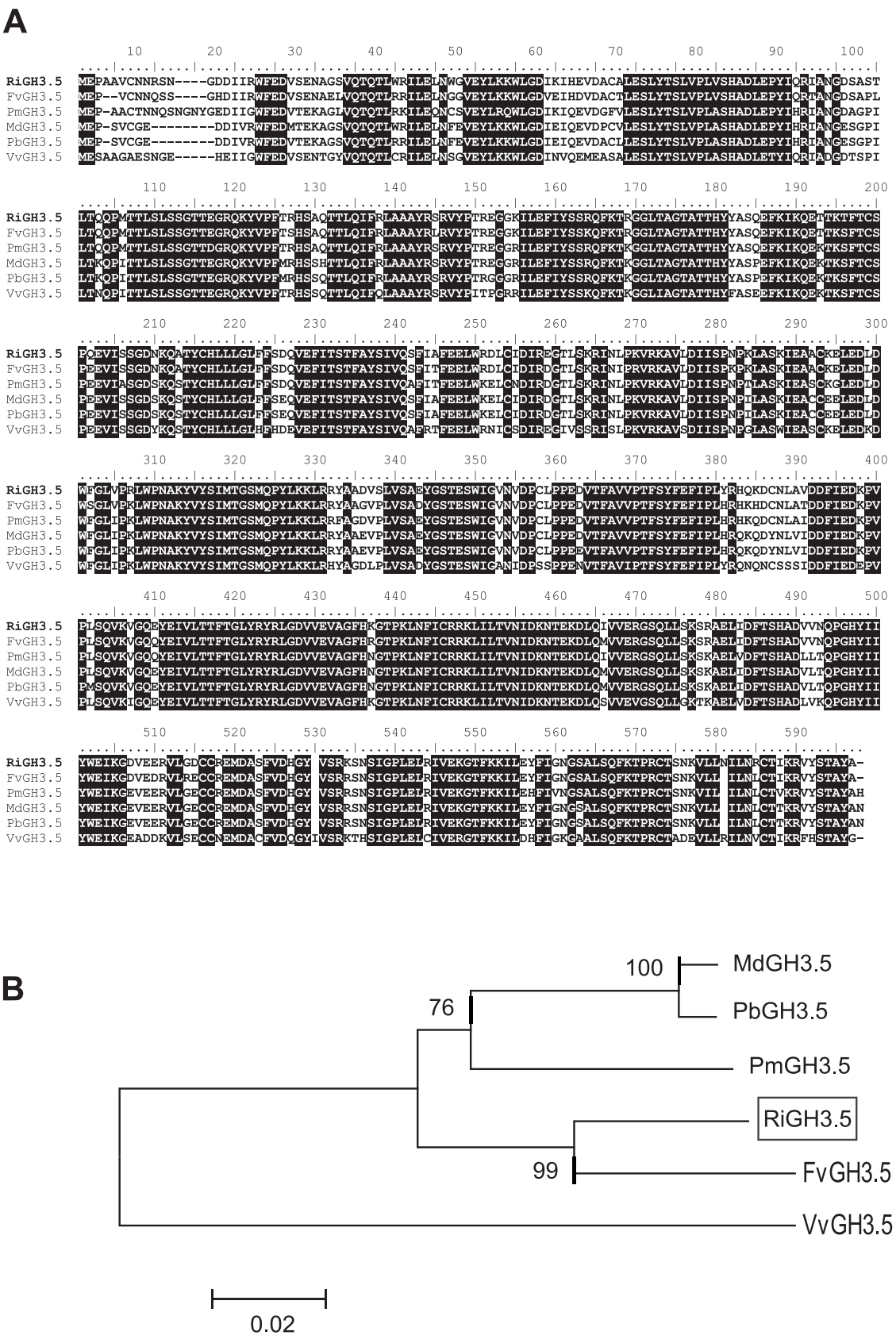


Fig. 3. (A) Alignment of the deduced RiGH3.5 sequence with other GH3.5 genome sequences from different floral and fruit species. Gaps are indicated by dashes, letters with black background are identical amino acids. Sequences were aligned using Bioedit Sequence Alignment Editor v7.0. At the end of alignment, the percentage of identity with RiGH3.5 is shown. (B) Phylogenic analysis of RiGH3.5. The phylogenic tree was built using MEGA software (version 6). The RiGH3.5 is indicated in black box. Numbers on branches indicate bootstrap values (as percentage).

of grape (*V. vinifera*); however, similarity with AtGH3.1 (*A. thaliana*) was only 77.8% (Fig. 2B). The conserved residues identified for RIGH3.1 (Fig. 2A) correspond to the binding motifs of ATP/AMP (^{107}SSGTSAGERK^{116}; ^{336}YASSE^{340}; ^{412}YRLGD^{416}) (Fig. 2A, black lines); to the formation of a hydrophobic pocket (T^{336}; F^{432}; I^{310}) (Fig. 2A, letters with gray background) that allows the union of the adenine ring, residues for binding of indole-3-acetic acid (IAA) (S$^{107;\ 108;\ 339}$) (asterisk); and finally an arginine residue (R^{115}) (black triangle) that participate in affinity for substrate, specifically to aspartic acid (Asp, D) (Peat et al., 2012).

Multiple alignments of the full-length predicted RiGH3.5 protein sequences (593 residues) revealed a high sequence homology to other GH3.5 described in the sequence genome of other *Rosaceae* species (Fig. 3B), principally with wild strawberry (*F. vesca*) (FvGH3.5), showed 93% identity, and with apple (*M. domestica*) (MdGH3.5) and pear (*P. bretschneideri*) (PbGH3.5) showed 89% similarity; and to plum (*P. mume*) (PmGH3.5) showed 88%, and to grape (*V. vinifera*) (VvGH3.5) showed 79% homology. Despite the high homology, the characteristic motifs for enzymes GH3.5 has not been reported (Fig. 3A).

3.3. Expression of Indole-3-acetic acid-amido synthetase transcripts during development and ripening of raspberry

The RiGH3 transcripts showed increased expression during development, from the SG to LG stage, with highest expression at LG stage (Fig. 4A). It is interesting to note that RiGH3.1 showed high levels of expression even in flowering (F) (Fig. 4A). On the other hand, compared to flowers (F), the expression of RiGH3.5 transcript in fruits was less pronounced (Fig. 3). Interestingly, both transcripts, RiGH3.1 and RiGH3.5 show a lesser expression during raspberry ripening, W to OR stages.

3.4. Quality parameter and GH3 expression under auxin treatment

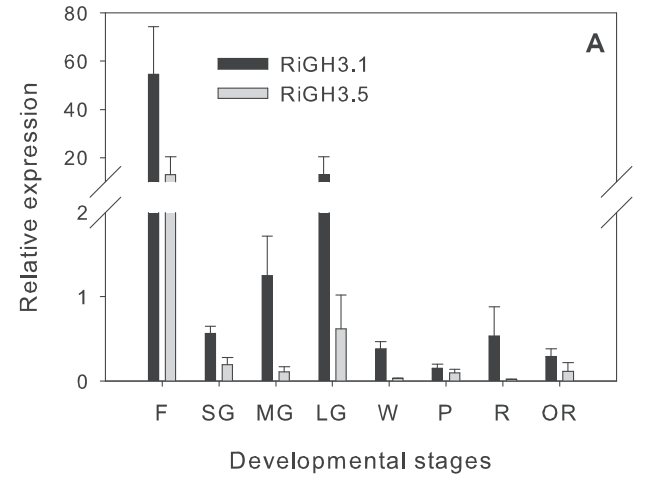
To understand the role of auxin conjugation during raspberry ripening an *in-vitro* assay was performed. No significant differences of firmness was observed between IAA-, IPA-treatments and control fruit during the times evaluated (in Data in Brief, Monsalve et al., 2018). Similarly, no changes of TSS and TA were observed during this assay (data no showed). Conversely, respiration was significantly increased at 18 and 36 h by IAA treatment compared to IPA and control conditions (in Data in Brief, Monsalve et al., 2018). Similarly, ethylene production was also significantly increased at 36 h by IAA treatment (in Data in Brief, Monsalve et al., 2018).

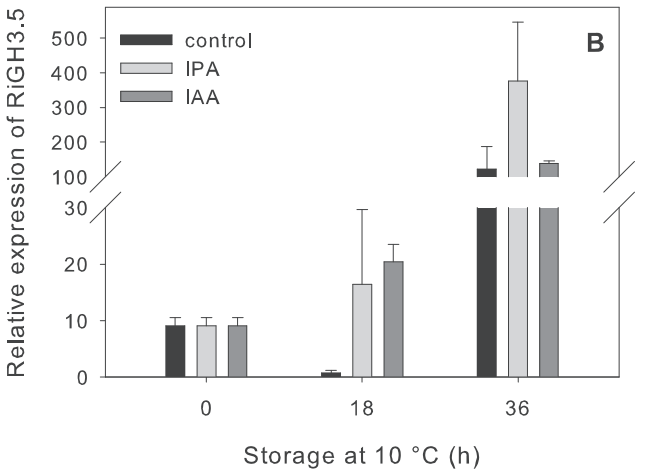
The expression of RiGH3.1 transcripts increased 45 times under indole-3-acetic acid (IAA) treatment after 18 h storage at 10 °C (Fig. 4B) compared to control fruits, suggesting a RiGH3.1 expression dependent of IAA levels. After 36 h of treatment it was not possible to identify significant differences between treatments. On other hand, RiGH3.5 transcripts (Fig. 4C) did not show significant differences in their expression after 18 h of treatment indicating that RiGH3.5 expression is independent of indole-3-acetic acid (IAA) action and control.

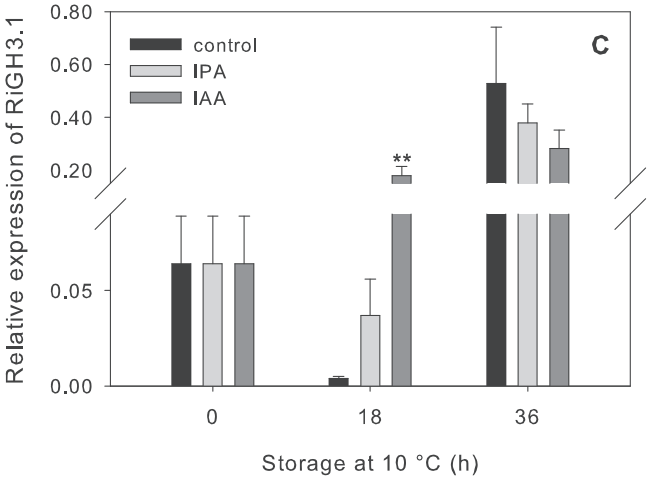
4. Discussion

4.1. Quality and physiological changes during fruit development and ripening

The quality parameter analyses indicate that raspberry fruit development is carried out from SG to LG stage and that ripening beginning from stage W, giving way to quality changes such as decrease in fruit firmness (Fig. 1B; in Data in Brief, Monsalve et al., 2018). During raspberry ripening, firmness is the one quality that changes most significantly, affecting the shelf-life of this fruit. Previously, we showed similar firmness decrease during ripening evaluated for two season,







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observing a delay of softening in fruits (W stage) treated with 1-MCP (Fuentes et al., 2015). The relationship between ethylene and firmness has a negative slope, which corroborates with what was observed during the fruit's maturity in the present (in Data in Brief, Monsalve et al., 2018) and previous studies (Fuentes et al., 2015), indicating that

Fig. 4. (A) Analysis of RiGH3.1 and RiGH3.5 transcripts during the development and ripening of raspberry fruit. Both transcripts were determined by qRT–PCR in whole fruits of each developmental stage. Flower (F) and developmental stages are shown as small green (SM), medium green (MG) large green (LG), white (W), pink (P), red (R) and overripe fruit (OR). Data are given as means ± SE from five replicates, normalized against Ri18S transcript abundance. Analysis of RiGH3.1 (B) and RiGH3.5 (C) transcripts during auxin treatment of white (W) stage. Both transcripts were determined by qRT–PCR in drupelets of each developmental stage. Data are means ± SE from five replicates (each one contains 10 fruits), normalized against Ri18S transcript abundance. Significant differences from control and IAA- and IPA treated fruits at the same time of treatment are indicated by ** for the probability levels $P \leq 0.01$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

ethylene may play a role during ripening, especially in determining the softening of raspberry fruit. This increase in ethylene during raspberry ripening is not in conformity with ethylene production reported in some other non-climacteric species. The ethylene production in strawberry was reported to be high in green fruit stage, and in decreased levels in white fruit, and then showed an increasing trend towards the red stage, concomitant with an enhanced respiration rate (Perkins-Veazie and Nonnecke, 1992; Iannetta et al., 2006).

4.2. Identification of characteristic motif of Indole-3-acetic acid-amido synthetase (GH3)

In the deduced amino acid sequence of RiGH3.1, it was possible to identify conserved residues sites (Fig. 2A), that has been described on characteristic motifs of enzymes GH3.1 family, as VvGH3.1 isolated from grape (*V. vinifera*) (Böttcher et al., 2011a) and *A. thaliana* (Ludwig-Müller, 2011). The conserved residues identified for RIGH3.1 (Fig. 2A) include arginine residue (R^{115}) that participate in affinity for substrate, specifically to aspartic acid (Asp, D) (Peat et al., 2012). Interestingly, this conjugation of IAA to aspartic acid and glutamic acid generates oxidized metabolites that follow degradation pathways, while the conjugation of IAA to alanine and leucine, leads to the formation of reservoirs of IAA, which are characterized by being composed hydrolyzables, allowing the generation of free IAA (Ludwig-Müller, 2011; Peat et al., 2012; Westfall et al., 2013). On the other hand, it has been reported that the conjugation of IAA to tryptophan acts as anti-auxin inhibiting the effects on the growth of plants, but without competing with IAA for binding to the auxin receptor such as transport inhibitor response 1(TIR1) (Westfall et al., 2013). Therefore, the presence of arginine residue (R^{115}) that participate in affinity for substrate, specifically to aspartic acid, is suggesting the relationship of RiGH3.1 activity with degradation of auxin, allowing the increases of ethylene and the ripening progress from W stage of raspberry.

Multiple alignments of the full-length predicted RiGH3.5 protein sequences revealed a high sequence homology to other GH3.5 described in the sequence genome of other *Rosaceae* species (Fig. 3B). Despite the high homology, the characteristic motifs for enzymes GH3.5 has not been reported (Fig. 3A) suggesting the need to perform targeted site mutagenesis to identify the key motives of this particular isoform.

4.3. GH3 expression during fruit development and ripening

The RiGH3.1 transcripts showed increased expression during development, from the SG to LG stage, with highest expression at LG stage (Fig. 4A). These high expression of GH3.1 transcript precedes with the onset of loss of firmness and the concomitant ethylene increase during ripening that take place from W stage. Similar to our results, the involvement of GH3.1 transcripts during fruit development and ripening was also reported in grape berries (*V. vinifera*) (Böttcher et al., 2010). It is interesting to note that RiGH3.1 showed high levels of expression even in flowering (F) (Fig. 4A), suggesting their important role in development processes. On the other hand, compared to flowers (F), the expression of RiGH3.5 transcript in fruits was less pronounced (Fig. 4B). Zhang et al. (2008) and Böttcher et al. (2011a,b) also reported the expression of AtGH3.5 and VvGH3.5 during flowering of *A. thaliana* and *V. vinifera*, respectively, suggesting a possible different role for each isoform of GH3 depending of tissue. Interestingly, both transcripts did not show significant expression during raspberry ripening, W to OR stages.

4.4. IAA conjugation and GH3 expression during ripening

The IAA levels has been reported to remain relatively constant throughout grape development and then decreases during the onset of ripening, and described to be of maximum concentration in flowers (Symons et al., 2006; Böttcher et al., 2011b; Ziliotto et al., 2012). A similar pattern has been observed in strawberry, with higher concentration in small green developmental stage and minimum in the ripe stage (Symons et al., 2012). In the present study, to understand the role of IAA conjugation during raspberry ripening an *in-vitro* trial was performed (Figueroa et al., 2009). During the IAA treatment, an increase of ethylene production and a less pronounced decrease in respiration were observed; however, it has been described that this ethylene increase may be due to stress response during hormonal treatment (Morgan and Drew, 1997; Böttcher et al., 2013). In soft fruits, the importance of IAA conjugation has been only reported during grapes ripening. Some years ago, Böttcher et al. (2011a,b) reported the presence of these genes in grapes, and, observed that the GH3.1 enzyme is involved in the process of berry maturation, and their transcript increases under exogenous-auxin treatment. Therefore, postharvest treatment of grape with IAA 0.5 µM, 12 and 22 days before the onset of maturation showed an increase in transcripts VvGH3.1 during the onset of maturation and an increase in the levels of IAA conjugated with aspartic acid (Asp, D), compared to the control group (Böttcher et al., 2010). Similar to the previous study, different expression pattern was observed for both RiGH3 isoform, the higher increase of RiGH3.1 transcripts was after 18 h of IAA treatment (Fig. 4B); conversely, the level of RiGH3.5 transcript (Fig. 4C) was independent of treatments. Although, the results suggest that RiGH3.1 could be induced during raspberry ripening under IAA treatment, in-plant assays during different developmental and ripening stages are necessary to determine the possible role of IAA conjugation in raspberry fruit.

In conclusion, during raspberry development the principal change is a sharp increase of fruit weight. Then, the firmness loss during fruit ripening is associated with increase of ethylene production. On other hand, a high expression of RiGH3.1 transcripts was observed before to onset of raspberry ripening. Thus, when the fruits at white stage (W) (the first stage of ripening), was treated with indole-3-acetic acid (IAA), an increase of RiGH3.1 transcript levels were observed, suggesting that IAA conjugation by RiGH3.1 might play a central role for onset of raspberry ripening. Further studies are underway to elucidate the possible auxin role and its crosstalk with other hormones, such as ethylene, during the fruit development and ripening transition of raspberry fruit and the differences between drupelets and receptacle tissues.

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