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Tocopherol Accumulation and Temporal Expression Analysis of *VTE1* and *VTE5* Gene Family in Fruit of Two Contrasting Avocado Genotypes

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Abstract

α-tocopherol is found in high concentrations in avocado fruit mesocarp, however, its accumulation and genetic control during maturation and ripening has not been elucidated. Based in the relevance of *VTE1* and *VTE5* genes in tocopherol biosynthesis and aiming to determine the association between tocopherol accumulation and expression of tocopherol biosynthetic genes, gene expression of *VTE1* and *VTE5* were evaluated through the time during three developmental stages: before harvest at 100, 160 and 220 days after flowering (DAF) and after harvest (220 DAF + 5) in two contrasting avocado genotypes (San Miguel and AVO40). San Miguel reached the highest levels at 220 DAF, whereas AVO40 increased α-tocopherol only after ripening (220 DAF + 5). A genome-wide search for *VTE1* and *VTE5* allowed to identify one and three genes, respectively. Both genotypes showed contrasting patterns of gene expression. Interestingly, AVO40 showed a highly positive correlation between α-tocopherol levels and gene expression of *VTE1* and all *VTE5* variants. On the other hand, San Miguel showed only a positive correlation between α-tocopherol level and *VTE1*gene expression.

Keywords Avocado · α-Tocopherol · Tocopherol cyclase · Phytol kinase

Introduction

Tocopherols are lipidic antioxidants with anti-inflammatory, antiaging, and antihypertensive activity [1]. They are chemically composed by a polar chromanol ring coming from the shikimate pathway and a phrenyl tail produced either via 2C-methyl-D-erylthritol 4-phosphate or by phytyl-phosphate pathway [2]. It is well known that a Phytol kinase, coded by the *VTE5* gene plays a central role on tocopherol biosynthesis [3]. *VTE5* expression levels are related with tocopherol levels in tomato [4], and its relation with phytol as by-product of chlorophyll degradation have been highlighted [3, 4]. On the other hand, *VTE1* codes for a tocopherol cyclase that catalyzes the conversion of phytyl quinol pathway intermediates to their corresponding tocopherol. Its activity has been largely evidenced in plants and cyanobacteria [5, 6], demonstrating that *VTE1* expression is tissue specific and regulated by the developmental stage of the fruit. To date, few studies report the relevance of *VTE1* and *VTE5* in plant species that accumulate tocopherol in fruit. In this sense, a previous report described α-tocopherol accumulation during olive fruit development with an evident correlation between α-tocopherol levels and *VTE1* and *VTE5* gene expression [7]. A similar study to elucidate how tocopherol is accumulated and how *VTE1* and *VTE5* genes are expressed during fruit maturation in avocado is still uncovered.

Most ripe fruits accumulate sugars; however, avocado fruit is characterized by the accumulation of lipids in the latest developmental stages, which are composed mainly by oleic acid and other lipidic secondary metabolites, including α-tocopherol. On the other hand, *Persea americana* is a highly diverse species derived from three races (Mexican, Guatemalan and West Indian) and the crosses between them [8, 9]. In this work, we analyzed two genetically contrasting genotypes [10] which are promising for their cultivation in tropical climates. Tocopherol content depends on environmental factors but also on the genotype [11]. In fact, α-tocopherol varies among varieties [11–13]. It is important to mention that tocopherol content in avocado has only been described after fruit ripening, but limited information exists about how α-tocopherol is accumulated during fruit

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maturation on the tree, before ripening. To date, a relation between phytol and α-tocopherol accumulation has been stablished [14], so it is proposed that α-tocopherol accumulation parallels lipid accumulation and is coordinated with the up-regulation of *VTE1* and *VTE5*.

Aiming to determine the association between fruit development and *VTE1* and *VTE5* gene expression levels, during α-tocopherol accumulation on avocado fruit, this research analyzed α-tocopherol content and gene expression levels during three developmental stages before and after harvest the avocado fruit, of two genotypes with contrasting α-tocopherol levels.

Materials and Methods

Material Fruit of two contrasting genotypes (AVO40 and San Miguel) [10], in three development stages before harvest (100, 160 and 220 DAF), and after harvest were also sampled at 220 DAF + 5 days (held at room temperature). The fruits were washed, the skin and seed were discarded, and pulp was frozen with liquid nitrogen and stored at −80 °C. The genotypes are growing in Northwestern Mexico (25°30′16”N, 108°27′15”W at an elevation of 16 m above sea level).

Methods: this section is described in Supplementary material.

Results and Discussion

Dry Matter, Oil and Tocopherol Contents

Dry matter, avocado mesocarp oil, and α-tocopherol levels were significantly higher in San Miguel genotype than in AVO40 (Fig. 1). In AVO40, dry matter did not increase significantly (16–18%) whereas in San Miguel reached 29% in 220 DAF + 5, in this sense, the increase in dry matter is related to the maturity of the fruit [15]. Oil content ranged from 3 to 19% and from 3 to 8% in San Miguel and AVO40, respectively (Fig. 1b), and both genotypes showed an increasing in oil content with time. There were also significant differences of oil levels between San Miguel and AVO40. The statistical analysis showed that the effect of Genotype and developmental stage as well as their interaction were significant ($p < 0.05$). A recent work also mentioned contrasting differences in oil between Hass and other genotypes growing in the same location [13], in this sense, a previous study suggested that San Miguel belongs to the Mexican race, whereas AVO40 could be a hybrid between West Indian and Mexican Races [10] thus confirming the relevance of the genotype in the oil accumulation trait.

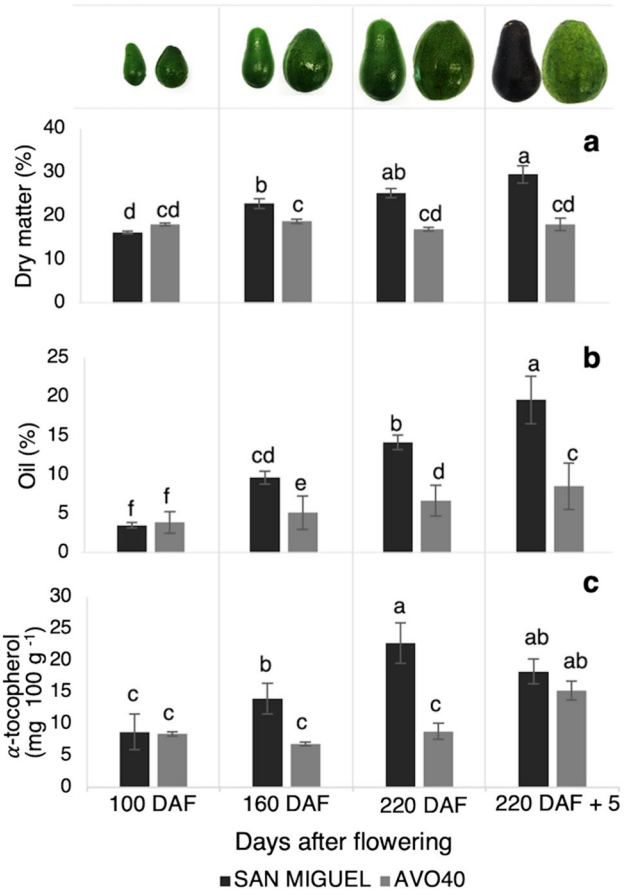

Fig. 1 Percent of dry matter (**a**), oil percentage (**b**), and content of **α**-tocopherol (mg 100 g^{-1} FW) (**c**) in avocado mesocarp of San Miguel and AVO40 genotypes at 100, 160, 220 and 220 + 5 days after flowering (DAF). Values correspond to the average of three biological replicates, bars represent the standard error. Values with different letters represent significant ($p \leq 0.05$) differences between interaction of factors in a Duncan test

α-tocopherol concentration in avocado mesocarp ranged from 8.7 to 18.2 mg 100 g^{-1} FW for San Miguel and 8.3 to 15.22 mg 100 g^{-1} FW for AVO40 (Fig. 1c). These values were higher than previously values reported in avocado growing in California (2.7 mg 100 g^{-1} FW), in Chihuahua, Mexico (0.39 to 1.47 mg 100 g^{-1} FW) and also in avocado from Spain (2.37 mg 100 g^{-1} FW) [12, 16, 17], these differences could be given by the growing altitude [18], in the location [12], also α-tocopherol role in responses to pant environmental stress [19]. Most of these works quantified α-tocopherol once mature fruits were harvested and reached the ripeness. In the present work, high levels of α-tocopherol were observed at the latest stages of fruit development. This in contrast to the pattern observed in olive fruits during fruit maturation on tree and after harvest, in which the highest concentrations occur at middle stages (32.45 mg 100 g^{-1} FW), followed by a drop [7].

Avocado mesocarp presented higher α-tocopherol levels (Fig. 1), than other vegetable oils including coconut, maize, olive, peanut and soybean [1], even in the first sampling points. Furthermore, α-tocopherol levels in ripe avocado mesocarp were also higher than other dietary sources such as almonds, hazelnuts, peanuts, spinaches, broccoli, kiwifruit, mango and raw tomato [1]. According to the Institute of Medicine (USA), the recommended dietary allowance is 15 mg *per* day of α-tocopherol, which suggests that intakes of 100 g of mature San Miguel and AVO40 could be enough to complete daily requirements.

Differences between genotypes in oil and α-tocopherol accumulation patterns were found (Fig. 1), these differences could be due to the genotype rather than to the environment [11], as described in genomic or transcriptomic studies in other plants that accumulate the metabolite in fruit, including olive [20]. Differences in α-tocopherol accumulation between San Miguel and AVO40 can also be explained in terms of its physiological roles. Regarding α-tocopherol accumulation, it correlated to lipid accumulation (Fig. 1). This might be explained by the antioxidant activity of tocopherol against lipid peroxidation. In this sense, tocopherols are mainly found in tissues that accumulate high amounts of poly unsaturated fatty acids, which could be oxidated [21]. It is well known that the main function of tocopherols is scavenging lipid peroxyl radicals, responsible for propagating lipid peroxidation [22].

Genome-Wide Identification of *VTE1* and *VTE5* Genes

A putative *VTE1* ortholog sequence was found in chromosome 2 of the avocado Hass genome with an ORF of 462 amino acids Table S2 and a complete domain that belongs to the Tocopherol cyclase superfamily (EC: 5.5.1.24). The cTP region, the chloroplastic localization sequence and the conserved YxEKNW and KPPGL motifs were also identified Fig. S2.

Several *VTE5*-like sequences were found in avocado genome and available transcriptomes (Table S2). These variations in *VTE5* sequences suggests tandem gene duplications in the avocado genome. Phylogenetic analysis suggests that avocado species have been subjected to whole genome duplication events in the past [23]. This gene duplication might enhance secondary metabolism and has been subjected to up and down-regulation, contributing to the avocado evolution from other basal angiosperms like Amborella. Here, three *VTE5* sequences, highly similar to phytol kinases, members of cytidylyltransferase protein family, were identified in chromosome 1 of the Hass genome (*VTE5–1, VTE5–2* and *VTE5–3)* with predicted ORF of 190, 326 and 243 amino acid residues, respectively (Fig. S2 and S3). Multiple alignment of VTE5 amino acid sequences of avocado and model plant species showed a non-conserved N-terminal, and a highly conserved C-terminal sequence. *VTE5–1,* has 4 transmembrane α-helix conserved domains, while *VTE5–2* and *VTE5–3* have 6 Fig. S3, which is characteristic of the cytidylyltransferases superfamily. The cTP and the protein localization domain indicate that *VTE5–1* and *VTE5–2* are located in the chloroplast Table S2, however, *VTE5–3* sequence did not present a cTP domain which could be due to an assembly error, since the phytol kinase activity has been demonstrated to be chloroplastic [3], and to date, there are no reports of VTE5 activity out of chloroplast. Discarding *VTE5–3*, the two *VTE5* like sequences here identified are supported by gene duplication [23] due to gene duplication of *VTE5* in avocado, which is absent in other model plants. As far as we know, this is the first time that *VTE5* gene family is described in plants, previous reports had only described a single *VTE5* gene in the genome of model plants [3, 4, 7].

Cluster analysis by Maximum Likelihood (Fig. S4) showed a close similitude among *VTE1* and *VTE5* family with *A. trichopoda VTE1* and *VTE5* genes, respectively, possibly because both *A. trichopoda* and avocado species come from basal angiosperms.

Expression Profile of *VTE1* and *VTE5* Genes

All three avocado *VTE5* and *VTE1* genes were expressed in both, San Miguel and AVO40 mesocarp during all fruit developmental stages. In AVO40 mesocarp, *VTE5* and *VTE1* genes reached the highest differential expression after harvest, whereas in San Miguel, *VTE5* genes showed the highest up-regulation before harvest whereas for *VTE1*, a significant up-regulation was not detected at any sampling point (Fig. 2).

VTE1 gene expression was contrasting between genotypes, reaching an up-regulation of 1.25 times in San Miguel and 8.95 times in AVO40 at 220 + 5 DAF (Fig. 2). Both, developmental stage and the interaction genotype by developmental stage factors were significant ($p \leq 0.05$). The observed patterns of *VTE1* relative expression in both San Miguel and AVO40 are contrasting to the observed in olive (with an up-regulation at earlier stages and a down-regulation at the end of development [7]) and tomato (with a constant expression level during fruit maturation followed by a down regulation at ripening [4]). Despite these differences, there is a positive correlation between α-tocopherol accumulation and *VTE1* up-regulation in olive, tomato [4, 5, 7] and avocado (Figs. 1 and 2 and Fig. S5), although the correlation was significant ($p \leq 0.05$) only in AVO40. The role of *VTE1*-coding protein on the last stages of tocopherol biosynthesis has been demonstrated through Arabidopsis mutants [6]. For *VTE5–1* expression, the ANOVA analysis revealed that the effect of genotype and the interaction of genotype by developmental stage were significant ($p \leq 0.05$). In San Miguel,

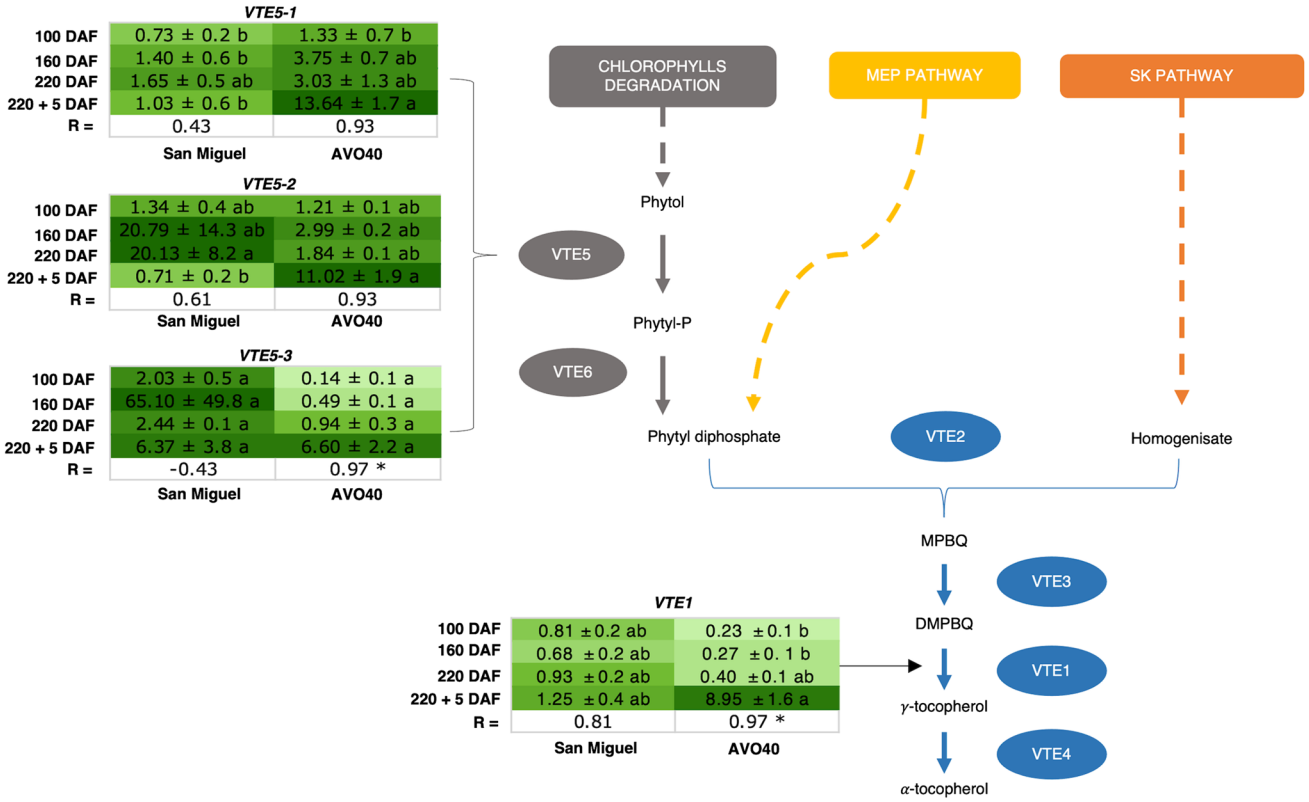

Fig. 2 Relative expression levels of *VTE5–1, VTE5–2, VTE5–3* and *VTE1* tocopherol biosynthesis genes in San Miguel and AVO40 genotypes, at 100, 160, 220 and 220 + 5 days after flowering (DAF). Values correspond to the average of three biological replicates ± the standard error. Values with different letters represent significant ($p \leq 0.05$) differences in a Duncan's test. Pearson's correlations among gene expression and α-tocopherol levels is shown. * Significant at $p \leq 0.05$

a slight increase was observed after 100 DAF. *VTE5–1* relative expression was slightly higher in AVO40 than in San Miguel at 160 and 220 DAF. After harvest (220 + 5 DAF), this difference increased largely. Regarding *VTE5–2*, the interaction of developmental stage by genotype in *VTE5–2*, was significant ($p \leq 0.05$). This gene was up regulated at 160 and 220 DAF in San Miguel, whereas in AVO40 the most important up-regulation was observed after harvest. Finally, *VTE5–3* was up regulated at all sampling points in San Miguel whereas in AVO40 the up-regulation was only observed after harvest, however, these differences were not statistically significant (Fig. 2).

In AVO40, the three *VTE5* genes showed a positive correlation with tocopherol levels (Fig. S5), although only the correlation with *VTE5–3* was significant ($p < 0.05$). In the same way, the temporal analysis of *VTE5* gene expression in olive fruit showed a down regulation along time, in parallel with a drop in tocopherol levels, at the latter development stages [7], pointing out that *VTE5* expression could be a marker gene for tocopherol accumulation in olive [7]. According to our results, avocado *VTE5–3* expression could be a positive marker for tocopherol accumulation but only for some genotypes.

Previous studies in other plants that accumulate this metabolite, including tomato [4] olive [7] and oil palm [24], have showed that tocopherol accumulation is regulated in a temporal and spatial way. In this sense, *VTE5* gene expression together with tocopherol accumulation patterns seem to be different among species with climacteric and non-climacteric fruits. In olive, a non-climacteric fruit (ripening occurs on tree), which presented higher tocopherol levels and *VTE5* up regulation at earlier developmental stages, was followed for a drop in tocopherol levels and gene expression [7]. By the other side, tomato, oil palm and mango are climacteric species that accumulate α-tocopherol together with *VTE5* up regulation along fruit development and ripening. These patterns are related with the ripening process triggered by ethylene production and linked with chlorophyll decrease in these species [4, 25]. In post harvested avocado fruit (post climacteric), α-tocopherol levels have been related with a decrease in chlorophyll, and as an ethylene response during ripening [14]. Then, it can be directed by phytol availability obtained from chlorophyll degradation during ripening.

Tocopherols play different roles in plants, α-tocopherol acts as a ROS quencher, limiting lipid peroxidation in photosynthetic membranes. Likewise, tocopherols have

an important role in response to plant environmental stress, including high-intensity light stress [22]. In this way, tocopherol levels are affected if stress is more severe and significant amounts of ROS are present [22]. In this sense, the presence of α-tocopherol is always related to its antioxidant activity, scavenging lipid peroxyl radicals [22]. Also, its structural function in the integrity of body oil membranes has been reported, relating oil content with tocopherol levels [26].

This work represents as far as we know the first evaluation of not only tocopherol accumulation in avocado fruit but also the relationship between tocopherol biosynthesis genes and metabolite accumulation during fruit maturation. Furthermore, oil percentage was also evaluated in every stage. Our results suggest a strong correlation between oil percentage ($r = 0.88$, $p \leq 0.01$) and tocopherol contents (Fig. S5), during fruit development. Several works have reported oil content and tocopherol accumulation in olive and palm mesocarp [20, 24]. Despite the differences between plant species in accumulation patterns along development, there is always a positive correlation between oil and tocopherol concentrations.

Tocopherol accumulation in plants is influenced by the developmental stage, environmental stress conditions [7, 22], and genotype [11, 20]. In this work, we selected two genotypes with contrasting fruit morphology and tree traits to analyze tocopherol accumulation during fruit development on tree, and our results confirm the relevance of the genotype and developmental stage effect and the interaction among these factors.

Conclusions

Both avocado genotypes showed an association between α-tocopherol content and gene expression of *VTE1* and *VTE5*, highlighting a significantly higher correlation in the genotype AVO40. The ANOVA analysis suggest that α-tocopherol accumulation is influenced by both genotype and developmental stage. Here, maximum levels of α-tocopherol were reached at different stages, but interestingly α-tocopherol showed a parallel accumulation with oil contents in both studied genotypes.

Our findings suggest the presence of three functional *VTE5* genes in avocado instead only one reported for other plants. This is valuable information to better understand and take advantage of oil and α-tocopherol in avocado genotypes. Furthermore, it would be useful for future avocado breeding programs focused on nutritional traits.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11130-022-00977-0.

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Data Availability Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

Conflict of Interest The authors declare no conflict of interest.

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