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Linking hormonal profiles with variations in sugar and anthocyanin contents during the natural development and ripening of sweet cherries

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ABSTRACT

Sweet cherries are highly appreciated by consumers worldwide and are usually cold-stored during postharvest to prevent over-ripening before distribution to the market. Sweet cherry is a non-climacteric fruit, for which ripening is known to be regulated by abscisic acid. Here we aimed to examine the hormone profiles, including measurements of abscisic acid, auxins, cytokinins and gibberellins by ultrahigh performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS), in relation to variations in sugar and anthocyanin contents, during growth and ripening of this fruit. Hormonal profiling revealed that indole-3-acetic acid, GA_1 and *trans*-zeatin levels decreased at early stages of fruit development, while GA_3 levels decreased at early stages but also later, once anthocyanin accumulation started. Conversely, abscisic acid levels rose significantly once the fruit started to synthetize anthocyanins, and isopentenyladenosine levels also increased during the ripening of sweet cherries. A strong negative correlation was found between GA_4 levels and both fruit biomass and anthocyanin levels, and between the levels of *trans*-zeatin and both fruit biomass and total sugar contents. In contrast, abscisic acid and isopentenyladenosine levels correlated positively with fruit biomass, anthocyanin and total soluble sugar content. Results suggest that auxins, cytokinins and gibberellins may act coordinately with abscisic acid in the regulation of sweet cherry development and ripening. Furthermore, it is shown that hormonal profile measurements by UHPLC–MS/MS may be a helpful tool to elucidate the timing of action of each specific hormonal compound during ripening, which has important applications in the agri-food biotechnological sector.

Introduction

Sweet cherry has become an important fruit worldwide due to its visual and organoleptic characteristics such as colour, sweetness and sourness. Nowadays, there is an increasing interest in improving fruit quality, mainly fruit size, and delaying maturity. Sweet cherry displays a characteristic three-stage growth pattern: (i) an initial phase of exponential growth driven by cell division and expansion, (ii) a relatively quiet second stage that correlates with endocarp hardening and embryo development, and (iii) a third, final stage of re-establishment of rapid growth, mainly characterised by cell expansion [1–3]. The ripening process starts with the accumulation of carbohydrates and anthocyanins during the second stage of growth and ends after the third growth phase has completed [4]. In contrast with climacteric fruits, such as tomato or apple, in which ethylene production and respiratory activity increase at the onset of ripening, sweet cherry is a non-climacteric fruit whose maturation process is known to be associated with abscisic acid (ABA) [5,6]. Most of the previous research on plant growth regulators on cherry fruit quality focused mainly on preharvest application of GA_3 in the second phase of growth, to increase fruit size and delay ripening [7,8]. Nonetheless, our knowledge about the possible use of other phytohormones, such as other gibberellins, and auxins or cytokinins, for improving sweet cherry quality is still very limited, despite its potential significance in the agri-food biotechnology sector.

In some non-climacteric fruits such as strawberries and grapes [9,10] and climacteric fruits such as apples [11], the role of auxins delaying the ripening process has been reported. With regard to gibberellins, GA_1 was found to be the most abundant at early stages of development in non-climacteric fruit (grapes), while GA_4 was only detected at later developmental stages. In sweet oranges, both GA_1 and GA_4 have been reported as biologically active gibberellins [12]. However, no information is yet available on the endogenous

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levels and role of other gibberellins in the growth and ripening of sweet cherries. Cytokinins, and particularly, its most active form, *trans*-zeatin, are involved in the regulation of cell division and sink strength in fruits, which is why their highest concentration is related to fruit set and early growth phases in some fruits [13]. Furthermore, a decrease in cytokinin levels before ripening in non-climacteric fruits, such as oranges and grapes, has been reported, suggesting that cytokinins may play an inhibitory role in fruit maturation [14]. On the other hand, 2-isopentenyladenine levels have been shown to increase during ripening in grapes, kiwifruit, tomato and strawberry, suggesting a role for this cytokinin in the ripening process [15]. Nevertheless, there is no information on either the concentration or effects of cytokinins on development and ripening of sweet cherries.

The aim of this study was to obtain insights into the variations in the endogenous levels of growth regulators, such as auxins, gibberellins and cytokinins, aside from those of the well-known ABA, in the growth and ripening of sweet cherries, focusing on the endogenous levels of these phytohormones during fruit development in orchard trees. In addition, to better understand the relation of the hormone profiles with fruit quality during ripening, we simultaneously measured various parameters associated with the maturation process, such as fruit biomass, malic acid levels, pH, fructose and glucose contents, and anthocyanin accumulation.

Material and methods

Experimental design and samplings

For the experiments, sweet cherries (*Prunus avium* L. *var.* Prime Giant) were obtained from trees growing in an exploited orchard at Partida Vall del Sector III (Lleida, NE Spain). Fruits were harvested at eight developmental stages (Fig. 1), which were visually characterised as follows: stage I, fully green and small-sized; stage II, green but larger in size; stage III, increased size with maximum of 30% red; stage IV, red-pink colour, with maximum 30% green; stage V, 90% red, 10% green; stage VI, almost red; stage VII, fully red, large-sized; and stage VIII, commercial harvest. Developing cherry fruits at stages I and II were sampled during 30th April 2015, fruits at stages III and IV were sampled during 7th May, fruits at stages V and VI were sampled during 15th May, fruits at stage VII were sampled during 22nd May, and fruits at stage VIII were sampled during 29th May. All samplings were performed early in the morning (between 9 and 10 a.m. local time) to minimise diurnal climatic variability between samplings. Six fruits per tree from a total of 8 trees were sampled at each development stage. Samples were immediately dipped in liquid nitrogen and stored at −80 °C until analyses.

Hormone profiling

Phytohormone levels were determined by ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) as described previously [16] with minor modifications. In short, fruit samples (100 mg) were extracted with 200 μL methanol:isopropanol:acetic acid, 50:49:1 (v/v/v), using ultrasonication and vortexing (Branson 2510 ultrasonic cleaner, Bransonic, USA) for 30 min. Deuterium-labelled internal standards, including d_5-indole-3-acetic acid, gibberellins (d_2-GA_1, d_2-GA_3, d_2-GA_4, d_2-GA_7), cytokinins (d_6-2-isopentenyl adenine, d_6-isopentenyl adenosine, d_5-*trans*-zeatin and d_5-*trans*-zeatin riboside) and abscisic acid (d_6-ABA) were added. After centrifugation, the pellet was re-extracted using the same procedure and the collected supernatants were merged and filtered through a 0.22 μm PTFE filter (Waters, USA) before analyses. Phytohormone levels were analysed by UHPLC-ESI–MS/MS. The system consisted of an Aquity UPLC™ System (Waters) quaternary pump equipped with an autosampler. An HALO™ C18 (Advanced Materials Technology Inc., USA) column (2.1 × 75 mm, 2.7 μm) was used. Solvent A was water with 0.05% glacial acetic acid and solvent B was acetonitrile with 0.05% glacial acetic acid. Flow rate was set at 0.6 mL/min. Quantification was made considering recovery rates for each sample by using the deuterium-labelled internal standards [16].

Fruit quality parameters

Sweet cherry quality was determined by measuring fruit biomass, levels of total anthocyanins and soluble sugars, and acidity. Fruit biomass was estimated by weighing the samples immediately at each harvest time point after transferring to the laboratory in bags (with high humidity to avoid desiccation). Total anthocyanins were determined as described [17]. Fruit samples (200 mg) were extracted with 1 mL methanol using ultrasonication and vortexing. Extracts were centrifuged at 1000 x *g* for 10 m at 4 °C and the pellet was re-extracted following the same procedure. Supernatants were collected and pooled before extract acidification. In order to acidify the extracts, 1% HCl was added and total anthocyanins measured spectrophotometrically at 530 nm. Total anthocyanins were calculated using cyanidin-3-glucoside as a reference, using a molar absorption coefficient of 34300 L cm^{-1} mol^{-1} as described [18].

For sugar analyses, samples (50 mg) were extracted with 1 mL ethanol 80% (v/v) at 80 °C. After centrifugation, the supernatants were pooled and dried completely under a nitrogen stream. The extracts were suspended in 1 mL of MilliQ water, passed through Sep-Pak Plus (100 mg) C18 cartridges (Waters, Milford, MA, USA), and then 0.5 mL of MilliQ water was added prior to injection into the HPLC system. Glucose, fructose and sucrose were isocratically separated on an Aminex HPX-87C column (Bio-Rad Carbohydrate Standard, Hercules, CA, USA) using water as a solvent at a flow rate of 0.6 mL/min. Detection was carried out with a differential refractometer (Knauer, Berlin, Germany) with the cell at 80 °C. Glucose, fructose and sucrose from Sigma (Steinheim, Germany) were used for quantification. Sucrose levels were always kept

Fig. 1. Sweet cherries during the study of natural development of fruits on the tree (from left to right, stages I to VIII).