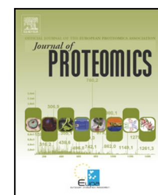
Journal of Proteomics xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot



Integrated analysis of metabolites and proteins reveal aspects of the tissue-specific function of synthetic cytokinin in kiwifruit development and ripening

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ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form 12 February 2016
Accepted 17 February 2016
Available online xxxx

Keywords:
Actinidia deliciosa
Cytokinin
Forchlorphenuron
Proteome
Firmness
Kiwifruit development
Kiwifruit ripening
Metabolite profiling

ABSTRACT

Fruit development and ripening depends on highly coordinated phyto-hormonal activities. Although the role of synthetic cytokinin N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) in promoting fruit growth has been established, knowledge regarding the underlying mechanism is still lacking. Here, we characterize the effect of CPPU application 20 d after full bloom at pre- and post-harvest biology of kiwifruit (*Actinidia deliciosa* [A. Chev.] C.F. Liang et A.R. Ferguson var. *deliciosa* cv. 'Hayward'). Data revealed that CPPU stimulates kiwifruit growth through the enlargement of small cells. During fruit development, the abundance of 16 proteins that are mainly related to defence was increased by CPPU while CPPU altered the expression of 19 polar metabolites in outer pericarp. Sugar homeostasis, cell wall modifications, TCA cycle and myo-inositol pathway were mostly affected by CPPU in kiwifruit during development. Upon postharvest ripening at 20 °C following 2 months of cold storage (0 °C), CPPU suppressed ethylene production and retained central placenta softening, indicating that CPPU induced tissue-dependent disturbances in climacteric ripening. Nineteen central placenta proteins and up to 15 metabolites of outer pericarp and central placenta tissues were affected by CPPU in ripened kiwifruits. These observations amplified our understanding in the regulation of fruit development and ripening by exogenously supplied cytokinins.

Biological significance: This study demonstrates that CPPU application, apart from fruit development, influenced also the kiwifruit climacteric ripening behaviour. An insight on the action of CPPU during kiwifruit development is provided, showing that it is partially based on a general stimulation of TCA cycle and myo-inositol pathway along with alternation in sugar and cell wall metabolism. Data also revealed that CPPU regulates ethylene biosynthesis and influences central placenta softening, indicating that this tissue may play a prominent role in kiwifruit ripening. Also, this work provides a first characterization of the ripening-affected central placenta proteins that offers insights into kiwifruit ripening. The current study provides a baseline of information for understanding the metabolic processes that are regulated by exogenous cytokinin during fruit development and ripening.

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1. Introduction

Fruit development and ripening are complex biological processes unique to plants [1]. Plant growth regulators coordinate multiple aspects of fruit development and ripening; accordingly, application of these regulators can induce fruit growth. For example, fruit enlargement to desirable size is largely achieved by application of synthetic cytokinins. The N-(2-chloro-4-pyridyl)-N′-phenylurea, forchlorphenuron (CPPU), a member of phenylurea synthetic cytokinin group, is among the most widely used worldwide. In fruits, CPPU acts mainly synergistically with endogenous auxins, induces parthenocarpy, increases size, fruit set and cluster weight of fruits [2]. It has been shown that CPPU and its derivatives act as cytokinin oxidase/dehydrogenase inhibitors and therefore play a role on endogenous cytokinin homeostasis but also they are recognized by plant cytokinin receptors [3]. However, the mechanism underlying the effect of CPPU on fruit growth is relatively unknown.

Commercial green-fleshed kiwifruit (*Actinidia deliciosa* (A. Chev.) C. F. Liang et A.R. Ferguson var. *deliciosa* 'Hayward') is a significant fruit crop that is highly appreciated by consumers due to its health-beneficial metabolites [4]. Kiwifruit industry interests in finding the way to increase fruit size at harvest and to maintain fruit quality during postharvest life. As a result, cultural practices, including synthetic cytokinins application, are employed to enlarge kiwifruit size [5].

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http://dx.doi.org/10.1016/j.jprot.2016.02.013
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Please cite this article as: A. Ainalidou, et al., Integrated analysis of metabolites and proteins reveal aspects of the tissue-specific function of synthetic cytokinin in kiwifruit..., J Prot (2016), http://dx.doi.org/10.1016/j.jprot.2016.02.013

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Interestingly, kiwifruit developmental metabolism is different from other fruiting species, since carbon is mainly stored as starch [6]. Indeed, net starch accumulation does not occur until the fruit has completed cell division [7], while net starch degradation mainly occurs after fruit harvest and, by the time the fruit is at eating ripe stage, almost all starch has been converted into soluble sugars [8]. At harvest, just after the physiological maturation stage, kiwifruits are characterized by high firmness and acidity and therefore they cannot be consumed before ripening is completed [9]. Compared with many other fruits, the ripening process in kiwifruit is also quite unusual. The 'Hayward' kiwifruit has been classified as a climacteric fruit, despite the fact that fruits ripen at postharvest stage without a marked increase in ethylene production [10].

In recent years, a number of analytical tools have been applied to study fruit ripening, targeting at monitoring genes, proteins and metabolites [11]. Research on the popular kiwifruit *A. deliciosa* 'Hayward' has been hampered by the lack of strong genomic tools due to its high ploidy and therefore genetic studies on this fruit have been applied to the diploid yellow-fleshed *Actinidia chinensis* genotypes. Huang et al. [12] sequenced and assembled a draft genome of *A. chinensis* whereas there are now complete nucleotide sequences of the chloroplast genomes for *A. chinensis* [13], a comprehensive genetic map of the 29 chromosomes of *A. chinensis* [14] and a considerable number of ESTs [15]. To support genetic and genomic studies of kiwifruit, the 'Hayward' proteome was recently developed to identify ripening-related proteins [16]. This study revealed that 'Hayward' ripening involves substantial turnover of existing and newly synthesized proteins, such as polyphenol oxidase, polygalacturonase, sucrose synthase, malate dehydrogenase and bet v 1 related allergen protein. However, apart from these proteins, kiwifruit ripening likely involves the up and down-regulations of hundreds of proteins which are not identified yet. In addition large-scale metabolite profiling has been applied to kiwifruit only recently during fruit development of *A. deliciosa* high- and low-starch-accumulating genotypes [6]. This study identifies some novel features of kiwifruit developmental biology, including changes in glucose levels and neutral invertase activity which marks the transition to net starch accumulation along with the involvement of β-amylase 9/α-amylase 2 in starch turnover. Nevertheless, there is still an extensive gap in our understanding in the regulation of kiwifruit development and ripening mechanism.

Evidences suggest that CPPU treatment has shown to be effective on enhancing kiwifruit growth [5,17]; however, there is currently little understanding on the underlying mechanism. Apart from enlargement in fruit size, the influence of CPPU on postharvest ripening process is not clear [18], although a possible connection to suppression of ethylene synthesis in kiwifruits was suggested [19]. Remarkably, current knowledge about the function of CPPU on fruit is derived from research on some physiological aspects of fruit growth and ripening, although there is a need for an overall molecular framework that can be used to better understand CPPU-associated fruit biology [20]. Fruit development and ripening relies on careful coordination of growth between distinct tissue types to allow protection of the fertilized seeds and their timely dispersal [21]. Hence, as a novel approach in this work, we examined using the kiwifruit as an experimental model, the physiology of the impact of CPPU in outer pericarp and central placenta (columella) coupled with a proteomic and metabolomic approach during pre- and post-harvest periods, which yields insight into both kiwifruit development and ripening syndromes.

2. Materials and methods

2.1. Orchard description, plant material and CPPU treatment

Field experiment was conducted during 2013 growing season in a mature commercial kiwifruit (*A. deliciosa*, cv. Hayward) orchard located at Meliki (Imathia, Central Macedonia, North Greece). Vines trained onto a T-bar trellis system were selected from the centre row of a typical block. Vines planted at 4 m within-row spaces and 4 m between rows. All applied field practices were as normal for commercial kiwifruit orchards in the area, with water supplied by drip irrigation. To investigate the effect of the synthetic growth regulator (N-(2-chloro-4-pyridyl)-N′-phenylurea) (CPPU) in kiwifruit physiology, six blocks consisted of 21 vines (three rows with seven vines each) were selected and labelled. Three of them were treated with CPPU using the commercial product 'Sitofex EC' (containing 1% CPPU w/v), while the other three served as control vines. The application of CPPU treatment was conducted according to commercial agricultural standard (i.e. 10 mg CPPU L^{-1} H_2O was prepared and 500 L ha^{-1} was sprayed in fruits 20 d after full bloom). The impact of CPPU was evaluated from the day of CPPU application (day 0) until harvest (day 125) as well as during ripening at 20 °C following 2 months of cold storage (0 °C, 95% humidity).

2.2. Fruit sampling procedure

2.2.1. During fruit growth period

During fruit growth, 10 kiwifruits were collected at random from the 4th vine of the second row in each block (replicate) of CPPU-treated and untreated (control) vines just before spraying (0 d) as well as after 2 or 30 d following CPPU application for phytochemical and protein analysis. The fruit of each replicate were divided into epicarp, outer pericarp, central placenta and seeds. Outer pericarp samples were snap frozen in liquid nitrogen and then stored at −80 °C, for determination of ascorbic acid, total antioxidant capacity and phenolic content, and for polar metabolite and protein analysis.

2.2.2. At harvest (maturity stage)

At commercial harvest stage (beginning of November; 125 d after CPPU treatment; DAT), kiwifruits were transferred to cold storage (0 °C, 95% RH), except for 10 fruits of each replicate which were prepared for phytochemical analysis as described above. At harvest stage, fruit mature factors (soluble solid content; SSC, outer pericarp and central placenta firmness) were also determined.

2.2.3. During postharvest ripening period

After 2 months of cold storage (0 °C, 95% RH), fruits were removed from the cold room and were allowed to ripen at room temperature (20 °C) for 18 d. During ripening period, ethylene production and respiration rate were daily monitored. Outer pericarp and central placenta firmness were recorded in whole fruits, while the juice of the same fruits was used for SSC determination. Ascorbic acid, phenolic content, and total antioxidant capacity were determined at 0, 2, 4, 6, 8, 10, 12, 15 or 18 d of ripening in the outer pericarp. The outer pericarp and central placenta samples were stored as described above. Protein analysis was performed for central placenta at 10, 12, 15 and 18 d of ripening, along with metabolite profiling for both outer pericarp and central placenta at 12 d of ripening.

2.3. Analysis of fruit growth rate

Fruit growth was monitored at 0, 2, 10, 18, 24 and 30 DAT. Since after 30 DAT, the major fruit cell division phase was ended [22] the growth rate was monitored every 10 d until harvest. The size–volume (V) of fruit was determined on 30 fruits per treatment by measuring their length (L), major (D_1) and minor diameter (D_2) with a calliper and calculated using the type $V = \pi L D_1 D_2/6$ [23]. Fruit weight was recorded at 30 DAT and at harvest using 30 fruits per treatment. Seed number per fruit was also measured at harvest.

2.4. Imaging fruit cell size by confocal laser-scanning microscopy (CLMS) and image analysis

To observe cell structure of fruit, cell walls were labelled by incubating cross-sections of the outer pericarp in propidium iodide [24]. Cell

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