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Novel insights of ethylene role in strawberry cell wall metabolism

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ABSTRACT

Due to its organoleptic and nutraceutical qualities, strawberry fruit (*Fragaria x ananassa*, Duch) is a worldwide important commodity. The role of ethylene in the regulation of strawberry cell wall metabolism was studied in fruit from Toyonoka cultivar harvested at white stage, when most changes associated with fruit ripening have begun. Fruit were treated with ethephon, an ethylene-releasing reagent, or with 1-methylcyclopropene (1-MCP), a competitive inhibitor of ethylene action, maintaining a set of non-treated fruit as controls for each condition. Ethephon treated-fruit showed higher contents of hemicelluloses, cellulose and neutral sugars regarding controls, while 1-MCP-treated fruit showed a lower amount of those fractions. On the other hand, ethephon-treated fruit presented a lower quantity of galacturonic acid from ionically and covalently bound pectins regarding controls, while 1-MCP-treated fruit showed higher contents of those components. We also explored the ethylene effect over the mRNA accumulation of genes related to pectins and hemicelluloses metabolism, and a relationship between gene expression patterns and cell wall polysaccharides contents was shown. Moreover, we detected that strawberry necrotrophic pathogens growth more easily on plates containing cell walls from ethephon-treated fruit regarding controls, while a lower growth rate was observed when cell walls from 1-MCP treated fruit were used as the only carbon source, suggesting an effect of ethylene on cell wall structure. Around 60% of strawberry cell wall is made up of pectins, which in turns is 70% made by homogalacturonans. Our findings support the idea of a central role for pectins on strawberry fruit softening and a participation of ethylene in the regulation of this process.

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1. Introduction

Primary plant cell wall is a complex and dynamic structure mainly formed by a cellulose-hemicellulose network embedded into a hydrophilic matrix of pectins, with a minor proportion comprised by structural and polysaccharide-modifying proteins (PMPs) [1,2]. Pectins are also the major component of middle lamella, the mediator of neighbor cells connection [3]. Furthermore, maintenance of cell adhesion is important to protect plant tissues integrity, but some developmental processes as dehiscence, organ abscission, pollen release and fruit ripening require cell separation, which in turn needs modifications of cell wall and middle lamella components. In that sense, it has been proved that the cooperative action of many PMPs is required for cell wall and middle lamella disassembly, being that process crucial to lead fleshy fruits softening [4,5]. Although changes in flesh fruit texture is required for both seeds spread and consumer's acceptance, an extensive postharvest softening drives to a higher susceptibility to pathogens attack, difficulties on shipping and a shortening of fruit shelf life. In this context, the effects of plant growth regulators on fleshy fruits ripening and cell wall disassembly have become a focus of considerable scientific attention [6–8].

Fruits are classified accordingly to the presence (climacteric) or absence (non-climacteric) of an increment on respiratory activity and a peak of ethylene biosynthesis during ripening [9]. The relationship between ethylene production and perception, and gene expression and accumulation of PMPs, has been studied both in

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climacteric and non-climacteric fruits by many research groups [8,10].

In strawberry, it is well known that auxins produced by achenes are essential for receptacle growth and expansion, but also these hormones constitute the key point to delay strawberry fruit ripening [11]. The expression of many genes encoding proteins involved in strawberry cell wall disassembly, as polygalacturonases (PGs) and β-xylosidases (β-Xyls), is down-regulated by treatments with exogenous auxins [12,13]. Nevertheless, the expression of *FaPME1*, a relevant gene in strawberry cell wall metabolism that encodes a pectin methylesterase (PME), is up-regulated by auxins [14]. On the other hand, it has been shown that abscisic acid (ABA) is a positive regulator of strawberry fruit ripening [12,15,16].

Regarding ethylene, a growing number of evidences support its participation in strawberry fruit ripening. Three strawberry cDNAs encoding different ethylene receptors were isolated, and authors reported an increased expression of *FaEtr1*, *FaErs1* and *FaEtr2* during fruit ripening [17]. Furthermore, *FaEtr1* and *FaEtr2* genes were more responsive to exogenous ethylene application in white fruit than in the red ones, in agreement with previous reports [18,19]. In that sense, it has been detected an increase in ethylene production both in ripe strawberries and when expanded fruit progress from green to white color, which suggests that ethylene might act as a signal for the progression of the ripening process [20]. Likewise, several studies have shown that gene expression or enzyme activities of strawberry fruit PMPs respond to ethylene, although the effect of this hormone on the metabolism of cell wall polymers is way far from being clear [12–14,21,22].

Therefore, the present study is aimed to contribute to the knowledge of ethylene's role in the cell wall and middle lamella metabolism of strawberry, an important agronomical crop and a model of non-climacteric fruit within Rosaceae family.

2. Materials and methods

2.1. Fruit material

Strawberry (*Fragaria* × *ananassa* Duch., cv Toyonoka) fruit were obtained from local producers (La Plata, Buenos Aires Province, Argentina). Fruit were harvested at white stage (W) with a mild pink tonality. The peduncle of each fruit was cut at 30 mm from the receptacle base, and fruit were washed, drained, classified according to shape and size, and used for plant growth regulators assays.

2.2. 2-chloroethylphosphonic acid and 1-methylcyclopropene treatments

Whole white strawberry fruit were submerged during 5 min in a solution of 2 mM 2-chloroethylphosphonic acid (ethephon; an ethylene-releasing agent) in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol (both used as surfactants), prepared immediately before use. Control fruit were submerged for the same time in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol. Immersion treatments were applied at room temperature, and then fruit were vertically placed on microcentrifuge tubes with their peduncles in contact with distilled water to avoid dehydration.

In the case of 1-methylcyclopropene (1-MCP; an inhibitor of ethylene perception) treatment, fruit with peduncles in contact with distilled water were treated with this inhibitor (1 $\mu l\, L^{-1}$) in a hermetic container for 10 h at 22 °C, while the control were kept under the same conditions without 1-MCP.

Immediately after treatments, fruit from both assays were placed in a growth chamber at 22 °C during 48 h. After storage, the calyx and peduncle were removed and fruit were cut apart, frozen in liquid nitrogen and stored at −80 °C until use. Thirty fruit were used for each condition and the entire experiment was repeated three times.

2.3. Isolation of cell wall polysaccharides

Cell wall polysaccharides were obtained as Alcohol Insoluble Residues (AIRs) according to díAmour et al. [23] with slight modifications. Five grams of frozen fruit were homogenized with 20 ml of boiling absolute ethanol and refluxed for 30 min. The homogenate was vacuum filtered and the residue was washed three times with 15 ml of absolute ethanol. After this, the residue was dried overnight at 37 °C and weighed. Three independent AIRs extracts were obtained for each treatment and control fruit.

2.4. Extraction and quantification of pectins

Polyuronides were isolated according to díAmour et al. and Nara et al. [23,24] with modifications. A 100 mg aliquot of AIRs was homogenized in 100 ml of distilled water and shaken overnight at 20 °C. The homogenate was vacuum filtered and the solid washed three times with 10 ml of distilled water. The filtrates were pooled and labeled as water-soluble pectins (WSP). The residue was then suspended in 50 ml of 0.05 M sodium acetate containing 0.04 M EDTA, pH = 4.5 and shaken during 4 h at 20 °C. The homogenate was vacuum filtered and the solid washed three times with 10 ml of the same buffer. The filtrates were pooled and labeled as EDTA-soluble pectins (ESP). Finally, the last residue was suspended in 50 ml of 0.05 M HCl and heated with agitation at 100 °C for 1 h. After cooling, the homogenate was vacuum filtered and the residue washed three times with 10 ml of 0.05 M HCl. The filtrates were pooled and labeled HCl-soluble pectins (HSP). Uronic acid concentrations of all fractions were estimated by the m-hydroxydiphenyl method using galacturonic acid (GalA) as standard [25]. Neutral sugars (NS) concentration was estimated by the anthrone method using glucose as standard [23].

2.5. Extraction and quantification of hemicelluloses and cellulose

The washed residue from pectin extraction was shaken for 8 h with 50 ml of 4 M NaOH at 20 °C. The homogenate was vacuum filtered and washed three times with 5 ml of 4 M NaOH. The filtrates were pooled and labeled as hemicellulose fraction. Finally, the remained solid was considered as cellulose. Quantification of hemicelluloses and cellulose was done after complete hydrolysis with 66% v/v H_2SO_4 at 37 °C during 1 h and estimated as glucose by using the anthrone method [23].

2.6. In vitro cell wall swelling

Five milligrams of AIRs were suspended in 4 ml of sterile distilled water and shaken horizontally for 24 h at 25 °C. Tubes were then placed vertically, and cell wall swelling was assessed based on the height of the sedimented AIRs layer [4]. Three independent AIR extracts were used for each condition analyzed.

2.7. RNA isolation and reverse transcription

Total RNA was isolated from 5 g of frozen fruit using the 2-butoxyethanol method [26], treated with DNAase I (Promega) and then purified with chloroform:octanol (24:1). First strand of cDNA was obtained by using the following mixture: 1 μg of total RNA, 0.03 mM dNTPs, 1 μl of Moloney murine leukemia virus RT (200 U μl^{-1}; Promega), 5 μl of 5× reaction buffer (250 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT, pH = 8.3), 330 pmoles of random primers (Biodynamics S.R.L., Buenos Aires, Argentina) and distilled water up to a total volume of 25 μl. The reaction mixture

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