Universidad de Málaga
Dpto. de Biología Vegetal
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Strawberry fruit softening and pectin disassembly:

Nanostructural characterization of fruit pectins by atomic force microscopy.

Role of the *β*-Galactosidase gene *FaβGal4.*

Candelas Paniagua Correas
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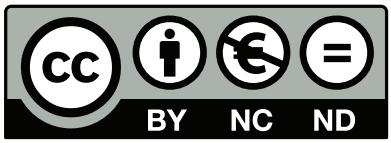

Memoria presentada por

Candelas Paniagua Correas

para optar al grado de Doctora por la Universidad de Málaga

Strawberry fruit softening and pectin disassembly:

I. Nanostructural characterization of fruit pectins by atomic force microscopy

II. Role of the *β*-Galactosidase gene *FaβGal4*

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Málaga, 2014

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que, Candelas Paniagua Correas ha realizado en este Departamento y bajo nuestra dirección el trabajo titulado **"**Strawberry fruit softening and pectin disassembly: I. Nanostructural characterization of fruit pectins by atomic force microscopy. II. Role of the *β*-galactosidase gene *FaβGal4,*" que constituye su memoria de Tesis Doctoral para aspirar al grado de Doctora Internacional en Biología. Y para que así conste, y tenga los efectos que correspondan, en cumplimiento de la legislación vigente, extendemos el presente informe.

En Málaga, a 10 de noviembre de 2014.

Dr. Miguel Ángel Quesada Felice Dr. José Ángel Mercado Carmona

A mis padres

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“Duda siempre de ti mismo,

hasta que los datos no dejen lugar a dudas”.

Louis Pasteur

INDEX

INDEX

CHAPTER V:FUNTIONAL CHARACTERIZATION OF *FAβGAL4*, A NOVEL β-GALACTOSIDASE GENE INVOLVED IN THE STRAWBERRY FRUIT SOFTENING

PREFACE

The structure of the present thesis manuscript is as follows.

Introduction has been divided into two main sections and it finishes with the proposal of the goals.

The first section addresses most of the fundamental aspects: botanical features and economical importance of strawberry fruits, cell wall and pectin structures and their relationship with fruit softening and, finally, main genes and enzymes involved in strawberry pectin processing during ripening.

The second section focuses on recent advances on pectin nano-structural modification assessed by atomic force microscopy. This section corresponds to a review published this year in Annals of Botany.

The **results and discussion** section is divided into three additional chapters. The third and fourth chapters focus on pectin changes during the ripening process and in selected transgenic genotypes with silenced pectinases, respectively. The fifth one describes the functional characterization of a novel transgenic line with a silenced galactosidase.

Finally, the **main conclusions are summarized** and **references** included.

Additionally, after the Conclusions, **a summary of the different chapters written in Spanish** is included to fulfill the presentation requirements stablished by the University of Málaga for International Thesis.

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ABBREVIATIONS:

A

AFM: atomic force microscopy
APG: antisense polygalacturonase
APEL: antisense pectate lyase
Ara: arabinose
ATR-FTIR: attenuated total reflectance FTIR

B

β-Gal: β-galactosidase
β-Xyl: β-xylosidase

C

cDNA: complementary DNA
CDTA: cyclohexane-trans-1,2- diamine tetraacetatic acid
CSP: CDTA-Soluble pectins
cv.: cultivar

D

DM: Degree of methylation
DMSO: dimethyl sulfoxide
DP: degree of polymerization
dRGII: dimer of RGII.

E

Exp: expansins

F

FTIR: Fourier transform infrared spectroscopy

G

Gal: galactose
GalA: galacturonic acid
GC: gas cromatography
Glc: glucose

H

HGA: homogalacturonan
HPSEC: High performance size exclusion chromatography

L

L_N: number-average contour length
L_W: weight-average contour length

M

ME: median
mRGII: monomer of RGII

P

PAW: phenol, acetic acid, water
PDI: polydispersity index
PEL: pectate lyase
PG: polygalacturonase
PGA: polygalacturonic acid
PME: pectin methyl esterase
PVPP: polyvinyl pyrrolidone

R

RGI: rhamnogalacturonan I
RGII: rhamnogalacturonan II
Rha: rhamnose
RT: room temperature

S

SEC: size exclusion chromatography
SEM: scanning electron microscopy
SSP:sodium carbonate soluble pectins

U

UA: uronic acid

W

WSP: water-soluble pectins

X

XGA: xylogalacturonan

INTRODUCTION: CHAPTER I

GENERAL CONCEPTS

1. THE STRAWBERRY

1.1 Systematics, taxonomy and morphology

The strawberry belongs to the *Fragaria* genus in the Rosaceae family, and it includes at least 23 species with varying ploidy levels (Folta and Davis, 2006). According to the ploidy level, strawberry species are classified in four groups: diploid (two sets of seven chromosomes), tetraploid, hexaploid and octoploid. The early varieties in Europe were derived from *F. vesca* (diploid) *and F. moschata* (hexaploid) and in American were derived from *F. chiloensis* (the octoploid species). Nowadays, the cultivated strawberry *(Fragaria* × *ananassa*) (Fig.1) (2n=8x=56) is a hybrid obtained from the octoploid species, *F. chiloensis* and *F. virginiana*, native to the Americas (Ahmadi and Bringhurst, 1991). These American species were introduced into Europe in 1714. They produce large, sweet and tasty fruits (Folta and Davis 2006). In addition, the hybrid improved agronomic aspects such as productivity (Senanayake and Bringhurst, 1967). *Fragaria* × *annanasa* is widely cultivated, and *F.vesca* L. and *F. moschata* Duch. also have a high commercial value. Nevertheless, both species have a narrow range of environmental adaptation.

The strawberry fruit is considered a soft fruit because it suffers bruises and flattening when small amount of pressure is applied (Bourne, 1979). The achene is the actual fruit of the strawberry plant, while the receptacle (the false fruit) is the edible part. The strawberry receptacle is composed of three types of tissue: the pith, the central cylinder, which is surrounded by the cortical tissue (composed by parenchymatic cells) and finally the epidermis (Harvis, 1943). The true fruits or achenes are embedded into the receptacle and are connected to the pith by the vascular system. Each strawberry may have from 20 to 500 achenes, depending on the environmental conditions and cultivars (Darrow, 1966). The achene is derived from one ovary and, when mature, it is composed of a thick pericarp, a thin testa, an endosperm and a small embryo (Winston, 1902)

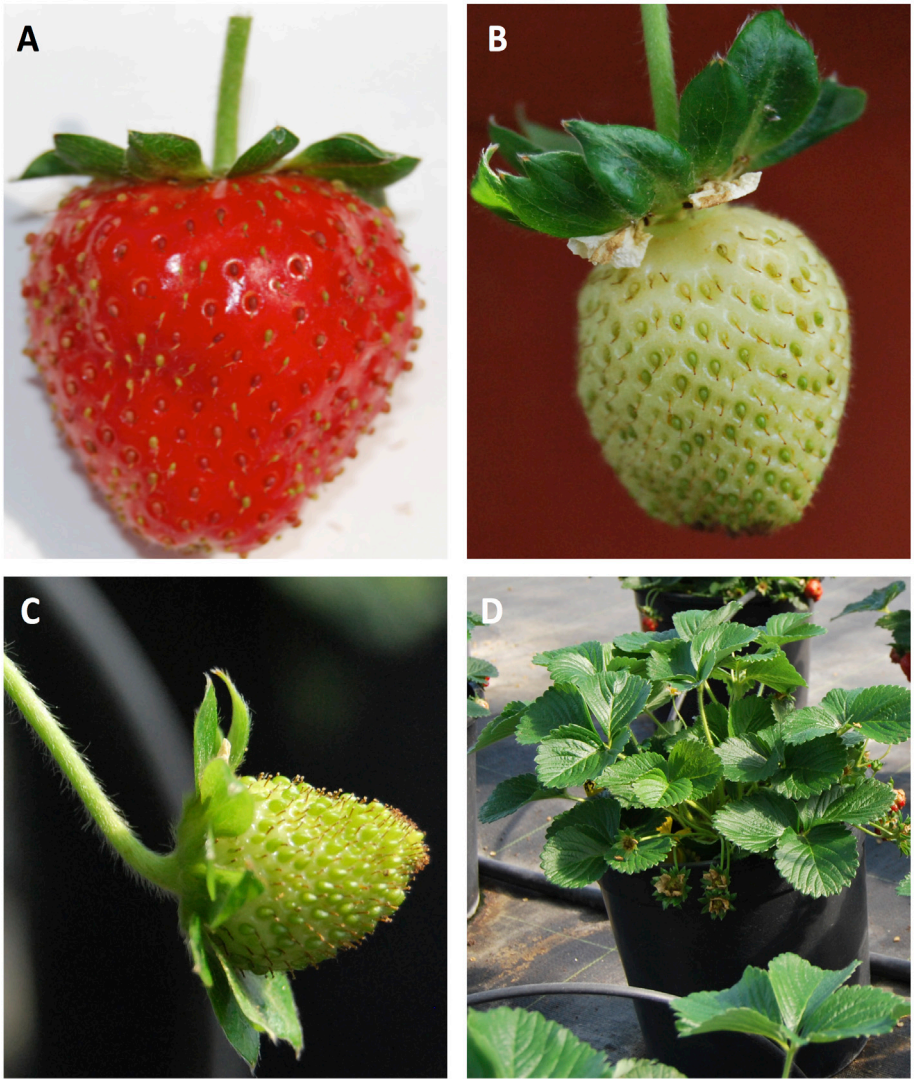

Figure 1. *Fragaria* × *ananassa* cv. 'Chandler'. **A**. Ripe fruit. **B**. White fruit. **C**. Green fruit. **D.** Strawberry plant.

1.2 Economic aspects

Fragaria × anannasa is one of the most economically important small fruits. The world production and consumer demands of strawberry fruits have been steadily increasing since the 1970s until now (Fig. 2). This could be due to its delicious flavour and taste, as well as its high nutritional value. Strawberries are enriched in essential minerals, organic acids, vitamins and antioxidant compounds which are benefit for human health.

The consumption of strawberries has also been associated with a decrease in the risk of cancer and heart diseases and with general protection of the immune system (Hannum, 2004)

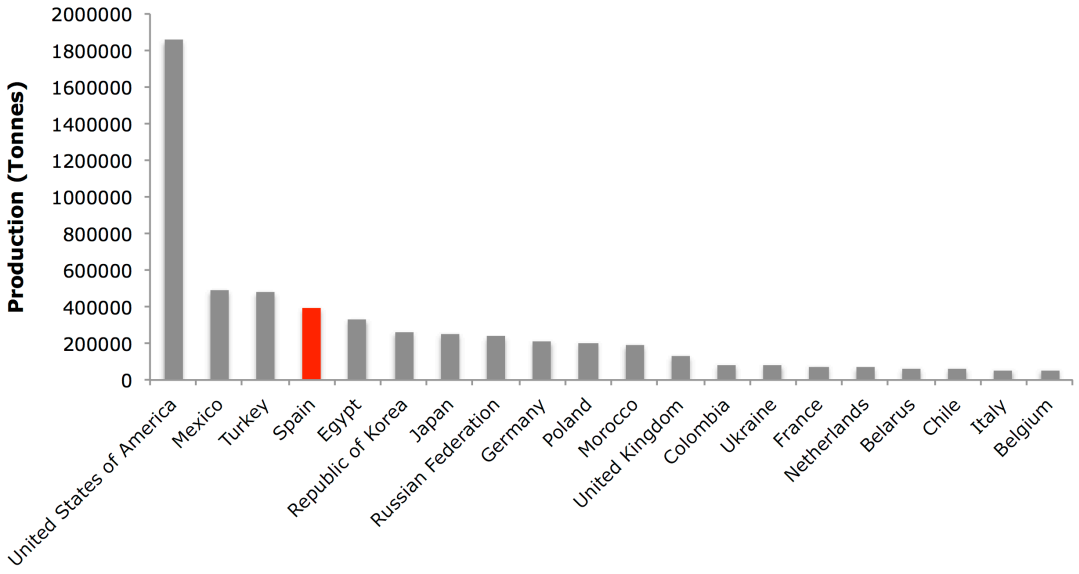

Figure 2. Strawberry production in 2012 (FAOSTAT 2014). This graph shows the 20 highest producing countries of strawberry fruits in 2012.

Spain is the fourth largest world producer of strawberries, after USA, Mexico and Turkey, producing around 400.000 tonnes per year. The national production has increased from 263.900 tonnes in 2008 to 393.475 tonnes in 2012 (FAOSTAT, 2014). Huelva, a province located in the southwest of Spain, contributes up to 80% of the national production, with more than 8000 hectares of monoculture. Nearly 50% of strawberry production is exported as fresh product, being France and Germany the main importer countries. In addition, strawberry is the non-climacteric fruit most used in the food industry (mainly in jams and juices).

1.3 Strawberry growth and ripening

Fresh weight, dry weight and fruit length or diameter are generally used to measure the growth of strawberry fruit (Perkins-Veazie, 1995). Two different growth models have been reported for this fruit. The first one

was characterized by an initial slow growth rate followed by an exponential phase and a final period of declining growth (Bollard, 1970). In a different growth model, strawberries show two periods of rapid growth separated by a slow growth phase (Coombe, 1992). The final shape of strawberry fruit is affected by the position of the flower, the duration of the cell division phase, the degree of cell enlargement, the number of cells and the size of the intracellular spaces, the number of pollinated achenes, the plant nutrition and the temperature (Darrow, 1966).

The ripening process is a genetically programmed process, where a large number of biochemical and physiological changes take place (flavour, fragrance, colour, texture) to facilitate seed dissemination. The fruits can be divided into two groups: climacteric, when the ripening is preceded by a peak on ethylene production and high respiration rates; and non-climacteric, when this peak is not necessary for initiating the ripening process. The strawberry is usually considered a non-climacteric fruit (Knee et al., 1977; Given et al., 1988), although recent studies show the presence of a peak of ethylene prior to ripening (Sun et al., 2013). There is controversy about the signal that starts the ripening in non-climacteric fruits. Recently, it has been suggested that the increase on the ratio abscisic acid (ABA)/auxin could trigger the ripening process in strawberry (Jia et al., 2011).

1.4 Fruit texture

Fruits can be classified as either "soft", which experience extreme softening during the ripening process, or "crisp", where the softening is moderate or null (Bourne, 1979). In general, fruit texture depends on multiple factors, such as tissue composition and architecture, cell shape, turgor pressure, mechanical properties of the cell wall and the strength and extension of adhesion areas between adjacent cells (Harker et al. 1997; Bourne, 2002). Softening during fruit ripening is a consequence of the dissolution of the middle lamella that reduces cell to cell adhesion, the

disassembly of parenchyma cell walls and the loss of cell turgor (Brummell and Harpster, 2001; Brummell, 2006), although this last process is considered of minor importance. In the case of strawberry fruit, softening is characterized by an almost totally dissolution of the middle lamella, a moderate pectin solubilisation and depolymerisation and a slight reduction of the molecular weight of hemicellulose. These processes will be more deeply discussed in the following sections.

2. THE CELL WALL

The plant cell wall is a common structure in all organisms belonging to the Plantae kingdom (Fig. 3). It is a three-dimensional complex mixture composed of polysaccharides, proteins and aromatic substances, which are secreted outside the cell. The cell wall experiences changes during cell division, expansion and differentiation (Carpita and Gibeaut, 1993). This structure determines the form of the plant, the growth and development, and it is also involved in the quality of many plant-based products. The cell wall components are assembled to form a complex network of covalent and non-covalent bounds, supplying the cell with mechanical strength and porosity, as well as regulating cell-cell adhesion and responding to pathogen attacks (McCann et al., 2001).

The cell wall is composed of three structural layers: the middle lamella, the primary cell wall and the secondary cell wall. The last one is not present in fruits, expect in some specialized types of cells like the xylematic bundle sheath. All the components are sequentially secreted out of the cell, creating the final organized structure. The middle lamella is the outer layer and the first to be synthetized, constituting the interface between the primary cell walls of neighbouring cells. After the process of cell division, the components of the cell wall build up forming the primary cell wall.

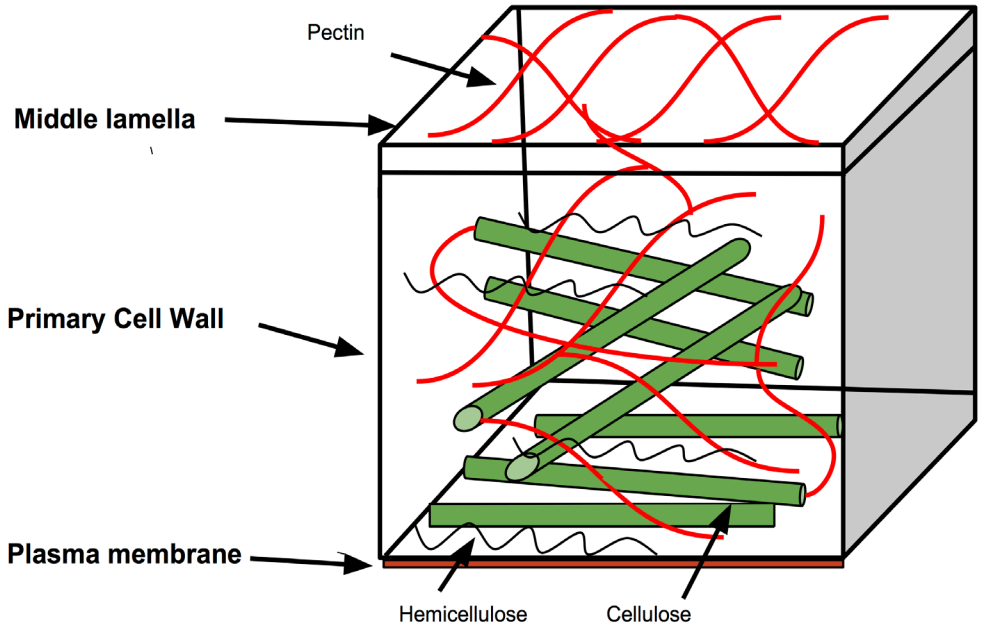

Figure 3. An outline of the plant cell wall.

2.1 Composition and structure of the primary cell wall

Primary cell walls are composed of pectin polysaccharides (35%), cellulose (30%), hemicellulose (25%), some structural proteins (10%) and a less representative group composed of esterified phenols. In fruit, the pectin component can reach up to 50% of the total polysaccharides (Fischer et al., 2001). Moreover, the wall is highly hydrated (65% water), and the aqueous phase contains various solutes, ions and soluble proteins, including enzymes. There are two phases in this wall, the microfibrillar and the matrix. The cellulose microfibrillar phase is highly crystalline and relatively homogeneous in its chemical composition. Hemicellulose and a complex of polysaccharides, which are highly hydrated, form the matrix.

According to its polysaccharide composition, the cell wall can be referred to as type I and type II. Type I primary walls are usually found in dicots, gymnosperms and non-commelinoid monocots, and consists of a cellulosic network embedded in a complex polysaccharide matrix. The wall referred to as Type II is found in Poales and Monocots and the predominant

components are glucuronoarabinoxylans (GAXs) and (1,3;1,4)-β-D-glucans (Carpita and Gibeaut, 1993). Nowadays, the distinction between Type I and II is questioned (Harris et al., 1997; Harris, 2005).

Different models have been proposed to explain how the cellulose, hemicellulose and pectins join to form the macromolecular network. Keegstra et al. (1973) described the first cell wall model in which all the matrix polymers were covalently linked together (Fig 4A). However, these links were not confirmed, and Hayashi et al. (1989) and Fry (1989) postulated the "Tethered Network Model" (Fig 4B), where the cellulose was tethered by xyloglucan and both components coexisted with an independent pectin network. Talbott and Ray published a variation of this model in 1992 (Fig 4C), in which the structure was the same as in the prior model but looser layers of matrix polysaccharides coated on the cellulose. A similar model with stratified layers was later suggested by Ha et al. (1997) (Fig 4D). Finally, Vincken et al. (2003) postulated a new hypothesis about the cell wall structure (Fig 4E) based on the observation of other researchers (Orfila et al., 2002; Ridley et al., 2001). In this model the cellulose/ xyloglucan framework would be embedded in a pectin matrix, and, therefore, the pectin matrix would have a more important role in the mechanical properties of the cell wall than the one proposed by the other models. This new cell wall view explains the formation of the middle lamella and microdomains, and the control of wall thickness.

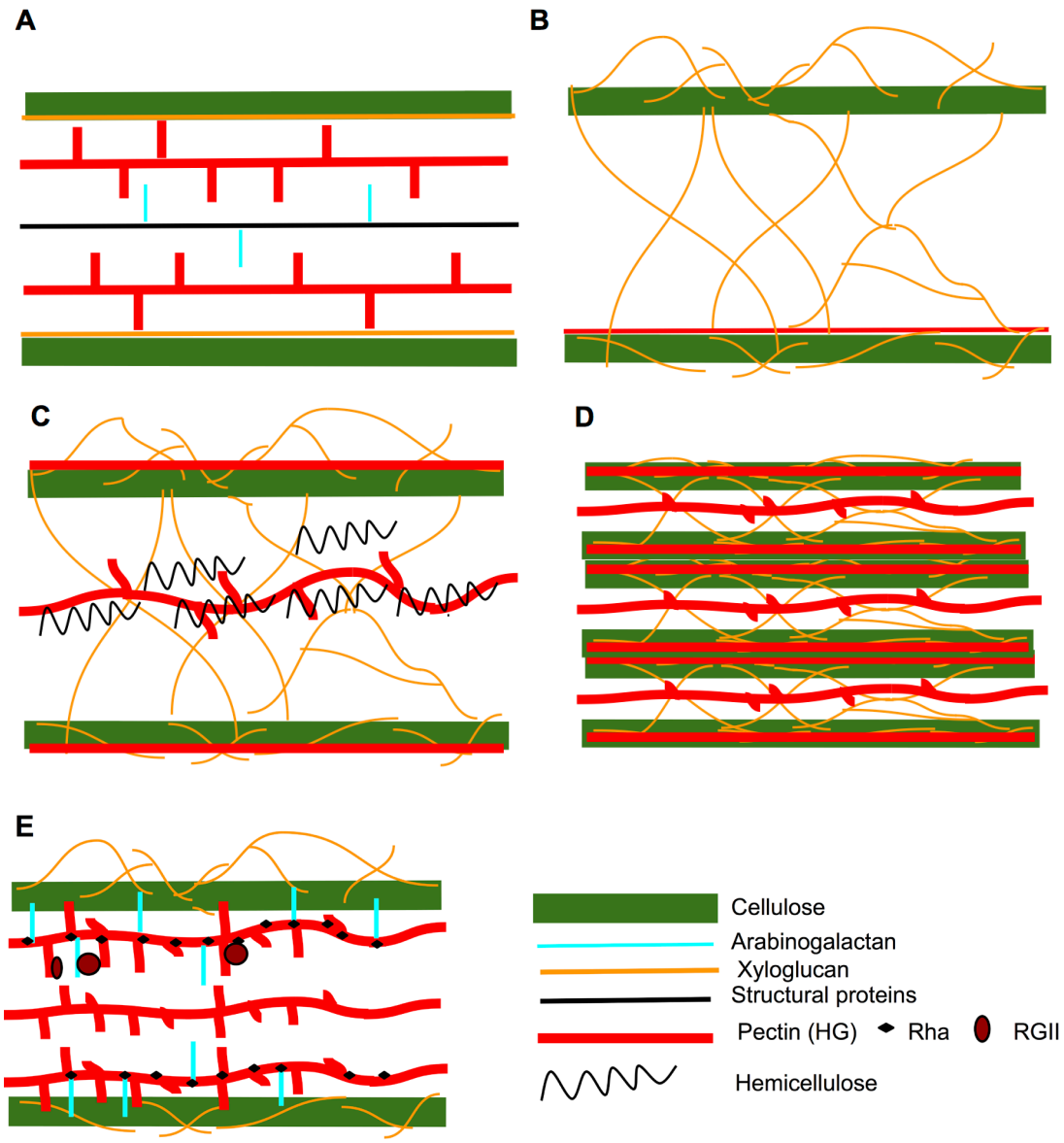

Figure 4. Alternative models of cell wall structure A. "Sticky model" of Keegstra et al. (1973). B. The "Tethered Network" C. "Multicoat Network" D. "Stratified Model". E. Model by Vincken et al. (2003).

2. 1.1 Pectins.

Pectins are one the main component in the primary cell wall and in the middle lamella and are involved in the firmness and changes of texture in the fruit during the ripening process (De Vries et al., 1984; Redgwell et al., 1992; Prasanna et al., 2007). Pectins also determine cell wall porosity. These polysaccharides have different domains (Fig. 5), associated with structural

elements, such as homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), arabinan, and arabinogalactan (Schols and Voragen 2002). HG is known as the 'smooth region' of the pectin, composed predominantly of a partially methyl-esterified (1-4)-linked α-D-galacturonic acid (GalA) homopolymer. When the HG appears with β-D-Xylp-(1-3) side chains, it is referred to as XGA. RGII is also a modification of HG, with about nine galacturonyl residues, containing clusters of five different chains with more than 20 uncommon sugar residues, e.g. 2-O-metilfucose, 2-O-metilxilose, apiose (Api), 3-C-carboxi-5-deoxi-L-xilose, 3- deoxi-mano-octulosonic acid (KDO) (Ridley et al., 2001; O'Neill et al., 2004); and 20 different links (O'Neill et al., 2004), constituting an oligosaccharide complex of 5-10 kDa (Whitcombe et al., 1995; Vidal et al., 2000; Strasser and Amadó, 2001). This polymer is usually found as dimmers with a borate cross-link (Ishii et al., 2001; Ishii et al., 1999). Early studies suggested that the RGII was a conserved dominium in plant evolution; however, some variations in the methy-esterification and methyl ether links, as well as in oxidation level, have been identified (Buffeto et al., 2014).

Another constituent of pectin, RGI or 'hairy region', is composed by a backbone of a repeating disaccharide unit [α 2)- α-L-Rhap-(1-4)-α-D-GalpA-(1-]n, where n can be larger than 100 residues (Visser and Voragen, 1996), with sugar side chains attached to the Rha residues (Maxwell et al.,2012). These side chains may contain galactan, arabinan and/or arabinogalactan residues (Willats et al.,2001). The abundance and nature of the side-chains can differ considerably from species to species (Vincken et al.,2003).

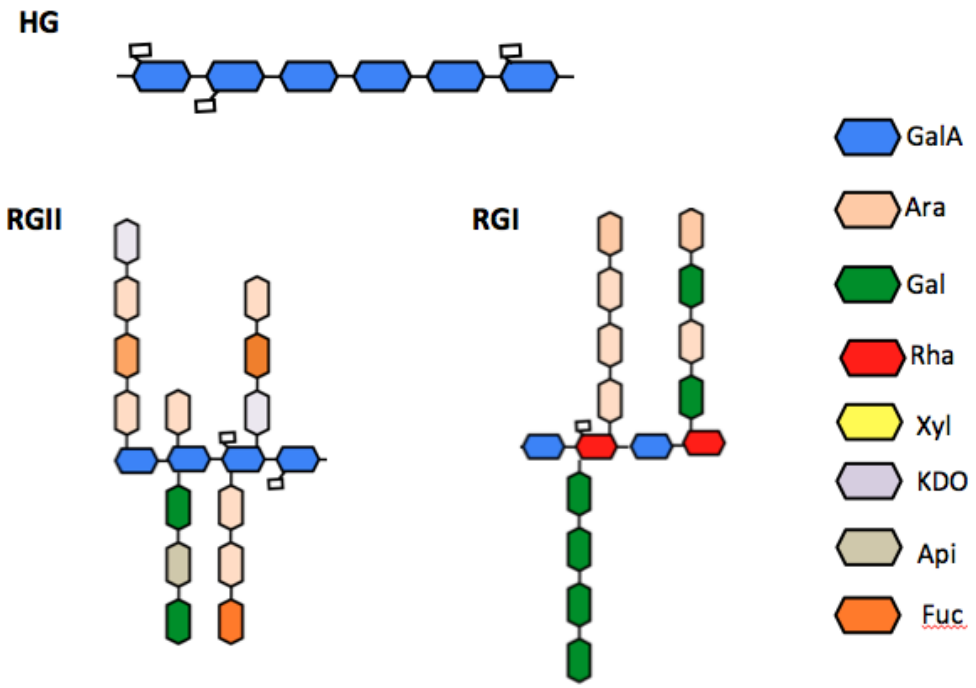

Figure 5. A simplified scheme of pectin components .

2. 1.1.1 Models for macromolecular structure of pectins

Although the chemical composition of pectins is well known, the links between the different kinds of polyuronides and their interactions with other cell wall components are poorly understood. It is widely accepted that pectin form an independent network in the cell wall flanked by the cellulose-xyloglucan network (Mccann et al., 1990; McCann and Roberts 1994; Ha et al., 1997; Vincken et al.,2003). However, there is not a consensus model for pectin structure *in muro.* Along this line, the hypotheses postulated by De Vries et al*.* (1982) and Vincken et al*.* (2003) are the most accepted. In the first model, the pectin backbone consists of (alternating) RGI and HG (Fig.6A). The second model postulates that RGI forms the pectin backbone, with HG and XGA as side chains interspersed with shorter neutral sugar side chains (arabinans, galactans) (Fig.6B). The exact distribution of these side chains, in block or randomly, is still not clear. The side chains distribution might have an important role in the properties of the cell wall (Vincken

et al., 2003). More recently, a third pectin model (Fig. 6C) has been proposed (Yapo, 2011). In this model, the pectin complex is formed by a RGI core connected with two unbranched HG blocks at the extremes. In each block, one of the HG elements would be linked to the RGI core via Rha whereas the other would be a side-chain of RGI. In the latter two models, the RGII would be found in the HG side chains linked by a covalent bond.

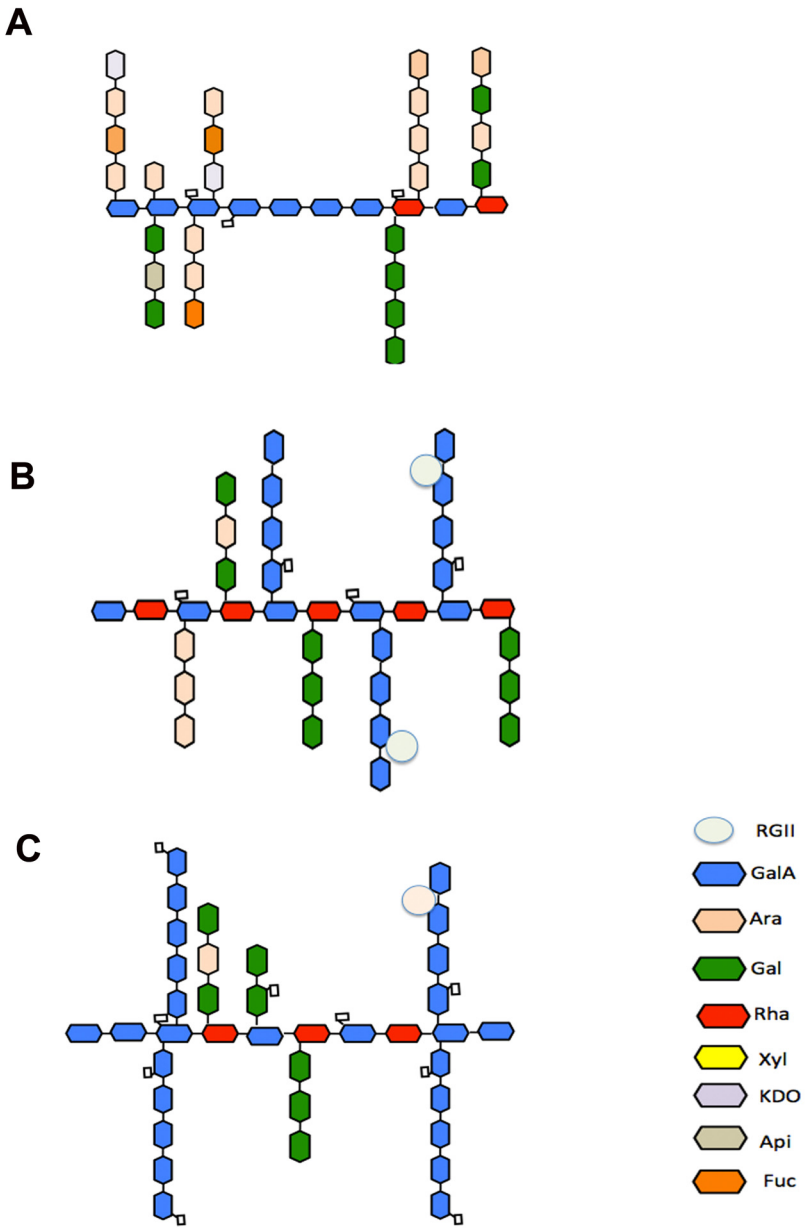

Figure 6. A scheme of pectin models. **A**.Vries´s model. **B**.Vincken´s model. **C**. Yapo´s model

2.1.1.2 Cross-links between pectin molecules result in the formation of networks

Different covalent and non-covalent bounds have been observed in pectin polymers, and are probably involved in intra- or intermolecular linkages. Among them, the calcium-pectin complex is the most studied. In this linkage, sections of two unesterfied pectic chains are held together by a number of calcium ions (Voragen et al., 2009). Ferulic acid esters have also been found in sugar beet pectins (Waldron et al., 1997). In addition, Lamport (1970) suggested that HG could be linked to relatively non-polar alcohols by uronyl esters. In pectins originating from cultured spinach cells up to approximately 5% of the GalA residues could be cross-linked in this way (Brown and Fry, 1993). Moreover, two molecules of RGII may form a complex with boron, linking two HG molecules

2.1.2 Cellulose

The chemical structure of the cellulose is a long linear chain of (1-4) β-D-glucan with a degree of polymerisation in the range from 5000 to 7500 glucose monomers, which form microfibrils, hydrogen bonded, with a parallel distribution. The cellulose chains have a repeating crystal conformation and around 30 to 200 independent chains are packed together to form the microfibril chains. The fibers are spread apart by 30 nm and polysaccharides, lignin and suberins are embedded in these spaces, providing high mechanical strength. The inner region of microfibrils is crystalline, which excludes water; however, the outer layers are amorphous (Pauly et al., 1999).

2.1.3 Hemicelluloses

Hemicelluloses are composed of long polysaccharides of non-cellulosic chains, which are bonded to neutral or slightly acidic side chains. These polymers are generally classified according to the main sugar residue in the backbone, e.g., xylans, mannans, and glucans, being xylans and mannans

the most common. The abundance of these polymers depends on the plant species, developmental stage, and tissue type. Hemicelluloses are involved in the regulation of wall elongation and modification and they have a strong effect on the interactions between cellulose microfibrils. Moreover, its side groups, as acetyl substituents, could have an effect on the solubility. Among the hemicelluloses the most important are xyloglucans, xylans, mannans and "mixed-linkage" glucans.

The xyloglugan (XGA) is a polysaccharide that is composed of linear chains of (1–4)β-D-glucan with numerous α-D-Xylose side chains, which could be substituted with α-L-Ara or β-D-Gal. This polymer is assembled by a polymerization degree of 6-11 residues, depending on the tissue and the species (Carpita and Gibeaut, 1993). The xylans (Fig 8C) are composed of β (1-4)-D-xylose linear chains, which are substituted by arabinose, glucuronic acid and methylglucuronic acid. This polymer is found primarily in the secondary cell wall (Wyman et al., 2004). XGA and xylans might form a link with cellulose and lignin by hydrogen bonding and by covalent linkages with other polysaccharides.

β(1-4) glucan or mixed-linkage glucans are composed of β(1-3) and β(1-4) D-glucose with a ratio 2:3. Finally, mannans are composed of linear polymers of β (1-4)-linked mannopyranosyl residues, with various combinations of galactose and glucose residues in the side chains, which are referred to as glucomannans, galactomannans, and galactoglucomannans.

3. CELL WALL MODIFICATIONS IN THE FRUIT DURING THE RIPENING PROCESS

The cell wall disassembly during ripening leads to fruit softening. This process involves a set of biochemical modifications of the cell wall components that are forming the primary cell wall and the middle lamella, mainly pectins and hemicelluloses, causing the weakening of the cell wall structure and the dissolution of the middle lamella (Brumell, 2006).

Cell wall modifying enzymes and proteins are involved in the modification of pectins (e.g. polygalacturonase, pectin methyl esterase, pectate lyase), and hemicelluloses, (e.g. cellulase, xyloglucanendotransglycosidase, expansin), during the ripening process (Brummel and Harpster, 2001). The cell wall disassembly process during strawberry ripening and the main proteins involved are discussed in the next sections.

3.1 Cell wall modifications during strawberry ripening

The solubilisation of pectins is the most common feature during the strawberry ripening. This process refers to an increase on the amount of pectins loosely bound to the cell wall. However, there is not a consensus about the role of pectin depolymerisation during the ripening. An early research, performed with chelated pectins, suggested that the depolymerisation process has not got an important role during ripening (Huber , 1984). However, Redgwell et al (1997b) showed a slight depolymerisation in pectins soluble in PAW(phenolic: acetic acid:water) o solvent used to inactivate enzyme during cell wall extraction. More recent studies displayed depolymerisation processes in covalently bound pectins as well (Rosli et al., 2004; Santiago-Doménech et al., 2008; Figueroa et al., 2010). These changes occur in different cultivars regardless of their texture (Koh and Melton, 2002; Rosli et al.,2004).

Regarding the neutral sugar content, it does not change significantly during strawberry ripening (Gross and Sam, 1984; Redgwell et al., 1997a; Koh and Melton 2002; Figueroa et al., 2010). Nevertheless, the galactose and arabinose content was reduced in the KOH 4M fraction during the ripening (Redgwell et al., 1997a; Koh and Melton 2002). The loss of the neutral sugar side chains produces an increase in the porosity (permeable pore size) and thus, this should facilitate the access of the cell wall modifying enzymes to their substrates (Brummell, 2006).

Table1. Summary of experimental results from transgenic strawberry plants with genes encoding cell wall proteins up regulated (FaPE1) or down-regulated (the rest of genes).

Protein family	Cell Wall target	Gene	Effect of gene suppression	References
Pectate lyase	Demethylated HGA	*plC*	Increased the fruit firmness and extended shelf life	Jiménez-Bermúdez et al. (2002); Youssef et al (2009)
Polygalacturonase1	Demethylated HGA	*FaPG1*	Increased the fruit firmness and extended shelf life	Quesada et al. (2009a); García-Gago et al.(2009)
Polygalacturonase2	Demethylated HGA	*FaPG2*	Increased the fruit firmnes s and extended shelf life	Paniagua et al. (unpublish result)
Pectin methylesterase	Methylated pectins	*FaPE1*	Increased fungal pathogen resistance	Osorio et al. (2008)
Endo-β-(1,4)-glucanase	Xyloglucan	*FaEG1*	No effect on firmness	Wolley et al. (2001), Palomer et al. (2006)
		FaEG3	No effect on firmness	Mercado et al. (2010)
Expansin	Xyloglucan-Cellulose	*FaExp2*	No effect on firmness Significant growth alterations	García-Gago et al (personal communication)
β-galactosidase	RGI-side chains	*FaβGal4*	Increased the fruit firmness	This thesis
Rhambogalacturonate lyase1	RGI		Increased the fruit firmness and extended shelf life	Molina-Hidalgo et al. (2013)
Pectin acetylesterase	HGA	*FaPAE1*	Increased the fruit firmness	Paniagua et al. (unpublish result)

The hemicellulose content diminished during strawberry ripening (Koh and Melton, 2002; Rosli et al., 2004). As occur for pectin depolymerisation, controversial results have been obtained regarding the molecular weight of hemicelluloses. Huber (1984) reported a significant depolymerisation of hemicelulosic polymers during fruit ripening. By contrast, Rosli et al. (2004) did not observe hemicellulose changes in other cultivars.

Several genes encoding cell wall modifying enzymes have been cloned in strawberry, and, for a few of them, their role on softening analyzed through genetic transformation. Table 1 shows the effect of the manipulation of the expresion of severals cell wall genes on fruit softening. Experimental results derived from these studies suggest a key role for pectin disassembly in fruit firmness.

3.1.1 Pectate lyase (PL)

The mechanism of activity for this enzyme involves the cleavage of glycosidic bonds in the unsaturated regions of homogalacturonan by β-elimination reactions in the presence of calcium ions (Posé et al., 2011). Three *pl* genes (*plA to plC*) have been identified in strawberry fruit whose expresion is up regulated during ripening (Medina-Escobar et al., 1997; Benítez-Burraco et al., 2003). The role of *pl* genes during ripening in strawberry fruit was demonstrated using a down-regulated transgenic line, in which the fruit firmness was greater with respect to non-transformed plants. In addition, cell wall analysis showed a lower degree of swelling, a reduction in the cell to cell adhesion and pectin solubilisation as well as a decreased depolymerisation in both chelated and covalently bound pectins (Jiménez-Bermúdez et al., 2002; Santiago-Doménech et al., 2008). The post-harvest behaviour displayed by these fruits improved showing longer shelf life (García- Gago et al., 2009a).Texture of processed fruit was also improved as result of *plC* silencing (Sesmero et al., 2007).

3.1.2 Polygalacturonase (PG)

PGs catalyse the hydrolytic cleavage of demethylated galacturonic linkages, and can be exo- or endo-type. The exo-PGs remove single galacturonic acid residues from the non-reducing terminal en of de-esterified polygalacturonic acid (Brummel and Harpster, 2001) and endo-type PGs, the most frequent form found in fruits, cleave these linkages at random. The endo-PGs have been described as ripening specific enzymes, and also play an important role in abscission zones, pollination and fruit growth (Hadfield and Bennett, 1998). These enzymes have been studied in a large number of species. Some fruits, such as tomato, avocado and peach, show an increase on PG activity during ripening (Hadfield and Bennett ,1998). However, PG activity is low in strawberry (Nogata et al., 1993; Lefever et al., 2004; Villarreal et al., 2008; Quesada et al., 2009a).

Three ripening-related polygalacturonases have been cloned in strawberry fruit: *FaPG1*, or *spG* (Redondo-Nevado et al., 2001), *FaPG2* or *B4* (Salentijn et al., 2003), and *T-PG* (Villarreal et al., 2008). *FaPG1* and *FaPG2* encode functional enzymes and are up-regulated during ripening (Villarreal et al., 2008; Quesada et al.,2009a; Villarreal et al., 2009). However, T-PG encodes an inactive protein due to a frame shift (Villarreal et al., 2008). The role of *FaPG1* on strawberry fruit softening has been tested by antisense transformation (Quesada et al., 2009a; García-Gago et al., 2009b). Ripe fruits from selected transgenic lines were significantly firmer than control. At the cell wall level, transgenic fruits showed a reduction in the solubilisation of pectins and an increase on pectins covalently bound to the cell wall (Quesada et al., 2009a). The down-regulation of *FaPG2* also reduces strawberry fruit softening (Paniagua et al., unpublished results)

3.1.3 β- Galactosidase (β- Gal)

β-Galactosidases are glycosyl hydrolases characterised by their ability to hydrolyse terminal, non-reducing β-D-galactosyl residues from numerous

β-D-galactoside substrates (Perez-Almeida and Carpita, 2006; Tateishi, 2008). In most fruits, β-Gals are encoded by small gene families with different patterns of expression that could play distinct roles in fruit development (Smith and Gross, 2000; Mwaniki et al., 2005; Tateishi et al., 2007; Othman et al., 2011). Functional analyses of β-Gal genes using transgenic plants have only been carried out in the climacteric tomato fruit model with contrasting results. Antisense *TBG4* tomato fruits displayed reduced *TBG4* mRNA levels and free cell wall galactose only at the onset of ripening but softening of ripe fruits decreased by 40% (Smith et al., 2002). By contrast, neither the silencing of *TBG1* (De Silva and Verhoeyen, 1998) nor *TBG3* (Carey et al., 2001) modified tomato fruit firmness. In *TBG6* antisense tomato plants, fruit firmness was also similar to control samples but these fruits showed structural alterations in the cuticle increasing fruit cracking (Moctezuma et al., 2003).

In strawberry, β-Gal activity increases during fruit development and remains high in ripe fruit (Trainotti et al., 2001; Figueroa et al., 2010). At the molecular level, Trainotti et al. (2001) isolated three full length cDNAs encoding β-Gal genes, *FaβGal1* to *FaβGal3*. Although all of them could be detected in fruit and vegetative tissues, only *FaβGal1* showed an expression pattern that could be related to the fruit ripening process, the other two genes being expressed mainly in green, immature fruits. Transcriptomic studies performed in our research group have enabled us to identify a new β-Gal gene, *FaβGal4*. In this thesis, the effect of *FaβGal4* down-regulation on fruit firmness and cell wall structure has been analysed (Chapter V).

3.1.4 Pectin methylesterase(PME)

PME catalyses the demethylesterification of homogalacturonan (Micheli, 2001; Pelloux et al., 2007). This process could play an opposing role in the softening process. On the one hand, block of pectins with free carboxyl groups stimulate the formation of calcium bridges (Micheli, 2001), so PME

contributes to cell wall stiffening; but these blocks are also necessary for the action of pectinases, such as polygalacturonase or pectate lyase (Draye y Van Cutsem, 2008), thereby PME contributes to the softening process.

In addition, there are some controversial about PME activity during strawberry ripening. Barnes and Patchett (1976) and Figueroa et al. (2010) showed an increase on PME activity during fruit development, from unripe (green) to ripe (red) stages. By contrast, Draye and Van Cutsem (2008) observed a continuous reduction in activity from the green to the overripe stage.

At the genetic level, four different PME genes have been described in the strawberry plant. Only one of them (*FaPE1*) is expressed in fruit, showing a peak of expression in the turning stage (Castillejo et al., 2004). Over-expressed transgenic lines showed a 20% reduction in the methyl esterification of soluble and chelated pectins, and a higher molecular mass of the Na_2CO_3 soluble pectin fraction, as well as greater resistance to *Botrytis cinerea* (Osorio et al., 2008).Comparatives studies carried out in commercial varieties of strawberries, suggested a correlation between fruit firmness and PME activity, with the exception of 'Camarosa' fruits (Lefever et al., 2004).

3.1.5 β-(1-4)Glucanase (EGase)

Endo-β-1,4-glucanases have been shown to be involved in abscission and in fruit softening; although their substrate is still unclear, it is believed that the xyloglucans and glucomannans are the targets of these enzymes (Brummell and Harpster, 2001).

Two EGase genes have been identified in strawberry *FaEG1* and *FaEG3* (Harpster et al., 1998; Llop-Tous et al., 1999; Trainotti et al., 1999a, 1999b) with a different pattern of expression during fruit development. *FaEG1* is fruit specific and its expression starts at the white stage; *FaEG3* is detected at an early stage, in vegetative tissue (Trainotti et al., 1999a). Studies carried out in transgenic lines with both genes silenced suggested

that there was no effect of the EGase on fruit firmness (Woolley et al., 2001; Palomer et al., 2006; Mercado et al., 2010). Nevertheless, cell wall analysis in transgenic plants displayed a higher amount of hemicellulose and larger molecular size of these polysaccharides as results of *FaEG3* silencing (Mercado et al., 2010).

3.1.6 Other cell wall genes

Other enzymes such as expansin, pectin acetyl esterase, rhamnogalacturonate lyase, arabinase, endo 1-4-β- xylanase and endo 1-4-β-mannanase, could contribute to the disassembly of the cell wall (Prasanna et al., 2007; Li, 2010; Molina-Hidalgo et al., 2013). In strawberry, the gene *rhamnogalacturonate lyase1* shows the same pattern of expression than other genes encoding cell-wall hydrolases. This gene seems to be involved in the dissolution of the middle lamella during strawberry ripening (Molina-Hidalgo et al., 2013).

Recent studies performed in tomato proposed that α-Man (α-manosidase) and β-Hex (β-D-N-acetylhexosaminidase) are also involved in the ripening process, and their silencing reduce fruit softening (Meli et al., 2010). These enzymes are glycosidases that act on short oligosaccharide chains from glycoproteins and other polymers (Badamaranahalli et al., 2004).

INTRODUCTION CHAPTER II:

FRUIT SOFTENING AND PECTIN DISASSEMBLY: AN OVERVIEW OF THE NANO-STRUCTURAL PECTIN MODIFICATIONS ASSESSED BY ATOMIC FORCE MICROSCOPY (AFM)

REVIEW- Annals of Botanny114:1375-1383. Special Issue "Cell Wall"

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1. SUMMARY

Background One of the main factors that reduce fruit quality leading to economically important losses is oversoftening. Textural changes during fruit ripening are mainly due to the dissolution of the middle lamella, the reduction of cell to cell adhesion, and the weakening of parenchyma cell walls as result of the action of cell wall modifying enzymes. Pectins, one major component of fruit cell walls, are extensively modified during ripening. These changes include solubilization, depolymerization and the loss of neutral side chains. Recent evidences in fruits with soft or crisp textures at ripening, strawberry and apple, suggest that pectin disassembly is a key factor in textural changes. In both fruits, the softening process was reduced as result of antisense down-regulation of polygalacturonase genes. Changes in pectic polymer size, composition and structure have traditionally been studied by conventional techniques, most of them relying on bulk analysis of a population of polysaccharides but studies focusing in the modifications at the nano-structural level are scarce. Atomic force microscopy allows the study of individual polymers at high magnification and with minimal sample preparation; however, AFM has scarcely been employed to analyze pectin disassembly during fruit ripening or postharvest storage.

Scope In this paper, firstly, the main features of the pectin disassembly process taking place during fruit ripening are discussed. Then, the nano-structural characterization of fruit pectins by AFM and its relationship with texture and postharvest fruit shelf life is reviewed. In general, fruit pectins are visualized under AFM as linear chains, few of them showing long branches, and aggregates. Number- and weight-average values obtained from these images are in good agreement with chromatographic analyses. Most AFM studies indicate a reduction in the length of individual pectin chains and in the frequency of aggregates as the fruit ripen, being pectins extracted with sodium carbonate, supposedly located within the primary cell wall, the most affected.

2. INTRODUCTION

Ripening of fleshy fruit is one of the last developmental phases of fruit ontogeny involving many genetic, biochemical and physiological modifications. Among others, changes include the accumulation of pigments and sugars, the production of aromatic compounds and flesh softening (Gapper et al., 2013). All these changes have evolved to make edible fruits more attractive to seed dispersal organisms. Ripening also enhances fruit organoleptic properties, making them adequate for human consumption. However, fruit quality diminishes as ripening reaches an advanced stage, mainly due to fruit oversoftening, increased pathogen susceptibility and the development of undesirable flavor and skin color, leading to important economic losses during postharvest fruit managing (Bapat et al., 2010). Thus, the rate of fruit softening not only determines the postharvest shelf life but also other economically important aspects such as the frequency of harvest, the handling procedures or the distance that the fruits can be transported. Therefore, the delaying of fruit softening is one of the major targets for breeding programs in most commodities. Based on the degree of softening during ripening, fleshy fruits are classified into two categories (Bourne, 1979), those that soften greatly as they ripen, acquiring a melting texture and usually having a short postharvest life (e.g., strawberry, peach, apricot, tomato) and those that soften moderately as they ripen, retain a crisp texture and have a long storage life (e.g., apple, pear, watermelon).

Firmness and juiciness are the most important textural components in the case of fleshy fruits (Toivonen and Brummell, 2008). Both features are largely determined by the characteristics of parenchyma cells (shape and size, cell wall thickness and strength, cell turgor) and the extension and strength of adhesion areas between adjacent cells (Harker et al., 1997). During ripening, parenchyma cell walls are extensively modified, altering their mechanical properties, and cell adhesion is significantly reduced as result of middle lamella

dissolution. Cell wall and middle lamella modifications leading to fruit softening result from the action of cell wall modifying enzymes (e.g., polygalacturonase (PG), pectin methyl esterase (PME), pectate lyase, β-galactosidase, cellulase) generally encoded by ripening-related genes (Brummell and Harpster, 2001; Goulao and Oliveira, 2008; Mercado et al., 2011). Other cell wall proteins with no hydrolytic enzymatic activity, such as expansins, also have a role in softening (Brummell et al., 1999). In general, the cell wall disassembly process responsible for softening involves the depolymerization of matrix glycans, the solubilization and depolymerization of pectins and the loss of neutral sugars from pectin-side chains (Brummell, 2006; Goulao and Oliveira, 2008). The extension of these changes greatly varies among different species (Mercado et al., 2011).

Recently, it has been suggested that the structural integrity of xyloglucan network performed by xyloglucosyltransferase/endohydrolase (XTH) could be important during fruit softening. This activity is generally higher during fruit expansion and then declines or remains constant during ripening (Goulao and Oliveira, 2008; Mercado et al., 2011). Miedes and Lorences (2009) suggested that XTH genes could be involved in the maintenance of cell wall structure rather than cell wall disassembly, and therefore, the decrease on XTH gene expression and activity could contribute to softening. Supporting this hypothesis, the overexpression of *SlXTH1* gene in tomato reduced fruit softening (Miedes et al., 2010). In the other hand, although generally considered of minor importance than cell wall disassembly, cellular turgor also plays a role in fruit softening (Harker et al., 1997). A loss of turgor pressure is generally observed during fruit ripening due to a regulated accumulation of apoplastic solutes (Wada et al., 2009), but transpirational water loss through the cuticle could also be involved (Saladié et al., 2007). Cell turgor may also be influenced by cell wall modifications taking place during fruit softening (Thomas et al., 2008), and it is therefore difficult to distinguish between causes and effects.

Pectin is the most abundant polymer in the middle lamella regulating intercellular adhesion (Willats et al., 2001), but primary fruit cell walls are also rich in polyuronides, accounting for up to 60% of cell wall mass in many fruits (Redgwell et al., 1997a). Probably, pectins are the cell wall components that change most during fruit softening, but their role in fruit firmness and softening is considered somehow controversial. This could be due to the results obtained with tomato fruit which has been extensively used as a model species to study fruit ripening. Ripening of this fruit is characterized by a high increase in PG mRNA levels, protein and enzymatic activity which correlates with the rate of softening (Brummell and Harpster, 2001). However, the down-regulation of a PG gene to reach a residual gene expression of 1% when compared with wild type fruits did not reduce softening, although transgenic overripe fruits showed an improved storage life (Smith et al., 1990; Kramer et al., 1992; Brummell and Labavitich, 1997). Even more, although depolymerization of solubilized pectins was suppressed in transgenic tomatoes, the solubility of pectins remained at wild-type levels (Smith et al., 1990). These early findings lead to the hypothesis that PG-mediated pectin disassembly is neither necessary nor sufficient to induce fruit softening, especially at early stages of the process (Hadfield and Bennett, 1998). This hypothesis was later supported by studies performed in the tomato mutant *rin* showing an altered ripening behavior (Giovannoni et al., 1989). However, more recent evidences in different species suggest an important role of pectin modifications in fruit softening. Thus, the down-regulation of a pectate lyase or a *PG* gene in strawberry significantly reduced fruit softening (Jiménez-Bermúdez et al., 2002; Quesada et al., 2009b). Similarly, silencing of *PG1* gene in apple, a crisp fruit with textural features completely different to strawberry, also diminished softening (Atkinson et al., 2012).

Pectin is one the most complex natural plant biopolymers (Vincken et al., 2003; Voragen et al., 2009). This complexity makes polyuronides difficult to characterize and to infer their role in the cell wall when analyzed by conventional techniques, most of them relying on measuring the colligative

properties and characteristics of a population of polysaccharides (Round et al., 2001). Atomic force microscopy (AFM) allows the analysis of individual polymer chains and to study the degree of heterogeneity within a sample with minimal sample preparation (Morris et al., 2010; Liu and Cheng, 2011). This technique has been used to determine the structure and functionality of polysaccharides from different sources, mainly focusing on aspects related to food characteristics (Kirby et al., 1995a; Morris et al., 2001). However, AFM has scarcely been used to study pectin modifications during fruit softening. The aim of this paper was to review the potential of AFM as a tool to gain insight into the pectin disassembly process during fruit ripening.

3. PECTIN DISASSEMBLY DURING FRUIT RIPENING

Traditional methodological approaches to study cell wall disassembly during ripening involve the purification of cell wall and the isolation of different cell wall fractions based on their solubility on different solvents (Brett and Waldron, 1996). In this approach, pectins are extracted from cell wall material after sequential treatments with water or PAW (phenol:acetic acid:water); chelating agents such as trans-1,2-diaminocyclohexane-N,N,N'N'-tetraacetic acid (CDTA), solubilizing pectins ionically bound to the cell wall; and sodium carbonate, releasing pectins covalently bound to the wall by ester linkages (Brummell, 2006). This pectin fractionation is arbitrary but it has been suggested that fractions represent different polymer moieties *in muro*. Thus, water extraction yields mainly pectins (water soluble pectins, WSP) freely soluble in the apoplast and already solubilized by processes occurred in vivo (Redgwell et al., 1992); chelated soluble pectins (CSP) present in the CDTA fraction are enriched in homogalacturonan (HGA) from the middle lamella; and sodium carbonate soluble pectins (SSP) show characteristically high ratios of neutral sugars to uronic acid, suggesting enrichment in rhamnogalactorunan I (RG-I) from the primary wall (Brummell, 2006). Significant amounts of polyuronides,

usually enriched in neutral sugars, still remain in the cell wall after this sequential extraction procedure (Redgwell et al., 1997*b*; Brummell et al., 2004).

Pectin solubilization is a common feature in fleshy fruit ripening. This process refers to an increase in the content of pectin loosely bound to the cell wall, mainly WSP but in some cases also chelated-soluble pectins, that is paralleled to a decrease in the amount of covalently bound pectins. It is thought that pectin solubilization occurs at the expense of polyuronides tightly bound to the wall (Wakabayashi, 2000; Brummell, 2006; Mercado et al., 2011). Pectin solubilization varies greatly among species. This process is very evident in fruits such as avocado (Wakabayashi et al., 2000), kiwifruit, tomato, persimmon (Redgwell et al., 1997*a*), strawberry (Figueroa et al., 2010), melon (Rose et al., 1998) or peach (Brummell et al., 2004), but, in general, it is less important in fruit with a crisp texture at ripening, such as apple, watermelon or nashi pear (Redgwell et al., 1997*a*; Hiwasa et al., 2004). Several processes have been proposed as responsible for pectin solubilization. The loss of arabinan and galactan side chains from RG-I could contribute to the solubilization because neutral chains might anchor pectins into the wall either through binding to matrix glycans or cellulose (Popper and Fry, 2005; Zykwinska et al., 2005) or by physical entanglement with other wall polymers. Supporting this hypothesis, many fruits show a marked loss of neutral residues, mainly arabinose and galactose, during ripening (Gross and Sams, 1984; Redgwell et al., 1997*b*). However, neutral sugar loss and pectin solubilization are not correlated in some fruits, e.g., plum shows an extensive depolymerization with any loss of arabinose or galactose, while apple and nashi pear show an extensive loss of galactose but a very slight pectin solubilization (Redgwell et al., 1997*b*). According to Redgwell et al. (1997*b*) pectin solubilization and loss of galactose take place mostly in different cell wall polysaccharides. The loss of neutral sugars could also induce pectin solubilization indirectly, by increasing the wall porosity allowing the access of other hydrolase enzymes to their substrate. This hypothesis has

been suggested by Smith et al. (2002) to explain the increase in firmness in transgenic tomato fruit with a β-Galactosidase gene silenced.

An alternative or complementary cause of solubilization would be the depolymerization of chelated and/or covalently bound pectins as result of the action of pectinase enzymes, such as polygalacturonase. Some fruits display a substantial loss of high molecular weight polymers coupled with an increase of intermediate and short pectins, e.g., avocado, tomato, peach, kiwifruit (Redgwell et al., 1992; Huber and O´Donoghue, 1993; Redgwell et al., 1997a; Brummell et al., 2004;). By contrast, other fruits show a low to moderate depolymerization of pectins, e.g. strawberry, blueberry, melon, apple (Redgwell et al., 1997a; Rose et al., 1998; Vicente et al., 2007). Although depolymerization is not as general as polyuronide solubilization, recent evidences obtained in fruits with crisp and soft texture when ripened support a key role of pectinases in softening. In strawberry, a soft fruit, Jiménez-Bermúdez et al. (2002) showed that the silencing of a pectate lyase, an enzyme that catalyzes the cleavage of glycosidic bonds of de-esterified pectins by a β-elimination reaction in contrast to the hydrolytic mechanism of PG, resulted in fruits firmer than the control at the ripening stage, without affecting other fruit quality traits such as soluble solids, color or anthocyanin content. Cell wall analyses of these fruits indicated a lower depolymerization of chelated and sodium carbonate soluble pectins, as well as a lower degree of pectin solubilization (Santiago-Doménech et al., 2008). More recently, Quesada et al. (2009a) obtained strawberry plants with a PG gene down-regulated. These plants displayed a significant increase in fruit firmness at ripening, even higher than those of plants obtained with the silencing of the pectate lyase gene (García-Gago et al., 2009b). Cell wall modifications detected in these fruits were similar to those of the antisense pectate lyase plants, i.e. a 42% decrease in polyuronide solubilization concurrently with a slightly lower depolymerization of chelated and covalently bound pectins, a decreased cell wall swelling and an increase in cell to cell adhesion (Posé et al., 2013). The silencing of a PG gene in apple, a fruit with a crisp texture, caused cell wall

changes similar to those described for strawberry and also improved fruit firmness and textural characteristics (Atkinson et al., 2012). These results challenge the previous notion that pectin depolymerization alone is not the determinant of softening, an idea mainly supported by the minor effect of PG silencing on tomato fruit firmness observed in earlier works (Haldfield and Bennett, 1998).

Progressive demethylesterification of HGA by pectin methyl esterase (PME) action is common to the ripening of many fruits, creating large regions of negatively-charged side groups (Prasanna et al., 2007; Goulao and Oliveira, 2008). In the absence of Ca^{2+}, this cell wall modification would contribute to the loosening of pectins by electrostatic repulsion (Brummell, 2006). However, Ca^{2+} concentration in the apoplast is high enough to allow cross-linking of unesterifed HGA chains by ionic interactions (Almeida and Huber, 1999). Furthermore, functional studies of PME genes in tomato (Tieman and Handa, 1994) and *Fragaria vesca* (Osorio et al., 2008) failed to find an effect of this gene in fruit firmness. However, the degree of pectin esterification could be related to tissue integrity and cell adhesion. Thus, transgenic tomato fruits with the PME gene silenced showed a reduced shelf life as result of an almost complete loss of tissue integrity in senescent fruits (Tieman and Handa, 1994). An altered pattern of methyl-esterification has also been observed in the *Cnr* (Colourless non-ripening) tomato mutant, which fails to soften but displays a severe reduction in pericarp cell adhesion (Orfila et al., 2002). Alternative hypotheses to explain the solubilization of pectins during fruit ripening are the synthesis of more freely soluble forms of pectin as the fruit ripens (Huber, 1984) or the degradation of RG-I backbone by rhamnogalacturonases (Molina-Hidalgo et al., 2013). These last authors found that transient silencing of a gene encoding a rhamnogalacturonate lyase prevented the dissolution of the middle lamella during strawberry fruit ripening, suggesting a key role of RG-I in fruit softening.

In conclusion, the processes that lead to solubilization of polyuronides in the wall during fruit softening are unclear. Recent evidences support a role for polyuronide depolymerization as the main mechanism involved, which had previously been discarded as a possible cause. Altogether, the involvement of different mechanisms depending on the species could be probable. As suggested by Brummell (2006), a better understanding of how HGA, RG-I and RG-II are interconnected to each other and to other polymers in the wall would help to explain this issue. The nano-structural characterization of polyuronides and their structural changes during ripening by AFM could be a powerful approach to reach a better explanation to this problem, as it is discussed in the next sections.

4. AFM ANALYSIS OF FRUIT PECTINS

4.1 AFM fundamentals

Atomic force microscope belongs to a family of instruments known as scanning probe microscopes that generate images of the samples by "feeling" rather than "looking" the specimen, allowing higher magnification than light microscopy and comparable magnification to electronic microscopy but obtainable under the more natural conditions used for light microscopy (Morris et al., 2010). In brief, a sharp tip is scanned relative to the sample surface and the changes in the magnitude of the force between tip and sample, are measured and used to produce a three dimensional image (profile) of the surface topography of the sample with subnanometer resolution. The sample is mounted in a liquid cell on top of a piezoelectric transducer controlling the motion of the sample in the three spatial dimensions at high resolution. The tip is positioned on a flexible cantilever allowing the tip to move up and down as it tracks across the sample. The cantilever deflection, and hence the displacement of the tip, is monitored using a laser beam focused onto the end of the cantilever and then, reflected off onto a four quadrant photodiode

detector. As the tip moves in response to the sample topography the angle of the reflected laser beam changes and the movement of the reflected spot on the surface of the photodetector is recorded as an electrical potential difference (error signal). This error signal can be used in a number of ways to generate a variety of images of the sample surface or of objects deposited onto this suface. A full description of this system and the different operation modes is described elsewhere (Kirby et al., 1995a; Morris et al., 2010). In addition to the molecular/sub-molecular resolution of this microscope for biological systems, AFM allows the visualization of samples with minimal preparation under natural or physiological conditions, in air or aqueous environments (Morris et al., 2010; Liu and Cheng, 2011).

Each type of sample requires the selection of the appropriate imaging mode. Two main methods are used for imaging polysaccharides (Morris et al., 2010). In the first case, polymers are drop-deposited onto mica, air dried and then imaged in the dc contact mode under alcohols. In this method, the AFM tip is brought into direct contact with the sample, and imaging under a liquid allows a greater precision in the control of the applied forces (Morris et al., 2010). Solvents such as propanol and butanol are the most frequently used. In this mode, as the probe is permanently in contact with the sample, a shear force can be generated causing damages to the probe but also to the sample in case of being greatly soft. In the second method used to image polysaccharides, samples are sprayed onto mica, air dried and then imaged in the ac non contact mode under air (McIntire and Brandt, 1997). Briefly, in this mode the cantilever is oscillated at a constant frequency above the sample and the weak attractive forces between tip and sample are monitored. As the sample is being viewed from a greater effective distance, there is a loss of resolution compared to conventional AFM. When the same samples have been studied with both techniques, similar results have been obtained (Morris et al., 2010). A variant of the non contact mode is known as tapping mode. In this case, during oscillation the probe contacts the surface of the sample intermittently at the lowest point, reducing lateral forces during imaging and

minimizing sample damaging (Morris et al., 2010; Liu and Cheng, 2011). The three AFM techniques above described have been used to study nanostructure of pectins isolated from fruits (Table 1). A typical image of pectins isolated from strawberry fruit and visualized under butanol in contact mode is shown in Fig. 1. This image clearly shows the complexity of extracted pectin structures that might reflect the intricacy of the original pectin networks in muro.

4.2 Fruit pectin nanostructure

Table 1 summarizes the AFM available data about pectin fractions extracted from several fruits, including comparison between different developmental stages and cultivars with different textural properties. Early AFM studies focused on the analysis of pectins from unripe tomatoes. Round et al. (1997) analyzed pectin samples extracted with sodium carbonate. Images showed a mixed population of single polymers and aggregates. Interestingly, 20% of single polymers showed long branches with about 30% of these having more than one branch. Lengths of the main backbone ranged between 30 and 390 nm and size distribution fitted a log-normal curve. The range of length sizes for the branches was narrower, ranging from 30 to 170 nm. Apparently, the removal of acetyl and methyl groups during alkaline extraction of SSP pectins does not modify its nano-structure, since tomato pectins extracted with CDTA and visualized by AFM were similar to SSP chains. However, these pectins were about 3 times longer than those extracted with sodium carbonate (Round et al., 2001; Kirby et al., 2008). Complementary analyses of neutral sugar composition and linkage suggested that these branches do not correspond to neutral sugars (Round et al., 2001), and it was postulated that the long branches observed in CSP, enriched in HGA, and SSP, enriched in RG-I, consist of polygalacturonic acid attached to the pectin backbone, with the neutral sugars present as short branches undetected by AFM (Round et al., 2001). This result has been supported by subsequent AFM experiments on pectins from apple (Zareie et al., 2003), peach (Yang et al., 2009) and red ripe strawberry

fruits (Posé et al., 2012a). In this last species, both CSP and SSP pectins showed a similar percentage of branched polymers, 8-9%, but SSP pectins showed smaller but more branches per backbone (Posé et al., 2012a). The lower size of SSP strawberry pectins was confirmed by size exclusion chromatography (Posé et al., 2012a). The polygalacturonic acid nature of the pectin branches observed by AFM has been strongly supported by experiments evaluating the effect of mild acid hydrolysis on SSP pectins from unripe tomato (Round et al., 2010). This treatment releases different sugars residues present in polyuronides at different rates, with galactose and arabinose linkages the most labile and the galacturonic acid the most resistant (Thibault et al., 1993). Round et al. (2010) observed that almost complete hydrolysis of galactose, arabinose and rhamnose has no significant effect on backbone and branch length distributions in individual pectins visualized by AFM. This result suggests that the observed single polymers were mainly formed by HGA and contain either no rhamnose or only small amounts located at the end of these chains. Besides isolated chains, complexes were also observed in these pectin samples, often possessing emerging strands with similar dimensions to isolated single chains. The size of the complexes observed in SSP samples by AFM decreased upon acid hydrolysis in parallel with the loss of neutral sugars. As the remnants of these complexes persisted after prolonged hydrolysis, Round et al. (2010) suggested that they may contain RG-I linked to irreducible HGA. Therefore, AFM offers a new view of sodium carbonate soluble pectins consisting of linear and sparsely branched HGA and aggregated complexes containing RG-I, irreducible aggregates of HGA and HGA polysaccharide chains (Round et al., 2010). The individual HGA chains observed either would be not linked to the RG-I or would be linked by bonds that are broken during extraction from the cell wall. In this picture, neutral sugars would be coiled or aligned along and around the rhamnogalacturonan regions. Pectin aggregates of similar size and shape have been observed in pectins from strawberry fruits as can be observed in Fig. 1.

In most AFM studies, pectin length varied in the range from 20 to 1000 nm, with pectin present in CSP in general longer than those observed in SSP

(Table 1). However, pectin lengths several times higher than the range above described have been reported for some fruits, e.g., peach (Yang et al., 2009), unripe jujube (Wang et al., 2012). In SSP pectins from ripe strawberry fruit, Posé et al. (2012a) also described the presence of large fibers within the background of individual molecules and aggregates, but these fibers disappeared when the samples were heated at 80ºC. Whether these large fibers represent novel supramolecular pectin structures, or whether they are artifacts formed during the extraction and/or deposition of the samples on the mica substrate, needs to be addressed. Besides pectin length, AFM allows the tridimensional measurement of pectin chains. Most reports indicate a height of the pectin chain in the range 0.5-2 nm. These measurements are in accordance with a helix structure of the chains with different aggregation status (Yang et al., 2009), since the vertical height of single-stranded polysaccharides is about 0.5 nm (McIntire and Brant, 1999). Contrary to height measurements, there is great variation in pectin chain widths amongst the different reports. Yang et al. (2009) observed that widths of pectins from peaches varied between 20 to 100 nm, not observing significant differences either among the pectin fractions analyzed (WSP, CSP and SSP) or peach genotypes. By contrast, in strawberry fruit the width of SSP chains was smaller than those of WSP or CSP, ranging the values between 15 to 78 nm (Chen et al., 2011). Xin et al. (2010) reported a mean width value of CSP pectin chains of 35 nm in unripe tomato fruit which decreased to 23 nm in turning fruit. Some regularity in the pectin chain width has also been reported for some fruits. For example, Zhang et al. (2012) described that chain width of the WSP and CSP fractions from peach followed three basic units, 54, 72 and 91 nm, and the widths of other pectin chains could be obtained by the sum of these basic units. In the same fruit, Yang et al. (2005) observed four basic width units for WSP but these units were smaller than those reported by Zhang et al. (2012) (11.7, 15.6, 19.5 and 35.1 nm). A pattern of pectin chain width distribution has also been observed in SSP pectin chains from cherries with four basic units (37, 47, 55 and 61 nm) (Zhang et al., 2008). These results suggest a natural structural conformation of pectins formed by intertwists between

these putative basic units. However, pectin width data should be considered with extreme caution and only used for qualitative comparison of objects within a particular sample. This is because the molecular width measured by AFM is subject to "probe broadening"; as the radius of the AFM tip is large relative to the diameter of the molecules being measured the image of the molecule being analyzed is convoluted with the tip profile and the measured dimension is considerably overestimated (Morris et al., 2010). Kirby et al. (1995b) observed that thickness of individual strands of the bacterial polysaccharide acetan was 5 times greater than the value obtained when the measurements were performed in aggregated regions where the molecular chains had become aligned and the broadening effect was not present. McMaster et al. (1999), observed the width of a cylindrical object as about three times greater than the actual value. Morris et al. (1997) and Fishman et al. (2007) described a geometrical correction to estimate the tip broadening effect. Probe broadening effects will vary with the actual radius of the tips used and can vary considerably from image to image. This might explain why chain width values reported are usually almost an order of magnitude higher than the observed pectin heights and, therefore the lack of correlation between pectin chain height and width, as well as the contradictory results obtained in different fruits and studies.

In general, nanostructural pectin features deduced from AFM images are in good agreement with data obtained using other approaches. Thibault et al. (1993), using high-performance size exclusion chromatography (HPSEC), reported that HGA pectins from different fruit sources hydrolyzed in 0.1M HCl contained approximately 72-100 galacturonic acid (GalA) residues. Similar degree of polymerization was found by Yapo et al. (2007) in HCl hydrolyzed citrus pectins using chromatographic techniques. According to Carpita and Gibeaut (1993), HGA contains up to 200 GalA units and are about 100 nm long. As above discussed, most AFM studies of isolated pectin chains reported a molecular length in the range 75-150 nm, which is in accordance with these data. The number of carbohydrate residues, as well as molecular weight, in individual pectins visualized by AFM can be estimated from contour length

parameters considering that extended chains adopt a 3^1 helical structure with a pitch of 1.34 nm, as deduced by Walkinshaw and Arnott (1981) based on X-ray diffraction data. Using this approach, Round et al. (2010) found that minimum native size of linear chains in SSP pectins from unripe tomato could be around 320 residues, slightly higher than the polymerization degree of HGA purified by mild acid hydrolysis (Thibault et al., 1993; Yapo et al., 2007). However, it should be taking into account that pectin length can be greatly influenced by the ripening stage of the fruit source, as discussed latter. As regard RG-I, the length of this polysaccharide is unknown (Carpita and McCann, 2000). McNeil et al. (1980) found that purified RG-I from sycamore cell walls treated with endopolygalacturonase had a MW of approximately 200 kDa, being the backbone formed by as many as 500 glycosyl residues. Interestingly, complexes found in AFM images of unripe tomato SSP pectins, which have been proposed to be composed mainly by RG-I, but also contains HGA chains, have a MW slightly higher, around 400 kDa (Round et al., 2010).

In summary, most nanostructural data of fruit pectins obtained by AFM analysis are in agreement with our previous knowledge on these polysaccharides, supporting the reliability of this approach to study pectin disassembly during fruit softening. The analysis of AFM published data differentiate two groups of reports, a first group describing pectin lengths below 1000 nm and widths between 10-50 nm, including data from tomato (Round et al., 1997; 2010), strawberry (Posé et al., 2012) and cherry (Zhang et al., 2008; Lai et al., 2013), and a second group which describe longer (>1000 nm) and wider (>50 nm) pectin chains, such as those observed in peach (Yang et al., 2009), jujube (Wang et al., 2012) and apricot (Chen et al., 2013). Applying the width correction described by Morris et al. (1997), the first group of pectins would correspond mainly to individual pectin chains while the big fibers would be highly packed pectins of several hundreds of laterally arranged chains. If this high level of packaging exists in muro or not needs to be addressed in the future. Similarly, the physicochemical nature of micellar aggregates and its relationship with isolated pectin chains should be further investigated.

Table 1. Summary of AFM nanostructural characterization of pectins isolated from different fleshy fruit.

Fruit	Cultivar	Stage	Pectin fraction	Pectin length (nm)	Pectin width (nm)	Image condition	Reference
Apricot *Prunus armeniaca* L.)	Jinhong	ripe	CSP	500-3000	23-234	Air; TP	Chen et al. (2013)
Cherry (*Prunus pseudocerasus* L.)	Caode Bende	unripe	WSP	ND	76-176	Air; DC	Lai et al. (2013)
			CSP	ND	37-61		
			SSP	448-749	37-140		Zhang et al. (2008)
		ripe	WSP	ND	37-82		Lai et al. (2013)
			CSP	ND	17-55		
			SSP	123-749	37-140		Zhang et al. (2008)
Jujube (*Zizyphus jujuba*)	Huanghua Zhanhua	unripe	CSP	500, >3000	23-98	Air; TM	Wang *et al*. (2012)
			SSP	500, >3000	35-156		
		ripe	CSP	<500, >3000	16-78		
			SSP	<500, 2500	16-78		

Table 1. Continuation.

Peach (*Prunus persica* L. Batsch.)	Jinxiu Milu	ripe	WSP	300-4200	20-100	Air; TM	Yang *et al*. (2006, 2009)
			CSP	100-3000	ND		
			SSP	20-900	35-70		
			WSP	ND	91-217	Air; NC	Zhang et al (2012)
			CSP	ND	91-181		
Strawberry (*Fragaria* x *annanassa* Duch.)	Chandler	ripe	CSP	20-500	10-15*	Butanol; DC	Posé *et al*. (2012) *This work
			SSP	20-320	10-15*		
	Shijixiang		WSP	88-3043	23-78	Air; TM	Chen *et al*. (2011)
			CSP	ND	23-78		
			SSP	87-1152	23-59		
Tomato (*Lycopersicon esculentum* Mill.)	Rutger		CSP	40-560	ND	Butanol; DC	Kirby et al. (2008)
			SSP	20-400	ND	Butanol; DC	Round et al. (2010)
			CSP	ND	19-117	Air; TM	Xin et al. (2010)
		ripe	CSP	ND	15-117		

*Width data correspond to non-corrected data and might be overstimated due to the probe broadening. See effect text for details.

ND, not described;DC, direct contact;

TM, tapping mode.

4.3 AFM analysis of pectins during fruit ripening and storage

Fruit texture is closely related to the structural integrity of fruit tissues and pectins play a key role in cell to cell adhesion and cell wall stiffness. Several enzymes acting on pectins have been related to the changes that suffer cell walls during softening, such as PG, pectate lyase, PME, pectin acetylesterase, β-galactosidase, β-xylosidase, α-arabinofuranosidase (Brummell and Harpster, 2001; Goulao and Oliveira, 2008; Mercado et al., 2011). Although there are numerous reports about the biochemical changes in cell walls and pectins during fruit ripening and postharvest, few studies have been devoted to analyze these processes at the nano-structural scale. Zhang et al. (2008) used AFM to study chelate-soluble pectins isolated from two Chinese cherry cultivars with crisp and soft textures. Two ripening stages were also compared; commercial ripe fruit vs. unripe fruit harvested 7 days before ripening. As expected, the firmer unripe fruits from both cultivars contained wider and longer chains than ripe fruits, as well as more polymers that were entangled together. However, comparison between cultivars did not support the same correlation, since firmness of the crisp cultivar was higher at both ripening stages, but its SSP fraction was richer in wider and shorter chains than the same fraction of soft fruits at both stages. A similar study was performed by Xin et al. (2010) in CSP pectins from tomato. In this case, turning stage fruits contained wider chains than light-red fruits and linear single and short chains were more frequently observed in light-red tomatoes. Yang et al. (2009) analyzed three kinds of pectins (WSP, CSP and SSP) in two peach cultivars with contrasting texture, soft and crisp. The most significant difference between the two cultivars was the length of the SSP pectins, with the SSP chains of the crisp fruit longer than those of the soft cultivar, average lengths 249 vs. 57 nm, respectively. Additionally, a small number of CSP and SSP chains of the crisp genotype had branches whilst few or no branched polymers appeared in the soft fruit pectin fractions. Accordingly, these authors suggested that differences in texture

in peach could be mainly related to the neutral-sugar rich pectin fraction from the primary cell wall. In Chinese jujube fruits, pectin length also decreased during ripening (Wang et al., 2012).Representative topographical AFM images of CSP pectins from strawberry fruit at two ripening stages are shown in Fig. 1 to illustrate the pectin modifications that frequently occur during fruit ripening. Qualitative analysis of these images showed that CSP samples from unripe fruits (Fig. 1-A) were enriched in long chains while shorter chains were more frequently observed in red fruits (Fig. 1-B). Branched polymers (Fig. 1-F) could be observed in both samples, unripe and ripe, but the frequency of branching decreased in ripe samples. Few aggregates with emergent polymer strands could also be observed in these samples (Fig. 1-E).

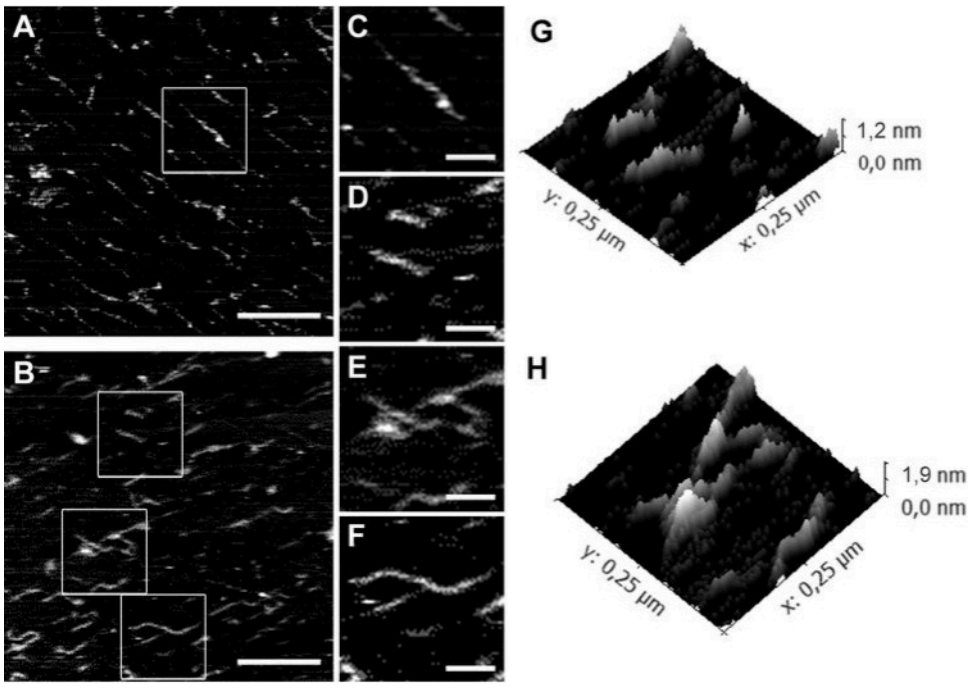

Figure 1: AFM topographical images representative of strawberry CSP pectins at unripe (A) and ripe (B) developmental stages, drop deposited onto mica and visualized by direct contact mode under butanol. Inlets (C-F) show zoomed areas from the A and B images showing examples of linear chains (C,D), multichain aggregates (E) and branched chains (F). (G) is equivalent to zoomed image D, and (H) is equivalent to zoomed image E showing the topographical details for linear chains and aggregates in 3D. Scale bars: for A and B are 250 nm; for C-F are 75 nm.

The nano-structural changes in pectins during fruit storage have been analyzed in a few numbers of species. In peach fruit stored at different temperatures (2, 8 and 15ºC) Zhang et al. (2010) observed an increase in the content of WSP and CSP and a decrease in SSP during storage in parallel to a decrease in fruit firmness, with this higher at higher temperature. However, fruit firmness was only correlated with the content of SSP pectin. An AFM study of this fraction showed that most SSP pectins formed large aggregates in fresh fruits that were reduced during the storage period, along with the rise of the storage temperature. Furthermore, the length and width of the pectin chains were reduced with storage time and higher temperatures. A similar result was observed when WSP and CSP were analyzed, although CSP pectins fruits at harvest were visualized mainly as single linear chains with a small number of aggregates (Zhang et al., 2012). The beneficial effects of controlled atmospheres, 2% O_2 and 5-10% CO_2, in the quality of peach stored at low temperature have also been related to a lower degradation of pectins at the nanostructural level (Yang et al., 2005; Yang et al., 2006a, 2006b). Interestingly, a decrease in the number of branched polymers was observed with storage time and it was concomitant to a reduction of pectin size and number of aggregates (Yang et al., 2005; Yang et al., 2006a, 2006b). In CSP pectins from apricots stored at 0ºC, Liu et al. (2009) also observed a decrease on the percentage of branched polymers with the storage time. Furthermore, the frequency of short CSP chains increased with time and the application of $CaCl_2$, a treatment that extends the postharvest life, supposedly due to the formation of calcium bridges between HGA chains, partially reduced the degradation of pectins (Liu et al., 2009). Length of WSP and SSP from strawberries decreased a 30-39% during the storage of the fruit at 4ºC for 15 days (Chen et al., 2011). As previously observed in apricot, $CaCl_2$ treatments partially reduced the depolymerization of strawberry SSP polyuronides (Chen et al., 2011).

Besides the study of individual pectin polymers, AFM provides a useful means to study their aggregation and the effect of factors affecting the

formation of gels, e.g. acid-induced and calcium-induced gelation, pectin concentration (Fishman et al., 2007; Morris et al., 2010; Morris et al., 2011). Most of these studies have been performed with fruit pectins extracted with chelating agents but they have not been related to the process of fruit softening. The structural information derived from gelation studies could be useful to understand the self-assembly process in the middle lamella (Morris et al., 2011), a region that is substantially modified during fruit ripening. AFM could therefore provide a new tool to unravel the role of pectin aggregation in the middle lamella dissolution process during fruit ripening.

5. CONCLUSIONS

Summarizing, the nano-structural characterization of pectins by AFM in fleshy fruits revealed significant modifications of the pectic chains and aggregates present in the pectin samples during fruit ripening and postharvest period. In general, these changes involve a decrease in the number of pectin aggregates and a reduction in the length of individual pectin chains and in the width of highly packed pectins, especially those extracted with sodium carbonate, supposedly located within the primary cell wall. Although additional work is needed to identify the physicochemical bases of the structures visualized by AFM, this technique could be a powerful tool to gain insight into the role of pectin disassembly in fruit softening.

OBJECTIVES:

The main goal of the present thesis was to study the role of pectin solubilization and depolymerisation in strawberry fruit softening during ripening by means of a functional approach. To achieve this goal we have characterized at biochemical and nanostructural levels pectins from control, non-transformed plants, as well as pectins from fruits of several transgenic lines with low expression levels of pectinase genes. This main objective was divided in three main sub-objectives:

1. Analysis of the cell wall modifications during strawberry fruit development, and nanostuctural characterization of pectins from unripe and ripe fruits by atomic force microscopy (Chapter III).
2. The study of fruit pectin nano-structure and cross linking of polymers by means of atomic force microscopy. Two transgenic lines were used for this objective, plants with the polygalacturonase gene *FaPG1* down-regulated and plants with the pectate lyase gene *FaplC* silenced. Their pectins were compared with those obtained from control non transgenic fruits (Chapter IV).
3. Functional characterization of *FaβGal4*, a novel β-galactosidase gene that could be involved in strawberry fruit softening, with special focus on the identification of pectin changes in fruits of these plants (Chapter V).

RESULTS AND DISCUSSION CHAPTER III:

NANOSTRUCTURAL CHANGES IN CELL WALL PECTINS DURING STRAWBERRY FRUIT RIPENING

1. SUMMARY

Strawberry (*Fragaria* × *anannasa* Duch.) is one of most important soft fruit. Rapid loss of firmness occurs during the ripening process, resulting in a short shelf life and high economic losses. To get insight into the role of pectin modifications in the softening process, cell walls from strawberry fruits at two ripening stages, unripe (green fruit) and ripe (red fruit), were extracted and fractionated with different solvents to obtain fractions enriched in a specific component. The yield of cell wall material as well as the different fractions decreased in ripe fruits. CDTA and Na_2CO_3 fractions underwent the largest decrements, being these fractions enriched in pectins supposedly located in the middle lamella and primary cell wall, respectively. Uronic acid content also decreased significantly during ripening in both pectin fractions, but the amount of soluble pectins, those extracted with phenol:acetic acid:water (PAW) and water increased in ripe fruits. Monosaccharide composition in CDTA and Na_2CO_3 fractions was determined by gas chromatography. In both pectin fractions, the amount of Arabinose and Galactose, the two most abundant carbohydrates, decreased in ripe fruits. The nanostructural characteristics of CDTA and Na_2CO_3 pectins were analysed by AFM. Isolated pectic chains present in the CDTA fraction were significantly longer and more branched in samples from green fruits than those present in samples obtained from red fruit. In spite of slight differences in lenght distributions, samples of Na_2CO_3 fraction from unripe samples displayed some longer chains at low frequency, that were not observed in ripe fruits. Moreover, the median of the lenght distributions were similar at both ripening stage. Branch characteristics were similar in both fractions. Concerning the presence of aggregates, their number was higher in green fruits samples of both fractions. These results support that pectic chain length and the nanostructural complexity of the pectin present in CDTA and Na_2CO_3 fractions diminish during fruit development, and these changes, jointly with the loss of neutral sugars, could contribute to the solubilization of pectins as shown by the higher pectic contents of the PAW and water fractions.

2. INTRODUCTION

The ripening process of fleshy fruits is a combination of physiological and biochemical processes that make edible fruits more attractive to seed dispersal organisms (Gapper et al., 2013). Softening is one of the most noticeable changes during the ripening process in soft fruits such as strawberry (Brummell and Harpster, 2001). The modification of the physical and chemical features of parenchyma cell walls is considered one of the major determinant of fruit softening (Smith et al., 2002). This process also involves the dissolution of the middle lamella (Perkins-Veazie, 1995) which is related to a decreased intercellular adhesion (Redgwell et al., 1997b). Nevertheless, the biochemical mechanisms underlying strawberry softening are not completely clear.

One of the cell wall changes that could be related to fruit softening is the solubilisation of pectins, a process which is characterized by an increase in water-soluble pectins, also in CDTA-soluble polyuronides in some fruits, and a decrease in the amount of covalently bound pectins, those supposedly located in the primary cell wall. Pectin solubilisation varies greatly among species, being very evident in species such as avocado (Wakabayashi, 2000), kiwifruit, tomato, persimmon (Redgwell et al., 1997b) strawberry (Figueroa et al., 2010) or peach (Brummell et al., 2004), but of less importance in fruits with a crisp texture at ripening, such as apple, watermelon or nashi pear (Redgwell et al., 1997a; Hiwasa, 2004).

Several hypotheses about the cause of pectin solubilisation have been proposed. One of these hypotheses relies on the loss of arabinan and galactan side chains from RG-I (Gross and Sams, 1984; Redgwell et al., 1997a), weakening the cell wall network. Moreover, the loss of neutral sugars might also increase the cell wall porosity, which could facilitate the access of pectinases to their substrates (Smith et al., 2002). However, not all the fruits show a correlation between the loss of neutral sugar residues and pectin solubilisation (Redgwell et al., 1997a). Alternatively or partially, pectin depolymerisation of quelated and/or covalently bound pectins (Redgwell et al., 1997b;Rose

et al., 1998; Vicente et al., 2005), as result of the action of pectinases, like polygalacturonase (PG), pectin methyl esterase (PME) or pectate lyase,may facilitate the solubilisation of pectic components not previously easily extracted in unripe fruit. Additionally, a seminal paper by Huber (1984) proposed the synthesis of new, more freely, soluble pectins in red fruits as the explanation of the increased pectin contents in the more soluble fractions at this development stage. None of these hypotheses are mutually exclusive and all these processes might influence pectin solubilisation to a greater or lesser extent.

Regarding to the role of depolymerization in the pectin solubilization process during strawberry ripening, previous papers reported controversial results. Huber (1984) and Redgwell et al., (1997a) showed minor variations in the average molecular weight of CDTA soluble polyuronides or pectins from the middle lamella, during strawberry ripening. By contrast, Rosli et al. (2004) found a significant depolymerization of pectins extracted with hydrochloric acid in 'Toyonaka' fruits, an extremely soft cultivar, but this modification was not detected in the other two cultivars analyzed, 'Camarosa' and 'Pajaro'. Finally, Santiago-Doménech et al. (2008) reported a slight decrease on the molecular weight of sodium carbonate soluble polyuronides during the ripening of the cv. 'Chandler'. In all these studies, polymer size was estimated by mean of size exclusion chromatography and the results correspond to a bulk behavior of the pectic fraction analyzed. A nanostructural characterization at the level of pectic isolated chains by AFM could provide a better understanding of this process.

The main objective of this research was to compare pectins of green and ripe red strawberry fruits at the nanostructural level to shed light on which structural changes could be related to softening. To this purpose, cell walls from unripe-green and ripe-red strawberry fruits were fractionated to obtain pectin fractions that were analyzed by AFM, as described in Posé et al. (2012a). FTIR and carbohydrate composition analyses were also performed to characterize strawberry pectins at these two developmental stages.

3. MATERIALS AND METHODS

3.1 Plant material

Strawberry (*Fragaria* × *ananassa* Duch., cv. 'Chandler') plants were grown in a greenhouse under natural photoperiod and temperature conditions. Fruits were collected from March to June. After harvest, fruits were immediately frozen in liquid nitrogen and stored at -80ºC until used. Two developmental stages were selected, green-unripe and red-ripe. Fruits from the green stage were about 1 cm in diameter displaying both green receptacle and green achenes. Ripe red fruits were characterized by its fully red receptacle and soft texture.

3.2 Cell wall extraction

Achenes from frozen fruits were removed and the cell wall from fruit receptacle was extracted following the method of Redgwell et al. (1992) as modified by Santiago-Doménech et al. (2008). This procedure yielded cell wall material (CWM) and polymers soluble in PAW (phenol:acetic acid:water, 2:1:1, w:v:v), solvent used to inactivate enzymes (PAW fraction). The CWM (80 mg) was sequentially fractionated following the procedure of Santiago-Doménech et al. (2008) to obtain pectin (water-, CDTA- and sodium carbonate- soluble fractions) and hemicellulose (KOH 1M and KOH 4M fractions).

3.3 Uronic acid and neutral sugar measurements

The uronic acid (UA) content in PAW and cell wall fractions was measured by the carbazol method as reported by Filisetti-Cozzi and Carpita (1991). Known concentrations of GalA were used as standard.

Neutral sugars were analysed by gas chromatography (Blakeney et al. 1983) with flame ionisation detection. In addition, the total neutral sugar

content was determined by the orcinol method (Rimington, 1931; Tillmans and Philippi, 1929) using glucose as standard.

3.4 Fourier transform infrared spectroscopy

Dried samples were used to capture the infrared spectra with an Attenuated Total Reflectance (ATR) accessory (MIRacle ATR, PIKE Technologies, USA) coupled to a Fourier transform infrared (FTIR) spectrometer (FT/IR-4100, JASCO, Spain), as described previously (Heredia-Guerrero et al.,2010). The samples were processed as described by Posé et al. (2012a) and the spectra were collected in the 4000–600 cm^{-1} range with a resolution of 4 cm^{-1} and averaged over 25 scans per sample.

3.5 Atomic force microscopy

The pectin-enriched fractions corresponding to CDTA and Na_2CO_3 soluble polymers were analysed by AFM as described by Posé et al. (2012a). The AFM was manufactured by ECS (East Coast Scientific Limited, Cambridge, UK). Short tip variety AFM probe model contact cantilevers (Budget Sensors, Bulgaria) were used with a quoted resonance frequency and force constant of 13 kHz and 0.4N m^{-1}, respectively. The imaging was operated using contact mode and with the same parameters used by Posé et al. (2012a) to allow results comparisons.

3.6 AFM image analysis

The individual chains were measured and characterized by their length distribution parameters (Posé et al. 2012a). The number-average (L_N), the weight-average (L_W) contour lengths and their ratio (L_W /L_N) or polydispersity index (PDI) was calculated (Odian, 2004). More than 350 individual measurements from independent images were employed to obtain the length distribution representations and related statistical parameters.

3.7 Statistical analysis

The GraphPad Prism software (v.5.0 b) was used for statistical analyses. Data were analyzed by Student t or Mann-Whitney U test, both at P = 0.05. Three independent replicates were used.

For AFM statistical analyses, thirty images per sample were collected to obtain representative data of the pectin samples. The median from the original data distributions were compared with the non-parametric Kruskal–Wallis test. Differences in the branching of the polymer chains, presence/absence of side chains, were analysed by Chi-square test at P = 0.05. The number of aggregates per micra were analysed using the Mann Whitney U test.

4. RESULTS

4.1 Cell wall yield.

The yield of CWM per weight of tissue (Table 1) was around three times higher in unripe fruit than in ripe fruit, as expected. In the same line, the yields of the different fractions enriched in pectins, i.e. water, CDTA and Na_2CO_3 fractions relative to weight of CWM, were higher in unripe fruits, being the differences statistically significant (Table 1). By contrast, the weight of fractions enriched with hemicellulosic material, KOH 1M and 4M, was similar at the two developmental stages. Concerning polymers soluble in PAW, no differences were observed between the yield of this fraction in unripe and ripe fruit (Table 1); however, the ratio PAW/CWM increased from 0.017 in unripe fruit to 0.67 in ripe fruit.

Table 1. Yields of PAW-soluble polymers and CWM relative to fruit fresh weight; and cell wall fractions contents after the sequential extraction from unripe and ripe fruit samples. Data correspond to the mean values±SD. Mean separation by Student *t*-test (CDTA, sodium carbonate and KOH 4M fractions) or Mann-Whitney *U*-test (water and KOH 1M fractions) both at P=0,05.

		Unripe	Ripe
CWM (g/100 g of fruit)		3.4 ±2.1	0.9 ±0.5
PAW (g/100 g of fruit)		0.06±0.01	0.06±0.008
Cell wall fractions (g/100 g CWM)	**Water**	6.2±0.5a	3.1±0.02a
	CDTA	22.6±5.8a	11.7±1.8b
	Na_2CO_3	24.0±1.5a	11.2±3.3b
	KOH 1M	7.4 ±1.2a	4.7±0.3a
	KOH 4M	2.6±0.9a	2.0±0.4a

Figure 1 shows the amount of galacturonic acid in the different cell wall fractions. In both types of fruit, the highest amount of galacturonic acid was observed in the CDTA fraction followed by the sodium carbonate and water fractions, respectively. As expected, the lowest pectic contents were those corresponding to KOH fractions. Galacturonic acid content of the water soluble fraction was slightly higher in red fruits. Conversely, all the other fractions displayed a decreased amount which was specially evident in CDTA fractions. The amount of GalA in PAW fraction increased from 16.6±3.2 to 53.3±5.5 mg GalA/100 g of fruit, from green to red stage, respectively. PAW and water polymers represent pectins solubilized by *in vivo* processes. These results clearly indicate a noticeable polyuronide solubilization during fruit ripening, being CDTA and sodium carbonate fractions the main sources of free pectins.

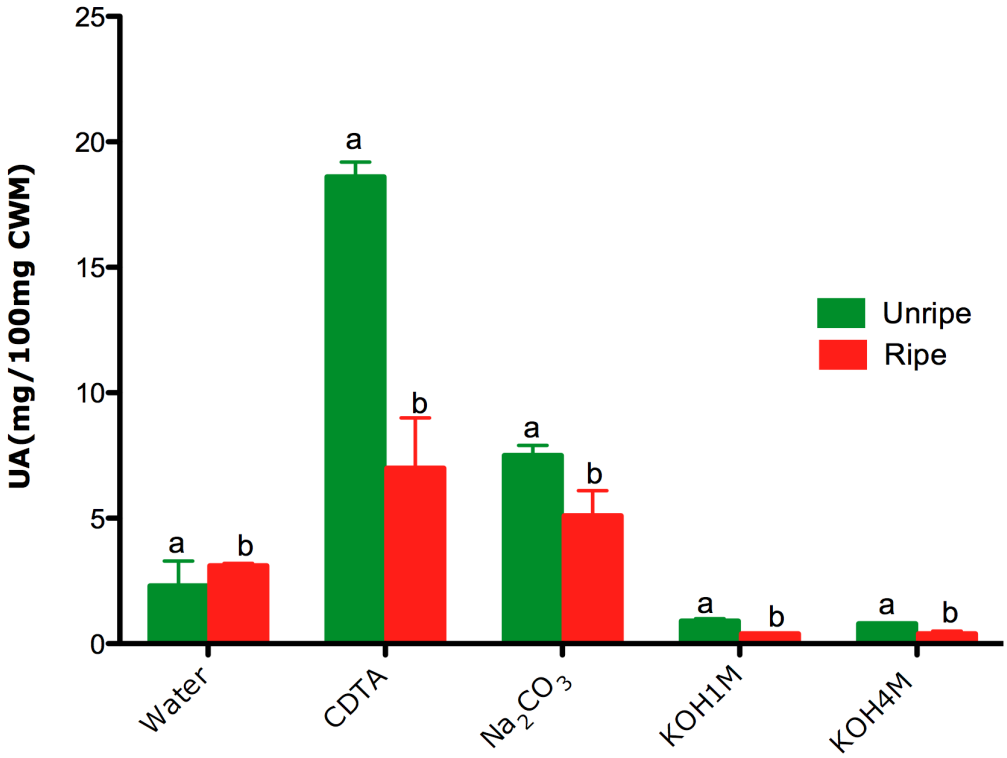

Figure 1. Uronic acid content in the different cell wall fractions. Mean separation between developmental stages by Student t-test at P=0.05.

Neutral sugar contents in CDTA and sodium carbonate fractions were analysed by gas chromatography. In both pectin fractions, Arabinose (Ara) and Galactose (Gal) were the most abundant monosaccharides, being the relative abundance of both sugars higher in carbonate fraction (Fig. 2). The most noticeable changes during fruit development were a decrease in the proportion of Ara in both pectin fractions and an increase on Gal in ripe fruits. However, when the amount of neutral sugars was expressed relative to the weight of CWM, Ara and Gal amounts decreased in both pectin fractions during ripening (Table 2). Large decreases on Rhamnose (Rha) were also observed as the fruit ripen (Table 2).

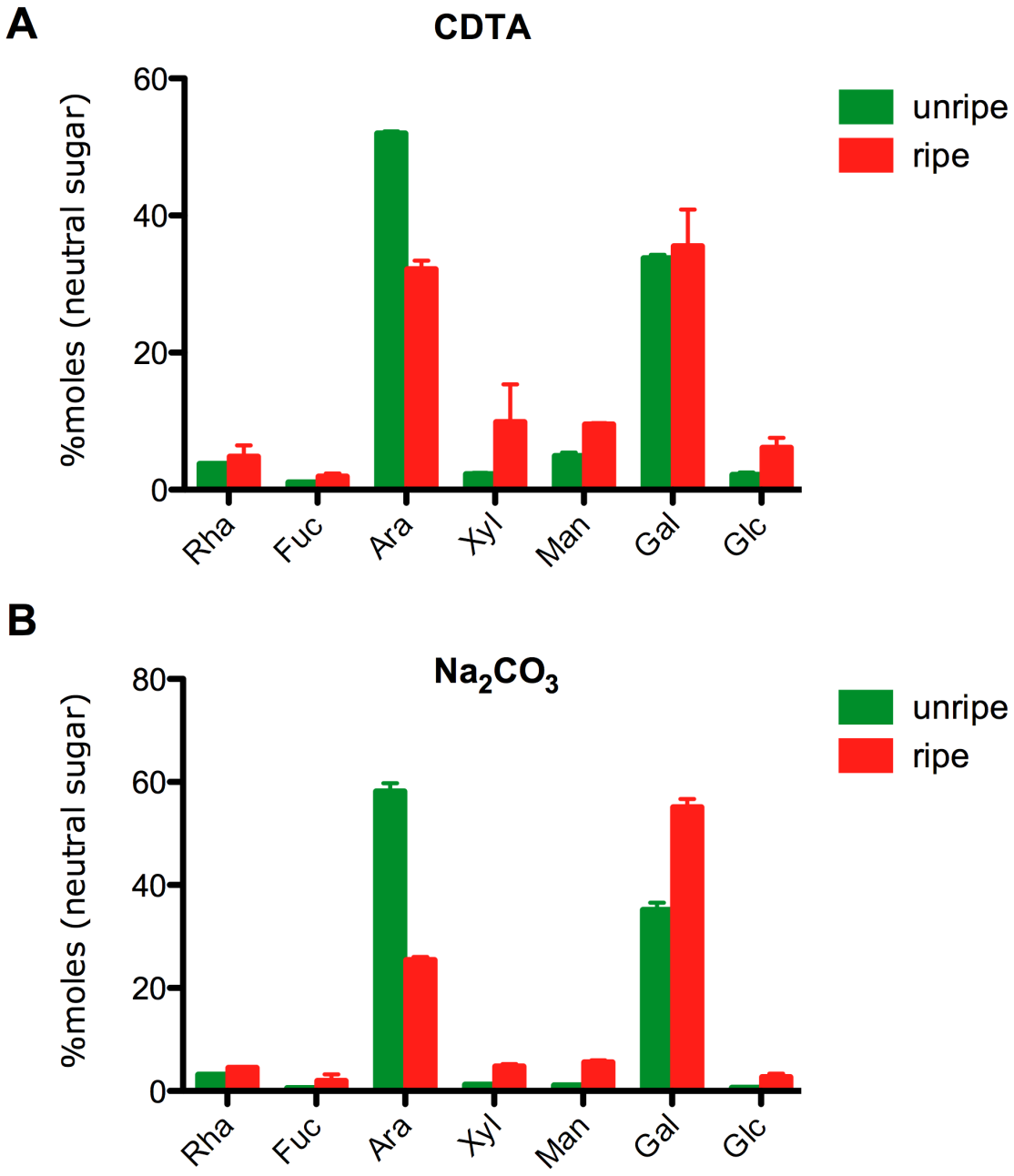

Figure 2. Percentage of neutral sugar content in unripe and ripe fruit of strawberry from CDTA (A) and Na_2CO_3 (B) by gas chromatography. The data correspond with the percentage of each sugar with respect to the total neutral sugar. Data from of two independent extractions.

Table 2. Amount of neutral sugar estimated by gas chromatography expressed in µg of monosaccharide /mg of CWM in CDTA and sodium carbonate fractions. Amount of galacturonic acid was estimated by colorimetric method.

	CDTA		Na_2CO_3	
µg/mg	**Unripe**	**Ripe**	**Unripe**	**Ripe**
Rhamnose	19.89	6.37	20.97	5.15
Fucose	5.59	2.56	3.63	2.22
Arabinose	249.11	38.51	351.87	26.34
Xylose	10.88	11.65	7.65	4.9
Mannose	29.12	13.97	8.08	7.02
Galactose	198.04	52.31	260.47	69.83
Glucose	12.79	8.97	4.68	3.42

4.2 FTIR analysis of cell wall fractions

The different pectin fractions from unripe and ripe fruits were analyzed by FTIR and the results obtained are shown in Fig. 3. In general, all fractions showed the typical fingerprint, between 1200-950 cm^{-1}, characteristic of polysaccharides.

The spectra of water soluble fractions showed some differences in unripe vs. ripe fruits (Fig. 3A). The peak at 1737 cm^{-1}, characteristic of esterified pectin, was higher in unripe samples, whereas the peak at 1625 cm^{-1}, characteristic of no-esterified pectins, showed the same absorbance at both developmental stages. The ratio of absorbance at these wavelengths can be used to estimate the degree of methylation (DM) of pectins (Lira-Ortiz et al., 2014). According to this, the DM was around 60% in ripe fruit and around 70-80% in unripe fruit. The peak at 1238 cm^{-1} is ascribed to acetyl groups (Kacurakova et al., 2000). This peak was higher in unripe fruit indicating that the higher degree of esterification on water soluble pectins in unripe fruits could be partially due to the acetylation of polyuronides. In the polysaccharide fingerprint region, samples from ripe fruits showed the characteristic spectrum of homogalacturonan with main peaks at 1016 and 1100 cm^{-1}. By contrast, peaks at 1078 and 1045 cm^{-1} were higher in unripe fruits suggesting enrichment in arabinogalactans.

The spectral profiles of CDTA fractions were similar in unripe and ripe fruits (Fig. 3B). In both samples, the ratio of absorbance at 1737/1625 cm^{-1} decreased, indicating a lower level of methylation, around 30%. The region 1200-900 cm^{-1} was typical of polygalacturonic acid. In sodium carbonate soluble samples, as expected, the peak at 1740 cm^{-1} disappeared whereas peaks at 1625 and 1415 cm^{-1} increased, due to the elimination of ester linkages during the alkaline extraction (Fig. 3C). However, the carbonate pectin fraction isolated from unripe fruits still maintained a small absorption band at 1740 cm^{-1}, indicating the presence of some ester bonds resistant to the mild alkaline conditions. The fingerprint region was similar at both developmental stages, and the stronger absorption bands at 1078 and 1045 cm^{-1} indicates a higher presence of neutral sugars in these samples when compared with CDTA fractions.

4.3 AFM analysis of fruit pectins

Figure 4 shows representative topographical AFM images of CDTA pectins from unripe (A) and ripe fruits (B). Linear filamentous chains with scarce side chains were mainly present in CDTA samples at both developmental stages. Apparently, the isolated pectis chains were longer in unripe fruit samples. In addition, polymer aggregates with micellar-like appearance were more frequent in unripe fruit samples.

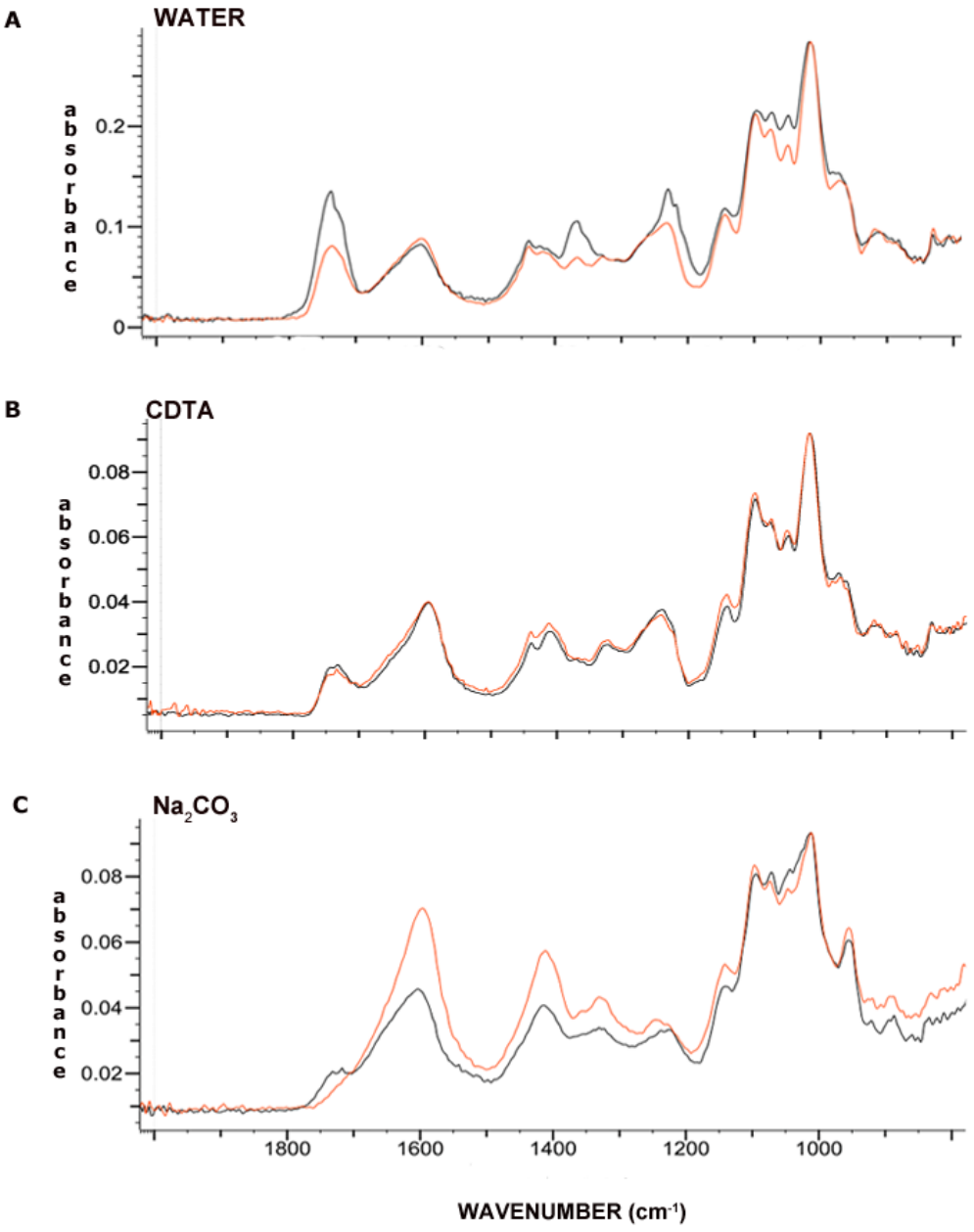

Figure 3. ATR–FTIR absorbance spectra of unripe (dark line) and ripe fruit (red line) of the difference pectic fractions in the 2000–800 cm^{-1} region.

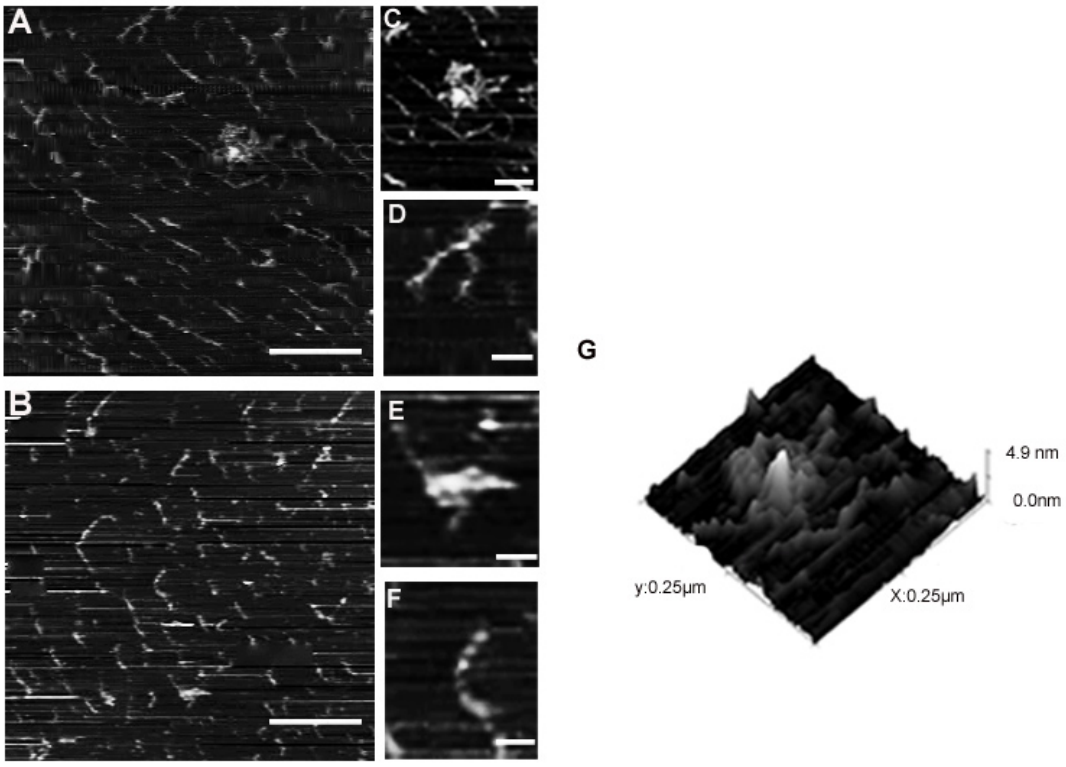

Figure 4. AFM topographical images representative of strawberry CDTA pectins at unripe (A) and ripe (B) developmental stages. A sample drop was deposited onto mica and visualized by direct contact mode under butanol. Inlets (C-F) show zoomed areas from A (C and D) and B (E and F) panels, and represent linear unbranched chain (F), branched chains (D) and micellar aggreagates (C and E). (G) 3D representation of a pectin aggregate. Scale bar for A-B is 250 nm; for C-H is 75 nm.

Contrary to CDTA pectins, topographical AFM images from Na_2CO_3 soluble pectin fractions does not show significant differences in the contour length of isolated pectic chain from unripe and ripe fruit samples (Fig. 5). However, branched chains and aggregates structures were more frequently observed in unripe samples.

Detailed images of these aggregates are shown in Fig. 5 (C and E) and a tridimensional representation in Fig. 5 (G). Moreover, pectins width ranged between 10 to 15 nm, and no differences were observed neither between pectin extract nor with the stage of fruit development.

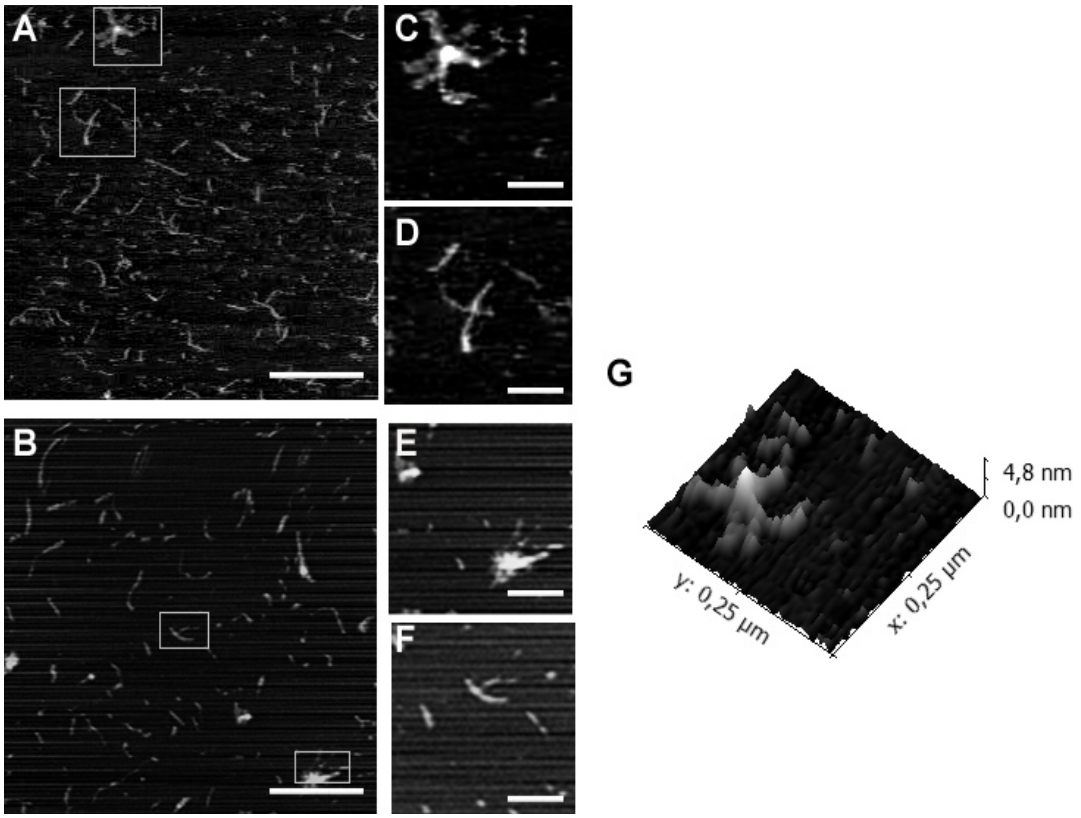

Figure 5. AFM topographical images representative of strawberry Na_2CO_3 pectins at unripe (A) and ripe (B) developmental stages. A sample drop was deposited onto mica and visualized by direct contact mode under butanol. Inlets (C-F) show zoomed areas from the A and B images showing examples of linear chains (C,D), multichain aggregates (E) and branched chains (F). (G) is equivalent to zoomed image C, to showing the topographical details for linear chains and aggregates in 3D. Scale bars: for A and B are 250 nm; for C-F are 75 nm.

Isolated polymer chains at both developmental stages were also characterized by its contour length distributions. The length distributions in CDTA fraction were in the range of 25 to 533 nm and 18 to 277 nm, in unripe and ripe fruit samples, respectively (Fig. 6A). Conversely the length distribution of Na_2CO_3 pectin samples ranged between 9 and 305 nm for unripe samples and from 10 to 255 nm for ripe (Fig. 6B). In both fractions, the range of length distribution was wider for unripe fruit samples, including pectic isolated chains, longer than 300 nm in CDTA fractions and longer than 275 nm in sodium carbonate fractions. These chains disappeared at the ripe stage. Furthermore, CDTA samples from unripe fruits showed a higher proportion of middle-size pectins in the range of 75 to 150 nm, being this range displaced to 50-74 nm at the ripe stage.

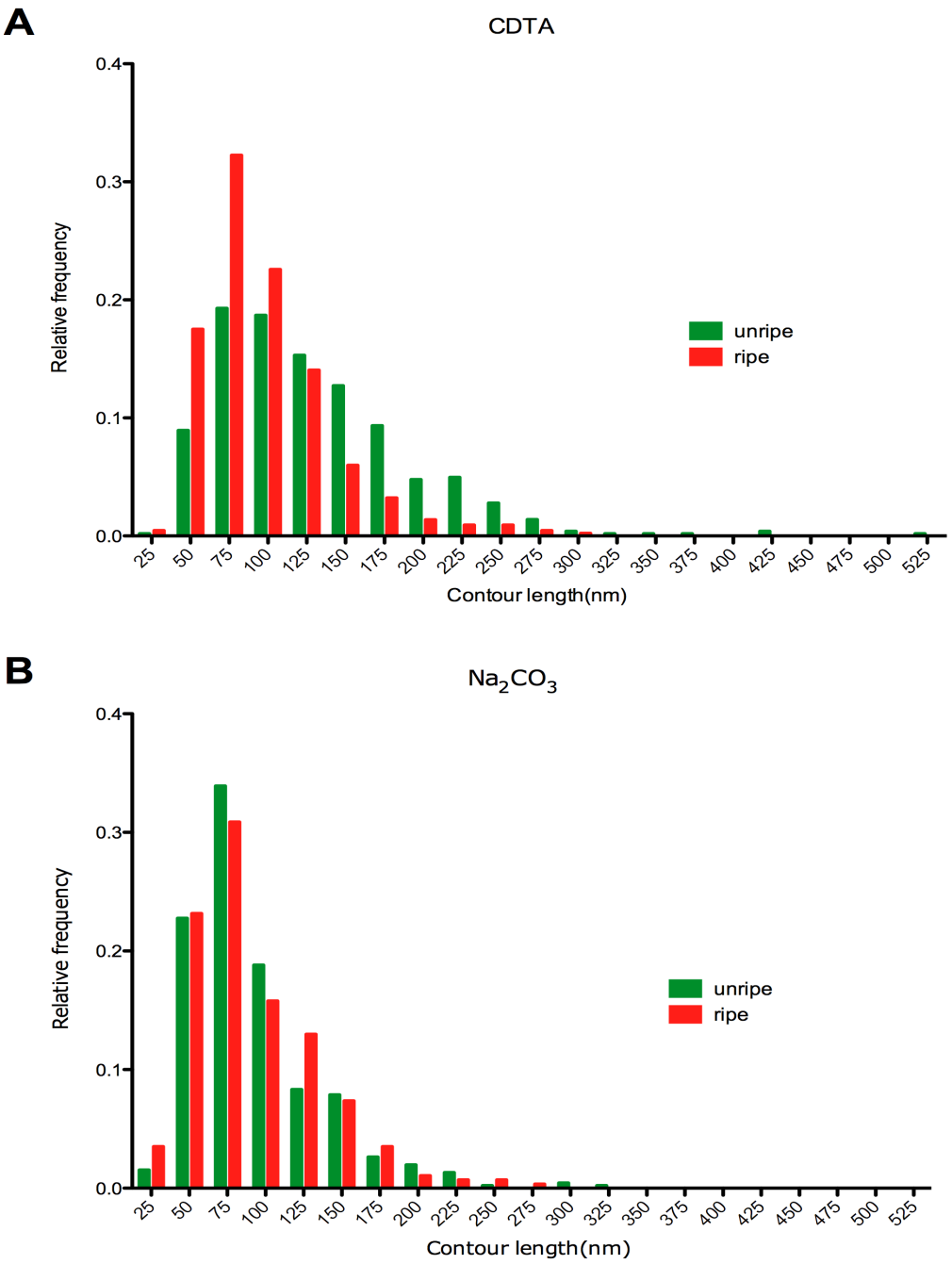

Figure 6. Contour length distribution from CDTA **(A)** and sodium carbonate **(B)** soluble polymers.

The pectin samples were also characterized by their number-average (L_N), weight-average (L_W) and polydispersity indexes (PDI) (Table 3) as well as by the medians of the original distributions. The results obtained showed a significant decrease of CDTA pectin length as the fruit ripen, from 103 nm

at unripe stages to 75 nm in ripe fruit. However, median values in Na_2CO_3 fraction were similar at both stages despite the presence at low frequency of longer pectin chains in the contour length distribution of unripe fruit samples.

Branched pectic chains were observed in both pectin fractions from either unripe or ripe fruits, however, its frequency decreased significantly during ripening (Table 4). L_N and L_W values of branches were not significantly affected by fruit development, being also similar in pectins from CDTA and sodium carbonate fractions. The slight differences in the branch length between ripe and unripe fruits in both fractions were not statistically significant.

Table 3. Descriptors of contour length distribution of CDTA and Na_2CO_3 pectins extracted from unripe and ripe strawberry fruits obtained from AFM images. ME corresponds to the median of the original data. For each pectin fraction, median values with different letters indicate significant differences by Kruskal-Wallis non-parametric median test at P=0.05.

		Unripe	Ripe
CDTA	**L_N(nm)**	116.3	90.9
	L_w(nm)	148.5	113.6
	PDI	1.3	1.2
	ME(nm)	102.87**a**	75.5**b**
Na_2CO_3	**L_N(nm)**	79.79	78.7
	L_w(nm)	104.12	102
	PDI	1.3	1.3
	ME(nm)	69.8**a**	69.2**a**

Table 4. Branching characteristics of the CDTA and Na_2CO_3 pectins extracted from unripe and ripe strawberry fruits obtained from AFM images. Branching percentages were statistically analyzed by Chi-squared test at P=0.05.

		Unripe	Ripe
CDTA	**Branching (%)**	12.92**a**	3.2**b**
	Multibranching(%)	-	0.6
	L_N(nm)	35.69	41.1
	L_w(nm)	51.08	47.3
	PDI	1.43	1.2
Na_2CO_3	**Branching (%)**	19.74**a**	3.4**b**
	Multibranching(%)	10**a**	2.5**b**
	L_N(nm)	35.83	28.6
	L_w(nm)	47.97	39.8
	PDI	1.34	1.4

5. DISCUSSION

5.1 Increase on soluble pectins and loss of neutral sugars during the ripening process

The amount of cell wall decreased significantly during strawberry ripening, as previously observed in other cultivars (Redgwell et al., 1997, Koh and Melton, 2002; Rosli et al., 2004). In general, the yield of all cell wall fractions disminished in ripe fruits when compared with those of unripe fruits, being the largest decreases observed in the case of CDTA and sodium carbonate fractions. Interestingly, the smaller decrease on fraction yield was observed in KOH 4M fraction which is enriched in hemicellulosic material. Contrary to the cell wall, the yield of PAW solubilised polymers was similar in both developmental stages.

Previous studies found an increase in the amount of soluble pectins, those extracted with PAW or water, during strawberry fruit development

(Woodward, 1972, Redgwell et al., 1997a, Koh and Melton, 2002). Redgwell et al. (1997a) observed that unripe strawberries contained 35-40% less PAW soluble pectins than ripe fruits. In addition, the suppression of a polygalacturonase gene, *FaPG1*, reduced the amount of PAW soluble pectins in red fruits while increasing the amount of bound pectins (Posé et al., 2013). Our results also indicate a solubilisation of pectins during fruit development. The increment in the polyuronide content in PAW soluble fraction, calculated as the difference in pectin content between unripe and ripe fruit, was 36.9 mg/100 g fruit, a value close to that reported by Redgwell et al. (1992) for the cv. Pajaro, 62 mg/100 g fruit. The amount of pectins soluble in water also increased in ripe fruits. The increase on soluble pectins (PAW plus water fraction) was parallel to a decrease on bound pectins, showing the ionically- and covalently-bound fractions the largest decrease.

Changes in the neutral sugar contents of the cell wall, mainly galactose and arabinose, also occur during the fruit ripening and they have been related to pectin solubilization (Smith et al., 1998; Kim et al., 1991; Isherwood and Jermyn 1956; Knee et al., 1977; Ahmed and Labavich, 1978). In this study, large decrements in the content of Ara and Gal were detected in CDTA and sodium carbonate fractions as the fruit ripen. Koh and Melton (2002) also described a decrease on Ara content in both pectin fractions during strawberry ripening. The amount of arabinose was particularly high in the Na_2CO_3- fraction at the unripe stage, suggesting the presence of highly branched arabinans. In grape berries, the decrease in β-(1-4) linked D-galactopyranosyl residues during ripening has been attributed to the loss of type I arabinogalactan (AG-I) and this modification was suggested to be a crucial initial step involved in softening (Nunan et al., 2001). However, Redgwell et al. (1997b) did not observe a correlation between the loss of neutral sugars and fruit softening.

5.2 Nanostructural analysis of pectins by AFM during the ripening process

In a previous paper we reported, the set up of the experimental conditions for AFM nanostructural characterization of CDTA and Na_2CO_3 polyuronides samples from ripe strawberry fruit (Posé et al., 2012a). Pectic chains were a 33% longer in CDTA than in Na_2CO_3 fractions, median lengths were 87 and 65 nm in CDTA and Na_2CO_3 fraction samples, respectively. The percentage of pectic chains with side chains was similar, around 9%, in both fractions (Posé et al., 2012a). Besides isolated pectic chains, micelle-like structures could be observed in the samples at a rate of 5-6 aggregates per 100 individual molecules (Posé et al., 2012a). In the present research paper, the medians of the pectic chains length distributions in red fruit samples were similar to those reported by Posé et al. (2012a), 75 and 69 nm in CDTA and Na_2CO_3 fraction, respectively. As expected, the CDTA soluble pectins in unripe fruit were significantly longer than those from ripe fruit, being 103 nm the median of the length distribution in unripe fruit. The percentage of branched chains also decreased significantly in ripe fruits. These results could suggest that ionically bound pectins are depolymerized during ripening. By contrast, the median values of the pectic chains present in carbonate fraction samples were similar at both developmental stages. Despite the similar values of L_N and L_W in unripe and ripe carbonate pectins, contour length distributions were slightly different, showing the unripe fruits a small proportion of large pectins, higher than 300 nm, not detect in ripe samples. However, the most noticeable change detected by AFM in carbonate fraction was the decrease in the percentage of branched chains, from 19 to 3% in unripe and ripe samples, respectively, and the reduction in the number of aggregates.

Size exclusion chromatography studies performed in our group showed a slight decrease on the molecular weight of sodium carbonate soluble polyuronides during the ripening of the cv. 'Chandler' (Santiago-Doménech et al., 2008). Similar results were obtained in 'Toyonaka' fruits, an extremely

soft cv., by Rosli et al. (2004). Furthermore, the downregulation of a pectate lyase or a polygalacturonase gene by antisense transformation increased the average molecular weight of carbonate pectins (Santiago-Doménech et al., 2008; Posé et al., 2013), suggesting that these pectins are depolymerized during ripening. These previous results are not in agreement with the lack of differences in the length of unripe and ripe carbonate chains observed by AFM in this study. However, a comparison of the average molecular mass obtained using chromatography with those deduced from AFM images is not entirely straightforward. Size exclusion chromatography monitors the volume of the molecules in mixture, based on hydrodynamic behaviour of polymers through a porous gel matrix. AFM depicts nanostructural details on isolated pectic chains. The increased complexity of carbonate pectins from unripe fruits, more branched and with a higher presence of micellar aggregates, could result in a higher MW when analysing these pectins by size exclusion chromatography.

Several studies have found changes in the width and length of pectin chains by AFM during fruit ripening. Zhang et al. (2008) observed that sodium carbonate soluble pectins of unripe cherry displayed wider and longer chains than those from ripe fruits. However, these results are questionable. The number of molecules measured in that study was low and probably the parameter values deduced did not represent adequately the values displayed by the population of polymers. On the other hand, the high values of pectin width reported in that paper indicate that these molecules likely correspond to a complex of cross-linked pectic polymers but not isolated pectic chains. In agreement with our results, Xin et al. (2010) observed longer and wider chains in CDTA, in samples from tomato fruits at the turning stage than in samples corresponding to red fruits. Moreover, longer and wider pectin chains with more branches have been related with fruit firmness in apricot (Liu et al., 2009).

6. CONCLUDING REMARKS

Table 5 summarizes the main changes detected in the cell walls during strawberry fruit ripening in the present study. In general, main features of the ripening process are the solubilisation of pectins, the decrease of Ara and Gal contents, the depolymerisation of CDTA pectins and the reduction of the structural complexity of both CDTA and sodium carbonate pectin fractions. Our results do not clarify if sodium carbonate pectins are depolymerize during ripening, as previously suggested by other authors, and further studies including size exclusion chromatography would be needed to address this question. Altogether, the results suggest that the modification of pectin nanostructure, supposedly due to the action of cell wall modifying enzymes such as PG or pectate lyase, and the loss of neutral side chains from rhamnogalacturonan could contribute to the large pectin solubilisation observed in strawberry fruit during ripening. These changes in the pectin matrix are related to the reduced mechanical strength of cell walls in ripe fruit, leading to a reduction in fruit firmness.

Table 5. Summary of the main changes in cell wall during the strawberry ripening process.

	Unripe	**Ripe**
Phenotype	Firm	Soft
Cell wall yield	High	Low
PAW/CWM	Low	High
Soluble pectins/Bound pectins	Low	High
Ara and Gal content	High	Low
Pectin length distribution (nm)	CDTA: 25-533 Na_2CO_3: 9-305	CDTA:18-277 Na_2CO_3: 10-255
Median value (nm)	CDTA:102,9 Na_2CO_3:69,8	CDTA:75,5 Na_2CO_3:69,2
% Branching	CDTA:12,9 Na_2CO_3:19,7	CDTA:3,2 Na_2CO_3:3,4
Numbre of Micellar aggregates	High	Low

RESULTS AND DISCUSSION CHAPTER IV:

UNRAVELLING FRUIT PECTIC CHAIN NANO-STRUCTURE AND PECTIC CHAINS INTERCONNECTIONS BY ATOMIC FORCE MICROSCOPY

1. SUMMARY

Pectins analyzed by atomic force microscopy (AFM) are usually visualized as isolated chains, branched or unbranched, and, in a lower frequency, as large aggregates. To gain insight into the nature of these structures, carbonate pectins from strawberry fruits were subjected to enzymatic digestion using fungal EndoPG or mild acid hydrolysis and visualized by AFM. Pectins from control, non-transformed plants, and two transgenic genotypes carrying antisense sequences of a polygalacturonase (APG) and a pectate lyase (APEL) genes were also included in this study. As result of 120 minutes (min) of fungal endo-PG digestion, contour length (L_N) values of isolated pectin chains decreased a 42, 61 and 39% in control, APG and APEL samples, respectively. The kinetic of endo-PG digestion was different in the three genotypes. Control and APG pectins showed a linear decrease on the pectin length with time of digestion, but pectins from antipectate lyase fruits only showed up the effects of enzymatic hydrolysis after reached the first 90 min of digestion. In the three genotypes, branches disappeared as result of endo-PG treatment. The number of micellar aggregates was also dramatically reduced as result of PG digestion in the case of control and APG pectins, but some aggregates were still present in APEL samples after 120 min of digestion. A treatment with 0.1M HCl at room temperature was applied to APG samples to release RGII borate diester bonds. This treatment neither reduced the length of pectin chains nor their branching. However, the number of aggregates was significantly reduced from 6 aggregates/μm^2 to 1.The results obtained suggest that chains, as well as branches, visualized by AFM are mainly composed by galacturonic acid and susceptible to Endo-PG digestion. It can be also deduced that the chain composition of some parts of these pectic structures would also contain residues which are not Endo-PG substrates. On the other hand, micellar aggregates also contain galacturonic acid, being these structures susceptible to PG digestion, and RGII could have an important structural role in the formation of these macrocomplex.

2. INTRODUCTION

Pectin is an important cell wall component in dicotyledonous plants and probably the most complex structural polysaccharide in nature (Vincken et al., 2003). Polyuronides influence the porosity and strength of the primary cell wall and the growth of the plant cell (Bacic et al., 1988). Pectin metabolism also plays an important role in the fruit ripening process, leading to a reduction in fruit firmness (Willats et al., 2001; Brummell, 2006). This process is really important in soft fruits like strawberry, where the loss of firmness during postharvest storage leads to high economic losses. Moreover, pectins have potential applications in areas such a bioenergy, food industry and medicine. An emerging field of clinical importance is the growing interest of pectins as natural components of the human diet and their effects on health, both as a source of fibres and anticarcinogenesis products (Glinsky and Raz, 2009; Maxwell et al., 2012).

Pectins are complex polysaccharides rich in galacturonic acid (Gal-A), which contain different domains varying backbones and their side chains such as homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), arabinan, and arabinogalactan (Schols and Voragen, 2002; Vincken et al., 2010; Burton et al.2010). HG is the main component, composed of a homopolymer of α-(1-4)-D-galacturonic acid (GalA), which can be methylesterified at C-6 carboxyl or acetylated at O2 or O3 (Sénéchal et al., 2014, Wolf et al., 2009; Gou et al., 2012). XGA, is a modification of HG with a β-D-Xylp-(1-3) side chains, which has been identified in reproductive tissues such as fruits and seeds (Albersheim et al., 1996; Schols et al., 1995).The degree of xylosidation vary between species (Yu et al.,1996). RGII is composed of nine galacturonyl residues, containing clusters of five different chains with more than 20 uncommon sugar residues and 20 different linkages (O'Neill et al., 2004). Finally, RGI is a backbone of repeating dimer of [->2)-α-L-Rhap-(1->4)- α-D-GalpA-(1->], which is substituted by arabinan, galactan and arabinogalactan side chains (Willats et al., 2001).

As discussed in the chapter one, the chemical composition of pectins is known, but the model for pectin structure in muro is not established. The first model showed the pectin as a complex backbone composed of both HG and the core of RGI with neutral sugar side chains (De Vries et al,. 1981; Schols and Voragen, 1996; Coenen et al., 2007). An alternative model, proposed by Vincken et al. (2003), postulates that the RGI is the main pectin backbone whereas HG and RGII are their side chains (Chapter I., Fig.6, page 18). More recently, Yapo (2011) suggested a novel model, in which the pectin complex is formed by a RGI core connected with two unbranched HG blocks at the extremes (Chapter I., Fig.6C, page 18).

There are a number of covalent and no covalent linkages in pectin matrix, which are involved in both in intra- and inter-molecular linkages. As detailed in chapter one, the calcium-pectin complex is the most studied. However, the RGII dimer, which is covalently crosslinked by a borate diester (Ishii et al., 2001), could have an important role in the cell wall structure. dRGII is involved in the formation of a three-dimensional pectic network in muro (Yapo, 2011) and this could contributes to the mechanical properties of the primary wall.

Despite of long lasting research focus on pectins, the entanglement and the role of the pectic polymers into their native environment are still not fully understood (Round et al., 2001). Techniques from many scientific disciplines have been used to study and characterize pectic polysaccharides for many years. Recent works have shown that Atomic Force Microscopy (AFM) could be an excellent tool to study pectin structure from different sources (Kirby et al., 1996; Round et al., 2001; Posé et al., 2012). AFM is an effective technique which can be used to characterize the different pectin domains from the cell wall (Round et al., 2001; Kirby et al., 2008; Yang et al., 2009a; Fishman and Cooke 2009; Posé et al., 2012a). Features as contour length, contour branches and number of ramifications can be studied through this technology.

In general, AFM studies of fruit pectins show individual polymers, unbranched or with a reduced number of sidechains, which can be connected to make up big fibers or aggregates. In ripe strawberry fruits, Posé et al. (2012a) found that both CDTA and Na_2CO_3 pectic soluble fractions showed a low but consistent presence of aggregates in the samples studied. This structures were not an artifact since they were visualized even at low sample concentration, discarding that they were formed by the superposition between polymers due to the reduction of solvent volume during drying down onto the mica support. Additionally, the possibility of the existence of residual calcium bridges was also discarded because when the Na_2CO_3 fraction was treated with imidazole these large aggregates did not disappear (Posé et al., 2012a). Furthermore, the silencing of a pectate lyase or a polygalacturonase gene increased pectin contour lengths and also the frequency of aggregates, especially in the case of sodium carbonate polyuronides from fruits with the PG gene down-regulated (Posé, 2012b). Thus, these aggregates could represent or be remains of supramolecular pectin structures present in muro. In sodium carbonate soluble pectins from unripe tomato, Round et al. (2010) found that the acid hydrolysis of these samples, a treatment that releases neutral sugars, did not affect individual pectin lengths but decreased the size of aggregates. These authors suggested that pectin aggregates were formed by a mixture of RGI and HG, while isolated chains were composed by galacturonic acid.

The aim of this study was to investigate the nature of isolated pectin chains and aggregates visualized by AFM. To this purpose, we have conducted two different experiments. In the first one, we have analyzed the effect of endo-PG digestion in pectin contour length and the number of aggregates in pectin samples of selected genotypes. In the second experiment, we have analyzed by AFM pectins subjected to mild acid hydrolysis. Since this treatment releases RGII from the cell wall (Kobayashi et al., 1996), the possible differences between control and treated samples could shed light on the presence of RGII in these aggregates and its putative role on their formation.

3. MATERIALS AND METHODS

3.1 Plant material

Control, non-transformed, strawberry (*Fragaria* × *ananassa,* Duch., cv. 'Chandler') plants, transgenic strawberry plants carrying antisense sequences of a pectate lyase gene, *FaplC* (line APEL39, described in Jiménez-Bermúdez et al. (2002) and Santiago-Doménech et al. (2008)), or a polygalacturonase gene, *FaPG1* (line APG29, described in Quesada et al. (2009a) and Posé et al. (2013)) were grown in a greenhouse under natural temperature and photoperiod. Transgenic ripe fruits showed a strong reduction in *FaplC* or *FaPG1* mRNA levels, higher than 95%. The fruits were harvested at the ripe stage, fully red, frozen in liquid N_2 and stored at -30ºC until used.

3.2 Cell wall isolation and pectin extraction

The procedure followed for the isolation of the cell wall material (CWM) from ripe strawberry fruit was based on the protocol of Redgwell et al. (1992) as modified by Santiago-Doménech et al. (2008). Briefly, 10-15 frozen fruits were ground to a powder in liquid N_2 and 20 g were homogenised in 40 ml of PAW (phenol: acetic acid: water, 2:1:1, w:v:v). The homogenate was centrifuged at 4000 g for 15 min and the supernatant filtered through Miracloth (Merck, Bioscience, UK). After centrifugation, the pellet obtained was treated with 90% aqueous DMSO to solubilise the starch. The extract was then centrifuged at 4000 g and the pellet washed twice with distilled water. The water fraction was discarded, and the de-starched pellet, which is considered the cell wall material (CWM), was lyophilised and weighed.

The CWM was fractionated as described previously (Santiago-Doménech et al., 2008) to obtain the Na_2CO_3 soluble pectin fraction. Briefly, CWM was washed overnight with deionised water, centrifuged at 6000 g for 15 min and the pellet was sequentially extracted with

0.05 M trans-1,2-diaminocyclohexane-N,N,N'N'-tetraacetic acid (CDTA) in 0.05 M sodium acetate buffer, pH 6, followed by 0.1 M Na_2CO_3 containing 0.1% $NaBH_4$. Sodium carbonate soluble fraction was exhaustively dialysed against distilled water until the pH of dialysed water was around 7. Aliquots of 5 ml were lyophilised and the rest of material was stored at -20ºC, as aqueous solution, until analyzed by AFM. Galacturonic acid was determined by the carbazole method (Filisetti-Cozzi and Carpita, 1991), using uronic acid (UA) as standard.

3.3 Acid hydrolysis treatment

To determine the effect of dRGII removal on pectin nanostucture, sodium carbonate soluble pectins were subjected to acid hydrolysis following the procedure of Kobayashi et al. (1996). Samples (1mg/ml) were incubated in 0.1 M HCL for 30 min at RT, neutralized with NaOH 1N and dialysed against pure water for 2 h. Then, samples were processed for AFM visualization as described in IV.3.5. Two types of controls were used in this experiment, non-treated samples and pectin samples treated with pure water instead of HCl.

3.4 Endo Polygalacturonase digestion

Sodium carbonate pectin samples diluted to a concentration of 0.5 mg/ml of galacturonic acid in 0.1 mM ammonium bicarbonate buffer, pH 5.5, were digested with a diluted endo-PG (280 u/mg; E.C. 3.2.1.15; from Aspergillus aculeatus; Megazyme) at 35ºC (optimum enzyme temperature recommended by the manufacturer). Enzimatic digestions were stopped at 0, 60, 90 and 120 min by boiling the samples for 10 min. Different dilutions of Endo-PG were tested to avoid artifacts and interferences in AFM images, being the optimal enzyme concentration found of $1:10^6$. Samples incubated with buffer without enzyme were used as control. After digestion, samples were visualized by AFM as described in the following sections.

3.5 Atomic force microscopy

Samples were diluted with water to a concentration of 2-4 µg/ml. Three µl of pectin solution was put down onto freshly mica and was dried into the blot at 37 ºC. The atomic force microscope used for the present studies was manufactured by ECS (East Coast Scientific, Cambridge, UK). The procedure was performed as described by Posé et al. (2012a), samples were imaged in a liquid cell under butanol. Triple distilled butanol (300 µl) was used as an imaging solvent with double purpose: it eliminates capillary condensation effects and, as a precipitant, it limits desorption during imaging (Kirby et al., 1996; Round et al., 2001; Posé et al., 2012a). Contact mode was selected to obtain images with high quality.

3.6 AFM image analysis

Contour length and branch pattern were analyzed and characterized by their length distribution parameters (Posé et al., 2012a). The number-average (L_N) and the weight-average (L_W) contour lengths and their ratio (L_W /L_N) or polydispersity index (PDI) were calculated (Odian, 2004). The effect of acid hydrolysis and endo-PG digestion on pectin aggregates was determined by recording the number of aggregates per µm^2. All experiments were performed by triplicate. Ten to 20 images per treatment were taken and 200-300 individual measurements from independent images were analyzed to obtain the length distribution representations and statistical parameters.

3.7 Statistical analysis

The GraphPad Prism software (v.5.0 b) was used for statistical analyses. The medians from the original length distributions were compared by non-parametric Kruskal–Wallis test. Differences in the branching of the polymer chains, presence/absence of side chains, were analyzed by Chi-square test. The number of aggregates per µm^2 of aggregate was analyzed by Mann Whitney U test. All statistical tests were performed at P = 0.05.

4. RESULTS

4.1 Effect of polygalacturonase digestion on nanostructure of sodium carbonate pectin samples

In this experiment, sodium carbonate pectin samples were treated with fungal endo-PG during different times, and visualized by AFM. Initially, several endo-PG dilutions were tested but only a low enzyme concentration, $1:10^6$, did not interfere with AFM images. Representative AFM images of samples from control, APG and APEL fruits after 0, 60, 90 and 120 minutes of enzymatic incubation ($1:10^6$) are shown in Figs. 1, 2 and 3. AFM provides both qualitative and quantitative information. Before enzymatic digestion, the qualitative analysis of these images showed isolated pectins containing long branches, some of which formed tridimensional structures or aggregates (Figs. 1A, 2A and 3A). In general, the isolated control chains were shorter (Fig. 1A) than those presents in transgenic samples (Figs. 2A and 3A), and the micellar aggregates were more abundant in APG samples, as described by Posé et al. (2012b). The zoomed images E-H in the Figs. 1-3 show details of the different pectin aggregates present in the samples. Three-dimensional images of aggregates are represented in the Fig. 4.

As results endo-PG treatment, a gradual degradation of the main backbone of the isolated pectins, their branches and aggregates was observed over time. This was specially evident in control and APG samples (images B to D in Figs. 1-3). However, pectin chains were not completely degraded after 2 h of treatment, probably due to the low fungal endo-PG concentration assayed. In the control (Fig. 1) and APG samples (Fig. 2), a reduction in the length of linear filamentous structures was clearly observed during enzymatic digestion, as well as a reduction in the number of branches. The PGase treatment also reduced the number of aggregates in the three genotypes, as already mentioned.

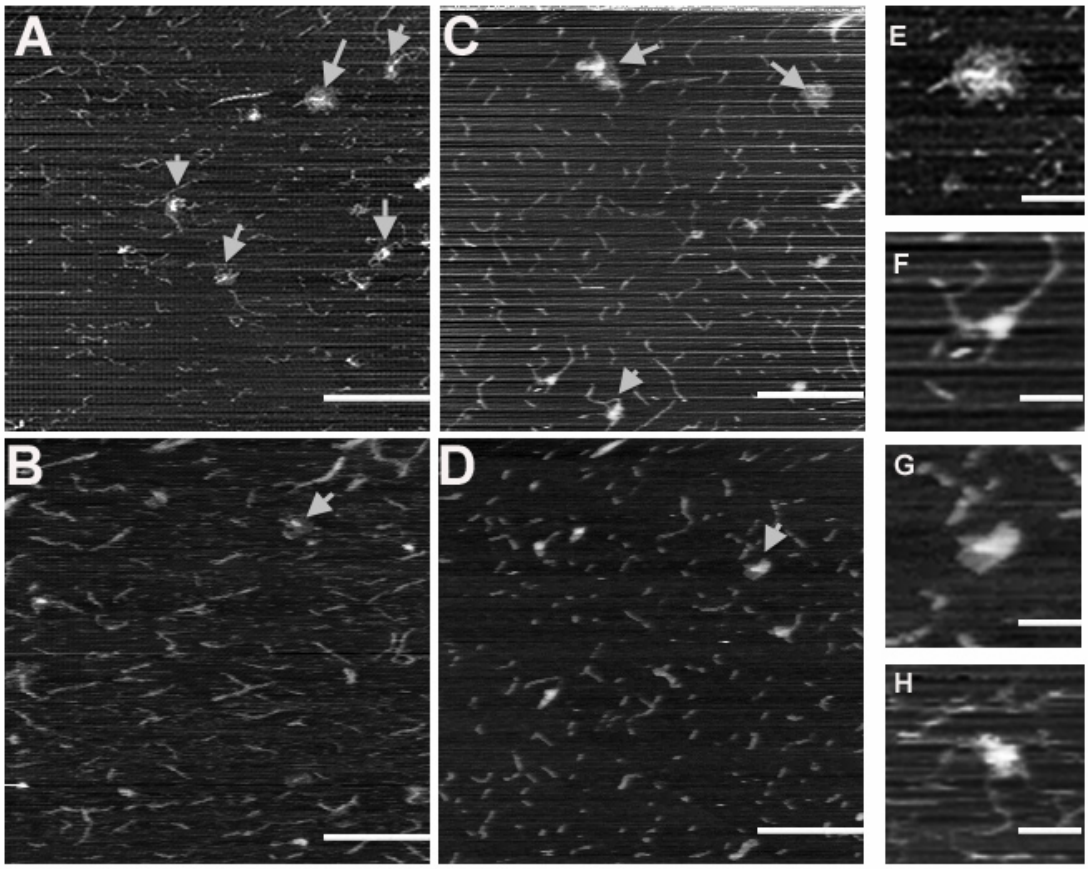

Figure 1. Representative topographical AFM images of Na_2CO_3 pectins from cell wall extracts of ripe control fruits incubated with PGase at time 0 (A), 60 min (B), 90 min (C) and 120 min (D). Inserts (E–H) show zoomed areas from the A, B, C or D. Zoomed images show examples of aggregates or multi-polymer complexes. The scale bars for A–D are 250 nm, whilst the scale bars for the inserts E-H are 50 nm. Arrows indicate micellar aggregates.

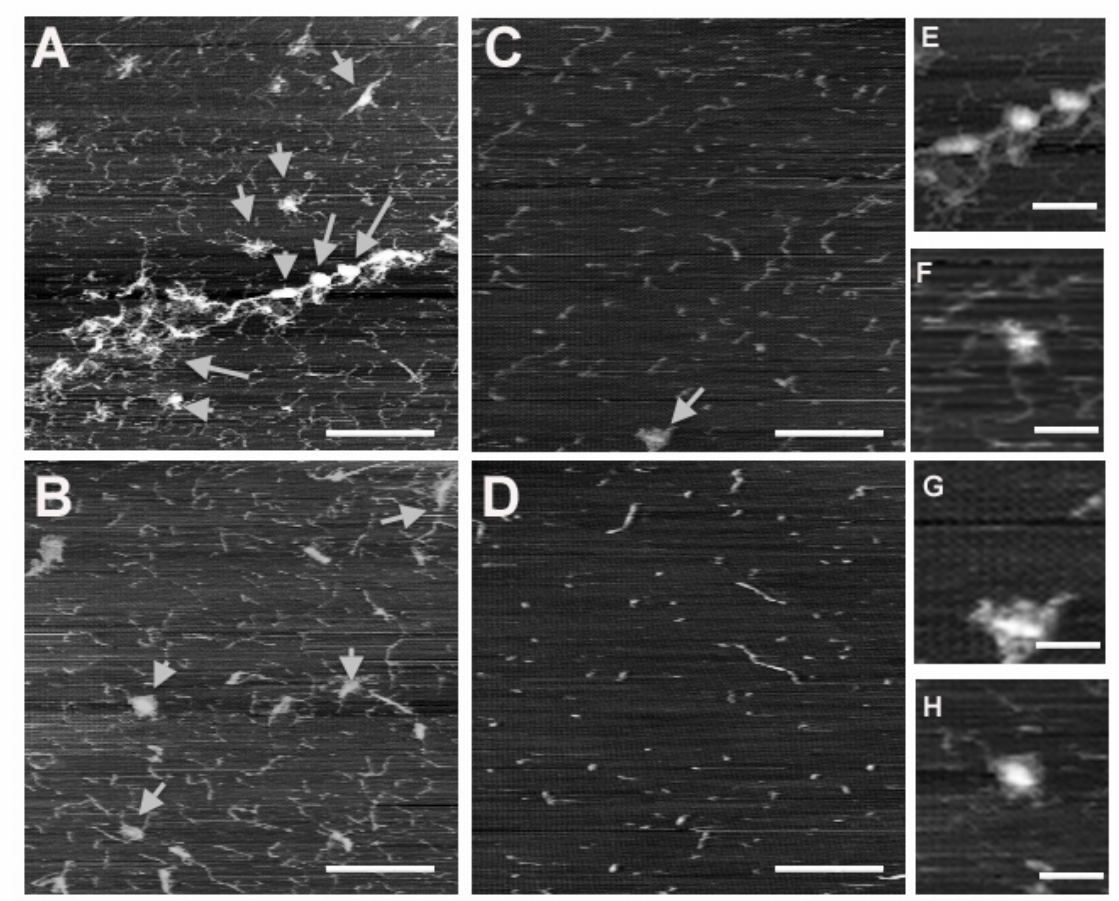

Figure 2. Representative topographical AFM images of Na_2CO_3 pectins from cell wall extracts of ripe APG fruits incubated with PGase at time 0 (A), 60 min (B), 90 min (C) and 120 min (D). Inserts (E–H) show zoomed areas from the A, B, C or D. Zoomed images show examples of aggregates or multi-polymer complexes. The scale bars for A–D are 250 nm, whilst the scale bars for the inserts E-H are 50 nm. Arrows indicate micellar aggregates.

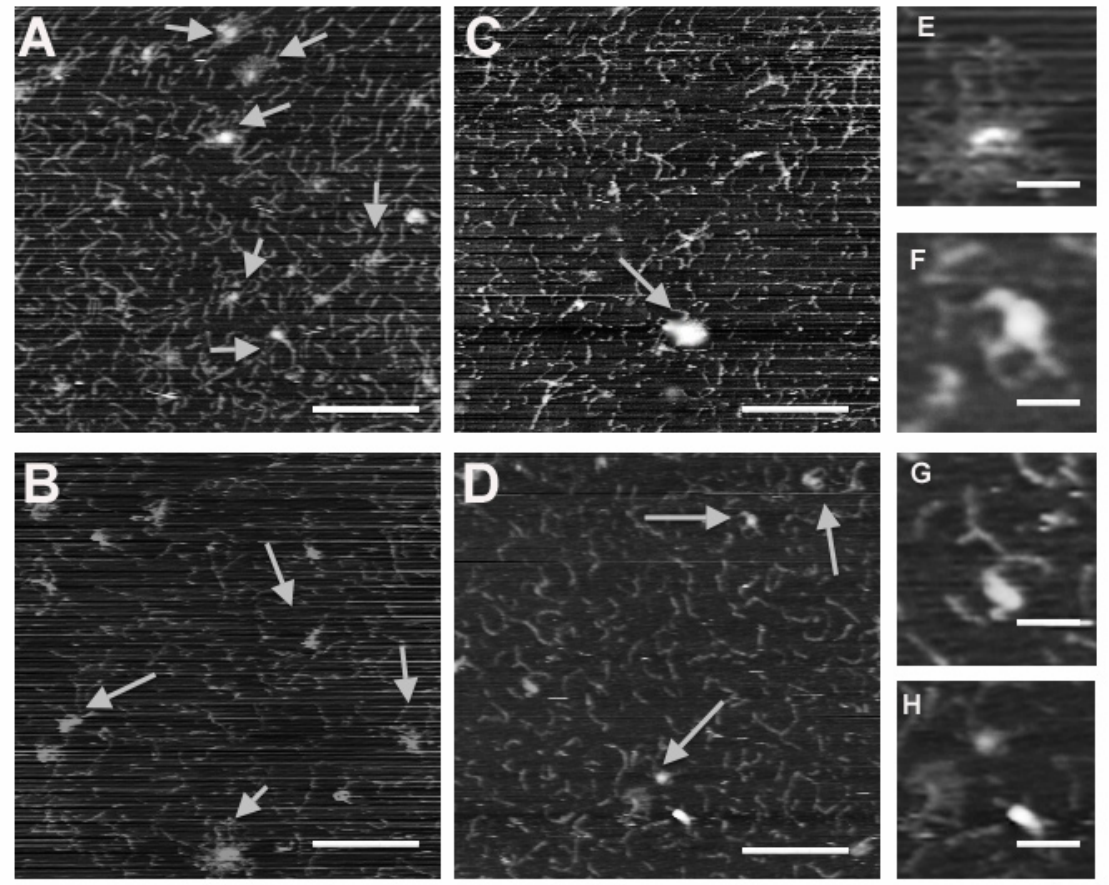

Figure 3. Representative topographical AFM images of Na_2CO_3 pectins from cell wall extracts of ripe APEL fruits incubated with PGase at time 0 (A), 60 min (B), 90 min (C) and 120 min (D). Inserts (E–H) show zoomed areas from the A, B, C or D. Zoomed images show examples of aggregates or multi-polymer complexes. The scale bars for A–D are 250 nm, whilst the scale bars for the inserts E-H are 50 nm. Arrows indicate micellar aggregates.

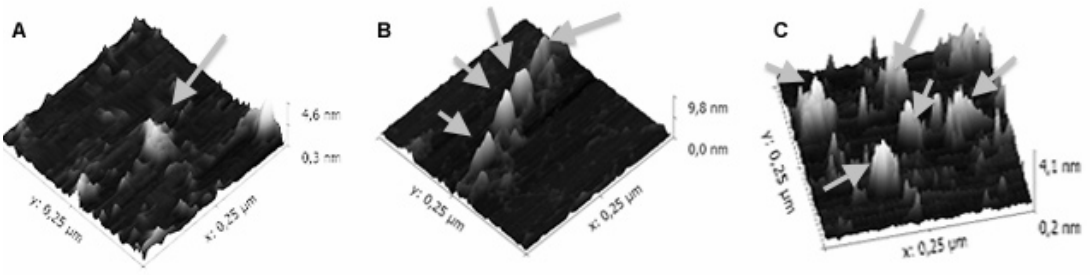

Figure 4. AFM three-dimensional images of the aggregates. **(A)** Control, **(B)** APG and **(C)** APEL. Arrows indicate micellar aggregates.

Fig. 5 shows the contour length distributions at different digestion times of isolated pectic chains from the three genotypes. Pectin length varied in the range 10-300 nm, being the more abundant contour length classes 50 and 75 nm in the three genotypes. After 90 min of enzymatic incubation, control sample distribution shifted significantly to the left and very minor changes in distribution were observed after 30 additional min of assay. This result suggests that most endo-PG hydrolysis of the pectic chains was achieved within the first 90 min of treatment (Fig. 5A). By contrast, length of APG pectic chains was continuously reduced during the 120 min of assay. It seems that further incubation time is required to obtain a stable distribution, as it occurred for control samples in 90 min (Fig. 5B). In the case of APEL, the pectin length distribution was similar at 0, 60 and 90 min, showing only a slight displacement to the left after two hours of PG digestion (Fig. 5C).

Table 1 shows several distribution parameters: the number-average (L_N), the weight-average (L_W) and the polydispersity index (PDI) of the pectic chains at different digestion times. The medians (ME) of the original data were also included and used for statistical comparison. In control and APG samples, the median of the length distribution decreased since the beginning of the incubation but the differences were only statistically significant after the first 90 min of digestion. The median of the APG length distribution was similar to the value obtained in control samples, 36 nm after 120 min of enzymatic treatment endo-PG digestion. This ocurred despite the significant difference in length distribution at time 0, with median for APG samples of 87 vs. 63.5 nm in control. Contour lengths of APEL samples at different digestion times did not show significant differences until reached 120 min of digestion, when the median decreased to 50 nm, higher than the values observed in the other two genotype samples.

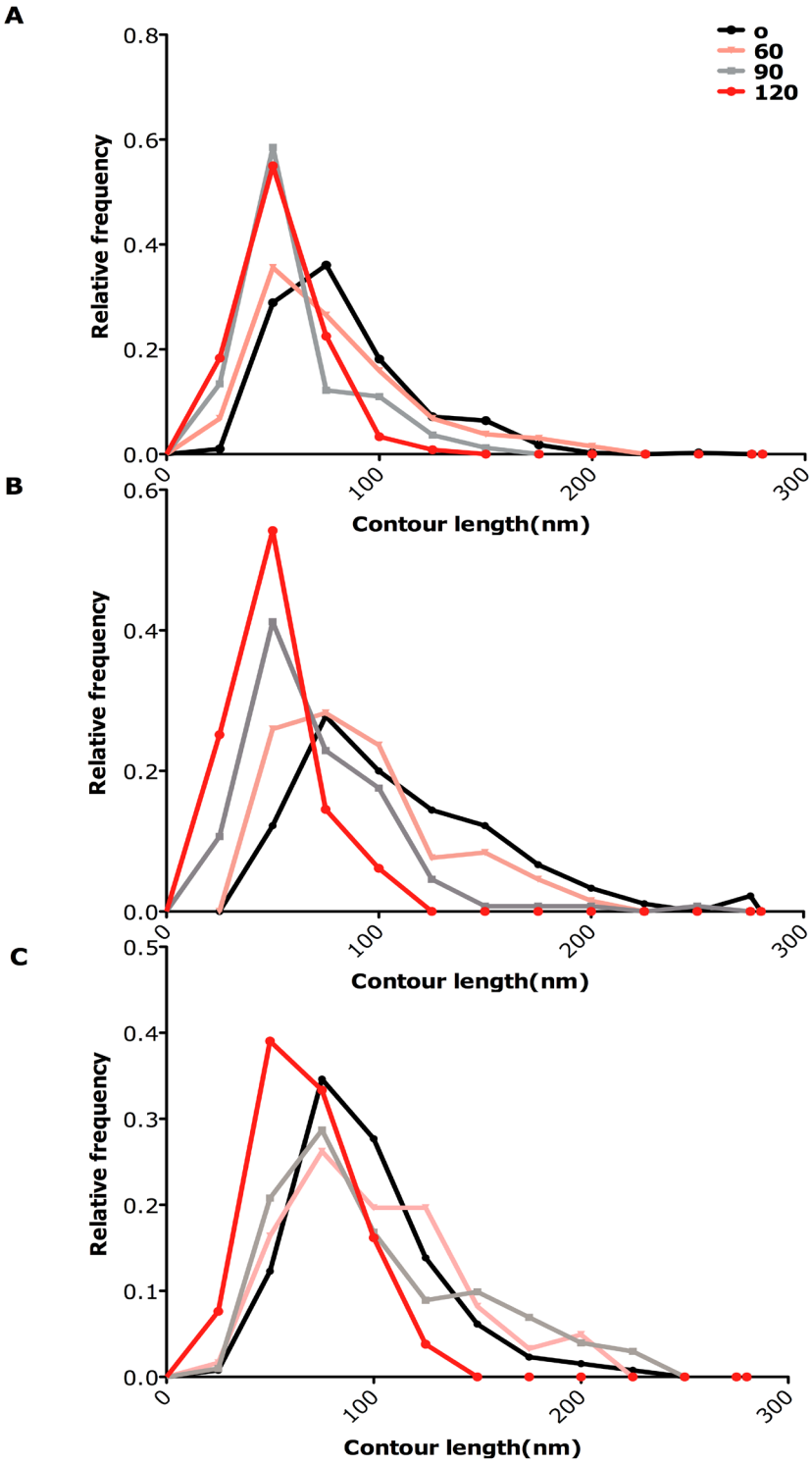

Figure 5. Contour lenght distribution of carbonate soluble pectins samples from control**(A)**, APG**(B)** and APEL **(C)** ripe fruits after 0, 60, 90 and 120 min of digestion with fungal endo-PG. A lineal representation was selected instead of histograms in order to facilitate comparison between times whithin each genotype.

Table 1 also shows the percentage of branching in the three genotypes after endo-PG digestion. At time 0, transgenic genotypes, especially APG samples, showed a higher percentage of branched polymers than control line, as previously described by Posé (2012b). The level of branching diminished after pectinase treatment, and at 120 min of treatment no branched pectic chains were observed in control and APG samples. However, APEL samples still maintained a low percentage of branched chains.

Table 1. Descriptors of contour length distributions of Na_2CO_3 pectins extracted from ripe strawberry fruits from control, APG and APEL lines at different times of digestion with PGase. L_N: number-average, L_W: weight-average, PDI: ratio L_W/L_N, ME=median of the original data.

	Time	T0	T60	T90	T120 min
Control	L_N(nm)	70,2	65,3	46,6	40,8
	L_w(nm)	85,8	86,9	61,2	50
	PDI	1,2	1,3	1,3	1,2
	ME(nm)	63,5**a**	58,6**a**	41,9**b**	36,6**b**
	Branching (%)	6,4%	3,0%	1,2%	0%
APG	L_N(nm)	97,8	78,5	57,2	37,9
	L_w(nm)	121,6	96,5	78	48,5
	PDI	1,2	1,2	1,4	1,3
	ME(nm)	87,7**a**	70,9**a**	48,9**b**	33,5**c**
	Branching(%)	33%	6,1%	1,5%	0%
APEL	L_N(nm)	89,8	87,9	89,2	54,8
	L_w(nm)	97,2	106,9	115,1	65,3
	PDI	1,1	1,2	1,2	1,2
	ME(nm)	86,2**a**	79,7**a**	78,6**a**	50,3**b**
	Branching(%)	7,5%	9,8%	8,9%	2%

To summarize, a linear decrease on pectin length as result of PG digestion was observed in control and APG samples, with linear regression coefficients of 0.95 and 0.99, respectively (Fig. 6). However, Endo-PG incubation was less effective when its substrate was the pectic chains

present in APEL samples. At least 120 min of treatment were required to diminish significantly the median of the size distribution and the side chains present were also more resistant to hydrolysis enzymatic .

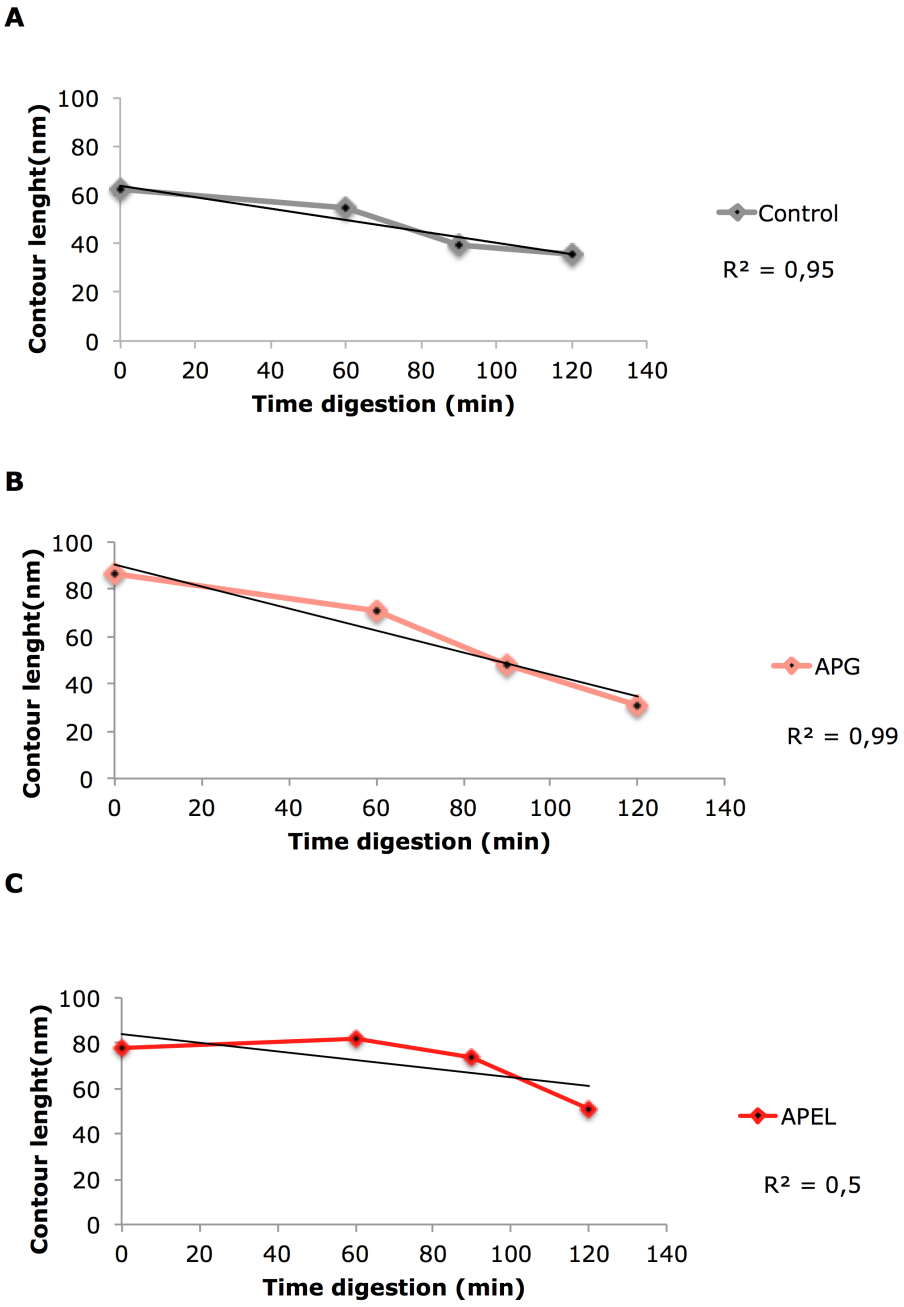

Figure 6. Kinetic of pectin chains digestion by fungal Endo-PG in control (A), APG (B) and APEL samples (C) . The medians of the length distribution at each incubation time is used as estimator of the chain size.

At the same time that pectic chain digestion occurred, a reduction in the number of aggregates in the samples of the three genotypes was also observed (Fig. 7). The mean number of aggregates at time 0 was 3, 8 and 4 aggregates per μm^2 for control, APG and APEL lines, respectively. In APG samples, the number of aggregates decreased linearly reaching a value of 0.1 aggregate per μm^2 after 120 min of digestion (Fig. 7A). A similar behaviour was observed in the control, although a residual amount of aggregates was found in some AFM images (Fig. 7B). In APEL, the reduction in the number of micellar aggregates occurred only after 120 min of enzymatic incubation, remaining between 2 or 3 aggregates/μm^2 (Fig. 7C).

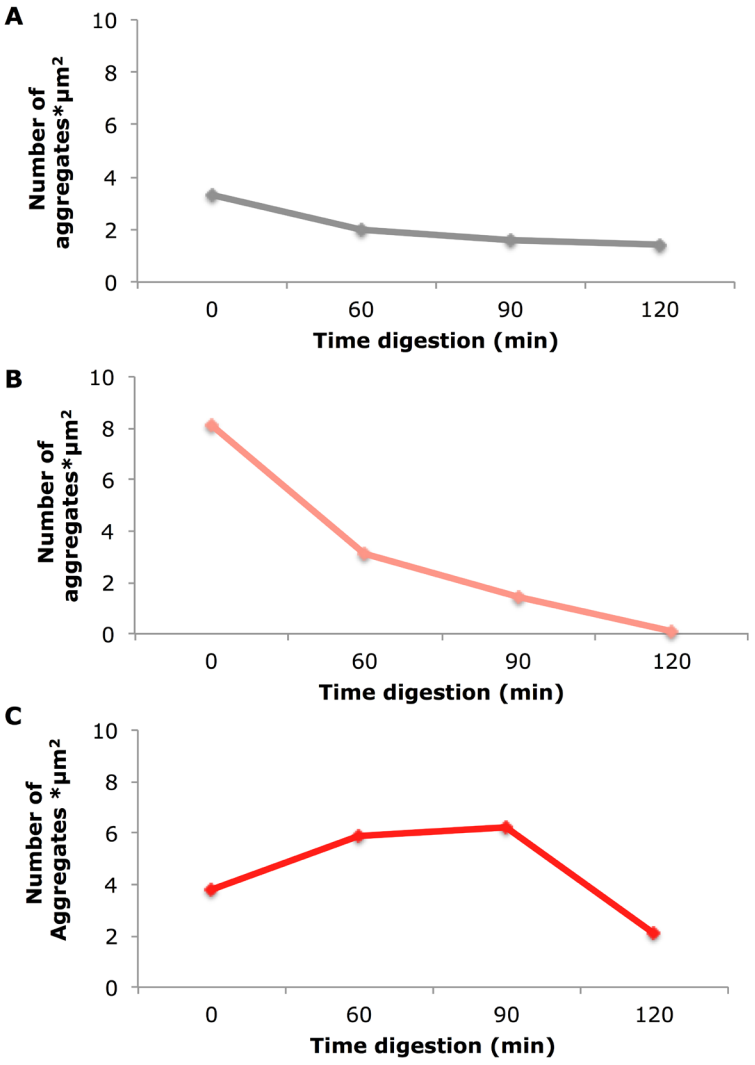

Figure 7. Effect of fungal Endo-PG digestion in the number of aggregates in pectin samples from control (A), APG (B) and APEL (C) samples.

4.2 Effect of acid treatment on the micellar aggregate structure

A mild acid hydrolysis treatment, 0.1 M HCl for 30 min at room temperature, was carried out in order to reveal if dimmers of RGII were related with the presence of aggregates in the samples. This treatment has been proposed to split dimmers of RGII and it was applied to Na_2CO_3 APG samples because this fraction displayed the highest number of aggregates. Fig. 8 shows a representative image of Na_2CO_3 soluble pectins prior to the chemical treatment (A), showing aggregates and long chains, and after mild acid hydrolysis (B), where the number of aggregates was notably reduced,whereas the length of isolated pectic chains was apparently unaffected. The quantification of the number of aggregates per image showed a reduction from 6 structures per μm^2 before hydrolysis to 1 after 30 minutes with 0.1M HCl treatment. Zoomed images (C-D) show the types of aggregates that were studied. These structures were characterized by an entanglement of pectins in the center with numerous emerging chains around, some of which were similar in size to isolated pectic chains.

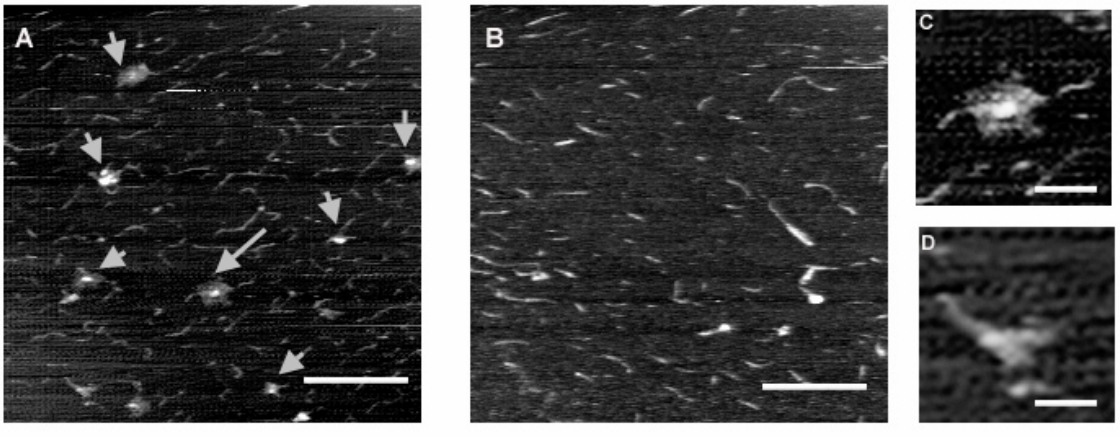

Figure 8. Representative topographical AFM images of Na_2CO_3 pectin extracts from antisense APG ripe fruit before (A) and after mild acid treatment (B). (C) and (D) show zoomed areas from the A and B images respectively. The scale bars for A–B are 250 nm, whilst the scale bars for C-D are 50 nm. Arrows indicate aggregates.

Isolated polymer chains were measured prior and after acid hydrolysis and their basic parameters calculated (Table 2). Although L_N and L_W values decreased slightly after acid treatment, the median of the length chain distributions prior and after hydrolysis were not statistically different. Similarly, the length of the chain branches was not affected by the acid hydrolysis treatment (Table 3), but the branching percentage decreased 3,2%.

Table 2. Descriptors of contour length distributions of Na_2CO_3 pectin extracted from ripe strawberry fruits from APG plants, after hydrolysis in 0.1M HCl for 30 min. Control corresponds to the sample treated with water. Values followed by different letters are significantly different at P = 0.05 by non-parametric median test. N=300 pectins in all the samples. L_N: number averange, L_W: weight average, PDI:polydispersity indexes and ME: median of the original date.

		APG	APG+HCL
Na_2CO_3	**L_N(nm)**	92	82
	L_w(nm)	127	112
	PDI	1.38	1.36
	ME(nm)	78.81**a**	70.08**a**

Table 3. Branching characteristics of the Na_2CO_3 pectins extracted from ripe strawberry fruits from APG plants, after hydrolysis in 0.1M HCl for 30 min. Control corresponds to the sample treated with water. Values followed by different letters are significantly different at P = 0.05 by non-parametric median test. N= 50-100 branched from 300 pectins.L_N: number averange, L_W: weight average, PDI:polydispersity indexes and ME: median of the original date.

		APG	APG+HCL
Na_2CO_3	**Branching (%)**	13.2 **b**	10**a**
	L_N(nm)	45.25	45.3
	L_w(nm)	61.81	57.2
	PDI(nm)	1.36	1.2

5. DISCUSSION

5.1 Pectinase silencing affects the endo-PG digestion pattern of sodium carbonate extracted pectins

Our results show that, at time 0, pectin chains from control line were shorter and less branched than those from the transgenic genotypes with pectinase genes silenced, as reported by Posé et al. (2012b). Both, *FaPG1* and *FaplC* genes are up-regulated during ripening, and therefore their silencing in the transgenic fruits could protect pectin from degradation *in vivo* (Santiago-Doménech et al., 2008; Posé et al., 2013). In spite of this shared characteristic, the transgenic samples behaved differently when were treated with fungal Endo-PG. In the case of APG, our results support that these pectin samples contain more sites susceptible to be recognize as substrate for Endo-PG than control samples and this could explain the different kinetic observed during the enzymatic treatment relative to control. This hypothesis is also supported on previous results obtained by polysaccharide analysis using carbohydrate gel electrophoresis (PACE) of APG carbonate samples (Posé et al., 2013). The APG samples were significantly more digested in vitro when they were incubated with Endo-PG enzyme than the control line. However, APEL pectins samples showed a certain degree of resistance to Endo-PG digestion, and pectin length was slightly reduced after 60 and 90 min of treatment, only after 120 min the differences were found statistically significant. Furthermore, this genotype showed the highest L_N and L_W values after 120 min digestion. It seems that the inhibition of *FaplC* expression *in vivo* results in carbonate fraction relatively resistant to Endo-PG hydrolysis. This is an unexpected result, since polygalacturonase and pectate lyase have the same target, demethylated pectins, but different mode of action (Marín-Rodríguez et al., 2002; Santiago-Doménech et al., 2008). The recalcitrance of APEL pectins to be digested suggests a different pectin structure and/or carbohydrate composition which might

interfere enzymatic activity. The presence of intercalated Rha residues or more neutral side branches might difficult endo-PG digestion. However, the sugar analysis of carbonate pectins did not reveal any difference between control and transgenics (results not shown). Alternatively, previous digestion of carbonate pectins by *FaplC* product could be necessary for the action of polygalacturonases and/or other cell wall modifying enzymes, and therefore its silencing would protect pectin chains to be degraded by this fungal Endo-PG. For example, the down-regulation of *FaplC* might protect the pectin against the action of xylanases, and the existence of xylose residues on the HG polymers prevents endopolygalacturonase digestion (Prade et al., 1999).

5.2 Isolated chains and branches visualized by AFM are mainly formed by galacturonic acid

Besides the above discussed differences in the digestion pattern between the transgenic genotypes analysed, the most important finding obtained in the PGase experiment is the confirmation of the galacturonic acid nature of the branches observed in isolated pectin chains by AFM. It must also be emphasized the presence of HG in the micellar aggregates. The almost totally elimination of chain branches and the reduction of pectin length by PGase treatment strongly support the hypothesis of Round et al. (2001). These authors suggested that galacturonic acid is the main component of the long pectin branches observed by AFM, being the smaller side chains formed by neutral sugars not detectable by AFM. The GalA nature of branches was also supported by experiments evaluating the effect of mild acid hydrolysis at high temperature (80ºC) on carbonate pectins from unripe tomato (Round et al., 2010). This treatment releases neutral sugars at different rates, being galactose, arabinose and rhamnose linkages the most labile. The loss of Rha during mild acid hydrolysis treatment did not have a significant effect on pectin backbone (Round et al., 2010), suggesting that isolated chains were formed by HG and not interrupted

by RGI. Nevertheless, Round et al. (2010) were not able to confirm this hypothesis due to the unspecific hydrolytic treatment used.

Despite the large decrease on pectin backbone length after PG treatment in all genotypes analysed, pectin backbone was not totally degraded after 120 min treatment. The partial digestion of strawberry pectins could be due to the low enzyme concentration used in this experiment to allow a clear visualization of isolated chains. Alternatively, some part of the backbone could not contain substrate specific domain for Endo-PG activity. The presence of ester groups blocking PG action should be discarded since these pectins were extracted with sodium carbonate at high pH, a treatment that demethylate pectins. However, neutral side-chains or the presence of intercalated Rha residues in the backbone could limit Endo-PG activity. In citrus pectin, a low frequency of single Rha interspersed in the HG structure was found (Zhan et al., 1998). Other articles also showed evidences of the presence of Rha that would be involved in the kinks between HG domains (Rees and Wright, 1971; Powell et al., 1982; Jarvis et al., 1984).

The number of carbohydrate residues of the pectic structures visualized by AFM can be estimated from L_N values considering a 3^1 helix structure with a pitch of 1.34 nm, as deduced from fiber diffraction analysis of polygalacturonic acid (Walkinshaw and Arnott,1981). According to this assumption, in the control samples, the degree of polymerization (DP) of the residual pectin backbone after 120 min of endo-PG digestion was approximately 91 DP, whereas the pectin fraction that has been degraded by the enzyme would contain about 66 residues. Interestingly, Thibault et al. (1993) and Yapo et al. (2007), using chromatographic techniques, reported a minimum length for HG of 72 GalA residues, a value close to the one found for the digested part of carbonate pectins. The presence of intercalated Rha residues in the pectin backbone does not agree with the previously discussed results obtained by Round et al. (2010), which did not observe a decrease in the length of isolated pectin chains after

acid hydrolysis. However, it is likely that the mild acid hydrolysis used in that work was partial and it caused selective release of neutral sugar side chains from RGI but the Rha-GalA linkages of the backbone were less affected by the treatment (Prade et al.,1999). Additional experiments with longer enzymatic incubation times are needed to confirm the indigestibility of part of the pectin structure as well as to determine the exact nature of the backbone residues which limit PG action.

5.3 RGII is involved in the formation of macromolecular pectin complex

Pectins are complex molecules that could form three-dimensional structures which might play an important role in the mechanical and physical properties of the primary cell wall in muro. In AFM images of strawberry pectins, micellar aggregates are frequently visualized, independently of the pectin dilution employed. Structures of similar form to those described in this paper have been observed by several authors in AFM studies (Morris et al., 1997; Round et al., 2010, Posé et al., 2012a). According to Round et al. (2010) these aggregates contain HG and RGI since the acid hydrolysis of pectins at high temperature to release neutral sugars decreased the size of these structures, although the aggregates were not fully removed after prolonged time of hydrolysis. The fungal Endo-PG digestion of strawberry pectins reduced the frequency of aggregates, suggesting that polygalacturonic acid is one of the components present in these aggregates, as reported by Round et al (2010). In the other hand, untreated samples from down-regulated *FaPG1* fruits showed a higher amount of aggregates than control and Apel samples. This result also indicates that polygalacturonase may be involved in the disassembly of the tridimensional structure of these aggregates.

Despite that RGII is a minor pectin component in the cell wall, it is believed that it could play an important role in the regulation of the pore size and other physical properties of the cell wall. It was also suggested that the thickness of the cell wall might depend of the number of RGII domains and

their exact position in the chain (Vincken et al., 2003). In the traditional pectin model of alternating "smooth" and "hairy" regions, RGII is integrated in the HG backbone. In the alternative pectin models proposed, RGII domains are included in HG domains (Ishii et al., 2001), which are covalently cross-linked to RGI. Additionally, RGII can cross-link by borate-diol ester two HG domains (Vincken et al., 2003). Yapo (2011) proposed that the formation of RGII dimers is a prerequisite for pectin macromolecular formation. This molecule is able to form dimmers via borate diester bridges between RGII molecules belonging to two different layers of pectins and also between pectic molecules within the same layer. Kobayashi et al. (1996) suggested the use of mild acid hydrolysis at room temperature for a short time (30 min) to break borate ester cross-linking without altering glycosidic linkages. This procedure has recently been used by Chormova et al. (2014) to release mRGII from dRGII. The use of this treatment in strawberry pectins diminished the number of aggregates without altering the length of isolated chains and only very slightly the branch pattern. These results indicate that dRGII could be a key component of micellar aggregates and participate in the tridimensional structure of these pectin complexes.

5. CONCLUSIONS

The enzymatic treatment of sodium carbonate soluble pectins from strawberry fruits with fungal Endo-PG decreased the length of pectin chains and the number of branches. This result corroborates the galacturonic acid nature of the isolated pectin strands visualized by AFM. This treatment also reduced the number of micellar aggregates per sample, indicating that these structures also contain HG, as suggested by Round et al. (2010). Contrary to Endo-PG treatment, hydrolysis with diluted HCl at room temperature did not modify the length of pectin strands, but significantly diminished the presence of aggregates. This treatment breaks borate diester bonds between RGII, and therefore, our results indicate that RGII has a structural role in the formation of these complexes.

RESULTS AND DISCUSSION CHAPTER V:

FUNTIONAL CHARACTERIZATION OF *FaβGal4*, A NOVEL β-GALACTOSIDASE GENE INVOLVED IN STRAWBERRY FRUIT SOFTENING

1. SUMMARY

Softening during ripening of fleshy fruits is mainly due to the disassembly of cell walls induced by cell wall-modifying enzymes. In strawberry, *Fragaria* × *ananassa* Duch. , solubilisation of the pectin polysaccharides and the loss of galactose from pectin side chains are the main processes occurring during ripening. β-Galactosidase activity increases in strawberry fruits during ripening. Three different β-gal genes have been described in strawberry fruit although only one of them showed a ripening-specific expression pattern. Our research group has isolated a novel β-gal gene, *FaβGal4*, which expression is enhanced during strawberry ripening. To assess the role of this gene in softening, we have obtained transgenic strawberry plants expressing an antisense sequence of this gene. Silencing of *FaβGal4* in transgenic fruits was close to 70%. Phenotypic analyses were carried out in transgenic plants during three consecutive growing seasons, using non-transformed plants as control. In two transgenic lines showing a significant *FaβGal4* silencing, fruits were slightly firmer than control at ripening. However, most transgenic fruits were smaller than control and deformed. Cell walls from these transgenic ripe fruits were isolated and sequentially extracted to obtain different cell wall fractions. An increase in the content of pectins covalently bound to the cell wall, extracted with sodium carbonate, as well as a slight increase in water soluble pectins, was observed in both transgenic lines. Neutral sugars were analysed by gas chromatography with flame ionisation detection. The amount of galactose was higher in all cell wall fractions in transgenic fruits when compared with controls, being the differences higher in the hemicellulosic fraction. The results obtained suggest that *FaβGal4* facilitates the solubilization of covalently bound pectins during the ripening process and this could reduce strawberry fruit firmness. Additionally, AFM characterization of pectins also supports a lower depolymerization of pectic chain at the molecular level.

2. INTRODUCTION.

Softening is one of the most characteristic developmental events of the fruit ripening process. In soft fruits, such as strawberry, the melting texture characteristic of ripe fruits is highly appreciated by consumers. Nevertheless, it poses a major problem for strawberry producers, determining the short postharvest shelf life of this fruit and limiting its storage and postharvest transport. During ripening, fruit cell walls are extensively modified, being these changes one of the most important factor leading to fruit softening. In general, cell wall modifications that frequently accompanied softening involve the solubilization of pectin polymers, the depolymerization of pectins and matrix glycans and the loss of neutral sugars from pectin side chains (Brummell, 2006; Goulao and Oliveira, 2008; Mercado et al., 2011). All these processes often occur concurrently during fruit ripening, although the extension of these modifications greatly depends on the kind of fruit (Brummell, 2006; Mercado et al., 2011).

Strawberry softening is characterized by a moderate increase on pectin solubilization, i.e. an increase on the amount of pectins loosely bound to the cell wall, and some pectin depolymerization (Posé et al., 2011). Functional studies of genes encoding pectinase enzymes, such as polygalacturonase (Quesada et al., 2009b) or pectate lyase (Jiménez-Bermúdez et al., 2002; Youssef et al., 2009; Youssef et al., 2013), support a key role of pectin enzymatic disassembly in strawberry softening. Transgenic fruits with low expression levels of these genes were significantly firmer than control and this correlated to a reduction in solubilization and depolymerization of polyuronides (Santiago-Doménech et al., 2008; Posé et al., 2013). The molecular mechanism underlying pectin disassembly is unclear. Many fruits, including strawberry, show a loss of neutral sugars, mainly arabinose and galactose, during ripening (Gross and Sams, 1984; Redgwell et al.,1997b). The removal of these carbohydrates from rhamnogalacturonan I (RGI) polyuronides by β-galactosidases (β-Gal) and α-arabinofuranosidases have

been suggested as one of the possible causes of the increase on soluble pectins (Koh and Melton, 2002; Brummell, 2006).

β-Galactosidases (EC 3.2.1.23) are glycosyl hydrolases characterized by their ability to hydrolyse terminal non-reducing β-D-galactosyl residues from numerous β-D-galactoside substrates (Pérez-Almeida and Carpita, 2006; Tateishi, 2008). In most fruits, β-Galactosidases (β-Gals) are encoded by small gene families with different pattern of expression that could play distinct roles during fruit development (Smith and Gross, 2000; Mwaniki et al., 2005; Tateishi et al., 2007; Othman et al. 2011). Functional analyses of β-Gal genes using transgenic plants have only been carried out in tomato, a climacteric model fruit, with contrasting results. Antisense *TBG4* tomato fruits displayed reduced *TBG4* mRNA levels and free cell wall galactose only at the onset of ripening but softening of ripe fruits decreased by 40% (Smith et al., 2002). By contrast, neither the silencing of *TBG1* (De Silva and Verhoeyen, 1998) nor *TBG3* (Carey et al., 2001) modified tomato fruit firmness. In *TBG6* antisense tomato plants, fruits firmness was also similar to control but their cuticle structure was affected and an increased fruit cracking was observed (Moctezuma et al., 2003).

In strawberry, β-Gal activity increases during fruit development and remains high in ripe fruit (Trainotti et al., 2001; Figueroa et al., 2010). At the molecular level, Trainotti et al. (2001) isolated three full length cDNAs encoding β-Gal genes, *FaβGal1* to *FaβGal3*. Although all of them could be detected in fruit and vegetative tissues, only FaβGal1 showed an expression pattern that could be related to the fruit ripening process, being the other two genes expressed mainly in green, immature fruits. Transcriptomic studies performed in our research group have enabled us to identify a large group of up regulated genes during strawberry fruit ripening. One of these genes, *FaβGal4*, displayed significant sequence homology with putative β-Gal from higher plants. The main goal of this study was the functional characterization of this gene. To this purpose, transgenic

strawberry plants carrying an antisense sequence of *FaβGal4* have been generated and the effects of *FaβGal4* down-regulation in fruit firmness and cell wall composition and nanostructure have been analyzed.

3. MATERIALS AND METHODS

3.1 Plant material

Strawberry plants (*Fragaria* × *ananassa* Duch., cv. `Camarosa´) were grown under field conditions in Huelva (S.W. Spain). Fruits were harvested at different developmental stages: small-sized green fruits (G1, 2-3 g), medium-sized green fruits (G2, 3-5 g), full-sized green fruits (G3, 4-7 g), white fruits (W, 5-8 g) and full-ripe red fruits (R, 6-10 g). Vegetative tissues including runners, flowers and expanding leaves were also harvested. All tissues and fruit samples were immediately frozen in liquid nitrogen and stored at -80 ºC.

The source of material for genetic transformations were in vitro micropropagated plants of cv.`Chandler´.

3.2 Auxin treatment

Achenes of two sets of 50 middle-sized green fruits (G2) each, still attached to the plant, were carefully removed using the tip of a scalpel blade. One set of deachened G2 fruits were treated with the synthetic auxin 1-naphthalenacetic acid (NAA) in lanolin paste. This paste included 1 mL of NAA, 1 mM, previously dissolved in 1 % (w/v) DMSO. The other set of G2 deachened fruits (control group) was treated with the same paste, but without NAA. Both treatments were applied over the whole fruit surface. All fruits were harvested 5 days after treatment, immediately frozen in liquid nitrogen and stored at -80 ºC. During the course of these assays, the fruits reached the G3-W developmental stage.

3.3 NDGA Treatment

Nordihydroguaiaretic acid (NDGA) is an ideal inhibitor of the NCED enzyme with regard to its permeating speed and its ability to block ABA biosynthesis (Creelman et al., 1992 ; Zhang et al., 2009). In tomato, the lowest NDGA concentration which completely blocks ABA accumulation is 100 µM (Zhang et al., 2009). Fruits were used at the mature G-W stages for this purpose. Twenty fruits were carefully injected with 1-2 mL of NDGA (100 µM) sterile solution or sterile water (control fruits) using a hypodermic syringe. Three replications were conducted for each treatment. The samples were harvested after 8 days of injection, when the fruits reached the R developmental stage, frozen in liquid nitrogen and stored at -80 ºC. Fruits treated with NDGA were white, while control fruits showed a red phenotype. These samples were used to evaluate relative expression of *FaβGal4* gene.

3.4 RNA isolation

Total RNA was isolated from independent pools of strawberry fruits at different growth and ripening stages and from vegetative tissues, in accordance with Asif et al. (2000). Achenes were always removed from fruit and only receptacle RNA was extracted and purified. RNA obtained was treated with RNase free DNase I (Invitrogen) and purified through the RNeasy Mini kit (Qiagen). RNA concentration and purity were evaluated using a NanodropTM spectrophotometer ND-1000 (Thermo Scientific) and by 1 % agarose gel electrophoresis.

3.5 Expression analysis by quantitative real time PCR (qRT-PCR)

Gene expression analysis of *FaβGal4* was performed by qRT-PCR through a iCycler (BioRad) device, as previously described (Benítez-Burraco et al., 2003). *FaβGal4* gene primer sequences for quantitative amplification were: 5´- CAG CCA CCC ACT CCT CTA TAA CCA GTT -3´ and 5´- GCG AAG CAG TAA AAT ACG AAG CAA AGC-3´. Each reaction was performed, at least, in triplicate and the corresponding C_t values were normalized using the C_t value corresponding to an *interspacer 26S-18S* strawberry RNA gene (housekeeping gene) (Benítez-Burraco et al., 2003; Raab et al., 2006; Encinas-Villarejo et al., 2009; Muñoz et al., 2010 and 2011; Cruz-Rus et al., 2011; Cumplido-Laso et al., 2012; Molina-Hidalgo et al., 2013). All of these values were subsequently used to determine the relative increase or decrease in *FaβGal4* gene expression in the samples in comparison to that in the control gene in accordance with Pedersen (2001). Interspacer *26S-18S* gene (primers: 5'-ACC GTT GAT TCG CAC AAT TGG TCA TCG-3' and 5'-TAC TGC GGG TCG GCA ATC GGA CG-3') was selected as control gene owing to its constitutive expression throughout all of the different experimental conditions tested. The efficiency of each particular qRT-PCR and the melting curves of the products were also analysed to ensure the existence of a single amplification peak corresponding to a unique molecular species. The expression levels of the different β-Gal genes in transgenic antisense *FaβGal4* fruits were measured by qRT-PCR as above described.

The gene expression analysis of *FaβGal4* during strawberry fruit development and after auxin or NAAG treatment were performed in colaboration with Drs. R. Blanco-Portales and J. Muñoz-Blanco (Universidad de Córdoba)

Due to both (target and reference) genes were amplified with efficiencies near 100% , a relative quantification was done following the $2^{-\Delta\Delta CT}$ (Livak) method.

3.6 Binary vector for antisense *FaβGal4* silencing and generation of transgenic strawberry plants

A 300 bp non-conserved region of the *FaβGal4* gene in antisense orientation was cloned into the pK7WG2 binary vector using Gateway technology (Invitrogen, Darmstadt, Germany) for antisense silencing of *FaβGal4* gene. The following forward primer 5'- AGA GGA GAT GCT CGG TCT CGG TAT C -3' and reverse primer 5'- TGG CAT AGC GCT TAA ATA GTT CAT TCA GTT -3' were used. The resulting fragment was cloned into pCR8/GW/TOPO (Invitrogen) and then transferred to the Gateway pK7WG2 vector by way of a specific recombination of both plasmids using LR clonase (Invitrogen). The resulting plasmid (pK7WG2-*FaβGal*) was tested through sequencing and restriction analyses prior to strawberry plant transformation. Plasmid was introduced into *Agrobacterium tumefaciens* strain AGL1 by electroporation.

Leaf discs of the micropropagated plants were used as explants for *Agrobacterium*-mediated transformation experiments, as described in Barceló et al. (1998). Explants were inoculated with a diluted culture of *A. tumefaciens* and putative transgenic material selected with 25 mg L^{-1} kanamycin. After 7-8 month of selection, kanamycin resistant shoots were acclimated and transferred to the greenhouse. The presence of the transgenes in these putative transgenic lines was confirmed by PCR amplification of a 220-bp fragment belonging to the *nptII* gene.

3.7 Phenotypic analysis of transgenic plants

Transgenic plants were evaluated during three consecutive growing seasons, using non-transformed plants, cv. `Chandler´, as control. Plants were grown in a greenhouse under natural temperature and light conditions and fruit collected from March to July. During the first year, ten independent antisense *FaβGal4* lines were analysed. Eight plants per line and a minimum of 10 ripe fruits per line were evaluated. During the second

and third years, two selected lines showing higher fruit firmness than control were evaluated. Thirty plants per line and 50-100 ripe fruits per line were analysed in both years. Fruits were harvested at the stage of full ripeness, when complete fruit surface was red, and the weight, size, color, soluble solids and firmness were recorded. Color was measured using a colorimeter (Minolta Chroma Metre CR-400, Osaka, Japan). The instrument was calibrated with a standard white and a standard black reflective plate before use. The L* a* b* color space parameters were recorded. Soluble solids were measured using a refractometer (Atago N1) and firmness by using a hand penetrometer (Effegi) with a cylindrical needle of 9.62 mm^2 area.

3.8 DNA extraction

Genomic DNA was extracted from young strawberry leaves using Qiagen DNeasy Plant kit. Previously, plant material had been washed three times with washing buffer solution consisting of 100 mM sodium acetate buffer (pH 5), 20 mM EDTA, 0.2 M Sorbitol, 2% polyvinylpolypyrrolidone PVP (mol. Wt. 40,000) and 1% β-mercaptoethanol (Mercado et al., 1999).

3.9 β-Galactosidase activity

β-Galactosidase activity was measured in ripe fruits according to Figueroa et al. (2010). Protein extraction was carried out by grinding 10 gr of strawberry fruits under liquid nitrogen into a fine powder. The powder was homogenized using an Ultra-Turrax device (Janke & Kunkel) with 20 mL of cold extraction buffer (0.05 M Na-acetate buffer, pH 6, 1M NaCl and 1% (w/v) PVPP). Homogenates were stirred at 4ºC for 3 h and then centrifuged at 11,000*g* for 30 min. The supernatant was used to determine β-Gal activity by using p-nitrophenyl-β-D-Galactopyranoside (Sigma) as *in vitro* substrate. β-Gal activity was measured in three independent extrations.

3.10 Cell Wall Analysis

The cell walls were extracted from frozen ripe fruits following the method of Redgwell et al. (1992) with some modifications as previously described by Santiago-Doménech et al. (2008). After extraction, the cell wall material (CWM) was sequentially fractionated following the procedure of Santiago-Doménech et al. (2008). Briefly, 300 mg of the CWM was sequentially extracted with deionized water; 0.05 M *trans*-1,2-diaminocyclohexane-*N*,*N*,*N'N'*-tetraacetic acid (CDTA) in 0.05 M acetate buffer (pH 6); 0.1 M Na_2CO_3 containing 0.02 M $NaBH_4$; 1M KOH containing 0.02 M $NaBH_4$; and 4M KOH containing 0.02 M $NaBH_4$. All fractions were extensively dialysed against distilled water. Then, the extracts were concentrated with a rotary evaporator and freeze-dried. Three independent fractionations per CWM sample were performed.

The uronic acid (UA) content was measured by the carbazole method (Filisetti-Cozzi and Carpita, 1991) using galacturonic acid (GalA) as standard. For neutral sugars analysis, CWM and the different cell wall fractions were hydrolyzed with 72% (w/w) sulphuric acid. After neutralization with ammonia, carbohydrates were derivatized to alditol acetates and analyzed by gas chromatography (GC) with flame ionization detection (Blakeney et al. ,1983). GC analysis was carried out in triplicate.

3.11 FTIR analysis

The dried samples were used to capture the infrared spectra with an Attenuated Total Reflectance (ATR) accessory (MIRacle ATR, PIKE Technologies, USA) coupled to a Fourier transform infrared (FTIR) spectrometer (FT/IR-4100, JASCO, Spain) as described previously (Heredia-Guerrero et al., 2010). The samples were processed as described by Posé et al. (2012a). The spectra were collected in the 2000–800cm^{-1} range with a resolution of 4cm^{-1} and averaged over 25 scans.

3.12 Atomic force microscopy

Samples were diluted with water to a concentration of 2-4 µg/ ml. Three µl of pectin solution was put down onto freshly mica and was dried into the blot at 37 ºC. The AFM used for the present studies was manufactured by ECS (East Coast Scientific, Cambridge, UK). The procedure was described by Posé et al. (2012a). Samples were imaged in a liquid cell under butanol. Triple distilled butanol (300 µl) was used as an imaging solvent to double purpose: it eliminates capillary condensation effects and, as a precipitant, it limits desorption during imaging (Kirby et al., 1996; Round et al., 2001; Posé et al., 2012a). Contact mode was selected to obtain images with high quality.

3.13 AFM image analysis

Contour length and branch pattern were analyzed and characterized by their length distribution parameters (Posé et al., 2012a). The number-average (L_N) and the weight-average (L_W) contour lengths and their ratio (L_W /L_N) or polydispersity index (PDI) were calculated (Odian, 2004). The aggregates were also determined by recording the number of aggregates per μm^2. All experiments were performed by triplicate. Thirty images per treatment were taken and 300 individual measurements from independent images were analysed to obtain the length distribution representations and statistical parameters.

3.14 Statistical analysis

The SPSS software was used for all the statistical analysis. The Levene test for homogeneity of variances was performed prior to ANOVA. In the case of non homogeneous variances, the non-parametric Kruskal-Wallis test was used. Mann-Whitney U test and Dunnett's Multiple Comparison Test were used for pair and multiple comparison; respectively.

For AFM study, the medians from original data distributions were compared by non-parametric Kruskal–Wallis test. Differences in the branching of the polymer chains, presence/absence of side chains, were analyzed by Chi-square test. The number of aggregates per micra was analyzed by Mann Whitney U test. All statistical tests were performed at $P = 0.05$.

4. Results

4.1 *FaβGal4* gene expression studies

Transcript analyses in fruits of the cultivated *F.* × *ananassa*, cv. `Camarosa´, showed that *FaβGal4* relative gene expression was low in the three green stages of fruit development followed by a statistically significant increase which starts at the white stage of receptacle ripening and reaches its highest level in red fruit (Fig. 1).

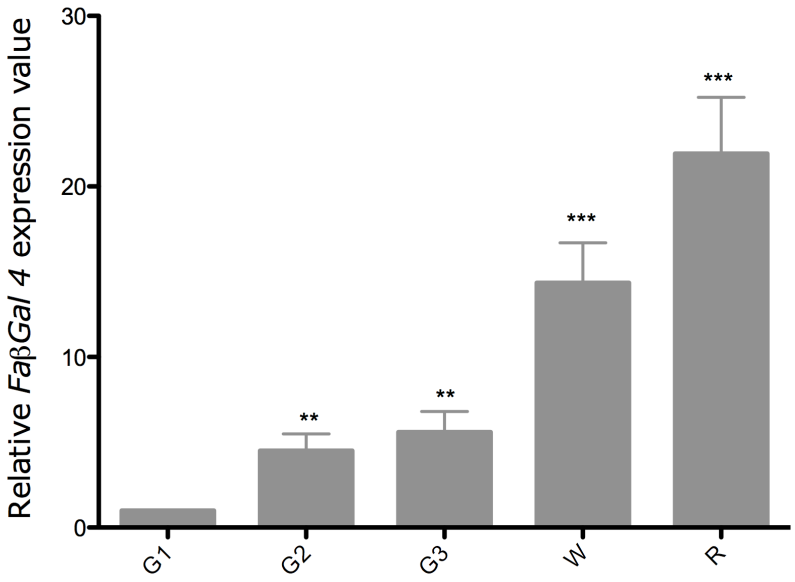

Figure 1. Developmental expression of the strawberry *FaβGal4* gene in fruit receptacle. The increase in the mRNA value was relative to the G1-Ct expression which was assigned an arbitrary value equal to unity. Mean values ± SD of five independent experiments are shown. G1: small-sized green fruit; G2: medium-sized green fruits; G3: full-sized green fruit (G1, G2, and G3: stages of development); W: white stage; R: red stage. Statistical significance with respect to the reference sample (G1 fruits) was determined by the Student´s t-test. (**) p-value < 0.01 and (***) p-value < 0.001.

FaβGal4 was expressed in achenes in all fruit ripening stages. Besides, *FaβGal4* was barely expressed in vegetative tissues (Fig.2) when compared with the relative abundance of *FaβGal4* transcripts in the ripe fruit receptacle. The increased level of expression observed in red fruit suggested a role in ripening for *FaβGal4*.

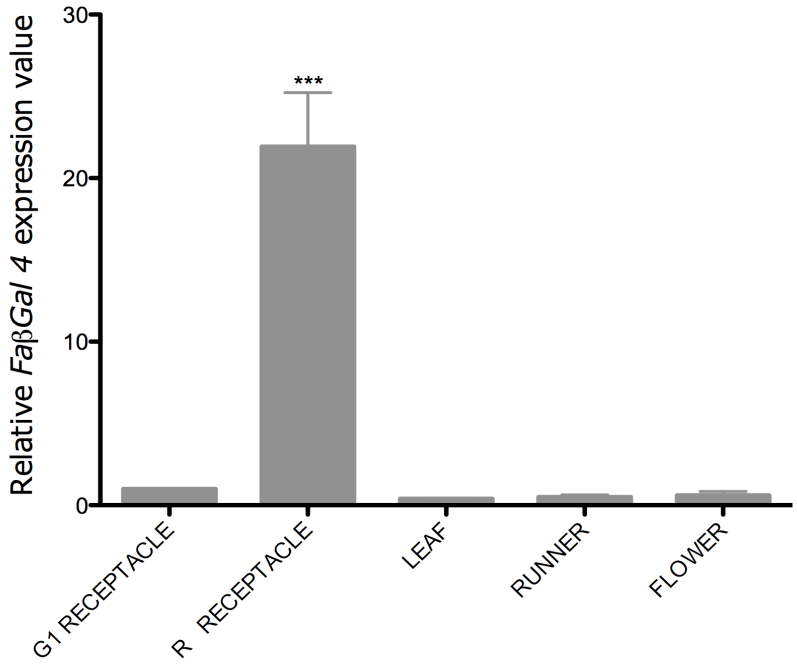

Figure 2. Analysis by qRT-PCR of FaβGal4 gene expression in different vegetative tissues against strawberry receptacles of mature (R: red) and immature (G1: green 1) fruits. The increase in the mRNA values was relative to G1 receptacle, which had the lowest FaβGal4 expression and was assigned an arbitrary value equal to unity. Statistical significance with respect to the reference sample (G1 receptacle) was determined by the Student´s t-test. (***) p-value < 0.001.

4.2 Hormonal regulation of the *FaβGal4 g*ene

It has been proposed that the ABA/auxin content ratio in strawberry fruit receptacles constitutes a signal that triggers the fruit ripening process (Perkins-Veazie, 1995). Thus, we decided to investigate whether *FaβGal4* gene expression was regulated by these two hormones. Since it is known that auxins present in strawberry receptacles are synthesized by the achenes, we performed a comparative gene expression analysis between

control and de-achened green fruits at the G2-stage that were externally treated with or without NAA. A clear increase of *FaβGal4* transcripts was detected in de-achened fruits (Fig. 3). This increase did not occur, however, when these de-achened fruits were treated with a lanonin paste containing NAA. These results clearly indicate that the expression of this gene was negatively regulated by auxins.

In addition, when we interfere negatively the receptacle ABA content by adding NDGA, an important reduction in the amount of *FaβGal4* and other cell wall hydrolases transcripts were observed when compared against control fruits. This indicates that *FaβGal4* expression could be activated by ABA.

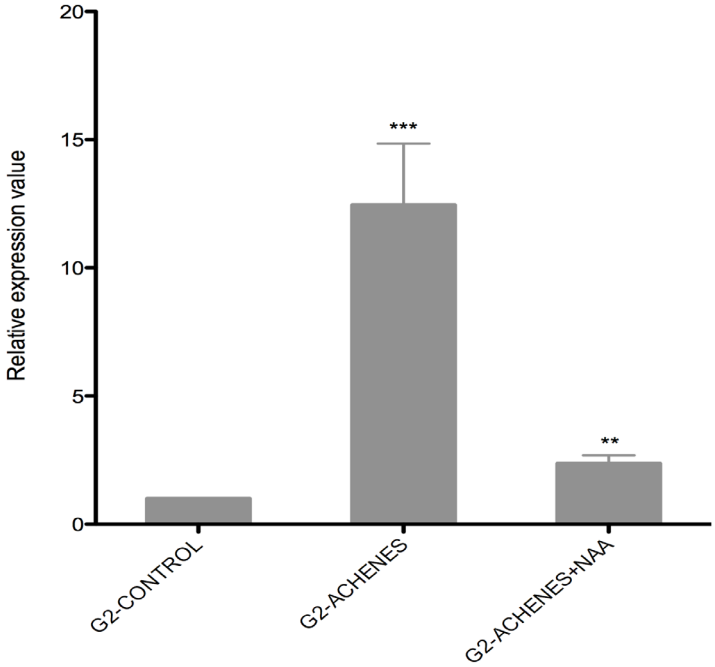

Figure 3. Analysis of the effects of removing achenes from G2 developing fruits on FaβGal4, gene expression by QRT-PCR. After auxin treatment, the increase in mRNA value was relative to G2 fruit (control), which was assigned an arbitrary value equal to unity. G2 Control: full-sized green fruit receptacle (G2 fruit); G2 - Achenes: G2 fruit receptacle without achenes for 5 days; G2 - Achenes + NAA: G2 fruit receptacle without achenes plus NAA for 5 days (added at day zero). Statistical significance with respect to the control sample (G2 fruits) was determined by the Student´s t-test. (***) p-value < 0.001 and (**) p-value < 0.01.

4.3 Obtaining *FaβGal4* antisense transgenic plants

Three independent *Agrobacterium*-mediated transformation experiments were performed to obtain transgenic strawberry plants, cv. `Chandler´, carrying the antisense sequence of the *FaβGal4* gene. After 20 weeks of selection in 25 mg L^{-1} kanamycin, 10 independent kanamycin-resistant shoots were recovered, yielding an average transformation rate of 2%. These shoots were micropropagated, acclimated to ex vitro conditions and transferred to the greenhouse. Transgenic mother plants were propagated by runners and daughter plants used for phenotypic analyses. The presence of the transgenes in these plants was confirmed by PCR amplification of a 220-bp fragment belonging to the *nptII* gene (results not shown).

4.4 Phenotypic analysis of antisense *FaβGal4* plants

Transgenic *FaβGal4* plants showed a vegetative growth pattern similar to that of the control plants (Fig. 4). However, fruit yield was reduced a 60-80% in most of the lines when compared with the control line, during the first year of analysis. Furthermore, the proportion of deform and small fruit in these lines was higher than in control (Fig.4 E-F). To estimate ripe fruit quality, the weight, length, width, color, soluble solids, and firmness were recorded in control and transgenic fresh fruits. Results obtained during the first year of analysis are summarized in Table 1. Mean fruit weight and size were significantly lower in most transgenic lines when compared with control fruits, with the exception of lines β-Gal21 and β-Gal27. Interestingly, fruit color, estimated by the L*, a* and b* color space parameters, was significantly different in most transgenic lines. Transgenic fruits showed an increase on L*, lightness, and b*, yellowness, while a*, redness, was significantly increased only in lines β-Gal28 and β-Gal37. Soluble solids increased significantly in three of the transgenic lines. Regarding fruit firmness, lines β-Gal28 and β-Gal37 yielded fruits significantly firmer than control, being the increase on firmness of 47% and 31%, respectively. Based on this observation, these two lines were selected for further studies.

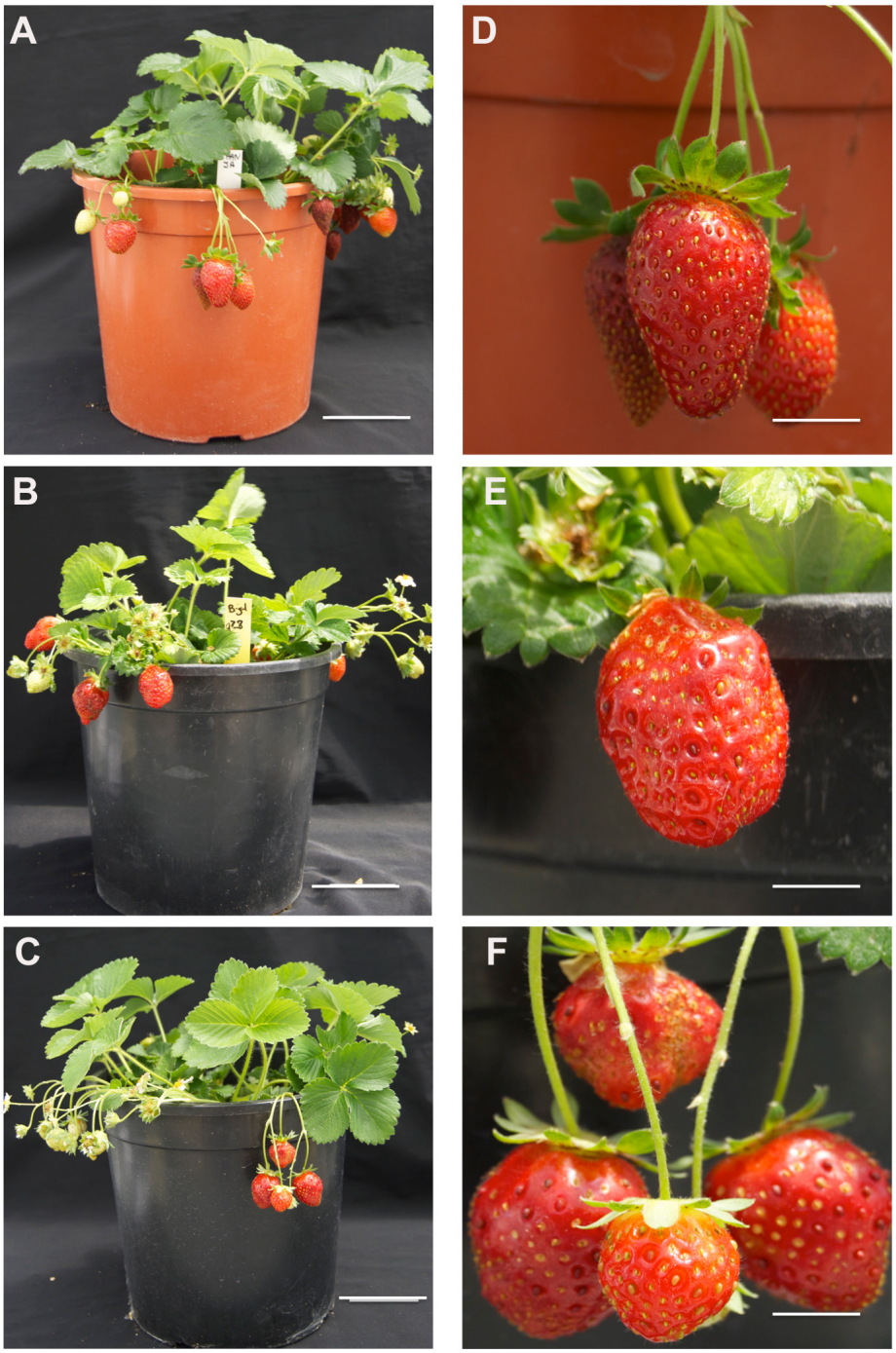

Figure 4. Strawberry non- transgenic control line cv` Chandler´ (A), plants from transgenic line βGal 28 (B), plant from transgenic line βGal 37 (C). Strawberry fruit from control (D), βGal28 (E) and βGal37 (F) lines.

Table 1. Characteristic of ripe fruit in control and transgenic β-Gal Plants. Data represent mean±SD of a minimum of 10 fruit per line, evaluated during the first year of analysis. Lines that are significantly different from the control by mann-whitney U test (weight, L*, b*, soluble solids and firmness) or Dunnet test(lenght, width and a*), both at P=0.05, are indicated with asterisks.

Genotype	Weight (g)	Length (mm)	Width (mm)	Color			Soluble solid (°Brix)	Firmness (N)
				L*	a*	b*		
Control	11.2±2.2	35.3±4.8	26.2±3.1	36.5±1.9	38.0±3.4	20.4±3.0	8.3±1.7	3.2±0.6
β-GAL15	9.5±1.8*	33.0±3.0	23.8±2.2*	38.7±3.3*	37.3±4.4	21.9±5.2	8.8±1.7	3.4±0.5
β-GAL18	7.8±1.6*	28.4±3.1*	24.4±2.2*	38.8±3.6*	39.5±5.1	21.3±3.3*	9.0±1.7	3.0±0.6
β-GAL19	7.8±1.8*	28.0±4.4*	24.0±2.6*	39.3±3.5*	37.8±3.6	22.3±3.5*	9.0±1.8	3.1±0.7
β-GAL21	10.2±3.1	32.2±5.4*	25.0±2.9	38.6±2.5*	37.9±3.3	23.1±3.9*	8.9±2.2	3.2±0.7
β-GAL24	7.3±2.1*	30.3±5.6*	22.9±3.4*	39.9±6.3*	39.8±5.5	26.0±5.5*	9.6±2.9	3.5±0.6
β-GAL25	5.2±2.0*	24.4±4.3*	22.6±3.1*	44.9±5.8*	40.7±4.7	28.1±4.2*	13.8±2.6*	3.5±0.9
β-GAL27	11.9±2.5	36.2±4.7	26.9±2.9	33.8±2.7*	36.9±3.2	18.1±3.5*	7.5±2.0*	3.4±0.6
β-GAL28	9.7±3.0*	28.8±4.1*	27.0±3.8	40.0±4.1*	43.2±3.8*	26.1±4.3*	10.0±2.7*	4.7±0.8*
β-GAL37	5.9±1.4*	22.7±3.8*	23.4±1.9*	40.5±3.2*	40.9±2.8*	25.8±3.2*	10.8±2.5*	4.2±0.9*

Fruit quality parameters in selected transgenic lines were evaluated during two additional growing seasons, using each year daughter plants derived from vegetative propagation by runners. Results obtained in the two growing seasons were similar to those observed during the first year of analysis (Table 2). Transgenic fruits were smaller than control and showed higher soluble solid contents, especially those from line β-Gal37. Color was also slightly modified in both transgenic lines. As regard to fruit firmness, both transgenic lines displayed a significantly higher firmness than control in the two years of analysis (Table 2), being the average increment on firmness of 28%. Fruit yield, estimated as g of fruit per plant, was significantly reduced in both transgenic lines. As previously mentioned, this decrement was due both to a reduction in fruit weight and to a decreased number of fruits per plant.

Table 2. Characteristics of ripen fruits in control and selected transgenic β-Gal plants. Data represent means±SD of a minimum of 50 fruits per line, evaluated during the second and third years of analysis. Different letters indicate statistically significant differences by Tamhane T2 test at *P*=0.05.

	Genotype	Weight (g)	Length (mm)	Width (mm)	Color			Soluble solids	Firmness (N)
					L*	a*	b*	(°Brix)	
Second year	Control	12.2±3.8a	36.2±4.8a	24.5±3.8a	36.1±3.5b	38.4±3.7b	21.0±4.2a	6.9±1.5c	2.6±1.0b
	β-Gal28	8.2±2.6b	29.6±3.7b	25.8±2.8b	36.8±4.2b	40.2±4.7a	22.5±5.4b	7.9±1.3b	3.4±0.6a
	β-Gal37	6.3±1.5c	24.0±2.5c	23.8±2.2c	38.8±4.1a	38.7±4.4b	23.5±4.8b	8.9±1.7a	3.5±0.5a
Third year	Control	14.3±5.3a	39.5±6.2a	29.3±4.6a	35.5±2.2b	36.6±3.7a	19.5±3.5b	6.4±1.4c	3.6±0.8b
	β-Gal28	9.9±3.4b	34.7±5.8b	25.7±4.1b	38.9±4.2a	42.9±4.1b	25.42±5.1a	8.1±1.5b	4.6±1.0a
	β-Gal37	7.3±2.3c	26.0±3.9c	24.9±3.2b	40.1±4.5a	38.6±4.9c	24.3±3.8a	9.2±1.9a	4.4±0.8a

4.5 *FaβGal4* gene expression and β-Galactosidase activity in transgenic fruits

The expression levels of *FaβGal4*, as well as the other three β-galactosidase genes described in strawberry fruit (*FaβGal1* to *3*), were analysed in red fruits from control and selected β-Gal28 and β-Gal37 transgenic lines by quantitative real-time PCR (QRT-PCR) (Fig. 5). A significant down-regulation of the *FaβGal4* gene was observed in ripe fruits from the two transgenic lines, being the percentage of silencing similar in both case and close to 70%. Despite the low similarity among *FaβGal4* sequence and those of the remaining β-galactosidase genes analysed, a similar level of silencing was observed for *FaβGal1* mRNA in both transgenic lines (Fig. 5). Furthermore, β-Gal28 transgenic fruits showed an additional silencing of *FaβGal3* gene. By contrast, mRNA levels of *FaβGal2* gene were not modified in transgenic lines.

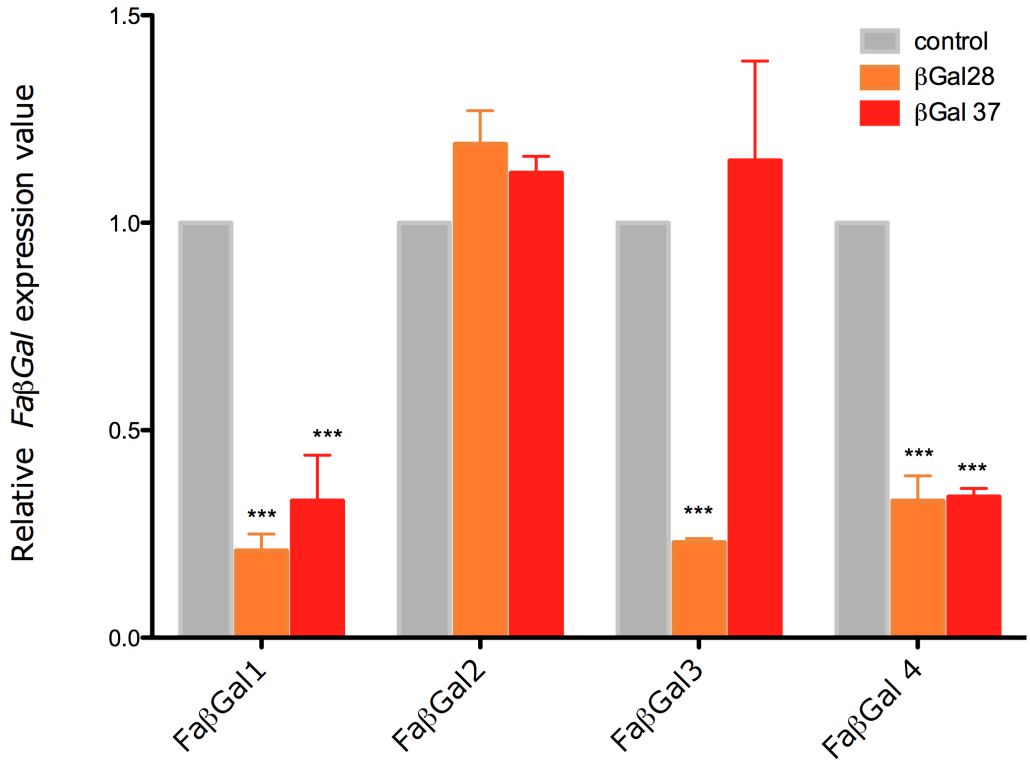

Figure 5. Relative gene expression of *FaβGal* 1, 2, 3 and 4, estimated by QRT-PCR, in ripe fruits from control and transgenic βGal plants. Bars represent mean±SD of three independent RNA quantifications. Statistical significance with respect to the control line was determined by the Student´s test (***) p-value < 0.001 and (**) p-value < 0.01.

β-Galactosidase activity was measured in protein extracts from ripe fruits of the two anti-*FaβGal4* selected lines following the protocol described by Trainotti et al. (2001). The enzymatic assay used nitrophenyl-β-D-galactopyranoside (NPG) as substrate. Total activity was similar in control and transgenic fruits, 78 pmol NPG.min^{-1}.μg of protein^{-1} in control fruits vs. 81 and 90 pmol NPG.min^{-1}.μg of protein^{-1} in fruits from β-Gal28 and β-Gal37, respectively.

4.6 Cell Wall Analysis

The yield of cell wall material (CWM) and soluble PAW fraction relative to 100 g of fruit fresh weight of fruit was similar in control and transgenic lines, with average values of 0.93 and 0.9 g, respectively. The cell wall material was sequentially fractionated with water, CDTA, sodium carbonate, and KOH (1 M and 4 M) to extract fractions enriched in water soluble pectins, ionically bound pectins, covalently bound pectins and hemicellulosic polymers, as described by Santiago-Doménech et al. (2008). The yields of the different cell wall fractions obtained were similar to those described by Santiago-Doménech et al. (2008) and Posé et al. (2013) in fruits of the same cv. There were not significant fraction yield differences among control and the two transgenic lines (data not shown).

Uronic acid (UA) content was measured in all fractions, and the results obtained are shown in Fig.6. In both transgenic lines, sodium carbonate fractions were enriched in UA when compared with control fraction. A slight but significant increase on UA was also observed in the transgenic water soluble fractions. The amount of UA was also higher in CDTA fraction from β-Gal37 line, but not in β-Gal28 line. Neutral sugars analysis by gas chromatography of the CWM was performed in control and β-Gal37. Transgenic CWM showed a 31% increased content of galactose and arabinose (Table 3). The values of rhamnose and fucose contents were also higher in transgenic fruits, although the differences were not statistically significant (Table 3).

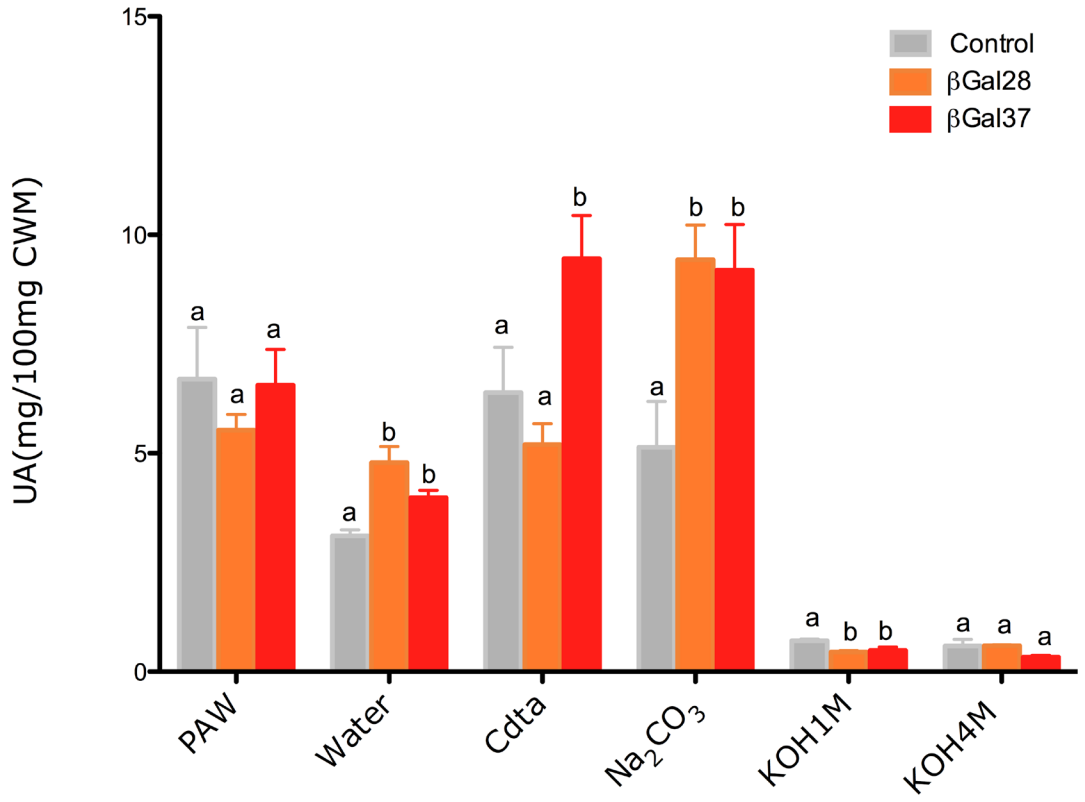

Figure 6. Uronic acid content, expressed as mg of UA per 100 mg of CWM, in PAW and the different fractions isolated from cell walls from control and transgenic β-Gal ripe fruit. Bars represent means±SD of five independent measurements.

Table 3. Neutral sugars content in cell wall material from ripe control and transgenic β-Gal28 fruits. Mean separation by Student´s t-test at P=0.05.

	Amount (mg.g CWM^{-1})						
	Rha	**Fuc**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**
Control	6.9a	5.7a	32.5**b**	48.4a	30.0a	78.5**b**	307.5a
β-Gal37	10.6a	8.2a	44.1**a**	45.5a	26.4a	103.4**a**	281.1a

In general, the trend described for the neutral sugar contents of the CWM was also observed in most cell wall fractions with some exceptions (Table 4). Control PAW fraction, which contains apoplastic free polymers solubilized by in vivo processes (Redgwell et al., 1997b), displayed the highest proportion of Rha, but this carbohydrate was not detected in transgenic PAW fraction. Additionally, this fraction, as well as KOH 4M,

showed a slight increase on glucose when compared with control. Polymers soluble in CDTA from transgenic fruit showed a slight decrease on Fucose and Arabinose. Similarly, sodium carbonate soluble pectins displayed a large decrease on Fuc.

Table 4. Neutral sugars composition of the different cell wall fractions extracted from control and transgenic β-Gal28 ripe fruits. The results are presented in mol % within each fraction to facilitate comparison.

	mol%	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
PAW	Control	9.4	1.8	16.3	18.5	7.1	33.2	13.8
	β-Gal	0.0	2.3	21.6	20.0	5.6	34.9	15.6
H_20	Control	2.2	1.4	27.5	21.3	14.2	20.1	13.3
	β-Gal	6.0	1.8	25.2	13.8	9.1	37.0	7.2
CDTA	Control	4.8	2.0	32.2	9.9	9.5	35.5	6.2
	β-Gal	8.1	1.7	27.6	4.0	8.7	46.3	3.6
Na_2CO_3	Control	4.5	2.0	25.4	4.7	5.5	55.1	2.7
	β-Gal	6.3	0.9	27.5	1.9	2.0	59.4	2.1
KOH 1M	Control	1.6	2.1	15.2	47.6	7.7	12.9	13.0
	β-Gal	3.3	2.1	19.4	23.2	2.8	37.2	12.1
KOH 4M	Control	1.1	4.0	12.1	32.4	6.3	18.5	25.7
	β-Gal	1.8	4.5	10.2	28.1	3.9	21.3	30.3
CWM-Residue	Control	0.7	0.2	1.8	3.3	5.6	3.0	85.5
	β-Gal	0.6	0.2	2.4	3.3	3.5	2.2	87.9

Regarding Galactose (Gal) contents, all cell wall fractions from transgenic fruits contained a higher proportion of this carbohydrate, being the highest increments observed in water and, especially, KOH 1M fractions (Fig. 7).

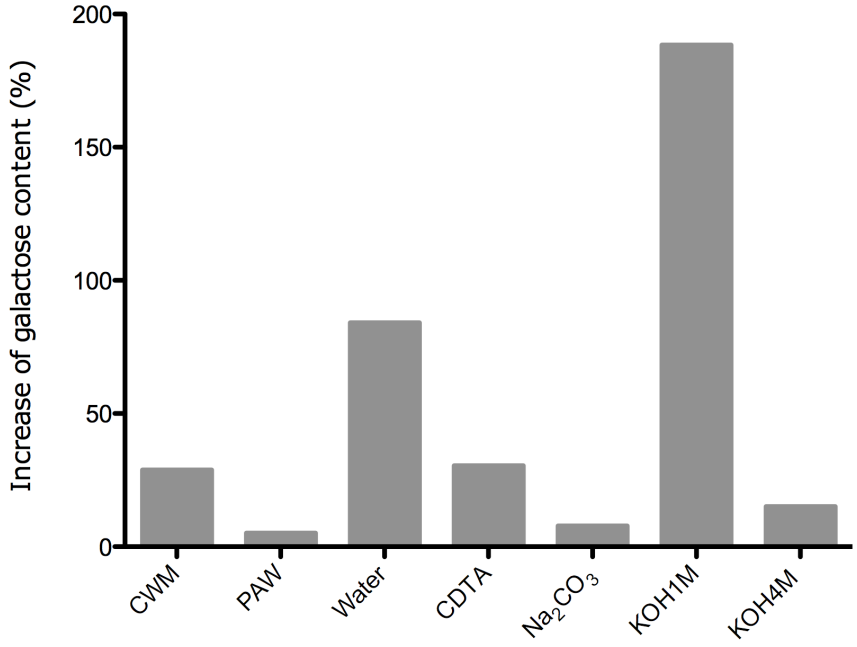

Figure 7. Increase on Gal content, expressed as percentage of control fruits, in CWM, PAW and the different cell wall fractions isolated from transgenic β-Gal37 ripe fruits.

Interestingly, the Gal increase in this last fraction was concomitant with large decrements on Xylose and Mannose and a high increment in Rhamnose (Table 4). The modification of the carbohydrate composition in KOH 1M fraction as result of *FaβGal4* silencing was confirmed by FTIR spectroscopy (Fig. 8). Control fruits showed a spectral profile in the mid-infrared region at 1200-800 cm^{-1}, corresponding to the fingerprint region of carbohydrates, with peaks at 1014, 1026, 1045, 1077 and 1095 cm^{-1}. According to Kacuráková et al. (2000) HGA pectin profiles have maximum absorption bands at 1100 and 1017 cm^{-1}, RGI shows the strongest peak at about 1070 and 1043 cm^{-1}, and the main xyloglucan absorption band is at 1041 cm^{-1}. The KOH1M profile from control fruits could be therefore assigned to a mixture of RG-I and xyloglucans. The KOH1M profile from transgenic fruits was quite different, showing a main peak at 1038 cm^{-1} and a shoulder at 1070 cm^{-1}. Accordingly to Kacuráková et al. (2000), these bands could be assigned to Ara and Gal, respectively, although the absorption IR bands of other neutral sugars overlap in this region.

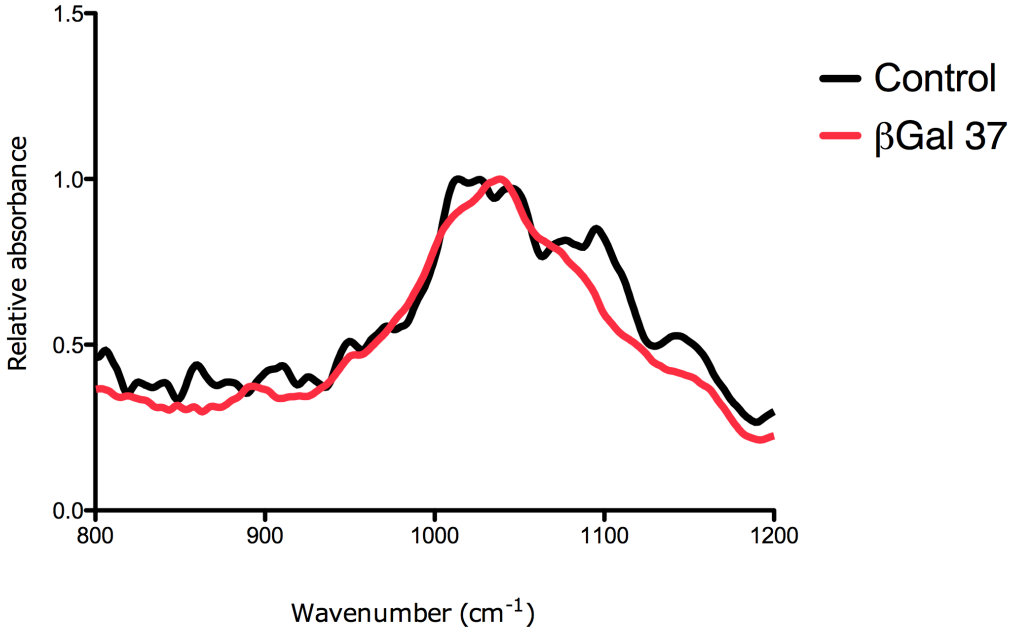

Figure 8. ATR-FTIR absorbance spectra of KOH 1M fraction from control and transgenic cell walls in the 1200-800 cm^{-1} region.

4.7 AFM analysis

Figure 9 shows representative topographical AFM images from CDTA fraction of the control (A) and βGal37 line (B), as well as zoomed areas to show up some characteristic of these samples (C to F). In both cases, linear filamentous structures corresponding to pectic chains were present with some proportion of them including side chains which correspond to chain branches (Fig. 9,C, D and F). This branching was more abundant in βGal samples (E and F) than in control images (C). The transgenic samples showed longer contour length than control samples.

Aggregates, distinguishable by height measurements, were also present in samples of both genotypes. These aggregates were more frequent and complex in transgenic samples (Fig.9.B,C, F, G and H) than in control ones (Fig.9.A, D). The higher complexity of the transgenic aggregates is better visualised when the three-dimensional images of aggregates from control and transgenic samples are compared (Fig 9, G-H).

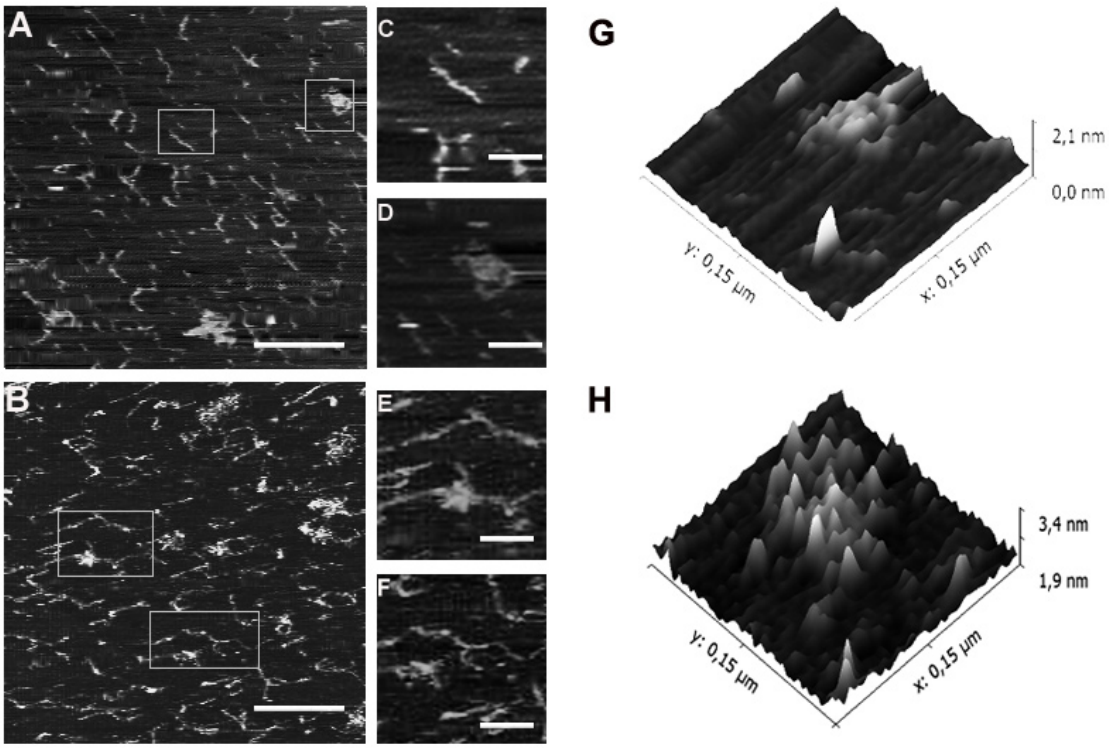

Figure 9. AFM topographical images representative of strawberry CDTAfraction of the CWM in ripe fruit of control (A) and βGal 37 plant(B) visualized by direct contact mode under butanol. Inlets (C-F) show twofold zoomed details from the A and B images. Zoom images are showing examples of linear chains (C), multibranched chains (E and F) and multichain aggregates (D). G and H correspond to 3D representation of pectin aggregate. Scale bars: for A-B are 250 nm; for C-F are 75 nm.

Figure 10 displays AFM images corresponding to sodium carbonate extracted from control(A) and βGal 37 line (B). Representative areas of these fraction were zoomed (C-F). Apparently, longer pectin chains were visualized in the case of the transgenic samples. Zoom of different aggregated structures are shown in Figure 10 C and F, as well as their three-dimensional aspect for βGal sample (G and H). In this fraction ,aggregates structures were similar in control and the transgenic lines but they were more frequent in the transgenic line images.

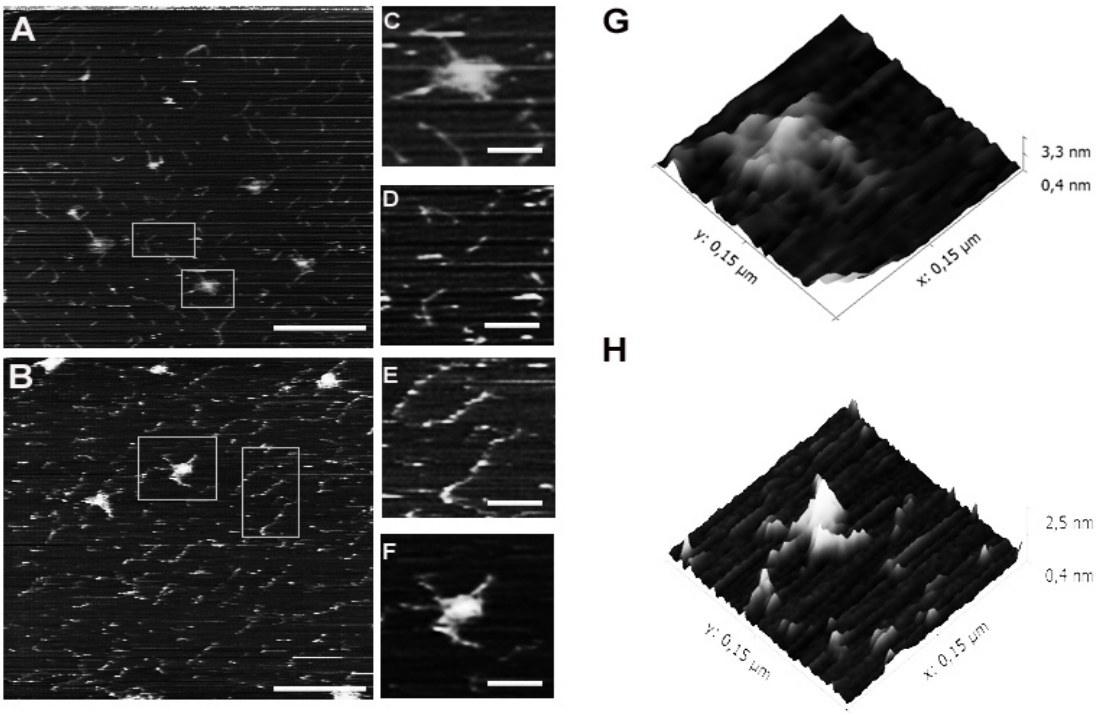

Figure 10. AFM topographical images representative of strawberry Na_2CO_3·fraction of the CWM in ripe fruit of control (A) and βGal37 plants (B) visualized by direct contact mode under butanol. Inlets (D-I) show zoomed from the A and B images. Zoom images showing examples of linear pectins and aggregate. G and H correspond to 3D representation of pectin aggregate Scale bars: for A-C are 250 nm; for D-I are 75 nm.

A quantitative study of these images was performed and the main results displayed in Figure 11 and Table 5 and 6. The contour length of isolated pectic chains was evaluated in the samples, as well as the number of branches per chain for both lines and fractions. The histograms obtained are shown in Figure 11. All the distribution obtained were right-skewed and well approximated by a Log normal, as represented in Figure11 (Posé et al., 2012a). In control samples, the contour length distribution for CDTA fraction (Fig. 11.A) was in the range of 18 to 277 nm and for βGal37 line it ranged from 21 to 437 nm. In the case of the sodium carbonate fraction(Fig. 11.B), contour length distributions ranged between 10 to 255 nm for control samples and from 15 to 393 nm for the transgenic line. In both fractions, the transgenic samples displayed wider contour length distributions reaching some chains higher length than control samples. The narrower length distribution of the control lines showed higher frequency of isolated molecules with smaller contour lengths.

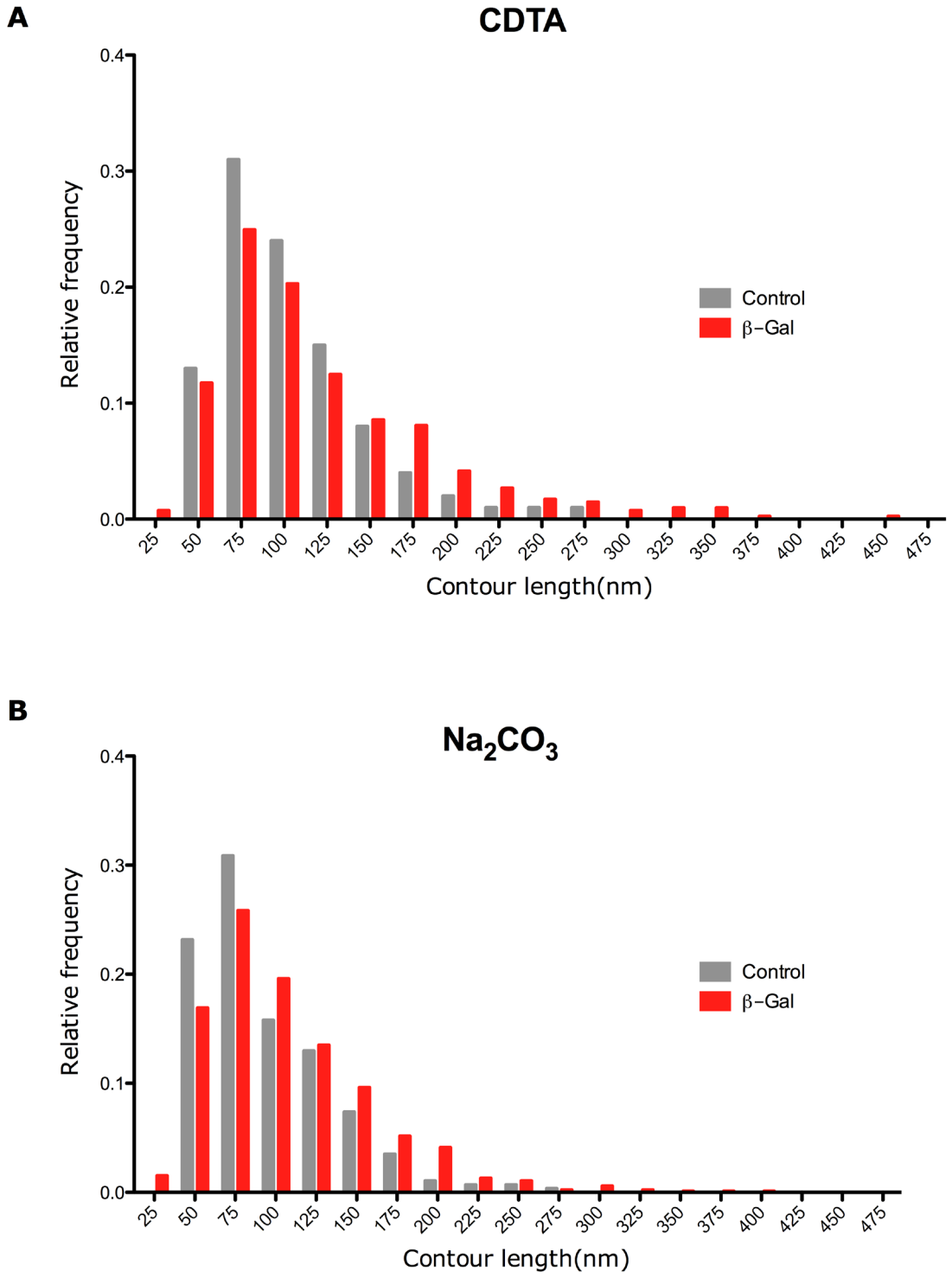

Figure 11. Contour length distribution of CDTA (A) and NA_2 CO_3 (B) soluble polymers from control (dark bars) and βGal37 line (red bars).

Table 5 shows the number-average ($L_{N)}$), the weight-average (L_W) and the polydispersity indexes (PDI) of the polymers present in both samples for both genotypes. The medians (ME) of these distributions were also obtained and included for statistical comparison using Kruskal–Wallis non parametric test (P = 0.05).

In CDTA samples, the three mentioned parameter (L_N, L_W and PDI) were higher in the case of the transgenic samples, confirming our qualitative observations. The medians of the contour length distributions were 92,8 nm in transgenic and 74,5 nm in control line, being this difference statistically significant. A similar trend was observed for the Na_2CO_3 fractions, except that the PDI was similar in control and transgenic samples. L_N, L_W and median values of this fraction were lower than those obtained in the CDTA fractions.

Table 5. Descriptors of contour length distribution of CDTA and Na_2CO_3 pectin extracted fraction from ripe strawberry fruits of control and βGal37 lines. ME corresponds to the median value of the contour length distributions. Values followed by different letters within the same row are significantly different at P = 0.05

		Control	β-Gal37
CDTA	**L_N(nm)**	90.9	108.6
	L_w(nm)	113.6	148.6
	PDI	1.2	1.4
	ME(nm)	75.5**b**	92.8**a**
Na_2CO_3	**L_N(nm)**	78.7	90.2
	L_w(nm)	102	115.6
	PDI	1.3	1.3
	ME(nm)	69.2**b**	79.7**a**

Furthermore, the branching pattern and the side chain characteristics were also analysed for both lines and fractions (Table 6). Transgenic samples displayed a significantly higher percentage of branched chains, 15 % in the CDTA fraction and 9% in the sodium carbonate fraction. The percentage of multi-branched polymers was also higher for transgenic samples, mainly

in CDTA fractions. In general, as previously reported (Posé et al., 2012), CDTA branches were longer than Na_2CO_3 branches. The transgenic samples displayed longer branches than control in both fraction, being their L_N and L_W values higher than those estimated in control samples.

Table 6. Branching characteristics of CDTA and Na_2CO_3 pectins extracts from ripe strawberry fruits in βGal37 and control lines as obtained from AFM images.

		Control	β-Gal37
CDTA	**Branching (%)**	3.2**b**	15**a**
	Multibranching(%)	0.6	3.7
	LN(nm)	41.1	47
	Lw(nm)	47.3	56.4
	PDI	1.2	1.2
Na_2CO_3	**Branching (%)**	3.4**a**	9.3**b**
	Multibranching(%)	0.35	0.7
	LN(nm)	28.6	36.6
	Lw(nm)	39.8	49.9
	PDI	1.4	1.4

5.DISCUSSION

A common feature of the cell wall disassembly process taking place during ripening of fleshy fruit is the loss of galactose from pectin side chains, supposedly due to the action of β-galactosidase enzymes (Smith and Gross, 2000; Brummell, 2006). The release of galactose has also been identified in other developmental processes involving the modification of cell wall structure, such as cell expansion or senescence (Buckeridge and Reid, 1994; Peña et al., 2004; O´Donghue et al., 2009). In strawberry, Trainotti et al. (2001) identified three β-Gal genes. All of them were expressed in young expanding leaves, stolons, flowers and green fruit, but only *FaβGal1* showed an increased expression during fruit ripening. Our comparative transcriptomic analysis between strawberry receptacles from immature

(green) *vs*. ripen (red) strawberry fruits, showed a sequence encoding an additional putative β-Gal gene, *FaβGal4,* whose expression was strongly up-regulated in red stage, suggesting that this gene could play a pivotal role in strawberry fruit cell wall disassembly and fruit softening.

5.1 *FaβGal4* is mainly expressed in ripe fruit receptacle and it is regulated by auxins and ABA

The expression pattern of *FaβGal4* gene, along receptacle fruit growth and maturation, was clearly ripening-related reaching a maximum of gene expression in red ripe fruits. This expression pattern was similar to those previously reported for other strawberry pectinases i.e. pectate lyases, polygalaturonases or rhamnogalaturonate lyase (Medina-Escobar et al., 1997; Redondo-Nevado et al., 2001; Benitez-Burraco et al., 2003; Quesada et al., 2009; Molina-Hidalgo et al., 2013).

During development of strawberry fruit, auxin levels, produced by achenes and released into the receptacle, decline and this invokes the ripening process (Perkins-Veazie, 1995). In accordance, several studies have shown that the expression of many strawberry fruit ripening-related genes encoding cell wall hydrolases was induced by removing the achenes from the fruit surface (Medina-Escobar et al., 1997; Redondo-Nevado et al., 2001; Benitez-Burraco et al., 2003). This was also the case for FaβGal4 gene which expression was also decreased as result of fruit treatments with synthetic auxins. More recently, Chai et al. (2011) and Jia et al. (2011) have also provided molecular evidences that indicate that ABA is a signal molecule that, at least, can promote the strawberry ripening related production of anthocyanins. In fact, ABA levels gradually increase concomitant with the ripening process in strawberry fruits (Chai et al., 2011; Jia et al., 2011). When strawberry fruits at the green stage were treated with NDGA, an inhibitor that blocks the synthesis of ABA, FaβGal4 was significantly down-regulated, as has been observed for other cell wall

hydrolases (Molina-Hidalgo et al., 2013). All together, these results indicate a putative co-regulation of FaβGal4 with genes encoding other pectin degrading hydrolases and supports a physiological role related with the enzymatic degradation of the cell wall in ripe fruits leading to fruit softening.

5.2 Antisense down-regulation of *FaβGal4* reduces strawberry fruit softening

Transgenic strawberry plants carrying a 300 bp antisense sequence of *FaβGal4* under the control of the constitutive promoter CaMV35S were obtained to get insight into the role of this gene on fruit softening and cell wall disassembly. In general, fruit yield and fruit weight were reduced in most transgenic lines, as have been observed in other transgenic lines with down-regulated pectinases, i.e. pectate lyase transgenic plants (Jiménez-Bermúdez et al., 2002; Youssef et al., 2009). Average fruit size and weight generally decreased when micropropagated strawberry plants are used directly for fruit production, but this side effect of the in vitro tissue culture phase disappears when the progeny of micropropagated plants is evaluated (Cameron et al., 1989; López-Aranda et al., 1994). In *FaβGal4* transgenic plants, the reduction on fruit weight was stably maintained after three rounds of runner propagation in the greenhouse suggesting that this effect was due to *FaβGal4* silencing rather than the in vitro micropropagation of plants. In strawberry, fruit size depends mainly on the number of achenes per fruit, the flower position and the receptacle sensitivity to auxin (Perkins- Veazie, 1995). In the case of the two transgenic lines selected, fruits showed a marked reduction on the average number of achenes per fruit, with mean values of 240.1, 105.59 and 69.5, in control, β-Gal28 and β-Gal37, respectively. However, the mean number of achenes per g of fruit was only statistically different to the control in the case of β-Gal28 fruits. These results suggest that the decrease on fruit size could be due to a deficient fertilization in transgenic plants as result of *FaβGal4* silencing. Early and late male gametophyte development genes with high homology

to β-Gal have been identified in pollen from tobacco, *Arabidopsis* and rice (Tursun et al., 2003; Hrubá et al., 2005). The products of these genes may participate in cell wall loosening during young pollen expansion after microspore mitosis and/or may be associated with pollen germination and pollen tube growth through the style (Hrubá et al., 2005).

Two out of the ten transgenic lines obtained produced fruits significantly firmer than control at the ripe stage. The firmer fruit phenotype was stably maintained during three growing seasons. Transgenic fruits of selected lines were in average a 30% firmer than control, an increment on firmness similar to the one obtained when down-regulating a pectate lyase (Jiménez-Bermúdez et al., 2002) or a polygalacturonase gene (Quesada et al., 2009). This increment of fruit firmness cannot be ascribed to the reduction in fresh weight, since no correlation between fruit weight and firmness was observed among the different transgenic lines obtained. Transcriptomic analysis of transgenic fruits from the selected lines showed a significant reduction, 70%, of *FaβGal4* mRNA levels when compared with control fruits. Although *FaβGal4* gene did not share significant sequence homology with the other three β-Gal genes previously described in strawberry, the constitutive expression of antisense *FaβGal4* sequence also reduced *FaβGal1* mRNA amounts on the two selected lines, and, additionally, *FaβGal3* on fruits from β-Gal28. By contrast, *FaβGal2* expression was not modified in transgenic fruits. These results indicate an intricate regulation of β-Gal genes during ripening of strawberry fruits. Additionally, despite the high reduction on β-Gal genes expression, β-galactosidase activity was not reduced in transgenic ripe fruits. Previous studies in transgenic tomato showed a complex relationship between the expression of the different β-Gal genes involved in fruit ripening and β-Gal activity. Antisense TBG4 tomato plants showed a 40% reduction in fruit softening although TBG4 silencing was only observed at the mature green stage (Smith et al., 2002). The expression of TBG3 gene was also altered in those antisense tomato fruit, down-regulating or up-regulating its expression depending on the ripening stage. Moctezuma

et al. (2003) found an altered expression of *TBG4* and *TBG5* in tomato fruits with *TBG6* down-regulated by antisense transformation. Contrary to the report of Smith et al. (2002), the suppression of *TBG6* was observed during the whole ripening process. Although it had no effect on softening, an increased fruit cracking and cuticle thickness, and a reduced locular space were observed in transgenic *TBG6* fruit (Moctezuma et al., 2003). As regard of β-galactosidase activity, neither Smith et al. (2002) nor Carey et al. (2001) found a decrease on total β-galactosidase activity in transgenic antisense *TBG4* or *TBG1* fruits, respectively. However, the silencing of *TBG4* reduced exo-galactanase activity, measured against a lupin galactan, at the breaker plus 3 days developmental stage, returning to wild control levels at breaker plus 7 days stage (Smith et al., 2002). The scenario was more complex when modifying the expression levels of *TBG6* (Moctezuma et al., 2003). In these antisense fruits, β-galactosidase activity was significantly higher than in control at early stages of development, returning to wild type levels at the later stages. However, exo-galactanase activity was reduced at the breaker stage (Moctezuma et al., 2003). These results could be explained by the large number of β-Gal isoforms expressed in fruit with different hydrolysing abilities against native cell wall polysaccharides and synthetic products (Tateishi, 2008)

5.3 Antisense *FaβGal4* down-regulation increases cell wall galactose levels and reduces pectin solubilization

According to Redgwell et al. (1997), Galactose levels decrease an 18% in strawberry ripe fruits when compared with green immature fruits. The silencing of *FaβGal4* increased Gal amount in a 30% in transgenic fruits, but also induces a significant increase in the Arabinose content. After cell wall fractionation, it was observed that all fractions contained higher amounts of galactose than controls, being the increments larger in water, CDTA and especially KOH 1M fractions. KOH-soluble fractions comprises mainly hemicellulosic polymers, but also contains pectins (Koh and Melton, 2002;

Santiago-Doménech et al., 2008; Posé et al., 2013). Interestingly, Redgwell et al. (1997) found that most Gal loss, 82.3% of total cell wall Gal loss, occurred in the KOH fraction and the CWM-residue, while only a 17.7% was lost from the CDTA/Na_2CO_3 polyuronide enriched fractions. This trend was observed in most fruit analysed, including species where an increase on β-galactosidase activity during fruit ripening had previously been reported, e.g. tomato, kiwifruit or avocado (Redgwell et al., 1997). The higher Gal content in KOH 1M fraction from antisense *FaβGal4* fruits could suggest that Gal loss takes place mostly in arabinogalactan pectins loosely bound to xyloglucans by covalent cross-links and/or physically entangled within the glycan matrix. It is noteworthy that this is the first report of a modification of cell wall Gal levels in fruits by suppression of a β-galactosidase gene. Total cell wall Gal content was not modified in antisense *TBG4* tomato fruit, although a decrease on free galactose levels was observed in transgenic fruit at mature green stage, returning the levels to wild type during ripening (Smith et al., 2002).

The relationship among neutral sugars loss, pectin solubilisation and fruit firmness is far from clear. Some fruits such as apple and nashi pear showed a marked loss of Gal but pectin solubilisation was slight (Redgwell et al., 1997). Conversely, plum did not show any Ara/Gal loss but an extensive pectin depolymerization (Redgwell et al., 1997). In *FaβGal4* silenced fruits, the increase on Gal cell wall levels was paralleled to a reduction in polyuronide solubilization, reflected in the higher amount of sodium carbonate soluble pectins. Previous studies in transgenic strawberry plants with pectate lyase or polygalacturonase genes silenced demonstrated that the increase on firmness in these transgenic fruits as result of hydrolases down-regulation was due to a reduced pectin solubilization and depolymerization (Santiago-Doménech et al., 2008; Posé et al., 2013). In both cases, cell walls from transgenic fruits displayed higher amounts of ionically and covalently bound pectins, those soluble in CDTA and sodium carbonate, respectively, than wild type. Whether the reduction in fruit softening in transgenic *FaβGal4*

fruits is directly due to the increment on cell wall Gal content or an indirect effect on pectin solubilisation needs to be elucidated. The reduced loss of Gal from hairy pectins could limit the access of other cell wall modifying proteins, such as polygalacturonase or pectate lyase, to their substrate therefore limiting pectin solubilization, and likely, depolymerization. AFM images revealed longer contour length distribution of the individual molecules, higher level of branching and branch length as well as higher number of supramolecular structures in βGal line than control line. These results strongly support a higher depolymerisation in control than transgenic lines of the polysaccharides present in both fractions. Taking into account the substrate of βGal enzyme and the composition of large side chains and backbone pectin, these results would also support a protective role *in muro* for the bound Gal residues. There presence would limit access to specific target of the cell wall enzyme which are directly involved in solubilisation/ depolymerisation processes. If β-Gal enzyme activity was necessary for the action of these others cell wall enzymes, then β-Gal should act earlier than them. Ogasawara et al. (2007) reported that the β-Gal activity is expresed early during bell pepper ripening.

As an alternative hypothesis, galactosyl residues could have a direct effect on mechanical properties of cell wall. In pea cotyledons, the increased content of pectin galactan at late developmental stages correlated with an increase on firmness (McCartney et al., 2000). *Arabidopsis* mutant *MUR3* lacks a xyloglucan-specific galactosyl transferase and their cell walls exhibited a reduced xyloglucan galactosidation (Peña et al., 2004). This modification markedly reduced cell wall strength during hypocotyl growth, although this effect could be related to a modulation of xyloglucan endo-transglycosidase/hydrolase activity (Peña et al., 2004). The ectopic expression of a fungal endo-β-galactanase in potato tuber reduced RG-I linear β-1,4-galactan and also modified the physical properties of the tissue, being more brittle when subjected to compression (Ulvskov et al., 2005). A putative role of *FaβGal4* on the release of galactosyl-containing

oligosaccharides that trigger the ripening process cannot be discarded. Gross (1985) found that mature green tomato fruit infiltrated with free Gal produced ethylene and ripened earlier than control fruits. More recently, Lahaye et al. (2013) found an indirect evidence of the role of Gal on fruit ripening, since they observed positive correlations between firmness and cell wall Gal content, galactosylated pectins and galactosylated xyloglucans in QTL tomato lines. This hypothesis could explain the pleiotropic effect of *FaβGal4* silencing on fruit color and also on the expression of the other β-Gal genes.

6. CONCLUSIONS

The results obtained support a key role of *FaβGal4* gene in the softening of strawberry fruit. Transgenic fruits with *FaβGal4* silenced showed a 30% increase on fruit firmness at the ripe stage. At the cell wall level, these transgenic fruits contained more Gal in all cell wall fractions than wild type, and also displayed a lower polyuronide solubilisation. Although the removal of Gal from cell walls is a general feature of the ripening process in many fruits, its relationship with pectin solubilisation and fruit firmness is controversial. Our results shed light on these processes and clearly indicate a close connection between galtose levels and polyuronide solubilisation. Additionally AFM results also support a lower depolymerisation of pectic chain at the molecular level. The reduction of Gal loss from galactosyl-containing side chains in the wall might result in decreasing wall porosity, obstructing access to their substrate to other cell wall hydrolases, leading to a reduction on softening. Alternatively, the increased content of galactosyl residues could have a direct effect on the mechanical properties of the cell wall. This work also indicates that *FaβGal4* is a good candidate for the biotechnological improvement of strawberry texture.

CONCLUSIONS:

1. The amount of pectins in the different cell wall fractions decreased during strawberry fruit development, with the exception of water fraction that showed a slight increment in the amount of polyuronides in fruits at the ripe stage. Similarly, the amount of pectins in PAW fraction notably increased in ripe fruits. CDTA fraction supported the largest decrease on pectin content during fruit development, followed by sodium carbonate fraction. These results clearly indicate a noticeable polyuronide solubilization during strawberry fruit ripening, being CDTA and sodium carbonate fractions the main sources of soluble pectins. Arabinose and Galactose contents disminished very significantly in CDTA and sodium carbonate fractions in red ripe fruits.
2. The nano-structural characterization of pectins by AFM during strawberry fruit ripening revealed a reduction in the length of pectic isolated chains from CDTA fraction samples. This solvent mainly extracts pectins ionically bound to the cell wall which are supposedly located in the middle lamella. However, length of pectic chains extracted with sodium carbonate, polyuronides covalently bound to the primary cell wall, did not diminished. Both kinds of pectins samples showed a reduction in their nanoestructural complexity in red fruits. The percentage of branching and the frequency of pectin aggregates decreased and these modifications were more conspicuous in the case of sodium carbonate soluble pectins.
3. The treatment of sodium carbonate soluble pectins from ripe strawberry fruits with fungal endo-PG decreased the length of pectin chains and the number of branches. This result corroborates the galacturonic acid nature of the isolated pectin strands and their side branches visualized by AFM. This treatment also reduced the number of micellar aggregates per sample, indicating that these structures also contain HG domains.

4. Mild acid hydrolysis of sodium carbonate soluble pectins from ripe strawberry fruits did modify neither the length of pectins nor the branching percentage, but significantly diminished the presence of aggregates. This treatment breaks borate diester bonds between RGII, and therefore, our results indicate that RGII has a structural role in the formation of these complexes.
5. *FaβGal4* gene encodes a putative β-Galactosidase enzyme which is expressed in ripe fruit receptacle. Silencing of *FaβGal4* gene reduces the softening process associated with the strawberry ripening process. Ripe fruits from antisense plants showed a 70% reduction in *FaβGal4* gene expresion levels *and* were 30% firmer than control. Transgenic fruits showed other phenotypic variations such as a reduction in fruit size and an increase on soluble solids content.
6. The analysis of cell wall extracts from antisense *FaβGal4* selected lines showed a slight reduction in pectin solubilisation and more amount of Gal in all cell wall fractions than wild type. At the nanostructural level, CDTA and Na_2CO_3 soluble pectins from antisense *FaβGal4* lines had pectins chains with longer contour lengths than control. In addition, the level of branching and the number of aggregate structures were higher in transgenic lines than in control samples. Overall, the results obtained provide evidences that the reduction of softening compared to control in the transgenic fruits was associated with a diminished pectin solubilisation and depolymerisation, likely due to a more difficult access of other enzymes to their cell wall targets. Then, the maintenance of a higher amount of bound Gal residues in the transgenic line would hamper the activity of other pectinase *in muro.* These results support a key role of the loss of Gal and *FaβGal4* gene in strawberry fruit softening during ripening.

RESUMEN DE LA MEMORIA :

La fresa se caracteriza por sufrir un rápido reblandecimiento durante el periodo de maduración, llegando a adquirir una textura semilíquida al final del desarrollo (Manning, 1993). Esta disminución de firmeza limita el periodo postcosecha considerablemente y provoca pérdidas de entre el 5 y el 25% de la producción. El reblandecimiento del fruto durante la maduración es consecuencia principalmente de cambios en las características y/o composición de las paredes celulares, siendo la pectinas uno de los polímeros de pared que más se modifican (Goulao y Oliveira, 2008; Mercado et al., 2011). El objetivo principal de esta tesis ha sido profundizar en el estudio del papel de las pectinas y las pectinasas en el proceso de maduración de la fresa, con el fin de avanzar en el conocimiento del proceso de reblandecimiento del fruto. Para ello, se han realizado estudios a nivel nanoestructural mediante microscopía de fuerza atómica (AFM) de las fracciones ricas en pectinas procedentes de frutos en diferente estadio de maduración. Además, se han llevado a cabo diferentes experimentos para determinar la estructura de los polímeros y complejos supramoleculares que se observan en las imágenes de AFM, utilizando para ello pectinas de frutos control y muestras procedentes de líneas transgénicas con genes que codifican pectinasas silenciados. Por último, se ha analizado el papel de un gen que codifica una β-galactosidasa, *FaβGal4*, mediante la generación de plantas transgénicas con dicho gen silenciado.

1. INTRODUCIÓN :

1.1 Capítulo I:

El cultivo de la fresa (*Fragaria* × *anannasa*) tiene gran importancia comercial y económica en el sector hortofrutícola. El fruto de la fresa pertenece al grupo de frutos blandos, caracterizado por sufrir un rápido reblandecimiento durante las fases finales de la maduración (Manning, 1993). Entre el 5 y el 25% de la producción de este fruto se pierde debido a

la sobremaduración y a las infecciones fúngicas asociadas a este proceso. El retrasar o disminuir el reblandecimiento del fruto es, por tanto, uno de los principales objetivos de los programas de mejora genética en fresa, ya que cualquier avance en este aspecto supondría una mejora de la rentabilidad y, consecuentemente, un importante aumento de los beneficios económicos al alargar la vida postcosecha del fruto.

Los cambios en la textura del fruto de fresa constituyen la principal causa de la pérdida de calidad de los frutos maduros y tienen como consecuencia la limitación del periodo postcosecha. La textura de los frutos carnosos es un carácter complejo que viene determinado por varios factores, como son las características de las células que lo constituye (forma y tamaño celular, grosor de la pared celular y turgencia celular), la forma en que las células se unen para formar el tejido carnoso del fruto (extensión y fuerza de las zonas de adhesión entre células) y, finalmente, de la presencia de células y tejidos no parenquimáticos (células epidérmicas, elementos vasculares, fibras) (Harker et al., 1997; Mercado et al., 2011). Entre los factores mencionados, es muy amplia la documentación que apoya que son los cambios en las características y/o composición de las paredes celulares que tienen lugar durante la maduración , los factores más determinante en la pérdida de firmeza del fruto (Goulao y Oliveira, 2008; Mercado et al., 2011). En el caso concreto de la fresa, existen abundantes datos acerca de las variaciones que sufren las paredes de los frutos maduros, aunque los mecanismos moleculares involucrados en dichos cambios no están claramente definidos (Manning, 1993; Perkins-Veazie, 1995; Posé et al., 2011). En este trabajo nos centramos en caracterizar los cambios que se producen en la textura debido a cambios en la pared celular, con el fin de obtener frutos de fresa de textura mejorada. Para ello, explicaremos con detalle los componentes de la pared celular y los cambios que se producen en ésta durante el proceso de maduración de la fresa.

La pared celular es una red tridimensional caracterizada por estar compuesta de polisacáridos, proteínas y sustancias aromáticas. Dichos compuestos son secretados por la célula y sufren cambios dinámicos durante la división, expansión y diferenciación celular (Carpita y Gibeaut, 1993). Los componentes de la pared celular y sus interacciones proveen a la célula de fuerza mecánica y regulan la porosidad y la adhesión entre células. Además de su función mecánica, la pared celular también presenta funciones biológicas, entre las que cabe destacar la respuesta ante ataques de patógenos (McCann et al., 2001).

En la pared de las células vegetales se distinguen hasta tres componentes estructurales: lámina media, pared primaria y pared secundaria. En los frutos, la pared secundaria solo está presente en algunos tipos celulares especializados como son los vasos conductores xilemáticos. La pared primaria, de forma general, es una compleja estructura de redes compuestas por polisacáridos pécticos (35%), celulosa (30%), hemicelulosa (25%) proteínas estructurales (10%) y algunos compuestos fenólicos de menor importancia a nivel cuantitativo. En frutos, el componente péctico puede alcanzar incluso el 50 % del total (Fischer et al., 2001).

Las pectinas son cuantitativamente el principal componente de la pared celular primaria y de la lámina media en frutos, contribuyendo a la consistencia de los frutos y siendo fundamentales en los cambios de la textura durante la maduración (Prasanna et al., 2007). Se considera que su presencia en las paredes celulares determina su porosidad, proporcionando superficies cargadas que modulan su pH y el balance iónico. Además, las pectinas son probablemente sustancias clave que participan en la resistencia mecánica de la pared celular primaria y son importantes para la estructura física de la planta (Sirisomboon et al., 2000). Su degradación durante el periodo de maduración parece ser la clave del reblandecimiento del fruto (De Vries et al., 1984; Redgwell et al., 1992). Las pectinas son polisacáridos altamente hidratados, ramificados y ricos en ácido D-galacturónico

(GalA). Según su composición podemos diferenciar Homogalacturonanos (HG), Ramnogalacturonano I (RGI) y Ramnogalaturonano II (RGII). Los homogalacturonanos (HG) son pectinas constituidas únicamente por moléculas de ácido D-galacturónico unidas mediante enlace α (1-4) (McNeil et al., 1984). El RGI, a diferencia del HG, tiene un esqueleto formado por la repetición de un dimero de de ramnosa (Albersheim et al., 1996; McNeil et al., 1980), con cadenas laterales compuestas principalmente por galactosa y arabinosa. Por último, el RGII tiene la peculiaridad de estar compuesto por un esqueleto de ácido galacturónico y cadenas laterales compuestas por azúcares neutros poco comunes como son 2-O-metilfucosa, 2-O-metilxilosa, apiosa, 3-C-carboxi-5-deoxi-L-xilosa y ácido 3- deoxi-mano-octulosónico (KDO). Es ampliamente reconocido que las pectinas son cadenas ricas en ácido galacturónico, pero hay gran controversia con respecto a su modelo estructural macromolecular. El modelo más aceptado sugiere que las pectinas estarían constituidas por regiones de HG alternadas con RGI. En un modelo alternativo, Vincken et al. (2003) postulan que el RGI formaría el esqueleto de la pectina, estando el HG en forma de cadenas laterales.

Por otro lado, a pesar de que sabemos que los componentes de la pared celular se encuentran entrelazados, formando una red muy compleja, mediante enlaces covalentes y no covalentes, el conocimiento de este tipo de interconexiones es muy limitado. Dichas conexiones pueden considerarse de gran importancia porque determinan las propiedades mecánicas y físicas de la pared celular. Entre las interconexiones más importantes destacamos la formación de geles por enlaces con Ca^{++}, ésteres de ferúlico, dímeros de RGII (ésteres con borato) y la formación de ésteres uronil.

Como se ha mencionado con anterioridad, la fresa es un fruto blando, cuya maduración se caracteriza por una drástica reducción de la firmeza en un corto periodo de tiempo. Las principales modificaciones que experimenta la pared celular del fruto de fresa durante la maduración son el incremento de la solubilización de pectinas, la despolimerización parcial de las pectinas

y de la fracción de hemicelulosa, una disminución de la cantidad de hemicelulosa (Huber, 1984; Rosli et al., 2004) y una disminución, aunque muy leve, en la cantidad de celulosa. Estos cambios ocurren en diferentes cultivares, independientemente de su firmeza (Koh y Melton 2002; Rosli et al., 2004). La pérdida de las cadenas laterales neutras en las pectinas también es un proceso clave en el desmantelamiento de la pared celular durante la maduración del fruto. Como consecuencia de este proceso se produce un incremento en el tamaño del poro de la pared, lo que facilitaría el acceso a su substrato de enzimas de pared que están involucradas en el desmantelamiento de la misma durante la maduración (Brummell, 2006).

Entre las enzimas implicadas en el proceso de maduración de la fresa identificadas podemos destacar poligalacturonasas, β-galactosidasas, β-(1-4) glucanasas y pectato liasas (Posé et al., 2011). Sin embargo, en este manuscrito se destacará el papel de tres de estas enzimas: la β-galactosidasa, la poligalacturonasa y la pectato liasa. Los papeles que juegan los diferentes enzimas en la maduración se resumen a continuación: 1) La β-galactosidasa (β-Gal) actúa sobre las cadenas laterales de galactanos presentes en el ramnogalacturonano I, siendo la disminución de residuos de galactosa y arabinosa uno de los cambios más comunes en la pared durante el proceso de maduración (Mercado et al., 2011). 2) La poligalacturonasa (PG) cataliza la rotura de cadenas de ácido poligalacturónico del homogalacturonano desmetiladas, mediante un mecanismo hidrolítico. 3) Por último, la pectato liasa presenta el mismo sustrato que la PG pero la rotura del HG se produce por β-eliminación, con un pH óptimo de 8 (Marín-Rodríguez et al., 2002). A nivel genético, en fresa se han identificado varios genes, específicos de maduración, que codifican estas tres enzimas. El análisis funcional de los genes *FaPG1* y *FaplC*, que codifican una poligalacturonasa y una pectato liasa, respectivamente, sugiere que estas pectinasas tienen un papel clave en el reblandecimiento de la fresa, ya que su silenciamiento incrementa la firmeza del fruto maduro.

1.2. Capítulo II:

Reblandecimiento del fruto y desmantelamiento de las pectinas. Aproximación a la caracterización nanoestructural de las pectinas mediante el uso de la microscopía de fuerza atómica (AFM).

Las pectinas, que son uno de los componentes principales de las paredes celulares de frutos carnosos, se modifican ampliamente durante la maduración. Estos cambios incluyen la solubilización, la despolimerización y la pérdida de las cadenas laterales neutras. Evidencias recientes en frutos con características texturales muy diferentes como son la fresa y la manzana, sugieren que el desmantelamiento de la matriz péctica es un factor clave en los cambios de textura (Atkinson et al., 2012: Posé et al., 2013). En ambos frutos, se ha demostrado que el silenciamiento de genes que codifican poligalacturonasas reduce notablemente el reblandecimiento del fruto.

El estudio de los cambios producidos en la pared celular que dan lugar a ese reblandecimiento suele realizarse mediante técnicas convencionales que dan una información poco precisa de las modificaciones estructurales en la población de polisacáridos. La microscopía de fuerza atómica (AFM) permite el estudio de polímeros individuales. Básicamente, la AFM escanea la superficie de la muestra generando una imagen tridimensional con una resolución comparable con la que proporciona la microscopía electrónica pero con una preparación mínima de las muestras (Morris et al., 2010; Liu y Cheng, 2011). A pesar de la potencialidad, hay muy pocos estudios que analicen el desmantelamiento de las pectinas durante la maduración del fruto utilizando la técnica de AFM. Por ello, el objetivo de este trabajo fue el analizar las principales características del proceso de desmantelamiento de las pectinas durante el proceso de maduración del fruto y, además, revisar los estudios de caracterización nano-estructural de las pectinas mediante AFM, realizados en diferentes frutos, así como su relación con la textura y postcosecha.

En principio, es importante aclarar qué se entiende en la literatura por el proceso de solubilización de las pectinas. Con este término se hace referencia al incremento de la cantidad de pectinas que están débilmente unidas a la pared celular y son por tanto, facilmente extraíbles. Dicha solubilización varía entre especies y es más pronunciada en frutos blandos. Las causas sugeridas para explicar este proceso han sido diversas, entre ellas caben destacar: la pérdida de azúcares neutros de las cadenas laterales del RGI (Popper y Fry, 2005), la despolimerización de las pectinas procedentes de las fracciones CDTA y carbonato sódico (Redgwell et al., 1992; Rose et al., 1998; Vicente et al., 2007), la síntesis de nuevas pectinas más libres (Huber, 1984) y, por último, la degradación del RGI mediante enzimas como ramnogalacturonasas (Molina-Hidalgo et al., 2013).

La mayoría de los datos nanoestructurales de pectinas de frutos obtenidos mediante AFM corroboran y enriquecen los datos obtenidos anteriormente con técnicas convencionales, apoyando de esta manera la fiabilidad de este enfoque para estudiar el desmantelamiento de las pectinas durante el reblandecimiento del fruto. En este capítulo se describen los datos obtenidos mediante el análisis por AFM (altura, ancho, longitud grado de polimerización) de pectinas aisladas de diferentes frutos, cultivares y estadios de desarrollo, así como de pectinas procedente de diferente fracciones (Capítulo II, Tabla 1). En general, se puede concluir que la longitud de las pectinas varía en el rango 20-1000 nm, siendo las pectinas extraídas de la pared mediante agentes quelantes, fracción CDTA, más largas que las pectinas unidas covalentemente a la pared primara y extraídas con carbonato sódico, fracción Na_2CO_3. Además, la mayoría de los trabajos indican una altura de estos polímeros en las muestras de 0,5-2 nm. Sin embargo, los resultados concernientes al ancho de las cadenas de pectinas no son tan concluyentes, encontrándose un amplio rango de resultados. Este hecho podría ser debido a una sobreestimación de la medida producida por el mayor diámetro de la sonda con respecto al de la muestra, lo que se conoce como "probe broadening effect".

El estudio de estos trabajos también permite diferenciar dos bloques de resultados bien diferenciados: un primer grupo que describe longitudes de pectinas por debajo de 1000 nm y con anchos comprendidos entre 10-50 nm, incluyendo datos de tomate (Round et al., 1997; Round et al., 2010), fresa (Posé et al., 2012) y cereza (Zhang et al., 2008; Lai et al., 2013), y un segundo grupo de artículos que describen pectinas más largas (> 1000 nm) y más anchas (> 50 nm), tales como las observadas en melocotón (Yang et al., 2009), azufaifa (Wang et al., 2012) y albaricoque (Chen et al., 2013). La aplicación de la corrección de la anchura descrita por Morris et al. (1997) nos indica que el primer grupo de pectinas correspondería principalmente a cadenas de pectinas individuales, mientras que el segundo grupo correspondería con grupos de cadenas de pectinas ensambladas.

Con respecto a la maduración y al periodo postcosecha, estudios realizados por Zhang et al. (2008) en las pectinas extraidas con quelantes de dos cultivares de cereza con diferente textura, en distintos estadios de desarrollo, mostraron pectinas más anchas y largas en frutos inmaduros. Sin embargo, no se encontró correlación entre los diferentes cultivares y su firmeza. Resultados similares fueron publicados por Xin et al. (2010) y por Wang et al. (2012). Yang et al. (2009) utilizaron cultivares de melocontón con distinta firmeza. Este estudio incluyó además las fracciones de pectinas solubles en agua y extraídas con carbonato, observando diferencias significativas en la longitud, principalmente en esta fracción, y una disminución del número de cadenas en las fracciones covalentes y extraídas con quelante durante la maduración del fruto. Estudios preliminares realizados por nuestro grupo de investigación en pectinas de fresa en diferentes estadios de maduración extraídas con quelantes, mostraron pectinas más largas, más ramificadas y con mayor número de agregados en frutos inmaduros (Capítulo II, Fig 1)

Resumiendo, la mayoría de los estudios de AFM indican una reducción en la longitud de las cadenas de pectinas individuales y en la frecuencia de

aparición de estructuras supramoleculares, agregados, siendo las pectinas extraídas con carbonato sódico, es decir, las pectinas unidas covalentemente a la pared celular primaria, las más afectadas. Aunque se necesita trabajo adicional para identificar la naturaleza físico-química de las estructuras visualizadas por AFM, esta técnica podría ser una herramienta poderosa para comprender mejor el papel de desmantelamiento de las pectinas en el reblandecimiento de los frutos.

3. RESULTADOS Y DISCUSIÓN:

3.1 Capítulo III:

Cambios nano-estructurales en las pectinas procedentes de las fracciones extraídas con quelantes y carbonato sódico durante la maduración del fruto de fresa.

En el Capítulo II se propone el AFM como una herramienta poderosa para el estudio del desmantelamiento de las pectinas que tiene lugar durante la maduración del fruto. Estudios preliminares realizados en nuestro laboratorio con pectinas de frutos de fresa en diferentes estadios de maduración, mostraron diferencias cualitativas importantes en la longitud y complejidad de las mismas entre los estadios analizados (Capítulo II, Fig. 1). En este capítulo, se realizó un exhaustivo estudio cuantitativo de las pectinas solubles en el quelante CDTA y en carbonato sódico de frutos de fresa inmaduros y maduros, además de un minucioso estudio de la pared celular de ambos tipo de frutos mediante técnicas convencionales. Esto se realizó con el propósito de conocer mejor los procesos de solubilización de pectinas que van asociados al reblandecimiento del fruto de la fresa.

Con el mencionado fin se recolectaron frutos de fresa (*F.* × *ananassa* Duch. cv. ´Chandler`) en diferentes estadios de desarrollo. Las paredes celulares se aislaron a partir de frutos rojo maduro y de frutos verdes, siguiendo el

protocolo de Redgwell et al. (1992), con leves modificaciones. A partir de los extractos de pared celular, se realizó un proceso de fraccionamiento secuencial siguiendo el protocolo de Santiago-Doménech et al. (2008), con algunas modificaciones, para obtener fracciones enriquecidas en los distintos polisacáridos que conforman la pared celular. La extracción secuencial se realizó tratando las sucesivos residuos de cada paso de extracción con agua, CDTA, Na_2CO_3, KOH1M y KOH4M. Posteriormente, se procedió al análisis de la composición de azúcares de las fracciones mediante medidas colorímetricas, , ácido galacturónico (Filisetti-Cozzi y Carpita,1991), así como con medidas de azúcares neutros mediante cromatografía de gases (Blakeney et al., 1983). Además, las fracciones ricas en pectinas CDTA y carbonato sódico, supuestamente las que sufren el proceso de solubilización, fueron analizadas por espectroscopía de infrarrojos por transformada de Fourier (FTIR) y por AFM. Para la visualización de dichas fracciones mediante AFM se siguió un procedimiento diferente según la naturaleza de la fracción. Para la fracción CDTA se utilizó el mismo protocolo utilizado en tomate (Kirby et al., 2008; Round et al., 2001) incluyendo un paso de 10 minutos de sonicación para facilitar la disgregación de las pectinas. Las muestras de carbonato se procesaron siguiendo el protocolo descrito por Posé et al. (2012).

La cantidad de pared celular obtenida en fruto verde fue el doble de la obtenida en fruto rojo. De igual forma, en todas las fracciones se observó una disminución de la cantidad relativa de fracción, expresada por mg de pared celular, durante la maduración (Capítulo IV, Tabla. 1). En cuanto al contenido en ácido galacturónico, las fracciones correspondientes a las pectinas solubles in vivo, fracciones PAW (fenol:ácido acético:agua) y agua, incrementaron su contenido en GalA de manera significativa en fruto rojo, especialmente la fracción PAW. Por el contrario, en el resto de las fracciones la cantidad de galacturónico fue mayor en fruto verde, principalmente en la fracción CDTA (Capítulo III, Fig. 1). Estos resultados ponen de manifiesto una solubilización de las pectinas durante la maduración. Asimismo, los análisis

de azúcares neutros realizados mediante cromatografía de gases (Capítulo III, Tabla. 2), referidos a la cantidad de fracción, revelaron una pérdida de azúcares a lo largo de la maduración, siendo la galactosa y arabinosa los azúcares que más se vieron afectados. Con respecto a los análisis realizados mediante FTIR, cabe destacar el mayor grado de esterificación en las pectinas solubles en agua en fruto verde, probablemente debido a acetilaciones. Además, la región denominada "huella péctica" indica enriquecimiento de homogalacturonanos en fruto rojo, mientras que en fruto verde prevalece el contenido en arabinogalactanos (Capítulo III, Fig. 3A). En la fracción CDTA no se observan diferencias en los espectros FTIR entre fruto verde y rojo, mientras que en la fracción carbonato, las muestras de fruto verde presentaron un pequeño pico de absorción correspondiente a enlaces tipo éster que no estaba presente en las muestras de fruto rojo.

Análisis realizados mediante AFM en fruto inmaduro y maduro de las fracciones CDTA (Capítulo III, Fig. 4) y carbonato (Capítulo III, Fig. 5) mostraron la existencia de una población de pectinas más largas en frutos verdes en ambas fracciones. Análisis estadísticos demostraron que la mediana de la distribución de longitudes de las pectinas era significativamente mayor en fruto verde que en fruto rojo en la fracción CDTA (Capítulo III, Tabla 3). Además, las pectinas de fruto verde presentaron una mayor frecuencia de ramificaciones simples y múltiples, tanto en la fracción CDTA como en la fracción carbonato (Capítulo III, Tabla 4). Estos resultados sugieren la existencia de una importante solubilización de las pectinas de la pared celular en el fruto de la fresa. La causa principal sería por la eliminación de las cadenas laterales de las pectinas tanto de las unidas iónicamente a la pared, como de las unidas covalentemente. Además, también habría una despolimerización de las pectinas unidas iónicamente. Por último, es evidente la pérdida de azúcares neutros, sobre todo en la fracción de pectinas que están unidas covalentemente a la pared.

3.2. Capítulo IV:

Profundizando en el estudio de la nano-estructura de las pectinas de frutos y sus interconexiones mediante el uso de microscopía de fuerza atómica

Como se ha demostrado anteriormente, la microscopia de fuerza atómica es una buena herramienta para la caracterización de pectinas (Round et al., 2001; Kirby et al., 2008; Yang et al., 2009; Fishman et al., 2007; Posé et al., 2012) debido a que dicha tecnología permite la visualización de cadenas de pectinas individuales, las posibles ramificaciones e incluso la caracterización de estructuras complejas. Las pectinas están compuestas por diferentes dominios, a los que están asociados diferentes elementos estructurales como el homogalacturonano (HG), xilogalacturonano (XGA), ramnogalacturonano I (RGI), ramnogalacturonano II (RGII), arabinano, y arabinogalactano (Schols y Voragen, 2002). A pesar de que la composición química de estos elementos es conocida, aún no está claro cuáles son las interacciones de estos elementos estructurales y su papel en la existencia de estructuras macromoleculares. Hay varias teorías al respecto pero las más aceptadas son las postuladas por Vries et al. (1981) y por Vincken et al. (2003). En el primer modelo, el esqueleto principal de la pectina estaría formado por zonas de RGI y HG alternándose. En el segundo modelo, el RGI formaría el esqueleto de la pectina y las ramificaciones podrían ser de HG, XGA y de azúcares neutros. Además, otro punto que debería ser estudiado en profundidad son los enlaces intermoleculares entre estos elementos, siendo hasta ahora los más estudiados los enlaces de puente de calcio entre moléculas de HG (Voragen et al., 2009) y los ésteres de ácidos ferúlicos (Waldron et al., 1997).

Estudios previos realizados mediante AFM en frutos de fresa (Posé et al., 2012) han mostrado, además de cadenas de pectinas individuales, grandes fibras o agregados de naturaleza desconocida. En muestras de tomate también se han descrito agregados de estructuras similar en la

fracción de pectinas solubles en carbonato sódico (Round et al.,1997). Una hidrólisis ácida prolongada de las pectinas, tratamiento que elimina los azúcares neutros, no disminuye la longitud de las cadenas individuales que se observan mediante AFM, pero sí da lugar a una disminución del tamaño de los agregados, aunque estos no llegan a desaparecer (Round et al., 2010). A raíz de estos resultados, Round et al. (2010) han postulado que las cadenas lineales visibles por AFM estarían formadas principalmente por ácido galacturónico mientras que los agregados serían estructuras compuestas por HG y RGI.

El objetivo principal de este capítulo fue la caracterización estructural de la fracción carbonato de los frutos de fresa, haciendo especial énfasis en la caracterización de moléculas agregadas. Para ello, fracciones carbonato de frutos de fresa control, sin transformar, y de frutos con genes de pectinasas silenciados, se sometieron a dos tratamientos diferentes. Por un lado, se realizó una digestión de las muestras de pectinas con una enzima endo-PG de hongos a distintos tiempos de digestión. Por otro lado, las muestras se sometieron a una hidrólisis ácida suave durante un corto tiempo. Tras ambos tratamientos, se procedió a la visualización de las muestras mediante AFM. Para el primer experimento se utilizaron pectinas procedentes de plantas no transgénicas (control) (Capítulo IV, Fig.1), plantas transformadas con la secuencia antisentido del gen de poligalacturonasa *FaPG1* (APG) (Capítulo IV, Fig.2), y, por último plantas, transformadas en antisentido con una secuencia del gen de pectato liasa *FaplC* (APEL) (Capítulo IV, Fig.3). Los valores de longitud de las cadenas de pectina se redujeron un 42, 61 y 39% en el control, APG y APEL, respectivamente, después de 120 min de digestión con Endo-PG (Capítulo IV, Tabla1). Además, la cinética de la digestión difirió en los tres genotipos (Capítulo IV, Fig.6). Las pectinas procedentes del control y APG mostraron una disminución de su longitud con el tiempo de digestión, mientras que en las muestras de la línea APEL solo se observó reducción después de 90 minutos. En los tres genotipos, las ramificaciones desaparecieron como resultado del tratamiento con

endo-PG (Capítulo IV, Tabla1). El número de agregados también se redujo drásticamente como resultado de la digestión con endo-PG en el caso del control y APG y, sin embargo, en la línea APEL todavía eran visibles después de 120 min de digestión (Capítulo IV, Fig. 7). Por otro lado, se realizó un tratamiento con HCl 0,1 M a temperatura ambiente durante 30 minutos en las muestras APG. Este tratamiento rompe los enlaces diéster de borato, liberando el RGII. La hidrólisis ácida no redujo ni la longitud de las cadenas de pectinas ni las ramificaciones (Capítulo IV, Tablas 2 y 3). Sin embargo, el número de agregados se redujo significativamente pasando de 6 agregados/ μm^2 a 2 agregados/ μm^2 , tras el tratamiento (Capítulo IV, Fig.8).

Este conjunto de resultados permite concluir que tanto el esqueleto de las pectinas, como las cadenas laterales visibles mediante AFM, están compuestos principalmente por ácido galacturónico. Aún así una parte de estas estructuras podría contener otros carbohidratos dificultando la digestión total con la endo-PG. Por otro lado, también se puede concluir que los agregados que se observan en las imágenes de AFM contienen ácido galacturónico, siendo estas estructuras susceptibles a la digestión por endo-PG. Por último, el RGII puede estar implicado en la existencia de estos agregados ya que estas estructuras macromoleculares disminuyen significativamente como resultado de la hidrólisis ácida suave.

3.3 Capítulo V:

Evaluación del gen *FaβGal4* en el reblandecimiento del fruto de la fresa mediante su caracterización funcional.

Uno de los cambios que ocurren durante la maduración de los frutos es la pérdida de residuos de galactosa (Gal) en las pectinas presentes en la pared (Gross y Sam, 1984). Esta disminución se adscribe a la actuación de las enzimas conocidas como β-Galactosidasas. Los cambios se detectan principalmente en las fracciones ricas en pectinas, aunque también se

pueden observar pequeños cambios en la matriz de hemicelulosas y en la fracción de celulosa (Gross y Sam, 1984).

La actividad responsable de la eliminación de la Gal de la pared celular es una exo-β-D-galactosidasa, enzima que elimina los residuos terminales no reductores de los β-D-galactósidos. Durante la maduración del tomate se ha visto que la cantidad de polímeros de galactosa disminuye mientras que se incrementa la cantidad de galactosa libre (Gross y Sam, 1984). En fresa, la actividad β-galactosidasa se incrementa durante el desarrollo del fruto y permanece alta en fruto rojo. Este incremento de actividad, al igual que en el tomate, se ha correlacionado con la pérdida de galactosa en la pared celular (Trainotti et al., 2001; Figueroa et al., 2010). Se han aislado tres genes de β-galactosidadasa en fresa *FaβGal1* hasta el *FaβGal3*. Solo uno de estos genes, *FaβGal1*, incrementa su expresión durante la maduración (Trainotti et al., 2001). Además *FaβGal1* y *FaβGal2* presentan un dominio de unión a lectinas, lo que se postula podría incrementar la eficiencia en la eliminación de los residuos de galactosa de los polímeros de la pared celular (Posé et al., 2011).

Estudios previos realizados en nuestro grupo de investigación, pusieron de manifiesto la existencia de un nuevo gen de β-galactosidasa, *FaβGal4*, con un patrón de expresión asociado a la maduración del fruto. Los estudios de expresión de este gen muestran niveles bajos de expresión en los primeros estadios del desarrollo, un incremento de la expresión a partir del estadio blanco y su máxima expresión en estadio rojo (Capítulo V, Fig.1). El análisis de expresión en diferentes tejidos mostró que su expresión es específica del receptáculo del fruto (Capítulo V, Fig.2). Tras esto, se demostró un efecto de bloqueo de la expresión del gen por las auxinas (Capítulo V, Fig.3). Este conjunto de resultados nos llevó a considerar como el principal objetivo de este trabajo el análisis funcional del gen *FaβGal4* para profundizar en su posible papel en los cambios en textura que sufren los frutos de fresa durante su maduración.

Para complimentar este fin, se obtuvieron plantas transgénicas de fresa conteniendo una secuencia de 300 pb de una región no conservada del gen *FaβGal4* en antisentido, empleando transferencia mediada por *Agrobacterium tumefaciens* (cepa AGL1) y siguiendo el protocolo descrito por Barceló et al. (1998). Después del crecimiento de las líneas transgénicas en medio de selección, las plantas fueron aclimatadas y transferidas al invernadero.

Las líneas transgénicas fueron evaluadas durante tres años consecutivos mostrando características fenotípicas similares a lo largo del tiempo. El crecimiento vegetativo de las líneas transgénicas fue similar al de las líneas controles, no transformadas (Capítulo V, Fig. 4). Sin embargo, la producción de frutos fue menor en la mayoría de las líneas transgénicas y se observó una proporción muy alta de frutos algo deformes y de menor tamaño (Capítulo V, Fig.4). Para estimar la calidad del fruto en estadio rojo maduro se midió peso, anchura, longitud, color, cantidad de sólidos solubles y firmeza de estos frutos. Con respecto a la firmeza, que es el factor más interesante en este estudio, dos líneas transgénicas (βGal 28 y βGal 37) mostraron un 30% más de firmeza con respecto al control durante los tres años de muestreo. Estas dos líneas fueron seleccionadas para estudios posteriores. Los análisis de expresión en frutos maduros mostraron un silenciamiento en torno al 70% del gen *FaβGal4*, así como del gen *FaβGal1*, en ambas líneas transgénicas, y un silenciamiento significativo del gen *FaβGal3* en la línea βGal 28 (Capítulo V, Fig. 5). Se extrajeron paredes celulares de frutos rojo maduro de las dos líneas transgénicas seleccionadas con el gen *FaβGal4* silenciado (βGal 28 y βGal 37), así como de frutos testigos sin transformar, siguiendo el protocolo de Redgwell et al. (1992) y se realizó un fraccionamiento de la pared tal y como describe Santiago-Doménech et al. (2008). El análisis de estas fracciones puso de manifiesto una mayor cantidad de ácido galacturónico en las muestras de ambas líneas transgénicas, tanto en la fracción de pectinas solubles en carbonato sódico como en la fracción soluble en agua. La línea βGal 37 mostró también altas cantidades de ácido

galacturónico con respecto al control en la fracción CDTA (Capítulo V, Fig. 6). Con respecto a las medidas de azúcares neutros realizada por cromatografía de gases, se observaron diferencias significativas con respecto al control en la pared celular completa, mostrando las líneas transgénicas altos valores de galactosa y arabinosa, a la vez que se detectó una disminución de la xilosa, manosa y glucosa (Capítulo V, Tabla 3) en estas mismas líneas. Además, el incremento de galactosa fue evidente en todas las fracciones, sobre todo en la fracción de pectinas extraída con KOH1M (Capítulo V, Fig.7). Estas diferencias en composición de azúcares como consecuencia del silenciamiento del gen fue también confirmada mediante FTIR, observándose en las líneas transgénicas un perfil característico de fracciones con altos valores en Ara y Gal.

Por último, análisis realizados utilizando microscopía de fuerza atómica revelaron una menor despolimerización tanto de la fracción de pectinas soluble en CDTA como de la fracción de pectinas solubles en carbonato sódico, en las líneas silenciadas (Capítulo V, Fig. 9 y 10). Por otra parte, análisis cuantitativos revelaron un aumento de la longitud del contorno de las cadenas aisladas de pectinas en la línea βGal 37 (Capítulo V, tabla 5), así como un aumento en la longitud de las cadenas laterales, el número de ramificaciones (Capítulo V, Tabla 6) y la presencia de agregados.

Con los resultados obtenidos se puede concluir que el gen *FaβGal4* juega un papel importante durante la maduración del fruto de la fresa. Las líneas transgénicas con el gen silenciado mostraron un incremento de la firmeza del 30% en frutos maduros. A nivel de la pared celular, los frutos transgénicos mostraron una mayor cantidad de Gal en todas sus fracciones, así como una reducción en la solubilización y despolimerización de las pectinas. Con estos resultados, postulamos que la disminución de la pérdida de los residuos de Gal durante el proceso de maduración en los frutos transgénicos disminuiría la movilidad a otras enzimas modificadoras de la pared, obstruyendo el acceso a sus sustratos, siendo ésta la causa de la mayor firmeza de los frutos de las líneas silenciadas.

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ANNEX: PUBLICATIONS

PANIAGUA C, POSE S, KIRBY AR, MORRIS VJ, QUESADA MA, MERCADO JA. FRUIT SOFTENING AND PECTIN DISASSEMBLY: AN OVERVIEW OF THE NANO-STRUCTURAL PECTIN MODIFICATIONS ASSESSED BY ATOMIC FORCE MICROSCOPY (AFM). ANNALS OF BOTANY. 114: 1375-1383. AÑO DE PUBLICACIÓN:2014

POSÉ S, **PANIAGUA C**, CIFUENTES M, BLANCO-PORTALES R, QUESADA MA, MERCADO JA. INSIGHTS INTO THE EFFECTS OF POLYGALACTURONASE *FAPG1* GENE SILENCING ON PECTIN MATRIX DISASSEMBLY, ENHANCED TISSUE INTEGRITY, AND FIRMNESS IN RIPE STRAWBERRY FRUITS. PUBLICACIÓN: JOURNAL OF EXPERIMENTAL BOTANY, 64, 3803-3815.AÑO DE PUBLICACIÓN: 2013.