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Survey

SURVEY

Contribution of cell wall-modifying enzymes to the texture of fleshy fruits. The example of apple

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Abstract: The cell walls of fleshy fruits consist of polysaccharide assemblies (pectin, hemicelluloses and cellulose), the structure and interactions of which vary depending on the genetics of the fruit, and its stage and conditions of development. The establishment and the structural reorganization of the assemblies result from enzyme/protein consortia acting *in muro*. The texture of fleshy fruits is one of the major criteria for consumer choice. It impacts also post-harvest routes and transformation processes. Disassembly of fruit cell wall polysaccharides largely induces textural changes during ripening but the precise role of each polysaccharide and each enzyme remains unclear. The changes of cell wall polysaccharides during fruit ripening have mainly emphasized a modulation of the fine chemical structure of pectins by hydrolases, lyases, and esterases. This restructuring also involves a reorganization of hemicelluloses by hydrolases/transglycosidases and a modulation of their interactions with the cellulose by non-catalytic proteins, such as expansin. Apple is the third most produced fruit in the world and has been the subject of studies about fruit quality. This paper presents some of the results to date about the enzymes/ /proteins involved in fruit ripening with particular emphasis on apple.

Keywords: hemicelluloses; ripening; softening; polysaccharide-hydrolases; xyloglucan-transglycosidase.

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1. INTRODUCTION

Fleshy fruits are defined as fruits consisting mainly of a soft succulent tissue made of water-rich parenchyma, named pulp or flesh. They include two main types of fruit: drupes and berries. Drupes are fleshy fruits with a stone (cherry, plum, peach, *etc*.) that have only one seed and the endocarp is thick and very hard. On the other hand, berries are fleshy fruits with a thin endocarp that contains generally several seeds scattered in the pulp.

Apple (*Malus domestica*) is a particular case of fleshy fruit where a core containing several seeds is surrounded by the fleshy edible tissue. Since the latter does not originate from the pistil tissue but from the inferior ovary, it is referred to as an accessory fruit, previously called false fruit. Among fleshy fruits, apple is the third world production, after tomato and grape.[1] In Europe, Italy, Poland and France are the three main producers and cumulate 5.8 Mt. Apple fruit texture is a key quality trait orienting consumer choice as well as agro-industrial processes, impacting post-harvest routes and resistance to diseases.[2,3] Firm, crispy, mealy, juicy, crunchy are among the words describing fruit texture. These add to the criteria of visual appearance, sweetness and sourness and aromatic perceptions to determine consumer preference. Texture is defined as a group of properties sensed by the feeling of touch (hand, mouth) and related to the deformation, disintegration, and flow of the food under force.[4] Fleshy fruits contain 85 to 95 % water. Consequently, texture results from the structure and organisation of about 1.0 to 4.0 % of the macromolecular dry matter in cell walls.

Cell walls play a key role in cell protection and in the regulation of intercellular exchanges. Their thickness, molecular organization and structure shape the cells, assure cell–cell adhesion and provide the mechanical support to the cell for withstanding turgor pressure. On the tissue scale, the cell density and organization, as well as the number, size and distribution of intercellular spaces have consequences on the mechanical properties of fruit. On the organ scale, the proportion and distribution of different tissues (parenchyma, conducting tissue, etc.) affect the perception of fruit texture. Particular combinations of these features on different scales define the mechanical and physicochemical properties at the origin of the various texture descriptors. The understanding and control of the origin and evolution of these different combinations in ripening fruit represent a major challenge for the fruit sector in order to provide fruits with the desired textures.[5]

Texture evolves early on, starting at fruit development,[6] but it particularly changes during fruit ripening as the result of major cellular metabolic modifications and cell wall disassembly. These phenomena lead to softening, the mechanism of which was the subject of numerous studies in tomato, taken as a fruit model.[7,8] However, despite common molecular features with other fruits, tomato largely differs in structural and mechanical aspects from others, such as apple. In

particular, its cell wall composition and the enzymatic machinery involved in wall disassembly differ. Nevertheless, the discovery of regions of genomic synteny across distant species, such as, for example, strawberry (*Fragaria*) and apple (*Malus*) in the Rosaceae family[9] enable analogies between the fruits to be addressed.

Although softening of a fleshy fruit involves changes in water content and compartmentalization, this review will highlight the cell wall contribution to the texture in apple. After an overview of its cell wall and its constitutive polymers, the main changes occurring in the wall of the ripening fruit and the key enzymes and proteins involved in this process will be summarized.

2. STRUCTURE OF PLANT CELL WALL

Cell wall is a nanocomposite surrounding each plant cell exterior to the plasmalema. It corresponds to an assembly of biopolymers, mainly polysaccharides but also proteins and phenolic compounds. Fleshy fruits such as apple mainly contain parenchyma cells where the cell wall is of a primary type. Primary cell walls are thin, flexible and highly hydrophilic (65 % water).[10] In apple cell wall, as in other fruit parenchyma, three main polysaccharide families interact to form the primary cell wall: cellulose, hemicelluloses and pectin. Cellulose is a 1,4-β-D-glucan, which is associated by numerous hydrogen bonds to form long and rigid microfibrils. It is essentially insoluble in dilute acid and alkali, is highly resistant to physical, chemical and enzymatic degradations, and ensures wall rigidity.

The hemicelluloses are composed of three groups of polysaccharides: xyloglucan (XyG), (galactogluco)mannan (GgM) and glucuronoxylan (GuX). They are predominantly formed of neutral sugars and are soluble in dilute alkali. In apple, the most abundant hemicellulose is xyloglucan,[11] which can account for 20–25 % of the primary cell wall.[12] Its backbone is a 1,4-β-D glucan on which 75 % of the glucose residues (Glc) are substituted by mono-, di- or tri-saccharide side chains. The first sugar of these side chains is always a α-D-xylopyranose (Xyl). In apple, this first residue can carry β-D-galactopyranose or α-L-fucopyranose-(1,2)-β-D-galactopyranose disaccharide. Acetyl-esterification can occur on XyG. A nomenclature was proposed by Fry *et al.*[13] for the different structures encountered in xyloglucans, whereby one letter codes for the differently-substituted β-D-Glc residues of xyloglucan-derived oligosaccharides (Fig. 1).

Xyloglucans are classified as ‘XXXG-type’ or ‘XXGG-type’ based on the number of backbone glucosyl residues that are branched.[14] Apple xyloglucan is of the XXXG-type with three Glc residues carrying a side chain and the fourth unsubstituted. In primary cell walls, xyloglucans extensively coat the cellulose microfibrils according to a reversible process involving hydrogen bonding (Fig. 2).[15]

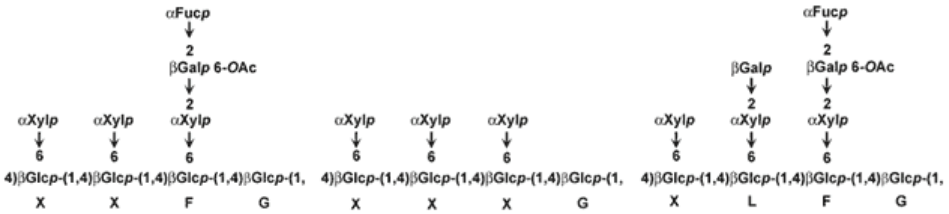

Fig. 1. Structure and nomenclature of apple xyloglucans.[13]

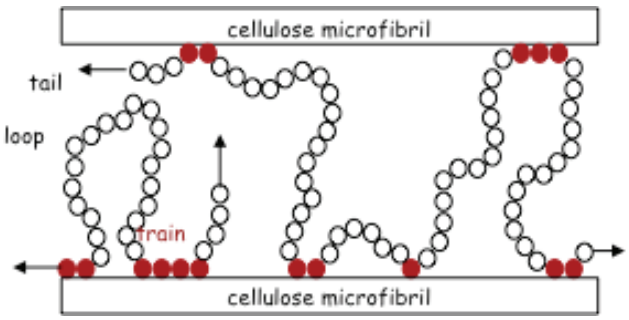

Fig. 2. Schematic representation of the interaction of xyloglucan and cellulose. Full circle, xyloglucan oligosaccharides that interact with cellulose microfibril (trains); empty circle, xyloglucan oligosaccharides that do not interact with cellulose microfibril (loops and tails), modified from Vincken *et al.*[15]

The second group of apple hemicelluloses is mannans. They are thought to account for only 3–5 % of the primary cell wall, as glucomannans or as galactoglucomannans (GgM). Glc and mannose (Man) form the backbone with an alternating or block-wise distribution, and the Man residues may carry Gal side chains.[12] Some structural data collected on GgM isolated from cultured tobacco or blackberry cells, or from kiwi fruit show a more or less alternating structure 1-4 linked β-D-Glc and β-D-Man. The Man residue can be substituted on *O*-6 with α-D-Gal or α-D-Gal (1-2) β-D-Gal and can be partially acetyl-esterified.[17–20] However, the fine chemical structure, properties and role *in planta* of GgM are still under discussion. They are able to form hydrogen bonds to cellulose[21,22] and are thus expected to share functions with XyG, although they are less abundant and generally shorter polysaccharides than XyG.[23]

The third group of hemicelluloses consists in glucuronoxylan. In tomato, GuX is co-extracted with GgM even under strong alkaline conditions, and enzymatic treatments of the extract provided evidence that GuX and GgM can be associated in a complex.[24] The function of GuX in the tomato wall remains to be established. The presence of GuX in apple has not been reported to date.

Pectin forms the third family of cell wall polysaccharides in the primary cell wall. Its high content in partly methyl-esterified galacturonic acid (GalA) and the

presence of rhamnose (Rha) are the distinctive features of this heterogeneous group of polysaccharides.[25] Pectin is a complex polysaccharide that can be envisioned as a multi-block co-biopolymer (Fig. 3). The simplest of these blocks is homogalacturonan (HG), an unbranched polymer of (1→4)-*α*-D-GalpA. Other minor galacturonans can be distinguished as, for example, rhamnogalacturonan II (RG-II) and xylogalacturonan (XGA), the latter being particularly present in apple pectin.[26] A second major block, rhamnogalacturonan I (RG-I), is composed of a repeating disaccharide unit [→2)-*α*-L-Rhap-(1→4)-*α*-D-GalpA-(1→]. RG-I is decorated primarily with arabinan and galactan side chains.

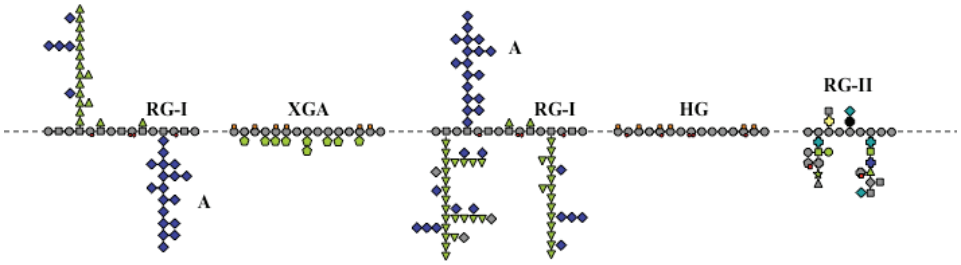

Fig. 3. Schematic representation of the main structural elements of pectin. HG: Homogalacturonan; RG-I: Rhamnogalacturonan I; XGA: Xylogalacturonan; RG-II: Rhamnogalacturonan II; A: Arabinan. Modified from Schols and Voragen.[25]

Homogalacturonan plays important roles in cell–cell adhesion and in controlling wall porosity. Depending on their degree of methylesterification and the distribution of the methyl-substituents, HG is able to dimerise *via* the presence of divalent cations, such as calcium. These interactions contribute to cell adhesion in the middle lamella region as well as to the control of wall porosity.[27] Rhamnogalacturonan I structural domains are also thought to contribute to the cell wall structure and cohesion through the ability of their side chains to interact with water.[28] On the other hand, it was shown that pectin side chains are able to link cellulose microfibrills. This was demonstrated *in vitro* by creating artificial composites by adsorption of extracted arabinan on the primary cell wall cellulose.[29] This demonstrated the ability of arabinan chains to stick to cellulose and a similar phenomenon is likely to occur *in planta*.

3. RIPENING-INDUCED CHANGES IN THE CELL WALL

Extensive depolymerisation and solubilization of pectins together with demethylation were correlated with the decrease in fruit firmness observed during ripening of many fruits.[30–32] Fruit ripening is also often accompanied with galactose loss.[31] On the other hand, no change in molecular weight was evidenced for hemicellulose groups such as GgM and GuX,[24] but could be observed for XyG.[7] In apple, galactose loss occurred mainly during maturation before ripening.[33] However, it was shown that in apple, the loss in galactose concerns not only pec-

tin, but also hemicelluloses. Indeed, during ripening, the galactose content decreased by 29 % in pectin and by 71 to 87 % in the hemicelluloses, according to the apple variety.[31] Nevertheless, there was no change in the molecular weight profile of the hemicelluloses and particularly xyloglucan.[34] More recently, galactose loss during ripening was confirmed in 14 and 17 apple genotypes collected over two years,[35] showing that it is a general feature of apple ripening. Moreover, important hemicelluloses modifications were observed during fruit construction and maturation. These results suggested that the galactose and mannose that disappeared during ripening originated from the same polysaccharide family. Simultaneously, acetyl-esterification decreased in xyloglucan.

3.1. Enzyme involvement in fruit ripening

Due to the complexity of cell wall polysaccharides, a large number of enzymes are involved in their modification or degradation. They can be grouped into three main categories. The first group corresponds to proteins that alter hydrogen bonds and is represented by expansins. The second category corresponds to depolymerases that cleave the polysaccharide backbone. This contains not only glycan hydrolases but also lyases that cleave uronic acid-containing polysaccharides by the β-elimination mechanism to generate an unsaturated uronic acid moiety at the new non-reducing end. This group also includes the transglycosidases that are specific for successively cleaving the polysaccharide backbone and transferring an oligosaccharide on the newly formed reducing end in a unique catalytic event, to allow chain extension. As an example, xyloglucanendotransglycosidase (XET) cuts and then ligates the XyG backbone during cell expansion.[36] The third category comprises the “shaving” enzymes that modify side chains or eliminate substitutions on the backbones of the polysaccharides. These include esterases that remove methyl- or acetyl-esters, and various glycosidases, such as galactosidases and arabinofuranosidases, which shorten the neutral side chains on pectin and hemicelluloses. These glycosidases catalyze the hydrolysis of glycosydic linkages to remove terminal residues specifically from the reducing or non-reducing end of oligosaccharides, polysaccharides or side chains.

To aid the occurrence of depolymerisation of matrix glycan, relaxation of the xyloglucan–cellulose network often occurs. This is thought to be the role of expansin, thanks to its ability to disrupt the hydrogen bonds between cellulose and xyloglucans.[37,38] This dissociation allows slippage of cell wall polymers, before the association reforms to restore the integrity of the cell wall network.[39] The resulting swelling of the cell wall is likely to improve diffusion of new polysaccharide components into the growing wall, as well as enzymes that cleave cellulose and pectins.[40] In tomato, overexpression or suppression of the ripening specific expansin *Exp*1 markedly affects texture.[38] In apple, the expansin activity pattern remains similar throughout all the developmental stages.[41]

Pectin depolymerisation during fruit ripening results from the combined action of endopolygalacturonase (PG, EC 3.2.1.15) and pectin methylesterase (EC 3.1.1.11) that were shown to increase drastically in several species.[42,43] In contrast, pectin-degrading enzymes (*i.e.*, endopolygalacturonase, pectin methylesterase and pectate lyase) are very low in apple.[41,44] Although the pectin content in apple is in the same range as in other fruits (1.5 to 2.5 % on a fresh weight basis),[45] it does not appear to be a major target for the ripening-involved enzymes. Nevertheless, *PG1* expression levels have been associated with softening in a range of cultivars,[46] and suppression of *PG1* in transgenic apple plants results in a firmer fruit.[47] Thus, the role of pectinolytic activities during apple ripening and softening remains to be elucidated. Other polysaccharide-degrading enzymes are likely involved in cell wall disassembly in apple.

Several different enzymes were followed from the apple fruit-set to the over-ripe stage.[41] In this study, xyloglucan endo-transglycosydase (XET) was found to be more active in the ripening fruit. This activity was shown to be particularly important in controlling tomato fruit softening.[48] In apple, the XET activity was shown to be consistent with the kinetics of XET genes expression during apple development, but it was not correlated with fruit growth.[49] In contrast, endoglucanase (EC 3.2.1.4), which contributes to cellulose and xyloglucan hydrolysis, seemed to be more prominent during growth than during ripening.[41] The study of the expression of cDNA encoding cell wall-modifying enzymes throughout the development process showed that many different enzymes, such as methylesterase, pectate lyase (EC 4.2.2.2), arabinofuranosidase (EC 3.2.1.55), endoglucanase and XET, were transcribed until late softening.[8] All the transcripts, except methylesterase, could be unambiguously detected by semi-quantitative RT-PCR in fruit during ripening. However, transcripts of endoglucanase were more abundant at fruit-set. Two XETs were detected in over-ripe fruit, one of them showing a ripening-related pattern. However, it has to be emphasised that none of the cDNAs identified in this work was fruit specific. It is also interesting to note that some hydrolases showed ripening-related expression patterns whereas limited or no polysaccharide depolymerisation was observed during apple softening.[34] Some of the hydrolases potentially involved in the softening process have been shown in other fruits to have only a limited effect on polysaccharide molecular weight. This is particularly true for a β-xylosidase (EC 3.2.1.37) in *Fragaria ananassa*, differently expressed in two strawberry cultivars with contrasted fruit firmness.[50] The softest cultivar showed an early accumulation of xylosidase transcripts and a higher translation to the protein during ripening. However, xylosidases are often described as bifunctional with both xylosidase and arabinofuranosidase activities. Consequently, it is rather difficult to know the precise role of these enzymes *in vivo*. Glycosidases such as β-galactosidase (EC 3.2.1.23) and α-L-arabinofuranosidase (EC 3.2.1.55) were related to the storability of

apple.[51] A high level of α-L-arabinofuranosidase activity was demonstrated to result from 3 different genes and was related to apple mealiness development during fruit storage.[52] The target cell wall polysaccharide of this activity and the resulting impact on the cell wall structure and mechanical properties involved in this texture defect remains to be established. As arabinan side chains in pectin are likely to interact with cellulose and contribute to the wall structure,[29] even a slight shortening of these chains by an arabinofuranosidase could affect this interaction and weaken the network. In addition, galactosidases, xylosidases, arabinofuranosidases and fucosidases potentially modify the XyG side chains structure and thus could control XET activity[53,54] and finally fruit texture.

3.2. CAZymes in Malus domestica

Previous results showed that variation of polysaccharide structure is associated with variations on some chromosomal regions that also control some variations in sensory as well as instrumental texture measurements.[55] However, the relationship between genetic variations, cell wall structure and apple texture are not known. Putative carbohydrate-active enzymes (CAZymes), including 26 glycosyl hydrolases (GH) and one polysaccharide lyase (PL) were identified in apple (www.cazy.org).[56] Among them, 20 GH and the PL could be involved in cell wall reorganisation (Table I). However, only 2 of them were identified at the protein level, the others being evidenced at the transcript level.

TABLE I. CAZymes in *Malus domestica*; GH: glycosyl hydrolase; PL: polysaccharide lyase

Enzyme	CAZy Family	Entry	Existence
β-Glucosidase	GH1	AEN94900	Evidenced at protein level
Endo-mannanase	GH5	C7A7X7	Evidenced at transcript level
Endo-mannanase	GH5	C7A7X8	Evidenced at transcript level
Endo-mannanase	GH5	C7A7X9	Evidenced at transcript level
Endo-glucanase	GH9	Q6V596	Evidenced at transcript level
XET	GH16	COIRH4	Evidenced at transcript level
XET	GH16	COIRH6	Evidenced at transcript level
XET	GH16	COIRH7	Evidenced at transcript level
XET	GH16	COIRH8	Evidenced at transcript level
XET	GH16	COIRH9	Evidenced at transcript level
XET	GH16	COIRI1	Evidenced at transcript level
XET	GH16	COIRI2	Evidenced at transcript level
XET	GH16	COIRI3	Evidenced at transcript level
XET	GH16	COIRI4	Evidenced at transcript level
β-1,3-Glucanase	GH17	Q6QCC8	Evidenced at transcript level
β-1,3-Glucanase	GH17	Q8RVM2	Evidenced at transcript level
β-1,3 glucanase	GH17	Q8RVM3	Evidenced at transcript level
Endo-polygalacturonase	GH28	P48978	Evidenced at transcript level
β-Galactosidase	GH35	P48981	Evidenced at protein level
α-Arabinofuranosidase	GH51	Q7X9G7	Evidenced at transcript level
Pectate lyase	PL1	Q6U7H9	Evidenced at transcript level

4. CONCLUSIONS

Cell wall softening is an essential feature of fruit ripening resulting from wall polysaccharide modifications. Diverse simultaneous structural changes in pectin, cellulose and hemicelluloses are thought to be responsible for the alteration of cell wall structure. These changes are the result of many different enzymatic activities, organized in consortia capable of a concerted action. However, the function of many of them has not yet been evidenced.

Further work is thus required to identify the enzymatic actors involved in cell wall remodelling and to link cell wall structure and apple texture. Now that the apple genome has been sequenced,[57] this will help the scientific community to identify the genes of interest for each mechanism.

ИЗВОД

УТИЦАЈ ЕНЗИМА КОЈИ МОДИФИКУЈУ ЋЕЛИЈСКИ ЗИД НА КОНЗИСТЕНЦИЈУ СВЕЖЕГ ВОЋА: ПРИМЕР ЈАБУКЕ

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Ћелијски зидови се састоје од полисахарида (пектин, хемицелулоза и целулоза) чије структуре и интеракције зависе од генетике воћа, као и стадијума и услова развоја. Успостављање и структурна реорганизација ових полисахаридних целина зависи од интеракција ензима/протеина који делују у зиду. Конзистенција свежег воћа је један од главних критеријума корисника приликом избора. Она зависи и од путева и процеса трансформације након брања. Разарање полисахарида ћелијског зида воћа веома утиче на његову конзистенцију током сазревања, али тачна улога сваког полисахарида и ензима се не зна. Промене полисахарида ћелијског зида током сазревања најизраженије су кроз промену хемијске структуре пектина дејством хидролаза, лијаза и естераза. Ово реструктурирање, такође, обухвата реорганизацију хемицелулозе дејством хидролаза/ /трансглукозидаза и модификацију интеракције ензима са целулозом посредством некаталитичких протеина као што је експанзин. Јабука је трећа воћка по величини производње у свету и њен квалитет се проучава. У овом раду су наведени подаци о ензимима/протеинима укљученим у сазревање воћа, уз посебан нагласак на јабуку.

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