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Could an Understanding of the Strawberry Softening Process Benefit from Aquaporins?

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ABSTRACT

The Major Intrinsic Protein (MIP) family includes a main group of key channels known as aquaporins (AQPs), described for sharing a very conservative structure with a pore that facilitates water and/or solute permeation. Since its first member was functionally reported in 1992, AQPs were found to be abundantly expressed in all kingdoms. Although many roles have been attributed to these small integral proteins, it is becoming evident that the number and type of AQPs within a membrane are major determinants of its water transport capacity. Thus, their presence is opening new perspectives to understand the role of plant cell membrane water transport in physiological and developmental processes. Strawberry is a fleshy fruit characterized by a rapid loss of firmness during ripening, limiting the shelf-life of these fruit. Even though fruit texture is influenced by various factors like structural integrity of primary cell wall, sugar accumulation, and the turgor pressure generated within cells by osmosis, main attention has been focused on degradation of cell wall polysaccharides. Turgor pressure, in spite of long being mentioned as a possible player in softening during fruit development, has not received proper consideration. In the light of AQP outbreak, it is worth questioning how these channels could contribute to strawberry fruit softening. In an attempt to answer this question, this review summarizes the current available information on plant AQPs, extending the knowledge to those specifically expressed in fruits to finally discuss the recent reported findings in strawberry, particularly those associated with ripening and softening processes.

Keywords: *Fragaria x ananassa*, water channel, turgor, ripening
Abbreviations: **AQP**, aquaporin; **ER**, endoplasmic reticulum; L_p hydraulic conductivity; **MIP**, major intrinsic protein; **NAA**, indole-3-acetic acid; **NIP**, nodulin26-like intrinsic protein; **PIP**, plasma membrane intrinsic protein; P_f, osmotic water permeability; **SIP**, small basic intrinsic protein; **TIP**, tonoplast membrane intrinsic protein

FRUIT RIPENING AND SOFTENING

Fleshy fruit ripening can be defined as a set of physiological and biochemical events leading to changes in fruit color, flavor, aroma and texture. In the case of fruits like strawberry, the division between development and ripening is not well defined, and ripening is considered as part of a continuous developmental process in which several physiological phases can be overlapped (Manning 1993). Strawberry fruit are very sensitive to postharvest decay, making storage difficult and leading to important economic losses. For these reasons the understanding of molecular mechanisms that regulate soft fruit ripening would be very useful.

In the present revision, the aspects which contribute to the final texture of ripe strawberry fruit will be revised with particular emphasis in water status and the role of the transcellular water pathway.

Strawberry fruit ripening

Strawberry is considered a non-climacteric fruit, being auxins produced by the achenes the main hormones that regulate the receptacle ripening process (Given *et al.* 1988). It has been reported that the expression of some genes associated with strawberry fruit ripening is down-regulated by auxins (Manning 1994; Aharoni *et al.* 2002a). At the present time, the role of ethylene in strawberry fruit ripening is not clear and several works have considered a revision of the possible role of this hormone (Bower *et al.* 2003; Iannetta *et al.* 2006). A number of observations have suggested that the low levels of ethylene produced by strawberry could be enough to regulate some ripening aspects (Tian *et al.* 1997; Trainotti *et al.* 2005; Villarreal *et al.* 2009).

As in other species, strawberry fruit development distinguishes several phases: fruit set, fruit growth associated in a first step to cell division and later to cell volume increase,

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and finally, the ripening phase, which overlaps with the last growth phase (Gillaspy *et al.* 1993). It was reported that receptacle growth follows either a single or a double sigmoid curve, depending on the cultivar (Havis 1943; Cheng and Breen 1992), while softening occurs continuously during fruit ripening, being the extension of this process cultivar-dependent (Salentijn *et al.* 2003; Rosli *et al.* 2004).

Loss of firmness in fleshy fruits has been mainly associated to solubilization and depolymerization of cell wall polysaccharides (Rose *et al.* 1998; Brummell and Harpster 2001; Brummell *et al.* 2004). The nature and extension of these processes vary between different species and even among cultivars of the same species. In the particular case of strawberry, the total amount of cell wall decreases during ripening, but no clear correlation was found between cell wall content and softening rate of cultivars with contrasting fruit firmness (Rosli *et al*. 2004).

Although cell wall loosening contributes greatly to texture variations during ripening, it is not the only factor that rules this process. Fruit softening is characterized by the loss of cell adhesion, which is produced by the degradation of the middle lamella. In addition, other aspects as cuticle integrity (Saladié *et al*. 2007) and cell turgor should be considered (Shackel *et al.* 1991; Shiota *et al.* 2006).

Cell wall metabolism

Cell wall disassembly has been traditionally considered as the principal process that leads to fruit softening (Fischer and Bennett 1991). Studies made mainly in climacteric fruits have shown that modification of cell wall polymers is a consequence of the coordinated action of cell wall-modifying proteins (expansins) and enzymes that catalyze the degradation of hemicelluloses: endo-1,4-β-D-glucanases, β-xyloxidases, endo-xylanases, xyloglucan endotransglycosylases, etc.; and enzymes that act on pectins: pectate lyases, polygalacturonases, pectin methylesterases, etc. (Brummell and Harpster 2001).

In strawberry fruit, softening has been suggested to be related to pectin solubilization (Rosli *et al.* 2004). Fruit with antisense suppression of a putative pectate lyase gene showed a significant reduction in pectin solubilization and softening (Jiménez-Bermúdez *et al.* 2002). In addition, polygalacturonase activity and gene expression have been associated to differences in the rate of softening observed between cultivars (Villarreal *et al.* 2008). Also, antisense down-regulation of a polygalacturonase gene (*FaPG1*) in transgenic strawberry plants leads to a diminution in post-harvest softening in comparison to wild type fruit (Quesada *et al.* 2009). Regarding hemicellulose metabolism, although strawberry lines with down-regulated expression of *cel1*, which encodes for an endo-(1,4)-β-glucanase, showed no appreciable reduction of ripening-related fruit softening (Woolley *et al.* 2001), it has been reported a correlation between β-xylosidase activity, *FaXyl1* mRNA, protein accumulation and firmness loss in two cultivars with contrasting firmness suggesting a role for β-xylosidases in strawberry fruit softening (Bustamante *et al*. 2006).

The main firmness reduction in strawberry fruit occurs between the large green and 25% red stages in different cultivars (Rosli *et al.* 2004; Villarreal *et al.* 2008). For example, 'Toyonoka' cultivar shows an intense softening, being its fruit very soft at 100% red stage. On the contrary, fruit from 'Camarosa' and 'Selva' cultivars are firm even at the end of ripening, which turn these varieties particularly apt for commercialization. As the modification pattern of cell wall polymers can vary among species, it is expected that the set of genes which are responsible for fruit softening might also differ.

Changes in water status

It is well known that cell turgor is function of the difference between apoplast total water potential and cell osmotic potential, therefore any increase in apoplastic solutes will negatively affect cell turgor. This is the case of the last phases of maturation of a fleshy fruit where solutes accumulate in the apoplast as ripening progress (Wada *et al.* 2009; Pomper and Breen 1995; Shackel *et al.* 1991). One of the earliest explanations for the loss of turgor in the ripe fruit was proposed by the compartmentation breakdown theory. This theory establishes that in the cell occurs a loss of compartmentation with the consequent solute leakage and loss of cell pressure turgor (Lang and Düring 1991). However, accumulating evidence of measurable cell pressure turgor during ripening (Thomas *et al.* 2006; Wada *et al.* 2009) and the expression of many genes including integral membrane proteins such as sugar transporters and aquaporins (AQPs) (Davies *et al.* 1999; Ageorges *et al.* 2000; Fillion *et al.* 1999; Picaud *et al.* 2003; Mut *et al.* 2008), have strongly lead to abandon this theory. Moreover, Thomas *et al.* (2008) speculated that the cell turgor could play a role as a signal for gene expression and metabolic changes that occur at the onset of ripening in grape berry.

It is important to remark that a reduction in cell turgor has been shown to stimulate sugar uptake in several plant sink tissues as a strategy to maintain this turgor (Daie and Wyse 1985; Wyse *et al*. 1986; Oparka and Wright 1988a, 1988b). In strawberry, solute levels in the apoplast increased as fruit developed from green-white to red while reaching, for instance, concentrations of approximately 50 mM of both glucose and sucrose, and the cell turgor values were shown to decline from 250 to 50 KPa for the green to pink fruit stage transition (Pomper and Breen 1995). This solute accumulation coexists with the progress of fruit softening. However, it was demonstrated that strawberry fruit cells do not have a sugar uptake system stimulated by a reduction in turgor (Pomper and Breen 1996). Therefore it would seem unlikely that strawberry fruit cells are able to respond to, or regulate, the levels of apoplastic solutes through sugar transport. This experimental observation suits with the loss of turgor reported for ripening fruits and supports the hypothesis that a fruit that softens does not maintain cell turgor pressure, not as an aberrant process but as a key physiological event.

In conclusion, it is accepted that the softening process is complex and involves, at the cellular level, cell wall disassembly as well as loss of cell turgor pressure. These events, that are juxtaposed and no yet clearly distinguished by biophysical parameters or gene expression profiles, would contribute to fruit softening.

THE CELL WATER PATHWAY AND THE RIPENING EVENTS

The multifaceted water channels

In certain mammalian organs, biophysical evidences for the regulatory properties of the water pathway through membranes were strongly supporting the idea of a pore-mediated water transport (Parisi *et al.* 1983, 1984a, 1984b) even decades before the discovery of the first water channel (Preston *et al.* 1992). In the particular case of fruit development and ripening, water movements are crucial in every phase since cell division, cell growth and loss of turgor require a strict control of water transport across membranes. However, the plant cell water pathway was never considered as crucial. This was probably due to the combination of two features: i) the water permeability of the lipid bilayer was considered enough and not a limiting factor to meet the cellular water movements requirements, ii) the apoplastic pathway was considered to govern water transport. Consequently, the role of water channels during fruit development received little attention. At the present times, awareness about the participation of water movements in fruit ripening events explains the growing interest to analyze the possible role of AQPs in this process (Hu *et al.* 2003; Picaud *et al.* 2003; Chervin *et al.* 2008; Fouquet *et al.* 2008; Mut *et al.* 2008; Alleva *et al*. 2010).

Why should AQPs be considered? The Major Intrinsic

Proteins (MIP) are a large ancient family of 28-30 kDa transmembrane protein channels (~800 members; http://mipdb.genouest.org) that are grouped together on the basis of sequence similarities. Functional data allows MIP division in at least two subfamilies: the AQPs, which mainly conduct water, and aquaglyceroporins, facilitating the passage not only of water but also of small uncharged molecules like polyols, urea, ammonia, boric acid, etc.

The movement of water and other solutes through AQPs is a passive mechanism driven by the concentration gradient of the transported molecule. Thus, these channels facilitate water and/or small neutral solute movements across biological membranes in a wide range of organisms. AQPs are abundant not only in plasma membrane but also in intracellular membranes (Jauh *et al.* 1998; Ishikawa *et al.* 2005; Uehlein *et al.* 2007) and evidences indicate that they can change the cell hydraulic conductance in a fast and reversible way by modulating membrane water permeability. All these features turn these channels relevant for all physiological cell processes where water movements must be rigorously controlled.

In plants, AQPs are traditionally classified into homology subclasses associated with the most common subcellular localization of each type of water channel (Johanson *et al.* 2001). The larger subclass includes the plasma membrane intrinsic proteins (PIP), which can be further divided into two clusters: PIP1 and PIP2. A second subclass is formed by the tonoplast membrane intrinsic proteins (TIP), while others are NIP (Nodulin26-like intrinsic proteins), or SIP (small basic intrinsic proteins, described as ER channels). However, the panorama cannot be straightforwardly analyzed, as accumulating evidence about unexpected subcellular localization of different type of AQPs is challenging this AQP classification originally based on sequence homology data (Wudick *et al.* 2009). Moreover, a new AQP subfamily has recently been reported, the XIP AQPs, with yet an unclear evidence for their localization (Danielson and Johanson 2008). Still, PIP and TIP remain as the most abundant plant AQPs.

All groups of AQPs are present in a same plant and each of them presents several members. This implies that a plant can express more than 30 AQPs in different tissues and organs, e.g. there are 35, 33 and 31 AQP homologs in Arabidopsis, rice and maize respectively (Chaumont *et al.* 2001; Johanson *et al.* 2001; Sakurai *et al.* 2005) and 28 were identified in the grapevine genome (Fouquet *et al.* 2008). Moreover, co-expression of several AQP types can occur in a single membrane. This high number of AQPs per plant species is probably reflecting tissue and cell specific regulation for water transport under different signals detected by the cell.

Despite this multifaceted character of plant AQPs, structural characteristics of water channels and its transport biophysics are shared between most of the members of the family, including AQPs of other kingdoms.

AQPs present six transmembrane helices linked by five loops (A-E). The N- and C-termini are cytosolic and the loops B and E have the conserved motives Asp-Pro-Ala (NPA) that form the channel pore. This structure, together with an aromatic/Arg (ar/R) motif determines its substrate specificity.

In addition, it has been established that water channels present a tetrameric assembly being each monomer an individual pore. In plants, this structure has been reported for PvTIP3;1 and SoPIP2;1 crystals (Daniels *et al.* 1999; Fotiadis *et al.* 2002; Kukulskia *et al.* 2005; Törnroth-Horsefield *et al.* 2006).

Undoubtedly, the discovery of water channels opened new research fields due to the skill of cells to control and regulate the diffusional water flow through membranes. As mentioned before, water (or solute) transport carried out by AQPs is bidirectional and follows the osmotic gradient through the membrane. However, as other channels, AQPs are subjected to regulatory processes. Consequently, AQPs confer a high and adjustable osmotic water permeability (P_f) or hydraulic conductivity (L_p) to the membranes.

The presence of AQPs should not be considered as relevant only for single cells, since at the tissue and organ levels many non-steady-state physiological processes involve water transport through membranes. As explained by Tyerman *et al*. (1999) time constants might involve longer periods if AQPs do not contribute to transmembrane water flow.

Beyond the accumulated evidence, the contribution of AQPs to water transport is still under study because of the complexity of the picture: a large number of isoforms, the expression control of each of them, and their short or long term regulatory mechanisms.

Controlling water transfer through the cell pathway

The osmotic water permeability (P_f) of a cell membrane is a parameter that reflects water transport capacity, i.e. the ability to conduct water across the membrane in response to a concentration gradient. Thus, the simple diffusion of water through the lipidic membrane is characterized by much lower P_f values when compared to the pore-mediated fast water exchange of AQPs (usually reflected in higher P_f values).

Regulation of water flow through cells can be achieved by either i) a rapid control of the protein itself, regulating its activity, gating or amount (e.g. modifying the pore aperture or by changing the number and/or the type of channel localized in the membrane); ii) a slower adaptive/developmental response, through the regulation of AQP gene expression.

A rapid way to control membrane water permeability is by regulating the activity of the AQP constitutively expressed. As far as from now, it is well known that plasma membrane water channel closure is achieved by dephosphorylation and modification of cell parameters such as cytosolic pH and divalent cation concentration, mainly calcium (Gerbeau *et al.* 2002; Alleva *et al.* 2006; Verdoucq *et al.* 2008). This reversible phenomenon, usually referred as gating, has been extensively studied by means of cytosolic acidification in PIP. The pH gating of PIP is dependent of the protonation state of a conserved histidine located in loop D (Tournaire-Roux *et al.* 2003). Structure-function analyses have also strongly contributed to elucidate this mechanism, based on the X-ray structures of the closed and open conformations of a plant AQP (Törnroth-Horsefield *et al.* 2006). These studies suggested that phosphorylation of two serine residues lying in consensus phosphorylation sites, one in loop B and the other in the C-termini might be involved in channel gating.

Recent evidence supports the idea that a fast water flow modulation could be accomplished by expression of different AQPs in a same membrane. This was particularly investigated in plasma membrane AQPs (PIP). In addition, the positive cooperation, i.e. an increase in the P_f, is obtained if more than one subgroup of PIP interacts physically within a single membrane.

Analysis using affinity-copurification and co-immunopurification techniques provided the first biochemical evidence that PIP1 and PIP2 physically interact in oocytes (Fetter *et al.* 2004). FRET imaging in living maize protoplasts co-expressing PIP1 and PIP2 further supports a model in which AQPs of the two classes directly interact to facilitate PIP1 trafficking (Zelazny *et al.* 2007). This cooperative effect in AQP activity could be the result of an enhanced plasma-membrane targeting of PIP1 promoted by PIP1-PIP2 interaction. However, the functionality of PIPs becomes more complex if we considered that interaction between isoforms is not restricted to PIP1-PIP2, since physical contact was also detected among different PIP1 or different PIP2 forming therefore PIP1-PIP1 and PIP2-PIP2 complexes (Fetter *et al*. 2004; Cavez *et al*. 2009).

Although much progress on plant AQP activity was achieved, is still unclear the functional relevance of these mechanisms. The above-mentioned report about protein-

protein interaction as determinant of AQP targeting is a step in the elucidation of these issues. There are evidences of protein modifications – e.g. glycosylation – that makes possible the relocalization of water channels after osmotic or salt stress (Vera-Estrella *et al.* 2004; Boursiac *et al.* 2008a, 2008b). Membrane proteomics is also assisting in a thorough description of the co- and post-translational modification pattern of AQPs, including the first report of a methylated membrane protein – dimethylation and monomethylation – in plants (Santoni *et al.* 2006).

Finally, gene expression is undoubtedly the longer-term way for the cell to answer to external and/or internal stimulus by adjusting the amount of available protein in the membrane. AQP gene expression is regulated developmentally – e.g. via hormones – and by environmental conditions, e.g. biotic and abiotic stress. As far as from now evidences are showing clearly the absence of a unique expression pattern, e.g. different AQP isoforms can be either up- or down-regulated depending on the stimulus and/or the organ studied. Difference in transcriptional regulation suggests that each isoform has a distinctive role, and this enhances the versatility to control water movements.

Fruit aquaporins

As far as from now, most of the AQP research in plants has been performed in *Arabidopsis thaliana*, *Zea mays* and *Oryza sativa*, predominantly in root or leaf systems, undoubtedly excellent organs not only to elucidate the role of these proteins in the hydraulic conductivity but also to study their contribution under physiological challenges (Forrest and Bhave 2007).

In recent years several laboratories have focused their work in identifying fruit AQPs seeking for their role in the physiology of the fruit. In tomato, it was reported that transgenic lines generated with reduced TRAMP mRNA, a membrane protein related to AQPs, showed increased organic acids and reduced sugar levels during fruit ripening (Chen *et al.* 2001). More recently, it was reported that the expression of genes encoding tomato PIPs changes during fruit development (Shiota *et al.* 2006). Moreover, a putative PIP1 AQP gene was related to fruit development and osmotic stress in apple (Hu *et al.* 2003). In those cases, the authors did not perform a functional characterization of the reported AQPs.

Strawberry and grape berry AQPs have also been reported as proteins involved in ripening and some of these AQPs have been functionally studied by heterologous expression in Xenopus oocytes (Picaud *et al.* 2003; Chervin *et al.* 2008; Fouquet *et al.* 2008; Mut *et al.* 2008; Alleva *et al*. 2010).

In strawberry, two full length cDNAs encoding a PIP1 and a PIP2 subtype AQPs were cloned: FaPIP1;1 (GQ390798) and FaPIP2;1 (GQ390799).

The expression of FaPIP1;1 was detected in fruit and ovaries, while no expression was found in other tissues and organs (Mut *et al.* 2008). The accumulation of FaPIP1;1 mRNA increases during strawberry fruit ripening, particularly in the case of cultivars that produce firm fruits. For example in firm cultivars as Selva and Camarosa, the expression level was low in green fruit, increased in white or 25% red stage and remained high until the end of ripening (Mut *et al.* 2008; Alleva *et al*. 2010). In the case of FaPIP2;1, the higher expression was detected early in ripening (white and 25% red stages), both in a firm and a soft cultivar. However, the expression level of FaPIP2;1 was markedly higher in the firm cultivar along ripening (Alleva *et al*. 2010).

The water transport activity of FaPIP1;1 and FaPIP2;1 was assayed by overexpression of both clones in *Xenopus* oocytes. The overexpression of FaPIP2;1 enhanced greatly water transport activity, while, FaPIP1;1 failed to contribute to water transport through the plasma membrane unless it was co-expressed with FaPIP2;1, suggesting that both AQP subtypes might require a functional interaction (Alleva *et al*. 2010). Although this kind of AQP regulatory mechanism was reported in other species and organisms, this was the first evidence in fruit PIPs. Functional characterization of isolated plasma membrane vesicles from red stage fruit demonstrated the presence of active water channels. As both PIP1 and PIP2 are expressed in PM vesicles, the results are in agreement with the reported water activity in FaPIPs co-expressed in *Xenopus* oocytes (Mut *et al.* 2008; Alleva *et al*. 2010).

AQPs during strawberry ripening were also identified by microarray multigenic expression analysis (Aharoni *et al.* 2002b). In this report, four genes annotated as AQPs (or membrane intrinsic proteins according to the putative function of its closest NCBI database homologues) have been detected as expressed preferentially in fruit receptacles. Three of these putative water channel genes seemed to have a barely higher expression in red stage in comparison with green stage. The fourth, in contrast, presented very different expression pattern, since it expresses at higher level in turning or white stage than in red stage.

Studies performed on other non-climacteric fruits have reported also different expression patterns for each AQP isoform during ripening. For instance, AQP gene expression investigated by means of microarrays during grape berry development indicates a heterogeneous profile: i- VvPIP1;1 showed no variation in expression during berry development, ii- VvPIP1;2, and VvPIP2;2 decreased their expression beginning at the veraison stage, iii- VvPIP1;3 showed a decrease in the amount of transcripts occurring after veraison, and iv- VvPIP2;1 and VvPIP2;3 genes showed an increase of expression at the veraison stage followed by a stabilization (VvPIP2;3) or a decrease of expression (VvPIP2;1) after veraison (Fouquet *et al.* 2008). Moreover, another report based on microarray experiments with various stages of berry development detected two groups of genes involved: cell wall structure and water exchange genes organized in three categories: i- those that remain high during the phase of berry diameter growth, where AQPs might be involved (AQUA1), ii- those that reach the maximum amount at the beginning of the second phase of diameter growth, and iii- genes whose expression was high over both expansion phases of berries, again involving here an AQP (AQUA2) (Chervin *et al.* 2008).

As mentioned previously, in the case of apple AQPs, only a PIP1 was studied, MdPIP1, whose increased expression is in accordance with the volume increase during fruit development (Hu *et al*. 2003).

Another important study relating AQPs to fruit development was reported on tomato PIPs (Shiota *et al.* 2006). As in the other cases, eight AQPs showed distinct expression patterns: LePIP1-1, LePIP1-2 and LePIP2-2 present stronger expression during the earlier phase of development, while LePIP1-5, LePIP2-1 and LePIP2-3 had higher expression level in the later phase; the expression of LePIP1-3 and LePIP1-4 was constant through tomato fruit development.

AQP expression was also analyzed in terms of hormone regulation. In the case of strawberry, auxins were reported as the hormones that mediate signals for the onset of fruit ripening. These hormones present maximum level at both the receptacle and achenes previous to the white stage, and then decline (Given *et al.* 1988). Interestingly, FaPIP1;1, as well as other ripening-related strawberry genes, presented an expression pattern repressed by the presence of auxins (Mut *et al.* 2008). In that work, endogenous auxins level was reduced by removing the achenes, which are the main source of strawberry fruit auxins. FaPIP1;1 mRNA expression was very high in fruits three days after the elimination of the achenes. By contrast, FaPIP1;1 mRNA levels were lower in the tissue where achenes were still present. Furthermore, exogenous application of NAA, a synthetic auxin, caused a decrease of FaPIP1;1 expression. Other AQPs were also described as proteins modulated by hormones during fruit development. For example: i- five AQP genes putatively involved in the regulation of berry ripening have

been detected as abscisic acid responsive proteins (Pilati *et al.* 2007) and, ii- also in grape berries, it was demonstrated that an ethylene treatment performed eight weeks after flowering generated changes in the expression pattern of various AQPs among other genes including those related to the establishment of the cell wall structure (Chervin *et al.* 2008).

The presence of precise patterns of AQP expression during fruit ripening suggests that water channels might be participating in specific processes during ripening. For example, water channels might be associated with the main mechanisms involved in cell division and cell expansion (Fouquet *et al.* 2008; Maurel *et al.* 2009) as in these events water must flow into cells to increase their volume. However, fruit softening involves the reduction of cell turgor, a process subjected to cell water efflux capacity that could include the participation of AQPs.

Brummell and Harpster (2001) have carried out an interesting comparison between fruit softening and fruit growth, as both events strongly require cell wall disassembly: in growth, to expand the cells and then to reconstruct the cell wall; in softening, to achieve the final fruit texture. However, while a progressive disassembly of the cell wall network occurs during softening and cell growth, changes in the cell wall are not equally achieved in both processes. For example, there are *ripening-related* expansins and *expansion-related* expansins, suggesting that each expansin isoform would be strictly involved in one of the wall modifying mechanisms.

A similar scenario seems to occur in the case of AQPs; a multigenic protein family including multiple isoforms, which play different roles and can interact to achieve a final physiological status in the fruit.

FINAL REMARKS

The complexity of the softening process arises from the variety of metabolic events occurring at the same time or at least as a very sophisticated temporal-organized process. Many enzymatic activities capable of cell wall disassembly have been investigated as responsible for the softening of fleshy fruits (Brummell and Harpster 2001). The enzymes known to be involved in this process are encoded, as also seems to be the case of AQPs, by multi-gene families (White 2002). This undoubtedly contributes to the difficulty of elucidating the molecular basis of softening.

Although much progress in the understanding of strawberry softening has been made, this interesting and important process is still poorly understood and remains an active research area. As it is evident from the reports up to date about AQPs in ripening, the pattern of expression of these channels is complex. Additionally, it is not clear yet if all AQP isoforms act as water channels or solute transporters. It is also important to consider that every fruit presents its own developmental phases. Taken all these aspects into account it is not straightforward that AQPs are channels involved in water transport only for cell expansion. As noted by Fouquet *et al*. (2008), spatio-temporal regulation along fruit development may be linked to particular physiological events which could involve specialized and different functions for some AQPs. Functional analysis of each isoform and real tissue location of them by – for instance, *in situ* hybridization techniques – are still needed to understand the role played by single AQPs in the whole process of ripening. Our proposal is to consider that AQPs could be key participants in the softening events since loss of turgor reported needs water and solute mobilization besides cell wall disassembly. AQPs provide a molecular basis for transmembrane water transport, thus not only they broaden water transport capacity through the membrane but also introduce its versatility through their regulatory mechanisms. The availability of strawberry cultivars with different softening rate turns as a very useful tool to understand the participation of AQPs and other softening-related proteins.

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