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ORIGINAL PAPER

Activity levels of six glycoside hydrolases in apple fruit callus cultures depend on the type and concentration of carbohydrates supplied and the presence of plant growth regulators

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Abstract Sucrose presence and concentration modulated in different ways and to different extents the activity of six plant glycoside hydrolases (PGHs) extracted from apple callus cultures, both in the water soluble fraction (WS-F) and in the NaCl-released fraction (NaCl-F). β-D-Glucosidase activity increased because of sucrose starvation and the addition of sucrose decreased both WS-F and NaCl-F β-D-glucosidase from calli grown in a Murashige and Skoog's basal medium with (MSH) or without (MS0) plant growth regulators (PGRs). WS-F and NaCl-F α-L-arabinofuranosidase, NaCl-F β-D-galactosidase and NaCl-F β-D-xylosidase activity reached a maximum when 0.045 M sucrose was added to the MS0 medium with an ensuing decline at higher sucrose concentrations. α-D-Galactosidase and α-D-xylosidase activity reached a maximum when 0.045 M sucrose was supplied and did not decline significantly in 0.09 M sucrose-supplied calli. When the effects of PGR presence or absence were analysed, NaCl-F β-D-glucosidase, α-D-galactosidase, β-D-galactosidase, α-D-xylosidase and β-D-xylosidase activities were found to be higher in MS0 than in MSH. To assess whether sugar effects were sucrose-specific, other sugars (glucose, fructose, galactose, maltose, lactose, raffinose, sorbitol and mannitol) were tested, with or without PGR supplementation. In general, sugar alcohols (mannitol, sorbitol) and some monosaccharides (fructose and glucose in particular) were better inducers of NaCl-F α-L-arabinofuranosidase, β-D-galactosidase and β-D-xylosidase activity than disaccharides (sucrose, maltose, and lactose) or the trisaccharide raffinose. This trend was not widespread to all PGHs assessed since sucrose-supplemented calli displayed higher NaCl-F α-D-galactosidase than those supplemented with glucose, galactose, sorbitol or mannitol. These results show that sugars supplied to callus tissue cultures as a carbon source can also modulate PGH activity. Modulation is different for each PGH, sugar-specific and, at least in the case of sucrose, concentration-dependent. Results also suggest the existence of regulatory interactions between PGRs and sugars as part of an intricate sensing and signalling network. Combination of PGR, sugar type and concentration should be taken into account to maximize each PGH activity for further enzyme studies.

Keywords *Malus* × *domestica* · α-L-Arabinofuranosidase · α- and β-D-Galactosidase · α- and β-D-Xylosidase · β-D-Glucosidase · Sugars

Abbreviations

α-Araf	α-L-Arabinofuranosidase
BA	6-Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
FRU	Fructose
GAL	Galactose
α-Gal	α-D-Galactosidase
β-Gal	β-D-Galactosidase
GLC	Glucose

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β-Glc	β-D-Glucosidase
LAC	Lactose
MAL	Maltose
MAN	Mannitol
MS0 medium	Murashige and Skoog (1962) medium; basal medium with no PGRs
MSH medium	MS medium + 1 mg l^{-1} 2,4-D + 0.1 mg l^{-1} BA
NaCl-F	Sodium chloride-released fraction
PGH	Plant glycoside hydrolase
PGR	Plant growth regulator
RAF	Raffinose
SOR	Sorbitol
SUC	Sucrose
WS-F	Water soluble fraction
α-Xyl	α-D-Xylosidase
β-Xyl	β-D-Xylosidase

Introduction

Plant cell-wall polysaccharides are the most abundant organic compounds found in nature and a large number of proteins identified by cell-wall proteomic analyses act on carbohydrates. Numerous enzymes and proteins are involved in the structural and metabolic regulation of the various cell-wall polymers (Showalter 1993). These include plant glycoside hydrolases (EC 3.2.1.; PGHs) which have several functions of great importance in cell-wall metabolism, plant defence, signalling, mobilisation of storage reserves and reorganisation of glycans (Goulao and Oliveira 2008; Henrissat et al. 2001; Minic 2008; Minic et al. 2007). Although great progress has been made in unravelling the multiple functions of PGHs, a good deal of other aspects remains unknown. The functions assigned to PGHs are provisional because their substrates in vivo, the consequences of their biological roles in muro and the potential entry of sugars from cell wall turnover into intracellular metabolic pathways have not yet been fully unravelled (Jamet et al. 2006).

Several PGHs are bound to the cell wall where they may potentially trim terminal non-reducing glycosyl residues off various pectic and hemicellulosic homo- and heteropolysaccharides as well as from different glycoconjugates (Rose et al. 2003; Sozzi et al. 2002). The structural complexity of cell-wall polysaccharides makes it difficult to determine the specific sites at which these enzymes may introduce functionally significant cell-wall changes. On the other hand, other PGHs are not bound to the cell wall, and may recognize moieties located in different molecules but their specific substrates and functions still remain to be defined. In addition, some PGHs seem to be multifunctional enzymes that possess catalytic domains belonging to different enzyme families.

During plant life, carbohydrates play an important role in various physiological functions. Sugars are not only crucial carbon and energy sources but also play roles such as signalling molecules and have acquired important regulatory functions early in plant evolution controlling metabolism, stress resistance, growth, and development. Lately, the pivotal role of sugars as signalling molecules and their profound effects on plant growth and development have become increasingly apparent (Koch 2004). In some cases, plant genes/enzymes are directly modulated by carbohydrates and appear to be part of a basic system responding to nutrient availability. In spite of comprehensive reviews being published on various aspects of sugar regulation in plants (Koch 1996, 2004; Rolland et al. 2006; Smeekens 2000), a great deal remains to be learnt about the exact mechanisms involved in the role of sugars as signalling molecules and their scope.

Not surprisingly, intricate regulatory interactions with plant hormones are an essential part of the sugar sensing and signalling network. Genetic analyses have revealed extensive interactions between sugar and plant hormone signalling (Rolland et al. 2006). In addition to photosynthesis and breakdown of sucrose (SUC) and starch, the hydrolysis of cell-wall polysaccharides likely generates sugar signals as well. Sugars themselves can signal alterations in gene expression and activity similar to the concepts developed for hormones. While hormones are purpose-built molecules, which are functional in the nano to micromolar range, sugars take part in intermediary metabolism and are present in the millimolar range. Sugar sensing can be defined as the interaction between a sugar molecule and a sensor protein in such a way that a signal is generated. This signal then initiates signal transduction cascades resulting in cellular responses such as altered gene expression and enzyme activity (Smeekens 2000).

Significant progress has been made in sugar sensing research lately but little progress has been made in identifying carbohydrates that modulate the PGH activity in plant tissues. In *Arabidopsis thaliana*, the expression of some PGH genes, which are assumed to release glycosyl residues from the cell wall, increases in response to sugar starvation (Lee et al. 2007). However, studies about conditions of sugar depletion or abundance are difficult to perform in whole plants. Besides, plant sugar signalling has proven to be difficult to study due to the complexity of source-sink interactions, responses to different sugar signals, and the close integration of a complex signalling network ruled by plant hormones, nutrients, and environmental conditions (Rolland et al. 2006). In fact, different problems have hindered research in this area: entry and distribution of PGRs and sugars are uncertain since studies

with intact plant organs require vacuum infiltrations, sprays or dips of the organ in different treatment solutions and each organ requires a singular treatment depending on its anatomy and morphology. Previous reports proved that apple callus tissue culture is an artificial biological system providing a convenient easy-to-handle source of plant material, which can be rapidly regenerated, and from which cell-wall enzymes may be obtained without difficulty (Alayón-Luaces et al. 2008; Liu et al. 2006). Since callus cultures can continue to grow in the basal medium with no plant growth regulators (PGRs) due to the so-called "habituation" phenomenon (Alayón-Luaces et al. 2008), this system allows comprehensive studies involving the addition of different carbohydrate types and/or concentrations with or without any addition of PGRs at physiological levels.

The objective of this work was to characterize the activity of six PGHs: α-L-arabinofuranosidase [α-Araf; EC 3.2.1.55], β-D-glucosidase [β-Glc; EC 3.2.1.21], α- and β-D-xylosidase [α-Xyl and β-Xyl; EC 3.2.1.- and EC 3.2.1.37, respectively] and α- and β-D-galactosidase [α-Gal and β-Gal; EC 3.2.1.22- and EC 3.2.1.23, respectively] from apple calli, as affected by SUC concentration with or without PGR supplementation. In addition, the effect of nine different carbohydrates, with or without PGR supplementation was also evaluated.

Materials and methods

Callus culture and chemicals

Immature fruit (75-day-old) of apple (*Malus* × *domestica* Borkh.) cv. Anna was collected and used. Primary callus explants were obtained from the enlarged fleshy receptacle. Each explant consisted of a 5-mm apple-tissue cube which was surface sterilized as previously described (Alayón-Luaces et al. 2008).

Primary callus explants were placed individually in 11-ml sterilized glass tubes containing 3 ml of basal Murashige and Skoog (1962) medium (MS medium) supplemented with 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg l^{-1} 6-benzyladenine (BA) as PGRs, and 3% SUC as the energy source (Skirvin et al. 1986). The pH of each medium was adjusted to 5.8 with either KOH or HCl prior to the addition of 0.65% agar (Sigma, A- 1296). Tubes were covered with aluminium foil and autoclaved at 0.101 MPa for 20 min. Tubes containing the explants were sealed with Resinite AF50® (Casco S.A.C. Company, Buenos Aires) and incubated at 27 ± 2°C in darkness for 30 days. Then, calli were subcultured at 30 day-intervals in a proliferation medium (Skirvin et al. 1986) consisting of fresh MS medium + 1 mg l^{-1} 2,4-D + 0.1 mg l^{-1} BA (MSH medium). All chemicals were from Sigma Chemical Co. (St. Louis, MO, USA).

Effects of different SUC concentrations

Since SUC is crucial as a plant carbon source and for the initiation of hexose-based sugar signals, it was used to evaluate the effect of sugar concentrations. Calli were subcultured three times at 30-day intervals. Then, the basal medium with no PGRs (MS0 medium) and the MSH medium were supplemented with 0, 0.045 or 0.09 M SUC. After 30 days, actively growing calli were harvested, frozen in liquid nitrogen and stored at −70°C until used.

Effects of different carbohydrates and callus growth

Calli were subcultured five times at 30-day intervals. Then, each sugar at a concentration of 0.09 M was added to the basal medium alone (MS0 medium) or supplemented (MSH medium) with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BA. Three monosaccharides [glucose (GLC), fructose (FRU) and galactose (GAL)]; three disaccharides [sucrose (SUC), maltose (MAL) and lactose (LAC)]; one trisaccharide [raffinose (RAF)]; and two sugar alcohols: [sorbitol (SOR) and mannitol (MAN)] were tested.

After 30 days, callus growth was assessed visually according to a four-stage scale, as follows: 1 (very low): callus size increased twofold; 2 (low): callus size increased threefold; 3 (moderate): callus size increased fourfold; 4 (high): callus size increased fivefold. Twenty independent replicates (tubes) per treatment were evaluated. Then, calli were harvested, frozen in liquid nitrogen and stored at −70°C until used.

α-L-Arabinofuranosidase, α- and β-D-galactosidase, β-D-glucosidase and α- and β-D-xylosidase activity

Glycoside hydrolases were extracted from 0.5 g of callus samples. Water-soluble fraction (WS-F) and NaCl-released fraction (NaCl-F) of enzyme solution were obtained as per Alayón-Luaces et al. (2008).

α-Araf, α-Gal, β-Gal, β-Glc, α-Xyl and β-Xyl activity was measured using the corresponding *p*-nitrophenyl derivatives as substrates, namely: *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-xylopyranoside, and *p*-nitrophenyl-β-D-xylopyranoside, respectively. The reaction mixture consisted of 125 µl of 100 mM citrate buffer, pH 4.5, 100 µl of 0.1% (w/v) bovine serum albumin, 100 µl of enzyme solution (or an appropriate dilution) and 100 µl of 20 mM substrate solution. The reaction was stopped after 60 min at 37°C, by addition of 425 µl of 0.13 M Na_2CO_3. Blanks

(0-time) were prepared by adding Na_2CO_3 prior to the addition of the substrate and absorbance was measured at 400 nm (Sozzi et al. 1999). Free *p*-nitrophenol was used as standard. One unit of enzyme activity was defined as the amount of enzyme that catalyses the liberation of 1 μg *p*-nitrophenol min^{-1} mg^{-1} protein. Enzyme activity calculations were based on enzyme dilutions having a linear increase in free *p*-nitrophenol over a 2-h incubation period. Three independent replicates per treatment were measured.

Protein concentration was assayed by the Coomasie Blue G dye-binding method using the Bio Rad reagent (Bio Rad, Richmond, CA, USA) and bovine serum albumin as standard.

Statistical analysis

Three independent replicates per treatment were prepared and measured for each glycosidase activity and results were shown as the mean ± SD of those three replicates. Statistical significance was determined by two-way ANOVA with the PC-SAS software package (SAS Institute Inc., Cary, NC, USA). The model assumptions of homogeneity of variance and normality were probed by means of Levene's and Shapiro–Wilk's tests, respectively. When these assumptions were not satisfied, data were transformed into ranks (Conover and Iman 1981) for further analysis. When a significant *F*-value was found, treatment means were compared using the Tukey's studentized range test ($P < 0.05$).

Results and discussion

Effects of different SUC concentrations on glycosyl hydrolase activity

To investigate the possible role of SUC concentration on the modulation of glycoside hydrolase activity, 0, 0.045 or 0.09 M SUC was added to the culture medium, with or without PGR supplementation (Table 1). α-Araf, β-Glc, β-Gal and β-Xyl activity was mainly detected in the NaCl-F as previously described (Alayón-Luaces et al. 2008). This activity was assumed to belong to "cell-wall enzymes", obtained from homogenisation with a saline buffer after successive washings of the cell-wall pellet with water to remove cytosolic proteins. On the other hand, WS-F and NaCl-F α-Xyl activity was hardly noticeable in apple callus cultures (Table 1), as previously stated (Alayón-Luaces et al. 2008). Because cell-wall glycosidases may be highly dynamic enzymes responsible for diverse aspects of cell wall properties, we analysed NaCl-F glycosidases with more detail.

When the effects of PGR presence or absence were examined, NaCl-F β-Glc, α-Gal, β-Gal, α-Xyl and β-Xyl

Table 1 Effect of different sucrose concentrations on the activity of six glycoside hydrolases: α-L-arabinofuranosidase; β-D-glucosidase; α-D-galactosidase; β-D-galactosidase; α-D-xylosidase; and β-D-xylosidase

Enzyme	Fraction	Sucrose concentrations					
		0 M		0.045 M		0.09 M	
		Medium		Medium		Medium	
		MS0	MSH	MS0	MSH	MS0	MSH
α-Araf	WS-F	2.1 ± 0.6 Ab	2.2 ± 0.6 Ab	5.9 ± 0.3 Aa	3.7 ± 0.6 Aa	2.5 ± 0.2 Ab	3.4 ± 0.0 Ab
	NaCl-F	23.8 ± 4.8 Ab	34.9 ± 10.5 Ab	128.5 ± 17 Aa	32.2 ± 6.6 Aa	7.3 ± 1.3 Ab	18.2 ± 2.2 Ab
β-Glc	WS-F	6.1 ± 2 Aa	5.7 ± 1.3 Aa	0.7 ± 0.1 Ab	0.7 ± 0.1 Ab	1.3 ± 0.1 Ab	0.7 ± 0.1 Ab
	NaCl-F	134.7 ± 16 Aa	125.8 ± 19 Ba	57.7 ± 7.7 Ab	27.3 ± 10.2 Bb	55.9 ± 3 Ab	15.5 ± 4.7 Bb
α-Gal	WS-F	3.9 ± 1.2 Ab	8.9 ± 2.7 Ab	32 ± 5.9 Aa	18.3 ± 5.4 Aa	22.6 ± 4.8 Aa	15.5 ± 1 Aa
	NaCl-F	21.7 ± 3.6 Ab	17.7 ± 2.5 Bb	51.6 ± 5.1 Aa	45 ± 4.7 Ba	62.3 ± 14 Aa	50.8 ± 6.1 Ba
β-Gal	WS-F	8.5 ± 2 Aa	24.9 ± 5.1 Aa	36 ± 2 Aa	18.4 ± 2.9 Aa	23.1 ± 3.9 Aa	16.4 ± 1.1 Aa
	NaCl-F	80.3 ± 20.2 Ab	76.9 ± 18.8 Bb	391.3 ± 38 Aa	93 ± 25 Ba	97.1 ± 22.3 Ab	46.8 ± 3.7 Bb
α-Xyl	WS-F	0.2 ± 0.1 Bb	0.2 ± 0.1 Ab	0.4 ± 0.1 Ba	0.7 ± 0.1 Aa	0.6 ± 0.2 Ba	0.8 ± 0.3 Aa
	NaCl-F	1.7 ± 0.8 Ab	0.1 ± 0.1 Bb	2.9 ± 0.9 Aa	1.3 ± 0.3 Ba	1.8 ± 0.4 Aab	1.4 ± 0.3 Bab
β-Xyl	WS-F	0.6 ± 0.2 Ab	0.4 ± 0.1 Bb	1.2 ± 0.1 Aa	0.5 ± 0.1 Ba	0.7 ± 0 Aab	0.7 ± 0.1 Bab
	NaCl-F	16.6 ± 2.8 Aab	13.6 ± 2.9 Bab	75.3 ± 16.1 Aa	10.4 ± 4.2 Ba	2.2 ± 0.5 Ab	4.1 ± 2.1 Bb

Enzyme activity was assessed in the water-soluble fraction (WS-F) and the NaCl-released fraction (NaCl-F) from apple calli treated (MSH) or not (MS0) with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BA in combination with different sucrose concentrations (0, 0.045 or 0.09 M)

Each value represents the mean ± SD of three replicates. Different small letters within each row indicate significant ($P > 0.05$) differences between treatments with different SUC concentration. Different capital letters within each row indicate significant ($P > 0.05$) differences between MS0 and MSH

activity was found to be higher in MS0 than in MSH, whereas no differences were found for α-Araf activity (Table 1). This agrees with previous results suggesting that the activity of glycoside hydrolases is differentially modulated by auxins, cytokinins, abscisic acid and gibberellic acid, and that 2,4-D and BA supplementation does not increase the activity of NaCl-F α-Araf, β-Glc or β-Xyl (Alayón-Luaces et al. 2008).

When sucrose concentration was analysed, WS-F α-Araf activity from MS0 callus supplemented with 0.045 M SUC was higher than with 0.09 M and 0 M (2.36- and 2.81-fold, respectively) (Table 1). NaCl-F α-Araf activity showed a similar trend, being approximately 1,660 and 440% higher in MS0 callus supplemented with 0.045 M SUC than in those with 0.09 M and 0 M SUC respectively (Table 1).

WS-F α-Gal activity reached a maximum when 0.045 M SUC was supplied but did not decline significantly when SUC concentration increased to 0.09 M regardless of the presence of PGRs (Table 1). NaCl-F α-Gal activity reached a maximum in a medium enriched with 0.045-0.09 M SUC (Table 1). As with other glycosidases (see below), SUC presence positively modulates α-Gal activity in callus tissue cultures and such behaviour may differ from that in other plant systems. Chrost et al. (2004) found that two genes encoding α-Gal isoenzymes in barley primary leaves are induced by senescence caused by darkness.

Cell-wall β-Gal catalyzes the hydrolysis of terminal non-reducing β-D-galactopiranosyl residues from various pectic (galactans and arabinogalactans, typical side-chains of rhamnogalacturonans) and hemicellulosic (galactomannans, galactoglucomannans, etc.) polysaccharides. In apple, β-Gal gene expression proved not to be tissue-specific (Goulao et al. 2008). Upon addition of different sucrose concentrations, NaCl-F β-Gal and NaCl-F β-Araf activity from calli grown in an MS0 medium displayed a similar trend (Table 1). In an MS0 medium, NaCl-F β-Gal activity rose ~4.9- and fourfold in cultures supplemented with 0.045 M SUC in comparison with those with no SUC and with 0.09 M SUC respectively.

Three classes of plant enzymes which degrade arabinoxylan have been identified: endoxylanases (EC 3.2.1.8), β-Xyl and α-Araf. Endoxylanases and β-Xyl may be responsible for cleavage of xylan backbone groups while α-Araf may remove side-chain arabinose substituents from xylan or oligoxylan (Minic 2008; Minic and Jouanin 2006). NaCl-F β-Xyl activity increased 4.52-fold in calli cultured in an MS0 medium treated with 0.045 M SUC, in comparison with those devoid of SUC. In contrast, NaCl-F β-Xyl activity decreased by 97% in MS0 cultured calli supplemented with 0.09 M SUC in comparison with those with 0.45 M SUC (Table 1). In any case, modulation of β-Gal and β-Xyl by sucrose seems to differ according to the species and the system utilized. In Arabidopsis, Lee et al. (2007) examined the expression of genes encoding putative β-Gal and β-Xyl proteins that could play roles in cell-wall polymer modifications. Induction of these genes was repressed in suspension cells grown in an MSH medium with 3% SUC, and an accumulation of β-Gal protein during sugar starvation was found.

On the contrary, β-Glc activity increased under SUC starvation conditions and the addition of SUC diminished both WS-F and NaCl-F β-Glc, regardless of the concentration applied (Table 1). Our results agree with those of Lee et al. (2003) reporting that the expression of an Arabidopsis β-Glc gene (At3g60140) was detected 12 h after sugar starvation. In addition, the expression of this gene (also reported as dark-inducible gene 2) encoding a putative cell wall-related β-Glc is up-regulated in response to darkness or leaf senescence (Fujiki et al. 2001) thus suggesting its relationship with stress conditions. In callus, β-Glc activity is also up-modulated under PGR starvation or Picloram addition (Alayón-Luaces et al. 2008) and could be engaged in utilizing β-glycoside conjugates as carbohydrate sources. This may also happen under other stress conditions such as leaf senescence or photosynthesis inhibition (Fujiki et al. 2001). β-Glc is widely found in prokaryote and eukaryote species and catalyzes the hydrolysis of the β-D-glycosidic bonds in a variety of natural β-glycoside conjugates (Poulton 1990).

Effects of different carbohydrates on glycosyl hydrolase activity

Genetic analyses have revealed extensive interactions between sugars and plant hormone signalling (Rolland et al. 2006). To assess whether sugar effects were SUC-specific, other sugars were tested with or without PGR supplementation (Tables 2, 3, 4).

PGH activity in callus cultures showed different trends in the MS0 and MSH medium. While no definite general glycosidase activity pattern was noticed when supplementing the cultures with sugars and PGRs simultaneously, calli grown in an MS0 medium displayed higher NaCl-F α-Araf, β-Glc, β-Gal and β-Xyl activity than those grown in an MSH medium (Tables 2, 3, 4).

In most cases, the type of sugar added to the medium changed the activity of α-Araf (Table 2), β-Gal (Table 3) and β-Xyl (Table 4). This became more apparent in the NaCl-F due to the higher activity levels of these PGHs. Not surprisingly, NaCl-F β-Xyl followed a trend similar to that of NaCl-F α-Araf (Tables 2, 4). Many plant α-Araf/β-Xyl genes encode bifunctional proteins displaying both α-Araf and β-Xyl activities in vitro but only capable of catalyzing arabinose release from native cell-wall polysaccharides (Tateishi et al. 2005).

Table 2 Effect of different carbohydrates on the activity of α-L-arabinofuranosidase and β-D-glucosidase

Sugar	Medium	α-Araf		β-Glc	
		WS-F	NaCl-F	WS-F	NaCl-F
SUC	MS0	4.4 ± 2.3 Aab	16.2 ± 5.2 Ad	2.5 ± 1.6 Ab	49 ± 27 Ab
	MSH	3 ± 0.3 Aab	6.2 ± 1.2 Bd	0.7 ± 0.1 Bb	28.3 ± 0.8 Bb
GLC	MS0	4.2 ± 1.4 Aab	49.8 ± 17.6 Abc	1.3 ± 0.4 Ab	40.2 ± 14.6 Ab
	MSH	4.1 ± 1.4 Aab	35.9 ± 3.1 Bbc	1.1 ± 0.2 Bb	32.1 ± 5.4 Bb
FRU	MS0	6 ± 2.8 Aab	81.3 ± 23.5 Aab	2.6 ± 0.2 Ab	111 ± 67 Aab
	MSH	5.7 ± 1.1 Aab	45 ± 1.7 Bab	1 ± 0.2 Bb	20.3 ± 3.5 Bab
GAL	MS0	4.1 ± 2.5 Aab	39.8 ± 22.2 Abc	2.9 ± 1.7 Ab	77 ± 15.2 Ab
	MSH	4.4 ± 0.4 Aab	39.1 ± 3.3 Bbc	2 ± 0.5 Bb	36 ± 7.5 Bb
MAL	MS0	3.9 ± 1.8 Aab	11.3 ± 6.1 Ad	3.3 ± 1.6 Ab	48.9 ± 16.7 Ab
	MSH	2.8 ± 0.2 Aab	6.5 ± 2.3 Bd	1 ± 0.4 Bb	22.6 ± 5.5 Bb
LAC	MS0	2.4 ± 0.3 Ab	39.8 ± 12.2 Acd	2.8 ± 0.4 Ab	44.5 ± 4.3 Ab
	MSH	3.2 ± 0.8 Ab	27.5 ± 7.4 Bcd	1.3 ± 0.2 Bb	24 ± 7.2 Bb
RAF	MS0	4.5 ± 1.2 Aab	36.3 ± 27.1 Acd	2.6 ± 1 Ab	50.5 ± 5.8 Ab
	MSH	3 ± 0.4 Aab	24.4 ± 5.7 Bcd	0.8 ± 0.1 Bb	33.9 ± 7.6 Bb
SOR	MS0	5.5 ± 1.8 Aab	53.4 ± 15.4 Abc	1.5 ± 0.3 Ab	53.1 ± 17 Ab
	MSH	3.7 ± 0.7 Aab	33.9 ± 8.7 Bbc	1.1 ± 0.1 Bb	28.9 ± 0.9 Bb
MAN	MS0	4.4 ± 2.1 Aa	76.8 ± 16.5 Aa	10.5 ± 3.5 Aa	154 ± 82 Aa
	MSH	8.4 ± 2.5 Aa	65.1 ± 8.6 Ba	8.2 ± 1.2 Ba	70 ± 17 Ba

Enzyme activity was assessed in the water-soluble fraction (WS-F) and the NaCl-released fraction (NaCl-F) from apple calli treated (MSH) or not (MS0) with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BA, in combination with 0.09 M of each of the following sugars: sucrose (SUC), glucose (GLC), fructose (FRU), galactose (GAL), maltose (MAL), lactose (LAC), raffinose (RAF), sorbitol (SOR) and mannitol (MAN)

Each value represents the mean ± SD of three replicates. Different small letters within each column indicate significant ($P > 0.05$) differences between sugar treatments. Different capital letters within each column indicate significant ($P > 0.05$) differences between MS0 and MSH

On the other hand, NaCl-F β-Glc activity was always higher in calli grown in the absence of PGRs but the extent of the increase in comparison with those grown with PGRs was largely dependent on the sugar added to the medium: GLC (26%), RAF (49%), SUC (74%), SOR (84%), LAC (85%), GAL (115%), MAL (116%), MAN (120%) and FRU (450%) (Table 2).

In most cases, some monosaccharides (FRU in particular) were better inducers of NaCl-F α-Araf, β-Gal and β-Xyl activity than disaccharides (SUC, MAL, LAC) or trisaccharides (RAF), and as good as sugar alcohols (MAN and/or SOR) (Tables 2, 3, 4). FRU and MAN were more effective ($P < 0.05$) to enhance NaCl-F α-Araf activity than SUC, MAL, LAC and RAF, while GLC and SOR were more effective than SUC and MAL (Table 2). FRU-, GLC- and MAN-supplemented calli displayed a higher NaCl-F β-Gal activity than those supplemented with SUC, MAL, or LAC (Table 3). Also, NaCl-F β-Xyl activity was higher in callus cultures supplemented with MAN, GLC, FRU or SOR, than in those supplemented with SUC, MAL, RAF or LAC (Table 4). Thus, results obtained using apple calli are in contrast with those found for other species and systems. Lee et al. (2007) reported that Arabidopsis β-Gal (At5g56870) and β-Xyl (At5g49360) gene expression is repressed in suspension cells grown with GLC, GAL, and FRU, an effect similar to that found using SUC. Our experiments showed that some of these monosaccharides can induce higher activities than SUC for NaCl-F β-Xyl and β-Gal in apple calli.

On the contrary, MAN induced higher NaCl-F β-Glc activity than SUC, GLC, GAL, MAL, LAC, RAF and SOR, and higher WS-F β-Glc than any other carbohydrate tested (Table 2). In Arabidopsis suspension cells, β-Glc (At3g60140) gene is repressed by GLC, FRU and GAL but not by MAN (Lee et al. 2007).

Although SUC is the major photosynthetic product and transport sugar in plants, many signalling effects on growth and metabolism can be attributed to the action of its hydrolytic hexose products –glucose and fructose– or their downstream metabolic intermediates (Rolland et al. 2006). SUC is the most widely used energy source in plant in vitro cultures but in our experiments SUC as well as the other two disaccharides tested did not induce the higher PGH activity, except for NaCl-F α-Gal (Table 3) and α-Xyl (Table 4). If some PGHs promote the disassembly of cell-wall polysaccharides or polysaccharide domains and contribute to the changes in cell-wall architecture, some monosaccharides such as GLC and FRU could be involved

Table 3 Effect of different carbohydrates on the activity of α-D-galactosidase and β-D-galactosidase

Sugar	Medium	α-Gal		β-Gal	
		WS-F	NaCl-F	WS-F	NaCl-F
SUC	MS0	18.9 ± 3.4 Aab	28.1 ± 7.1 Ba	16.1 ± 4.6 Ac	65.8 ± 18.6 Ab
	MSH	7.5 ± 0.9 Bab	54 ± 2.1 Aa	11.3 ± 0.9 Bc	25.4 ± 4.9 Bb
GLC	MS0	20.4 ± 4.7 Aab	19 ± 4.5 Bbc	28 ± 6.1 Abc	158 ± 16.9 Aa
	MSH	11.3 ± 2.5 Bab	32 ± 5.8 Abc	15.7 ± 5.9 Bbc	128 ± 8.8 Ba
FRU	MS0	24 ± 10.6 Aa	20.7 ± 9.6 Babc	43.6 ± 30.1 Aab	181 ± 75.6 Aa
	MSH	27.3 ± 6.7 Ba	40.3 ± 8.9 Aabc	23.7 ± 5.9 Bab	108 ± 16.4 Ba
GAL	MS0	31.3 ± 11.3 Aab	25.2 ± 11.2 Bbc	15 ± 7 Ac	78.9 ± 21 Aab
	MSH	14.8 ± 2.2 Bab	22.6 ± 1.9 Abc	9.9 ± 2.9 Bc	105 ± 17.1 Bab
MAL	MS0	14.5 ± 1.3 Aab	31.7 ± 6.3 Babc	15.1 ± 3.4 Ac	56.6 ± 17.4 Ab
	MSH	11.7 ± 2.4 Bab	34.4 ± 2.8 Aabc	10.5 ± 1.3 Bc	27 ± 4 Bb
LAC	MS0	15.1 ± 2.4 Ab	17.2 ± 1.8 Babc	19.9 ± 1 Abc	75 ± 2.4 Ab
	MSH	8.5 ± 1.9 Bb	36.5 ± 4.4 Aabc	11.4 ± 6.5 Bbc	49.7 ± 15.1 Bb
RAF	MS0	18 ± 5.1 Aab	30.6 ± 7.4 Bab	26.9 ± 10.5 Abc	117 ± 26.2 Aab
	MSH	15 ± 7.2 Bab	44.6 ± 9.7 Aab	13.4 ± 6.2 Bbc	57.8 ± 4.8 Bab
SOR	MS0	26 ± 5.9 Aab	19.9 ± 8.2 Bcd	30.7 ± 4.4 Abc	156 ± 76.6 Aab
	MSH	10.5 ± 1.2 Bab	22.3 ± 1 Acd	15.6 ± 3.2 Bbc	44.2 ± 7.5 Bab
MAN	MS0	9.9 ± 6 Aab	8.6 ± 7.7 Bd	49 ± 23.6 Aa	153 ± 10.9 Aa
	MSH	20.3 ± 5.9 Bab	6.2 ± 3.6 Ad	48.7 ± 10.2 Ba	99.8 ± 17.6 Ba

Enzyme activity was assessed in the water-soluble fraction (WS-F) and the NaCl-released fraction (NaCl-F) from apple calli treated (MSH) or not (MS0) with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BA, in combination with 0.09 M of each of the following sugars: sucrose (SUC), glucose (GLC), fructose (FRU), galactose (GAL), maltose (MAL), lactose (LAC), raffinose (RAF), sorbitol (SOR) and mannitol (MAN)

Each value represents the mean ± SD of three replicates. Different small letters within each column indicate significant ($P > 0.05$) differences between sugar treatments. Different capital letters within each column indicate significant ($P > 0.05$) differences between MS0 and MSH

in mechanisms modulating PGHs metabolism in apple callus. Disaccharides such as SUC, MAL and LAC consist of two monosaccharides joined covalently by an *O*-glycosidic bond, which is formed when a hydroxyl group of one sugar reacts with the anomeric carbon of the other. Differences in NaCl-F α-Araf; β-Xyl and β-Gal activity found in calli treated with an equimolar concentration of monosaccharides and disaccharides suggest that the explant cells were incapable of fully metabolizing disaccharides, probably for lack of cleavage.

RAF is considered a transport and storage carbohydrate in many plants (RAF series of oligosaccharides) but may also play a role as a protective agent during maturation and drought, and may act as cryoprotectant in frost-hardy species (Bachmann and Keller 1995). In our experiments, RAF-treated calli showed levels of PGH activity statistically comparables with those in SUC-treated calli (Tables 2, 3, 4). Interestingly, both RAF- and SUC-treated calli displayed a relatively high NaCl-F α-Gal activity (Table 3). It is likely that the α-Gal activity levels of RAF-treated calli are due to the release of SUC since most of α-Gal isolated from plant organs have a high affinity towards RAF and can hydrolyse it to GAL and SUC (Carmi et al. 2003; Keller and Pharr 1996).

MAN and SOR are two sugar alcohols broadly distributed among plants: they play a key role in their metabolism and are primary photosynthetic products involved in the response to different kinds of biotic and abiotic stress (Moing 2000). Since several cell-wall PGHs have been found to be up-regulated under stress conditions such as darkness, sugar depletion, senescence or infection (Contento et al. 2004; Fujiki et al. 2001; Lee et al. 2004), a response to these alditols could be expected. The occurrence of SOR in the *Rosaceae* family is well-known but MAN caused a more profound response in the PGHs studied, particularly in WS-F and NaCl-F β-Glc activity (Table 2). Metabolically speaking, MAN is considered almost inert in tissue cultures because of its low consumption and catabolism rates. Thus, the enhanced activity levels of different cell-wall PGHs (NaCl-F α-Araf, NaCl-F β-Xyl, and WS-F and NaCl-F β-Glc; Tables 2 and 4) in MAN-supplied calli may also be attributed to cellular solute potentials. As other sugars such as GLC are rapidly and highly metabolized in tissue cultures, differences in cell-wall glycosidase activities could be related to cellular activity and/or osmotic pressure of the growth medium throughout the 30 day-experiment. Positive modulation of β-Glc activity by MAN was strong: in MAN-treated calli,

Table 4 Effect of different carbohydrates on the activity of α-D-xylosidase and β-D-xylosidase

Sugar	Medium	α-Xyl		β-Xyl	
		WS-F	NaCl-F	WS-F	NaCl-F
SUC	MS0	0.4 ± 0.2 Aa	1.1 ± 0.2 Ba	1 ± 0.1 Ab	5.4 ± 0.8 Ad
	MSH	0.8 ± 0.1 Aa	1.4 ± 0.2 Aa	0.6 ± 0.1 Ab	3.6 ± 0.9 Bd
GLC	MS0	0.6 ± 0.2 Aa	0.4 ± 0.2 Bab	0.6 ± 0.2 Ab	34.8 ± 4.6 Aab
	MSH	0.8 ± 0.1 Aa	1 ± 0.1 Aab	0.8 ± 0.1 Ab	29.1 ± 3.6 Bab
FRU	MS0	0.8 ± 0.3 Aa	0.6 ± 0.6 Bab	0.9 ± 0.2 Ab	33.3 ± 2.9 Aab
	MSH	1 ± 0.2 Aa	0.9 ± 0.3 Aab	0.6 ± 0 Ab	24.1 ± 4.8 Bab
GAL	MS0	1.2 ± 0.6 Aa	0.4 ± 0.4 Bab	0.8 ± 0.1 Aab	16.1 ± 4.7 Abc
	MSH	0.8 ± 0.2 Aa	1 ± 0.4 Aab	1.1 ± 0 Aab	30.5 ± 4.8 Bbc
MAL	MS0	0.7 ± 0.1 Aa	0.1 ± 0.1 Bab	0.9 ± 0.2 Ab	7.1 ± 5.8 Ad
	MSH	0.8 ± 0.1 Aa	1.2 ± 0.1 Aab	0.5 ± 0.1 Ab	3 ± 0.4 Bd
LAC	MS0	0.7 ± 0.1 Aa	0.6 ± 0.2 Bab	0.6 ± 0.1 Ab	13.9 ± 3 Acd
	MSH	0.9 ± 0.1 Aa	1 ± 0.4 Aab	0.7 ± 0.2 Ab	7.6 ± 2 Bcd
RAF	MS0	1 ± 0.3 Aa	0.4 ± 0.7 Bab	1 ± 0.1 Ab	8.1 ± 2.2 Ad
	MSH	0.9 ± 0.1 Aa	1.3 ± 0.1 Aab	0.6 ± 0.1 Ab	7.4 ± 1.7 Bd
SOR	MS0	0.7 ± 0.3 Aa	1 ± 0.7 Ba	1.2 ± 0.5 Ab	37.2 ± 20.2 Ab
	MSH	0.7 ± 0 Aa	1.1 ± 0.3 Aa	0.6 ± 0.2 Ab	14.4 ± 1.6 Bb
MAN	MS0	0.4 ± 0.2 Aa	0.4 ± 0.4 Bb	1.2 ± 0.4 Aa	42.1 ± 9.7 Aa
	MSH	0.8 ± 0.3 Aa	0.2 ± 0.1 Ab	1.7 ± 0.3 Aa	38.1 ± 4.8 Ba

Enzyme activity was assessed in the water-soluble fraction (WS-F) and the NaCl-released fraction (NaCl-F) from apple calli treated (MSH) or not (MS0) with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BA, in combination with 0.09 M of each of the following sugars: sucrose (SUC), glucose (GLC), fructose (FRU), galactose (GAL), maltose (MAL), lactose (LAC), raffinose (RAF), sorbitol (SOR) and mannitol (MAN)

Each value represents the mean ± SD of three replicates. Different small letters within each column indicate significant ($P > 0.05$) differences between sugar treatments. Different capital letters within each column indicate significant ($P > 0.05$) differences between MS0 and MSH

WS-F β-Glc activity rose 4.2- (MS0 medium) and 11-fold (MSH medium) in comparison with SUC-treated calli while NaCl-F β-Glc increased 3.14- (MS0 medium) and 2.5-fold (MSH medium) respectively (Table 2). β-Glc may be positively associated with certain stress conditions such as the presence of Picloram and/or PGR starvation (Alayón-Luaces et al. 2008). A gene homologous to a β-Glc was found to be expressed in apples exclusively during the infection process of the fruit by *Penicillium expansum* (Sánchez-Torres and González-Candelas 2003). Even though MAN is usually associated with a stress response, it has also been related to cell-wall protoplast regeneration (Blaschek et al. 1981; Ochatt and Power 1988) thus suggesting that β-Glc may play a role in cell-wall metabolism through MAN modulation. Anyhow, modulation by MAN is PGH-specific since MAN-treated calli showed a decrease in NaCl-F α-Gal (Table 3) and α-Xyl (Table 4) activity in comparison with SUC-treated calli.

While callus growth was lower in the MS0 proliferation medium (Table 5), calli grown in such medium displayed NaCl-F α-Araf, β-Glc, β-Gal and β-Xyl activity higher than those grown in an MSH medium (Tables 2, 3, 4). Also, calli grown on a MAN-enriched medium showed poor

Table 5 Effect of different carbohydrates on callus proliferation competence

Sugar	Callus growth	
	MS0	MSH
SUC	3.05 ± 0.60	3.85 ± 0.37
GLC	3.15 ± 0.67	3.90 ± 0.31
FRU	2.15 ± 0.67	3.90 ± 0.31
GAL	2.00 ± 0.65	2.95 ± 0.60
MAL	2.10 ± 0.64	3.85 ± 0.37
LAC	2.05 ± 0.69	2.05 ± 0.60
RAF	3.10 ± 0.55	3.15 ± 0.59
SOR	3.00 ± 0.65	3.85 ± 0.37
MAN	1.25 ± 0.44	1.15 ± 0.37

Apple calli were treated (MSH) or not (MS0) with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BA, in combination with 0.09 M of each of the following sugars: sucrose (SUC), glucose (GLC), fructose (FRU), galactose (GAL), maltose (MAL), lactose (LAC), raffinose (RAF), sorbitol (SOR) and mannitol (MAN). After 30 day-incubation, callus growth was assessed visually according to a four-stage scale, as follows: 1 (very low): callus size increased twofold; 2 (low): callus size increased threefold; 3 (moderate): callus size increased fourfold; 4 (high): callus size increased fivefold. Each value represents the mean ± SD of twenty independent replicates

growth (Table 5) and high NaCl-F α-Araf, β-Glc, β-Gal and β-Xyl activity. However, no definite correlation could be found between callus growth (Table 5) and the activity of these cell-wall glycosidases (Tables 2, 3, 4) for the different sugars tested. For instance, callus growth was higher using SOR than MAN, but no significant differences were detected in NaCl-F β-Gal activity when calli were supplemented with those sugars. Calli supplemented with SUC and SOR showed a similar growth, but NaCl-F α-Araf, α-Gal and β-Xyl activity levels proved to be significantly different. Growth displayed by SUC- and GLC-supplemented calli was similar but NaCl-F α-Araf, α-Gal, β-Gal and β-Xyl activity levels were different. Whether the activity of these cell-wall glycosidases has to do more with the quality of the cell wall than with callus proliferation is a hypothesis deserving further research.

Conclusion

This study gives an insight on the modulation and signalling associated with hydrolase activity in plant tissues. Our results show that sugars supplied to callus tissue cultures as carbon sources can also modulate PGH activity. This modulation is sugar-specific, different for each PGH and, at least in the case of sucrose, concentration-dependent. Results also suggest the existence of regulatory interactions between PGRs and sugars, as part of an intricate sensing and signalling network. This study can be useful for the definition of "tailor-made" culture media for specific purposes since the levels of glycosidase activity could modify the configuration of callus cell-wall polysaccharides. Combination of PGRs, sugar type and concentration should be taken into account to optimize each PGH activity towards further enzyme research.

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