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RESEARCH PAPER

Towards understanding vacuolar antioxidant mechanisms: a role for fructans?

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Abstract

Recent *in vitro*, *in vivo*, and theoretical experiments strongly suggest that sugar-(like) molecules counteract oxidative stress by acting as genuine reactive oxygen species (ROS) scavengers. A concept was proposed to include the vacuole as a part of the cellular antioxidant network. According to this view, sugars and sugar-like vacuolar compounds work in concert with vacuolar phenolic compounds and the 'classic' cytosolic antioxidant mechanisms. Among the biologically relevant ROS (H_2O_2, $O_2^{\cdot-}$, and ·OH), hydroxyl radicals are the most reactive and dangerous species since there are no enzymatic systems known to neutralize them in any living beings. Therefore, it is important to study in more detail the radical reactions between ·OH and different biomolecules, including sugars. Here, Fenton reactions were used to compare the ·OH-scavenging capacities of a range of natural vacuolar compounds to establish relationships between antioxidant capacity and chemical structure and to unravel the mechanisms of ·OH–carbohydrate reactions. The *in vitro* work on the ·OH-scavenging capacity of sugars and phenolic compounds revealed a correlation between structure and ·OH-scavenging capacity. The number and position of the C=C type of linkages in phenolic compounds greatly influence antioxidant properties. Importantly, the splitting of disaccharides and oligosaccharides emerged as a predominant outcome of the ·OH–carbohydrate interaction. Moreover, non-enzymatic synthesis of new fructan oligosaccharides was found starting from 1-kestotriose. Based on these and previous findings, a working model is proposed describing the putative radical reactions involving fructans and secondary metabolites at the inner side of the tonoplast and in the vacuolar lumen.

Key words: Fructan, phenols, radicals, ROS, stress, sugars, vacuole.

Introduction

Carbohydrates are essential molecules of life. They are polyhydroxy aldehydes and ketones with different degrees of polymerization (DP) and consisting of carbon, hydrogen, and oxygen. Monosaccharides (DP1), disaccharides (DP2), oligosaccharides (DP ≤10), and polysaccharides (DP >10) can be distinguished. They can fulfil structural (Peshev and Van den Ende, 2013), signalling (Jang and Sheen, 1994; Smeekens *et al*., 2010), transport, and storage functions. The dynamics between polymerized carbohydrates (e.g. starch and fructan) and small, soluble carbohydrates play a major role in plant stress responses. Under stress, soluble carbohydrates [e.g. hexoses, sucrose, fructans, raffinose family oligosaccharides (RFOs); commonly referred to as sugars from this point on] and sugar-like compounds (e.g. mannitol, sorbitol, etc.) can assist in osmotic adjustments as well as in membrane and protein stabilization (Tarczynski *et al*., 1993; Amiard *et al*., 2003; Hincha *et al*., 2003). Together with hormones, small sugars form an integral part of the plant signalling network

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regulating stress and defence responses (Rolland *et al*., 2006; Gomez-Ariza *et al*., 2007).

Reactive oxygen species (ROS) are an integral part of aerobic life, and may act both as initiators and propagators of oxidative stress in plants (Elstner and Osswald, 1994). Both *in vitro* (Nishizawa *et al*., 2008; Stoyanova *et al*., 2011) and *in vivo* (Nishizawa *et al*., 2008) experiments strongly suggest that sugar-(like) compounds counteract oxidative stress by acting as genuine ROS scavengers. An independent theoretical approach (Hernandez-Marin and Martínez, 2012) also predicted that common sugars can act as antioxidants. Nishizawa *et al*. (2008) and Schneider and Keller (2009) suggested a role for RFOs as a part of the chloroplast antioxidant system, protecting plant cells from oxidative damage (Foyer and Shigeoka, 2011). In addition, a concept to include the vacuole as a part of the cellular antioxidant network was proposed (Van den Ende and Valluru, 2009; Peshev and Van den Ende, 2013). According to this view, sugars and sugar-like compounds, accumulating to a large extent in the vacuole, may act as ROS scavengers, working in concert with vacuolar phenolic compounds and the 'classic' cytosolic antioxidant mechanisms. Together with other systems involving organellar and cytosolic invertases and hexokinases, such mechanisms might contribute to overall cellular ROS homeostasis (Xiang *et al*., 2011). It was speculated that any sugar-(like) compound accumulating in the vicinity of any cellular membrane has the potential to act as a ROS scavenger contributing to membrane stability under stress (Bolouri-Moghaddam *et al*., 2010). For instance, trehalose, a well-known sugar involved in yeast stress responses, is considered as an important player in the yeast antioxidant system (Jamieson, 1998; Benaroudj *et al*., 2001).

In plants, ROS are mainly produced during respiration and photosynthesis (Moller, 2001). As in all aerobic organisms, part of the electrons participating in electron transfer processes can flow to oxygen, resulting in the formation of superoxide ($O_2^{\cdot -}$), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Wiseman and Halliwell, 1996). ROS are highly reactive molecules; they may induce lipid peroxidation and oxidize proteins and DNA. In addition to mitochondria and chloroplasts, other organelles in plant cells can be involved in generation of ROS, including the vacuole. Despite its generally neglected and poorly understood role in this process, the vacuole may also be an important site for ROS production due to the activity of H_2O_2-dependent class III peroxidases at the inner face of the tonoplast (Sottomayor *et al*., 2004; Costa *et al*., 2008) and NADPH oxidases that might be located in the tonoplast (Carter *et al*., 2004; Whiteman *et al*., 2008) but using cytosolic NADPH to release $O_2^{\cdot -}$ in the vacuole (Van den Ende and Valluru, 2009). Among the biologically relevant ROS (H_2O_2, $O_2^{\cdot -}$, and ·OH), hydroxyl radicals are the most reactive and dangerous species. The ·OH moiety is known to react with almost all biomolecules at rates the same as those occurring in diffusion-controlled reactions (Hernandez-Marin and Martínez, 2012) and, as a consequence, there are no enzymatic systems known to neutralize them in any living beings (Gechev *et al*., 2006). Therefore, it is of particular importance to study in more detail the radical reactions between ·OH and different biomolecules, including carbohydrates. Reactions between cell wall polysaccharides and ·OH have been proposed to be important for cell wall loosening, a process that is essential during germination, elongation growth, and fruit ripening (Fry *et al*., 2001; Müller *et al*., 2009).

As well as their damaging properties, ROS [as well as reactive nitrogen species (RNS)] are now recognized as an integral part of cellular signalling mechanisms both in plants and in animals (Wojtaszek, 1997; Apel and Hirt, 2004; Lambeth, 2004; Nuhse *et al*., 2007). Therefore, cellular ROS homeostasis forms a central concept. In a broader context, the importance of ROS for all aerobic life forms should be extended to their impact on human health. Excess ROS can affect food quality and/or initiate ROS-based diseases, and this might be counteracted by natural antioxidants (Kazak *et al*., 2011; Stoyanova *et al*., 2011; Van den Ende *et al*., 2011). In plants, (a)biotic stresses also result in ROS increases (Apel and Hirt, 2004). Abiotic stress is estimated to be the leading cause of crop loss, exceeding 50% worldwide (Boyer, 1982; Cramer *et al*., 2011). Therefore, it is of great importance to study the mechanisms controlling ROS production, neutralization, and ROS signalling pathways under stress. This is expected to aid the development of stress-tolerant crops in the future.

In order to better understand and test the proposed 'sugars as antioxidants' concept (Peshev and Van den Ende, 2013), it is essential first to compare an *in vitro* array of sugars with other well-known natural antioxidants (e.g. phenolic compounds). Moreover, mechanistic insights are needed into the predominant chemical reactions between sugars and ·OH *in vitro*, before further efforts can be undertaken to investigate the importance of such reactions *in vivo*. Here, Fenton reactions were used to compare the ·OH-scavenging capacities of a range of natural compounds occurring in plant vacuoles in order to uncover relationships between antioxidant capacity and chemical structure. The major focus was on sucrose and 1-kestotriose, the smallest inulin-type fructan (Vergauwen *et al*., 2000). Both are essential molecules to initiate fructan synthesis in plants (Livingston *et al*., 2009). Importantly, the splitting of disaccharides and oligosaccharides emerged as a predominant outcome of these radical-based reactions, creating opportunities to use the formed non-radical products as future markers to uncover the existence of non-enzymatic sugar–radical interactions *in vivo*.

Material and methods

Fenton reagent–carbohydrate reactions

Pure carbohydrates with 10 mM end concentration were mixed together with 1 mM H_2O_2, 1 mM ascorbate (AsA), 100 µM $FeSO_4$, and 100 µM Na-EDTA in 50 mM Na-phosphate buffer pH 7.2. The reaction mixture was vortexed and incubated at 30 °C for 24 h.

Purification of products derived from Fenton reagent–carbohydrate reactions

From the above-mentioned reactions, 400 µl was injected onto a semi-preparative Dionex BioLC system. The Carbopac™

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PA-100 column (22 × 250 mm) was equilibrated with 90 mM NaOH for 2 h (flow rate 5 ml min^{-1}). A Na-acetate gradient was applied as follows: 0–60 min, linear gradient from 100% 90 mM NaOH to 80% NaOH (20% 0.5 M Na-acetate); 60–90 min 100% 0.5 M Na-acetate and 30 min column regeneration (100% 90 mM NaOH). Fractions of 5 ml (= 1 min) were collected and stored at 4 °C. The samples were analysed with analytical high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described below. Activated charcoal columns (300 µl) were prepared in Pasteur pipettes and calibrated as follows: 1 ml of H_2O (HPLC grade), 1 ml of 10% ethanol, 1 ml of 15% ethanol, 1 ml of 20% ethanol, 1 ml of 25% ethanol, 1 ml of 30% ethanol, 1 ml of 40% ethanol, 1 ml of 50% ethanol, 1 ml of 75% ethanol, 1 ml of 100% ethanol, and finally 10 ml of H_2O (HPLC grade). Selected samples were loaded on these calibrated activated charcoal columns. The flow-throughs were discarded. The columns were eluted with 1 ml of H_2O, 10% ethanol, 15% ethanol, 20% ethanol, 25% ethanol, 30% ethanol, and 40% ethanol, respectively. All fractions were analysed with HPAEC-PAD (see below). Purified fractions were placed in a speed vac (AES2010 Savant) to evaporate ethanol. Finally, the samples were dissolved in 100 µl of H_2O (HPLC grade).

Mass spectometry (MS) analysis

The analysis was carried out at ProMeta (KU Leuven, Belgium) with a Bruker Daltonics Apex-Qe FT mass spectrometer containing a 9.4 Tesla magnet. Electron spray ionization (ESI) was used (with an ES Nebulizer needle from Agilent Technologies). Capillary voltage was 1800 V. The second set of samples was analysed with a Bruker Daltonics microTofQ mass spectrometer.

Plant material and sucrose induction experiment

For sap isolation, chicory was sown in May in a local field as described (Van den Ende *et al*., 1996). Mature leaves (end of July) were cut into pieces of 4 cm and mixed. One half of these leaf pieces were incubated in Milli-Q water and the other half in 250 mM sucrose for 2 d at 20 °C and under continuous light (60 µmol m^{-2} s^{-1}). For comparison, an untreated control sample was also taken at day 0 and immediately frozen under liquid nitrogen and kept at –80 °C. After 2 d, the treated leaves were washed, dried, and frozen under liquid nitrogen. Forced chicory roots were derived from the Nationale Proeftuin voor Witloof, Herent, Belgium. *Cynara cardunculus* var scolymus (globe artichoke) crude extract was kindly provided by Andrea Moglia (DISAFA-Plant Genetics and Breeding, University of Turin, Italy).

Hydroxyl radical-scavenging assay

Hydroxyl radical-scavenging activity was determined by measuring the ability of the various sugar, phenolic, and other components to inhibit the formation of the strongly fluorescent 2-hydroxyterephthalate (HTPA) in a reaction between terephthalic acid (TPA; 1,4-benzenedicarboxylic acid) and ·OH generated in a Fenton reaction. The protocol of Stoyanova *et al*. (2011) was used with modifications. A 900 µl reaction mixture was prepared containing 125 µM TPA, 2.5 µM Na-EDTA, 2.5 µM $FeSO_4$, 25 µM AsA, and 25 µM H_2O_2 in a 50 mM Na-phosphate buffer (pH 7.2). The reaction was vortexed and incubated overnight at 30 °C. HTPA fluorescence was measured at room temperature with a RF-10AXL fluorescence detector (Shimadzu Corporation, Kyoto, Japan; 315 nm excitation and 420 nm emission), coupled with a Smartline Autosampler 3950 (Knauer, Berlin, Germany) and a Smartline Pump 1000 (Knauer, Berlin, Germany). The elution (1 ml min^{-1}) was done with H_2O (HPLC grade). The injection volume was 300 µl. Hydroxyl radical scavenging of each compound was characterized by its half-inhibitory concentration for HTPA formation as before (Stoyanova *et al*., 2011).

Superoxide radical assay

Superoxide scavenging was measured according to the assay used in Stoyanova *et al*. (2011). In this study, sucrose-fed and non-fed chicory saps were added to inhibit the $O_2^{\cdot-}$-induced reduction of nitroblue tetrazolium (NBT) to formazan. Superoxide was generated from a xanthine/xanthine oxidase source. Formazan production was measured on the basis of ΔA_{560} nm. The 1.0 ml reaction mixture contained 0.025 U of xanthine oxidase in 50 mM K-phosphate buffer (pH 7.5; 0.3 mM Na-EDTA, 0.1 mM xanthine, and 1 mg ml^{-1} NBT). Boiled plant saps were added at various, increasing concentrations, and their superoxide radical scavenging was characterized by concentrations inhibiting the increase in the 560 nm absorption by 50%.

Carbohydrate extraction and HPAEC-PAD analysis

The root material was first washed with tap water and then additionally with ddH_2O, dried with a paper towel, and homogenized using a mortar and pestle under liquid nitrogen. The frozen leaf samples were directly homogenized using a mortar and pestle under liquid nitrogen. Nine volumes of ddH_2O were added to the homogenate and the mixture was boiled for 10 min at 90 °C. The extract was centrifuged at 16 000 *g* for 5 min. Subsequently, 200 µl of the supernatant was added to a mixed bed Dowex column (300 µl of Dowex H^+ and 300 µl of Dowex Ac^-; both 100–200 mesh; Acros Organics, Morris Plains, NJ, USA). The column was eluted six times with 200 µl of ddH_2O (Vergauwen *et al*., 2000).

For plant sap isolation, Milli-Q water was added (up to 30% of the fresh weight) and the homogenate was heated immediately at 99.9 °C for 12 min. After cooling, the mixture was centrifuged for 5 min at 16 000 *g*. The supernatants were used for testing ROS-scavenging capacities as described above and for carbohydrate analysis with HPAEC-PAD as described below. The protein content of the extracts was determined according to Bradford (1976).

The samples from Fenton reactions were centrifuged at 16 000 *g* for 5 min. From the supernatant, an aliquot was analysed using HPAEC-PAD as described (Vergauwen

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et al., 2000). Peak quantification and identification was performed using the external standards method (Shiomi et al., 1991). Co-injection studies were performed for peak identification.

Phenol extraction and measurement

Phenolic compounds were extracted as follows: finely ground freeze-dried leaf tissue (0.010 g) was mixed with 990 µl of 80% aqueous methanol and incubated for 20 min in an ultrasonic bath. Afterwards, the samples were centrifuged for 5 min at 16 000 *g*. The total phenolic content in the supernatant was determined by Folin–Ciocalteu assay (Singleton *et al.*, 1999) with minor modifications. In brief, 50 µl of extract was added to 450 µl of 10% Folin–Ciocalteu reagent, the solution was mixed, and after 5 min of incubation 500 µl of 7% Na_2CO_3 and 200 µl of H_2O were added. The mixture was incubated for 90 min. The adsorption at 595 nm (Multiskan Ascent, Thermo Labsystems, Beverly, MA, USA) was subsequently measured. To calculate the total phenolic contents, a standard curve with gallic acid was used.

Chemicals

The origin of the chemicals is listed in Supplementary Fig. S1 available at *JXB* online. *Cynara scolymus* phenolic compounds were kindly provided by Andrea Moglia.

Statistics

Statistical analysis was performed with the analysis of variance (ANOVA) single factor test. Values with $P < 0.05$ were considered significantly different.

Results and Discussion

Due to great concerns for the safety of chemical or artificial food supplements and antioxidants, plant-derived natural products are becoming increasingly popular these days (Van den Ende *et al.*, 2011). So far, sugars have been mostly neglected as putative antioxidants and, to date, research has mainly focused on the antioxidant capacity of plant-derived phenolic compounds. Moreover, a plethora of different methods have been used to measure the antioxidant capacity of certain compounds (Awika *et al.*, 2003), which has led to variable results. Here, a single method (Stoyanova *et al.*, 2011) is used to compare the scavenging capacity of a list of naturally occurring sugars and phenolic compounds in aqueous solution against ·OH radicals at neutral pH (7.2). This focus arose for two major reasons (i) carbohydrates in general have a much better scavenging capacity against ·OH radicals as compared with $O_2^{\cdot-}$ (Stoyanova *et al.*, 2011) and (ii) although living cells contain numerous enzymatic and non-enzymatic systems to control H_2O_2 and $O_2^{\cdot-}$ levels (Blokhina *et al.*, 2003), the only way to deal with ·OH radicals is by scavenging them with higher concentrations of non-enzymatic antioxidants or to rely on mechanisms that prevent their formation (Gechev *et al.*, 2006).

Antioxidant hierarchy among vacuolar compounds: an in vitro study

Table 1 ranks the *in vitro* ·OH radical-scavenging capacity of natural compounds of plant origin, with IC_{50}-·OH values expressed in millimolar (mM). Two major groups can be distinguished: (i) carbohydrates (excluding the polysaccharide inulin) and (ii) phenolic compounds (or phenolic-like compounds) and inulin. Monosaccharides such as glucose and fructose are the least efficient scavengers. Most disaccharides, some monosaccharides, and raffinose form a transit group, followed by the trisaccharides maltotriose and 1-kestotriose. The best scavenger among soluble carbohydrates is inulin, which has scavenging properties intermediate between those of dihydrocaffeic acid and chicoric acid. It can be argued that, when expressed on a molar basis, inulin has excellent scavenging properties since it is a long soluble polymer with many places where ·OH can attack to derive a proton and an electron to form water (in the case of hydrogen abstraction, see below). Referring to the situation *in planta*, inulin shows high affinity for membranes (Vereyken *et al.*, 2003), where dangerous ROS such as ·OH radicals can be produced by the action of membrane-associated class III peroxidases (Passardi *et al.*, 2004; Bolouri-Moghaddam *et al.*, 2010). Therefore, inulin is expected to scavenge these ·OH radicals, preventing membrane damage (see also below). Huge

Table 1. Comparison of the ·OH-scavenging capacity of different (vacuolar) sugars and phenolic compounds. The fructans inulin and 1-kestotriose are indicated in bold. SE: standard error of the mean.

Compounds	IC_{50} (mM)	SE
Phenolic compounds and inulin		
1,5-Dicaffeoylquinic acid	0.00009	0.00002
1,3-Dicaffeoylquinic acid	0.00011	0.00002
3,4-Dicaffeoylquinic acid	0.00101	0.00002
4,5-Dicaffeoylquinic acid	0.00105	0.00006
3,5-Dicaffeoylquinic acid	0.00116	0.00010
Tannic acid	0.00346	0.002
Caffeic acid	0.00595	0.001
Chicoric acid	0.02	0.0003
Inulin	0.05	0.004
Dihydrocaffeic acid	0.08	0.001
Gallic acid	0.13	0.015
Sugars		
1-Kestotriose	0.30	0.033
Maltotriose	0.31	0.044
Maltose	0.37	0.04
Raffinose	0.41	0.044
Sucrose	0.44	0.048
Xylose	0.45	0.022
Mannose	0.48	0.016
Trehalose	0.55	0.04
Glucose	0.79	0.07
Fructose	1.15	0.075
Artichoke leaf extract in methanol (mg DW ml^{-1})	0.000007	0.000002
Inulin (mg ml^{-1})	0.23	0.019

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variation is observed when considering the ·OH-scavenging capacity of plant phenolic compounds (Table 1). The most superior scavengers were a series of phenolic compounds derived from *C. cardunculus* varscolymus (Supplementary Fig. S2 at *JXB* online). These compounds share exactly the same structural elements but differ in the site of phenolic substitutions which lead to different spatial conformation. The difference between the worst and the best of these compounds is ~10-fold (Table 1).

The weakest phenolic compound in the test is gallic acid (Table 1), although it is considered as a standard antioxidant (Habila *et al*., 2010). It is only twice better than 1-kestotriose, while caffeic acid is ~50 times better. The variation in this group of phenolic compounds is much more extensive than that observed among the soluble carbohydrates. The most superior antioxidants show a C=C bond in their side chains. Three main mechanisms are devoted to the direct scavenging of ·OH radicals: electron transfer ($\cdot OH+R \rightarrow OH^- + R^+$); hydrogen abstraction ($RH+\cdot OH \rightarrow R\cdot + H_2O$) and addition to an aromatic ring or to a double bond yielding an addition product ($\cdot OH+R=R \rightarrow HO\text{-}R\text{-}R\cdot$). If double bonds are present, addition is the preferable reaction (Soylemez and von Sonntag, 1980; Czapski, 1984; Hernandez-Marin and Martínez, 2012). This hypothesis was tested further by comparing dihydrocaffeic acid and caffeic acid, only differing by the presence of a double bond in the side chains of the latter (Supplementary Fig. S2 at *JXB* online). Indeed, caffeic acid showed a 10-fold better scavenging capacity compared with dihydrocaffeic acid (Table 1). First, the presence of an extra side chain double bond provides one more possibility for ·OH addition and thus these phenolic compounds may neutralize a higher number of radicals as compared with those lacking that extra double bond in their side chain. Secondly, it can be speculated that substitutions at the aromatic ring obstruct the attack by ·OH, making the side chain double bound the preferential point of attack. This obstruction may also explain the difference between gallic acid and dihydrocaffeic acid, the latter having fewer OH groups.

Functional foods include, for instance, probiotics (living bacteria), prebiotics (compounds selectively stimulating 'beneficial' intestinal bacteria), and antioxidants (Van den Ende *et al*., 2011). In this study, inulin (a well-known pre-biotic), expressed on a mg ml^{-1} basis, showed inferior scavenging properties as compared with the crude extract from the well-known Mediterranean vegetable *C. cardunculus* var scolymus with good antioxidant properties (Wang *et al*., 2003; Table 1). This result suggests that the *C. cardunculus* var scolymus extract contains a mixture of superior antioxidants, such as the artichoke phenols listed in Table 1, and, probably, other compounds with perhaps even better scavenging capacities.

To screen for putative synergistic effects between phenolic compounds and carbohydrates, mixtures at different ratios were compared *in vitro* (Table 2). No synergistic effects were observed in any case. In 50/50 mixtures, the best out of the two antioxidants determined the antioxidant capacity of the whole mixture (Table 2). Even a 16-fold excess of 1-kestotriose in a 1-kestotriose/gallic acid mixture resulted in an IC_{50}-·OH that was closer to that of the most powerful antioxidant (gallic acid in this case, Supplementary Table S1 at *JXB* online). These are important results that should be taken into consideration when the antioxidant capacities of plant saps are compared *in vitro* (see below). However, the situation *in vivo* might be much more complicated when considering the action and co-existence of carbohydrates and phenolic compounds (e.g. in the vacuole). *In vivo*, two pools should be discerned, a pool associated with membranes (e.g. the tonoplast) and a pool with the free solution (e.g. vacuolar lumen) (see below).

Fate of sugars in reaction with ·OH radicals

As a necessary next step in understanding sugar–·OH radical reaction processes, the *in vitro* generated products were investigated in more depth. Therefore, Fenton reactions were applied in which the decomposition of hydrogen peroxide occurs in the presence of transition metals (e.g. Fe^{2+}) yielding ·OH (Walling, 1975). Subsequently, the fate of di- and trisaccharides (Supplementary Fig. S3 at *JXB* online) before and after the Fenton reaction was followed with HPAEC-PAD (Fig. 1). In all cases, a partial breakdown of the sugars to smaller mono- and disaccharide entities was observed. For instance, sucrose is partially broken down to glucose and fructose. Trehalose and maltose lead to the formation of glucose. Raffinose (Gal-Glc-Fru) leads to the formation of melibiose (Gal-Glc), sucrose, fructose, and galactose plus glucose (the latter two are not separated on HPAEC-PAD). The fructan 1-kestotriose (Glc-Fru-Fru) splits into inulobiose (Fru-Fru or F_2), sucrose, glucose, and some fructose. Intriguingly, a new putative trisaccharide peak 'F_3' was also formed during the Fenton reaction (Fig. 1). In all cases, newly unidentified peaks with a putative higher DP appeared at later retention times (see also below).

Theoretical studies by Hernandez-Marin and Martínez (2012) predicted that carbohydrates scavenge ·OH through hydrogen atom transfer (hydrogen abstraction). They calculated that the occurrence of the abstraction is much more likely to happen from C-H rather than from O-H, yielding carbon-centred radicals. Both the present data and previous research on galactomannans and cell wall polysaccharides (Fry *et al*., 2001; Tudella *et al*., 2011) show that one prominent outcome of ·OH interaction with carbohydrates consisting of more than one moiety is a scission of these molecules into entities with a lower molecular weight. Based on the present, unique observations on the breakdown of sugars in the Fenton reagent–sugar reactions in solution, and existing literature on sugar radiation chemistry (for a

Table 2. ·OH-scavenging capacity of mixtures of different sugars and phenols. SE: standard error of the mean.

Compounds	IC_{50} (mM)	SE
Inulin–caffeic acid (1 mM)	0.011	0.001
Inulin–chicoric acid (1 mM)	0.023	0.001
Sucrose–chicoric acid (1 mM)	0.037	0.004
Gallic acid+1-kestotriose (10 mM)	0.154	0.013
Sucrose+1-kestotriose (10 mM)	0.296	0.010

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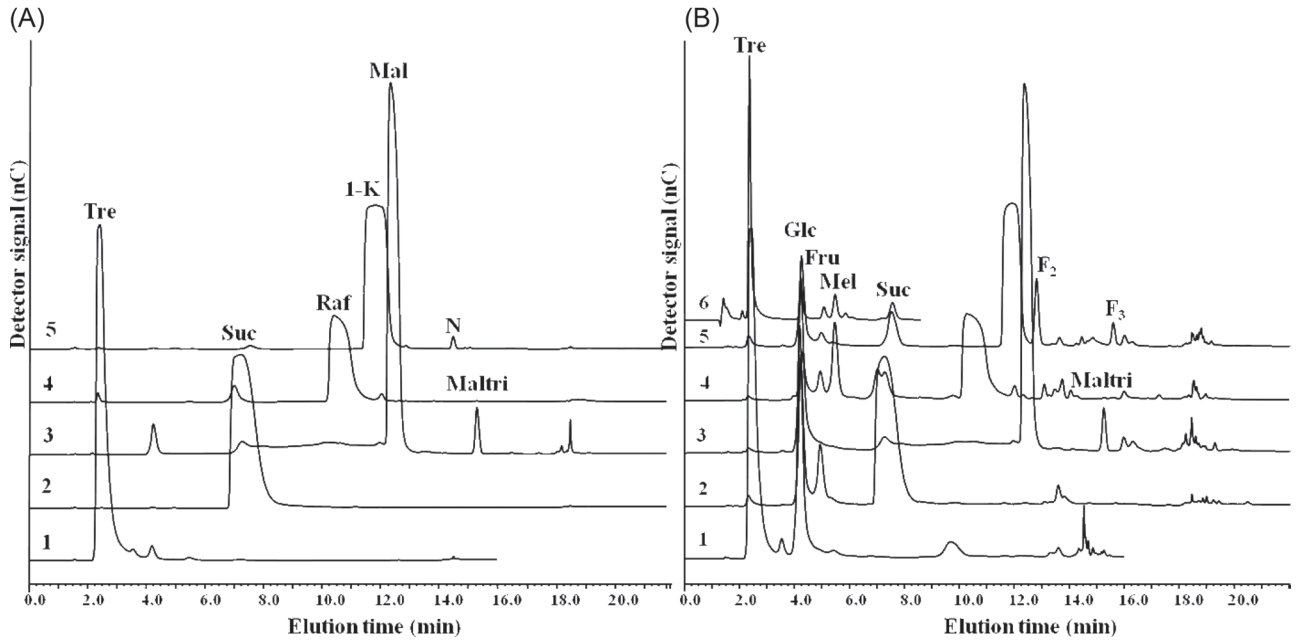

Fig. 1. HPAEC-PAD chromatograms showing the products derived from *in vitro* Fenton reagent–carboydrate reactions. (A) The tested carbohydrates before the onset of the Fenton reactions are as follows: 1, trehalose (Tre); 2, sucrose (Suc); 3, maltose (Mal), maltotriose (Maltri); 4, raffinose (Raf); 5, 1-kestotriose (1-K); 1,1-nystose (N). (B) The carbohydrate mixtures derived after the Fenton reactions: 1, trehalose; 2, sucrose; 3, maltose; 4, raffinose; 5, 1-kestotriose; 6, a reference containing trehalose (Tre), glucose (Glc), fructose (Fru), melibiose (Mel), and sucrose (Suc).

review, see von Sonntag, 2006), a detailed scheme is proposed showing some putative reactions between ·OH and sucrose (Fig. 2) leading to the production of carbon-centred radicals and free hexoses. Scission of the glycosidic bond might occur directly (Fig. 2, reaction 2) or indirectly (Fig. 2, reactions 1 and 3). If the attack of ·OH occurs at the C-H in the glucose moiety next to the glycosidic bond, this leads to a direct scission resulting in the formation of a glucose radical and fructose (Fig. 2, reaction 2). Reactions 1 and 3 describe the scission of the glycosidic linkage when the radical attack does not occur at the C1 position. In this scenario, a ring opening occurs followed by a splitting of the glycosidic bond. Obviously, many more radical reactions are possible than those presented in Fig. 2, for instance when the attack occurs at the C3 of the fructose moiety of sucrose, a point of preferential attack as predicted by Hernandez-Marin and Martínez (2012). Although less probable, H abstraction from O-H groups might also occur to a certain extent. Furthermore, it should be noted that all the reactions, those leading to a splitting and those that do not, generate a new radical as an end-product. Probably, such radicals are less reactive (or dangerous) than the original ·OH radicals.

Since fructans are known to be important prebiotics (Van den Ende *et al*., 2011) and proposed as new players involved in vacuolar antioxidant mechanisms (Van den Ende and Valluru, 2009), the fate of 1-kestotriose during Fenton reactions *in vitro* and the identity of the formed products was studied more thoroughly. These investigations provided a deeper understanding of the fate of the organic radicals produced after the initial splitting of the sugar molecules. The suspected F_2 (inulobiose) and F_3 (inulotriose, DP3), already observed in Fig. 1, are typical reducing-type fructans formed by fructan:fructan fructosyl during the chicory forcing process (Van den Ende *et al*., 1996). Co-injection of 1-kestotriose-derived Fenton reactions and forced chicory extracts confirmed that the *in vitro* formed F_2 and F_3 elute at exactly the same time as inulobiose and inulotriose in forced chicory extracts (Supplementary Fig. S4 at *JXB* online). Additional evidence was generated with enzyme reactions containing either chicory 1-FEH (fructan-1-exohydrolase) IIa (Le Roy *et al*., 2007) or sugar beet 6-FEH (Van den Ende *et al*., 2003) (Fig. 3). A complete degradation of the peak consistent with the F_2/inulobiose was detected with chicory 1-FEH IIa, whereas only a partial degradation of the putative F_3/inulotriose peak was found (Fig. 3), indicating that the F_3 peak might contain other fructose-only fructan trisaccharides besides inulotriose. Since sugar beet 6-FEH shows a considerable side activity with 1-kestotriose (Van den Ende *et al*., 2003), it was not unexpected to find that F_2 also served as a substrate, but to a lesser extent as compared with chicory 1-FEH IIa. Overnight incubation also showed a limited degradation of F_3. When both enzymes were combined, a further decrease in F_3 was observed (Fig. 3). Taken together, these results strongly suggest that F_2 is inulobiose whereas F_3 most probably consists of a mixture of fructose-based oligosaccharides with different types of linkages. To confirm further the identity of these compounds, extensive peak purification was performed and isolated compounds were analysed by MS. In the fraction containing F_2, a molecular weight corresponding to the DP2-Na adduct (365 Da) was found. Accordingly, in the F_3 fraction, a molecular weight of 527 Da was detected (Fig. 4). Further, a molecular weight corresponding to the DP4-Na adduct (689 Da) was detected in several fractions (data not

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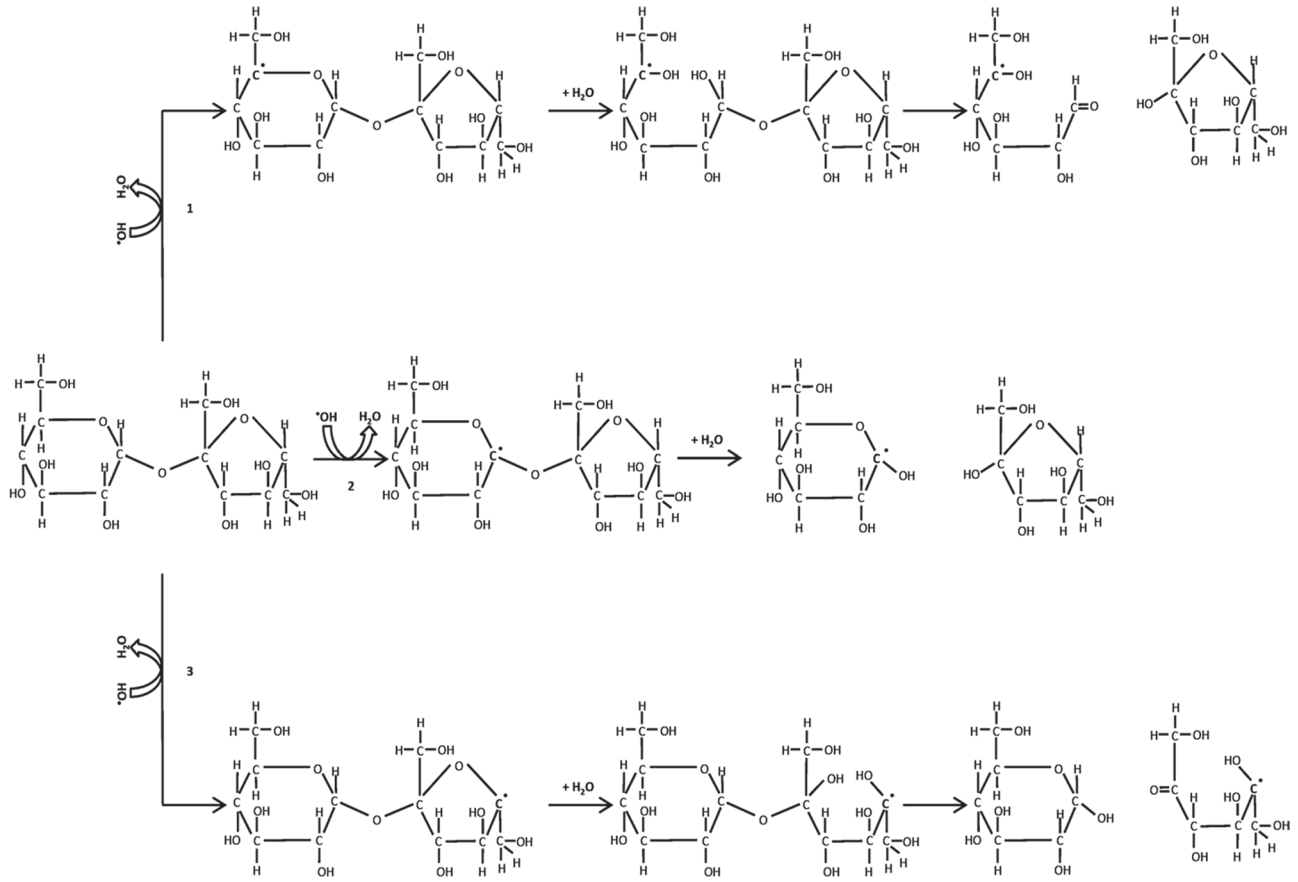

Fig. 2. Some possible reactions between sucrose and the ·OH radical leading to the scission of the glycosidic bond. The ·OH radical abstracts a hydrogen atom (H) from sucrose yielding a sugar radical and a free hexose. The abstraction may occur at the glucose or fructose moiety, respectively. Three possible reactions are presented: (i) The radical attack occurs on the glucose moiety next to the glycosidic bond (reaction 2). In this case a direct scission of the bond occurs and a glucose radical and fructose are released. (ii) The H is abstracted from the fructose or glucose moiety but not in the neighbourhood of the glycosidic linkage (reactions 1 and 3). Probably, first a ring opening takes place in the corresponding moiety followed by the scission of the glycosidic bond. In all cases, a free hexose and a hexose radical are formed.

shown), but unfortunately the amount of the product was too low for further purification. The spreading of DP4 compounds over several fractions also suggested that they may have a diverse structure. Therefore, it can be concluded that, next to sugar degradation, non-enzymatic, radical-based *de novo* synthesis of fructosyl oligosaccharides was found, through a recombination of carbohydrate radicals. Novel higher DP saccharides are formed not only from 1-kestotriose, but also from all other sugars when exposed to a Fenton reaction (Fig. 1). The chemical nature (e.g. relatively high reactivity) of the radicals indicates that they undergo more than just one type of reaction. This means that even when (a) dominant reaction path(s) is/are followed, probably considerable numbers of side reactions may occur. Moreover, oxygen may participate in those reactions, modifying the nature of the sugar moieties. For all these reasons, it can be assumed that the newly formed DP3 oligosaccharides from 1-kestotriose will not be the only products formed. Accordingly, in most of the fractions collected after the elution time of 1-kestotriose, molecular weights >400 Da were observed during MS (data not shown). To rule out that the newly formed 'F_3' peak is not derived from the small 1,1-nystose impurity in 1-kestotriose (Fig. 1), a Fenton reaction in the presence of ultrapure 1-kestotriose (see the Materials and methods) was performed. These results confirmed the synthesis of F_3 from 1-kestotriose (Supplementary Fig. S5 at *JXB* online). The *de novo* formation of oligosaccharides *in vitro* can be explained by a termination of the radical chain reaction process by a bimolecular interaction between sugar radicals forming a stabile non-radical product as depicted in Fig. 5. The proposed scheme does not take into account that carbon-centred radicals can also undergo an interaction with molecular oxygen.

Antioxidant capacity of plant saps: additive roles for fructans and phenolic compounds?

To test whether increasing fructan and polyphenol contents in saps correlate well with increased ROS-scavenging capacities, excised chicory leaves were fed with sucrose specifically to induce fructan accumulation (Fig. 6). Total fructan concentrations increased >100-fold in sucrose-fed leaves, whereas sucrose concentrations increased >3-fold. Table 3

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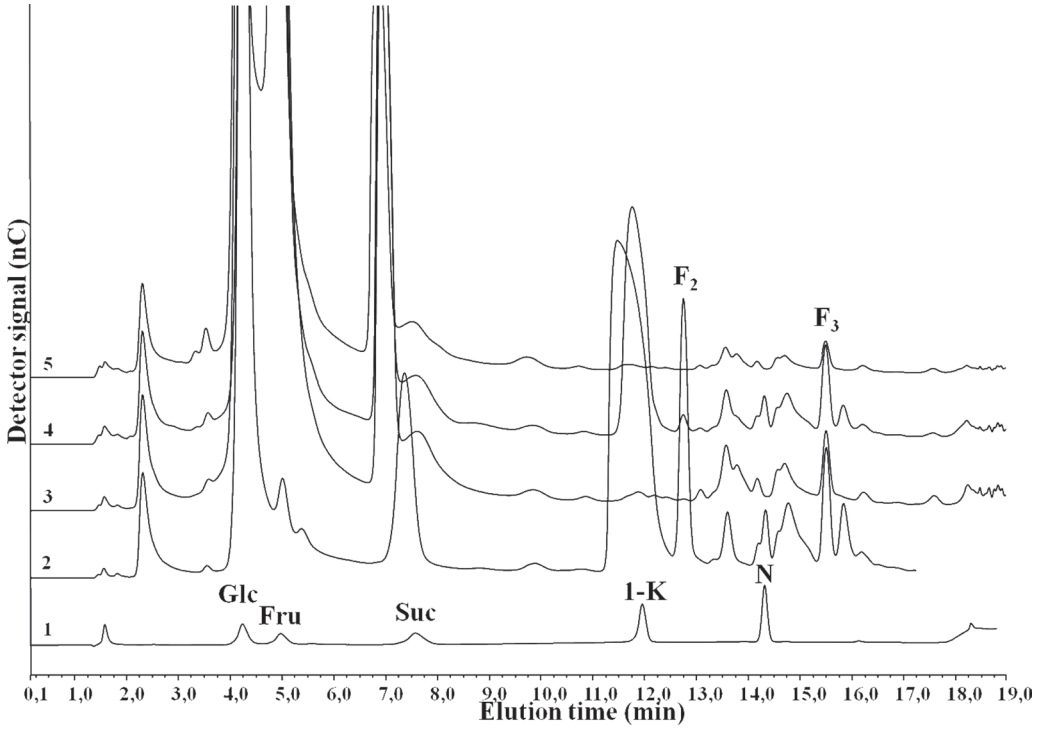

Fig. 3. Effect of 1-FEH and 6-FEH enzyme activity on the products generated during a Fenton reaction with 1-kestotriose. (1) A reference containing glucose (Glc), fructose (Fru), sucrose (Suc), 1-kestotriose (1-K), and 1,1-nystose (N). (2) Products generated by a Fenton reaction with 1-kestotriose (F_2 and F_3). (3) Products as depicted under 2 were further incubated with chicory 1-FEH IIa for 24 h. (4) Products as depicted under 2 were further incubated with sugar beet 6-FEH for 24 h. (5) Products as depicted under 2 were further incubated with chicory 1-FEH IIa and sugar beet 6-FEH for 24 h.

shows the IC_{50}-$O_2{\cdot}^-$ and IC_{50}-·OH data of plant saps derived from sucrose-treated leaves together with saps derived from untreated and water-fed control leaves. All samples were better scavengers of ·OH than $O_2{\cdot}^-$ radicals, as shown by the higher IC_{50}-$O_2{\cdot}^-$ values. Sucrose feeding led to increased scavenging capacities. Water-fed samples were not significantly different from untreated samples. Total phenolic compounds were also compared between these tissues (Table 4). Like fructans, total phenol levels were significantly higher in sucrose-fed leaves compared with control leaves. This was not unexpected since the synthesis of both fructans and some phenolic compounds (such as anthocyanins) is triggered by a specific sucrose signalling pathway (Solfanelli *et al.*, 2006; Ritsema *et al.*, 2009). Total phenol content increased by ~40% and, at the same time, IC_{50}-$O_2{\cdot}^-$ and IC_{50}-·OH dropped by 30%, suggesting that the total phenol quantity is an important player in determining the overall scavenging capacity. However, it cannot be excluded that fructans, or perhaps other (unknown) compounds present in the sap of sucrose-induced plants, can also partly contribute to the increased scavenging capacity.

It should be noted that studying the antioxidant capacity of soluble plant saps has major drawbacks. First, the most powerful antioxidant may mask less effective ones as described in Table 2. Secondly, soluble saps do not reflect the actual cellular context *in vivo* with the presence of intact (organellar) membranes, the tonoplastic membranes being available for strong interactions with fructans (Vereyken *et al.*, 2003). Therefore, the absence of hard evidence for a synergistic action between sugars and phenolic compounds *in vitro* (Table 2) does not exclude the possibility of a putative synergistic action *in vivo*. The *in vitro* ROS-scavenging capacities of inulin and chicoric acid, the major phenolic compound in chicory (Innocenti *et al.*, 2005), are rather similar. Here, a novel speculative concept for a possible collaboration between fructan and phenolic pools in plant vacuoles (e.g. chicory) is proposed (Fig. 7). This working model suggests that vacuolar inulins and phenols (e.g. chicoric acid) perform additive roles, working consecutively in two different spatial environments (the membrane and its vicinity on the one hand, and the vacuolar lumen on the other hand), and exchanging molecules. Unlike chicoric acid, inulins are known to insert deeply into the tonoplast. This means that inulin concentrations can be very high in this environment, part of the inulin chain being inserted in the membrane and the other end probably extending into the lumen (Fig. 7). Thus, compared with chicoric acid, inulin is more ideally positioned to scavenge ·OH radicals produced by the hydroxylic cycle of type III peroxidases (Passardi *et al.*, 2004; Van den Ende and Valluru, 2009; Ferreres *et al.*, 2011). A sucrose radical originating from the interaction between 1-kestotriose (the smallest inulin) and ·OH might be stimulated to diffuse into the lumen when it is recycled there. It was recently predicted that sucrose interacts closely with some secondary metabolites (Wohlert *et al.*, 2010; Tavagnacco *et al.*, 2012). It can be assumed that sucrose radicals would also take part in such complexes, the intimate reaction between the sugar

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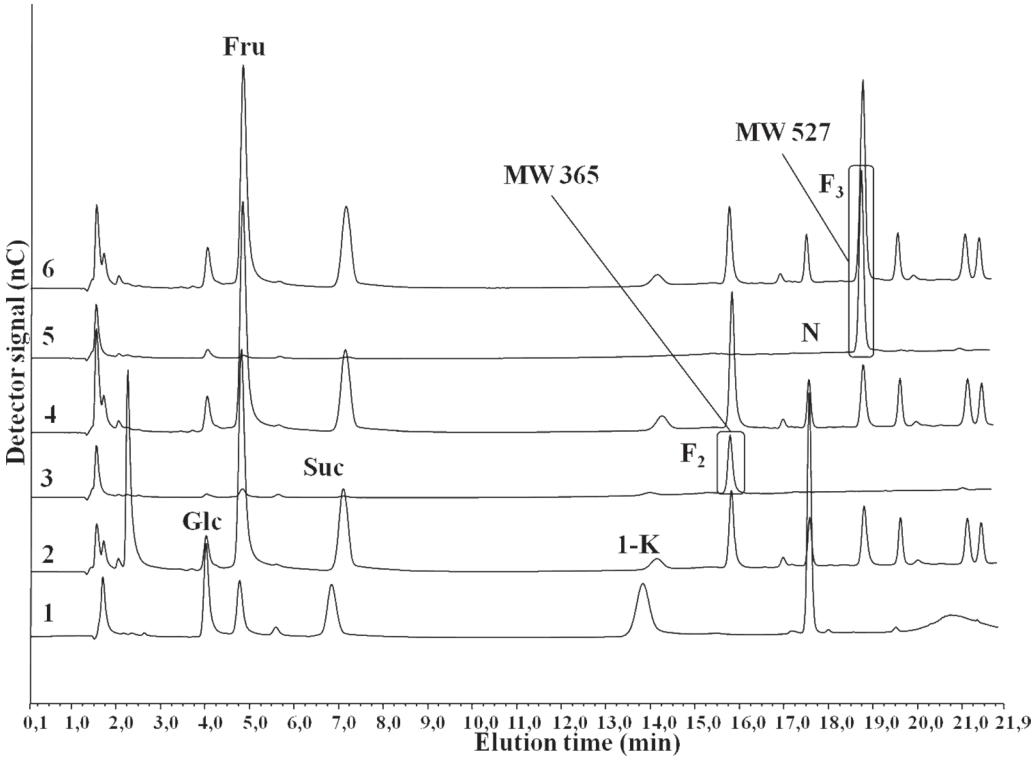

Fig. 4. Purification of F_2 and F_3 from the 1-kestotriose Fenton reaction mixture. The molecular weight (MW) detected by mass spectroscopy is indicated on the HPAEC-PAD profiles. (1) A reference containing glucose (Glc), fructose (Fru), sucrose (Suc), 1-kestotriose (1-K), and, 1,1-nystose (N). (2) A forced chicory root extract. (3) The HPAEC-PAD profile of purified F_2; the MW obtained by mass spectrometry and corresponding to the disaccharide-Na adduct is shown. (4) Co-injection of purified F_2 with a forced chicory root extract. (5) The HPAEC-PAD profile of purified F_3: the MW obtained by mass spectrometry analysis corresponding to the trisaccharide-Na adduct is presented. (6) Co-injection of purified F_3 with a forced chicory root extract.

radical and the secondary metabolite allowing the transfer of the radical from the sugar to the secondary metabolite, recycling the sugar. These recycling mechanisms were suggested before for sugars with oxidation of phenolic compounds (Van den Ende and Valluru, 2009) and for phenolic compounds with oxidation of AsA (Takahama, 2004).

Theoretically, other possible reactions in the vacuolar lumen include (i) the recombination of sugar radicals with phenols to yield sugar–phenol compounds (such as those reported by Choudhary *et al.*, 2006); (ii) the recombination of sugar radicals with each other to form higher DP oligosaccharides; and (iii) the recombination of phenolic radicals with each other into entities with a higher molecular weight. It was speculated before that oxidized phenolic compounds may be reduced by AsA and/or glutathione (GSH), integrating vacuolar antioxidant mechanisms within the overall cellular antioxidant network (Peshev and Van den Ende, 2013). So far the connection between the vacuole and the rest of the cellular antioxidant network through AsA and GSH has remained poorly supported. However, Queval *et al.* (2011) recently demonstrated that GSH can accumulate in stressed vacuoles in its oxidized form, suggesting that this is part of a general response to excess H_2O_2 in plants. Moreover, AsA was found in vacuoles of *Arabidopsis thaliana* and *Nicotiana tabacum*, but in lower concentrations compared with other cellular compartments. However, the strongest proportional increase in AsA levels was observed in vacuoles under high light conditions (Zechmann *et al.*, 2011). Further research is needed to characterize tonoplastic GSH and AsA transporters and study the exact fate of these metabolites in vacuoles.

Possibly, the same concept can be extended to non-fructan-accumulating plants and tissues accumulating other types of vacuolar compounds. For instance, in sugar cane and sugar beet and phloem-associated tissues, the quenching of ·OH by sucrose might be of particular importance (Van den Ende and Valluru, 2009). Supporting this, exogenous sucrose elicited protective effects prior to or upon exposure to oxidative stress-inducing components such as herbicides (Sulmon *et al.*, 2006; Nishizawa *et al.*, 2008; Ramel *et al.*, 2009).

A picture is emerging in which cell wall-derived ·OH seems necessary for proper cell wall-loosening processes (Fry *et al.*, 2001; Schopfer, 2001; Müller *et al.*, 2009). Although the origin of cell wall-derived ·OH is under debate, recent data suggest that peroxidase-mediated Haber–Weiss reactions and plasma membrane NADPH oxidases are producing most of the $O_2^{\cdot -}$ that is used as a source for the production of ·OH (Müller *et al.*, 2009). Their tonoplastic/vacuolar counterparts probably contribute to the synthesis of ·OH in the vicinity of the tonoplast, necessitating ·OH-scavenging processes (Figs. 1, 5, 7) to prevent membrane damage. In fact, any vacuolar compound, present at higher concentrations and with substantial affinity for the tonoplast membrane, would be a suitable candidate to fulfil such a role.

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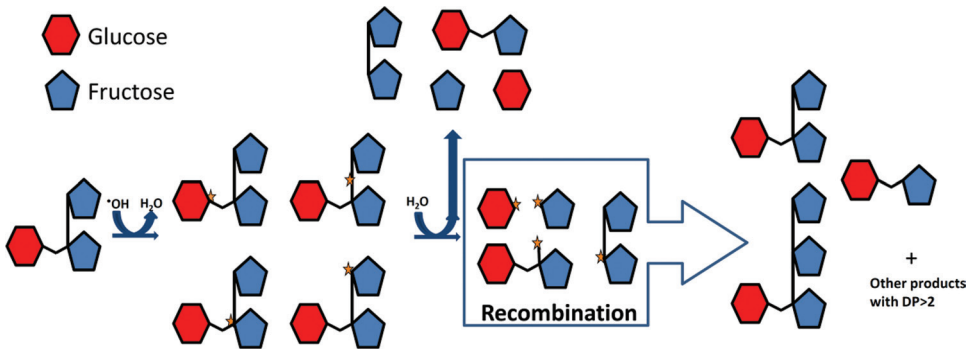

Fig. 5. Schematic presentation of non-enzymatic oligosaccharide synthesis from 1-kestotriose in a Fenton reaction. The ·OH radical abstracts a hydrogen (H) atom from 1-kestotriose, leading to a formation of a 1-kestotriose radical which (partially) fragments into lower DP sugars and sugar radicals. The latter might recombine with each other forming novel, higher DP oligosaccharides. Thus, both breakdown and synthesis of new oligosaccharides can occur in radical reactions involving ·OH and 1-kestotriose. Free radical molecules are indicated by stars.

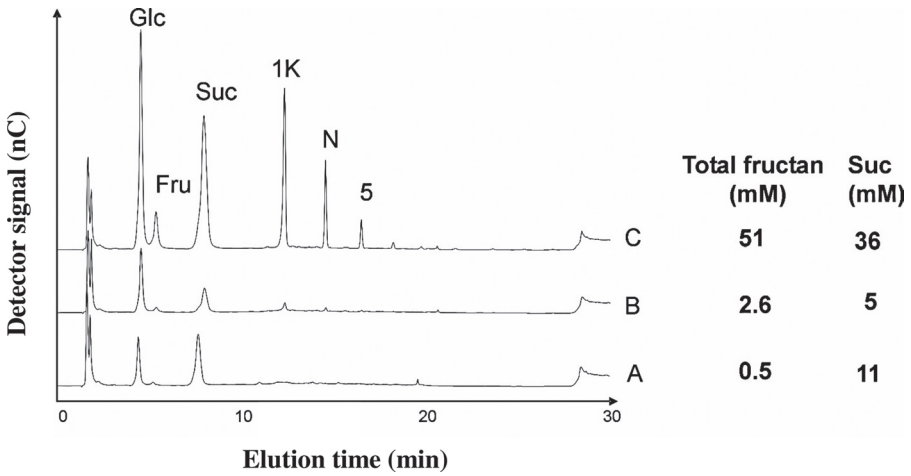

Fig. 6. HPAEC-PAD sugar profiles of saps derived from chicory leaves. (A) Control leaves. (B) Excised leaves incubated for 2 d in water. (C) Excised leaves incubated for 2 d in 250 mM Suc. Glc, glucose; Fru, fructose; Suc, sucrose; 1K, 1-kestotriose; N, 1,1-nystose; 5, 1,1,1-kestopentaose.

Conclusion and perspectives

The *in vitro* work on the ·OH-scavenging capacity of sugars and phenolic compounds revealed a correlation between sugar structure (particularly DP) and ·OH-scavenging capacity, in line with previous research (Nishizawa *et al.*, 2008; Hernandez-Marin and Martínez, 2012), further validating the concept of sugar as an antioxidant. The number and position of C=C-type linkages in phenolic compounds also greatly influences the antioxidant properties. The radical reactions between sugars and ·OH were studied in great detail *in vitro* in Fenton reactions with 1-kestotriose, a DP3 inulin-type fructan. The present data fit with a hydrogen abstraction model leading to random sugar scission and synthesis of diverse new oligosaccharides by a random recombination of sugar radicals. Similar reactions were observed for other stress tolerance-related oligosaccharides (Fig. 1) such as, for instance, raffinose. Considered together with the

Table 3. Scavenging capacity against ·OH and $O_2^{\cdot -}$ radicals of plant saps derived from untreated (Unt), H_2O (H_2O-fed), and sucrose fed (Suc-fed) chicory leaves. Significant differences are indicated with different letters.

	IC_{50}-·OH (ng protein)	IC_{50}-$O_2^{\cdot -}$ (ng protein)
Unt[a]	2.93 ± 0.15	25.02 ± 2.28
H_2O-fed[a]	3.14 ± 0.22	22.79 ± 1.95
Suc-fed[b]	1.95 ± 0.17	18.29 ± 2.12

Table 4. Total phenol content (as gallic acid equivalents: GAE) in chicory leaves fed with sucrose (Suc-fed) and H_2O (H_2O-fed) compared with an untreated control (Unt). Significant differences are indicated with different letters. SE: standard error of the mean.

	mg GAE g^{-1} DW	SE
Unt[a]	16.3	1.5
H_2O-fed[a]	19.2	1.3
Suc-fed[b]	22.9	0.9

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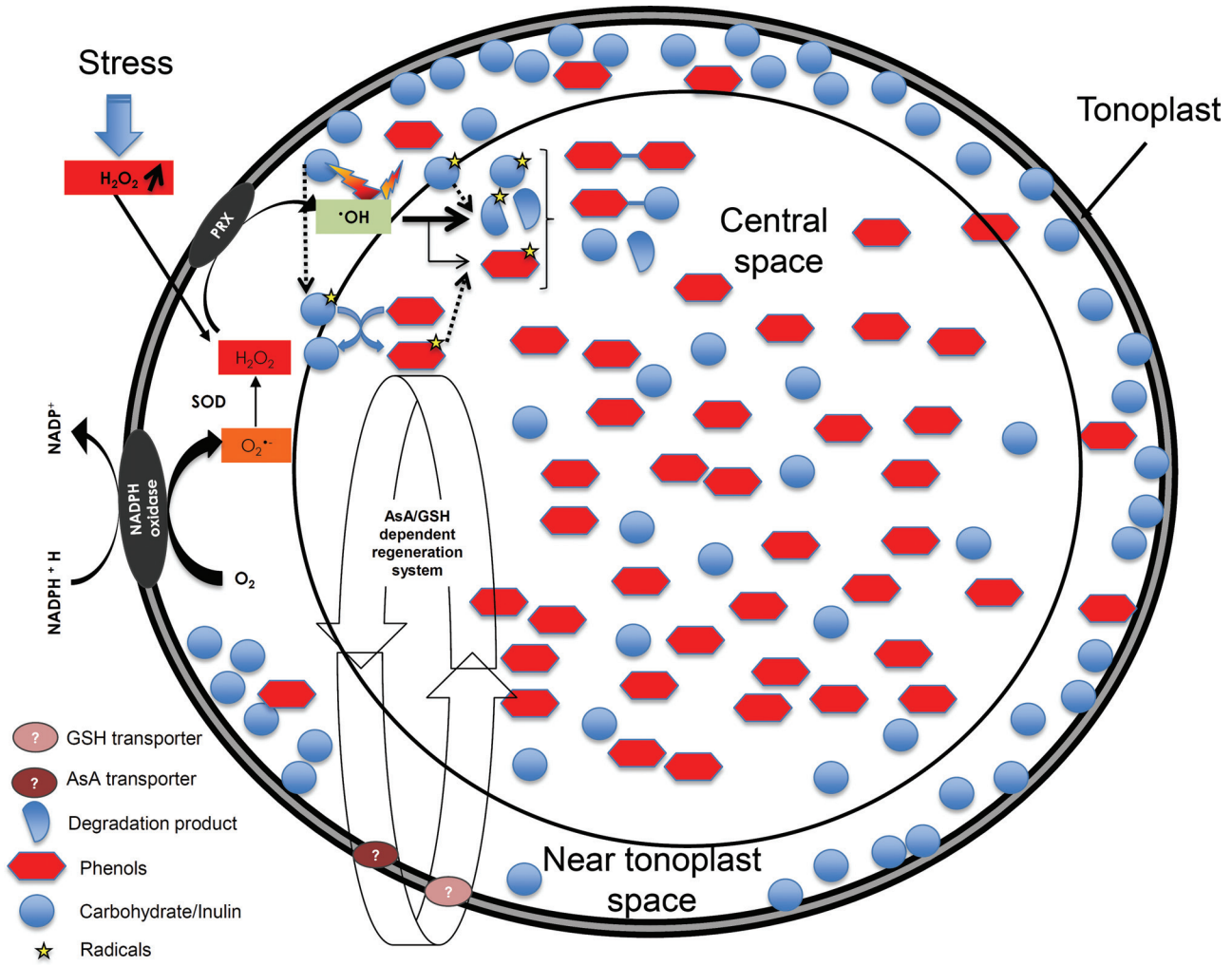

Fig. 7. Model for the role of fructans in the vicinity of tonoplastic membranes and integration in the cellular antioxidant network. Two distinct areas can be distinguished in vacuoles: the near tonoplast inner space and the central vacuolar lumen. Fructans and phenolics are both vacuolar compounds. Fructans strongly interact with membranes and thus a higher concentration can be expected in the near tonoplast environment, while phenolic compounds might predominate in the vacuolar lumen. Under stress, excess cytosolic H_2O_2 might pass through the tonoplast (either by diffusion or assisted by aquaporins) and enter the vacuole. Additionally, superoxide radicals ($O_2^{\cdot-}$) may be produced by tonoplast-resident NADPH oxidases and transformed to vacuolar H_2O_2 by superoxide dismutase (SOD). H_2O_2 is a substrate of type III vacuolar peroxidases (PRXs) associated with the tonoplast. PRXs may produce ·OH through the hydroxylic cycle. Inserting deep into the tonoplastic membrane, fructans are ideally positioned to react with this radical, resulting in the formation of new carbohydrate radicals. As deduced from our *in vitro* experiments, these radicals may undergo scission, splitting up into smaller radical and non-radical components that tend to diffuse away from the tonoplastic membrane into the central vacuolar lumen. Here, sugar radicals might be recycled to sugars and/or radical recombination reactions may occur, resulting in the formation of sugar–phenol compounds, higher DP neutral carbohydrates, or phenolics. Furthermore, sugar recycling might occur at the expense of secondary metabolites (e.g. phenolic compounds) that need subsequent recycling on their own with ascorbate (AsA) and/or glutathione (GSH) that seem to be present at least in some vacuoles. However, further research is needed to demonstrate the presence of AsA and GSH transporters in the tonoplast.

convincing *in vivo* data on the greatly improved antioxidant properties of *Arabidopsis* galactinol synthase overexpressors with strongly increased raffinose levels (Nishizawa *et al.*, 2008), these data strongly suggest that fructans as well as other sugars might be implicated in (organellar) antioxidant mechanisms *in vivo*. A new hypothetical model is proposed for putative vacuolar antioxidant mechanisms in fructan plants (such as chicory), with a central role for ·OH scavenging by fructans at the tonoplast. Providing direct *in vivo* evidence for the proposed ·OH radical reactions near the tonoplast is an extremely difficult task and a huge challenge. Following up specific carbohydrate breakdown or a build-up of novel oligosaccharides as a result of the above-mentioned radical reactions under stress treatments is a possibility, but unfortunately the same components can be generated by endogenous enzymatic activities as well (e.g. in chicory F_2, F_3, and F_4 can be generated by the combined action of chicory 1-FEH and 1-FFT) and usually no knockdown or knockout

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plants are available for this purpose (chicory is not a model plant). Even shifting to a model plant (such as *Arabidopsis*, an RFO accumulator) presents great challenges since RFO breakdown relies on multigenic families of α-galactosidases (Peters *et al*., 2010) and β-fructosidases (Van den Ende *et al*., 2009). Immersing tissues of the non-fructan accumulator *Arabidopsis* in 1-kestotriose seemed a plausible way out, but seems compromised by recent findings that both 1-kestotriose and inulobiose can be detected in *Arabidopsis* (Bolouri Moghaddam and Van den Ende, 2012, and references therein). Needless to say, further research is needed to unravel the exact origin of 1-kestotriose and inulobiose in *Arabidopsis* and on the origin of the intriguing fructo-phenolic compounds in *Lindelofia stylosa* (Choudhary *et al*., 2006). Such approaches would be likely to provide more evidence for the proposed hypothetical model *in planta*. In addition to the radical reactions between ·OH and vacuolar compounds such as sugars and phenolic compounds, the radical reactions between such compounds and other types of ROS, such as superoxide (Table 3), need further attention. Furthermore, the results presented here urge further research on the reactions between carbohydrates and RNS as equally important reactive species in plants.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Origin of chemicals used.

Figure S2. Chemical structures of the tested phenolic compounds (derived from Neveu *et al*., 2010).

Figure S3. Chemical structures of the tested carbohydrates.

Figure S4. HPAEC-PAD chromatograms showing the co-injection of a 1-kestotriose Fenton reaction with a forced chicory root extract.

Figure S5. HPAEC-PAD chromatograms of a Fenton reaction with ultrapure 1-kestotriose (1-K) and its controls.

Table S1. ·OH-scavenging capacity of gallic acid/1-kestotriose mixtures at varying ratios.

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