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Levans in Excised Leaves of *Dactylis glomerata*: Effects of Light, Sugars, Temperature and Senescence

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***Dactylis glomerata* (orchardgrass) accumulates a single series of levans and the high DP polymers might be correlated with an increased stress resistance. A single levan series could be induced in excised orchardgrass leaves, without any 1-kestose accumulation, strongly suggesting that fructan synthesis occurs independently of 1-SST activity. This elegant excised leaf system was used to study fructan metabolism regulation as affected by environmental conditions and exogenous sugar treatments. In contrast to the well-studied barley excised leaf system, fructan biosynthesis could not be rapidly induced in the light without exogenous sugar and only a limited fructan synthesis was observed in the dark with sugar. It can be concluded that both light and sugar are needed to achieve an optimal fructan synthesis. To induce fructan biosynthesis, sucrose could be replaced by a combination of glucose and fructose. Fructans were found to be a surplus pool of sucrose when a threshold sucrose concentration is surpassed. A metabolic switch to fructan degradation was observed when induced orchardgrass leaves were incubated in the dark at 30°C. Interestingly, fructans persisted during senescence of sugar-induced orchardgrass leaves. On the longer term, these fundamental regulatory insights might help to create superior grasses for future feed and/or biomass production.**

Key words: *Dactylis glomerata*, fructan, levan, orchardgrass, regulation, senescence

Fructans, fructose polymers derived from sucrose, are produced by several bacteria and about 45000 species of angiosperms (Hendry, 1993). They become increasingly popular as healthy food compounds and as a source for non-food applications. Suggested roles for fructans *in planta* include long and short-term storage, stress protection and osmoregulation (Van Laere and Van den Ende, 2002). Fructans are believed to accumulate in the vacuole (Frehner et al. 1984), although both fructans and the enzymes degrading them (fructan exohydrolases or FEHs) have been reported to occur in the apoplast (Livingston and Henson, 1998).

Fructan biosynthesis has been intensively studied in a number of model plants including chicory, Jerusalem artichoke, onion, *Lolium*, wheat and Asparagus (Edelman and Jefford, 1968; Fujishima et al., 2005; Shiomi et al., 2007; Van den Ende and Van Laere, 2007; Prud'homme et al., 2007; Yoshida et al., 2007). In all cases, at least two or three fructan biosynthetic enzymes (fructosyl transferases) are necessary to build the fructans found in these species. Fructan breakdown is catalyzed by FEHs sequentially hydrolyzing terminal fructosyl units. Many different types of FEHs have now been characterized and cloned, fulfilling a number of important physiological functions both in fructan plants and non-fructan plants (De Coninck et al., 2007).

Dicotyledonous plants accumulate fructans that consist mainly of β2-1 bound fructose units (inulins), whereas in monocotyledonous species fructans with β2-6 linkages (levans and graminans) are prevalent. Among grasses, fructans occur predominantly in C3 species (Hendry, 1993). So far research efforts mainly focused on economically important grasses such as barley (*Hordeum vulgare* L), wheat (*Triticum aestivum* L.) and *Lolium* species (*Lolium perenne*, *Lolium temulentum*). Wheat and barley synthesize lower degree of polymerization (DP) graminan-type fructans containing both β2-6 and β2-1 linkages (Yoshida et al., 2007) while *Lolium perenne* accumulates low DP inulin and inulin neoseries fructans and higher DP levan neoseries fructans (Pavis et al., 2001). *Dactylis glomerata*, *Phleum pratense*, *Poa ampla* and a few other grass species (*Phalaris aquatica*, *Puccinellia stricta*) are special since they are known to accumulate only a single series of linear β2-6 linked fructans with a terminal glucose (Yamamoto and Mino, 1985; Spollen and Nelson, 1988; Chatterton et al., 1993; Bonnett et al., 1997). Despite the presence of one simple, dominant levan series, only a few attempts (Cairns et al., 1999; Wei et al., 2002) have been undertaken to unravel the fructan anabolism in some of these species. However, it remains unclear how many (one or two) fructosyl transferases are needed to biosynthesize the levans accumulating in these plants. Yamamoto and Mino (1985) purified and characterized a 6-fructan exohydrolase (6-FEH) involved in the degradation of levans in *Dactylis glomerata*.

Some dicot plant species like *Viguiera discolor* (Isejima and Figueiredo-Ribeiro, 1993) and *Echinops ritro* (Vergauwen et al., 2003) produce considerably higher DP inulin-type fructans than all other species. Since both of these species are drought-tolerant, it can be hypothesized that high DP fructans may be directly or indirectly involved in resistance to abiotic stress. It was demonstrated that the high DP inulin profile observed in *Echinops ritro* could be attributed to the presence of a "high DP" fructan: fructan 1-fructosyltransferase (1-FFT). A kinetic comparison with a "low DP" 1-FFT from chicory revealed that the chicory 1-FFT had a higher affinity for short carbohydrates (sucrose, fructose and 1-kestose) while the high DP 1-FFT preferred longer inulin chains as acceptors (Vergauwen et al., 2003). Recently, the high DP 1-FFTs from *Echinops ritro* and *Viguiera discolor* have been cloned and heterologously expressed (Van den

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Ende et al., 2005, 2006).

Interestingly, and similar to what is observed among dicot species, some monocots developed the capacity to synthesize high DP levan-type fructans. The longest fructans ever described in plants are observed in *Dactylis glomerata* (orchardgrass) where they can reach a DP of up to 314, depending on the tissue and environmental conditions (Yamamoto and Mino, 1985). Like ryegrass, orchardgrass is an economically important species cultivated for hay making and grazing in temperate zones. Strikingly, some varieties of *Dactylis* and *Lolium* are able to survive severe summer droughts in Mediterranean areas, and this was reported to be correlated to their higher "high DP" fructan contents (Volaire et al., 1998). The fructan concentration in autumn was also found to be closely related to winter hardiness in orchardgrass (Sanada et al., 2007).

Under ideal growth conditions, most fructan accumulating plants contain no or very low amounts of fructans in their source leaves. However, fructan synthesis can often be induced in leaf blades under certain physiological conditions. Therefore, excised leaf blades became a very popular system to study regulation of fructan metabolism (Morcuende et al., 2004; Nagaraj et al., 2004). In particular high DP fructan biosynthesis could be induced by incubating leaf blades of *Echinops ritro* in sucrose (Van den Ende et al., 2006).

There are many good reasons to choose orchardgrass as a model plant to study fructan metabolism: i) the species accumulates a single series of levan-type fructans, so it can be expected that the number of enzymes involved in their biosynthesis is more limited compared to wheat, barley and *Lolium* all accumulating a complex mixture of oligo- and polysaccharide type fructans ii) the species can accumulate very high DP fructans which might be related to stress resistance in plants iii) the limited number of fructan biosynthetic genes (maybe only one gene) could be of great biotechnological value when introduced in economically important crop plants iv) so far studies on fructan metabolism regulation are mainly restricted to barley, and it would be very useful to investigate the regulation in orchardgrass and other grasses as well, in order to detect common mechanisms or, alternatively, marked differences between species v) these deeper insights in fructan metabolism regulation might lead to a further increase in water-soluble carbohydrates (WSC) in grasses which is related to digestibility and fermentation quality of silage.

As a first step towards future work on enzyme purification and characterization, gene cloning and biotechnological applications, we report here on the accumulation of levan-type fructans in excised orchardgrass leaf blades as affected by different (environmental) factors such as photoperiod, temperature and exogenous sugar treatment. Furthermore, the fate of levan-type fructans was followed during leaf senescence.

MATERIALS AND METHODS

Plant Material

Mature, photosynthesizing source leaves were collected from a 4-year old stand of *Dactylis glomerata* derived from a natural population in Heverlee, Belgium. The leaves were cut with a sharp scalpel and the cut end was immediately placed in water. Three leaves were immediately frozen in liquid nitrogen (untreated control) and stored at –80°C until analyzed. For treatments, three groups of three leaves were taken.

Different Light Regimes

Excised leaves were placed in a growth chamber at 20°C in Milli Q water supplemented with 0.01% chloramin (water control) or in 200 mM sucrose supplemented with 0.01% chloramin. Leaves were treated for 32h under 3 different conditions: i. continuous light (350 µmol photons m^{-2} s^{-1}) ii. 12h of light, 12h of dark, 8h of light and iii. continuous dark. Thereafter, part of the leaves were extensively washed with Milli Q water and subsequently frozen in liquid nitrogen and stored at -80°C until further analysis. The remaining leaves of the three conditions were transferred to Milli Q water supplemented with 0.01% chloramin. Half of them received full light while the other half were kept in the dark. After 24h, the leaves were washed with Milli Q water and subsequently frozen in liquid nitrogen and stored at -80°C until further analysis.

Exogenous Sugar Treatments

Excised leaves were placed in a growth chamber for 32h at 20°C in Milli Q water supplemented with 0.01% chloramin (water control) and in 200 mM sucrose, 200 mM glucose, 200 mM fructose, 100 mM glucose + 100 mM fructose and 200 mM mannitol, all supplemented with 0.01% chloramin. Experiments were performed under continuous light (350 µmol photons m^{-2} s^{-1}) and in the dark. After 24h, the leaves were washed with Milli Q water and subsequently frozen in liquid nitrogen and stored at -80°C until further analysis.

Effect of Temperature

Excised leaves were induced to accumulate fructans as described above (continuous light, 32 h, 200 mM sucrose) and were then transferred to growth chambers at 20°C, 25°C an 30°C and incubated for 24h in the dark or in continuous light as described (Geuns et al., 1997). The leaves were washed with Milli Q water and subsequently frozen in liquid nitrogen and stored at –80°C until further analysis.

Leaf Senescence

Excised leaf blades were incubated in 200 mM sucrose in continuous light and followed on a daily basis during 1 week. Daily samples were washed with Milli Q water and subsequently frozen in liquid nitrogen and stored at –80°C until further analysis.

Carbohydrate Analysis

Excised leaf blades were ground in liquid nitrogen with mortar and pestle until a fine powder was obtained. Five volumes of Milli Q water were added and the mixture was incubated at 90°C for 15 min. After cooling, the homogenate was centrifuged at 3000 g for 5 min. A 200 µL sample

of the supernatant was passed through a 0.5 mL bed volume of Dowex®-50 H^+ and a 0.5 mL bed volume of Dowex®-1-Acetate. The resins were rinsed six times with 200 µL distilled water. The pH of the eluate was adjusted to pH 6.0 with a small amount of an unbuffered Tris solution (1.0 M). The eluate was diluted twice and centrifuged at 3000 g for 5 min. From this neutral fraction 25 µL was analyzed with high-pressure anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described (Vergauwen et al., 2000). Quantification was performed on the peak areas with the external standards method for glucose, fructose and sucrose. A short HPAEC-PAD method was developed in which all levans (DP 3 and higher) were eluted from the column with 500 mM Na-acetate. These fructan peaks were manually collected and subjected to mild acid hydrolysis as described (Vergauwen et al., 2003). The total amount of fructose in fructans (TF) was subsequently quantified with the external standard method as described above.

RESULTS AND DISCUSSION

Levan Synthesis Can be Induced in Excised Orchardgrass Leaf blades

We propose *Dactylis glomerata* (orchardgrass) as a model plant to study high DP levan metabolism in grasses since only one prominent fructan series is present. Moreover, the highest DP ever reported in plants was observed in orchardgrass (Yamamoto and Mino, 1985). Typically, the lower parts of the plant (stubble) accumulate higher fructan concentrations compared to the upper parts (foliage) and total fructan levels strongly vary among ecotype and environmental conditions (Volaire and Lelièvre, 1997; Sanada et al., 2007). Research efforts focused on fructan metabolism in stubble because these fructans are believed to act as an energy reserve allowing fast re-growth after defoliation (Prud'homme et al., 2007). To our knowledge, no specific research was ever dedicated to fructan metabolism in excised mature leaf blades of *Dactylis glomerata*. Leaf blades from healthy, well-watered plants only contain glucose, fructose and sucrose and no fructans could be detected (Fig. 1). However, after incubation of excised orchardgrass leaves in 200 mM sucrose for 32h, a single fructan series became apparent in these induced leaves, similar to the one occurring in the stem base of the plant (not shown). Accumulation of low DP fructans was limited and most of the material eluted from the HPAEC-PAD column only when using 500 mM Na Acetate, already indicating the presence of higher DP fructans (arrow in Fig. 1). Manual collection and mild acid hydrolysis of these peaks from sucrose-induced leaves (4 days) resulted in a mean DP of about 20 (as calculated from the fructose to glucose ratio). We show here that oligofructans do not accumulate to a great extent in *Dactylis*, suggesting that higher DP fructans are even better acceptor substrates for the enzyme(s) involved in the biosynthetic process. The absence of consistent oligofructan accumulation was also reported in *Phleum* (Spollen and Nelson, 1988). By using 6-kestose and a complex wheat fructan pattern as a reference (the position of 6,6 nystose, 6,6,6 kestopentaose and 6,6,6,6 kestohexaose is known in wheat; Yoshida et al., 2007), it could be confirmed that the fructan series in orchardgrass is indeed of the levan-type, as previously reported (Yamamoto and Mino, 1985). Furthermore, the levan-type nature was re-confirmed by manual collection of these "higher DP fructan peaks" and treatment with sugar beet 6-FEH (Van den Ende et al., 2003) and with α-amylase (not shown). These peaks disappeared after treatment with 6-FEH but not after treatment with α-amylase. Moreover, mild acid hydrolysis removed sucrose and the levan type fructans (Fig. 1). Starch is known to be insensitive to mild

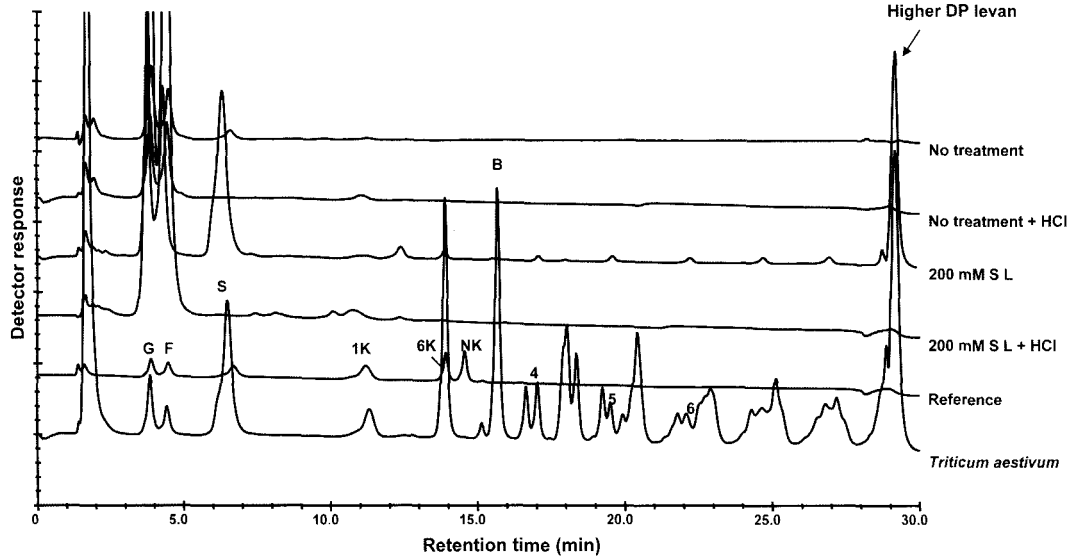

Figure 1. HPAEC-PAD chromatograms showing carbohydrates in excised orchardgrass leaf blades before and after induction of fructan biosynthesis (200 mM sucrose, 32h, continuous light, 20°C) and also after additional mild acid hydrolysis with HCl as described in Vergauwen et al., 2003. For comparison, HPAEC-PAD chromatograms of carbohydrate standards and a wheat stem are included as references. G: glucose; F: fructose; S: sucrose; 1K: 1-kestose; 6K: 6-kestose; NK: neokestose; B: bifurcose; 4: 6,6 kestotetraose; 5: 6,6,6 kestopentaose; 6: 6,6,6,6 kestohexaose. The arrow shows the position of higher DP levan.

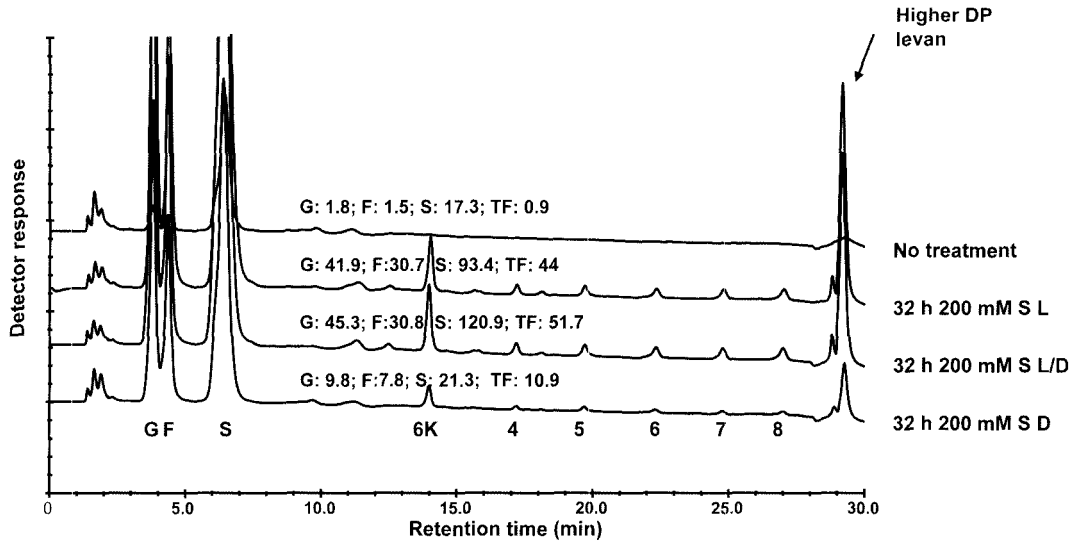

Figure 2. HPAEC-PAD chromatograms showing carbohydrates in excised orchardgrass leaf blades incubated in 200 mM sucrose under different light regimes at 20°C. L: continuous light (32h); L/D: 12h of light, 12h of dark, 8h of light; D: continuous dark (32h). G: glucose; F: fructose; S: sucrose; 6K: 6-kestose; 4: 6,6 kestotetraose; 5: 6,6,6 kestopentaose; 6: 6,6,6,6 kestohexaose; 7: 6,6,6,6,6 kestoheptaose; 8: 6,6,6,6,6,6 kestooctaose. TF: total fructan. A control carbohydrate pattern (no treatment) is also presented. The arrow shows the position of higher DP levan. The concentrations (in µmoles g FW $^{-1}$) of G, F, S and TF are shown.

acid hydrolysis. It can be concluded that the compounds in the chromatograms represent fructans and not starch, but it cannot be ruled out that starch is present (perhaps in lower concentrations) in induced leaves of orchardgrass.

Strikingly, neither 1-kestose nor bifurcose could be detected in the induced orchardgrass leaves, strongly suggesting that fructan biosynthesis is independent on 1-SST activity. In case 1-SST would occur, the absence of 1-kestose accumulations could only be explained by the presence of a 6-FFT type enzyme with an extremely high affinity for 1-kestose as fructosyl donor. However, this seems highly unlikely since i. no 6-FFT type enzymes have ever been reported in plants ii. so far fructan biosynthetic enzymes generally show a very low affinity (high K_m) for their substrates (Van Laere and Van den Ende, 2002), a noticeable exception being the high DP 1-FFT from *Echinops ritro* (Van den Ende et al., 2006) showing an apparent donor K_m of 15 mM for 1-kestose. Taken together, these observations suggest that fructan biosynthesis in orchardgrass is independent of 1-SST and FFT-type enzymes but might occur by a combination of 6-SST and 6-SFT enzymes or even by a single 6-SST/ 6-SFT enzyme with characteristics that might resemble those of some bacterial levansucrases.

As a first step towards unravelling the enzymology of fructan biosynthesis in orchardgrass and better understanding the regulation of this fructan metabolism, we further used the elegant excised leaf system to follow fructan patterns under various environmental conditions.

Combining Light and Exogenous Sucrose Maximizes Fructan Accumulation

Untreated plants accumulate up to 17.3 µmoles sucrose g FW^{-1} but this concentration seems not high enough to induce fructan synthesis. Fig. 2 shows the carbohydrate patterns in excised orchardgrass leaves upon incubation in 200 mM sucrose under different light regimes. The introduction of a 12h dark period in the treatment did not compromise fructan accumulation compared to a continuous light treatment. It is striking that fructan accumulation is greatly affected (5 times lower) in the dark. Dark abolishes the production of endogenous sucrose so the total concentration of sucrose in the tissue might fall below a threshold sucrose concentration (as proposed by Pollock et al., 2003) needed to efficiently induce fructan biosynthesis at the gene level and/or provide enough substrate for the fructan biosynthetic machinery. Many fructan biosynthetic genes contain both sucrose responsive elements (SURE) and light responsive elements in their promoters (Nagaraj et al., 2004 and own observations), suggesting that the presence of both light and sugar signals are needed for an efficient fructan production, as we observed (Fig. 2). However, it should be noticed that light is not indispensable for induction of fructan biosynthesis in excised barley leaves since equal amounts of fructans could be generated in continuously illuminated leaves (incubated in water) compared to leaves held in the dark but incubated in 0.5 M of sucrose (Wagner et al., 1986). However, it is questionable whether the use of 0.5 M sucrose can be considered as a "physiologically relevant" concentration. It is well known that such high sugar concentrations cause osmotic stress. Moreover, it is unlikely that experiments using such high concentrations are still suitable to study sugar signalling events and therefore we decided not to use sugar concentrations higher than 200 mM. Up to date there is still a lot of debate on the localization of fructan biosynthesis (vacuolar versus vesicular) and the putative sucrose concentration near the active sites of the fructosyl transferases involved in the process (Prud'homme et al., 2007).

We pre-treated excised orchardgrass leaves under 3 different conditions as shown in Fig. 2 and then half of the leaves were transferred to continuous dark for 24h in water

whereas the other half were kept under continuous light in water (Fig. 3). As expected, leaves kept in the light further accumulated fructans. Interestingly, leaves that were pre-induced in continuous or discontinuous light and sucrose further slightly increased their fructan contents during subsequent incubation in the dark and in water. Apparently, the endogenous concentration of sucrose in these leaves (12.3 and 9.7 µmoles sucrose g FW^{-1}) was still sufficient to continue fructan synthesis with the enzymes induced during the pre-treatment. Leaves that were pre-treated in the dark could be stimulated to accumulate fructans (up to 81 µmoles fructose g FW^{-1}) when they were transferred to water in the light for 24h. However, further incubation in water in the dark resulted in a decrease of sucrose and fructans. It is likely that the sucrose concentration in these leaves dropped below a critical concentration (3.8 µmoles sucrose g FW^{-1}), resulting in a downregulation of fructosyl transferase transcripts and/or enzyme activities as described in excised barley leaves (Nagaraj et al., 2004). These researchers showed a strong decrease in fructan concentration when

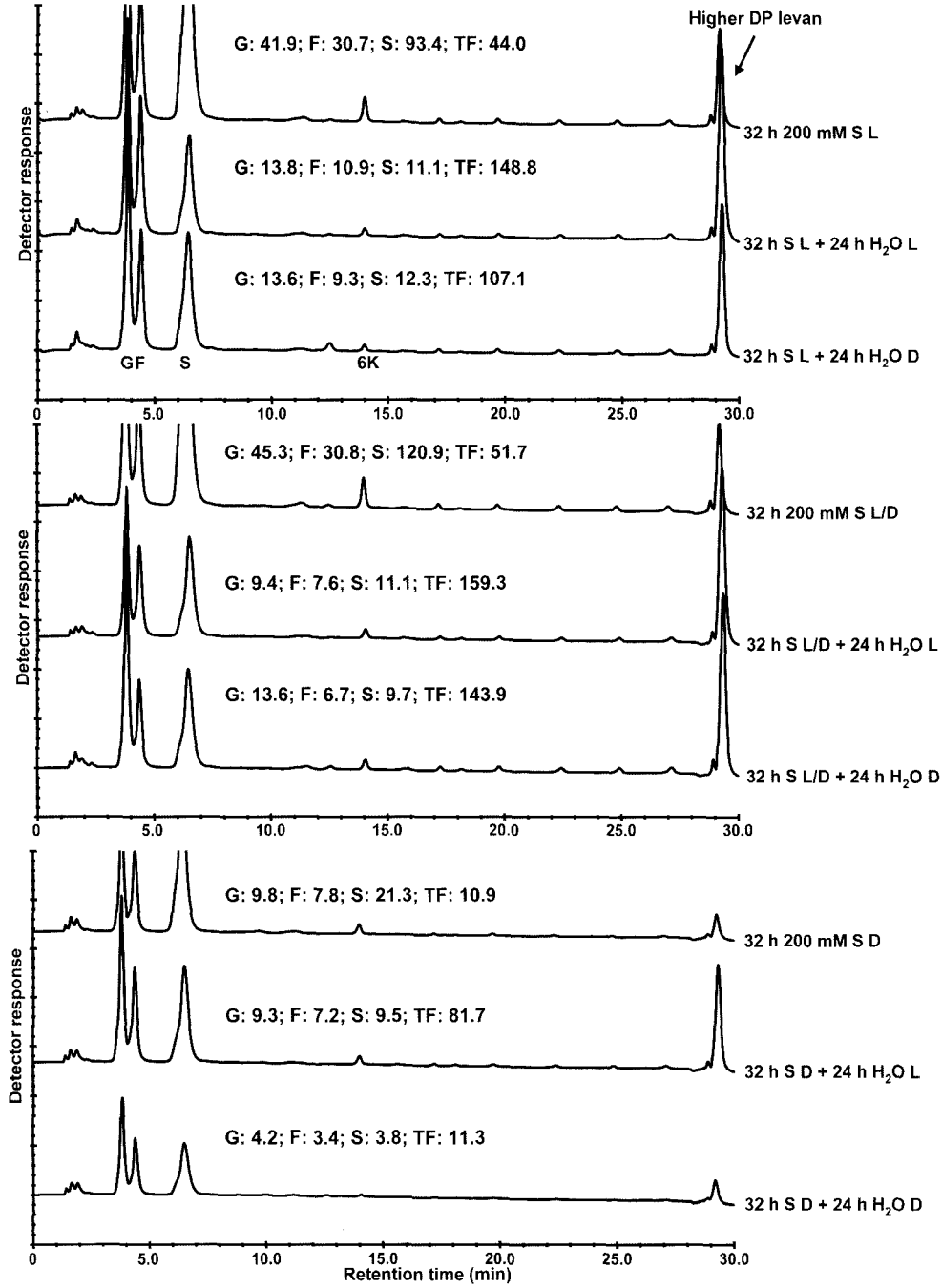

Figure 3. HPAEC-PAD chromatograms showing carbohydrates in excised orchardgrass leaf blades first incubated in 200 mM sucrose under different light regimes (cfr Figure 2) and then transferred to water for 24 h in continuous light (L) and dark (D). G: glucose; F: fructose; S: sucrose; 6K: 6-kestose; TF: total fructan. The arrow shows the position of higher DP levan. The concentrations (in µmoles g FW^{-1}) of G, F, S and TF are shown.

illuminated barley leaves (24h) were placed in the dark for a period of 24h. This was explained by a downregulation of both 1-SST mRNA transcripts and 1-SST enzymatic activity.

It is possible that low sugar concentrations could induce 6-FEH gene transcription. It was demonstrated before that the induction of 6-FEH in excised stem base of orchardgrass was suppressed by exogenously supplied glucose, fructose and sucrose (Yamamoto and Mino, 1987). On the other hand, low sucrose concentrations might activate sucrose-inhibited FEHs as reported in wheat (Van den Ende et al., 2003a) and *Lolium* tissues (Lothier et al., 2007), even during the period of active fructan biosynthesis.

Effect of Hexoses and Sucrose on Fructan Accumulation in Excised Orchardgrass Leaves

Figure 3 demonstrated that a combination of light and sucrose drive efficient fructan synthesis in excised leaves of orchardgrass. What is the effect of absence of exogenous sugar in this system? Can sucrose be replaced by other metabolizable sugars and what is the effect of a non-metabolizable sugar in this system? In severe contrast to the excised leaf system of barley, showing a very rapid accumulation of sucrose and fructans under continuous light and without addition of exogenous sugar (Nagaraj et al., 2004), no fructan synthesis could be induced when excised orchardgrass leaves are placed in the light for 32h (Fig. 4). This observation strongly suggests that the threshold sucrose concentration to induce fructan biosynthesis in orchardgrass is considerably higher than in barley. Compared to 200 mM sucrose, a combination of 100 mM glucose and 100 mM fructose led to a similar amount of fructans, while 200 mM glucose or 200 mM fructose resulted in lower amounts (Fig. 4). It is well known that grass source leaves contain very high activities of sucrose anabolic and catabolic activities (Kingston-Smith et al., 1999). This means that exogenously applied hexoses can be rapidly transformed into sucrose and that exogenously applied sucrose can be rapidly broken down to hexoses, making it difficult to fully discriminate between hexose and sucrose-mediated signalling in such systems. However, studies with hexose analogues on barley and wheat leaves (Müller et al., 2000) strongly suggested that induction of fructan biosynthesis occurs independently of hexokinase but very likely occurs via a specific interaction of sucrose with specific transporters/sensors that remain to be identified. Compared to glucose, fructose and sucrose, fructan synthesis was very low with mannitol in the incubation medium, excluding the possibility that fructan synthesis could be induced by osmotic stress. When excised orchardgrass leaves were incubated in the dark in the presence of 200 mM of these sugars, proportionally similar results were obtained but the total fructan concentrations were much lower (see also Fig. 2) and more difficult to quantify. These results suggest that light might be a more prominent effector in the orchardgrass leaf system compared to the barley leaf system and this is an interesting subject of further investigation.

Reversing Fructan Accumulation

Thorsteinnson et al. (2002) reported that intact *Phleum* plants accumulated more high DP fructans when transferred from higher to lower temperatures, and explained this observation by a change in source/sink balance within these plants. Higher concentrations of fructan were also reported during late autumn in several orchardgrass varieties (Yamamoto et al., 1999; Sanada et al., 2007). These observations on intact plants prompted us to investigate whether excised orchardgrass leaves, after induction of fructan biosynthesis by sucrose and light, would arrest fructan accumulation by transferring them to water in the dark and at increasing temperatures (20, 25 and 30°C). We detected a clear metabolic switch in the dark at 30°C, resulting in much lower fructan concentrations than in the light. Interestingly, this even

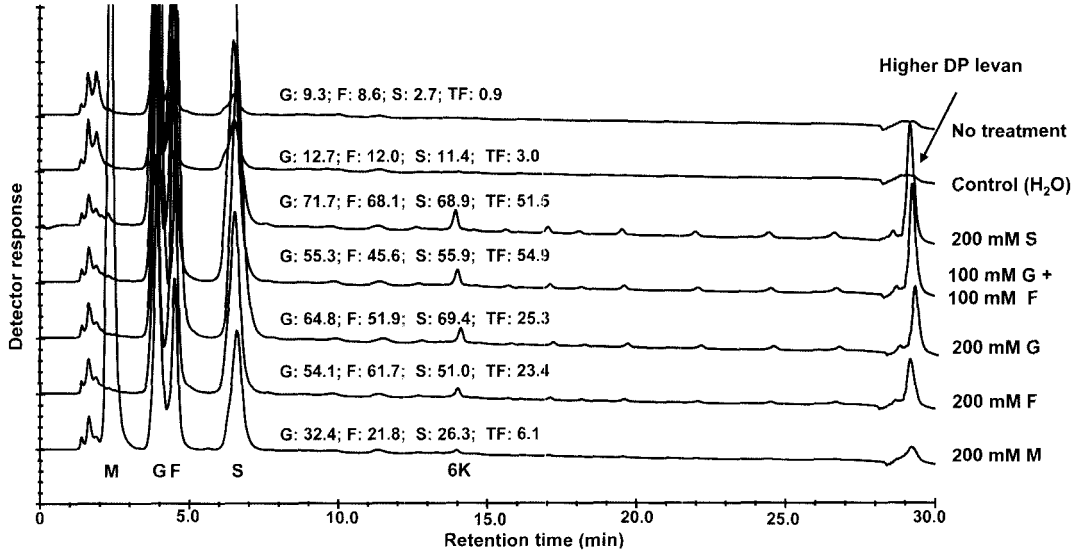

Figure 4. HPAEC-PAD chromatograms showing carbohydrates in excised orchardgrass leaf blades incubated in 200 mM of different sugars for 32h at 20°C under continuous light. Chromatograms of untreated and water incubated leaves are presented as controls. G: glucose; F: fructose; S: sucrose; M: mannitol; 6K: 6-kestose; TF: total fructan. The arrow shows the position of higher DP levan. The concentrations (in μmoles g FW^{-1}) of G, F, S and TF are shown.

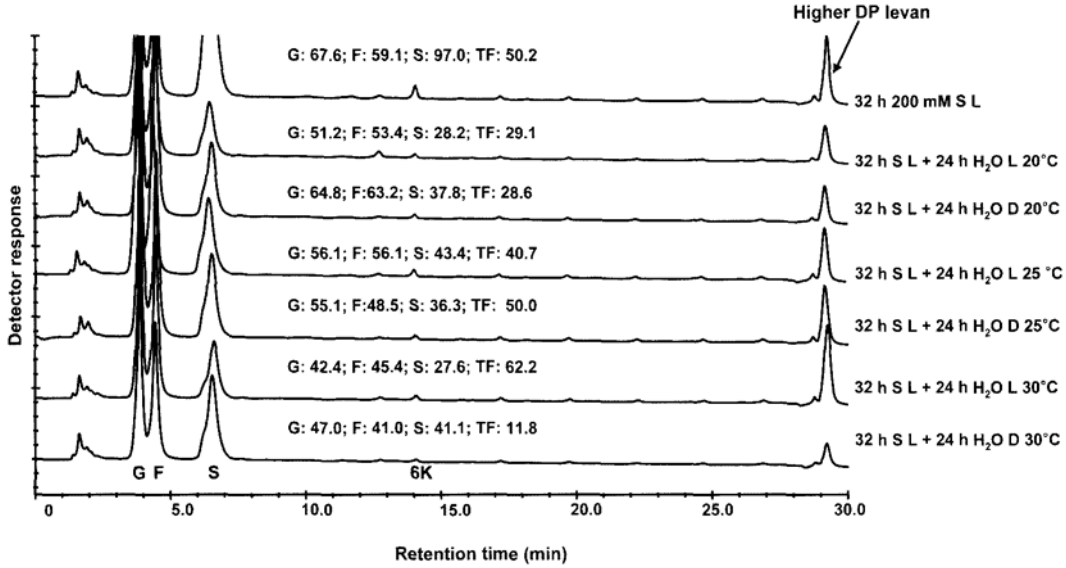

Figure 5. HPAEC-PAD chromatograms showing carbohydrates in excised orchardgrass leaf blades first incubated in 200 mM sucrose under continuous light at 20°C and then transferred to water at different temperatures (20°C, 25°C and 30°C) in the light (L) and in the dark (D). G: glucose; F: fructose; S: sucrose; 6K: 6-kestose; TF: total fructan. The arrow shows the position of higher DP levan. The concentrations (in μmoles g FW^{-1}) of G, F, S and TF are shown.

occurred despite the fact that the total sucrose concentration (41.1 μmoles g FW^{-1}) was higher compared to the light condition (Fig. 5). Like in barley, it can be speculated that fructosyl transferase transcripts or enzymes are much more sensitive to degradation in the dark at elevated temperatures. Obenland et al. (1991) postulated that 1-SST is continuously subjected to constant proteolytic degradation in the vacuole of barley leaves, and that the observed enhancement of 1-SST activity in the light or upon feeding sucrose is due exclusively to *de novo* protein synthesis. Likely, high temperatures (30°C) in the dark might further accelerate vacuolar protein degradation but not promote the *de novo* protein synthesis to the same extent, since energy requirements are needed to sustain the strongly increased respiration and compensate for the absence of photosynthesis. On the other hand, it cannot be excluded that FEHs are specifically induced to degrade the fructans and provide energy to cope with the dark treatment and the increased respiration at 30°C.

Fate of Fructans During Leaf Senescence

Leaf senescence is characterized by a decline in chlorophyll content and photosynthetic activity. There is debate whether *natural* leaf senescence is induced by sugar starvation or by sugar accumulation. Perhaps different types of senescence need to be distinguished (Buchanan-Wollaston et al., 2005). Dark or starvation-induced senescence is observed when seedlings are transferred to a glucose-free medium under a low light intensity limiting photosynthesis, and this senescence can be delayed by overexpressing KIN 10 (Baena-Gonzalez et al., 2007). On the other hand sugar-induced senescence has been demonstrated and the senescent-specific gene SAG 12 is over 900 times upregulated by glucose (Pourteau et al., 2006). Hexokinases have been implicated as sugar sensors, since mutants affected in hexokinase did not accumulate hexoses and showed delayed

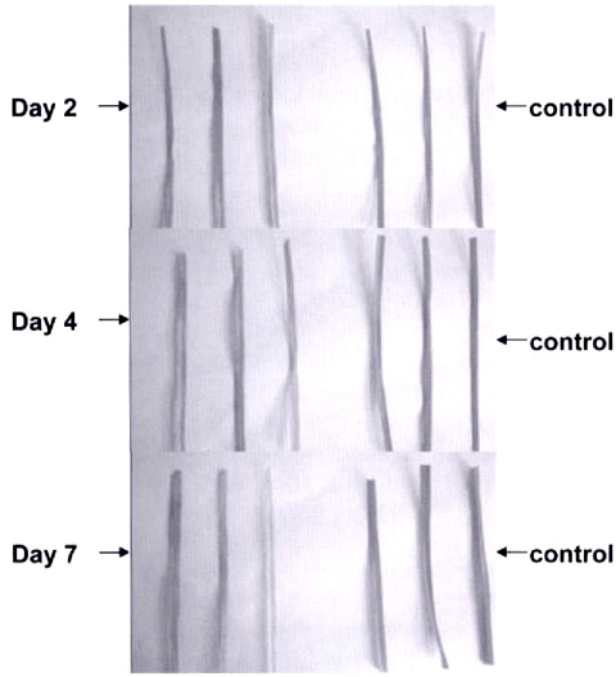

Figure 6. Pictures of excised leaves of orchardgrass showing progress of leaf senescence. At the left, three leaves are shown after 2, 4 and 7 days of incubation in 200 mM sucrose. At the right, three untreated control leaves are shown for comparison.

senescence (Moore et al., 2003; Pourteau et al., 2006). Parrott et al. (2007) prevented phloem but not xylem transport by steam girdling of barley leaves. Such leaves accumulated sugars to a high extent and this was associated by accelerated leaf senescence in the steam-girdled leaves. The yellowing of the leaves correlated with increased sugar levels and drastic changes in senescent specific proteins as

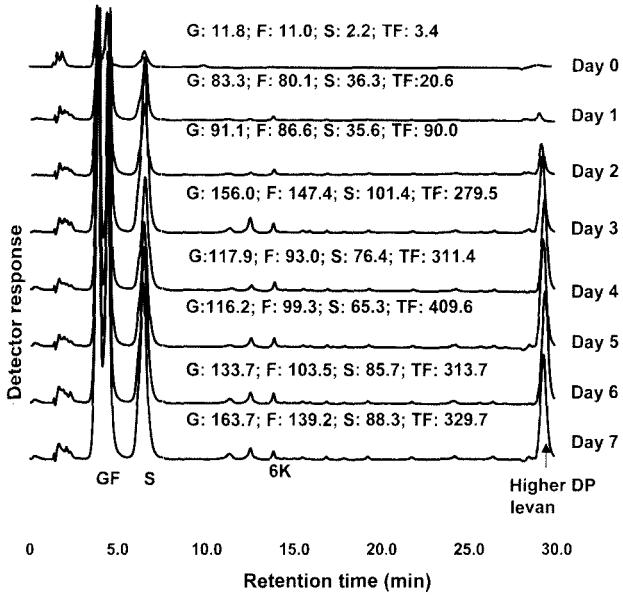

Figure 7. HPAEC-PAD chromatograms showing carbohydrates in excised orchardgrass leaf blades incubated in 200 mM sucrose in continuous light at 20°C and followed as a function of time (day 0-day 7). Leaf senescence became apparent at day 5. G: glucose; F: fructose; S: sucrose; 6K: 6-kestose; TF: total fructose fructan. The arrow shows the position of higher DP levan. The concentrations (in μmoles g FW^{-1}) of G, F, S and TF are shown.

revealed by elaborated transcriptomic analyses. One of the typical characteristics of senescing leaves is the degradation of biopolymers to monomers that, after export, can be used to sustain other parts of the plant (sink leaves, seeds). Gregersen and Holm (2007) demonstrated that α-amylase, invertase and sucrose synthase are upregulated in senescing flag leaves of wheat while sucrose phosphate synthase was downregulated, in accordance with a degradation of sucrose and starch in senescing leaves. These observations prompted us to follow leaf yellowing and the fate of fructan, hexose and sucrose concentrations in excised leaves of orchardgrass during senescence in the presence of sucrose. Onset of leaf yellowing became apparent after 4 days (Fig. 6), which is comparable to the girdled leaves in Parrott et al., 2007. However, fructans were not subjected to extensive hydrolysis in senescing leaves incubated in 200 mM sucrose (Fig. 7) suggesting that FEHs are absent or inhibited by sugars during sugar-induced leaf senescence. Overall, fructans tend to accumulate when sucrose concentrations exceed 30 μmoles g^{-1} FW (day 1, Fig. 7). This is in line with the point of view that fructans can be considered as a true extension of sucrose.

General Conclusions and Further Research

Besides high DP inulin accumulators (*Viguiera, Echinops*), some monocots such as *Dactylis glomerata* accumulate a single series of levans reaching high DP in some parts of the plant (Yamamoto and Mino, 1985). The presence of these polymers might be correlated with an increased stress resistance. Levans could be induced in excised orchardgrass leaves incubated with sugar in the light. We used the elegant excised leaf system to study the regulation of fructan metabolism as affected by light, temperature and different small sugars. Most of our results are in line with the general point of view that fructans can be considered as a surplus pool of sucrose and that a threshold sucrose concentration is needed to induce fructan synthesis. In contrast to the well-studied barley excised leaf system, dark and sugar only result in a negligible fructan synthesis. Moreover, no rapid fructan accumulation was observed in the light without adding extra sugar to the incubation medium. These observations strongly suggest that both sugar and light are necessary to achieve an optimal fructan synthesis in orchardgrass. Fructans persisted during senescence of sugar-induced orchardgrass leaves. However, a metabolic switch to fructan degradation was observed when induced leaves were incubated in the dark at 30°C.

The absence of 1-kestose in the carbohydrate patterns suggests that levan biosynthesis in orchardgrass might occur by a single 6-SST/6-SFT enzyme. Now that we know the environmental conditions needed to maximize fructan concentrations in orchardgrass, we can generate optimal material for purification and further characterization of the fructosyltransferase(s) involved. Generating peptide sequence information will lead to cDNA cloning and functional analysis by heterologous expression in *Pichia pastoris*. It will be interesting to introduce the cDNA(s) of orchardgrass in agronomically important species (such as rice) to make them more resistant to drought or cold-stress. Moreover, the availability of the cDNA and the enzyme will allow much deeper studies on the regulation of fructan metabolism in orchardgrass. These fundamental insights in fructan metabolism regulation might lead to a further increase in WSC in grasses. Miller et al. (2001) demonstrated the benefit of using high WSC cultivars for milk production. Other authors argue that grasslands hold great promises for biodiesel and biomass production (Tilman et al., 2006).

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