Fructan biosynthesis in transgenic plants

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Abstract

Data from plants transformed to accumulate fructan are assessed in the context of natural concentrations of reserve carbohydrates and natural fluxes of carbon in primary metabolism: Transgenic fructan accumulation is universally reported as an instantaneous endpoint concentration. In exceptional cases, concentrations of 60–160 mg g⁻¹ fresh mass were reported and compare favourably with naturally occurring maximal starch and fructan content in leaves and storage organs. Generally, values were less than 20 mg g⁻¹ for plants transformed with bacterial genes and <9 mg g⁻¹ for plant–plant transformants. Superficially, the results indicate a marked modification of carbon partitioning. However, transgenic fructan accumulation was generally constitutive and involved accumulation over time-scales of weeks or months. When calculated as a function of accumulation period, fluxes into the transgenic product were low, in the range 0.00002–0.03 nkat g⁻¹. By comparison with an estimated minimum daily carbohydrate flux in leaves for a natural fructan-accumulating plant in field conditions (37 nkat g⁻¹), transgenic fructan accumulation was only 0.00005–0.08% of primary carbohydrate flux and does not indicate radical modification of carbon partitioning, but rather, a quantitatively minor leakage into transgenic fructan. Possible mechanisms for this low fructan accumulation in the transformants are considered and include: (i) rare codon usage in bacterial genes compared with eukaryotes, (ii) low transgene mRNA concentrations caused by low expression and/or high turnover, (iii) resultant low expression of enzyme protein, (iv) resultant low total enzyme activity, (v) inappropriate kinetic properties of the gene products with respect to substrate concentrations in the host, (vi) in situ product hydrolysis, and (vii) levan toxicity. Transformants expressing bacterial fructan synthesis exhibited a number of aberrant phenotypes such as stunting, leaf bleaching, necrosis, reduced tuber number and mass, tuber cortex discoloration, reduction in starch accumulation, and chloroplast agglutination. In severe cases of developmental aberration, potato tubers were replaced by florets. Possible mechanisms to explain these aberrations are discussed. In most instances, the attempted subcellular targeting of the transgene product was not demonstrated. Where localization was attempted, the transgene product generally mis-localized, for example, to the cell perimeter or to the endomembrane system, instead of the intended target, the vacuole. Fructosyltransferases exhibited different product specificities in planta than in vitro, expression in planta generally favouring the formation of larger fructan oligomers and polymers. This implies a direct influence of the intracellular environment on the capacity for polymerization of fructosyltransferases and may have implications for the mechanism of natural fructan polymerization in vivo.

Key words: Fructan, fructosyltransferase, FFT, inulin, levan, levansucrase, oligosaccharide, polysaccharide, SST, vacuole.

Introduction

Fructan (oligo- and poly- fructosyl sucrose) rather than starch, occurs naturally as the primary reserve carbohy-
Fructan is synthesized directly from sucrose as the sole precursor, apparently without the involvement of phosphorylated sugars or nucleotide co-factors. Its synthesis is extrachloroplastic, it is water soluble, and it accumulates in the vacuole of both photosynthetic and storage cells. In addition to its reserve role, it is thought by some authorities to confer stress resistance (to drought and cold) on plant tissues, although this remains controversial (Vijn and Smeekens, 1999). Fructan occurs in many economically important species, for example, in the vegetative tissues of the temperate forage grasses and cereals and in the perennating organs of chicory, artichoke, asparagus, dahlia, and the onion family. Fructan is also elaborated as an extracellular polysaccharide by some bacteria. With reported molecular masses of 1–5×10⁶ Da, bacterial fructan, termed levan, is larger than plant fructans by 2–3 orders of magnitude. The non-specialist reader is referred to previous recent papers for detailed accounts of the structure, physiology, biosynthesis, and enzymology of fructan (Vijn and Smeekens, 1999; Cairns et al., 1999, 2000, and references therein), a knowledge of which is assumed here. This review will focus on the physiological aspects of artificial fructan-accumulating plants produced by transformation with bacterial and plant-derived fructosyltransferases.

An earlier critique (Cairns, 1993) dealt with the interpretative difficulties which arise when conclusions from enzymological studies performed in isolation, are placed into the framework of the known physiology of natural fructan accumulation in vivo. When parameters such as substrate concentration, enzyme affinities, carbon flux, accumulation rates, enzyme activity, enzyme concentrations, and the structure of fructan products were considered in parallel in vitro and in vivo, a number of ambiguities were identified. These were associated with two central factors: (i) the unusually low affinity (high apparent Kₘ) of plant fructosyl transferases for their substrates, (ii) the difficulty in distinguishing physiologically relevant in vitro fructan synthesis from artefactual side-reactions of invertase. Substrate affinity and invertase continue to be important considerations in the assessment of results from transgenic fructan accumulators. Cairns (1993) also identified some confusion in the definition of the term ‘synthesis’ and how this can be distinguished from the related concepts of ‘primer glycosylation’ and ‘non-synthetic fructosyl transfer’ (disproportionation). The distinctions between these concepts are of utility in interpreting results from some fructan-transformants. Despite an extensive literature describing the enzymology of what is loosely described as plant fructan synthesis, to date, there remain only a few reports of its de novo enzymatic polymerization (Cairns et al., 1999).

Considerable progress has been made in understanding plant fructan synthesis since 1993, notably in the areas of in vitro synthesis, enzyme purification, gene cloning, and transgenesis. Recent work has amply confirmed the peculiarity of plant fructosyl transferases with regard to their kinetic properties (low affinity) and high enzyme concentration is emerging as an important, though perhaps unexpected, requirement for the in vitro polymerization of plant fructan. Enzyme properties determined in vitro have interesting physiological consequences for transformants in planta. Clearly, the subcellular environment in which a transgene is expressed will have consequences for its function since, for example, host plants may not contain the necessary high intracellular concentrations of sucrose. Such physiological considerations may have been overlooked in the haste to produce transgenic plants and may, in part at least, explain the low rates of fructan synthesis reported for some transformants. It has become apparent that both the quantitative and qualitative functions of fructosyltransferases are affected by the in vitro and in vivo conditions in which the activity is expressed. As with the earlier enzymology, results from molecular biology need to be carefully assessed with regard to physiological consequences, in order to determine their significance adequately. The purpose of this review then, is to update the earlier critique and take account of developments in current knowledge of fructan synthesis, especially in the emerging area of transgenic fructan-accumulating plants.

**Units, assumptions and calculations**

The basis of expression for quantities of fructan product varies widely in the literature, the choice of units clearly being influenced by the type of information to be communicated. In molecular biology there has been a tendency to present results in the form of ‘plus or minus’, ‘single end point’ values. In such studies, the smallest trace of oligosaccharide is ‘positive’, regardless of its significance in terms of physiological fluxes. Physiologists and biochemists, in contrast, have an interest in the details of concentrations, affinities, rates, and fluxes. In many cases it is difficult and in some cases impossible, to estimate such parameters from data reported in studies of transgenesis. Units in current usage include in the numerator: mass, moles, equivalent (e.g. to hexose) and ‘arbitrary’. In the denominator, fresh and dry matter, unit soluble protein, unit chlorophyll, and unit leaf area are found and, in some cases, none. Physiological rates of transgenic product formation (i.e. including time and some measure of tissue quantity in the denominator) have not been reported.

In this study, where possible, values have been recalculated from the source literature into a common format. Data for net flux of carbohydrate were compiled...
using the conventional enzymological unit nkat (nanomoles s\(^{-1}\)) for reserve polymer synthesis. Because fructan polysaccharides are of disperse \(M_r\), molar fluxes were estimated from mass data by dividing by the \(M_r\) of anhydrohexose (162 Da). Where possible, rate values were related to physiological processes by expression on the basis of fresh mass of tissue. Where not reported in the source study, tissue water content of 80%, of which 90% was vacuolar, was assumed. For conversion of product accumulation periods expressed in months, one month was taken to equal 30 d. In the absence of indications to the contrary, transgenes were assumed to be expressed constitutively and the gene products active throughout the entire growth period of the organ/plant examined. PFD is expressed here in \(\mu\)mol m\(^{-2}\) s\(^{-1}\). Photosynthetic integral (PI) was calculated as PFD multiplied by daily photoperiod.

**A physiological context for transgenic fructan synthesis**

Reports of ‘high-level’ fructan accumulation in transgenics are common, but suffer from a lack of definition of what ‘high-level’ might mean. Tissue fructan concentrations are rarely compared with those of natural endogenous reserve polysaccharide accumulation and absolute rates of transgenic fructan accumulation have, to date, never been reported. To provide a physiological context, Fig. 1 summarizes field data for a clear sunny day in mid-May (5.5 weeks before the Summer Solstice) in Aberystwyth, UK. The figure illustrates variation in photon flux for an ‘average temperate summer day’ and the corresponding instantaneous carbohydrate concentrations for leaves of *Lolium perenne*, a NFA. Integrating under the PFD curve (Fig. 1a) gives a photosynthetic integral of 29 mol m\(^{-2}\) quanta. The maximum and mean irradiances were 1200 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) and 510 \(\mu\)mol m\(^{-2}\) s\(^{-1}\). Figure 1b shows changes in leaf carbohydrates. Integrating between ‘X’ and ‘Y’ for total WSC gives 260 mg g\(^{-1}\) which was accumulated, and then subsequently exported, in a 12 h period and is equivalent to 37 nkat g\(^{-1}\) as anhydrohexose. This gives a minimum flux of carbohydrate through the leaf, since export would have taken place continuously throughout the day and the daytime flux would have been invisible to measurements of instantaneous concentration. The difference between the sucrose and total WSC curves represents fructan content, which transiently reached a maximum of 60 mg g\(^{-1}\) at midday. The maximal rate of fructan accumulation was 22.5 mg g\(^{-1}\) h\(^{-1}\) (39 nkat g\(^{-1}\)), during which sucrose substrate concentration was 22 mg g\(^{-1}\), equivalent to a vacuolar concentration of c. 90 mM. Mean tissue water contents were c. 80% corresponding to c. 0.2 g dry matter g\(^{-1}\). These figures are consistent with published values for reserve polysaccharide accumulation in a range of species (Gay and Thomas, 1995; Koroleva *et al*., 1997; Cairns *et al*., 2002a).

**Plants transformed with bacterial fructosyltransferase genes**

Historically, fructan biosynthesis was elucidated earlier in bacteria than in higher plants. By the early 1990s...
the bacterial fructan polymerase, levansucrase, had been purified and characterized, antibody probes were available and the genes were isolated, sequenced and cloned. By contrast, the first plant fructosyl transferase genes did not become available until the mid-1990s. It was a logical early step to incorporate the available bacterial gene into the available, transformable, fructan-non-accumulating plants. The stated rationales for the production of the bacterial transformants were: (a) experimental: to produce plants with altered source–sink balance for the experimental investigation of carbon partitioning, (b) experimental: to examine the effects of differential subcellular location of exogenous levan synthesis (Röber et al., 1996; Caimi et al., 1997), (c) industrial: to provide sources of fructan qualitatively and quantitatively improved by comparison with that from natural sources (Ebskamp et al., 1994), (d) aimed at crop improvement: via enhanced carbohydrate retention and hence yield (Caimi et al., 1997) or (e) aimed a crop improvement via enhanced stress tolerance (Pilon-Smits et al., 1995, 1999). The extent to which these aims have been achieved is indicated below.

### Table 1. Summary of parameters associated with fructan accumulation for plants transformed with bacterial levansucrase

Except where otherwise indicated, the structural gene was *SacB* from *Bacillus subtilis* under the control of a constitutive promoter. The fructan concentrations are expressed on a fresh mass basis. Where several values were reported, the maximum is presented. Data are ranked in decreasing order of levan accumulation rate.

<table>
<thead>
<tr>
<th>Host</th>
<th>Tissue/putative compartment</th>
<th>PFD (μmol m⁻² s⁻¹)</th>
<th>Photoperiod (h)</th>
<th>Photo-synthetic integral (mol m⁻² d⁻¹)</th>
<th>Accumulation period (d)</th>
<th>Endpoint fructan (mg g⁻¹)</th>
<th>Calculated rate (nkat g⁻¹)</th>
<th>Estimated sucrose substrate (mM)</th>
<th>Reference</th>
</tr>
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<tr>
<td>Maize</td>
<td>Kernel/vacuole⁷</td>
<td>NR</td>
<td>NR</td>
<td>ID</td>
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<td>18</td>
<td>0.03</td>
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<td>NR</td>
<td>ID</td>
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<td>66</td>
<td>0.03</td>
<td>ID</td>
<td>Gerrits et al., 2001</td>
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<td>NR</td>
<td>ID</td>
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<td>17.5</td>
<td>0.02</td>
<td>ID</td>
<td>Röber et al., 1996</td>
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<td>NR</td>
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<td>Droughted leaf</td>
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<td>24</td>
<td>3.6</td>
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<td>0.35</td>
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<td>Pilon-Smits et al., 1995</td>
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<td>14</td>
<td>5.0</td>
<td>101</td>
<td>1</td>
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<td>ID</td>
<td>Pilon-Smits et al., 1999</td>
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<tr>
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<td>42⁵</td>
<td>16</td>
<td>2.4</td>
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<td>ID</td>
<td>van der Meert et al., 1994</td>
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<td>8.4</td>
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<td>NR</td>
<td>ID</td>
<td>2.8</td>
<td>ID</td>
<td>5</td>
<td>Ebskamp et al., 1994</td>
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<tr>
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<td>42</td>
<td>NR</td>
<td>ID</td>
<td>10</td>
<td>ID</td>
<td>32</td>
<td>Gerrits et al., 2001</td>
<td></td>
</tr>
</tbody>
</table>

⁷ Levansucrase gene from *Bacillus amyloliquifaciens*: patatin promoter:cultured tubers: assumes 80% water.
⁶ Levansucrase gene from *Bacillus amyloliquifaciens*: SSU12–2 promoters.
⁵ Glasshouse: winter.
⁴ Glasshouse: spring.
³ 3000 lux: converted according to McCree (1972).
² Levan sucrase gene from *Erwinia amylovora*: B33 tuber specific promoter.
¹ Glasshouse.
⁰ Zein promoter.
NR, appropriate data not reported.
ID, Insufficient data for calculation.

**Quantitative aspects of non-fructan plants transformed with bacterial fructosyltransferases**

Levansucrases, mainly the *SacB* gene from *Bacillus* spp., generally under the control of CaMV 35S and a putative subcellular targeting sequence, were used to produce stable transformation of SA such as potato, maize and tobacco. Table 1 summarizes for these transformants a number of parameters pertinent to reserve carbohydrate accumulation. The initial striking feature of Table 1 is how little attention has been given to the reporting of the cultural conditions which determine primary productivity. This is in studies where reserve accumulation (i.e. photosynthesize in excess of demand for maintenance and growth) and biomass productivity were of primary concern. Irradiance and/or photoperiod, hence PI, were ignored as important experimental variables in 12 out of 16 studies. In two of the four instances where such information was provided (for potato), PFD was low at 42 μmol m⁻² s⁻¹. This approximates to the light compensation point for C₃ photosynthesis (Milthorpe and Moorby, 1974), where net carbon fixation should have been zero. It is surprising that any reserve or biomass accumulation took place under these
conditions. Interestingly, the highest reported PI produced the lowest levan concentration at 0.04 mg g⁻¹ (Ye et al., 2001) whilst the lowest reported PIs of 3.6 and 2.4 gave endpoint levan concentrations of 0.35 and 10 mg g⁻¹, respectively. Given that the PFD was near the compensation point, it is not clear where this carbohydrate originated in the latter two studies.

In terms of the endpoint concentrations of levan, reports varied between 0.04 and 160 mg g⁻¹ (c. 0.02–80% of dry biomass) with the majority lying between 5–20 mg g⁻¹ (c. 2.5–10% of dry biomass). The maximum value was obtained for leaves grown in vitro under unspecified conditions, but presumably involved an exogenous carbon source. This value is remarkable because it is roughly twice the maximal reserve carbohydrate concentrations observed in leaves in nature (Fig. 1b; Cairns et al., 2002a) and is more similar to the generally reported total dry matter content of leaves. The maximal endpoint concentrations for autotrophic transformants were 66 and 20 mg g⁻¹ (c. 33% and 10% of dry biomass) obtained by the same group. Overall, these values for leaf tissue were very impressive, comparing well with the maximum fructan concentration shown in Fig. 1 (60 mg g⁻¹), with the high concentrations of endogenous starch accumulated in leaves (e.g. in Trifolium pratense: 62 mg g⁻¹; Jones, 1990) and with natural fructan accumulation in tubers of H. tuberosus (150 mg g⁻¹; Schubert and Fuerle, 1997). The data superficially suggest a marked shift in carbon resource allocation in the transformants.

**Endpoint concentrations and fluxes**

The plants were generally grown for weeks or months with the transgene under the control of a constitutive promoter (Table 1). Hence the endpoint fructan concentrations were the sum of levansucrase activity occurring over the entire growth period and represent the total flux of carbon into levan. This interpretation is confirmed by one data set (van der Meer et al., 1994), where levan concentration in the leaves increased steadily from young, to middle-aged, to old leaves (absolute age was not specified, hence rates could not be calculated). Interestingly, these authors (and others; Turk et al., 1997; Ebskamp et al., 1994) took care to point out that the leaves were ‘harvested at the end of the light period’. This could be misleading to an uncritical reader, because it could be taken to imply that the recorded levan contents were the product of photosynthesis in that single photoperiod only, rather than the sum of (constitutive) accumulation over the entire growth period. The likelihood of the reader adopting this interpretation is increased because, in general, levan accumulation was presented together with instantaneous concentrations of other endogenous carbohydrates (sucrose, starch, monosaccharides). Such presentation does not compare equivalent parameters because the endogenous sugars are subject to turnover and transport on hourly and daily time-scales (Fig. 1), whilst the levan accumulated over weeks and months. To assess the magnitude of levan accumulation as a proportion of primary metabolism, it is necessary to compare it with the flux of sugar through photoassimilation, rather than with instantaneous pools of endogenous sugars.

When endpoint concentrations of levan were expressed as a function of constitutive accumulation period, the rates of accumulation were low, at between 0.0007–0.03 nkat g⁻¹ (Table 1). These values are 1200–53 000-fold lower than the minimum daily flux of carbon from leaves calculated from Fig. 1b (37 nkat g⁻¹). When proportionally expressed, flux into levan was equivalent to 0.0002–0.08% of this minimum primary flux. By comparison with natural fluxes, the levan-transformations did not cause a significant diversion of photosynthate flux, but rather, a quantitatively minor leakage into a metabolically inert compartment. This view contrasts with the emphasis in such statements such as ‘we observed that bacterial fructosyltransferase can compete with this process [the sink demand of starch synthesis] and divert normal carbohydrate flow’ (van der Meer et al., 1994). Clearly, in some cases and over long accumulation periods, such minor leakage can cumulatively cause substantial instantaneous concentrations, but this does not imply a significant modification of flux or partitioning. Thus, one of the primary experimental aims has not been achieved. By contrast, there is one report, for tobacco leaves, where levan detectable by TLC was detected within 36 h of the chemical-induction of levansucrase expression (Caimi et al., 1997). This was suggestive of rapid accumulation, but since levan concentrations were not reported, the calculation of a rate was precluded. This apparent rapid accumulation was accompanied by equally rapid tissue necrosis (discussed below).

**Explaining the low rates of product accumulation in the levan transformants**

It is of interest to speculate on the cause of these low rates of levan accumulation. One possibility is product turnover. However, a generally stated reason for the choice of host was that they were not expected to contain enzymes of fructan hydrolysis (van der Meer et al., 1994; Pilon-Smits et al., 1995, 1999; Caimi et al., 1996; Turk et al., 1997) or were shown not to contain extractable fructan hydrolases (Ebskamp et al., 1994). Where tobacco tissue was subjected to extended incubation in the dark, accumulated transgenic levan did not breakdown (Ebskamp et al., 1994). Turnover of levan would not appear to explain the low accumulation rates.

That tissue sucrose substrate concentration has an influence on levan accumulation rate is clear, since transformation of high-sucrose mutants of maize resulted in increased levan accumulation in kernels, though the sucrose concentrations were not quantified and related to
levansucrase activity (Caimi et al., 1996). Hence, a possible explanation for low levan accumulation rates is inappropriate (limiting) substrate conditions. In enzymology, a primary determinant of rate is the relationship between substrate concentration and the enzymes' affinity for that substrate. For the levansucrase from Bacillus subtilis, the sucrose concentration for half maximal activity (apparent $K_m$) is 20 mM (Dedonder, 1966). Estimates of sucrose concentrations at the putative site of levansucrase expression, the vacuole, are listed in Table 1. Six of the eight values were at c. 20 mM or above. Although the rate of activity would have been limited by, and sensitive to, substrate concentrations, the activity would have been substantial since it was functioning at greater than half of the maximal rate. Hence substrate limitation per se is unlikely to provide a general explanation for the observed low rates of accumulation in these transformants. Table 1 also shows that in two reports, leaf tissue sucrose concentrations were lower than 5 mM, at which the enzyme activity would have been c. <7% of $V_{max}$. These low sucrose concentrations corresponded with lower endpoint concentrations and lower accumulation rates and may have adversely influenced total accumulation in these transformants.

Whilst not explicitly emphasizing low absolute rates of accumulation, many studies of levansucrase transformants tacitly alluded to the issue by reference to other, related expression parameters. It was generally noted that (a) levansucrase activity could not be detected by conventional enzyme assays. Instead, sensitive radiochemical detection was necessary using $^{14}$C-labelled sucrose. Where this was successful, only qualitative data were presented (as TLC autoradiographs). Trace amounts were synthesized and the structure of the enzyme product was not determined. Given that milligram quantities of polymeric fructan, sufficient for chemical, chromatographic and structural analyses, can be synthesized in several hours by endogenous enzymes from NFA (Cairns et al., 1999), by comparison, the levansucrase activity in the transformants was extremely low. (b) Levansucrase protein could not be detected in the transformants by immunological probes. (c) In all cases, mRNA for the levansucrase could not be detected by Northern blots, although low level expression of the mRNA was detected in transformed ryegrass by the more sensitive method of RT-PCR (Ye et al., 2001). Taken together, the evidence suggests that the primary reasons for the low rates of levan accumulation were low message expression and ultimately low enzyme quantity.

**Attempts to increase levansucrase expression in transformants**

In tacit acknowledgement of the low rates, a number of approaches to increase expression were attempted, such as the use of tandem CaMV 35S promoters and a translational enhancer AlMV RNA4 (Ebiskamp et al., 1994). In addition, different promoters such as maize ubi (Ye et al., 2001), B33 tuber specific, maize 2–2 inducible, zein seed-specific promoter (Caimi et al., 1996), and a variety of putative subcellular targeting sequences have also been used with levansucrase genes. Compared to the generally observed levansucrase expression rates, conspicuous improvements have not been demonstrated by the use of different combinations of these sequences. This has been interpreted to indicate a more fundamental problem resulting from the characteristics of the structural gene. In this connection, codon usage is different in eukaryotes compared to the gram-positive bacteria such as B. subtilis (Röber et al., 1996; Ye et al., 2001). It is possible, in a eukaryotic host, that the activity of the gene product is impaired due to inappropriate amino acid substitutions. Further, localized regions of AT richness and the presence of mRNA destabilizing ATTA sequences, may contribute to the rapid turnover of the Bacillus message (Ye et al., 2001). All of these factors could contribute to the observed low expression rates. To avoid some of these difficulties, Röber et al. (1996) used a levansucrase gene from the gram-negative bacterium Erwinia amylovora which does not have the rare codon usage, arguing that it should improve expression rates in a eukaryotic background. Table 1 compares the performance of the E. amylovora transformant with those using levansucrase from Bacillus spp. At 17.5 mg g$^{-1}$ the endpoint concentration of levan fell in to the general range of 5–20 mg g$^{-1}$ with an accumulation rate of 0.02 nkat g$^{-1}$. These values were amongst the highest for all the transformants, but not better than the best results obtained using the B. subtilis SacB gene. Hence the substitution of a gram-negative for a gram-positive structural gene did not appear to enhance the rates of expression of the transgene significantly or provide a general explanation for low rates of transgene expression.

When putatively cytosolic expression of the levansucrase gene was placed under the control of a chemically-induced promoter, apparently high rates of levan accumulation were induced in leaves, but caused rapid tissue destruction (within 36 h). By contrast, no plants transformed with a (normally successful) constitutive promoter were recovered (Caimi et al., 1997). This may indicate that, in general, only plants with low rates of expression survive, thus explaining the occurrence of low levan accumulators only, as a function of lethal negative selection of high-rate transformants.

As indicated, attempts to the explain low expression rates have focused on the bacterial origin of the structural gene, but as will be seen, the problem is not exclusive to the bacterial genes. Plant-derived fructosyl transferases are also expressed at low rates in transformants (Table 2 discussed below). Hence, hypotheses based specifically on the characteristics of bacterial genes do not provide a general explanation. Expression rates of all transgenes are low in all transformants and this is indicative of a
Table 2. Summary of parameters associated with fructan accumulation in plants transformed with plant-derived fructosyltransferases

<table>
<thead>
<tr>
<th>Host/tissue</th>
<th>Gene/source</th>
<th>Reference</th>
<th>PFD (mg g⁻¹ s⁻¹)</th>
<th>Calculated Kₘ (mM)</th>
<th>Estimated Kₘ of product (mM)</th>
<th>Accumulative integral (mol m⁻² s⁻¹)</th>
<th>Endpoint (h)</th>
<th>Calculated rate (nkat g⁻¹)</th>
<th>Estimated fructan (mM)</th>
<th>Fresh mass basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar beet/root</td>
<td>SST/ H. tuberosus</td>
<td>Seavenet et al., 1998</td>
<td>0.6 0.0002 1 280</td>
<td>0.075</td>
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<td>Hellewege et al., 2000</td>
<td>0.4 0.0004 28 280</td>
<td>0.075</td>
<td>15</td>
<td>22</td>
<td>62 3</td>
<td>6 2 4</td>
<td>18 0</td>
<td>1</td>
</tr>
<tr>
<td>Tobacco/root</td>
<td>6-SFT/ H. vulgare</td>
<td>Sprenger et al., 1997</td>
<td>0.1 0.00002 2 280</td>
<td>0.075</td>
<td>15</td>
<td>22</td>
<td>62 3</td>
<td>6 2 4</td>
<td>18 0</td>
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</tr>
<tr>
<td>Petunia/leaf</td>
<td>SST/ H. tuberosus</td>
<td>van der Meer et al., 1998</td>
<td>0.5 0.0003 1 280</td>
<td>0.075</td>
<td>15</td>
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<td>62 3</td>
<td>6 2 4</td>
<td>18 0</td>
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</tr>
<tr>
<td>Sugar beet/leaf</td>
<td>SST/ H. tuberosus</td>
<td>Seavenet et al., 1998</td>
<td>0.5 0.0002 1 280</td>
<td>0.075</td>
<td>15</td>
<td>22</td>
<td>62 3</td>
<td>6 2 4</td>
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<tr>
<td>Tobacco/leaf</td>
<td>6-SFT/ H. vulgare</td>
<td>Sprenger et al., 1997</td>
<td>0.5 0.00002 1 280</td>
<td>0.075</td>
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<td>6 2 4</td>
<td>18 0</td>
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</tr>
<tr>
<td>Petunia/leaf</td>
<td>SST+FFT/ H. tuberosus</td>
<td>van der Meer et al., 1998</td>
<td>0.5 0.00002 1 280</td>
<td>0.075</td>
<td>15</td>
<td>22</td>
<td>62 3</td>
<td>6 2 4</td>
<td>18 0</td>
<td>1</td>
</tr>
</tbody>
</table>

Except where otherwise indicated, the structural gene was under the control of a constitutive promoter. Fructan concentrations are expressed on a fresh mass basis. Where several values were reported, the maximum is presented. Data are ranked in decreasing order of fructan accumulation rate.

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Fructan biosynthesis in transgenics

Structural identification of levan in transformants

Bacterial levan is a very large polymer and this presents some difficulties for routine identification. In the absence of detailed derivatization and structural analysis (Cairns et al., 1999; 2002b) or the use of direct physical measurement techniques such as Low Angle Laser Light Scattering (Fishman et al., 1996), the assessment of molecular size is not straightforward. Techniques reported for the identification of transgenic levan have been TLC, size exclusion chromatography (SEC) and NMR spectroscopy. Levan does not migrate from the origin on TLC and this has been used widely to show its presence in transformants or in enzyme digests. The size threshold for non-migration is DP₅=₆c. 10 for 2,6-linked fructans (Cairns et al., 1999). Hence, strictly interpreted, non-migration on TLC is evidence only for the presence of levan larger than 1.6×10³ Da and when presented alone (Cai et al., 1996, 1997; Pilon-Smits et al., 1999; Gerrits et al., 2001) is not indicative of large polymers equivalent to those formed naturally by bacteria (1–5×10³ Da). SEC on materials such as Sepharose 4B have been used to estimate molecular size, in some cases by reference to protein size standards and/or an excluded volume marked by blue dextran. However, it is noteworthy that, in the study of Ye et al. (2001), Sepharose CL-4B did not separate transgenic levan from endogenous fructan of Lolium multiflorum, both were apparently present in the excluded volume. The large fructan of Lolium spp. has a maximum size of 6.5×10³ Da as shown by HPAEC (Turner et al., 2002). The conclusion of levan size by exclusion from SEC appears ambiguous.

NMR data have been presented in the form of comparisons of spectra obtained for standard levan and levan isolated from transformants. The transgenic materials gave similar spectral patterns to standards, though potentially significant chemical shifts of 3–4 ppm in primary signals were observed in the study of Röber et al. (1996), which could indicate structural differences. NMR spectra give information about the electronic configurations of individual atoms within a compound. Whilst the spectra are indicative of bond structure, they give no indication of molecular size for polymers unless, for example, signals from atoms in terminal sugars are determined in a ratio with signals from equivalent atoms in the linkage sugars. It is concluded that, for the majority of studies of transgenic levan synthesis in plants, co-identity with the bacterial polymer, certainly with respect to size, has not been unambiguously demonstrated.
The study of Ye et al. (2001) used TLC and SEC to analyse transgenic levan, but obtained contradictory results using the two methods. TLC showed that non-transformed controls contained no high M_r fructan. By contrast, quantitative analyses of Sepharose CL-4B eluates showed high M_r material was present, at between 37% and 75% of the concentrations in the transformant. Hence, a serious criticism of this work is that Sepharose CL-4B, the primary method for quantitation of the transgenic product, was unable to differentiate endogenous grass polyfructan from the putative bacterial levan. In this study, the elevated concentration of high M_r fructan in the transformants is explicable in terms of endogenous fructan accumulation alone: Since the transformants exhibited reduced growth, it is possible that lowered demand for assimilates caused an up-regulation of reserve accumulation and enhanced endogenous fructan accumulation. If so, the observed enhanced putative levan could simply be a side-effect of the altered endogenous source–sink balance in favour of endogenous fructan accumulation.

Has subcellular targeting been established unequivocally in bacterial levan transformants?

The native SacB gene contains a bacterial secretion sequence which defaults to the extracellular compartment in plants (Caimi et al., 1997). Plant vectors have been designed to contain targeting sequences to replace or override this internal signal and send the levansucrase to different subcellular locations: the vacuole, cytoplasm, extracellular matrix, endomembrane system, and, most recently, to plastids. In general, however, the actual targeting of the transgene product has been assumed rather than demonstrated.

The most convincing evidence for actual targeting to predetermined locations was reported by Gerrits et al. (2001). Using subcellular fractionation, levan was unambiguously shown to be associated with chloroplasts isolated from protoplasts of tobacco plants transformed with SacB fused with a ferredoxin chloroplast targeting sequence. In control transformants targeted with the sporamin vacuolar targeting sequence (shown to target the GUS reporter gene to the vacuole, but to target levansucrase to the cytoplasm, cytosolic target may have placed the gene product at the cell surface, was not discussed, although for a separate study it was made explicit that the internal bacterial secretion sequence was removed from the gene (Caimi et al., 1997). The latter has been shown to mis-localize the levansucrase in the endomembrane system in tobacco (Turk et al., 1997). Neither were shown actually to target the gene product to the vacuole, although the results were discussed under this assumption. A third construct expressed by Caimi et al. (1996) contained no targeting sequence. This was assumed to target the gene product to the cytoplasm, again in the absence of any demonstration. The description of the construction of the vector made no reference to the internal bacterial secretion sequence. The possible consequence, that the putative cytosolic target may have placed the SacB gene product at the cell surface, was not discussed, although for a subsequent study it was made explicit that the internal sequence was removed from the gene (Caimi et al., 1997). The actual subcellular localization of the transgene products was not established in these studies.

Although immunological detection of levansucrase has failed because of the low expression of the enzyme protein (discussed above), sufficient polysaccharide product did accumulate in some transformants for visualization by immunofluorescence microscopy. Röber et al. (1996) used putative apoplastic and putative vacuolar targeting sequences with levansucrase to transform starch-deficient potato. Micrographs convincingly showed enhanced fluorescence in tuber cell walls of apoplastically-targeted levan transformants, relative to non-levan transformed controls. However, in similarly transformed leaves there was considerable additional signal from cell interiors, implying the additional presence of levan in vacuoles. Further, in the vacuole-targeted transformants there was a strong signal in the cell walls similar to that in the apoplastically-targeted tissue and the signal was clearly present in the vacuoles of only three of the 12 cells in the image. Clear segregation of independently targeted levan synthesis was not demonstrated in these experiments.

Similar fluorescence experiments performed by Pilon-Smits et al. (1996) clearly showed that transgenic levan from a yeast CPY vacuolar-targeted enzyme did not localize in the vacuole, but rather, at the cell perimeter in potato leaves. Hence, work from two independent laboratories indicate mis-localization of the transgene product, regardless of the putative specificity of the targeting sequence. The CPY construct has been used in subsequent studies and described as containing ‘the CPY yeast vacuolar targeting sequence’ (Pilon-Smits et al., 1999). Whilst strictly this statement is accurate, to an uncritical reader this could be taken to imply that the ‘gene product went to the vacuole’. Subcellular localization was not demonstrated in this study.

Caimi et al. (1996) used a seed-specific promoter and, separately, vacuolar targeting sequences for barley lectin and sweet potato sporamin. The latter has been shown to mis-localize the levansucrase in the endomembrane system in tobacco (Turk et al., 1997). Neither were shown actually to target the gene product to the vacuole, although the results were discussed under this assumption. A third construct expressed by Caimi et al. (1996) contained no targeting sequence. This was assumed to target the gene product to the cytoplasm, again in the absence of any demonstration. The description of the construction of the vector made no reference to the internal bacterial secretion sequence. The possible consequence, that the putative cytosolic target may have placed the SacB gene product at the cell surface, was not discussed, although for a subsequent study it was made explicit that the internal sequence was removed from the gene (Caimi et al., 1997). The actual subcellular localization of the transgene products was not established in these studies.

The modified SacB gene, without the internal secretory sequence, was used under the control of the chemically-controlled maize 2–2 gene in the absence of a targeting
sequence and had a presumed cytosolic localization. When levan accumulation was induced in tobacco leaves containing this construct, instead of their usual position at the cell perimeter, the chloroplasts aggregated at the cell centre. This could be explained if levan in the cytoplasm interacts with the chloroplast outer membrane, cross-linking and thereby agglutinating chloroplasts. This is consistent with recent reports which indicate that levan interacts directly with membranes (Demel et al., 1998; Vereyen et al., 2001).

If it is hypothesized that cytoplasmically-synthesized levan can bind to and agglutinate chloroplasts as indicated, then the levan in the chloroplastic fraction of Turk et al. (1997, discussed above) need not have been synthesized within the chloroplast but may have occurred by surface interaction of the outer membrane with, for example, cytoplasmically-synthesized levan. This could invalidate the conclusions that (i) the levansucrase was actually targeted to, and active in, the chloroplast and (ii) that a sucrose pool exists in this organelle. The control was the targeted accumulation of levan within the endomembrane system and the resulting absence of levan in the chloroplast fraction. This result is consistent with the hypothesis, because accumulation within, and/or binding to, the endomembrane system would have sequestered the levan away from the chloroplasts, resulting in the absence of a levan signal in the control chloroplast fraction.

In conclusion, there are no unambiguous demonstrations of successful predetermined targeting, but some good indicators that the levansucrase has been inadvertently sent to the cell surface and to the endomembrane system. For the other studies it is not clear where the enzyme was localized. The observation that levan can be synthesized in the endomembrane system sets an interesting precedent for the organization of endogenous fructan polymerization, since there are indications of the microsomal localization of this process (Kaeser, 1983; Cairns et al., 1999).

Aberrant development of plants transformed with bacterial levansucrase

As already noted, in some studies it proved impossible to recover levansucrase transformants using an otherwise successful constitutive transformation system indicating that expression of the levan sucrase can be lethal (Caimi et al., 1996). In other reports of successfully recovered transformants, levan accumulated, but phenotypic problems were not noted. Some of these studies used low irradiance (Pilon-Smits et al., 1995) and it is possible that the resulting low levan accumulation rate precluded the attainment of concentrations sufficient to cause developmental problems. In subsequent reports, a number of development aberrations, some quite dramatic, were associated with the expression of levan sucrase activity and, as a general observation, higher concentrations of levan correlated with more severe phenotypic problems.

On the whole plant level, above-ground stature was often reported to be diminished and root size reduced relative to controls. In leaves, tissue exhibited bleaching and/or necrosis and, in some instances, specific subcellular aberrations such as chloroplast agglutination. Often, effects on leaf morphology occurred in older tissues which contained relatively higher levan concentrations and a gradient of severity of the effect was clearly visible from lower (older) to higher (younger) leaves (Gerrits et al., 2001). In some instances, the occurrence of the leaf lesions correlated with specific developmental phases, for example, the onset of tuberization in potato plants (Röber et al., 1996) and at the switch to reproductive development in ryegrass (Ye et al., 2001). Leaf tissues accumulating levan generally exhibited lowered starch concentrations relative to controls and, in one instance, diurnal turnover of starch and sucrose was reported to be abolished (Pilon-Smits et al., 1996). Conversely, Turk et al. (1997) reported a decreased translocation of photoassimilates from leaves and an associated increase in both starch and hexose.

Because of its potential as an industrial source of transgenic products, the potato tuber has been of particular interest for levan transformation. Transgenic tubers also exhibit unusual phenotypes. In general, transformants produced lower total yield (by up to 80–90%: Gerrits et al., 2001), fewer, smaller tubers with reduced starch concentration and, in some cases, modified starch granule morphology. In the study of Caimi et al. (1997) a major developmental shift caused the formation of florets instead of microtubers, under microtuber-inducing conditions. A brown cortex was a common phenotype in levansucrase transformants (Röber et al., 1996; Pilon-Smits et al., 1996; Caimi et al., 1997).

Using a seed-specific promoter Caimi et al., (1996) elegantly showed that, in maize transformed with levansucrase fused to a putative vacuolar targeting sequence, seeds developed normally and accumulated fructan, whilst a putatively untargeted levansucrase caused devastating retardation of seed development to <10% of the dry mass of controls. In the latter case, the levansucrase was presumed to be targeted to the cytosol. The results were interpreted to show that levan accumulation in the cytosol is disruptive whilst vacuolar localization sequesters levan into a space where its disruptive effects are minimized. However, the study is open to criticism because (a) actual localization was not demonstrated for either subcellular compartment, (b) because in another system, the sporamin vacuolar targeting sequence is known to mis-localize levansucrase into the endomembrane system rather than the vacuole (Turk et al., 1997), and (c) because the bacterial secretion sequence was not taken into account and it is possible that the untargeted (putative cytosolic) gene product was actually localized elsewhere, such as the...
cell perimeter, as shown by Röber et al. (1996) and Pilon-Smits et al. (1996). Regardless of the actual location, the study does provide strong indications that differential localization modulates the detrimental effects of levansucrase transformation.

**Mechanism of levansucrase-mediated developmental effects**

The mechanism by which tissues are damaged by transgenic levansucrase accumulation is by no means clear. However, a useful starting point is the observation that levansucrase has been used as a (negative) selectable marker in bacterial transformation. Levansucrase contains an internal restriction site. When other genes of interest are inserted within the levansucrase structural gene, its function is abolished. Where insertion does not disrupt the structural gene, the activity is expressed, killing the cell. In this system, survivors have an insertion in the levansucrase structural gene. The expression of a large levan polymer in the cytoplasm presumably disrupts cellular spatial organization so drastically that the cell dies. This could provide a model for the inability to recover certain classes of transformants and for the occurrence of necrosis in those which slowly accumulate levan to a threshold damaging level. Vacular localization would negate this effect and explain survival of transformants so targeted. However, as indicated it is not clear that targeting has been successful. Inadvertent targeting to the cell perimeter or inadvertent sequestration within the endomembrane could also avoid direct effects on cytoplasmic organization and reduce toxicity. Clearly, transformants are able to accumulate sub-lethal concentrations of levan regardless of putative localization.

Based on the water-solubility of commercially available levan of c. 1% (w/v), *in situ* precipitation has been proposed as a mechanism, for example, to explain the browning of potato tuber cortex (Pilon-Smits et al., 1996). This model has the virtue that it could explain the threshold effects, where phenotypic aberration occurs only after substantial accumulation. However, ryegrass plants reported to contain a maximum of 0.04 mg g⁻¹ (c. 0.005% w/v) levan exhibited adverse phenotypic effects (Ye et al., 2001) whilst potato plants reported to contain 160 mg g⁻¹ (c. 20% w/v) levan had no reported aberrant phenotype (van der Meer et al., 1994). It would not appear that levan concentration and/or precipitation per se explains all of the observations.

The necrotic lesions formed in levan transgenics are reminiscent of the hypersensitive response of plants to pathogen infection. Some levan-producing bacteria belong to taxa such as *Erwinia*, which include plant pathogens. It is possible that levan, levansucrase or the message, triggers the hypersensitive response in transformants and explains the necrosis. When levan was injected or supplied to cut petioles in potato, necrosis was not induced. Injecting the levansucrase caused some necrosis but the results were inconclusive since controls from bacteria without levansucrase also produced necrosis (Röber et al., 1996). It would appear that exogenous levan or levansucrase alone do not trigger necrosis and that occurrence within the living cell is necessary, at least in potato.

Because levan is a soluble polymer, its detrimental effects have been attributed to its osmotic effect (Caimi et al., 1996), although no analyses of tissue osmotic adjustment were reported. However, assuming the transgenic levan to be as large as is generally assumed, the molar concentration of the polymer would be negligible compared with an equivalent mass of sucrose or hexose, especially in view of the low mass generally accumulated. The osmotic contribution of levan and its physiological effect should, in theory, be minimal. A further suggestion to explain developmental effects is metabolic disruption of sucrose metabolism caused by the diversion of carbon flow from primary metabolism (Caimi et al., 1996). Given the estimates of flux into levan of between 0.0002–0.08% of primary carbon metabolism (above), it is unlikely that leakage into levan *per se* had a significant detrimental impact on the steady-state sucrose pool or fluxes of photosynthate.

A number of reports found a negative correlation between levan and starch concentrations. The latter were generally depressed when compared to wild-type or empty-vector controls. For example, van der Meer et al. (1994) found a greater than 10-fold reduction in starch accumulation in potato. The highest levan accumulator contained c. 12 mg g⁻¹ levan and an equivalent concentration of starch. They hypothesized that the mechanism for the reduced starch concentration was efficient competition for carbon by levansucrase compared with starch and a resulting diversion of the flow of photoassimilate. As discussed above, the low *rates* of levan synthesis make metabolic competition an unlikely mechanism for reduced starch accumulation. Further, reduction in starch accumulation was non-stoichiometric. The sum of fructan and sucrose in the transformant was 24 mg g⁻¹ whilst the mean starch concentration in the wild-type control was 65 mg g⁻¹ with a maximum of 110 mg g⁻¹. Clearly the transformation resulted in a reduction in total assimilation rather than a simple diversion by competition.

To conclude the assessment of the aberrant phenotypes, it is noteworthy that (a) the highest reported levan concentrations were obtained in culture, presumably at low light with an exogenous carbon source, (b) plants maintained near the light compensation point were not reported to have aberrant phenotypes, (c) increased likelihood of developmental problems occurred at higher carbohydrate concentrations (correlating with higher irradiance?), and (d) one of the clearest, striking and most rapid physiological effects of fructan accumulation...
was chloroplast agglutination. Could these phenomena be linked?

Beneficial phenotypes resulting from levan accumulation

One of the rationales for levan transgenesis is rooted in the suggestion that fructan accumulation confers stress resistance, particularly to drought. For example, tobacco plants expressing levan accumulation are reported to show improved performance (determined as growth rate, fresh mass, root size, and dry mass) under polyethylene glycol-mediated osmotic stress (Pilon-Smits et al., 1995). Physiological aspects of these experiments are discussed elsewhere (Cairns et al., 2000). Similar experiments were reported for sugar beet (Pilon-Smits et al., 1999) with similar results. Whilst parameters such as biomass and leaf area were quantified for the plants used in the beet drought trial, levan content was not. Likewise, conventional measurements of osmotic adjustment have not been reported for these transformants (Blum et al., 1996). It is difficult mechanistically to assess the involvement of levan in the claimed drought resistance, in the absence of such measurements.

Plants transformed with plant fructosyltransferase genes

The enzymology of plant fructan synthesis is more complex than bacterial levan synthesis. Plant fructans exhibit a wide range of species-specific linkage structures and size distributions. Multiple enzymes appear to be involved in polymerization, the enzymes generally catalyse more than one fructosyltransferase and/or hydrolease reaction and hence the interpretation of enzymological data can be ambiguous (Cairns, 1993). Partly for these reasons, plant enzymes were isolated, sequenced and used for transformation later than their bacterial counterparts. The stated reasons for the production of plant–plant fructan transformants have mainly been to (i) confirm an established two-enzyme model for fructan biosynthesis (Hellwege et al., 1997, 1998, 2000) and (ii) create crops of biotechnological interest (Sénévé et al., 1998). This section examines the results of these transformations and the primary technique used for their analysis.

High performance anion exchange chromatography-pulsed amperometric detection

Fructans in higher plants are smaller than bacterial levan, are more amenable to chromatographic analyses and, unlike levan, can be fractionated by TLC and sized by SEC. In the last 15 years, high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD, Dionex Corporation) has revolutionized the study of plant fructan polymers and has become a standard technique. Before consideration of the results obtained for transgenic plants in which HPAEC-PAD has been used, it is instructive initially to consider its strengths and weaknesses.

The Carbopac columns have exquisitely high resolution, giving separations of individual oligo- and polysaccharides differing in size by only one hexose moiety (162 Da). The technique can reproducibly resolve members of homologous series and distinguish between structural isomers differing in chain linkage type or branching. Manipulation of elution conditions permits resolution of glycans in the range of DP between 1 and c. 100. The PAD data capture system has a wide sensitivity/attenuation range and quantities of polymer in the low nanogram-range can be clearly detected with a high signal-to-noise ratio. The quantitative response of the PAD is different for each individual carbohydrate species and the detector response factor decreases with increased DP. Hence, in the absence of calibrated responses for each individually detected compound, the method is at best semi-quantitative. The units of the output from the PAD are the Coulomb or the Ampere, though PAD data are sometimes reported in arbitrary units, often without indication of the total mass of carbohydrate or tissue fresh mass equivalent injected or the inclusion of standards. In consequence, reports of HPAEC-PAD data can be difficult to interpret quantitatively.

High sensitivity per se can be problematic and this is illustrated in Fig. 2. The analyte contained sucrose in a 1×10^4-fold excess over the individual fructans (eluuting at retention times >9 min), the mean mass of each was c. 25 ng. At low sensitivity (Fig. 2a) these fructans were not detected. The same trace presented at high sensitivity (Fig. 2b) revealed a striking high-resolution chromatogram showing a population of fructan species in the range of DP=c. 10–50. Clearly, trace quantities of carbohydrate can produce apparently large signals which could appear to an uncritical reader to be quantitatively very impressive.

A further problem resulting from high-sensitivity is that the masses of carbohydrate detected and isolated by HPAEC-PAD can be insufficient for subsequent analysis by other confirmatory methods. For example, TLC and structural determination by glycosyl linkage analysis/GC-MS require c. 1–10 µg minimum, whilst Nuclear Magnetic Resonance Spectroscopy requires masses in the low mg range. In the absence of confirmatory data, the basis for the conclusions is often totally dependent on HPAEC-PAD. Because of the unarguable power of HPAEC-PAD, there is a risk of being seduced into regarding it as an absolute determination of fructan, which it is not. Albeit powerful, it is only a relative technique and data should be interpreted with appropriate caution. The demerits of the technique are especially pertinent when considering reports of plant–plant transgenesis, where rates of fructan product accumulation and instantaneous con-
centrations, both in planta and in vitro are generally low.

Quantitative aspects of non-fructan plants transformed with plant fructosyltransferases

Table 2 summarizes quantitative parameters of relevance to reserve accumulation in plant–plant transformants. As for the bacterial transformants, more than half the studies did not report conditions of irradiance. Two of the transformants were apparently grown close to the light compensation point for C₃ plants and the criticisms set out above may apply. The instantaneous concentrations of transgenic fructan were generally below 9 mg g⁻¹ and as such are lower than the maxima found for endogenous reserve accumulation (60–150 mg g⁻¹, above). The exception was roots of sugar beet transformed with SST from *H. tuberosus*, where 62 mg g⁻¹ of oligofructan of DP=3–5 accumulated. This concentration parallels the maximal natural concentrations of starch and fructan in photosynthetic tissue (Fig. 1; Cairns et al., 2002a), although in storage roots, for example, of *Helianthus tuberosus*, natural fructan content can reach 160 mg g⁻¹ (Schubert and Fuerle, 1997). As with levan accumulation, when the rates of accumulation were estimated, values were low, in the range 0.00002–0.025 nkat g⁻¹. This range is equivalent to 0.00005–0.07% of the minimum flux calculated from Fig. 1. The maximal value was exceptional, all others were below 0.005 nkat g⁻¹ (0.014% of flux). Although the maximal value compared well with the maxima obtained with the levan transformants, on the whole, rates of fructan accumulation were lower in the plant–plant transformants than for the bacterial transformants.

Explaining the low rates of product accumulation in the plant–plant transformants

With the exception of chicory and onion, the host plants were chosen because they lacked endogenous fructan metabolism. As reasoned above, it was assumed that fructan hydrolase was absent in these plants and that product hydrolysis does not provide a general explanation for the observed low rates of accumulation. However, invertase is ubiquitous in higher plants. Small oligofructans (DP=3–7) are degraded by invertases (Cairns, 1993) and could hydrolyse transgenic fructan products. It is pertinent that acid invertases are naturally present in the vacuole, the putative site of natural and much of the transgenic fructan accumulation. Fructan hydrolysis certainly occurs in some instances where enzyme preparations from transformants and controls have been tested against oligofructan (van der Meer et al., 1998). From the available data it is not clear if this is a problem in planta.

Unlike the levan transformants, accumulation rates are likely to be adversely influenced by the kinetics of the plant-derived synthetic enzymes. Figure 3 shows substrate–activity curves for trisaccharide synthesis by SST from *H. tuberosus*, and for polymer synthesis by a fructan polymerase from the grass, *Phleum pratense*. The data illustrate the universally low sucrose sensitivity and affinity of plant fructosyltransferases, which are effectively unsaturable. Table 2 lists the approximate substrate affinities of the plant fructosyltransferases which have been used for plant transformation. All values were 280 mM or higher. These are compared with estimates of substrate concentration in the transformants, all of which were 38 mM or lower. Of ten values, eight concentrations were ≤22 mM, i.e. <8% of $K_m$. The enzymes would all have been severely substrate-limited in the transformants, the rate of fructan synthesis would have been sensitive to changes in substrate concentration and the limitation
would have contributed significantly to the observed low rates of product accumulation.

In common with the levan transformants, other measures of expression such as detection of enzyme activity, immunological detection of enzyme protein and detection of expressed message were generally low or non-existent in the plant-plant transformants. Hence, absolute concentrations of gene products and total enzymatic activity were also likely to be low. In combination with the substrate limitation of synthesis and the possibility for invertase-mediated hydrolysis of product, this provides circumstances favouring extremely low product accumulation rates. It is noteworthy that plant-plant transgenics are not the result of expression SST and FFT activities. An explanation not considered is as follows: The pattern from the control. By definition, these are not products of SST. Strictly interpreted, the petunia SST-transformant did not function in the absence of SST. Given the apparent presence of trisaccharide in the untransformed plants, it could have been interesting to see what fructan products accumulated in planta in the FFT-only transformant used as the source of the enzyme.

**Petunia** plants containing both of the SST and FFT constructs did not contain fructan in any tissues, except for trace quantities in yellow senescent leaves. No explanation for this pattern of expression (of constitutively-controlled genes) was offered. The fructan was detected by sensitive HPAEC-PAD in plant extracts concentrated 10-fold relative to the samples from the SST transformants, hence instantaneous product concentration must have been extremely low. The discontinuous size distribution pattern of the fructan products was interesting. There was a general reduction in abundance of products with size in the range DP=3–8, followed by an abrupt increase in abundance at DP 9–10, in turn followed by a smooth pattern of size decrease to c. DP 40. This overall pattern is quite distinct from the chicory inulin standard which exhibited a smooth decay over the whole size range of DP 1–40. (It is not clear why chicory rather than *H. tuberosus* inulin was used as standard.) The authors offer a number of plausible scenarios to explain the pattern based on invertase hydrolysis of the smaller products and/or differences in the balance of expression SST and FFT activities. An explanation not considered is as follows: The pattern from DP 9–40 in the transformant is strongly reminiscent of the water-soluble products of hydrolysis of starch during debranching (Tomlinson et al., 1997; Cairns et al., 2002b). It is conceivable that the pattern of sugars reported for the

Fructan biosynthesis in transgenics

Transformation of plants with fructosyl transferases from Helianthus tuberosus

The most pervasive model for fructan synthesis in plants was based on studies of *H. tuberosus* and involves two monofunctional enzymes: (a) SST, proposed to generate trisaccharide only, from sucrose and (b) FFT, which by disproportionation, was proposed to elongate/shorten polymers, using trisaccharide as the entry point for substrate. Corresponding enzymes were purified, the genes were obtained by RT-PCR and subsequently transformed into *Petunia* by Koops and co-workers (Koops and Jonker, 1996; van der Meer et al., 1998). Sequence analysis indicated the presence of signal sequences in both genes with the implication of vacuolar localization for the gene products. SST and FFT were separately introduced into petunia and later combined into the same plant by crossing. Significantly, when analysed with the sensitive HPAEC-PAD as the sole analytical method, an equivalent signal corresponding to trisaccharide was detected in both the SST-transformant and the untransformed control (Koops and co-workers’ Figs 5a, b, 7; van der Meer et al., 1998). As such, the data provided no evidence for transformation with a monofunctional SST. Traces of material co-eluting with inulin of DP=4 and 5 in the transformant were the primary differences between it and the control. By definition, these are not products of SST. Strictly interpreted, the petunia SST-transformant did not provide evidence for transformation with SST, but rather it indicates that SST functioned as an FFT-type activity acting upon endogenous trisaccharide as substrate.

Transformation with the FFT was clearly demonstrated in vitro since enzyme extracts formed traces of product of up to DP=8 by disproportionation from tetrasaccharide. Only enzyme extracts and not tissue sugars from transformants were assessed for FFT expression because of the authors’ expectation that the enzyme could not function in the absence of SST. Given the apparent presence of trisaccharide in the untransformed plants, it would have been interesting to see what fructan products accumulated in planta in the FFT-only transformant used as the source of the enzyme.

Transformation of plants with fructosyl transferases from Helianthus tuberosus

The most pervasive model for fructan synthesis in plants was based on studies of *H. tuberosus* and involves two monofunctional enzymes: (a) SST, proposed to generate trisaccharide only, from sucrose and (b) FFT, which by disproportionation, was proposed to elongate/shorten polymers, using trisaccharide as the entry point for substrate. Corresponding enzymes were purified, the genes were obtained by RT-PCR and subsequently transformed into *Petunia* by Koops and co-workers (Koops and Jonker, 1996; van der Meer et al., 1998). Sequence analysis indicated the presence of signal sequences in both genes with the implication of vacuolar localization for the gene products. SST and FFT were separately introduced into petunia and later combined into the same plant by crossing. Significantly, when analysed with the sensitive HPAEC-PAD as the sole analytical method, an equivalent signal corresponding to trisaccharide was detected in both the SST-transformant and the untransformed control (Koops and co-workers’ Figs 5a, b, 7; van der Meer et al., 1998). As such, the data provided no evidence for transformation with a monofunctional SST. Traces of material co-eluting with inulin of DP=4 and 5 in the transformant were the primary differences between it and the control. By definition, these are not products of SST. Strictly interpreted, the petunia SST-transformant did not provide evidence for transformation with SST, but rather it indicates that SST functioned as an FFT-type activity acting upon endogenous trisaccharide as substrate.

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transformant could be explained as a combination of the oligosaccharides apparently occurring naturally in petunia, those resulting from ‘SST’ transformation (DP=4 and 5) and by starch mobilization in senescing leaves. Since HPAEC data is the sole evidence presented, it is not possible to distinguish these possibilities and decide whether the evidence actually represents polyfructan synthesis and, hence, a role for FFT in planta, in the petunia transformants. Since it is also difficult to decide if SST (trisaccharide synthesis) was actually expressed in either the single- or double-transformant, and the evidence for fructan polymerization is open to alternative interpretation, their view that they had cloned the pathway of fructan biosynthesis in Jerusalem artichoke, may have been premature.

The *H. tuberosus* SST structural gene was subsequently used to transform sugar beet (Sévenier *et al.*, 1998). From the perspective of enzyme kinetics, the sugar beet vacuole is a highly appropriate environment for a low-affinity plant fructosyl transferase, because sucrose concentrations may reach 500 mM (Saftner *et al.*, 1983). The transformant was convincingly shown to accumulate small fructans to high instantaneous concentration, though at a low rate (Table 2). This demonstrated SST activity (i.e. trisaccharide synthesis), but since roughly half of the mass of product was present as oligofructan of DP=4 and 5, the enzyme was not monofunctional, exhibiting additional FFT activity. Although no data were shown, independent enzyme incubations with substrates of DP=3, 4 and 5 produced higher homologues, confirming that ‘SST’ had FFT activity. Enzyme extracts from wild-type controls were stated to contain invertase activity and to hydrolyse small fructans (data not shown). Unfortunately, rates of enzymatic hydrolysis were not presented, so its potential quantitative contribution to net fructan accumulation in planta could not be assessed. Invertase was implicated as the primary cause of low fructan concentrations in leaves, even though the SST activity could not be detected in protein extracts of this tissue. Invertase may certainly have had an effect, but low expression is equally likely to have caused low accumulation in leaves. No phenotypic aberrations were reported for beet, but the tubers shown were described as ‘young’ and were grown for two months in the glasshouse, in winter (R Sévenier, personal communication). Since the primary aim of the work was ostensibly the industrial production of fructan, results from mature beet grown under agricultural conditions would have been more informative.

*Transformation of plants with fructosyltransferases from Cynara scolymus*

One consequence of the advent of PCR is that genes can be isolated from a given species and transformed into another without any necessity for the *in vitro* enzymological analysis of the gene product. An example is the work of Hellwege *et al.* (1997, 1998). Primers designed using the sequences of *H. tuberosus* fructosyltransferases were used to obtain genes from *C. scolymus*. The genes were subsequently transformed into non-fructan plants. There are no published kinetic data for these enzymes or direct indication of their substrate and product specificities, responses to inhibitors, etc. The working assumption was that the model for fructan synthesis proposed for *H. tuberosus* holds for *C. scolymus* and that the enzymes would have similar properties.

When *C. scolymus* SST was transiently expressed in tobacco protoplasts, lysates incubated with sucrose synthesized small amounts of trisaccharide during 20 h incubations. The same gene stably transformed into potato resulted in the accumulation of trisaccharide and oligomers of DP 4 and 5. Hence, in common with SST from *H. tuberosus* in sugar beet, the gene product had substantial additional FFT or fructan-sucrase activity. The gene also had a 61% homology with acid invertases of dicots and could have additional hydrolytic activity. The authors felt that they had ruled out this possibility on the basis of differential sensitivity of SST and (the largely endogenous) invertase to pyridoxal-HCl, and because fructose did not accumulate in transformed potato tubers. The pyridoxal experiment observed a stimulation of trisaccharide synthesis of the SST, but this does not imply a lack of effect on any hydrolytic activity (Cairns and Ashton, 1991). Indeed, the enhancement of SST activity could simply be a function of the maintenance of high sucrose concentration caused by inhibiting the endogenous tobacco invertase. Inhibition of total invertase activity (endogenous plus transgenic) reveals little about the proportion which could be due to the SST. Similarly, the lack of accumulation of fructose in tubers may simply reflect turnover. These experiments failed to establish if the *C. scolymus* SST has invertase activity or not.

*C. scolymus* FFT was transiently expressed in tobacco protoplasts. Lysates were incubated with substrates of DP 3–7 for 4 d at 22 °C. Activity was thus extremely low and only traces of oligoinulin products of DP up to 20 could be detected by sensitive HPAEC-PAD (Hellwege *et al.*, 1998). The experiment was repeated using the FFT from *H. tuberosus* and the results were similar except that the products reached only DP 12. This was used as evidence that the product specificity of the two enzymes was different and that it explained the natural product size distribution in vivo: larger in *C. scolymus*, smaller in *H. tuberosus*. Clearly the FFT activity in both lysates was extremely low and since no quantification of product was reported, rates of fructosyltransfer could not be calculated or normalized. This is important, as the authors point out, since it has been shown that maximal fructan size is related to enzyme concentration (Cairns, 1995; van den Ende and van Laere, 1996; Cairns *et al.*, 1999). The FFT gene was isolated by PCR without enzymological analysis of the
gene product. It was assumed to have properties similar to the enzyme from *H. tuberosus* but this is not necessarily so. As has been seen, SST has FFT activity. By analogy, could the *C. scolymus* FFT have SST activity in planta? The *C. scolymus* SST individually and FFT and SST simultaneously were expressed in potato plants (Hellwege et al., 2000). In the SST-only transformants, fructans of DP up to 8 accumulated in tubers and up to DP=5 in leaves. Results from both tissues confirm ‘SST’ as a multifunctional SST/FFT enzyme. The sucrose concentration was higher in the tubers than the leaves and may reflect the larger products and higher concentrations in the tuber. The double transformant accumulated primarily oligofructan of DP 3, 4 and 5 (Hellwege et al., 2000). Traces of polymer of up to DP=40 (6.5 kDa) were also present, as shown by HPAEC. Fructans up to DP=90–100 can be resolved by HPAEC (Cairns et al., 1999) and would have been detected, if present, in the double transformant. In roots of *C. scolymus* maximum native polymer size has been reported elsewhere to reach DP=200 (32.5 kDa: Pražnik and Beck, 1985). Uncalibrated SEC was used in an attempt to show that fructan of DP \( \leq 200 \) was present in both wild-type tubers of *C. scolymus* and the double-transformant potato tubers. The SEC profiles of tubers and transformants were identical (as they were on HP AEC). In the absence of a size calibration for SEC and with no measure of the size of the native polymers used as standards, the SEC gave no size information additional to that from HPAEC. The evidence shows that the *C. scolymus* tubers contained DP up to 40 only, and this was the maximum size attained in the transformant. As in the previous studies, the double transformant was shown to manufacture traces of high \( M_f \) fructan, but did not address the question of sufficiency of FFT expressed alone.

Transformation of non-fructan plants with 6-sucrose fructosyl transferase from barley

Fructan biosynthesis in grass leaves is inducible in response to sucrose accumulation (Cairns et al., 2002a). The parallel induction of a fructosyltransferase led to the identification and purification of 6-SFT from barley leaves (Simmen et al., 1993; Duchateau et al., 1995). With sucrose as sole substrate, roughly 80% of its activity is hydrolytic (i.e. invertase) and c. 20% is fructosyl transferase, catalysing the formation of 6-kestose, a trisaccharide isomeric in which a fructosyl residue is glycosidically linked to the 6-carbon of the fructose residue of sucrose. The sucrose affinity for invertase activity is c. <10 mM and for trisaccharide synthesis is >300 mM. 6-SFT has a third activity in vitro: it transfers fructose from sucrose to the 6-carbon of fructosyl residues of inulin (2,1-linked) oligosaccharides, i.e. it catalyses 6-branching of small linear inulin primers. As a consequence of these properties in vitro, transforming 6-SFT into a non-fructan plant where sucrose would be the sole substrate, 6-kestose accumulation would be expected. Transformation into an inulin accumulator such as chicory, should result in primer glycosylation to form branched oligofructans. Both transformations would also be expected to co-introduce invertase activity to the site of transgenic fructan accumulation.

The 6-SFT enzyme has been cloned and expressed in heterologous plant systems. When transiently expressed in tobacco (Sprenger et al., 1995), incubations of intact protoplasts allowed detection of trisaccharide synthesis from sucrose and 6-branch glycosylation of inulin trisaccharide. High background invertase in the protoplasts precluded determination of transgenic sucrose hydrolysis (N Sprenger, personal communication). The enzyme was subsequently stably expressed at low rates in tobacco. This species contained sucrose as sole substrate and, as predicted, accumulated traces of 6-kestose (Sprenger et al., 1997). Endpoint concentrations were low, but were higher in roots than in leaves and positively correlated with sucrose concentration. Yellow senescing leaves contained the highest product concentrations and highest sucrose concentrations of the leaves sampled. Analyses of the products of roots by HPAEC revealed as predicted, 6-kestose, but also traces of two branched tetrasaccharides and a series of higher oligomers up to DP=12. Hence, in planta 6-SFT clearly has a fourth activity which could be either 6-FFT or a 6-fructan sucrase (6-polymerase). In addition, one of the branched tetrasaccharide products was identified as bifurose. This contains a 2,1 glycosidic linkage which 6-SFT does not form in vitro. The enzyme appears to have fifth activity in planta, that of 1-SST.

Transformation of an inulin-accumulating plant with barley 6-sucrose fructosyl transferase

Chicory naturally accumulates 2,1-linked inulin of DP \( \leq 40 \) in roots. In 6-SFT transformants of chicory, no transgenic fructan products occurred in the roots. Sprenger et al. (1997) demonstrated that the formation of traces of inulins up to DP \( \leq 16 \) can be induced in excised, illuminated leaves of wild-type chicory. When chicory leaves expressing the 6-SFT gene were similarly induced, additional trace shoulders occurred in the HPAEC peaks of the natural inulins of DP 4–7. This was indicative of limited 6-branch fructosylation (primer glycosylation) of the endogenous oligosaccharides.

Based on sequence homologies between fructosyltransferases, 6-SFT was used to isolate a fructosyltransferase gene from onion. Oligofructans of the onion family naturally contain 6-fructosylated glucose residues, collectively termed the neoseries after the parent trisaccharide ‘neokestose’. When transformed into chicory, the onion gene resulted in the formation of trace quantities of neoseries fructans of up to DP7 by primer
glycosylation of the natural linear inulins (Vijn et al., 1997).

**Has subcellular localization of plant transgene products been established?**

Fructosyltransferase genes from plants naturally contain putative signal and targeting sequences. Hence, heterologous targeting sequences have not been included in vectors constructed for plant–plant transgenesis. It has generally been assumed that vacuolar targeting took place because fructan synthesis occurred in transformants. However, in only one instance has this been demonstrated, for onion 6G-FFT in tobacco, where 70–100% of transgenic 6G-FFT activity in protoplasts was reported to be found in isolated vacuoles. Indirect evidence for vacuolar localization was adduced from the studies of transformed chicory because of the primer glycosylation of endogenous fructans. The logic of the argument is as follows: Because natural inulin synthesis is thought to be vacuolar and branched and neo-series inulins were synthesized, natural synthesis and transgenic primer glycosylation must have been co-localized in the vacuole. The possibilities that inulin synthesis or inulin synthesis plus transgenic primer glycosylation could have taken place in the endomembrane system prior to entering the vacuole (Kaeser, 1983; Cairns et al., 1999), were not considered. Endomembrane-associated synthesis in transit to the vacuole would not affect results showing vacuolar localization of fructan or fructosyltransferases. Clearly, conditions permitting levan synthesis exist in the endomembrane system of tobacco (Turk et al., 1997). It is worth noting that (i) the evidence for vacuolar localization of natural fructan synthesis rests on only a few studies (Wagner et al., 1983; Frehner et al., 1984; Cairns et al., 1989; Darwen and John, 1989), (ii) with the exception of the study of Darwen and John (1989) the protoplasting enzymes used contained fructosyl transferases which may interfere with the interpretation of data (Winters et al., 1992), and (iii) the evidence for vacuolar localization is for the synthesis of only the smallest fructans, the trisaccharides, and not the full range of natural fructans. Recent evidence based on the requirements for high enzyme and substrate concentrations to enable polymerization of fructan, indicate that the vacuole may not be an ideal compartment for fructan synthesis (van den Ende and Van Laere, 1996; Cairns et al., 1999).

**Phenotypic aberrations are not reported for plant–plant transgenics**

Unlike the bacterial transformants, no aberrant developmental effects have been observed in transgensics containing plant-derived fructosyl transferases. In the case of beet tap root, very high endpoint concentrations of oligofructan were obtained and the lack of phenotypic effects was attributed to vacuolar localization (although this was not demonstrated). Equally, it could be a function of the small size, chemical and structural similarity of the transgenic oligosaccharides to sucrose. Further, the plants were apparently grown at low irradiance and were harvested young, as were the potato tubers in the study of Hellwege et al. (1998). Developmental problems in the bacterial transformants correlated with older tissues and longer accumulation periods. All other plant–plant transformants contained only low concentrations of transgenic fructan and the lack of phenotypic effects may simply reflect this fact.

**Do results from plant–plant transgenesis advance current understanding of natural fructan synthesis?**

Overall, the understanding acquired from plant–plant transgenesis is largely confirmatory of that previously known from enzymology. A general criticism of work in plant–plant transgenesis is that the experiments have been performed with the emphasis on confirming, rather than testing, the SST/FFT hypothesis. Thus some authors appear surprised that that, by synthesizing higher oligofructans in planta, sucrose-fructosyltransferases do not behave as expected, observations for which they cannot offer an explanation (Hellwege et al., 1997; Sévenier et al., 1998). The answer is straightforward: the data do not fit the theory and the theory needs to be changed. Further, theory dictates that FFT cannot function in the absence of the product of SST. Hence, no attempt has yet been made to observe the products, in planta, of FFT-only transformants. Given the apparently anomalous behaviour of sucrose-fructosyltransferases in planta, why not also FFT? Besides, the experiment is intrinsically of value since it tests the hypothesis: A negative result would strengthen evidence for a monofunctional FFT. Despite these limitations and the extremely low rates of expression, the work of Hellwege et al. (2000) provides the best evidence yet to support a (modified) two-fructosyltransferase model of fructan biosynthesis for Jerusalem and globe artichokes.

The results of transgenesis with 6-SFT confirmed two of the activities observed in vitro; namely, 6-kestose synthesis and 6-primer fructosylation. However, transgenesis provided no information about the expression and effects of its major activity, invertase. This is of interest because, in conditions where sucrose is the sole or predominant substrate (as during the initiation of natural fructan synthesis in vivo), hydrolysis predominates. Under these conditions, total invertase activity can be twice the rate of sucrose synthesis and it is not clear how sucrose is able to accumulate in the vacuole if, as is generally accepted, these hydrolytic enzymes are also vacuolar. The invertase activity of 6-SFT could be an additional factor explaining the low rates of expression in transgensics.

Two additional activities of 6-SFT, namely, 6-polymerase (sucrase or 6-FFT) and 1-SST were also manifested in planta. The transgenic results confirm in vitro data and
extend the view of 6-SFT as a multifunctional enzyme with a wide product specificity. Whilst the physiological studies of the transformants do not provide information about the hydrolytic functions of the fructosyltransferases, sequence data certainly do show universally agreed similarities with invertases. One wonders how the sequences would have been identified and named, had the genes been isolated without the preconception that they were involved in fructan synthesis? In view of the continued inability to distinguish 6-SFT from invertase and consideration of the quantitatively minor proportion of its activity which is synthetic in vitro, it still seems pertinent to ask if some plant fructosyltransferase polypeptides are primarily invertases. There is no doubt that the 6-SFT transgene product catalyses trisaccharide synthesis and primer fructosylation, but equally, so does the 6-SFT and a variety of other invertases in vitro. Neither observation rules out the possibility that the enzyme involved is an invertase or provides proof of its role in natural fructan synthesis. In this context, no studies of antisense repression or sense co-suppression have yet been reported for any plant fructosyltransferase.

**Cellular effects on transgene expression**

The interaction between essentially hydrolytic polypeptides and the environment in which they function may be a significant factor in determining the extent of fructan polymerization. From the authors’ perspective, the most exciting observations from fructan transgenics concern the differential patterns of product formation observed in vitro and in planta. As alluded to above, ‘SST’ enzymes from *H. tuberosus* and *C. scolymus* synthesize larger fructans in planta than in vitro. The 6-SFT, which generates only trisaccharide from sucrose in vitro, catalyses the formation of 2,6 linked fructans of up to DP=12 from sucrose, i.e. it acts as a polymerase in transgenic potato tubers. Finally, a mutant levansucrase which synthesizes only trisaccharide in vitro, forms a polymer of apparent high molecular mass when expressed in planta (Chambert and Petit-Glatron, 1993; Caimi et al., 1996). Taken together these observations indicate that the fructosyltransferases have the capacity for polymerization and that expression in intracellular conditions favour the expression of this activity.

These observations in planta have parallels with the effects of enzyme concentration in in vitro studies, and it is well documented that conditions of reaction affect the qualitative function of fructosyltransferases (Cairns and Ashton, 1991; Cairns, 1995; van den Ende and van Laere, 1996; Cairns et al., 1999). In the authors’ view, the most convincing demonstrations of fructan polymerization require high-enzyme and high-substrate concentrations and the mechanism in vitro may be a function of (i) increased rate of intermediate synthesis (ii) closer spatial proximity of enzymes substrate and intermediates (iii) reduced water availability, leading to reduced invertase activity of fructosyltransferases (i.e. reduced competition caused by fructosyl transfer to water, Cairns et al., 1999).

In addition, it is known that reduction of water content in enzyme reactions by increased solvent content, also favours polymerization (Chambert and Petit-Glatron, 1993). It is possible that similar physico-chemical effects modify the activity of transgene products in planta and may explain the historical difficulty in establishing in vitro polymerization with plant enzymes.

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