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The rice genome encodes two vacuolar invertases with fructan exohydrolase activity but lacks the related fructan biosynthesis genes of the Pooideae

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Summary

- Fructans are believed to contribute to cold and drought tolerance in several plant families (Poaceae, Asparagaceae and Asteraceae), but it is not clear why the ability to accumulate these polymers is found in some genera (e.g. *Triticum*) but not in others (e.g. *Oryza*).
- As fructan biosynthesis enzymes (FBEs) evolved from vacuolar invertases (VINs), we searched the rice genome sequence for genes related to both FBE and VIN genes of wheat and other members of the Pooideae. We compared them at the levels of exon–intron structure, protein sequence, and the enzymatic properties of recombinant proteins after expression in the yeast *Pichia pastoris*.
- We found that rice possesses two VIN genes (*OsVIN1* and *OsVIN2*) and no FBE genes. FBE genes appear to have arisen in the Pooideae by a series of gene duplications from an ancestor of wheat *TaVIN3*. Recombinant TaVIN2, OsVIN1 and OsVIN2 behaved as invertases with no FBE activity, but possessed high fructan exohydrolase activity, especially OsVIN1.
- The engineering of fructan accumulation into rice for greater stress tolerance could founder on endogenous exohydrolases, but the fact that *OsVIN1* transcripts are absent from peduncles of well watered and drought-stressed plants removes one potential obstacle to this endeavour.

Key words: drought, evolution, fructans, *Pichia pastoris*, rice, vacuolar invertase, wheat.

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Introduction

The principal storage forms of carbohydrate in flowering plants are sucrose (Winter & Huber, 2000) and starch (Tetlow *et al.*, 2004). In addition, sucrose has well established roles in carbon metabolism and cellular signalling (Koch, 2004), while the starch of columellar cells participates in gravitropism (Takahashi *et al.*, 2003). Fructans, a third storage form, are found in approx. 15% of plants (Pollock & Cairns, 1991; Hendry, 1993; Hendry & Wallace, 1993; Vijn & Smeekens, 1999). Fructans have been implicated in protecting plants from cold stress and water deficit (Pilon-Smits *et al.*, 1995; Puebla *et al.*, 1997; Hincha *et al.*, 2002; Konstantinova *et al.*, 2002) and in maintaining proper sucrose concentrations for

photosynthesis and transport (Pollock *et al.*, 2003). They have been studied principally in the eudicot family Asteraceae and the monocot families Asparagaceae, Liliaceae and Poaceae.

Fructans are synthesized by the action of specialized fructosyltransferases, which we refer to here collectively as fructan biosynthesis enzymes (FBEs). Sucrose:sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) transfers the fructosyl group of one sucrose molecule to the fructose of a second sucrose molecule to form 1-kestose (Vijn *et al.*, 1998). Sucrose: fructan 6-fructosyltransferase (6-SFT) is presently designated EC 2.4.1.10, like bacterial levansucrase, but has a distinct main activity. Whereas levansucrase creates long, linear levans, 6-SFT transfers a fructosyl group to the intermediate fructose of 1-kestose to produce bifurcose (1 & 6-kestotetraose), a branched fructan. 6-SFT shows, in addition, a 6-SST side activity (Sprenger *et al.*, 1995). Fructan:fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100) catalyses the formation and extension of β(2,1)-linked fructans in the Asteraceae (Bonnett *et al.*, 1997; Vergauwen *et al.*, 2003) and the Poaceae (Kawakami & Yoshida, 2005). A distinct FBE found in the Asparagaceae is fructan:fructan 6^G-fructosyltransferase (6G-FFT, EC 2.4.1.-), which catalyses a transfructosylation from fructo-oligosaccharides to C6 of the glucose residue of sucrose or fructo-oligosaccharides (Shiomi, 1989; Ritsema *et al.*, 2003; Ueno *et al.*, 2005).

Sequence analysis has established that FBEs evolved from vacuolar invertases independently in the monocots and eudicots (Vijn & Smeekens, 1999). Very much earlier in the history of plants, vacuolar invertases had evolved from cell-wall invertases (Sturm, 1999; Ji *et al.*, 2005a). Both vacuolar and cell-wall invertases hydrolyse sucrose irreversibly and are designated EC 3.2.1.26. In the evolution of 1-SST from vacuolar invertase, water was replaced as the fructosyl group acceptor by a second sucrose molecule (Vijn *et al.*, 1998). In the further evolution of FBEs, as suggested above, there were changes in the donor substrate and/or the acceptor substrate. Because fructan accumulators contain multiple cell-wall invertases, vacuolar invertases and FBEs, these evolutionary events must have been underpinned by various types of gene-duplication events (Vijn & Smeekens, 1999; Bowers *et al.*, 2003; Ji *et al.*, 2005a).

Reliable identification of fructan accumulators and nonaccumulators among plant species depends on the use of suitable fructan assay methods (Pollock & Cairns, 1991) and appropriate plant growth conditions, especially temperature and nutrition (Wang & Tillberg, 1996; Yang *et al.*, 2004; Morcuende *et al.*, 2005). The inability of plants to accumulate fructans could, in principle, be caused by the suppression, mutation or absence of FBE genes. In the Gramineae (Poaceae), fructan accumulation is associated with temperate rather than tropical species (Chatterton *et al.*, 1989), but the genetic basis of this distinction is unclear. Fructan metabolism and FBE genes have been studied intensively in the three main temperate tribes of the subfamily Pooideae, including cereals such as *Avena*, *Hordeum* and *Triticum*, and forage grasses such *Lolium* and *Poa*. By contrast, rice (*Oryza*) of the subfamily Ehrhartoideae is not known to possess fructans.

Here we use analytical methods recommended by Pollock & Cairns (1991) to determine whether rice accumulates fructans in the peduncle, a major site of accumulation in wheat (Gebbing, 2003). In addition, the completion of the sequencing of the rice genome (International Rice Genome Sequencing Project, 2005) allowed us to conduct a comprehensive search for genes encoding putative FBEs. A complete inventory of rice invertase genes (Ji *et al.*, 2005a) identified nine genes resembling cell-wall invertases, two genes resembling vacuolar invertases and eight genes resembling neutral/alkaline invertases. We report now on the phylogenetic and enzymatic relationship between the two vacuolar invertases of rice (OsVIN1 and OsVIN2) and authentic invertases and FBEs of the Pooideae. We measured the invertase, fructan 1-exohydrolase and FBE activities of recombinant OsVIN1 and OsVIN2 expressed in and secreted from the yeast *Pichia pastoris*. We studied the regulation of *OsVIN1* and *OsVIN2* gene expression in four tissues in response to reproductive-stage drought stress. Our data suggest that the conversion of rice into a fructan accumulator by the introduction of FBE genes from wheat or other natural accumulators must take into account the possibility of futile cycling caused by the enzymatic properties of OsVIN1 and OsVIN2.

Materials and Methods

Plant materials

Rice (*Oryza sativa* L. indica cv. IR64) seeds were obtained from Genetic Resources Center at the International Rice Research Institute (IRRI). The experiments were conducted in the dry season of 2005 (January–June; average daily radiation 17.2 MJ m^{-2}; average daily sunshine 7.6 h; average humidity 84%; average max. and min. temperatures 31.2 and 22.8°C). Plants were grown in an IRRI glasshouse under natural daylight in buckets containing 9 kg soil and basal fertilizer consisting of ammonium sulfate (18 g N), sodium dihydrogen phosphate (9 g P) and KCl (18 g K). Plants were watered twice daily. Supplementary fertilization was provided at panicle initiation (20% of basal), or withheld to provide N deficiency at flowering.

Carbohydrate extraction and chromatography

Water-soluble carbohydrates were extracted from duplicate samples of nine peduncles harvested 2 d after heading from both N-sufficient and N-deficient plants. Samples were ground in liquid N, extracted with 10 volumes of hot water and boiled for 15 min. After cooling, the extract was centrifuged at 10 000 ***g*** for 10 min and the supernatant was freeze-dried for analysis by high-performance anion-exchange

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chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex, Sunnyvale, CA, USA; Vergauwen *et al.*, 2003) with and without acid hydrolysis. Sucrose, glucose, fructose and raffinose were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fructans for use as standards were extracted from chicory root (Van den Ende & Van Laere, 1996). Raffinose was included as a standard because it occurs in rice plants and appears on the chromatograph near 1-kestose.

Drought stress

IR64 plants were grown individually to maturity in pots in the IRRI phytotron (12 h day, 29°C : 12 h night, 21°C). The pots contained 3 kg soil and basal fertilizer consisting of ammonium sulfate (6 g N), sodium dihydrogen phosphate (3 g P) and KCl (6 g K). Plants were watered twice daily. Supplementary fertilization was provided at panicle initiation (20% of basal). Primary tillers were marked on each plant and the progress of reproductive development was monitored in terms of the interauricle length (distance between auricle of flag leaf and that of penultimate leaf). At 3 d before heading, when the interauricle distance reached 13.0 ± 0.5 cm, drought stress was applied by withholding water for 2 d. Samples were collected from well watered plants and stressed plants at 3 d before heading (dbh) and 1 dbh. Flag leaves, panicles, anthers and peduncles were collected in liquid N_2 and stored at −80°C until needed for RNA extraction as described below. The relative water content of the flag leaves was calculated as:

$$((W - DW)/(TW - DW)) \times 100$$

where W is the sample fresh weight, TW is the sample turgid weight and DW is the sample dry weight (www.plantstress.com/methods).

Phylogenetic analysis

Phylogenetic analysis included the two vacuolar invertases of rice (OsVIN1 and OsVIN2), 17 other angiosperm vacuolar invertases and 12 angiosperm FBEs. The phylogeny was outgroup-rooted using an *Arabidopsis* cell-wall invertase (AtCIN = AtcwINV1) and a rice cell-wall invertase (OsCIN1) (Ji *et al.*, 2005a). The GenBank accession numbers for all 33 proteins are given in the legend to Fig. 2.

ClustalX (Thompson *et al.*, 1997) was used to produce a preliminary protein sequence alignment. This alignment was inspected visually, and uncertain or highly gapped positions were excluded from the analysis, resulting in a data matrix of 536 aligned positions. Three complementary methods of analysis were used to assess confidence in phylogenetic relationships. First, neighbour-joining bootstrap analysis with 1000 bootstrap replicates was performed in paup 4.0b10 (Swofford, 2002). Second, parsimony bootstrap analysis was performed in paup 4.0b10 with 300 bootstrap replicates; for each replicate the most parsimonious tree was searched heuristically with 10 stepwise random addition replicates and five trees held at each step. Third, we performed Bayesian phylogenetic inference with model averaging, using MrBayes 3.1 (Ronquist & Huelsenbeck, 2003). The chains ran for two million generations and trees were sampled every 100th generation. Chains were judged to converge by using the standard deviation for split frequencies, and burn-in was set to 10 000 trees.

To obtain a second estimate of phylogenetic relationships between the sequences, we analysed the corresponding cDNA sequences. For VvVIN1 and VvVIN2, the nucleic acid sequence was not available in GenBank, so these sequences were not included in the analysis. The coding DNAs were aligned in translated form using RevTrans 1.4 (Wernersson & Pedersen, 2003). Again, uncertain positions were excluded from analysis, resulting in a data matrix of 1573 positions. Both neighbour-joining and parsimony bootstrap analyses were performed using paup 4.0b10, each with 1000 bootstrap replicates. As three of the available protein sequences (AF069309, AJ532551, AJ563384) were incomplete at the N-terminus, we conducted ClustalX analysis with sequences beginning 24 amino acids upstream from the first D of the active site (DPN).

5′ and 3′ rapid amplification of cDNA ends (RACE)

RACE was performed using the SMART RACE cDNA Amplification Kit according to the manufacturer's instructions (Clontech, Palo Alto, CA, USA). Gene-specific primers were as follows:

OsVIN1 3′-end: 5′-CCGAGGACAGTTATGCTG-GACACG-3′
OsVIN1 5′-end: 5′-TAGAAGAAGACAGCCGTC-CGCTCC-3′
OsVIN2 3′-end: 5′-CTTCCCTACTCCTACTCGC-CGCTCC-3′
OsVIN2 5′-end: 5′-CCATCAGCCTCACCATCTCCTCC-3′
TaVIN2 5′-end: 5′-AGTCTGGAGTCTCGGAGAAGAC-3′
TaVIN2 3′-end: 5′-CGACCTTCCTTCGGTCTAAA-3′.

The cDNA fragments were cloned individually using the pGEM-T Easy Vector System (Promega, Madison, WI, USA) for OsVIN1 and 2 and the TOPO TA cloning system (Invitrogen, Groningen, the Netherlands) for TaVIN2 and fully sequenced.

Heterologous expression in yeast

We constructed the expression plasmids pXMJOsVIN1, pXMJOsVIN2 and pXMJTaVIN2 to allow expression and secretion of these vacuolar proteins (Kawakami & Yoshida, 2002; Ueno *et al.*, 2005). These plasmids were based on the

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pPICZαA vector, which contains an *Eco*RI–*Not*I (OsVIN1 and 2) cloning site or an *Eco*RI–*Xba*I (TaVIN2) downstream from the methanol-inducible *AOX1* promoter and the α-factor secretion signal (Invitrogen). The plasmid also contains the zeocin resistance gene for direct selection of multicopy integrants. *Eco*RI–*Not*I (OsVIN1 and 2) and *Eco*RI–*Xba*I (TaVIN2) inserts encoding the respective mature protein regions were prepared by RT-PCR using the primers:

OsVIN1-F: 5′-CCG**GAATTC**CGGGACTACGCCT-GGACCAA-3′
OsVIN1-R: 5′-GAAT**GCGGCCGC**GACAAATCAT-TCATTCCCATTAC-3′
OsVIN2-F: 5′-CCG**GAATTC**GAGGCGTTCCCGT-GGAGCAA-3′
OsVIN2-R: 5′-GAAT**GCGGCCGC**CAGGCCAGCAG-CTAGCAGCAG-3′
TaVIN2-F: 5′-AATGACC**GAATTC**CCGTGGAGCA-ATGCCA-3′
TaVIN2-R: 5′-TGATGTAA**TCTAGA**CGACCTTC-CTTCGGTCTAAA-3′.

Bold letters indicate the positions of the *Eco*RI, *Not*I and *Xba*I sites in the 5′ ends of the forward and reverse primers, respectively. Underlined bases in the *OsVIN1*-F primer encode amino acids 101–106 (RDYAWT); those in the *OsVIN2*-F primer encode amino acids 113–118 (EAFPWS); and those in the *TaVIN2*-F primer encode amino acids 120–124 (PWSNA). The RT-PCR amplicons and the pPICZαA vector were digested with *Eco*RI/*Not*I or *Eco*RI/*Xba*I and gel-purified. After dephosphorylation of the vector, the RT-PCR products were ligated into the vector. The ligation mix was transformed into *Escherichia coli* (strain: TOP10F′) competent cells as described by Van den Ende *et al.* (2001). Cells were plated on a 2 × yeast tryptone medium supplemented with zeocine as selection agent. The resulting plasmids, pXMJOsVIN1, pXMJOsVIN2 and pXMJTaVIN2, were checked by DNA sequencing then transformed into *P. pastoris* strain X33 using electroporation, and selected as described by Kawakami & Yoshida (2002). Methanol (2%, v/v) was included in the *Pichia* expression media. After transformation and 5 d methanol induction, yeast cells were removed by centrifugation, the secreted proteins in the supernatants were freed of low-molecular weight contaminants by dialysis, then glycosylated proteins (including recombinant invertase) were concentrated and purified by passage through a ConA–Sepharose column (25 × 100 mm) before analysis by SDS-PAGE (2 µg per lane) and staining with Coomassie Brilliant Blue.

Q-TOF mass spectrometry

The SDS-PAGE protein bands (75 kDa) of the fully functional recombinant OsVIN1 and OsVIN2 enzymes were subjected to mass spectrometric (MS) identification. The stained bands were excised, trypsinized, extracted, desalted and analysed on Q-TOF MS as described earlier (Van den Ende *et al.*, 2003a). Sequence information was derived from the MS/MS spectra with the aid of the MaxEnt 3 (de-convoluting and de-isotoping of data) and PepSeq software from the Micromass BioLynx software package (Matrix Science Ltd, London, UK).

Enzyme assays

The glucosyl-fructosidase (invertase) and fructosyl-fructosidase (fructan exo-hydrolase) activities of recombinant OsVIN1, OsVIN2 and TaVIN2 were assayed by measuring fructose release from substrates. Glucosyl-fructosidase was assayed using 2–250 mM sucrose or raffinose as substrate, while fructosyl-fructosidase was assayed using 2–250 mM 1-kestose, in a final volume of 30 µl 50 mM sodium acetate buffer (pH 5.0). Fructose formation was determined by HPAEC-PAD. Enzymatic activity was calculated in units (U) defined as the amount of enzyme that formed 1 µmol fructose min^{-1} at 30°C. The amount of recombinant protein in each reaction was 50 ng, 1.7 µg and 9.6 ng, respectively, for OsVIN1, OsVIN2 and TaVIN2, as determined by the method of Sedmak & Grossberg (1977). V_{max} and K_m(sucrose) were calculated over the range 2–125 mM sucrose. Activities against other substrates are reported as a percentage of activities against sucrose. 1-Kestose synthesis was assayed under the same incubation conditions, but with 60–1500 mM sucrose. Products (1-kestose, 6-kestose, neokestose and fructose) were quantified by HPAEC-PAD. 1-Kestose and 6-kestose were generous gifts from Dr M. Iizuka (Iizuka *et al.*, 1993). Neokestose was kindly provided by Dr N.J. Chatterton (Utah State University, Logan, UT, USA).

RNA isolation and RT-PCR analysis

Total RNA was isolated using Trizol reagent as described by the manufacturers (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed using Superscript II reverse transcriptase-Taq polymerase enzyme mix (Invitrogen), 1.0 µg DNase I-treated total RNA, and the following gene-specific forward and reverse primers:

OsVIN1-F: 5′-TGGAGCAGCAGCATACAGC-3′
OsVIN1-R: 5′-CGGATGTAAGCAGAGTTCAGC-3′
OsVIN2-F: 5′-GACATCGTCAAGAGGGTCG-3′
OsVIN2-R: 5′-CCATCCATGATCCATCATCC-3′
OsG3PDH-F: 5′-GCAGGAACCCTGAGGAGATC-3′
OsG3PDH-R: 5′-TTCCCCCTCCAGTCCTTGCT-3′.

Amplification conditions were as follows: reverse transcription at 50°C for 30 min; preamplification denaturation at 92°C for 2 min, 35 cycles of denaturation at 92°C for 30 s, primer annealing at 56°C for 30 s and primer extension at 68°C for 1 min, and a final extension of PCR products at

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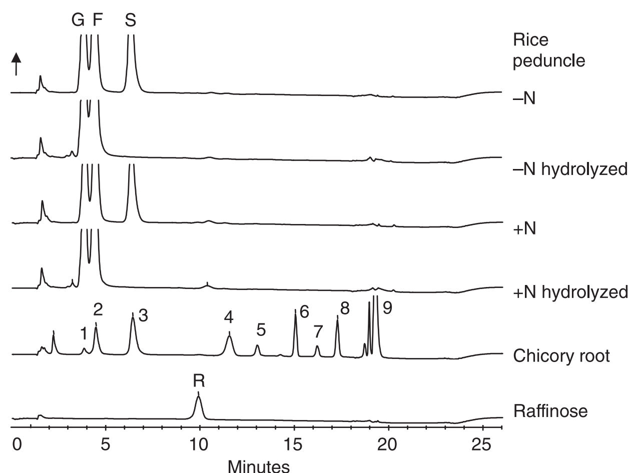

Fig. 1 Rice peduncles lack fructans. Water-soluble carbohydrates were extracted from peduncles of rice plants growing in the presence and absence of nitrogen fertilizer. Extracts were analysed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Elution positions are shown for glucose (G), fructose (F), sucrose (S), raffinose (R) and nine identified components of chicory root extract (Van den Ende *et al*., 1996). Components 1–9: glucose, fructose, sucrose, 1-kestose, inulobiose, 1,1 nystose, inulotriose, 1,1,1 kestopentaose and fructans of higher degrees of polymerization. The vertical arrow represents the detector response in arbitrary units.

68°C for 10 min. RT-PCR products were separated by electrophoresis on 1.2% agarose gels. The *OsG3PDH* primers were used to confirm that the DNAase I-treated RNA was free from contamination by genomic DNA.

Results

IR64 fails to accumulate fructans in the peduncle

To test whether rice accumulates fructans in the peduncle, we extracted the water-soluble carbohydrates from this tissue in well watered IR64 plants at 2 d after heading. The plants had been fertilized at panicle initiation with or without N; N deficiency is known to enhance the diversion of photosynthate from growth-related processes into fructans in wheat and barley (Wang & Tillberg, 1996; Yang *et al*., 2004) and chicory (Van den Ende *et al*., 1999). Chromatography (HPAEC-PAD) showed that the peduncles accumulated glucose, fructose and sucrose but lacked fructans (Fig. 1). The very small peaks seen at elution times of 19–20 min were not hydrolysed by acid, unlike fructans, and no evidence was found for fructans of lower degree of polymerization, including 1-kestose (corresponding to peak no. 4 in the chicory standard). The levels of glucose, fructose and sucrose increased by approx. 50% in response to N deficiency. These data support the classification of rice as a nonaccumulator of fructans.

Table 1 *OsVIN2* gene is the rice genome sequence with greatest similarity to Ta-1-SST

Class of target	Top hits	Score	Gene	Protein
Full-length cDNAs	AK072245	777	OJ1359_D06.20	OsVIN2
	AK099312	668	OSJNBb0020O11.6	OsVIN1
BAC clones	AP004851	405	OJ1359_D06.20	OsVIN2
	AL662998	378	OSJNBb0020O11.6	OsVIN1

Query sequence for tBLASTn: protein sequence of wheat cDNA clone AB029888. Target sequences: nr database for *Oryza sativa*. L. cv. Nipponbare.

Evolution of vacuolar invertases and FBEs in monocots and dicots

To explain nonaccumulator status, we employed tBLASTn analysis (Altschul *et al*., 1997) to determine whether the rice genome contains any genes encoding FBEs. We used FBEs of the Pooideae (wheat, barley, *Poa*, *Lolium*) as query sequences, and the rice genome and rice cDNAs as the target sequences. Table 1 illustrates these results for Ta-1-SST as query; the target sequences were in the 'nr' database of the temperate japonica cv. Nipponbare (http://www.ncbi.nlm.nih.gov/BLAST). The scores were higher for full-length cDNAs than for the corresponding genes because of the presence of introns in the latter (see Fig. 3). For Ta-1-SST and all other FBE genes, the best hit was the full-length cDNA of *OsVIN2*, followed by the full-length cDNA of *OsVIN1*, suggesting that no entry in the rice full-length cDNA database encodes a protein with greater similarity to the FBEs. More significantly, for a collection of BAC clones covering 95.3% of the rice genome and 98.8% of the euchromatin (International Rice Genome Sequencing Project, 2005), the best hit was again the *OsVIN2* gene within the relevant BAC clone, followed by *OsVIN1*. We repeated the tBLASTn analysis with target sequences in the database of the Beijing Genomics Institute, derived from the tropical indica cv. 9311 (http://rise.genomics.org.cn/rice/index2.jsp), and obtained identical hits and scores. Our conclusion is that *OsVIN2* is the rice sequence most closely related to the FBE genes of the Pooideae.

This relationship was clarified through phylogenetic analysis of 19 angiosperm vacuolar invertases and 12 angiosperm FBEs (Fig. 2). Because vacuolar invertases evolved from cell-wall invertases early in the evolution of green plants (Sturm, 1999; Ji *et al*., 2005a), we used cell-wall invertases of rice (OsCIN1) and *Arabidopsis* (AtcwINV1) as outliers. The analysis showed that the FBEs of the Asteraceae, Asparagaceae and Poaceae evolved independently and comparatively recently from specific groups of vacuolar invertases. In the Asteraceae (dicot), sequences were available for 1-SST and 1-FFT, while in the Asparagaceae sequences were available for 1-SST and 6G-FFT, and in the Poaceae for 1-SST, 1-FFT and 6-SFT.

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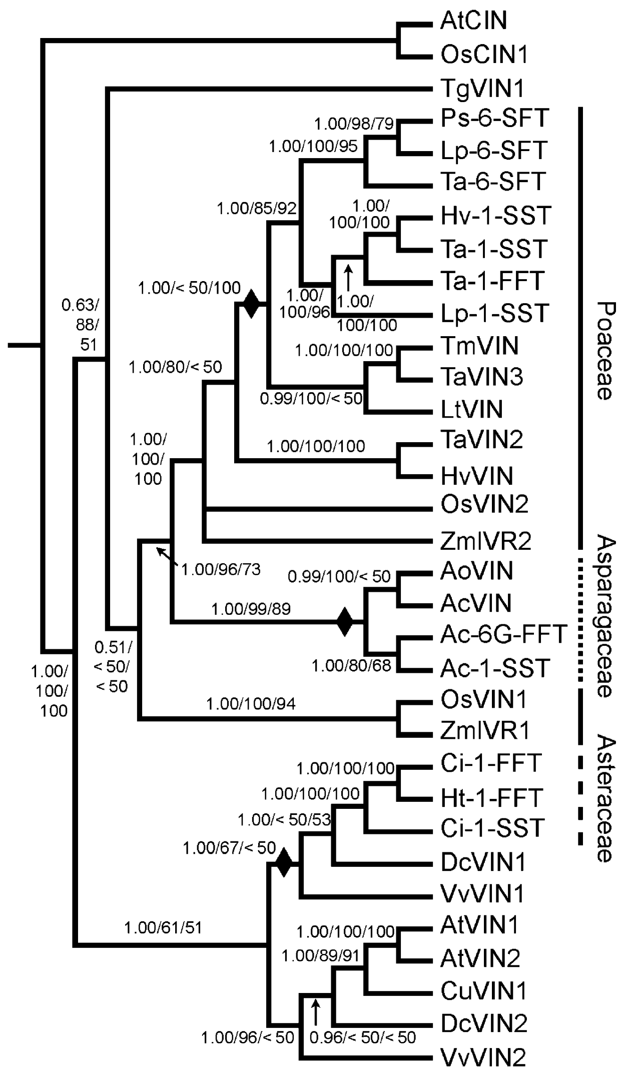

Fig. 2 Phylogenetic tree of the rice vacuolar invertases (OsVIN1 and OsVIN2) and selected vacuolar invertases and fructan biosynthesis enzymes. ClustalX analysis was performed on the known or deduced amino acid sequences beginning 24 aa upstream from the first conserved D of the active site and ending at the C-terminus. Confidence in phylogenetic relationships was assessed by (1) neighbour-joining bootstrap analysis with 1000 bootstrap replicates (paup 4.0b10; Swofford, 2002); (2) parsimony bootstrap analysis with 300 bootstrap replicates (paup 4.0b10); and (3) Bayesian phylogenetic inference (MrBayes 3.1, Ronquist & Huelsenbeck, 2003). The three confidence estimates are given as fractions or percentages. The NCBI database accession numbers of the proteins or their cDNAs were as follows. Vacuolar invertases: *Allium cepa*, AJ006067 (AcVIN); *Asparagus officinalis*, AF002656 (AoVIN); *Hordeum vulgare*, AJ823275 (HvVIN); *Lolium temulentum*, AJ532551 (LtVIN); *Oryza sativa*, AF276703 (OsVIN1); *O. sativa*, AF276704 (OsVIN2); *Triticum aestivum*, AF069309 (TaVIN3); *T. aestivum*, AJ635225 (TaVIN2); *Triticum monococcum*, AY575717 (TmVIN); *Tulipa gesneriana*, X95651 (TgVIN1); *Zea mays*, U16123 (ZmIVR1); *Z. mays*, AJ563384 (ZmIVR2); *Daucus carota*, X75352 (DcVIN1); *D. carota*, X67163 (DcVIN2); *Vitis vinifera*, AAB47171 (VvVIN1); *V. vinifera*, AAB47172 (VvVIN2); *Citrus unshiu*, AB074885 (CuVIN1); *Arabidopsis thaliana*, AY142666 (AtVIN1); *A. thaliana*, AY114066 (AtVIN2). Sucrose:sucrose 1-fructosyl transferase (1-SST): *A. cepa*, AJ006066 (Ac-1-SST); *H. vulgare*, AJ567377 (Hv-1-SST); *L. perenne*, AF492836 (Lp1-SST); *T. aestivum*, AB029888 (Ta-1-SST); *Cichorium intybus*, U81520 (Ci-1-SST). Sucrose:fructan 6-fructosyltransferase (6-SFT): *L. perenne*, AF494041 (Lp-6-SFT); *Poa secunda*, AF192394 (Ps-6-SFT); *T. aestivum*, AB029887. Fructan:fructan 1-fructosyltransferase (1-FFT): *H. tuberosus*, AJ009756 (Ht-1-FFT); *C. intybus*, U84398 Ci-1-FFT); *T. aestivum*, AB088409 (Ta-1-FFT). Fructan:fructan 6G-fructosyltransferase (6G-FFT): *A. cepa*, ACY07838 (Ac-6-GFT). The outliers were two cell-wall invertases: *A. thaliana* AtcwINV1, AY079422 (AtcwINV1 = AtCIN); *O. sativa*, AY342319 (OsCIN1). TaVIN1 is not included in the ClustalX analysis because only a partial cDNA sequence is available through wheat EST BQ744016. Diamonds mark the ancestral vacuolar invertases from which the fructan biosynthesis enzymes of the Asteraceae, Asparagaceae and Poaceae appear to have arisen.

We used neighbour-joining bootstrap analysis, parsimony bootstrap analysis and Bayesian phylogenetic inference to characterize the relationship between the FBEs and the vacuolar invertases, especially in the Poaceae. The numerical results are reported in Fig. 2. The data suggest that an ancient *VIN* gene duplication within the monocots separated the common ancestor of OsVIN1, ZmIVR1 and TaVIN1 from the common ancestor of all other monocot vacuolar invertases and FBEs. A later duplication appears to have separated TaVIN2 and HvVIN from LtVIN, TmVIN, TaVIN3 and the FBEs of the Pooideae. A third duplication then appears to have separated LtVIN, TmVIN and TaVIN3 from the FBEs, evolution of which to 1-SST, 1-FFT and 6-SFT required additional gene duplications during the radiation of the Pooideae. Thus the data suggest that the FBEs of the Pooideae evolved from a particular vacuolar invertase (marked with a diamond in Fig. 2) that was also an ancestor of TaVIN3, LtVIN and TmVIN, but was not an ancestor of OsVIN2 or ZmIVR2. The same conclusion was reached by analysis of cDNA sequences; the crucial branching at the diamond was supported by values of 100 and 93 for neighbour-joining bootstrap and parsimony bootstrap methods, respectively. Diamonds also mark the ancestral vacuolar invertases from which the FBEs of the Asteraceae and Asparagaceae appear to have arisen.

Exon–intron structure of genes

Additional support for the phylogeny of FBEs in the Poaceae comes from a provisional analysis of the exon–intron structure of some of the corresponding genes (Fig. 3). Genes encoding OsVIN1 and ZmIVR1 possess six introns; genes encoding OsVIN2, ZmVIN2 and TaVIN2 contain two introns; and those encoding Ta-1-SST and Ta-6-SFT contain three introns. As discussed by Ji *et al.* (2005a), the six introns of *OsVIN1* were reduced to two in *OsVIN2*, probably in two steps: (1) concerted precise loss of introns 4–6 through RNA-mediated homologous replacement, and (2) precise loss of

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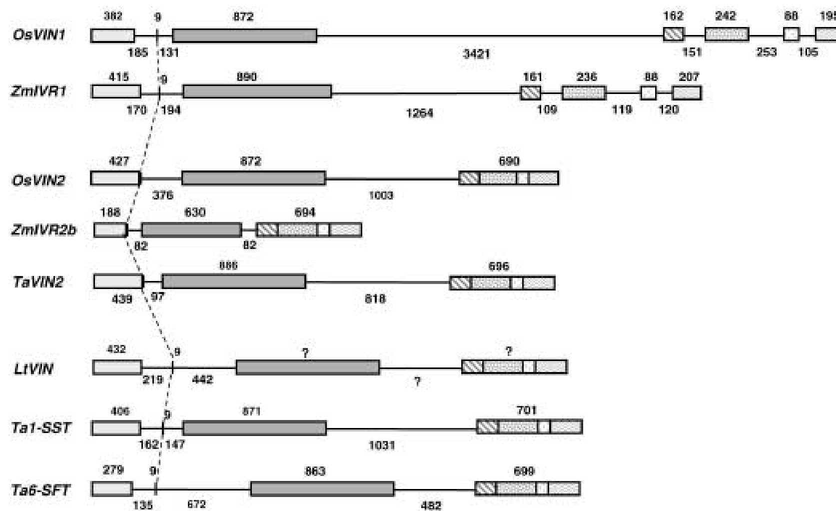

Fig. 3 Exon–intron structure of genes encoding selected vacuolar invertases and fructan biosynthesis enzymes (FBEs). Boxes, exons; lines, introns. The lengths of exons and introns are shown above and below the gene, respectively. Question marks denote incomplete sequence data. The vertical line represents the nine-base mini-exon encoding the conserved DPN of the active site (see Fig. 6 for the amino acid sequences around DPN in selected vacuolar invertases and FBEs). The exon–intron structures are based on the following sequences: OsVIN1 (AF276703), OsVIN2 (AF276704), ZmIVR1 (U16123), ZmIVR2b (AJ563423), TaVIN2 (L.S. and W.v.d.E., unpublished), LtVIN (AJ532552), Ta-1-SST (AB159786), Ta-1-SFT (L.S. and W.v.d.E., unpublished). Because the complete sequence of ZmIVR2 is not available in the database, the missing segment was introduced from ZmIVR2b, a closely related pseudogene, to complete the provisional exon–intron structure.

intron 1 through a similar mechanism, leaving only introns 2 and 3. *Ta-1-SST* and *Ta-6-SFT* possess introns 1–3 and appear to have arisen from a six-intron precursor by step (1) without step (2). A crucial finding is that a sequenced fragment of *LtVIN* contains introns 1 and 2, indicating that the FBE genes are related at the exon–intron level to *LtVIN* and presumably to *TaVIN3* and *TmVIN*. These results should be considered provisional, at least until the genomic sequence of *TaVIN3* is available. See Discussion for further comments.

In summary, the FBEs evolved from vacuolar invertases at least three times in flowering plants – within the Asteraceae, the Asparagaceae and the Poaceae. Among those members of the Poaceae for which there is information, only the Pooideae contain FBEs; these enzymes appear to have arisen by a series of gene duplications that occurred only in the Pooideae. The tBLASTn results (Table 1) can now be understood in terms of *OsVIN2* being the rice gene most closely related to the lineage that, within the Pooideae, leads to the FBEs.

Secretion of recombinant OsVIN1 and OsVIN2 proteins from *Pichia pastoris*

The fact that rice lacks FBE genes goes a long way towards explaining the absence of fructans in this crop. However, we checked whether OsVIN1 and OsVIN2 themselves possessed any FBE activity, including the capacity to synthesize the simple fructan 1-kestose. We adopted the well validated approach of cloning the cDNAs for OsVIN1 and OsVIN2 into the yeast *P. pastoris* (Cereghino & Cregg, 2000) and using its methanol-induction, glycosylation and secretion systems to prepare large quantities of recombinant OsVIN1 and OsVIN2 for enzymatic analysis.

The expression vector included the secretion signal peptide from yeast α-factor, and this peptide replaced the putative vacuolar-targeting N-terminal region of OsVIN1 and OsVIN2 discussed by Ji *et al.* (2005a). Exposure of transformed *P. pastoris* cultures to methanol induced transcription, translation, signal peptide removal, glycosylation and secretion of OsVIN1 and OsVIN2. After 5 d, yeast cells were removed by centrifugation and the secreted proteins concentrated and purified by ConA–Sepharose affinity column chromatography, and analysed by SDS-PAGE. Staining with Coomassie Brilliant Blue revealed for each recombinant protein a prominent band of the expected size (75 kDa) (Fig. 4). The identities of these proteins were confirmed by cutting the bands from the gel, digesting the proteins with trypsin and analysing them by Q-TOF mass spectrometry. There was excellent agreement between the observed tryptic fingerprint of the excised proteins and that predicted from the sequences of OsVIN1 (Table S1 in Supplementary Material), OsVIN2 (Table S2) and TaVIN2 (not shown). We used dilutions of the purified recombinant proteins for assays of fructose release from substrates during invertase action (glucosyl-fructosidase) and during fructan exohydrolase action (fructosyl-fructosidase), and for assays of 1-kestose synthesis.

Invertase and 1-FEH activities

Recombinant OsVIN1, OsVIN2 and TaVIN2 were compared with respect to their capacity to hydrolyse sucrose (Fig. 5a–f).

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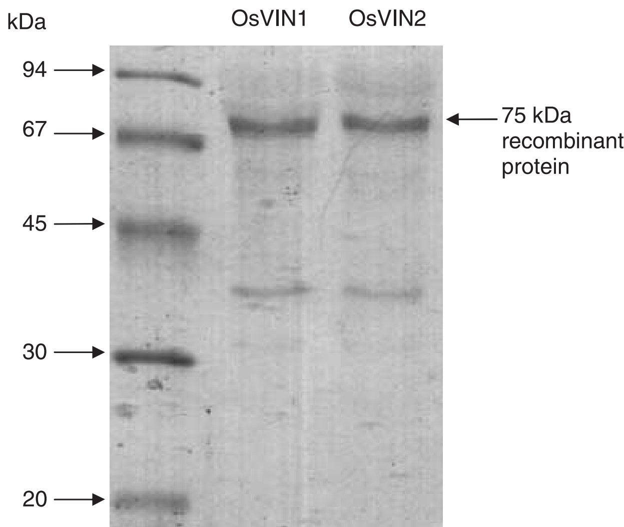

Fig. 4 Detection of recombinant rice vacuolar invertases OsVIN1 and OsVIN2 secreted from transformed cells of yeast *Pichia pastoris*. Secreted proteins were concentrated by affinity column chromatography on Concanavalin A–Sepharose, analysed by SDS-PAGE and stained with Coomassie Brilliant Blue. kDa, molecular weights of markers; arrow on right, recombinant OsVIN1 and OsVIN2.

Table 2 Activities† of recombinant OsVIN1, OsVIN2 and TaVIN2 against sucrose (invertase), 1-kestose (fructan 1-exohydrolase) and raffinose (glucosyl-fructosidase) as functions of substrate concentration

		Substrate concentration (mM)			
Enzyme	Substrate	2*	10*	50*	250**
OsVIN1	Sucrose	100	100	100	100
	1-Kestose	36.8	34.1	25.5	31.7
	Raffinose	19.5	18.8	14.8	22.8
OsVIN2	Sucrose	100	100	100	100
	1-Kestose	11.0	9.5	10.5	14.6
	Raffinose	19.8	17.7	15.8	22.5
TaVIN2	Sucrose	100	100	100	100
	1-Kestose	1.8	2.2	6.0	4.1
	Raffinose	6.1	6.6	8.2	12.0

†Activities expressed as percentage of the rate of fructose released with sucrose as substrate. Absolute rates of recombinant enzymes with sucrose as substrate are shown in Fig. 3(a–c).
Data are mean of duplicate assays (*, error < 10%; **, error < 20%).

For sucrose in the range 2–125 mM, the three enzymes adhered to the classical Michaelis–Menten model. TaVIN2 showed the highest V_{max} (2910 nkat mg^{-1} protein), followed by OsVIN1 (510 nkat mg^{-1}) and OsVIN2 (20.5 nkat mg^{-1}). The K_m for sucrose of the three enzymes was 22.6, 13.2 and 7.4 mM, respectively.

The ability of the recombinant enzymes to hydrolyse raffinose and 1-kestose was also evaluated (Table 2). For substrates in the range 2–250 mM, the three enzymes preferred sucrose (glucosyl-fructosidase activity) over raffinose (also glucosyl-fructosidase activity) by five- to 10-fold. The preference for hydrolysing sucrose rather than 1-kestose (fructosyl-fructosidase activity) was much more variable, ranging from *c.* threefold for OsVIN1, to *c.* 10-fold for OsVIN2, to > 20-fold for TaVIN2. The high intrinsic fructan 1-exohydrolase (1-FEH) activity of OsVIN1 would tend to mask any capacity for 1-kestose synthesis *in vitro* and *in vivo*. By contrast, the low 1-FEH activity of TaVIN2 might be an important adaptation to coexistence between invertase activity and fructan accumulation in wheat.

1-Kestose synthesis

Recombinant OsVIN1, OsVIN2 and TaVIN2 were tested for their ability to synthesize 1-kestose in the range 60–1000 mM sucrose (Fig. 5g–i). It was not possible to calculate accurate K_m(sucrose) for these activities, but they were certainly > 1 M and hence higher than the likely physiological levels of

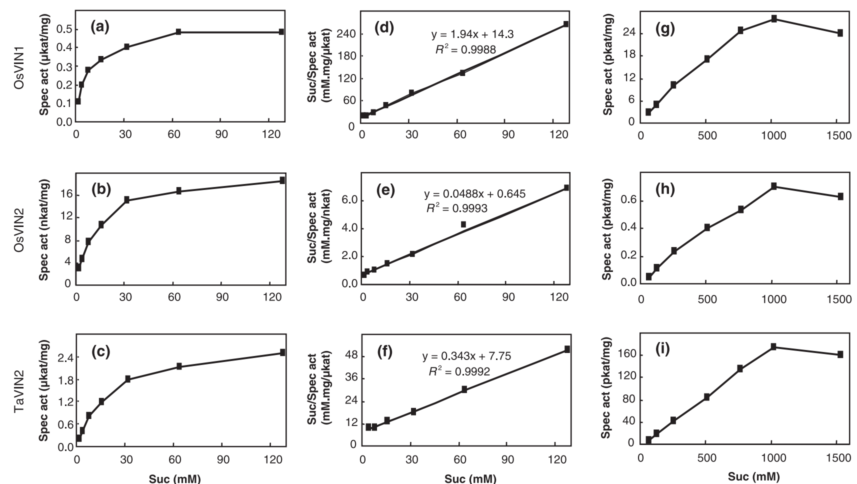

Fig. 5 Enzymatic characterization of recombinant OsVIN1, OsVIN2 and TaVIN2. (a–c) Plots of specific invertase activity vs [sucrose]; (d–f) plots of [sucrose]/specific invertase activity vs [sucrose]; (g–i) plots of specific 1-SST activity vs [sucrose].

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OsVIN1 MMDGAAPLLPETSPESRQQRDPERGKRRTPVLPAVVASAVVLLGLAALFLVYGF--30--SEKST--34--NWMNDPNG--26--GHAV
Ivr1 MIPAVADPTTLDGGGARRPLLPETDPRGRAAAGAEQKRPPATPTVLTAVVSAVLLLVLVAVTVLASQ--30--SEKST--32--NWMNDPNG--26--GHAV
TgVIN1 MGGRDLESSTPLLHHEPYSPRKTITTIVSSIVAAALLLSLITLLNT--23--SEKST--30--NWMNDPDG--26--GHAV
OsVIN2 METRDDVADASALPYSYSPLPAGDAASADLAAARRSRRRPLCVALFLASAAVILAVAVLSGV--30--SEKTS--38—NWMNDPNG--27--GHAV
TmVIN MESRAISPGETALPYAYAPLAPSDVAEERRGGGGGVRWRACVAVLAASAVVVLVVASALAGSG--30--SEKTS--39--NWMNDPNG--26--GHAV
HvVIN MPTMDTTDRGSYAQLPDDAEAGSAHRRRTGPLCAAILLTSAALLLAVAALAGV--37--SEKTS--37--NWMNDPNG--27--GHAA
Hv1-SST MARRSSGPAWPEVARVRRRACRLGRAGARRRRRGLRAGVAGRDAVAGSVPSIPAT--12--SEKTS--32--NDPNG--27--GHAV
Ta1-SST MDSSRVILIPGTPPLPYAYEQLPSSSADAKGIEEERAGGGGLRWRACAAVLAASAVVALVVAAAVFGASGAGW--25--SEKTS--31-NDPNG--27--GHAV
Ta1-FFT MESSRGILIPGTPPLPYAYEPLPSSSADANGQEDRRITGGVRWRAWAAVLAVGALVVAAAVFGAS--19--LEKAS--32--NDPNG--26--GHAV
Lp1-SST MESSAVVVQGTTAPLLPYAYAPLPSSADDARENQSSGGGVRWRACAASALVVLLVVVGFFAGG--27-SEKES--28--MNDPNG--26--GHAV
Ta6-SFT MGSHGKPPLPYAYKPLPSDADGERAGCTRWRVCAVALTASAMVVVVVGATLLAGF--38--DPNG--27--GHAV
Ps6-SFT MDSRGITPGAYAPLPSSGDDQRGGGVRYCLACVATVLAASAVVVVVAFVSVGGV--41--DPNG--27--GHVV
Lp6-SFT MESRAIPSAAYAPLLPSAADDVALAKQDRPSVGWRGFLTVLAASGVVVLLVGATLLAGS--44-DPNG--27--GHVV
AoVIN MASSRDVESPPTSYAPLPSDDEQRPGSAPPRSRLRLIAIAMPPILLLALAAALFLSGSGAVT--25--SSKSS--35--NWMNDPNG--26--GHAV
AcVIN MSSDDLESPPSSYLPIPPSDEFHDQPPPLRSWLRLLSIPLALMFLLFLATFLSNL--31--SDKTS--35--NWMNDPNG--26--GHAV
Ac1-SST MESRDIESSPALNAPLLQASPPIKSSKLKVALLATSTSVLLLIAAFFAV--47--DPN--28-GHAV
Ac6G-FFT MDAQDIESRHPLIGARPRRRALRSLSILLAAALLLGLVLFYANGTGSGTAV--31--MNDP--28--GHAV
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Fig. 6 Conserved motifs near the N-terminus of vacuolar invertases and fructan fructosyltransferases of monocots. The subterminal transmembrane segment predicted by the PSORT program (http://psort.ims.u-tokyo.ac.jp) is underlined. The PSORT prediction was negative only in the case of 1-SST of *Hordeum vulgare*, where a charged RD dipeptide interrupted the hydrophobicity of the segment. Shaded boxes highlight the basic region upstream from the transmembrane segment and the first two partially conserved motifs downstream from this segment.

sucrose. Moreover, for each enzyme V_{max} as 1-SST was very low relative to V_{max} as invertase (Fig. 5a–f). Even so, the V_{max} for 1-kestose synthesis was *c.* sixfold higher for TaVIN2 than for OsVIN1, a possible consequence of the difference in 1-FEH activity observed above for these two enzymes.

Vacuolar targeting mechanism

Ji *et al.* (2005a) pointed out that the vacuolar invertases of rice and *Arabidopsis* possess a distinct N-terminal motif closely resembling the N-terminal motif that targets alkaline phosphatase to the vacuolar membrane in yeast (Piper *et al.*, 1997; Vowels & Payne, 1998). The main feature of the motif is a sub-N-terminal hydrophobic domain that is generally 20–29 residues in length, except in barley 1-SST, where it is only 19 residues long and interrupted by a pair of oppositely charged residues. The hydrophobic domain is predicted by the PSORT program (http://psort.ims.u-tokyo.ac.jp) to orient the protein such that the short N-terminus faces the cytosol and the long C-terminus faces the vacuolar lumen. There is also a rather variable basic region immediately upstream from the hydrophobic domain. The conservation of these features of the N-terminal motif between vacuolar invertases and FBEs suggests that these two groups of enzymes are targeted to the similar vacuolar compartments, unless subtle differences in the sub-N-terminal motif or elsewhere in the polypeptide chain of VINs and FBEs provide for spatial separation within the complex vacuolar domain.

In approx. 60% of the proteins there is another consensus sequence, SEKTS, located downstream from the transmembrane domain. The functions of the basic and SEKTS motifs are unknown. Also highlighted in Fig. 6 is a conserved motif, NWMNDPNG, known as the sucrose-binding box (Ritsema *et al.*, 2005). It is characteristic of vacuolar invertases but is not well conserved in FBEs, including the FBEs of the Pooideae and the Asparagaceae (Fig. 2). The only component of the sucrose-binding box that is conserved throughout the vacuolar invertase/FBE family is the DPN encoded by the mini-exon (see Discussion; Fig. 3 legend).

Drought-responsiveness of *OsVIN1* and *OsVIN2* expression

An alternative mechanism for preventing futile cycling by VINs and FBEs could be differential expression of the corresponding genes. We examined the expression of *OsVIN1* and *OsVIN2* by RT-PCR to see whether their expression patterns might create the chance of avoiding futile cycling in transgenic plants. We also examined the drought-responsiveness of these genes, as fructan synthesis shows a complex relationship to water status in plants (see Discussion). Figure 7 shows the expression of *OsVIN1* and *OsVIN2* in flag leaves, panicles, anthers and peduncles of well watered and drought-stressed plants of IR64. Drought stress was initiated by withholding water from pot-grown plants at 3 d before heading; samples were taken 2 d later. The relative water content of the flag leaves declined from ~93% to ~80% over the 2 d of stress. Transcript levels for *OsVIN1* and *OsVIN2* were examined by 35 cycles of RT-PCR. In well watered and drought-stressed plants, *OsVIN1* was highly expressed in flag leaves, panicles and anthers, but *OsVIN1* transcripts were reproducibly undetectable in peduncles. By contrast, in all four tissues *OsVIN2* transcripts were readily detectable in well watered plants and increased markedly in response to drought stress. These results suggest that, in transgenic rice plants expressing wheat FBE genes, the vacuoles in the peduncle will experience little or no futile cycling attributable to the invertase and 1-FEH activities of OsVIN1.

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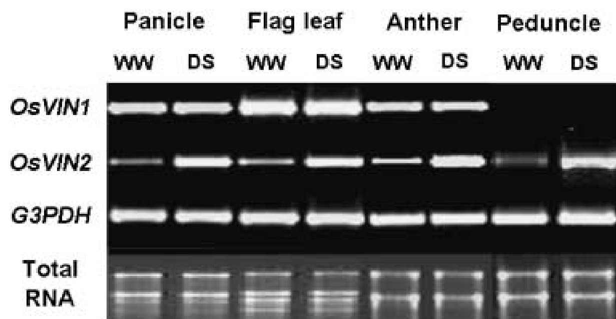

Fig. 7 Detection of transcripts of *OsVIN1* and *OsVIN2* in four tissues of well watered and drought-stressed plants of rice (*Oryza sativa* L. cv. IR64) using RT-PCR. Water was withheld from pot-grown plants for 2 d, starting 3 d before heading. At 1 d before heading, RNA was extracted from the flag leaves, panicles, anthers and peduncle of well watered and drought-stressed plants. Transcripts were detected by 35 cycles of RT-PCR. Transcripts of cytosolic glyceraldehyde-3-phosphate dehydrogenase were included to control for absence of genomic DNA. Ribosomal RNA content was used to normalize the reactions. Data represent two technical replicates of two biological replicates.

Discussion

Rice lacks FBE genes

Chatterton *et al.* (1989) showed that fructan accumulation in the Gramineae (Poaceae) is associated with temperate rather than tropical species. They included members of the Pooideae in their analysis and confirmed that they were fructan accumulators, but rice was not included. Our chromatographic analysis of soluble carbohydrates extracted from the peduncle of cv. IR64 supports the notion that rice is a nonaccumulator.

An explanation for the nonaccumulator status of rice was provided by tBLASTn analysis. The genome of the temperate japonica rice cultivar Nipponbare lacks orthologues of the FBE genes of the Pooideae and contains only two homologues in the form of vacuolar invertases, *OsVIN1* and *OsVIN2*. Identical hits and scores were obtained when tBLASTn was conducted on the genome of the tropical indica rice cultivar 9311. Furthermore, expression of recombinant *OsVIN1* and *OsVIN2* in the yeast *P. pastoris* yielded proteins that were authentic invertases with no FBE activity of their own. We conclude that, because it lacks FBE genes, rice cannot be a fructan accumulator.

A caveat to this conclusion arises from the existence of physical and sequence gaps in the rice genome sequence, amounting to *c.* 18.1 Mb out of the total of 389 Mb (International Rice Genome Sequencing Project, 2005). However, 78% of the missing genome is in heterochromatin rather than euchromatin; it is estimated that 98.8% of the genes have been sequenced. The fact that FBE orthologues were not found in either the Nipponbare genome or the 9311 genome strengthens our conclusion.

Broader phylogenetic analysis suggests that the FBEs of the Asteraceae, Asparagaceae and Poaceae originated independently from specific vacuolar invertases, in agreement with Vijn & Smeekens (1999). Thus the evolution of FBEs from vacuolar invertases occurred at least three times in flowering plants. The data suggest that the vacuolar invertase from which the FBEs of the Poaceae evolved was an ancestor of TaVIN3, rather than TaVIN1 or TaVIN2, which are orthologues of OsVIN1 and OsVIN2, respectively. There is no rice orthologue of TaVIN3. The ancestor of TaVIN3 appears to have been produced by duplication of the ancestral gene of TaVIN2. This duplication, along with subsequent gene duplications generating the FBEs (1-SST, 1-FFT and 6-SFT), appears to have been limited to the Pooideae.

A comparison of cDNA and genomic sequence data for the Poaceae supports the above conclusion at the level of exon–intron structure. OsVIN1 and ZmIVR2 contain six introns, the most common number for vacuolar and cell-wall invertases (Ji *et al.*, 2005a). OsVIN2, ZmIVR2 and TaVIN2 contain two introns (2 and 3); while Ta-1-SST and Ta-6-SFT contain three introns (1–3). A fragment of LtVIN, which is closely related to TaVIN3, also contains introns 1 and 2; complete sequencing of LtVIN is needed to confirm that it contains three introns.

It is likely that the last common ancestor of the 2- and 3-intron genes contained three introns, and that intron 1 was lost in the lineages to OsVIN2, ZmIVR2 and TaVIN2. Intron 1 has also been lost in other species, possibly because of the mini-exon that lies between introns 1 and 2. The mini-exon codes for three amino acids (DPN) that form an essential component of the invertase active site. Being only 9 bp long, the mini-exon is sometimes overlooked *in silico* by algorithms and *in vivo*, including under cold stress in potato (Bournay *et al.*, 1996; Simpson *et al.*, 2000). It does not appear essential for a plant to encode DPN in the form of a mini-exon (exon 2): *OsVIN2* is an example where intron 1 is lost and the mini-exon becomes the last nine bases of the preceding exon; 1-SST of *Lolium perenne* (Chalmers *et al.*, 2003) is an example where intron 2 is lost and the mini-exon becomes the first nine bases of the following exon.

In addition to the Asteraceae, Asparagaceae and Poaceae, the Liliaceae also accumulate fructans, but more sequence information is needed about FBEs and vacuolar invertases of members of the Liliales, such as tulip (*Tulipa gesneriana*), before we can conclude that FBEs also evolved independently in this order. The fact that tulip produces the inulin neoseries (Shiomi, 1989) may indicate that the FBEs of the Asparagaceae and the Liliaceae share a common origin.

In summary, the absence of FBE genes from rice is a sufficient reason for the nonaccumulator status of rice. The FBE genes appear to have evolved within the early Pooideae, after this subfamily of the Poaceae separated from lineages leading to rice and maize.

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Invertase, 1-FEH activity and potential for futile cycling

The evolution of FBEs from vacuolar invertases involved the loss of glucosylfructosidase and fructosylfructosidase activities and the gain of fructosyltransferase activity (Obenland *et al.*, 1993; Simmen *et al.*, 1993; Kawakami & Yoshida, 2002). It is clear that OsVIN1, OsVIN2 and TaVIN2 have not made this evolutionary advance. They display very low ratios of FBE activity to invertase activity (< 1 : 10 000). Their V_{max} as FBEs (specifically 1-SST) is in the range 0.7–170 pkat mg^{-1} protein, whereas their V_{max} as invertases is in the range 20.5–2910 nkat mg^{-1} protein. Similarly, their K_m for sucrose as 1-SST is > 1 M, whereas their K_m for sucrose as invertases is in the range 7.4–22.6 mM. The fact that the recombinant enzymes display 1-FEH activity (hydrolysis of 1-kestose) is undoubtedly an important factor in the virtual absence of 1-SST activity. It is intriguing to note that the replacement of water as second substrate by sucrose (Vijn *et al.*, 1998) is a key event in the evolution of FBEs, because at high sucrose concentrations the two hydrolase activities are replaced by fructosyltransferase activity. Vacuolar invertases possess the ability to hydrolyse both the substrate (sucrose) and the product (fructan) of FBEs. Colocalization of these two types of enzyme in the vacuole would risk futile cycling.

The ability of vacuolar invertases to show 1-FEH activity is paralleled by the evolution of 1-FEHs from cell-wall invertases (Van den Ende *et al.*, 2000). 1-FEHs of the latter class lack invertase activity and are believed to be located in the vacuole rather than, or in addition to, the cell wall (Wagner & Wiemken, 1986; Van den Ende *et al.*, 2000). Their targeting to vacuoles is presumably different from the targeting of vacuolar invertases because they possess a hydrophobic N-terminal signal peptide rather than the unusual N-terminal targeting motif shown in Fig. 6 (Ji *et al.*, 2005a). The functional roles, if any, of the 1-FEH activity of vacuolar invertases may be different from those of the 1-FEHs themselves, which include modulation of the degree of polymerization of fructans (Van den Ende *et al.*, 2003b).

Cairns (2003) reviewed attempts to enhance fructan accumulation in plants through transformation with bacterial or plant FBE genes. He noted that, in most cases, the percentage of photosynthetic flux diverted into fructans in such transgenic plants was < 0.08%. He put forward six possible explanations for this low percentage, including *in situ* product hydrolysis. It is clear that *in situ* fructan hydrolysis could be caused by 1-FEH or 6-FEH activity, depending on whether the FBEs introduce 2,1- or 2,6-linkages. Before transforming rice with heterologous FBE genes, we should characterize the target tissues for 1-FEH and 6-FEH activity. It is encouraging that the rice peduncle has no detectable transcripts of the *OsVIN1* gene, a major source of 1-FEH activity. It will be important to find out how fructan accumulators such as wheat minimize or completely avoid futile cycle involving vacuolar invertases.

Drought stress

Our RT-PCR data show clearly that *OsVIN2* is upregulated by drought stress in four tissues (flag leaf, panicle, anthers, peduncle). Similar results have been reported for *Ivr2* and *Ivr1* of maize (Pelleschi *et al.*, 1999; Kim *et al.*, 2000; Trouverie *et al.*, 2003). This response may allow rice to increase the osmotic potential of the vacuoles to maintain turgor under water deficit (Pelleschi *et al.*, 1999; Kim *et al.*, 2000), as all four tissues experience a decline in water status on drought stress (results not shown). Alternatively, increased *OsVIN2* expression may help mobilize stored sucrose for use in metabolic processes (Koch, 2004; Roitsch & Gonzalez, 2004).

Yang *et al.* (2004) found that, in wheat stems, levels of 1-SST were reduced under drought stress, whereas levels of 1-FEH increased and allowed depolymerization of fructans. These results run contrary to the drought-induced increase in transcript levels for *OsVIN2* (this paper) and the drought-induced decrease in transcript levels for all the rice cell-wall invertase genes (Ji *et al.*, 2005b). Stress responsiveness may have been reversed as the vacuolar invertases evolved into FBEs and the cell-wall invertases evolved into 1-FEHs. However, there are also reports of fructan accumulation in monocots and dicots under drought or cold stress (De Roover *et al.*, 2000; Kawakami & Yoshida, 2002; Amiard *et al.*, 2003; Kerepesi *et al.*, 2004). Adjustments in the degree of polymerization of fructans may be as important as the changes in abundance (Van den Ende *et al.*, 1998). Hence the balance of fructan synthesis and breakdown may be more important than complete separation of the two processes.

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References

Altschul SF, Madden TL, Schäffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.

Amiard V, Morvan-Bertrand A, Billard JP, Huault C, Keller F, Prud'homme MP. 2003. Fructans, but not the sucrosyl-galactosides, raffinose and loliose, are affected by drought stress in perennial ryegrass. *Plant Physiology* **132**: 2218–2229.

Bonnett GD, Sims IM, Simpson RJ, Cairns AJ. 1997. Structural diversity of fructan in relation to the taxonomy of the Poaceae. *New Phytologist* **136**: 11–17.

Bournay AS, Hedley PE, Maddison A, Waugh R, Machray GC. 1996. Exon skipping induced by cold stress in a potato invertase gene transcript. *Nucleic Acids Research* **24**: 2347–2351.

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Bowers JE, Chapman BA, Rong J, Paterson AH. 2003. Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events. *Nature* **422**: 433–438.

Cairns AJ. 2003. Fructan biosynthesis in transgenic plants. *Journal of Experimental Botany* **54**: 549–567.

Cereghino JL, Cregg JM. 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris. FEMS Microbiological Reviews* **24**: 45–66.

Chalmers J, Johnson X, Lidgett A, Spangenberg G. 2003. Isolation and characterisation of a sucrose:sucrose 1-fructosyltransferase gene from perennial ryegrass (*Lolium perenne*). *Journal of Plant Physiology* **160**: 1385–1391.

Chatterton N, Harrison JPA, Bennett JH, Asay KH. 1989. Carbohydrate partitioning in 185 accessions of Gramineae grown under warm and cool temperatures. *Journal of Plant Physiology* **134**: 169–179.

De Roover J, Van den Branden K, Van Laere A, Van den Ende W. 2000. Drought induces fructan synthesis and 1-SST (sucrose:sucrose 1-fructosyl transferase) in roots and leaves of chicory seedlings (*Cichorium intybus* L.). *Planta* **210**: 808–814.

Gebbing T. 2003. The enclosed and exposed part of the peduncle of wheat (*Triticum aestivum*) – spatial separation of fructan storage. *New Phytologist* **159**: 245–252.

Hendry G. 1993. Evolutionary origins and natural functions of fructans. A climatological, biogeographic and mechanistic appraisal. *New Phytologist* **123**: 3–14.

Hendry GAF, Wallace RK. 1993. The origin, distribution, and evolutionary significance of fructans. In: Suzuki M, Chatterton NJ, eds. *Science and technology of fructans.* Boca Raton, FL, USA: CRC Press, 119–139.

Hincha DK, Zuther E, Hellwege EM, Heyer AG. 2002. Specific effects of fructo- and gluco-oligosaccharides in the preservation of liposomes during drying. *Glycobiology* **12**: 103–110.

Iizuka M, Yamaguchi H, Ono S, Minamiura N. 1993. Production and isolation of levan by use of levansucrase immobilized on the ceramic support SM-10. *Bioscience, Biotechnology and Biochemistry* **57**: 322–324.

International Rice Genome Sequencing Project. 2005. The map-based sequence of the rice genome. *Nature* **436**: 793–800.

Ji XM, Van den Ende W, Van Laere A, Cheng SH, Bennett J. 2005a. Structure, evolution and expression of the two invertase gene families of rice. *Journal of Molecular Evolution* **60**: 615–634.

Ji XM, Raveendran M, Oane R, Ismail A, Lafitte R, Bruskiewich R, Cheng SH, Bennett J. 2005b. Tissue-specific expression and drought responsiveness of cell-wall invertase genes in flag leaves, panicles, anthers and peduncles of rice. *Plant Molecular Biology* **59**: 945–964.

Kawakami A, Yoshida M. 2002. Molecular characterization of 6-SFT and 1-SST associated with fructan accumulation in winter wheat during cold hardening. *Bioscience, Biotechnology and Biochemistry* **66**: 2297–2305.

Kawakami A, Yoshida M. 2005. Fructan:fructan 1-fructosyltransferase, a key enzyme for biosynthesis of graminan oligomers in hardened wheat. *Planta* **21**: 1–15.

Kerepesi I, Banyai-Stefanovits E, Galiba G. 2004. Cold acclimation and abscisic acid induced alterations in carbohydrate content in calli of wheat genotypes differing in frost tolerance. *Journal of Plant Physiology* **161**: 131–133.

Kim JY, Mahé A, Brangeon J, Prioul JL. 2000. Maize vacuolar invertase, IVR2, is induced by water stress. Organ/tissue specificity and diurnal modulation of expression. *Plant Physiology* **124**: 71–84.

Koch K. 2004. Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Current Opinion in Plant Biology* **7**: 235–246.

Konstantinova T, Parvanova D, Atanassov A, Djilianov D. 2002. Freezing tolerant tobacco, transformed to accumulate osmoprotectants. *Plant Science* **163**: 157–164.

Morcuende R, Kostadinova S, Perez P, Martinez-Carrasco R. 2005. Fructan synthesis is inhibited by phosphate in warm-grown, but not in cold-treated, excised barley leaves. *New Phytologist* **168**: 567–574.

Obenland DM, Simmen U, Boller T, Wiemken A. 1993. Purification and characterization of three soluble invertases from barley (*Hordeum vulgare* L.) leaves. *Plant Physiology* **101**: 1331–1339.

Pelleschi S, Guy S, Kim JY, Pointe C, Mahe A, Barthes L, Leonardi A, Prioul JL. 1999. *Ivr2*, a candidate gene for a QTL of vacuolar invertase activity in maize leaves. Gene-specific expression under water stress. *Plant Molecular Biology* **39**: 373–380.

Pilon-Smits EAH, Ebskamp MJM, Paul MJ, Jeuken MJW, Weisbeek PJ, Smeekens SCM. 1995. Improved performance of transgenic fructan-accumulating tobacco under drought stress. *Plant Physiology* **107**: 125–130.

Piper RC, Bryant NJ, Stevens TH. 1997. The membrane protein alkaline phosphatase is delivered to the vacuole by a route that is distinct from the VPS-dependent pathway. *Journal of Cell Biology* **138**: 531–545.

Pollock CJ, Cairns AJ. 1991. Fructan metabolism in grasses and cereals. *Annual Reviews of Plant Physiology and Plant Molecular Biology* **42**: 77–101.

Pollock C, Farrar J, Tomos D, Gallagher J, Lu C, Koroleva O. 2003. Balancing supply and demand: the spatial regulation of carbon metabolism in grass and cereal leaves. *Journal of Experimental Botany* **54**: 489–494.

Puebla AF, Salerno GL, Pontis HG. 1997. Fructan metabolism in two species of *Bromus* subjected to chilling and water stress. *New Phytologist* **136**: 123–129.

Ritsema T, Joling J, Smeekens S. 2003. Fructan patterns synthesized by onion fructan:fructan 6G-fructosyltransferase (6G-FFT) expressed in tobacco BY2 cells – is fructan:fructan 1-fructosyltransferase (1-FFT) needed in onion? *New Phytologist* **160**: 61–67.

Ritsema T, Verhaar A, Vijn I, Smeekens S. 2005. Using natural variation to investigate the function of individual amino acids in the sucrose-binding box of fructan:fructan 6^G-fructosyltransferase (6G-FFT) in product formation. *Plant Molecular Biology* **58**: 597–607.

Roitsch T, Gonzalez MC. 2004. Function and regulation of plant invertases: sweet sensations. *Trends in Plant Science* **9**: 606–613.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Sedmak JJ, Grossberg SE. 1977. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Analytical Biochemistry* **79**: 544–552.

Shiomi N. 1989. Properties of fructosyltransferases involved in the synthesis of fructan in liliaceous plants. *Journal of Plant Physiology* **134**: 151–155.

Simmen U, Obenland D, Boller T, Wiemken A. 1993. Fructan synthesis in excised barley leaves (identification of two sucrose-sucrose fructosyltransferases induced by light and their separation from constitutive invertases). *Plant Physiology* **101**: 459–468.

Simpson CG, Hedley PE, Watters JA, Clark GP, McQuade C, Machray GC, Brown JW. 2000. Requirements for mini-exon inclusion in potato invertase mRNAs provides evidence for exon-scanning interactions in plants. *RNA* **6**: 422–433.

Sprenger N, Bortlik K, Brandt A, Boller T, Wiemken A. 1995. Purification, cloning, and functional expression of sucrose:fructan 6-fructosyltransferase, a key enzyme of fructan synthesis in barley. *Proceedings of the National Academy of Sciences, USA* **92**: 11652–11656.

Sturm A. 1999. Invertase: primary structures, functions, and roles in plant development and sucrose partitioning. *Plant Physiology* **121**: 1–7.

Swofford DL. 2002. *Paup*. Phylogenetic analysis using parsimony (*and other methods).* Sunderland, MA, USA: Sinauer Associates.

Takahashi N, Yamazaki Y, Kobayashi A, Higashitani A, Takahashi H. 2003. Hydrotropism interacts with gravitropism by degrading amyloplasts in seedling roots of Arabidopsis and radish. *Plant Physiology* **132**: 805–810.

Tetlow IJ, Morell MK, Emes MJ. 2004. Recent developments in understanding the regulation of starch metabolism in higher plants. *Journal of Experimental Botany* **55**: 2131–2145.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence

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alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876–4882.

Trouverie J, Thevenot C, Rocher JP, Sotta B, Prioul JL. 2003. The role of abscisic acid in the response of a specific vacuolar invertase to water stress in the adult maize leaf. *Journal of Experimental Botany* **54**: 2177–2186.

Ueno K, Onodera S, Kawakami A, Yoshida M, Shiomi N. 2005. Molecular characterization and expression of a cDNA encoding fructan:fructan 6G-fructosyltransferase from asparagus (*Asparagus officinalis*). *New Phytologist* **165**: 813–824.

Van den Ende W, Van Laere A. 1996. Variation in the *in vitro* generated fructan pattern from sucrose as a function of the purified chicory root 1-SST and 1-FFT concentrations. *Journal of Experimental Botany* **47**: 1797–1803.

Van den Ende W, Van Hoenacker G, Moors S, Van Laere A. 1998. Effect of osmolytes on the fructan pattern in feeder roots produced during forcing of chicory (*Cichorium intybus* L.). *Journal of Plant Physiology* **153**: 290–298.

Van den Ende W, De Roover J, Van Laere A. 1999. Effect of nitrogen concentration on fructan and fructan metabolizing enzymes in young chicory plants (*Cichorium intybus*). *Physiologia Plantarum* **105**: 2–8.

Van den Ende W, Michiels A, De Roover J, Verhaert P, Van Laere A. 2000. Cloning and functional analysis of chicory root fructan 1-exohydrolase I (1-FEH I): a vacuolar enzyme derived from a cell-wall invertase ancestor? Mass fingerprint of the 1-FEH I enzyme. *Plant Journal* **24**: 447–456.

Van den Ende W, Michiels A, Van Wonterghem D, Clerens SP, De Roover J, Van Laere AJ. 2001. Defoliation induces fructan 1-exohydrolase II in Witloof chicory roots. Cloning and purification of two isoforms, fructan 1-exohydrolase IIa and fructan 1-exohydrolase IIb. Mass fingerprint of the fructan 1-exohydrolase II enzymes. *Plant Physiology* **126**: 1186–1195.

Van den Ende W, De Coninck B, Clerens S, Vergauwen R, Van Laere A. 2003a. Unexpected presence of fructan 6-exohydrolases (6-FEHs) in non-fructan plants: characterization, cloning, mass mapping and functional analysis of a novel 'cell-wall invertase-like' specific 6-FEH from sugar beet (*Beta vulgaris* L.). *Plant Journal* **36**: 697–710.

Van den Ende W, Clerens S, Vergauwen R, Van Riet L, Van Laere A, Yoshida M, Kawakami A. 2003b. Fructan 1-exohydrolases: β(2,1) trimmers during graminan biosynthesis in stems of wheat? Purification, characterization, mass mapping and cloning of two fructan 1-exohydrolase isoforms. *Plant Physiology* **131**: 621–631.

Vergauwen R, Van Laere A, Van den Ende W. 2003. Properties of fructan:fructan 1-fructosyltransferases from chicory and globe thistle, two Asteracean plants storing greatly different types of inulin. *Plant Physiology* **133**: 391–401.

Vijn I, Smeekens S. 1999. Fructan: more than a reserve carbohydrate? *Plant Physiology* **120**: 351–360.

Vijn I, van Dijken A, Luscher M, Bos A, Smeets E, Weisbeek P, Wiemken A, Smeekens S. 1998. Cloning of sucrose:sucrose 1-fructosyltransferase from onion and synthesis of structurally defined fructan molecules from sucrose. *Plant Physiology* **117**: 1507–1513.

Vowels JJ, Payne GS. 1998. A dileucine-like sorting signal directs transport into an AP-3-dependent, clathrin-independent pathway to the yeast vacuole. *EMBO Journal* **17**: 2482–2493.

Wagner W, Wiemken A. 1986. Properties and subcellular localization of fructan hydrolase in the leaves of barley (*Hordeum vulgare* L. cv. Gerbel). *Journal of Plant Physiology* **123**: 429–439.

Wang CW, Tillberg JE. 1996. Effects of nitrogen deficiency on accumulation of fructan and fructan metabolizing enzyme activities in sink and source leaves of barley (*Hordeum vulgare*). *Physiologia Plantarum* **97**: 339–345.

Wernersson R, Pedersen AG. 2003. RevTrans – constructing alignments of coding DNA from aligned amino acid sequences. *Nucleic Acids Research* **31**: 3537–3539.

Winter H, Huber SC. 2000. Regulation of sucrose metabolism in higher plants: localization and regulation of activity of key enzymes. *Critical Reviews of Biochemistry and Molecular Biology* **35**: 253–289.

Yang J, Zhang J, Wang Z, Zhu Q, Liu L. 2004. Activities of fructan- and sucrose-metabolizing enzymes in wheat stems subjected to water stress during grain filling. *Planta* **220**: 331–343.

Supplementary Material

The following supplementary material is available for this article online:

Table S1 Fragment ions detected in Q-TOF after tryptic digest of OsVIN1, with calculated matches to theoretical digest of virtual cDNA derived protein, and confirmation of identity by tandem MS/MS sequencing

Table S2 Fragment ions detected in Q-TOF after tryptic digest of OsVIN2, with calculated matches to theoretical digest of virtual cDNA derived protein, and confirmation of identity by tandem MS/MS sequencing

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