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Molecular Cloning and Functional Identification of Invertase Isozymes from Green Bamboo *Bambusa oldhamii*

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Three *Boβfruct* cDNAs encoding acid invertases were cloned from shoots of the green bamboo *Bambusa oldhamii*. On the basis of the amino acid sequences of their products and phylogenetic analyses, *Boβfruct1* and *Boβfruct2* were determined to encode cell wall invertases, whereas *Boβfruct3* encodes a vacuolar invertase. The recombinant proteins encoded by *Boβfruct2* and *Boβfruct3* were produced in *Pichia pastoris* and purified to near homogeneity using ammonium sulfate fractionation and immobilized metal affinity chromatography. The pH optima, pI values, and substrate specificities of the isolated enzymes were consistent with those of plant cell wall or vacuolar invertases. The growth-dependent expression of *Boβfruct1* and *Boβfruct2* in the base regions of shoots underscores their roles in sucrose unloading and providing substrates for shoot growth. Its high sucrose affinity suggests that the *Boβfruct2*-encoded enzyme is important for maintaining the sucrose gradient between source and sink organs, while the predominant expression of *Boβfruct3* in regions of active cell differentiation and expansion suggests functions in osmoregulation and cell enlargement.

KEYWORDS: Bamboo (*Bambusa oldhamii*); cell-wall invertase; vacuolar invertase; cDNA cloning; recombinant invertase; gene expression

INTRODUCTION

In higher plants, sucrose is a principal product of photosynthesis, a major form of translocated carbon, and an important substrate for growth. Cleavage of sucrose, which in plants is catalyzed by either sucrose synthase (UDP-glucose:D-fructose 2-α-D-glucosyl transferase, EC 2.4.1.13, SuS) or invertase (β-D-fructofuranoside fructohydrolase; EC 3.2.1.26), serves as a starting point for various carbohydrate metabolic pathways. The products (hexoses) of sucrose cleavage and sucrose itself can also act as direct or indirect regulators that modulate expression of diverse genes, including SuS and invertase genes (*1*−*4*), making both SuS and invertase crucial for plant development, growth, and carbon partitioning (*5*, *6*).

Invertase, which irreversibly hydrolyzes sucrose into fructose and glucose, is present in plants as a group of isozymes (*7*, *8*). On the basis of their pH optima, solubility characteristics, and subcellular localization, these isozymes have been categorized as vacuolar, cell wall-associated, and cytoplasmic invertases (also referred to as soluble acid invertase, insoluble acid invertase, and neutral/alkaline invertase, respectively). The two types of acid invertase are both glycoproteins that show optimal activity at acidic pH, although their pI values differ; all known vacuolar enzymes have an acidic pI, while most of the cell wall enzymes have a basic pI. Unlike the enzyme in vacuoles or apoplasts, cytoplasmic invertases are not glycosylated and have an optimal pH of 7.0−7.8 and an acidic pI value (*7*, *8*).

The possible physiological roles of cell wall and vacuolar invertases have been analyzed in many plants. The proposed functions of cell wall invertases include regulation of phloem unloading, sucrose partitioning, cell differentiation, and plant development, as well as regulation of the responses to signals from various biotic and abiotic stresses (*5*−*11* and references therein). The proposed functions of vacuolar invertases include regulation of turgor and cell enlargement, control of the sugar composition in fruits and storage organs, and involvement in the response to wounding, cold, drought, hypoxia, and gravitropism (*5*−*8*, *11*, and references therein). In contrast to acid invertases, the function of cytoplasmic invertase is unclear, but it is thought to be involved in channeling sucrose into catabolism (*5*).

Although studied in numerous plants, little is known about the expression and function of invertases in bamboo. Bamboo belongs to the family Poaceae (Gramineae) and is distinguished by its unique flowering habit and rapid growth. The plants typically form clumps or spread via rhizomes. As shoots emerge from buds on the rhizomes, they rapidly develop into new culms and reach their full height within a single growth season. Before the new culms are able to assimilate carbon, they depend entirely upon sucrose supplied from the mature bamboos through the rhizomes to support their rapid growth. This makes sucrose

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unloading and subsequent catabolism within shoots critical for culm growth and highlights the importance of invertases in this plant. Our aim in the present study, therefore, was to investigate the molecular and biochemical properties of invertases in growing bamboo shoots. To that end, we cloned three cDNAs encoding cell wall invertase and vacuolar invertase from shoots of green bamboo *Bambusa oldhamii* (synonyms, *Leleba oldhami* and *Dendrocalamopsis oldhami*), which is the most widely cultivated species in Taiwan and is valued for its shoots as a vegetable. By characterization of the heterologously expressed recombinant invertases and analysis of the levels of each invertase mRNA in shoots at different growth stages, the roles played by the three isoforms during the growth of this plant were suggested.

MATERIALS AND METHODS

Plant Materials. Fresh shoots of *B. oldhamii* (Munro) were harvested from a bamboo farm in Taipei, Taiwan. Etiolated shoots (15 cm tall on average) and three groups of green shoots (30, 60, and 100 cm tall, on average) were collected. After the outer layers of the sheaths were removed, each shoot was divided into three parts (top, middle, and base) for separate analysis. The base was an area 5−10 cm above the point where the shoot joins the rhizome, the middle consisted of the developing nodes and internodes, and the top consisted of a series of overlapping sheaths surrounding the developing nodes and internodes. Unexpanded leaves and mature leaves were collected from mature bamboo plants. Each sample was immediately frozen in liquid nitrogen and stored at −80 °C until used.

RNA Isolation. The frozen bamboo samples were ground to a fine powder in liquid nitrogen, after which the total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Poly(A)$^+$ RNA was purified from the total RNA with biotinylated-oligo(dT) using a PolyATract mRNA isolation system (Promega, Madison, WI).

Isolation of Partial-Length Invertase cDNA Using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). First-strand cDNA was synthesized from poly(A)$^+$RNA isolated from etiolated bamboo shoots using an oligo-dT$_{15}$ primer and RT (Superscript II, Invitrogen). The resultant cDNA was then used as a template for PCR with primers 5′-AAAAACTGGATGAACCATCCTAATGGT-3′ and 5′-TCTTCCACCTTGAGCAAAGCTTTCAAC-3′, which were designed based on conserved regions within plant acid invertase sequences. The amplified 1.4 kb DNA fragment was cloned into plasmid pGEM-T (Promega), and its identity was confirmed by sequencing.

Cloning Invertase cDNA by Screening a cDNA Library. The PCR-amplified 1.4 kb DNA fragment was ^{32}P-labeled using a Random Prime Labeling System (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and used as a probe for screening a cDNA library from etiolated bamboo shoots (*12*). The positive plaques were isolated, subjected to in vivo excision, and amplified as phagemids for further analysis.

DNA Sequencing and Sequence Analysis. Both DNA strands were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with an ABI 3730 XL DNA Analyzer (Applied Biosystems, CA). Sequence analysis, alignment of the amino acid sequences, and phylogenetic analysis were carried out using Vector NTI Suite 9 Sequence Analysis Software (Invitrogen). The cleavage site for the leader sequence was predicted by the SPScan program in the Wisconsin Package (Accelrys, Inc., San Diego, CA).

Construction of Expression Plasmids. The expression plasmids pBoIT1, pBoIT2, and pBoIT3 were constructed using similar strategies. The regions of the three *Boβfruct* cDNAs encoding the predicted mature proteins were amplified by PCR. The amplified *Boβfruct1* DNA fragment was digested with *Pst*I and *Xba*I and then ligated with *Pst*I-*Xba*I-digested pPICZαB (Invitrogen) to form pBoIT1. The amplified *Boβfruct2* and *Boβfruct3* DNA fragments were digested with *Eco*RI and *Xba*I and respectively ligated into the *Eco*RI/*Xba*I site of plasmid pPICZαB, yielding pBoIT2 and pBoIT3.

Production and Purification of Recombinant Invertase in *P. pastoris*. *P. pastoris* strain X-33 was transformed with pBoIT1, pBoIT2, or pBoIT3 using a *Pichia* EasyComp Kit (Invitrogen). Growth of the transformed cells and induction of expression with methanol were carried out according to the manufacturer's instructions. The induction times for recombinant BoIT2 and BoIT3 were 48 and 72 h, respectively. The cultures were then centrifuged for 30 min at 6000*g*, after which the proteins in the centrifugal supernatant were precipitated by 80% saturation of ammonium sulfate. After centrifugation, the precipitates were dissolved in PB-7.0 (50 mM sodium phosphate, pH 7.0) and dialyzed against PB-7.0 containing 0.3 M NaCl. The resultant enzyme solution was mixed with cobalt-based immobilized metal affinity chromatography (IMAC) resin (BD TALON Resin, BD Biosciences Clontech, Palo Alto, CA) and incubated for 30 min at 4 °C. The enzyme−resin mixture was then packed into a column, washed with PB-7.0 containing 0.3 M NaCl and 10 mM imidazole, and then eluted with PB-7.0 containing 0.3 M NaCl and 150 mM imidazole. Fractions containing invertase activity were collected.

Enzyme Assay and Protein Analytic Methods. The invertase activity was assayed at pH 5.0 as described by Sung and Huang (*13*). The protein concentration was determined using the protein−dye binding method (*14*), with bovine serum albumin serving as the standard protein. For deglycosylation of the recombinant invertase, 10 μg of the purified enzymes (in PB-7.0) was incubated with 10 units of N-glycosidase F (PNGase F, Roche Applied Science, Mannheim, Germany) at 37 °C for 16 h.

Proteins were separated by 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (*15*), and the proteins on the gels were stained with Coomassie Blue R-250 or silver nitrate or were transferred onto PVDF membranes (Millipore, Bedford, MA) for immunodetection using an anti-(His)$_6$ antibody (Amersham Pharmacia Biotech).

Semiquantitative RT-PCR. Reverse transcription reactions were carried out using total RNA isolated from the various bamboo samples collected. The resultant cDNAs were then used as templates for PCR with gene-specific primers to amplify each *Boβfruct* cDNA. The sequences of the gene-specific primer pairs were as follows: *Boβfruct1*, 5′-GAAGAGGATCTTCCTTCGTG-3′ (forward; nucleotides 1880−1899) and 5′-CCTAGAGGCATACAAATTGGC-3′ (reverse; nucleotides 2028−2048); *Boβfruct2*, 5′-CACCACTCTGCTAACTCAT-3′ (forward; nucleotides 1764−1782) and 5′-ACAGTGCGAAATTAG-CACC-3′ (reverse; nucleotides 1952−1970); and *Boβfruct3*, 5′-CCAAGCCTACATGGCTTAA-3′ (forward; nucleotides 2017−2035) and 5′-TGCTTGCATCATGGCATCT-3′ (reverse; nucleotides 2171−2189). An actin cDNA fragment was also amplified as an internal control using primers 5′-TGGCATCACACCTTCTACAA-3′ (forward) and 5′-ACCTGGATCTTCATGCTGCT-3′ (reverse), which were designed from the conserved regions of monocot actin sequences. The amplified cDNA fragments were sequenced to confirm their specificity; moreover, for each cDNA, the linearity of the amplification was confirmed by carrying out the PCR for different numbers of cycles.

RESULTS

Cloning and Characterization of Invertase cDNA from Bamboo Shoots. A partial-length cDNA clone, presumably encoding invertase, was obtained by RT-PCR using a set of primers designed from the conserved regions of plant invertase sequences and poly(A)$^+$RNA isolated from bamboo shoots. The deduced amino acid sequence was highly homologous to the corresponding regions of acid invertases from other plants (data not shown), so the cDNA was used as a hybridization probe to screen a bamboo shoot cDNA library, and nine potential clones were selected and subjected to further analysis. Nucleotide sequence analysis revealed that all nine clones contained a 5′-UTR, an open reading frame, a 3′-UTR, and a poly(A) tail and could be divided into three distinct types: *Boβfruct1*, *Boβfruct2*, and *Boβfruct3*. The characteristics of the three cDNAs and their deduced amino acid sequences are summarized in **Table 1**. The amino acid sequences deduced from *Boβfruct1* and *Boβfruct2*

Table 1. Summary of the *Boβ*fruct cDNA Sequences[a]

	Boβfruct1	*Boβfruct2*	*Boβfruct3*
	cDNA		
5′-UTR (bp)	113	24	58
ORF (bp)	1761	1731	1977
3′-UTR (bp)	274	270	225
	protein		
amino acid (no. of residues)	586	576	658
molecular mass (kDa)	65.6	64.4	71.9
pI	8.87	7.25	5.69
leader sequence (no. of residues)	22	22	57
glycosylation site (no.)	3	3	4
molecular mass of mature protein (kDa)	63.2	62.1	66.3
pI of mature protein	8.66	7.23	5.60

[a] The accession numbers for *Boβfruct1*, *Boβfruct2*, and *Boβfruct3* are DQ267823, DQ267824, and DQ267825, respectively.

were 81% identical but showed lower identity to the sequence deduced from *Boβfruct3* (52 and 51%, respectively). BLAST analysis revealed that the predicted proteins encoded by *Boβfruct1*, *Boβfruct2*, and *Boβfruc3* had the highest identity to rice cell wall invertase OsCIN2 (accession number AY578159, 85.3%), rice cell wall invertase OsCIN1 (AB073749, 83.8%), and rice vacuolar invertase INV3 (AF276704, 78.3%), respectively.

Figure 1 shows the alignment of the amino acid sequences. All three sequences contain the typical motifs of plant acid invertases, including a β-fructosidase motif "NW[M/I]NDPN-[G/A]", a putative catalytic domain "WEC[V/I/P]D", and a conserved "S[V/I]VESF" sequence (*7*, *8*, *16*). Moreover, the three sequences all contain leader sequences in their N terminus, as are found in all plant acid invertases (*7*, *8*). Although plant fructan-metabolizing fructosyltransferases also share motifs conserved among acid invertases and both are highly homologous to one another (*17*, *18*), acid invertases can be differentiated from fructan-metabolizing enzymes based on an amino acid triplet, WMN or WIN, in the β-fructosidase motif (*16*, *19*). The presence of WIN in the amino acid sequences deduced from *Boβfruct1* and *Boβfruct2* (amino acid residues 61−63 and 60−62, respectively) and WMN in the sequence deduced from *Boβfruct3* (112−114) (**Figure 1**) confirms that the three genes encode acid invertases and not fructan-metabolizing enzymes.

As shown in **Figure 2**, the proteins encoded by *Boβfruct1* and *Boβfruct2* have a closer evolutionary relationship to cell wall invertases than to vacuolar invertases, whereas the protein encoded by *Boβfruct3* appears to belong to the vacuolar invertase family. Several features in the three *Boβfruct*-encoded sequences also support this classification: (i) The fourth residue of the conserved catalytic domain WEC[V/I/P]D in the *Boβfruct1*- and *Boβfruct2*-encoded proteins is proline, which is characteristic of cell wall invertases, and the corresponding amino acid residue in the *Boβfruct3*-encoded protein is isoleucine, which has been seen in some vacuolar invertases (*7*). (ii) The predicted leader sequences for the *Boβfruct1*-, *Boβfruct2*-, and *Boβfruc3*-encoded proteins are 22, 22, and 57 amino acids in length, which is consistent with that the leader sequences of vacuolar invertases are longer than those of cell wall invertases (*7*, *8*, *19*). (iii) The pI values for the mature proteins of the three isoforms were calculated to be 8.66, 7.23, and 5.60, respectively, which are consistent with the previous observations that cell wall invertases have basic pI values and vacuolar invertases have acidic ones (*7*, *8*, *11*).

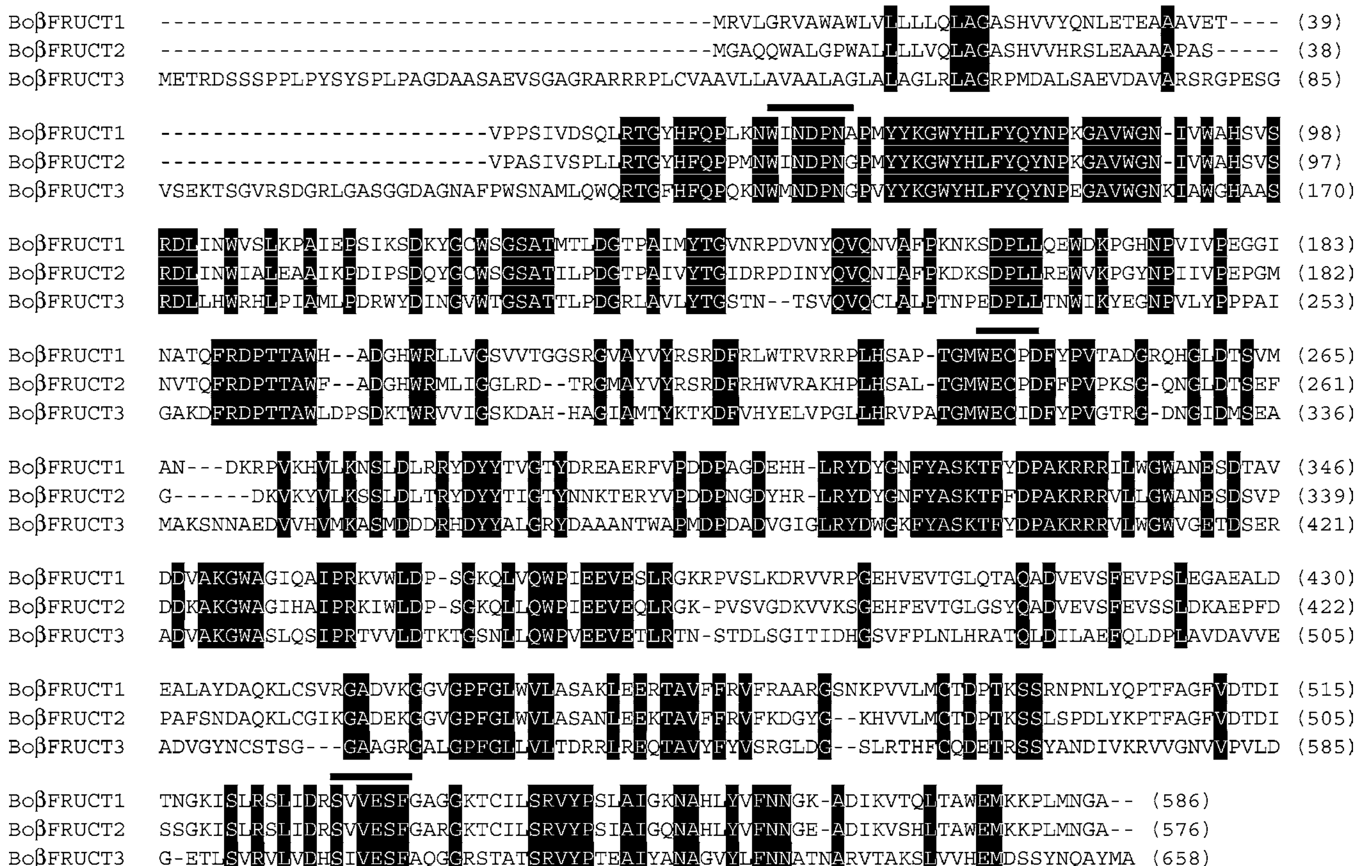

Figure 1. Alignment of the deduced amino acid sequences derived from bamboo *Boβfruct* cDNAs. Identical residues are shown on a black background. The characteristic conserved elements of acid invertases are indicated by lines above the sequences.

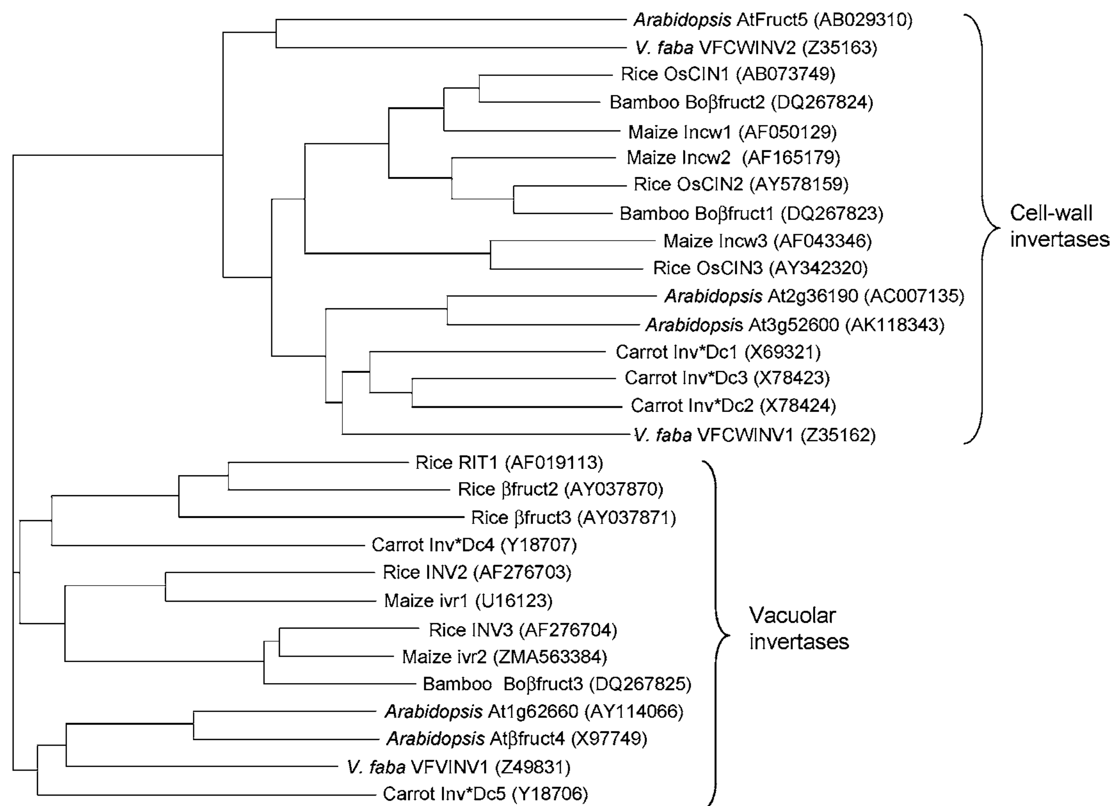

Figure 2. Phylogram of the amino acid sequences of invertases from various plants.

Expression of the *Boβfruct*-Encoded Proteins in Yeast. To further characterize the proteins encoded by the three *Boβfruct* genes, the cDNA sequences encoding the putative mature protein regions were cloned into a secreted and His-tag fusion expression vector (pPICZαB), which was then used to transform *P. pastoris*. Strains harboring *Boβfruct1*, *Boβfruct2*, and *Boβfruct3* (YIT1, YIT2, and YIT3, respectively) were grown at 30 °C in the presence of methanol. Acid invertase activity and 77 kDa His-tagged proteins were detected in the growth medium of strains YIT2 and YIT3 (**Figure 3A**,**B**). However, the invertase activity in the growth medium of strain YIT1 was barely detected and the His-tagged proteins were undetectable. It remains to be determined whether the expression level of recombinant *Boβfruct1*-encoded proteins was low or the proteins were unstable under our experimental conditions.

The recombinant *Boβfruct2*- and *Boβfruct3*-encoded proteins (designated rBoIT2 and rBoIT3, respectively) were purified to near homogeneity from the growth media of methanol-induced strains YIT2 and YIT3, respectively, using ammonium sulfate fractionation and cobalt-based IMAC (**Figure 4**). The specific activity of the purified rBoIT3 was 1.9 times that of rBoIT2 (**Table 2**).

Biochemical Properties of the Recombinant Invertases. The molecular masses of rBoIT2 and rBoIT3 were estimated to be 70.1 and 72.6 kDa, respectively, using gel filtration chromatography, which is very close to those estimated by SDS-PAGE (77.5 kDa for both, **Figure 4**), suggesting that the native form of the two enzymes is a monomer. After PNGase F treatment, the resultant deglycosylated forms exhibited the electrophoretic mobility of a 68.2 kDa protein (**Figure 4**), which is very close to the calculated molecular masses for the c-myc and His tag-fused recombinant enzymes (62.5 and 64.5 kDa, respectively). The pI values estimated by IEF were 9.2 for rBoIT2 and 6.5 for rBoIT3 (data not shown). Although these values are higher than the calculated pI values for the nonglycosylated recombinant enzymes, they are consistent with the fact that plant cell wall invertases have basic pI values, while vacuolar invertases have acidic ones.

The optimum reaction temperatures were found to be 60 °C for rBoIT2 and 50 °C for rBoIT3. Both enzymes had similar thermostabilities. They were stable within the temperature range of 0−30 °C, and their activities were completely lost at 50 °C (data not shown). The pH stability profiles of the two recombinant enzymes are also similar. Both were stable at acidic pH (**Figure 5A**). rBoIT2 had a pH optimum of 3.0, while rBoIT3 showed the highest activity within a pH range of 4−5 (**Figure 5B**). At pH 2.0, rBoIT2 still retained 90% activity, but rBoIT3 exhibited no detectable activity. Their affinities for sucrose differ substantially: the apparent K_m values of rBoIT2 and rBoIT3 for sucrose were 0.42 and 22.9 mM, respectively.

In addition to acting on sucrose, rBoIT2 and rBoIT3 also hydrolyzed raffinose, exhibiting activities that were 92.1 and 24.6% of that toward sucrose, respectively. No hydrolytic activity was detected with maltose, lactose, or cellobiose. This substrate specificity is consistent with that for *β*-fructofuranosidases.

Table 3 shows the effects of metal ions on the activities of rBoIT2 and rBoIT3. The activities of both enzymes were inhibited by $HgCl_2$, which is known to act as a sulfhydryl inhibitor of plant invertases (*8*). rBoIT2 was activated by Ca^{2+} and Co^{2+} and was inhibited by the other ions tested. The activity of rBoIT3 was not affected by K^+ and Ca^{2+} significantly but was inhibited by the other ions tested. The enzyme was more sensitive to Cu^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+}, and Zn^{2+} than was rBoIT2.

Expression of *Boβfruct* Genes in the Growing Bamboo and Leaves. The expression of the three *Boβfruct* genes in growing bamboo shoots and leaves was examined using semiquantitative RT-PCR (**Figure 6**). In shoots, levels of *Boβfruct1* mRNA were higher in the base region than the other two regions and increased as the shoots grew. Levels of *Boβfruct2* mRNA in different regions of the same shoot did

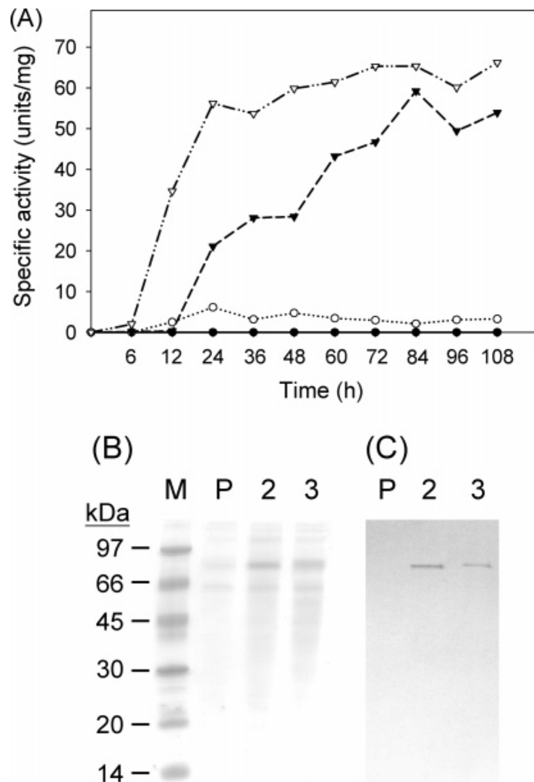

Figure 3. Expression of recombinant invertases in *P. pastoris.* (**A**) Strains YIT1 (○), YIT2 (▼), and YIT3 (▽), which, respectively, harbored *Boβfruct1*, *Boβfruct2*, and *Boβfruct3*, or cells transformed with empty pPICZαB vector (●), were grown in media without methanol at 30 °C until the A_{600} values reached 1.0, after which expression of recombinant proteins was induced by adding methanol to the cultures. Samples were collected at various times after induction. Proteins secreted into the culture medium and those present in the cellular fraction were assayed for invertase activity at pH 5.0 (**A**). The secreted proteins of cells harvested after 48 h of induction were separated on 10% SDS-polyacrylamide gels, which were then stained with Coomassie blue R-250 (**B**) or transferred onto a PVDF membrane and immunodetected with an anti-(His)$_6$ antibody (**C**). M, molecular mass markers; P, cells transformed with pPICZαB; 2, strain YIT2; and 3, strain YIT3.

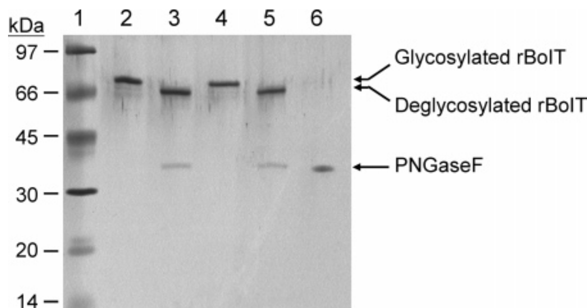

Figure 4. Analysis of purified and deglycosylated recombinant invertases. Purified rBoIT2 and rBoIT3 were incubated with PNGase F for 16 h at 37 °C and then separated on 12.5% SDS-PAGE. The resolved proteins were stained with silver nitrate: lane 1, molecular mass markers; lanes 2 and 4, purified rBoIT2 and rBoIT3; lanes 3 and 5, purified rBoIT2 and rBoIT3 treated with PNGase F; and lane 6, PNGase F.

not show the distinct differences seen with *Boβfruct1*, but the accumulation of *Boβfruct2* mRNA in the base region was also growth-dependent. The highest level of *Boβfruct2* mRNA was seen in 100 cm green shoots. *Boβfruct2* mRNA was also abundant in leaves, especially in source leaves, whereas there was little expression of *Boβfruct1* in both sink and source leaves. In contrast to *Boβfruct1* and *Boβfruct2*, there was only a low level of *Boβfruct3* mRNA in the base region of shoots; levels were highest in the middle and the top regions of 60 cm green shoots. The transcript of *Boβfruct3* was barely detectable in leaves.

DISCUSSION

We have isolated three cDNA clones encoding acid invertases from bamboo shoots. On the basis of their amino acid sequences and phylogenetic analysis, *Boβfruct1* and *Boβfruct2* were determined to be cell wall invertase genes, while *Boβfruct3* was a vacuolar invertase gene. The proteins encoded by *Boβfruct2* and *Boβfruct3* were successfully expressed and purified from *P. pastoris*. The two recombinant enzymes (rBoIT2 and rBoIT3) were both stable at acidic pH and had acidic optimal pH values, which is consistent with the acidic environment of apoplasts and vacuoles. Moreover, the finding that the pH optimum for rBoIT2 was lower than that for rBoIT3 is consistent with earlier observations that in some plants cell wall invertases have lower pH optima than vacuolar invertases from the same species (*20*–*22*). This difference in pH optima has been attributed to a single amino acid substitution in the catalytic domain WEC[V/I/P]D: Cell wall invertases contain a proline residue, while vacuolar invertases contain valine or isoleucine (*23*). Substituting the proline residue in the catalytic domain of cell wall invertase with a valine shifts the pH optimum toward more basic values and decreases the rate of raffinose cleavage (*23*). The difference in the rates on raffinose cleavage exhibited by different invertases was also observed in the present study; i.e., rBoIT2 (cell wall enzyme) showed a greater activity toward raffinose than rBoIT3 (vacuolar enzyme). This is consistent with the result of Goetz and Roitsch (*23*) who proposed that the proline and valine/isoleucine residues play a key role in determining the substrate specificity of the two enzymes. However, involvement of other amino acid residues cannot be excluded.

In an earlier study, two soluble acid invertases (type I and type II) and two cell wall isoforms (type III and type IV) were purified from green bamboo shoots (*24*). The K_m value of rBoIT3 was nearly identical to the type II enzyme (22.5 mM) but higher than the type I enzyme (7.9 mM) from green bamboo shoots and other soluble acid invertases from maize endosperms (2 and 10.5 mM for invertases I and II, respectively, *25*), wheat coleoptiles (3.5 mM, *26*), barley elongating stem tissue (12.0 mM, *20*), potato leaves and tubers (2.4 and 7.9 mM, respectively, *27*), rice grains (0.94 and 12.1 mM for IT4 and IT5, respectively, *28*), *Arabidopsis* leaves (5, 12, and 5 mM for INV1, INV2, and INV3, respectively, *22*), pear fruit (3.33 and 4.58 mM for AIV I and AIV II, respectively, *29*), and suspension-cultured cells of a different bamboo species (*Bambusa edulis*) (3.7 mM, *30*). The K_m value of rBoIT2 was lower than the cell wall invertases from green bamboo shoots (1.3 mM for type IV isoform, *24*) and other plants such as maize endosperm (8 mM, *25*), sugar beet seedlings (1.33 mM, *31*), *Lilium* pollen (0.98 mM, *32*), wheat coleoptiles (1.7 mM, *26*), suspension-cultured cells of sugar beet (0.56 and 4.2 mM for saline-released and EDTA-released enzymes, *33*), barley elongating stem tissue (5.0 mM, *20*), and rice grains (5.39 mM, *13*). It is noteworthy that rBoIT2 had the highest affinity for sucrose among known cell wall invertases. This characteristic could be related to its physiological function within this remarkable plant, which exhibits a high growth rate. The average elongation rate of bamboo culms is 9.7 cm/day for *B. oldhamii* (*34*) and can be over 100 cm/day for *Phyllostachys edulis* (*35*). To support the

Table 2. Purification of the Recombinant Invertases rBoIT2 and rBoIT3 from Transformed *P. pastoris*[a]

purification step	enzyme	total activity (units[b])	total protein (mg)	specific activity (units/mg)	yield (%)	purification (fold)
centrifugal supernatant	rBoIT2	9616.4	164.77	58.4	100	1.0
of the culture	rBoIT3	10936.4	157.26	69.5	100	1.0
ammonium sulfate	rBoIT2	3942.7	82.25	47.9	41	0.82
fractionation	rBoIT3	3385.3	30.61	110.6	31	1.6
Co-IMAC	rBoIT2	1538.6	1.74	884.3	16	15.1
	rBoIT3	2542.0	1.50	1694.5	23	24.3

[a] The data were obtained from the centrifugal supernatant of a 1 L culture of methanol-induced *P. pastoris* harboring *Boβfruct2* or *Boβfruct3*. [b] One unit of invertase was defined as the amount of enzyme that catalyzed the formation of 1 μmol of reducing sugar from sucrose per minute at 37 °C and pH 5.0.

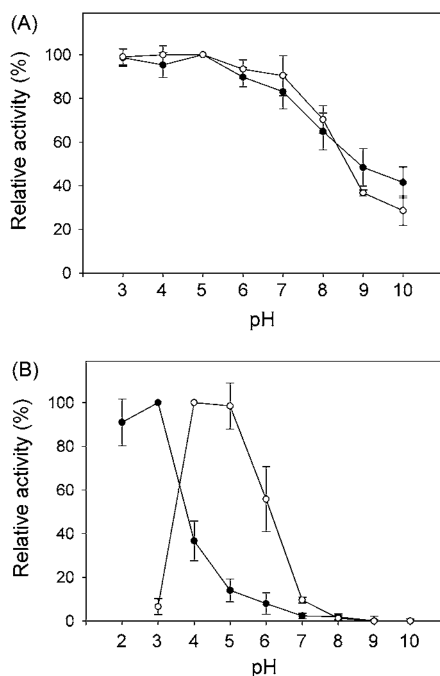

Figure 5. Effects of pH on the activity of recombinant invertase. (**A**) pH stability profile: purified rBoIT2 (●) and rBoIT3 (○) were incubated at various pH values for 30 min, after which the residual invertase activity was determined at pH 5.0. (**B**) pH optima: purified rBoIT2 (●) and rBoIT3 (○) were assayed at various pH values.

Table 3. Effect of Metal Ions on the Activity of Recombinant Invertases

addition (5 mM)	relative activity (%) rBoIT2	relative activity (%) rBoIT3	addition (5 mM)	relative activity (%) rBoIT2	relative activity (%) rBoIT3
none	100	100	$FeCl_2$	41.5	3.7
KCl	87.4	95.1	$HgCl_2$	29.5	2.1
LiCl	76.6	85.9	$MgCl_2$	90.9	88.9
$CaCl_2$	108.7	96.7	$MnCl_2$	84.5	48.6
$CoCl_2$	129.4	11.3	$ZnCl_2$	82.1	16.2
$CuCl_2$	32.8	0.6			

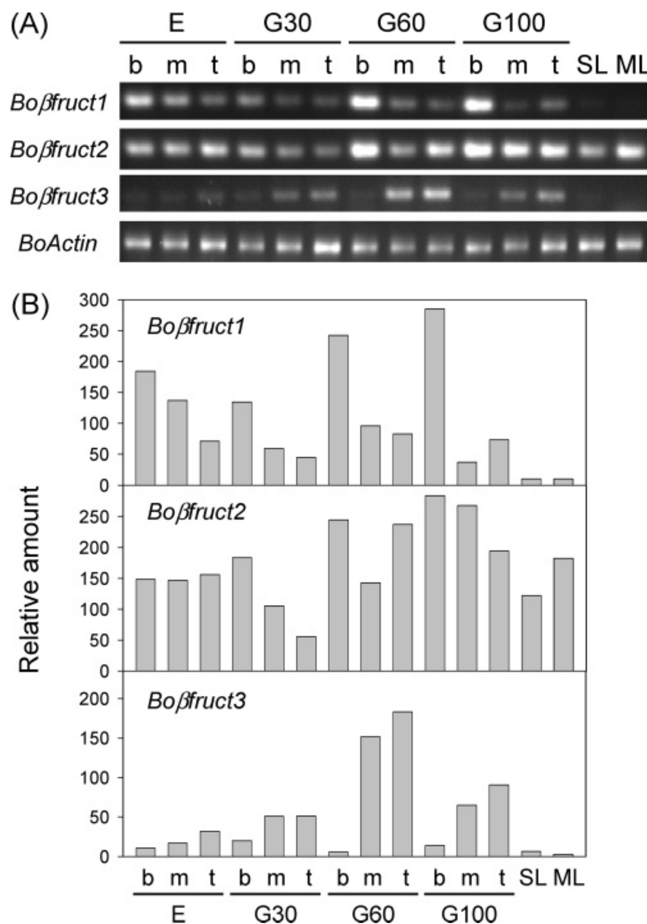

Figure 6. Expression of *Boβfruct* genes in bamboo. Total RNA was isolated from 15 cm etiolated shoots (E), 30 cm green shoots (G30), 60 cm green shoots (G60), 100 cm green shoots (G100), sink leaves (unexpanded leaves, SL), and source leaves (mature leaves, ML). Each shoot was divided into three parts: the base (b), middle (m), and top (t). The relative abundance of mRNA was analyzed by RT-PCR using specific primers for each gene. Amplification of actin transcript (*BoActin*) was carried out in parallel as an internal control. (**A**) RT-PCR products analyzed by agarose gel electrophoresis. (**B**) The densities of the cDNA bands in panel **A** were quantitated using an image analyzer and normalized to the actin cDNA level. The figures show a representative example from three independent experiments having similar results.

rapid growth, effective translocation of sucrose from source organs of mature bamboo to shoots must be required. The presence of high sucrose affinity invertases that readily bind sucrose within the apoplasts of sink cells would be expected to facilitate such translocation by helping to sustain a sucrose gradient between the source and the sink organs.

The above postulation that *Boβfruct2*-encoded invertase is involved in sucrose unloading is further supported by the growth-dependent expression pattern of *Boβfruct2* in the base regions of the shoots. The base of the shoot is the area just above the point where the shoot joins the rhizome and is the site at which sucrose unloading occurs. Furthermore, the highest levels of *Boβfruct2* mRNA were found in the base of 100 cm green shoots, which is the stage at which the rates of culm elongation are higher (*34*). Similarly, the level of *Boβfruct1* mRNA in the base region paralleled the growth of shoots, which is indicative of the importance of both of these cell wall invertases in sucrose translocation. However, the two isoforms

likely have different functions in different tissues, as the relative abundance of their mRNAs differed in the various shoot tissues and in the leaves. In contrast to the predominant expression of *Boβfruct1* in the base of the shoot, the expression of *Boβfruct3* was higher in the top and middle regions of the shoots, where rapid cell differentiation and expansion are ongoing. The pattern of *Boβfruct3* expression is consistent with the involvement of vacuolar invertases in osmoregulation and cell enlargement.

While our results show a close correlation between the abundance of the three invertase mRNAs and bamboo growth, how the gene expression and enzyme activity are regulated and whether these enzymes play regulatory roles in sugar-mediated modulation of gene expression remain unknown. We will focus on these important issues in the future with the aim of gaining additional insight into the growth of this remarkable plant.

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