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

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Three *Bo*fruct 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, *Bo*fruct1 and *Bo*fruct2 were determined to encode cell wall invertases, whereas *Bo*fruct3 encodes a vacuolar invertase. The recombinant proteins encoded by *Bo*fruct2 and *Bo*fruct3 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 *Bo*fruct1 and *Bo*fruct2 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 *Bo*fruct2-encoded enzyme is important for maintaining the sucrose gradient between source and sink organs, while the predominant expression of *Bo*fruct3 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 growth, development, 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...
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 oldhamii and Dendrocalamopsis oldhamii), 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 Taipeh, 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 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-d(T) primer and RT (Superscript II, Invitrogen). The resultant cDNA was then used as a template for PCR with primers 5′-AAAAACCTGGATGAACCATCCTAATGGT-3′ and 5′-TCTTCCCCACCTTGAGCAAAAGCTTCCAAC-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 32P-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 sequences, and phylogenetic analysis were carried out using Vector NTI Suite 9 Sequence Analysis Software (Invitrogen). The cleavage sequences, and phylogenetic analysis were carried out using Vector NTI Suite 9 Sequence Analysis Software (Invitrogen). The cleavage sequences, and phylogenetic analysis were carried out using Vector NTI Suite 9 Sequence Analysis Software (Invitrogen). The cleavage sequences, and phylogenetic analysis were carried out using Vector NTI Suite 9 Sequence Analysis Software (Invitrogen).

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: Bojfruct1, Bojfruct2, and Bojfruct3. The characteristics of the three cDNAs and their deduced amino acid sequences are summarized in Table 1. The amino acid sequences deduced from Bojfruct1 and Bojfruct2
were 81% identical but showed lower identity to the sequence deduced from Bo\textsubscript{fruct}3 (52 and 51%, respectively). BLAST analysis revealed that the predicted proteins encoded by Bo\textsubscript{fruct}1, Bo\textsubscript{fruct}2, and Bo\textsubscript{fruct}3 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 \(\alpha\)-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 \(\alpha\)-fructosidase motif (16, 19). The presence of WIN in the amino acid sequences deduced from Bo\textsubscript{fruct}1 and Bo\textsubscript{fruct}2 (amino acid residues 61–63 and 60–62, respectively) and WMN in the sequence deduced from Bo\textsubscript{fruct}3 (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 Bo\textsubscript{fruct}1 and Bo\textsubscript{fruct}2 have a closer evolutionary relationship to cell wall invertases than to vacuolar invertases, whereas the protein encoded by Bo\textsubscript{fruct}3 appears to belong to the vacuolar invertase family. Several features in the three Bo\textsubscript{fruct}-encoded sequences also support this classification: (i) The fourth residue of the conserved catalytic domain WEC[V/I/P]D in the Bo\textsubscript{fruct}1- and Bo\textsubscript{fruct}2-encoded proteins is proline, which is characteristic of cell wall invertases, and the corresponding amino acid residue in the Bo\textsubscript{fruct}3-encoded protein is isoleucine, which has been seen in some vacuolar invertases (7, 16). (ii) The predicted leader sequences for the Bo\textsubscript{fruct}1-, Bo\textsubscript{fruct}2-, and Bo\textsubscript{fruct}3-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).

### Table 1. Summary of the Bo\textsubscript{fruct} cDNA Sequences

<table>
<thead>
<tr>
<th>Bo\textsubscript{fruct}1</th>
<th>Bo\textsubscript{fruct}2</th>
<th>Bo\textsubscript{fruct}3</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′-UTR (bp)</td>
<td>113</td>
<td>24</td>
</tr>
<tr>
<td>ORF (bp)</td>
<td>1761</td>
<td>1731</td>
</tr>
<tr>
<td>3′-UTR (bp)</td>
<td>274</td>
<td>270</td>
</tr>
<tr>
<td>protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>amino acid (no. of residues)</td>
<td>586</td>
<td>576</td>
</tr>
<tr>
<td>molecular mass (kDa)</td>
<td>65.6</td>
<td>64.4</td>
</tr>
<tr>
<td>pI</td>
<td>8.87</td>
<td>7.25</td>
</tr>
<tr>
<td>leader sequence (no. of residues)</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>glyclosylation site (no.)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>molecular mass of mature protein (kDa)</td>
<td>63.2</td>
<td>62.1</td>
</tr>
<tr>
<td>pI of mature protein</td>
<td>8.66</td>
<td>7.23</td>
</tr>
</tbody>
</table>

\[ a \] The accession numbers for Bo\textsubscript{fruct}1, Bo\textsubscript{fruct}2, and Bo\textsubscript{fruct}3 are DQ267823, DQ267824, and DQ267825, respectively.

Figure 1. Alignment of the deduced amino acid sequences derived from bamboo Bo\textsubscript{fruct} cDNAs. Identical residues are shown on a black background. The characteristic conserved elements of acid invertases are indicated by lines above the sequences.
Expression of the *Bo* fruct-Encoded Proteins in Yeast. To further characterize the proteins encoded by the three *Bo* fruct genes, the cDNA sequences encoding the putative mature protein regions were cloned into a secreted and His-tag fusion expression vector (pPICZα B), which was then used to transform *P. pastoris*. Strains harboring *Bo* fruct1, *Bo* fruct2, and *Bo* fruct3 (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 *Bo* fruct1-encoded proteins was low or the proteins were unstable under our experimental conditions.

The recombinant *Bo* fruct2- and *Bo* fruct3-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 HgCl₂, which is known to act as a sulphydryl inhibitor of plant invertases (8). rBoIT2 was activated by Ca²⁺ and Co²⁺ and was inhibited by the other ions tested. The activity of rBoIT3 was not affected by K⁺ and Ca²⁺ significantly but was inhibited by the other ions tested. The enzyme was more sensitive to Cu²⁺, Fe²⁺, Hg²⁺, Mn²⁺, and Zn²⁺ than was rBoIT2.

Expression of *Bo* fruct Genes in the Growing Bamboo and Leaves. The expression of the three *Bo* fruct genes in growing bamboo shoots and leaves was examined using semiquantitative RT-PCR (Figure 6). In shoots, levels of *Bo* fruct1 mRNA were higher in the base region than the other two regions and increased as the shoots grew. Levels of *Bo* fruct2 mRNA in different regions of the same shoot did not show significant differences.
and immunodetected with an anti-(His)

with Coomassie blue R-250 ( ).

were separated on 10% SDS-polyacrylamide gels, which were then stained

5.0 ( ).

times after induction. Proteins secreted into the culture medium and those

by adding methanol to the cultures. Samples were collected at various

reached 1.0, after which expression of recombinant proteins was induced

YIT1 ( ), YIT2 ( ), and YIT3 ( ), which, respectively, harbored Bojfruct1, Bojfruct2, and Bojfruct3, or cells transformed with empty pPICZ

vector ( ), were grown in media without methanol at 30 °C until the A600 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.

not show the distinct differences seen with Bojfruct1, but the accumulation of Bojfruct2 mRNA in the base region was also growth-dependent. The highest level of Bojfruct2 mRNA was seen in 100 cm green shoots. Bojfruct2 mRNA was also abundant in leaves, especially in source leaves, whereas there

was little expression of Bojfruct1 in both sink and source leaves. In contrast to Bojfruct1 and Bojfruct2, there was only a low level of Bojfruct3 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 Bojfruct3 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, Bojfruct1 and Bojfruct2 were determined to be cell wall invertase genes, while Bojfruct3 was a vacuolar invertase gene. The proteins encoded by Bojfruct2 and Bojfruct3 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/IP]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 Km 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 Km 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, 33). 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
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 \( \text{Bofruct2} \)-encoded invertase is involved in sucrose unloading is further supported by the growth-dependent expression pattern of \( \text{Bofruct2} \) 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 \( \text{Bofruct2} \) 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 \( \text{Bofruct1} \) 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

Table 2. Purification of the Recombinant Invertases rBoIT2 and rBoIT3 from Transformed \( P. \) pastoris

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

\( ^a \) The data were obtained from the centrifugal supernatant of a 1 L culture of methanol-induced \( P. \) pastoris harboring \( \text{Bofruct2} \) or \( \text{Bofruct3} \). \( ^b \) One unit of invertase was defined as the amount of enzyme that catalyzed the formation of 1 \( \mu \)mol of reducing sugar from sucrose per minute at 37 °C and pH 5.0.

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

<table>
<thead>
<tr>
<th>addition (5 mM)</th>
<th>relative activity (%)</th>
<th>relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rBoIT2</td>
<td>rBoIT3</td>
</tr>
<tr>
<td>none</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>KCl</td>
<td>87.4</td>
<td>95.1</td>
</tr>
<tr>
<td>LiCl</td>
<td>76.6</td>
<td>85.9</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>108.7</td>
<td>96.7</td>
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<tr>
<td>CoCl(_2)</td>
<td>129.4</td>
<td>11.3</td>
</tr>
<tr>
<td>CuCl(_2)</td>
<td>32.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

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.

Figure 6. Expression of \( \text{Bofruct} \) 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 (\( \text{BoActin} \)) 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.
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 Bofruct1 in the base of the shoot, the expression of Bofruct3 was higher in the top and middle regions of the shoots, where rapid cell differentiation and expansion are ongoing. The pattern of Bofruct3 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.

LITERATURE CITED

(35) Ueda, K. Studies on the physiology of bamboo; with reference to practical application. Forests Bulletin; Kyoto University: Kyoto, Japan, 1960; Vol. 30.