Planta (2008) 227:1063–1077
DOI 10.1007/s00425-007-0681-1

ORIGINAL ARTICLE

Carbohydrate mobilization and gene regulatory profile in the pseudobulb of *Oncidium* orchid during the flowering process

Chih-Yu Wang · Chung-Yi Chiou · Heng-Long Wang · Ramanarayan Krishnamurthy · Shripathi Venkatagiri · Jun Tan · Kai-Wun Yeh

Received: 16 October 2007 / Accepted: 7 December 2007 / Published online: 9 January 2008

Abstract The pseudobulb of *Oncidium* orchid is a storage organ for supplying water, minerals and carbohydrates to the developing inflorescence. Different patterns of mannan, starch and pectin metabolism were observed in the pseudobulb of three developmental stages by histochemical staining and high performance anion exchange chromatographic (HPAEC) analysis. Copious pectin was strongly stained by ruthenium red in young pseudobulbs demonstrating that mannan and pectin were preferentially accumulated in the young pseudobulb sink at inflorescence pre-initiation stage. Concomitant with the emergence of the inflorescence, mannan and pectin decreased gradually and converted to starch. The starch, synthesized at the inflorescence developing stage, was eventually degraded at the floral development stage. A systematic survey on the subtractive EST (expression sequence tag) library of pseudobulb in the inflorescence pre-initiation stage revealed the presence of five groups of gene homologues related to sucrose, mannan, starch, pectin and other carbohydrate metabolism. The transcriptional level of 13 relevant genes related to carbohydrate metabolism was characterized from pseudobulbs of three different developmental stages. The specific activities of the enzymes encoded by these genes were also assayed. The expression profiles of these genes show that the transcriptional levels largely correlated with the enzyme activities, which were associated with the respective carbohydrate pools. These results demonstrated a novel functional profile of polysaccharide mobilization pathway as well as their relevant gene expression in the pseudobulb of *Oncidium* orchid during the flowering process.

Keywords Carbohydrate metabolism · EST (expressed sequence tags) · Inflorescence · *Oncidium* · Pseudobulb

Abbreviations

HPAEC	High performance anion exchange chromatography
EST	Expression sequence tags

C.-Y. Wang · C.-Y. Chiou · R. Krishnamurthy · J. Tan · K.-W. Yeh (✉)
Institute of Plant Biology, National Taiwan University, Taipei, 106, Taiwan
e-mail: ykwbppp@ntu.edu.tw

H.-L. Wang
Department of Life Science, National University of Kaohsiung, Kaohsiung, Taiwan

J. Tan
College of Bioinformatics, Chongqing University of Posts and Telecommunications, Chongqing 400065, China

Present Address:
S. Venkatagiri
Department of Botany, Karnatak University, Dharwad, India

Introduction

Carbohydrates are important nutritients and energy sources in living organisms. The structural components of the cells are largely made up of carbohydrates. During plant growth and development, photoassimilates produced by the source leaf are translocated toward different sinks for utilization or accumulation. The source leaves accumulate large foliar carbohydrate pools to buffer variations in the rate of photosynthesis and to liberate sufficient sucrose at night to ensure a more or less continuous supply of sucrose for sink organs in many plant species (Geiger 1987). Recent evidence has

shown that carbohydrates are able to modulate gene expression. High levels of carbohydrate could inhibit the expression of genes involved in photosynthesis (Van Oosten and Besford 1994), and increase the expression of genes involved in nitrate assimilation, growth, storage and starch remobilization (Thomas and Rodriguez 1994; Baier et al. 2004). It implicates that carbohydrates can perform more complex functions involved in plant growth and development, in addition to structural and nutritional functions (Rook et al. 2006).

Oncidium "Gower Ramsey" has a long erect and brilliant inflorescence consisting of many small, yellow flowers. A marked morphological feature of this orchid, like many other epiphytic orchids, is the presence of pseudobulb that plays the role of a strong sink for the storage of carbohydrates (Yong and Hew 1995; Hew and Ng 1996). Its pseudobulb is of the heteroblastic type and with a single internode (see Fig. 1). The plant requires about 1 year of vegetative growth to develop into a mature pseudobulb. In the vegetative growth period, photosynthates transported from epiphytic leaves are converted and accumulated in the developing pseudobulb organ. When the tissues of the developed pseudobulb are full of glutinous polysaccharides, the plant may shift into the reproductive stage from the vegetative stage, and a flower bud initiates at the base of the pseudobulb. It requires about 2–3 months for complete inflorescence development from flower bud. Accompanying with the flower development, the carbohydrate content in the pseudobulb is used up. So far, the environmental and endogenous physiological factors triggering the flowering process are still ambiguous.

During the past 10 years, *Oncidium* hybrid, such as Goldiana was reported as a C_3 shade plant (Hew and Yong 1994). The carbohydrate pool in the pseudobulb during inflorescence development has also been observed (Ng and Hew 1996). Reserved carbohydrates, such as sucrose, glucose and fructose accumulate in the pseudobulb of Gower Ramsey before the initiation of inflorescence (Wang et al. 2003). The sucrose level was transiently elevated to 20 mg g^{-1} DW at inflorescence developing stage (B_1 stage), relative to ~10 mg g^{-1} DW at the inflorescence pre-initiation stage (Wang et al. 2003). Then, it declined to ~10 mg g^{-1} DW, accompanying the gradual decrease of glucose and fructose level during the late inflorescence developing stage. Recently, a polysaccharide from the pseudobulb of the inflorenscence developing stage was

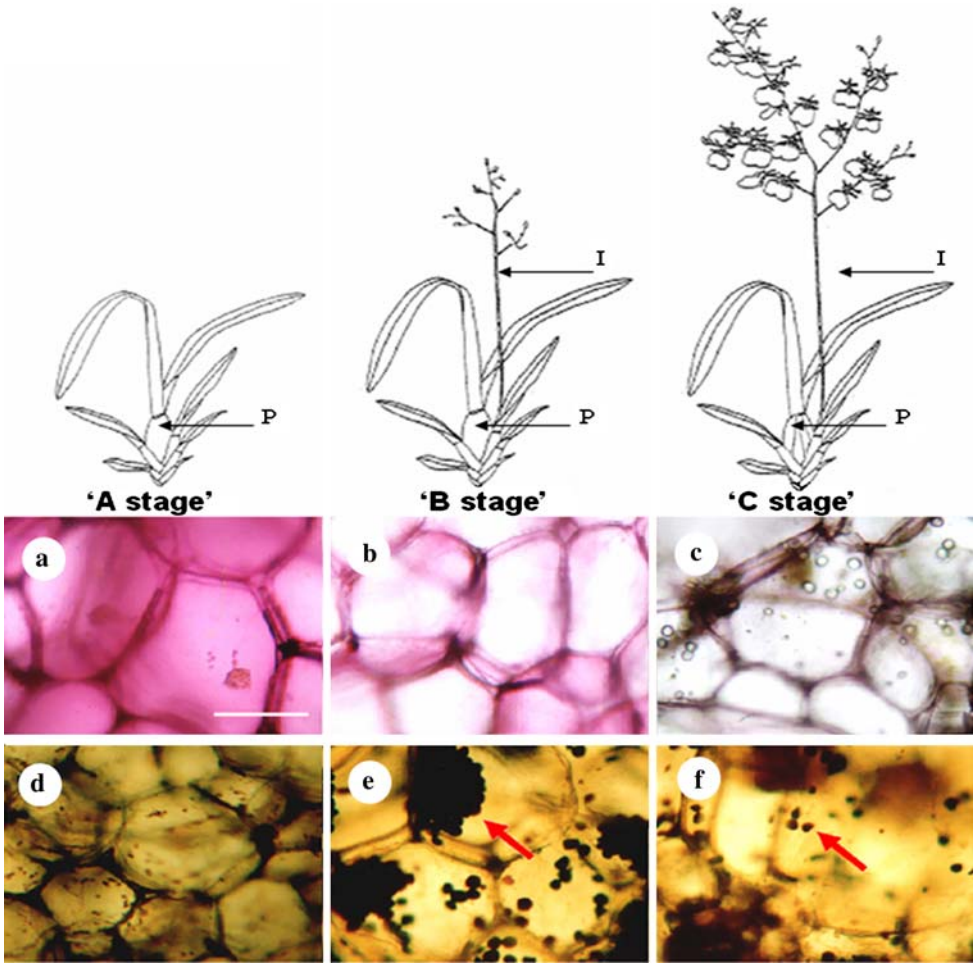

Fig. 1 Transverse sections of the pseudobulb stained with ruthenium red for pectin (**a**–**c**), and iodine for starch (**d**–**f**) at different stages of inflorescence development. **a**, **d** Inflorescence pre-initiation stage (*A stage*). **b**, **e** Middle inflorescence developing stage (*B stage*). **c**, **f** Late inflorescence stage (*C stage*). *I* inflorescence, *P* pseudobulb. *Embedded arrows* indicate starch grains. Plant pictures are redrawn from the original diagram of Hew and Yong (1994)

identified and characterized as pure mannan, consisting of more than 95% mannose and a high degree of uniformity in structure (Wang et al. 2006). Although a direct link between the biochemical changes of carbohydrate metabolism in the pseudobulb and in the flowering process has been proposed (Hew and Ng 1996; Wang et al. 2003), the molecular evidence for the metabolic control of the flowering process is still scanty. To understand the accumulation, interconversion and degradation of different polysaccharides in the developmental pseudobulb more precisely, each polysaccharide form should be analyzed individually. As the first molecular approach to demonstrate carbohydrate metabolism and gene regulation correlated with the flowering process in the pseudobulb, we focus on the identification and expression pattern of the functional genes related to carbohydrate metabolism in the pseudobulb of *Oncidium* hybrid Gower Ramsey. The carbohydrate metabolism in the pseudobulb was monitored by determining the expression level and the enzymatic activity of the relevant genes. In this work, a novel profile of polysaccharide mobilization in *Oncidium* pseudobulb, which starts from sucrose to mannan, then to starch, was clarified. Furthermore, the functional role of carbohydrate mobilization during the flowering process of *Oncidium* pseudobulb has been discussed.

Materials and methods

Plant materials

Oncidium Gower Ramsey plants were obtained from Shih-Dong orchid nursery in Taiwan, and were grown in 30 cm diameter pots under controlled conditions at 25–32°C, and a 14 h photoperiod in a glasshouse. Natural irradiation was supplemented with artificial illumination (high pressure sodium lamps of 400 W) to maintain a regular photon flux density of 1,000 μmol $m^{-2}s^{-1}$.

Histochemical staining of polysaccharides of the pseudobulb

Free-hand sections of the pseudobulb were taken at different stages, as indicated in the legend to Fig. 1. Pectin was localized in the tissues following the method described by Willats et al. (2001). The sections were stained with 0.02% (w/v) aqueous (deionized water) solution of ruthenium red and incubated in the dye for 10 min before microscopic observation. Starch was localized following potassium iodide and iodine staining (Caissard et al. 2004). The sections were directly stained for 15–45 min in 0.5% iodine water containing 1% potassium iodide. The stained sections were observed under a compound microscope (Olympus, IMT-2, Japan) and then the images were photographed.

Extraction and determination of polysaccharides from the pseudobulb

Extraction and determination of starch was performed as described by Wang et al. (2003) using potato starch as standard and 2, 2′-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) for the color reaction. Starch was estimated as the amount of glucose released by measuring the absorbance of the reaction at 405 nm.

Mannans were isolated from the pseudobulb according to the method described by Mulimani and Prashanth (2002) with little modification. The pseudobulb powder was stirred in water at 80°C for 5 h and filtered through two layers of Miracloth. The residue was extracted once again with hot water (5 ml/g tissue) for 1 h and filtered. The filtrates were combined and TCA was added to the final concentration of 5% (w/v) while stirring the solution at low speed. The contents were kept at 4°C for 1 h for the precipitation of the protein and centrifuged at 20,000*g* for 30 min. The supernatant was collected and filtered through two layers of Miracloth. The mannans were precipitated by the addition of chilled ethanol to a final concentration of 80% (v/v) and keeping it at 4°C overnight. The precipitate was collected by centrifugation at 20,000*g* for 30 min at 4°C. Mannans pellet was washed with a mixture of ethanol and ether (1:1, v/v) and dried in vacuum. The quantity of mannans was determined gravimetrically.

Pectin was isolated from the alcohol insoluble residue of the pseudobulb (Sobry et al. 2005). The fine powder of pseudobulb was dispensed in 80% ethanol (5 ml/g tissue) and boiled for 40 min and filtered (Stancato et al. 2001). The residue was washed twice with 80% ethanol followed by acetone and air dried to get alcohol insoluble residues (AIR). Starch was removed from the AIRs by suspending it in 90% (v/v) dimethylsulfoxide for 16 h at 20°C and centrifuging at 20,000*g* for 20 min (Stolle-Smits et al. 1999). The pectic polysaccharide was extracted from the starch-free AIR following the method described by Western et al. (2001) with minor modification. The AIR was stirred in 0.5% ammonium oxalate solution (25 ml/g AIR) at 80°C for 1 h and centrifuged at 20,000*g* for 20 min. The supernatant was collected and ethanol was added five times the volume of the extract to precipitate pectic polysaccharides. The fibrous precipitate was collected by filtration through four layers of Miracloth and vacuum dried and weighed.

Analysis of mannans and pectins by high-performance anion exchange chromatography

Mannans and pectins were dissolved in water and analyzed on high performance anion exchange chromatography (HPAEC) chromatographic equipment (Dionex Corp., Sunnivale, CA, USA) fitted with CarboPac PA-100 anion

exchange column (2 × 250 mm; Dionex; with the PA-100 guard column) and electrochemical detector ED-50 (Dionex). Samples were eluted with 0.1 M NaOH (A) and 0.5 M NaOAc in 0.1 M NaOH (B) gradient with the following scheme: 0 min, 90% A and 10% B; 30 min, 10% A and 90% B; 30–40 min, 10% A and 90% B; and 40–50 min, 100%B with a flow rate of 0.25 ml/min. The column was equilibrated to initial conditions for 10 min between each run. Mannan from *Saccharomyces cervevisiae* (Sigma, M7504) and pectin from citrus fruit (Sigma, P9135) were used as standard in HPAEC identification.

Acid hydrolysis and HPAEC-PAD analysis

The monosaccharide composition of pectin and mannans was determined by acid hydrolysis followed by HPAEC-PAD on Carbopak PA10 column (Dionex). At 30°C for 30 min, 10 mg of pectin/mannan was pre-incubated in 0.1 ml of 72% H_2SO_4 and then the mixture was diluted to 2 N H_2SO_4 by the addition of 1.1 ml of MilliQ water. The hydrolysis was carried out at 120°C in an autoclave for 1 h. After acid hydrolysis, the mixture was neutralized with 2 N NaOH (prepared from 50% NaOH; Fisher Scientific, Pittsburgh, PA, USA) before HPAEC analysis.

The monosaccharides were analyzed by HPAEC-PAD with ED 50 detector (Dionex) and CarboPak PA-10 analytical column (2 × 250 mm) with PA-10 guard column (Dionex). Acid hydrolysates of mannans were analyzed by eluting the sugars isocratically with 20 mM NaOH for 20 min followed by 200 mM NaOH for 10 min with a flow rate of 0.25 ml/min. The column was equilibrated with 20 mM NaOH for 10 min between each run of samples in a sequence. Acid hydrolysates of pectins were analyzed mainly according to the method described by Talaga et al. (2002) with a convenient modification. The gradients of NaOH and NaOAc were used simultaneously to elute the sugars by mixing NaOH, NaOAc and water appropriately to generate the following gradient of NaOH: 0–15 min, 18 mM; 15–18 min, 18–100 mM; 18–45 min, 100 mM; 45.1–50 min, 18 mM. The simultaneous gradient of NaOAc was: 18–35 min, 0–250 mM; 35.1–45 min, 500 mM. The column was equilibrated for 10 min with 18 mM NaOH between each run of samples in a sequence. The PAD was set to the waveform as specified in the manufacturer's (Dionex) instructions. Peaks were identified based on the relative retention time of peaks of authentic monosaccharide standards (Sigma). Sugars were quantified after calibration of PAD response to concentration of different sugars.

RNA preparation and Northern-blot assay

The total RNA extraction and Northern-blot analysis were carried out as described earlier (Tan et al. 2005). In brief, the total RNAs of the pseudobulb and its upper leaf were extracted following the pine tree method (Chang et al. 1993). A weight of 10 μg each of total RNAs from the pseudobulb and its upper leaf were separated on 1% agarose/formaldehyde gel and blotted onto nylon membranes (Amersham, Bucks, UK). The EST cDNA fragments were randomly labeled with α-p-^{32}P-dCTP (R*edi*prime^{TM} II Kit, Amersham) as probe to hybridize the RNA-blotted membranes and the membranes were washed following the standard protocol. The membrane was exposed to fluorescent plate for 12 h (Typhoon 9400, Amersham). Band intensities were normalized with respect to the amount of mRNA loaded.

Extraction and quantification of key carbohydrate metabolizing enzymes from different developmental stages of *Oncidium* pseudobulb

Plant protein from *Oncidium* pseudobulb was extracted using P-PER plant protein extraction kit (Pierce Chemical Co., Rockford, IL, USA), according to the manufacturer's instructions. In brief, 0.5 g of pseudobulb tissue at different development stages was ground into fine powder in liquid nitrogen and dissolved in 2–3 ml of Hepes extraction buffer (pH 7.5). After centrifugation, the supernatant was eluted through Amicon Ultra filter column (Millipore, Bellerica, MA, USA). The concentration of the total protein was estimated by Bradford method (Bradford 1976) and the protein was adjusted to 1 μg per μl. The protein was immediately used for different enzymatic assay or immediately aliquoted and stored frozen at −80°C until further use. The quality of the crude protein was checked by SDS-PAGE resolution and by using RPN8 antibody for Western-blot assay as an internal standard.

Assay of invertase activity

The invertase activity was assayed following the method described by Dreier et al (1998), with some modification. Pseudobulb tissues were ground in liquid nitrogen and suspended in 1 ml of pre-cooled (4°C) separation buffer. The samples were centrifuged at 15,000*g* for 15–30 min at 4°C and both soluble invertase (supernatant) and wall-bound invertase (pellet) enzymes were assayed separately. The pellets were resuspended in 1 ml of the same solution and used for subsequent enzymatic tests. For each sample, 100 μl of the solution was diluted with 0.2 M Na acetate buffer (pH 4.0 for soluble invertase assays and pH 5.5 for wall-bound invertase assays) to a final volume of 600 μl. The enzymatic reaction was triggered by adding 800 μl of 0.225 M sucrose and supplemented with 50 μl of 1 g/l glucose to avoid oxygen interference at low reducing sugar concentration. An aliquot of 700 μl was pipetted off to a

fresh tube as the A_1 sample and was incubated at 30°C for 1 h. To the remaining mixture, designated as A_0 sample, was immediately added 1 ml of 1% (w/v) DNSA, 0.5 M KOH and 1 M Na/K tartrate, and heated for 10 min at 100°C to stop reaction. The reaction of sample A_1 was stopped as described above after 1 h. The absorbance of the reaction samples was measured at 560 nm. The invertase activity was calculated as the difference in reduced sugar levels between the samples A_1 and A_0. Invertase activities were represented as µg of glucose formed in 1 ml of extract per hour and mg of total protein.

Assay of sucrose synthase activity

The assay mixture contained 50 µg of enzyme along with the substrate 0.1 M sucrose and 500 µM UDP (480 µl) at pH 6.4. The mixture was incubated at 37°C for 60 min. This reaction was terminated by adding 60 µl 1 M Tris buffer (pH 8.8) and boiling the reaction mixture (1 ml) at 80°C for 10 min. To this coupling enzyme reaction buffer containing 0.2 U/ml UDP-glucose dehydrogenase, 0.03 M NAD^+ and water were added. NAD^+ reduction was measured at 340 nm using a U-3200 Hitachi spectrophotometer (Hitachi, Tokyo, Japan). The initial absorbance at 340 nm prior to the addition of NAD^+ to the reaction mixture was taken as reference blank. The reduction in A_{340} by the addition of NAD^+ was noted every minute until no further change in the absorbance was observed.

Assay of GDP-mannose pyrophosphorylase

GDP-mannose pyrophosphorylase (EC 2.7.7.13) was assayed according to Marolda and Valvano (1993). The reaction was started by the addition of Na PP_i (freshly prepared) to give a final concentration of 1 mM. The enzyme activity was monitored by measuring the A_{340} using Hitachi U-3200 spectrophotometer (Hitachi). One unit of enzyme activity was defined as the activity that reduced 1 µM of NADP min^{-1} at 25°C.

Assay of β-mannosidase

β-Mannosidase enzyme assay was performed by taking 60 µl (50 µg protein) of the extract and incubating it with 90 µl of 2 mM *p*-nitrophenyl β-D-mannopyranoside (Sigma) in McIlvaine buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 5.0) for 2 h at 37°C. The reaction was terminated by the addition of 75 µl of 0.2 M aqueous sodium carbonate. The absorbance of the color developed by the reaction is read at 405 nm, considering the extinction coefficient of *p*-nitrophenyl to be 18,400 to calculate the enzyme activity in terms of pmol min^{-1} g^{-1} fresh weight.

Assay of β-1,4-mannose endohydrolase

A known quantity of the enzyme (50 µg) was added to 800 µl of the reaction mixture containing 200 mM acetate buffer (pH 5.0) with 100 mM NaCl, 1% insoluble substrate (AZCL-galactomannan; Megazyme, Bray, County Wicklow, Ireland). The reaction was stirred continually at 37°C. Aliquots (200 µl) were taken at different times and heated for 5 min at 100°C. This was further centrifuged at 12,000*g* for 5 min and absorbance of the supernatant was read at 595 nm on a microplate reader. Enzyme activity is expressed as Δ595 mg^{-1} min^{-1} (Marraccini et al. 2001).

Assay of granule-bound starch synthase (GBSS)

The reaction mixture (200 µl) consisted of 20 µl (50 µg) of the enzyme extract, 100 mM Bicine (pH 8.5), 25 mM potassium acetate, 10 mM DTT, 5 mM EDTA, 1 mg amylopectin and 1 mM ADP [U-^{14}C] glucose (Amersham) at 1.15 GBq/mol. The reaction mixture was incubated for 10 min at 25°C and the reaction was terminated by heating to 90°C for 2 min. Control assays were terminated immediately after the addition of amylopectin. To the reaction mixture, 3 ml of 75% aqueous methanol containing 1% (w/v) KC1 was added and incubated at room temperature for 5 min. It was further centrifuged at 2,000*g* for 5 min and the supernatant was discarded. The pellet obtained was redissolved in 0.3 ml water. This washing and resuspension after centrifugation was repeated twice and the radioactivity was determined by liquid scintillation counting (Denyer et al. 1997).

Assay of starch phosphorylase

The reaction mixture contained 50 mM sodium acetate, pH 5.4, 0.3% (w/v) potato soluble starch (Sigma S-2630), 8 mM potassium Glc-1-P, and the enzyme sample in a final volume of 100 µl. After incubation at 37°C for 10 min in a microtiter plate, 200 µl of ferrous sulfate molybdate solution was added to each well, and the inorganic phosphate released was determined according to the procedure of Fiske and Subbarow (1925). The mixture was incubated for 10 min at room temperature and the absorbance at 650 nm was measured by an ELISA reader.

Assay of β-amylase

β-Amylase activity was determined as described by Wang et al. (1995) by adding 50 µg of the protein extract to 50 µl β-amyl reagent (Megazyme) followed by incubation at different time intervals. The reaction was terminated by the addition of 300 µl of 1% Tris to 20 µl of the reaction mixture. After thorough mixing, 150 µl of the solution was

pipetted on to a microplate and the A_{410} was determined. One unit of activity was defined as the quantity of enzyme that released 1 mM of *p*-nitrophenol min^{-1} (Gana et al. 1998).

Results

Changes in polysaccharide composition in *Oncidium* pseudobulb during inflorescence development

In order to investigate the changes in carbohydrate quality and content in the pseudobulb, three stages of pseudobulbs were selected (Fig. 1): "A stage" (inflorescence pre-initiation stage), "B stage" (inflorescence developing stage) and "C stage" (late inflorescence stage, when flowers develop). The accumulation of starch and pectin content in the pseudobulb tissues was determined by iodine and ruthenium red staining. Since ruthenium red can stain carboxyl groups of acidic sugars of demethylated pectins (Iwai et al. 1999), intense staining indicates that the mucilaginous material of the pseudobulb was largely made up of demethylated pectins at the inflorescence pre-initiation stage (A stage). Transverse sections of pseudobulb for pectin showed that the tissue was intensively stained at inflorescence pre-initiation of A stage (Fig. 1a). A decrease in the stain intensity during the later stages of the pseudobulb development was observed (Fig. 1b, c). Consistently, a reduction in the total was also observed (Fig. 2a). Further, the amount of mannan was also determined and the maximum amount of mannan was present in the pseudobulb at inflorescence pre-initiation (Fig. 2c).

Transient accumulation of starch in the pseudobulb of the inflorescence developing stage (starting from B_1 to B_2 stage) was found by iodine staining (Fig. 1e) and quantification of starch in the pseudobulb (Fig. 2b). This accumulation occurred after the sucrose peak (Wang et al. 2003). Lower level of starch was subsequently observed in the late inflorescence development stage (C stage; Fig. 1f). These data showed that mannan and pectin highly accumulated in the inflorescence pre-initiation stage (A stage), but gradually degraded in the inflorescence emergence and developing stages (B and C stage). On the other hand, starch was notably accumulated in the inflorescence developing stage (B stage), but decreased strongly in the late inflorescence stage (C stage).

Compositional changes in polysaccharides at three different developmental stages of pseudobulb were detected and quantified by HPAEC

The changes in the polysaccharide pool of the pseudobulb during three different developmental stages of pseudobulb were analyzed by HPAEC using pulsed amperometric detection (PAD) and UV detection. The purity of mannans was ascertained by the appearance of a single prominent peak in the HPAEC chromatogram (Fig. 3a). Monosaccharides of mannans in the pseudobulb are mannose, galactose, glucose and a small percentage of arabinose as determined by HPAEC. The results showed that there was no significant change in the mannan content and galactose and glucose level from the young to the older stage of pseudobulb (data not shown), indicating a prominently quantitative change, accompanied by a very slight qualitative change in mannans.

The qualitative change in pectin was more prominent during the flowering process as seen in the HPAEC profile of pectin (Fig. 3b). This qualitative change was further observed as a compositional change in the monosaccharides of pectin during the flowering process (Fig. 4).The relative percentages of rhamnose, arabinose and galactose of pectin were found to decrease in the pseudobulbs, whereas, those of glucose and galacturonic acid increased in the pseudobulb with the growth of the inflorescence axis (Fig. 4). An increase in the ratio of homogalacturonan to rhamnogalacturonan was also evident in the pseudobulb during flowering.

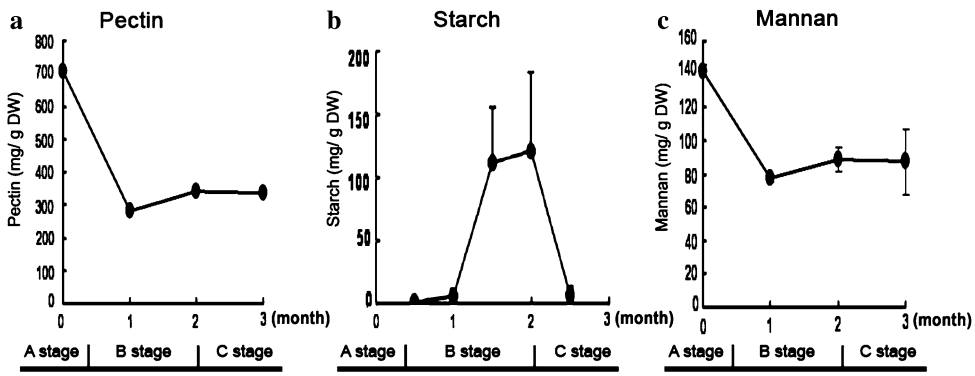

Fig. 2 Changes in pectin (**a**), starch (**b**), and mannan (**c**) levels in the pseudobulb of *Oncidium* at different times during the development of inflorescence. *Vertical bars* indicate standard error of the mean of two independent observations

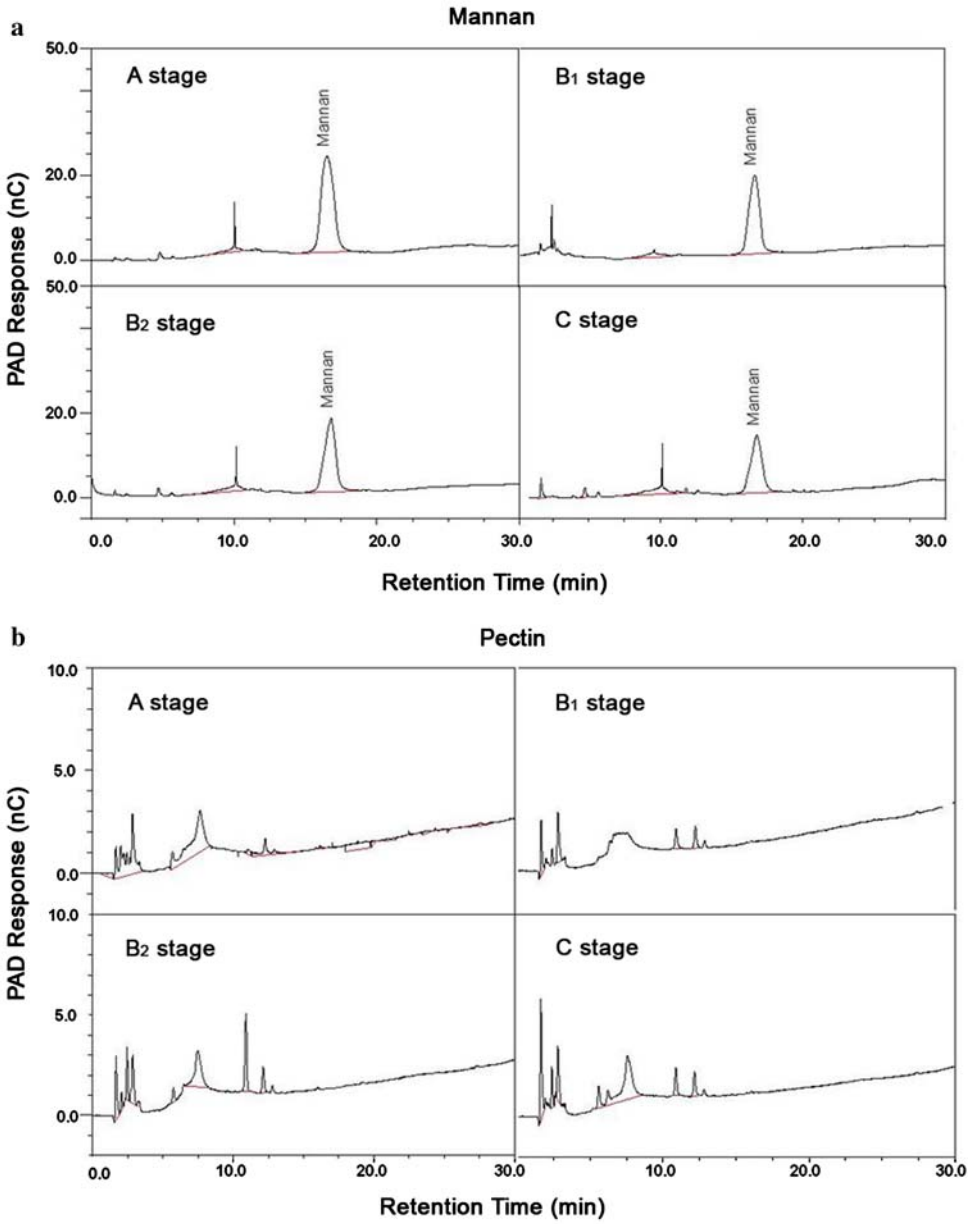

Fig. 3 HPAEC profile of polysaccharides of *Oncidium* pseudobulb at three different developmental stages of inflorescence. **a** HPAEC chromatogram showing qualitative alternation in mannans. **b** HPAEC chromatogram showing qualitative alteration in pectin. The stages are: *A* inflorescence pre-initiation stage, B_1 early inflorescence developing stage, B_2 middle inflorescence developing stage and *C* late inflorescence stage

Expression pattern of genes related to carbohydrate metabolism in the pseudobulb during three different developmental stages of inflorescence

To investigate the functional genes related to the carbohydrate metabolism in *Oncidium* pseudobulb, we searched in a subtractive EST library, which was previously generated by subtracting leaf ESTs from pseudobulb EST of A stage pseudobulb (Tan et al. 2005). Consequently, 40 EST clusters related to carbohydrate metabolism were identified and grouped (Table 1). They were classified into sucrose, mannan, starch, pectin and other carbohydrate-related enzyme groups (Table 1). The EST category of genes involved in carbohydrate metabolism suggests that *Oncidium* pseudobulb is an enclave of carbohydrate metabolic network of sucrose–mannan–starch–pectin interconversion.

Based on the EST gene information (Table 1), the temporal expression pattern of four groups of genes related to sucrose, mannan, starch and pectin metabolism was investigated by Northern-blot analysis. Altogether, 13 EST gene representatives for the key enzymes in carbohydrate metabolism were selected to analyze their abundance in pseudobulb at three different stages of inflorescence development (Fig. 5). The selection of these genes for mRNA blot analysis was based on their key positions in driving the reactions in the metabolic network, which connects sucrose, starch, mannan and pectin metabolism. Among them, sucrose synthase (EC 2.4.1.13) and invertase (EC 3.2.1.26) are

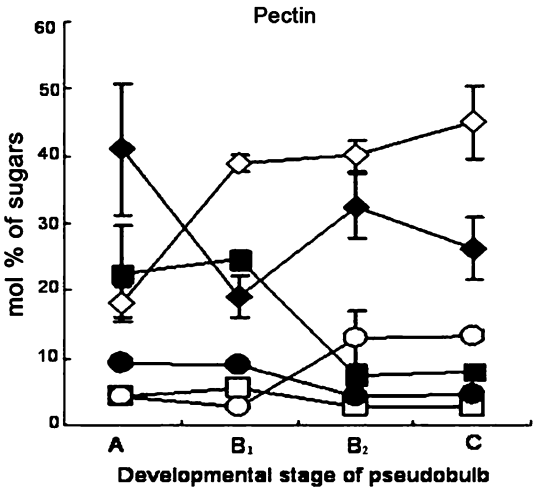

Fig. 4 Compositional change in pectin at three different developmental stages of pseudobulb. Sugar composition of pectin in the pseudobulb was analyzed by HPAEC (*open squares*, rhamnose; *filled circles*, arabinose; *filled squares*, galactose; *open circles*, glucose; *filled diamonds*, xylose/mannose and *open diamonds*, galacturonic acid). The stages are: *A* inflorescence pre-initiation stage, B_1 early inflorescence developing stage, B_2 middle inflorescence developing stage and *C* late inflorescence stage

involved in sucrose metabolism; mannose-6-phosphate isomerase (EC 5.3.1.8), GDP-mannose pyrophosphorylase 1 (EC 2.7.7.13), GDP-mannose pyrophosphorylase 2 (EC 2.7.7.13), mannosyltransferase (EC 2.4.1.32), β-mannosidase (EC 3.2.1.25) and β- (1, 4)-mannan endohydrolase (3.2.1.78) belong to mannose-related metabolic pathway. Granule-bound starch synthase (EC 2.4.1.21) and starch phosphorylase (EC 2.4.1.1) catalyze starch synthesis and degradation, respectively. Pectin esterase (EC 3.1.1.11), polygalacturonase (EC 3.2.1.15) and pectate lyase (EC 4.2.2.2) are involved in pectin metabolism (Table 1).

Both, sucrose synthase and invertase genes, were actively expressed in young and developing pseudobulb (A stage, Fig. 5a, b). The genes encoding mannan biosynthetic enzymes such as mannose-6-phosphate isomerase, GDP-mannose pyrophosphorylase-1, GDP-mannose pyrophosphorylase-2 and mannosyltransferase were active in the pseudobulb of inflorescence pre-initiation (A stage, Fig. 5c–f), and early inflorescence developing stage (B_1 stage). Genes for mannan degrading enzymes such as β-mannosidase and β-(1, 4)-mannan endohydrolase, however, were active in the middle inflorescence developing stage of the pseudobulb (B_2 stage, Fig. 5g, h). The expression level of granule-bound starch synthase peaked at the inflorescence-developing stage (B_1 and B_2 stage, Fig. 5i). However, the mRNA transcripts of starch phosphorylase were abundant in the later inflorescence stage of the pseudobulb (B_2 and C stage; Fig. 5j). On the other hand, the expression of pectin esterase, polygalacturonase and pectate lyase genes were simultaneously observed in the pseudobulb inflorescence pre-initiation and in the early stage of inflorescence development (Fig. 5k–m).

Enzyme activity of the key carbohydrate metabolizing enzymes in the pseudobulb during three different developmental stages of inflorescence

Northern-blot analysis on mRNA transcripts provides no direct evidence for metabolic activity. To link the transcription level with biochemical function, the specific activities of the above-mentioned carbohydrate metabolizing enzymes in different stages of inflorescence development have been investigated. To confirm the correspondence of the transcriptional and translational levels, several key enzymes were selected to assay enzyme activity. They include: (1) two sucrose hydrolytic enzymes, invertase and sucrose synthase; (2) one mannan biosynthetic enzyme, GDP-mannose pyrophosphorylase; (3) two mannan degrading enzymes, β-mannosidase and β-1,4-mannose endohydrolase; (4) one starch biosynthetic enzyme, granule-bound starch synthase; (5) one starch degrading enzyme, starch phosphorylase. In addition, activity assay of β-amylase was performed. Although the β-amylase EST gene was not discovered in the EST library, it is considered as an essential enzyme catalyzing the production of maltose from linear glucans. It is abundant in both photosynthetic tissues and in starch storage organs of different plant species. Our present results showed that the biochemical activity patterns of these selected enzymes largely correlated with their transcription profiles. The invertase activity was mostly related to the wall-bound enzyme (Fig. 6a). The highest level of invertase activity, both wall-bound and soluble invertase, was found in the stage of inflorescence pre-initiation (A stage), the same as was found for the gene transcriptional level (Fig. 5a). The enhanced enzymatic activity of sucrose synthase (Fig. 6b) during the inflorescence pre-initiation stage was in accordance with the high transcription profile of sucrose synthase gene (Fig. 5b) indicating the role of the enzyme in the breakdown of sucrose for storage and development of pseudobulbs. However, its activity was again high during the late inflorescence stage (Fig. 6b), possibly due to a low turnover rate of protein degradation or increased translation. The distinctly decreased activities of invertase and sucrose synthase appearing at B_1 stage of the inflorescence development (Fig. 6a, b) were evidently in accordance with a transient higher level of sucrose accumulation in the pseudobulb (Wang et al. 2003). It indicated that both the cell wall and soluble invertases responded to the physiological state and metabolic needs. Likewise, the specific enzyme activity of another key enzyme, GDP-mannose pyrophosphorylase, was higher at the inflorescence pre-initiation stage (Fig. 6c). This elucidates the preferential accumulation of mannan during the early stage of the

Table 1 EST annotations of genes related to carbohydrate metabolism in the *Oncidium* pseudobulb during flowering stage

GI number	Putative identification of gene	Reference organism	*E* value	ESTs[b]
Sucrose-related				
22347630	Sucrose synthase	*Oncidium*	1.00E−96	6
22347630	Sucrose synthase	*Oncidium*	2.00E−27	2
22347630	Sucrose synthase	*Oncidium*	1.00E−38	2
22347630	Sucrose synthase	*Oncidium*	4.00E−30	1
1352468	Beta-fructofuranosidase 1 (invertase)	*Zea mays*	2.00E−31	1
Mannose-related				
15225896	Phosphomannomutase	*Arabidopsis thaliana*	2.00E−16	2
15225553	GDP-mannose pyrophosphorylase 1	*Arabidopsis thaliana*	2.00E−23	2
29893656	GDP-mannose pyrophosphorylase 2	*Oryza sativa*	1.00E−13	2
17226270[a]	Beta-mannosidase	*Lycopersicon esculentum*	1.00E−13	1
15220627	Beta-1,4 mannan endohydrolase	*Arabidopsis thaliana*	6.00E−36	1
15232927	Mannose-6-phosphate isomerase	*Arabidopsis thaliana*	8.00E−50	1
11275529	Mannose-6-phosphate isomerase	*Oryza sativa*	6.00E−45	1
22326970	Mannosyltransferase	*Arabidopsis thaliana*	4.00E−15	1
Starch-related				
30699056	ADP-glucose pyrophosphorylase	*Arabidopsis thaliana*	4.00E−66	2
20042976	Epimerase/dehydratase	*Oryza sativa*	1.00E−56	3
15242099	Aldose-1-epimerase	*Arabidopsis thaliana*	3.00E−65	2
12585316	Phosphoglucomutase (cytoplasmic)	*Solanum tuberosum*	2.00E−84	1
25408401	Glucosyltransferase	*Arabidopsis thaliana*	2.00E−26	1
15626365	Granule-bound starch synthase	*Pisum sativum*	4.00E−86	1
2351056	Phosphoglucose isomerase	*Dioscorea septemloba*	2.00E−80	1
29893646	Nucleoside-diphosphate-sugar pyrophosphorylase	*Oryza sativa*	6.00E−68	4
13195430	Alpha 1,4-glucan phosphorylase L isozyme	*Oryza sativa*	1.00E−43	1
12658431	Starch phosphorylase	*Ipomoea batatas*	2.00E−31	1
Pectin-related				
6174913	Pectin esterase 1	*Lycopersicon esculentum*	1.00E−13	4
13958032	Polygalacturonase	*Pisum sativum*	3.00E−57	3
15231926	NAD-dependent epimerase/dehydratase	*Arabidopsis thaliana*	2.00E−63	1
7406669	Ripening-related protein	*Vitis venifera*	4.00E−79	3
18412253	Polygalacturonase	*Arabidopsis thaliana*	2.00E−22	1
20161185	Pectin esterase	*Oryza sativa*	1.00E−12	2
10177179	Pectate lyase	*Arabidopsis thaliana*	4.00E−23	1
Others				
20514290	Beta-galactosidase	*Oryza sativa*	7.00E−63	1
18461259	Beta-galactosidase	*Oryza sativa*	2.00E−49	1
5441877	Glycogenin glucosyltransferase	*Oryza sativa*	5.00E−84	1
5441877	Glycogenin glucosyltransferase	*Oryza sativa*	7.00E−93	1
11264291	Alpha-galactosidase	*Arabidopsis thaliana*	3.00E−33	1
20161490	Beta-1,3 glucanase	*Oryza sativa*	7.00E−39	1
9998899	Cinnamyl alcohol dehydrogenase	*Populus balsamifera*	3.00E−38	1
21594350	dTDP-glucose 4-6-dehydratase	*Arabidopsis thaliana*	1.00E−16	1
30686654	*N*-acetylglucosamine-phosphate mutase	*Arabidopsis thaliana*	8.00E−38	1
1351279	Triosephosphate isomerase, cytosolic (TIM)	*Petunia hybrida*	4.00E−27	1

EST clusters are grouped into sucrose-related, mannose-related, starch-related, pectin-related and other sugars hydrolytic enzymes. Databank of Oncidium EST library was deposited in the website http://plantbio.lifescience.ntu.edu.tw/english/estdatabase.htm

[a] Full length cDNAs were completed by RACE and deposited in the Genebank (Accession no. DQ 289592; DQ 289593; DQ 289594; DQ 289595)

[b] The frequency of EST contig

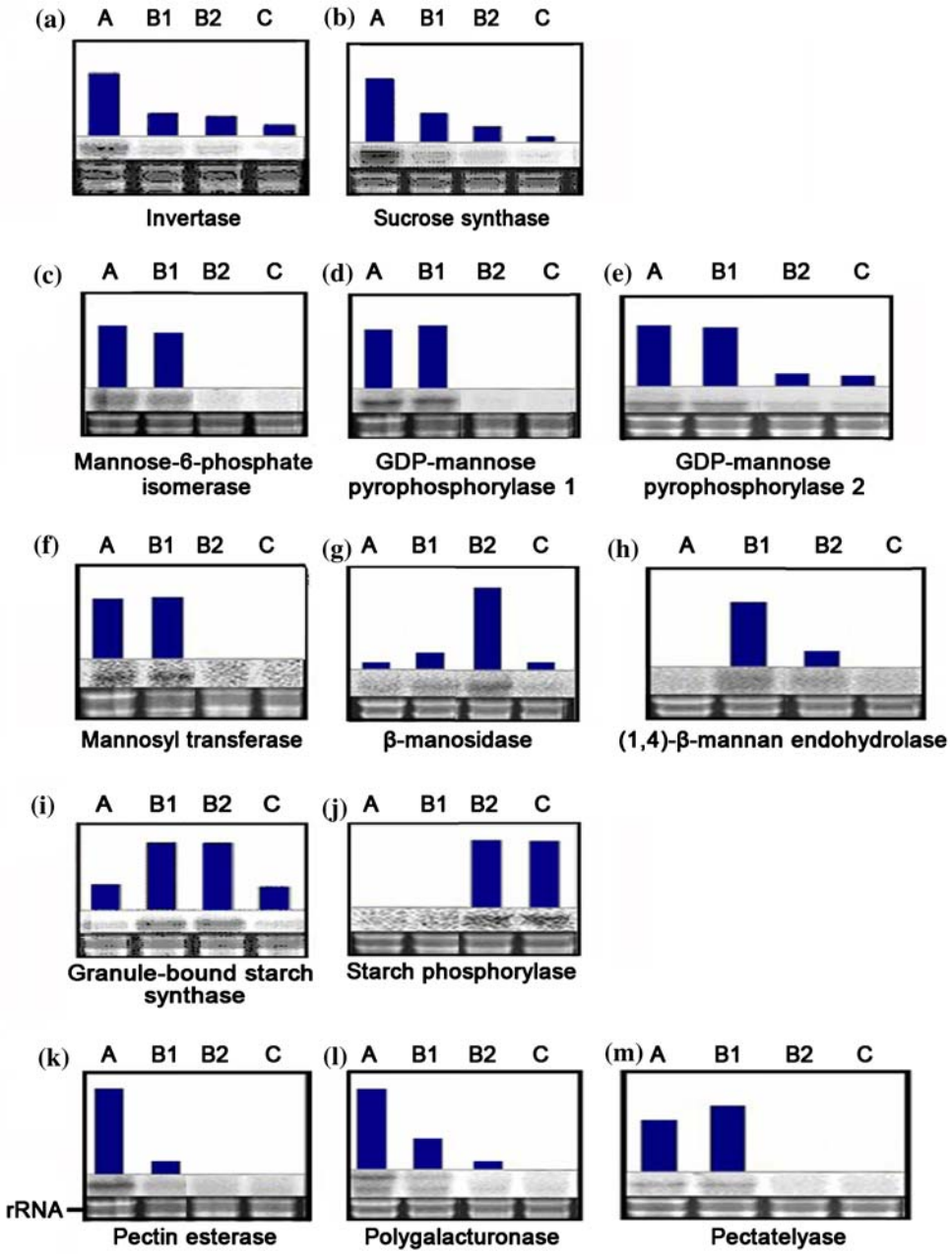

Fig. 5 Northern-blot analysis of mRNA transcripts related to sucrose, mannan, starch and pectin metabolism in the pseudobulb at different stages of inflorescence development: Relative intensities of Northern blots are represented by *histograms*. Stages: *A* inflorescence pre-initiation stage, B_1 early inflorescence developing stage; B_2 middle inflorescence developing stage, C_1 late inflorescence stage

pseudobulb development for further mobilization. The accumulated mannans were degraded by β-mannosidase and β-1,4-mannose endohydrolase to yield galactose, mannose, glucose, etc. The activities of β-mannosidase and β-(1,4)-mannan endohydrolase also correlated with their transcript levels and with increased enzymatic activities observed at early or middle stage of inflorescence developing stage (Fig. 6d, e). This shows that these enzymes facilitate the mobilization of mannans towards the synthesis of starch. The synthesis of starch through the degradation of mannan during the inflorescence development stage may be due to the higher enzymatic activity of granule-bound starch synthase (Fig. 6f). A correspondent increase in the transcript level of this gene encoding the enzyme was also found (Fig. 5i). The activity of starch phosphorylase in the pseudobulb of *Oncidium* was observed to be higher during the flowering stage (B_2 and C stage, Fig. 6g). This provides evidence for the role of starch phosphorylase in replenishing glucose-1-phosphate, a key compound in carbohydrate metabolism for energy supply to flower development. Furthermore, β-amylases are known to hydrolyze α-1,4-glucosidic linkages from the reducing ends of polysaccharide chains to form maltose (Beck and Ziegler 1989). Although it was not discovered from substractive EST categories, its function was predictable. From the β-amylase activity assay, it was evident that the enzyme activity was relatively

higher at the middle and late inflorescence developmental stage (Fig. 6h), which explains its prominent role in starch degradation. Although no enzyme activity data of mannose 6-phosphate isomerase, mannosyl transferase, ADP-glucose pyrophosphorylase, pectin esterase, polygalacturonase and pectatelyase were shown in this work, we suppose that the results are similar to those of the enzymes that we have assayed above.

Discussion

The pseudobulb of *Oncidium* orchid is a strong sink for partitioning the photosynthates during vegetative development. During its growth, the photosynthates produced in the upper younger leaves are transported to the developing pseudobulb sink. In our present study, carbohydrates in *Oncidium* pseudobulb of different developmental stages were analyzed by histochemical staining and HPAEC. Polysaccharides of mannan and pectin are extremely abundant in young and developing pseudobulbs (Figs. 1, 2, 3) at the inflorescence pre-initiation stage (designated as A stage). It is well known that plants at the vegetative stage always convert sugar photosynthates into polysaccharides and accumulate them in sinks as nutritional and energy sources. As the inflorescence initiates from the pseudobulb base (designated as B stage), *Oncidium* switches its life cycle from the vegetative stage to the reproductive stage. Concurrently with inflorescence initiation, the polysaccharides of mannan and pectin start to mobilize in the pseudobulb.

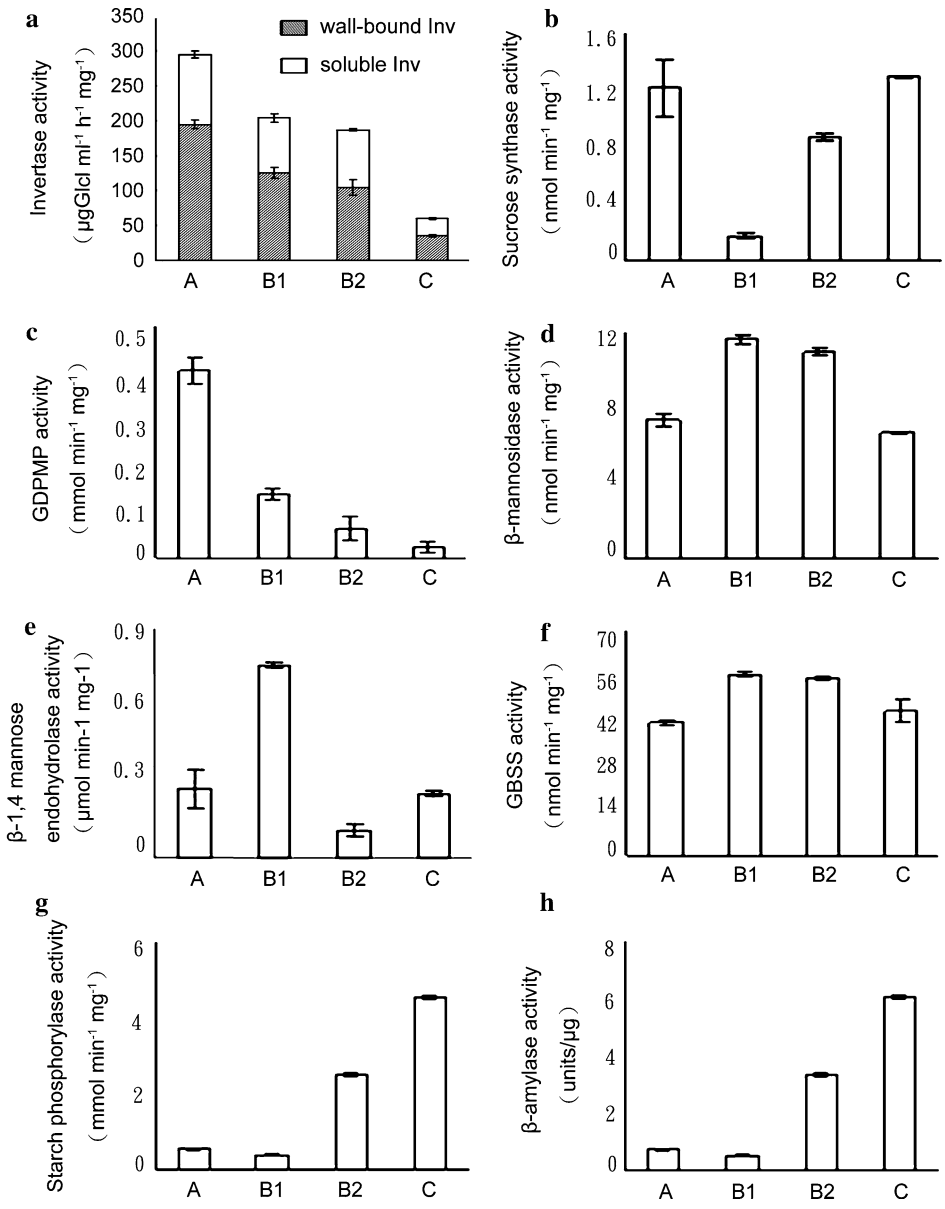

Fig. 6 Biochemical activities of several key carbohydrate metabolizing enzymes in the *Oncidium* pseudobulb at different developmental stages. The enzymes assayed are invertase (**a**), sucrose synthase (**b**), GDP-mannose pyrophosphorylase (**c**), *β*-mannosidase (**d**), *β*-1, 4 mannose endohydrolase (**e**), granule-bound starch synthase (**f**), starch phosphorylase (**g**), *β*-amylase (**h**). The enzyme activity was calculated after 60 min of reaction with the appropriate substrate. The unit of enzyme-specific activity of the respective enzymes is described in "Materials and methods". Developmental stages: *A* inflorescence pre-initiation stage, B_1 early inflorescence developmental stage, B_2 middle inflorescence developmental stage, *C* late inflorescence stage. The data represent the combined results of two independent experiments. *Error bars* indicate the variability between duplicate assays of duplicate extracts

This results in the appearance of starch grains from the early inflorescence developing stage (B_1 stage). Concomitant with the growth and development of inflorescence (B_2 stage), starch grains are gradually degraded for energy supply and eventually used up in the late inflorescence stage (designated as C stage) during which the floral organs are complete developed.

In accordance with the previous reports describing that sucrose, mannan and starch occurred in the carbohydrate pool of the pseudobulb in the inflorescence pre-initiation stage (Wang et al. 2003, 2006), our present results now demonstrate carbohydrates starting from sugar to mannan/pectin and to starch in the pseudobulb during inflorescence development. At stage A of the inflorescence pre-initiation, high-level activities of invertase and sucrose synthase function to cleavage sucrose for mannan and pectin synthesis (Fig. 6a, b). A drop-off of sucrose synthase and invertase activities subsequently occurs in B_1 stage of the pseudobulb (Fig. 6a, b), concomitant with a transient peak of the sucrose level (Wang et al. 2003). Thus, the transient accumulation as well as degradation of sucrose at an early inflorescence stage correlates with the transient decline in sucrose synthase activity, while invertase activity remains low. The starch grains start to accumulate at the B_1 stage and peak at B_2 stage (Fig. 2b). This metabolic network implicates that sucrose plays a functional role to regulate starch biosynthesis during the inflorescence developmental stage of pseudobulb. It is well known that elevated sugars can up-regulate genes for enzymes involved in starch biosynthesis, including granule-bound starch synthase and branching enzyme (Salehuzzaman et al. 1994). In our previous work (Wang et al. 2003), sucrose content was monitored at an elevated level during early inflorescence developing stage (correspondent to B_1 stage). Coincidentally, the high expression level of starch synthase was detected at the same stage (Figs. 5i, 6f), and starch grains were apparently observed (Figs. 1e, 2b). This confirms that a highly elevated sucrose level precedes the start of starch biosynthesis.

Even though the *Oncidium* orchid has become a valuable commercial product in the flower market, comprehensive information concerning its physiology and molecular biology by systematic research is still scarce and is awaited. In this regard, in addition to the above-mentioned biochemical data, our subtractive EST data bank provided an informative knowledge base for functional gene studies (Tan et al. 2005). The subtractive EST genes, which are preferentially expressed in pseudobulb could be functionally categorized into sucrose-, mannan-, starch-, and pectin-metabolizing groups in this work. Further, Northern-blot data demonstrate that the expression pattern of each gene varied, but the expression levels correlated with the pseudobulb and inflorescence development at a relative level (Fig. 5). The gene transcriptional patterns were confirmed/complemented by biochemical activity assay of the gene-encoded enzymes (Fig. 6). These molecular data indicate that carbohydrate mobilization in flowering pseudobulb involves extensive transcriptional regulation of sucrose, mannan, pectin and starch metabolic pathways. This metabolic gene regulatory network and the associated carbohydrate mobilization are a novel finding in this orchid plant.

The presence of an apparent metabolic gene regulatory network for carbohydrate metabolism in the *Oncidium* pseudobulb during the flowering process

Northern-blot analysis of mRNA transcripts as well as biochemical activities of the enzymes related to carbohydrate metabolism and various patterns of carbohydrate levels confirm carbohydrate metabolic interconversion in the *Oncidium* pseudobulb. As shown in Fig. 7, sucrose produced in the leaves is transported to the pseudobulb before the onset of inflorescence initiation and is immediately metabolized. It is widely accepted that sucrose utilization in plant sink organs depends on its cleavage through the action of either sucrose synthase or invertase. The latter group of enzymes, including wall located and cytoplasmic form, catalyzes the hydrolysis of sucrose and other β-fructose-containing oligosaccharides (i.e., raffinose) into glucose and fructose. Sucrose synthase often functions in the degradative direction, interchangeably converting sucrose and UDP to fructose and UDPGlc. UDP-glucose thus synthesized could be a possible central metabolite for mannan or pectin metabolism. Activity of sucrose synthase and invertase are closely related to the extent of sucrose import in many systems. Sucrose synthase is considered to be a reasonable indicator of "sink strength" in a number of instances (Sung et al. 1994). As shown in Fig. 6a, invertase activity was mostly related to the wall-bound enzyme, suggesting that the function of invertase likely was more closely correlated with sucrose partitioning /import (Tymowska-Lalanne and Kreis 1988; Sung et al. 1994). The high expression level of sucrose synthase and invertase indicates that the young developing pseudobulb is a strong sink at the inflorescence pre-initiation stage (Fig. 5a, b). Our data suggest that the expression of both genes encoding sucrose-cleaving enzymes is developmentally regulated. In addition, high-level expression of mannose-6-phosphate isomerase, phosphomannomutase, GDP-mannose pyrophosphorylases and mannosyltransferase (Fig. 5c–f) with a concomitant increase in mannan content (Figs. 2, 3) before/at inflorescence pre-initiation stage (Fig. 5a–f) implies that sucrose is metabolized for the synthesis of mannans or pectin preferentially to starch (Fig. 7). Although accumulation of mannans for nutrition supply or storage is very seldom in nature, it appears that the storage of mannans in the *Oncidium*

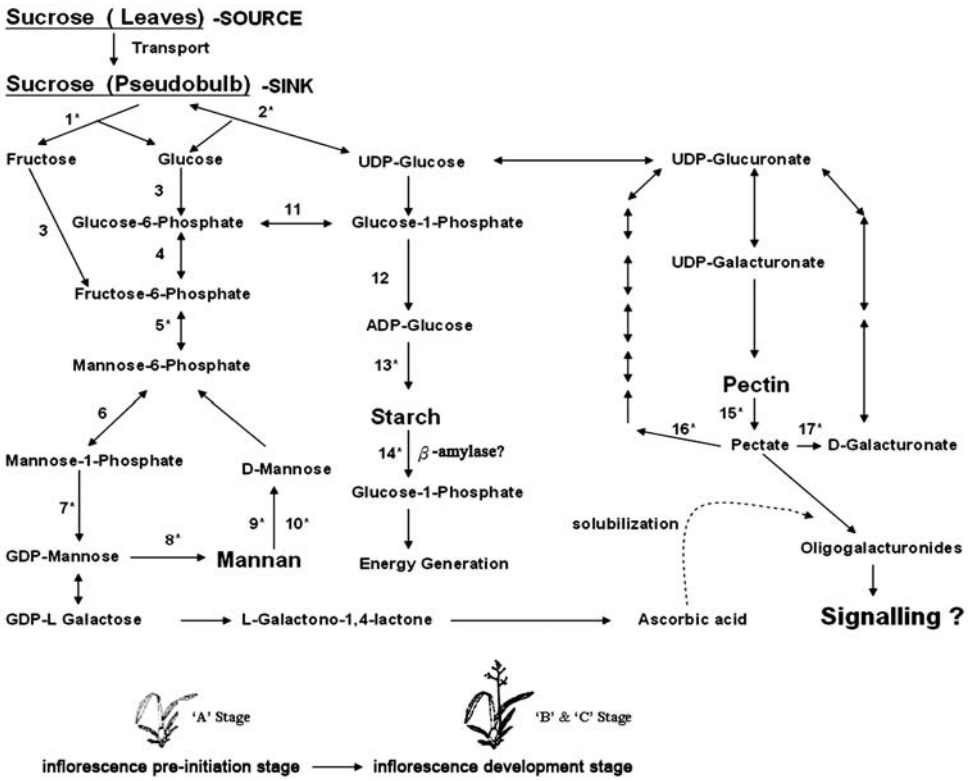

Fig. 7 Carbohydrate metabolic pathway in the pseudobulb of *Oncidium* during the flowering process (see text for details). Sucrose, which is transported from leaves to the pseudobulb is preferentially metabolized to yield precursors of mannan and pectin. Accumulation of mannan and pectin takes place at the inflorescence pre-initiation stage (*A stage*), and there is a transient accumulation of starch after the inflorescence is about 1 month old (*B stage*). The large amount of energy required to sustain floral development (*C stage*) is provided by the degradation of starch in the pseudobulb at an inflorescence age of about 2 months. The model interrelates sucrose, mannan, starch and pectin metabolism in order to explain the probable links in the generation of signals inducing flowering. Enzymes: *1* invertase, *2* sucrose synthase, 3 hexokinase, *4* glucose-6-phosphate isomerase, *5* mannose-6-phosphate isomerase, *6* phosphomannomutase, *7* GDP-mannose pyrophosphorylase, *8* mannosyltransferase, *9* β-mannosidase, *10* β-(1,4)-mannan endohydrolase, *11* phosphoglucomutase, *12* ADP-glucose pyrophosphorylase, *13* granule-bound starch synthase, *14* starch phosphorylase, *15* pectin esterase, *16* pectate lyase, *17* polygalacturonase. * Northern-blot analysis was performed for these enzymes

pseudobulb is a preparatory step to face the metabolic outburst during the flowering process. It is possible that mannans also play some functional roles in plant metabolism toward abiotic stress tolerance, such as low water availability/water storage capacity and pathogen resistance (Buckeridge et al. 2000; Ni et al. 2004). The accumulated mannan is eventually mobilized by β-(1, 4)-mannan endohydrolase and β-mannosidase (Figs. 5, 7) into D-mannose (Fig. 7, step 9 and 10), and subsequently starch is synthesized via intermediates of Glu-6-P, Glu-1-P and ADP-Glu in the pseudobulb (Fig. 7, step 11 and 12). Transient accumulation of starch in the pseudobulb of the inflorescence developing stage (B Stage, Figs. 1, 2b) is exemplified by increased mRNA transcripts for granule-bound starch synthase (Fig. 5i). Mobilization of sucrose to mannan and then to starch requires the role of another two key enzymes, phosphoglucose isomerase and phosphoglucomutase, and the correspondent genes have been found in the EST library (Table 1). Ultimately, starch is mobilized to meet the energy requirement of floral development by the action of starch phosphorylase and/or β-amylase releasing glucose-1-phosphate as observed by a high level of mRNA transcripts (Fig. 5j) and enzyme activities (Fig. 6g, h), respectively, at the late inflorescence stage.

The pectin metabolism is also linked to the mobilization of sucrose in the pseudobulb before initiation of inflorescence (Fig. 7) as shown by the increased pectin content in this stage (Figs. 1, 2c). A qualitative change in pectin was more prominent in the pseudobulb than in mannan during inflorescence development (Figs. 3b, 4). This qualitative change in pectin is probably brought about by the action of pectin esterase (pectin methylesterase) into demethylated pectin. The mRNA transcripts for pectin modifying enzymes were at a higher level during the inflorescence pre-initiation stage (Fig. 5k, l) and early inflorescence developing stage (Fig. 5m). This modification was evident on ruthenium red staining (Fig. 1a–c).

Physiological significance of the sucrose–mannan–starch metabolic pathway in the *Oncidium* orchid

The metabolic network occurring in the *Oncidium* orchid appears to be a complicated cycle. An intermediate polysaccharide reservoir of mannan seems a redundant step in the biological utilization. However, mannan has also a function in stress resistances to drought and pathogen infection.

Photosynthetic production of sugars and starch is regulated by the demand for a sink. Carbohydrate mobilization is required for the transition from vegetative to reproductive growth in most plants. Some evidence has been provided that sucrose is an early and essential component of flowering stimulus in most species. Soluble sugars have been implicated in the regulation of developmental processes, such as the timing of flowering (Bernier et al. 1993). The increased supply of sucrose triggers the start of its activation, suggesting that the extra sucrose plays a signaling role in stimulating flowering (Corbesier et al. 1998), and the extra sucrose usually comes from reserve (starch) mobilization. In the *Oncidium* pseudobulb, starch mobilization was monitored at the inflorescence developing stage (B_2 stage) for the energy supply of floral development. Although starch mobilization is thought to be critical for floral induction in most plants (Corbesier et al. 1998), it seems that the transition mechanism from vegetative to reproductive growth in *Oncidium* is not correspondent to the onset of starch mobilization in the pseudobulb. This suggests that the mechanism of irregular flowering time of *Oncidium* might be affected/ controlled by some more factors rather than sucrose alone.

In addition to this, we have observed high levels of ascorbic acid in the pseudobulb just before inflorescence initiation (results not shown). Previous reports have shown that L-ascorbate precursor, galactono-1,4-lactone accumulates in the pseudobulb of *Oncidium* (Wang et al. 2003). In the present context, it should be inferred that the expression of GDP-mannose pyrophosphorylase (Fig. 5d and e) is involved in the production of GDP-mannose, a key intermediate in the production of L-ascorbic acid through the Smirnoff-Wheeler biosynthetic pathway (Smirnoff et al. 2001) in the early stage of inflorescence development. In addition to its antioxidant action, L-ascorbic acid plays a role in the non-enzymatic solubilization of pectins into OGAs (Dumville and Fry 2000, 2003), and causes the compositional change of pectin. This appears coincident with the finding of the substantial alternation of pectin composition (Figs. 3b, 4). OGAs have been considered as non-traditional plant growth regulators with the ability to induce flowering along with other functions (Creelman and Mullet 1997; Etzler 1998). OGAs are also implicated in the induction of oxidative burst as a defense response of plants to pathogen attack, ethylene response and fruit ripening (Dumville and Fry 2000). Although OGAs have not been detected in this work, the physiological function is still predictable.

This numerous interactions and intersections, which occur are potentially important for the modulation and balancing of various inputs from different signaling cascades so that plants can integrate all this information to execute the proper developmental responses. The differences in the transcript levels and enzymatic activity are important, but for many biological processes in plants the interrelated metabolic pathways seem to be enigmatic and provide a better understanding of the underlying mechanisms. Therefore, the regulatory metabolic network occurring in *Oncidium* pseudobulbs could be a source of developmental signals for inflorescence induction. Further investigation of the specificity and physiological significance of this carbohydrate metabolic gene regulatory network and the carbohydrate mobilization in the pseudobulb tissue of *Oncidium* orchids is needed.

Acknowledgments The authors are grateful to the National Science Council, Taiwan for the financial support granted to Professor Kai-Wun Yeh under the project NSC-93-2317-3-002-012. The authors are also grateful to Professor Zhang, R-H and Professor Wang A-Y, at the Department of Biochemistry, NTU, for their assistance in the enzymatic activity assays of starch phosphorylase and sucrose synthase, respectively. Appreciation is expressed to Associate Professor Hsieh H-L, at the Institute of Plant Biology, NTU for his kind provision of RPN8 antibody.

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