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Cloning and Characterization of Novel Soluble Acid Invertase Which is Responsible to JA, ABA and GA During Tip Growth of Pea Seedlings (*Pisum sativum*)

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Abstract − The enzyme invertase contributes to sugar unloading, pathogen defense, differentiation and development in plants. We cloned the complete cDNA of a soluble acid invertase from pea seedlings (*Pisum sativum*) via RT-PCR and the rapid amplification of the cDNA end (RACE) technique. The full-length cDNA of the soluble pea invertase comprised 2237 bp and contained a complete open reading frame encoding 647 amino acids. The deduced amino acid sequence showed high homology to soluble acid invertases from various plants. Northern blot analysis demonstrated the soluble acid invertase gene of *P. sativum* was strongly expressed in sink organs such as shoot tips and root tips, and induced by abscisic acid, gibberellic acid and jasmonic acid in shoots. Especially, gibberellic acid enhanced the gene expression of the soluble acid invertase in a time-dependent manner. This study presents that the gene expression patterns of a soluble acid invertase from pea are strongly consistent with the suggestion that individual invertase gene product has different functions in the growing plant.

Key words : gene expression, gibberellic acid, pea, soluble acid invertase

INTRODUCTION

Plant growth is regulated by many different factors, for example, the availability of minerals or organic nutrients, plant hormones and growth regulators. Organic nutrients derived from photosynthetic products are generated in tissues that are involved in carbon and energy fixation, called 'sources' The initial products from these sources are small carbohydrate molecules, which are converted into translocatable compounds in cells. These compounds flow through the plant vascular system to non-photosynthetic or growing tissues, called 'sinks'. Among these translocatable compounds, sucrose, disaccharide fructosyl glucose, is pre-eminent (Frommer and Sonnewald 1995; Fernie *et al*. 2002; Koch 2004). During sucrose transport, the pathway from source to sink may involve the cleavage of sucrose into its derivatives, glucose and fructose, or direct movement of sucrose. Sucrose utilization in sink tissues also involves the cleavage of sucrose into its component hexoses. These hexoses or resultant hexose derivatives affect plant growth in a variety of ways. They play critical roles as a source of energy in respiration and general carbohydrate metabolism, contributing to the building of the plant cell wall, and to the synthesis of energy storage molecules such as starch. Their presence as simple molecules in a solution also contributes to the control of turgor pressure, which is related to the elongation of cells and the growth of the plant (ap Rees 1988; Anderson *et al*. 2002; Proels *et al*. 2003; Mitsuhashi *et al*. 2004).

The invertase (beta-fructofuranosidase: E.C.3.2.1.26) is a key enzyme for the cleavage of sucrose, hydrolyzing sucrose to its two components: glucose and fructose. According to the pH optimum and cellular location, plant invertases can be separated into four groups: soluble acid invertase (vacuo-

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lar), insoluble acid invertase (extracellular), insoluble alkaline/neutral invertase, and soluble alkaline/neutral invertase (Fernie *et al*. 2002; Koch 2004). The term 'insoluble' identifies activity that is extractable only by high ionic strength buffers. Soluble acid invertases have optimum activity between pH 3 and pH 5 with a vacuolar location (Roitsch *et al*. 1995) while soluble alkaline invertases can have optimal activity between pH 7 and 9 with a cytoplasmic location (Copeland 1990). Little is known about insoluble alkaline invertases. Several studies have suggested that soluble acid invertases are confined to the vacuole (Unger *et al*. 1994) and other studies have suggested that they are also located in the free space of cytoplasm (Fahrendorf and Beck 1990). Since invertase is the first enzyme in sucrose utilization it is not surprising that invertases are associated with a variety of cell activities such as stem and root elongation, phloem unloading, regulation of sink strength, and transition of source tissues to sink tissues (Tymowska-Lalanne and Kreis 1998a). Invertase activity has also been implicated in pathogen and wounding responses (Sturm and Chrispeels 1990) and the determination of sugar composition in fruit (Sonnewald *et al*. 1997). Invertase activity has been reported to be regulated by cytokinin (Godt and Roitsch 1997), ethylene (Linden *et al*. 1996), gibberellic acid (Wu *et al*. 1993), abscisic acid (ABA) and jasmonic acid (Zhang *et al*. 1996).

The present study was initiated to continue earlier work on pea invertases, specifically to investigate a gene and gene expressions involved in the production of soluble acid invertases and promoted by recurring reports that increased invertase activity results from GA-induced growth of dwarf pea seedlings (Moll 1971) as well as carbohydrate repartitioning caused by environmental changes such as drought resistance in bean (Cuellar-Ortiz *et al*. 2008). In the current study we determined the full-length cDNA sequence of a soluble acid invertase from pea, characterizing the gene expression in specific tissues as well as a transcriptional regulation by gibberellic acid.

MATERIALS AND METHODS

1. Plant material

Garden pea (*Pisum sativum*), Little Marvel (dwarf) was employed in this study. To obtain etiolated tissue, pea seeds were surface-sterilized by washing in 10% Clorox solution for 10 min before rinsing in sterile distilled water and planted in autoclaved vermiculite. Seedlings were grown in a greenhouse at Sungkyunkwan University, Korea under natural light conditions. The stems, leaves, roots and root tips of 14-day-old seedlings were harvested for RNA extraction. Tissues were collected in liquid nitrogen and stored at −70°C for RNA extraction.

2. GA, JA and ABA application

An ABA sol ution [100 μM ABA (Sigma) in sterile water with 0.01% (v/v) ethanol and 2 drops Tween-20/L], and GA solutions [15 μM GA_3 (Sigma) with 2 drops Tween-20/L] were applied to 12-day-old dwarf pea seedlings by aerial spraying until the entire surface of each plant was uniformly moistened. JA (Sigma) was dissolved in *N*, *N*-dimethylformamide (100 mM stock solution) and subsequently diluted in water to a 100 μM solution. The JA solution was also applied to each plant as described for the ABA and GA solutions. Water (with 2 drops Tween-20/L)-sprayed pea plants served as controls. Shoot tissues were harvested at 24 hr after H_2O, ABA, GA, and JA treatments and RNAs were extracted from the tissues immediately after harvest. For a time-course induction of invertase mRNA by GA treatment, shoot apexes were harvested at 3 hr, 6 hr, 12 hr, and 24 hr after GA treatment.

3. cDNA cloning

Total RNA was isolated by an RNeasy Plant Kit (QIAGEN) according to the manufacturer's description. Poly-A RNA was collected from the total RNA using the Poly (A)Tract mRNA Isolation System (Promega). Double-stranded cDNA was synthesized using a Marathon cDNA Amplification Kit (Clontech) and PCR was performed using a SMART RACE cDNA Amplification Kit (Clontech) with a 3′ RACE primer (5′-GATAGTAACGGCGTTTGGACAGGCTCTG-3′) and a 5′ RACE primer (5′-TCCTTCGGTAGTAAGCCAAGC-TGTCGTC3-′).

4. Northern blot analysis

Twenty micrograms of RNAs extracted from various tissues were separated by formaldehyde-agarose gel electrophoresis and blotted onto nylon filters. A 560 bp of a partial cDNA in-

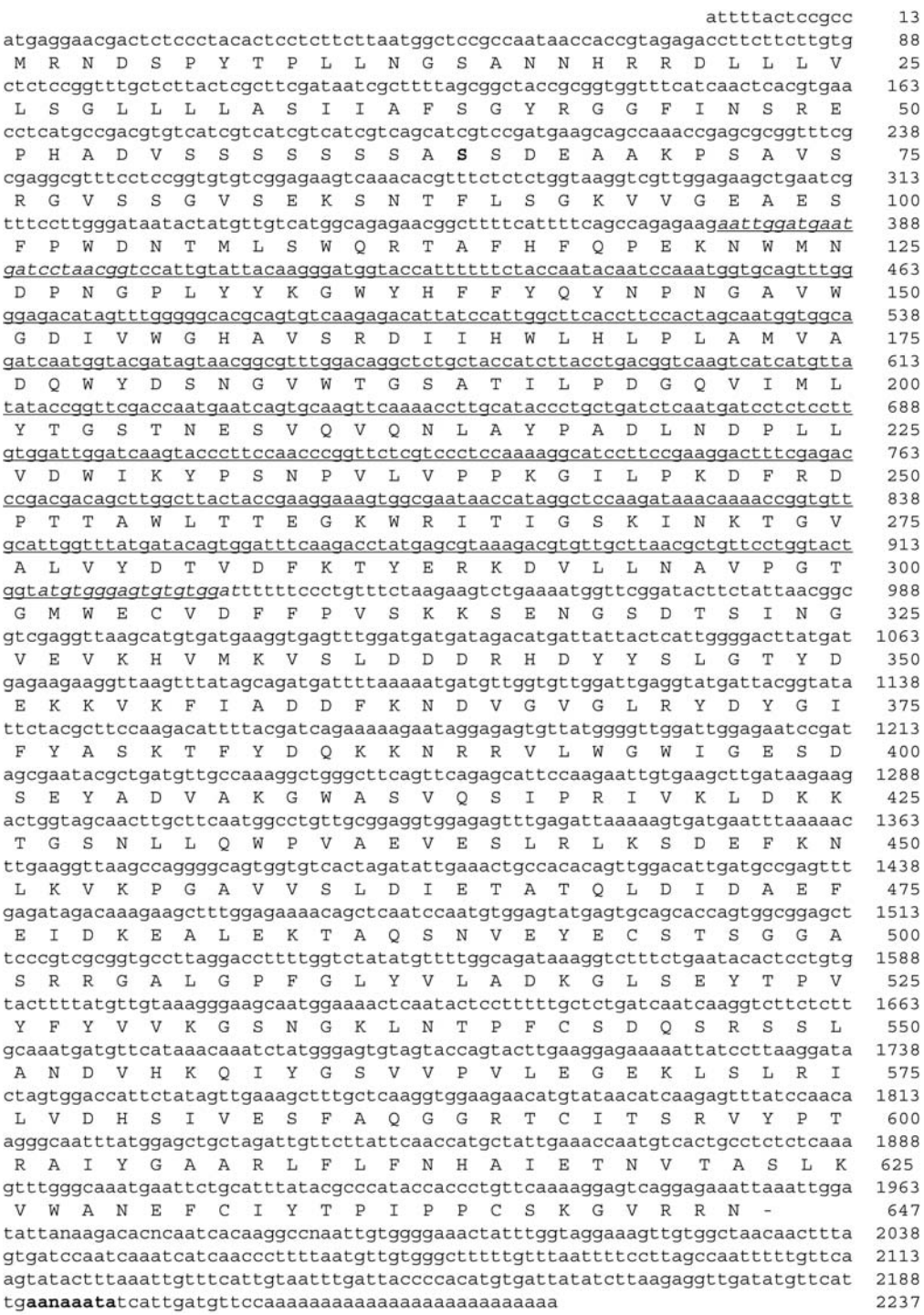

Fig. 1. The nucleotide and deduced amino acid sequences of *P. sativum* soluble acid invertase cDNA. The PCR primer sequences used by Zhang *et al.* (1996) are italicized. The sequence of PsI-P used as a probe in northern blotting is underlined. The amino acid sequences are in one-letter code. A potential poly-adenylation signal is bold.

sert probe (PsI-P, Fig. 1) was labeled with alkali-labile digoxigenin (DIG)-dUTP using a DIG HIGH Prime Kit (Boehringer Mannheim) according to the manufacturer's protocol. Hybridization and detection were performed according to The DIG User's Guide (Boehringer Mannheim) with anti-digoxigenin-alkaline phosphatase-Fab fragments with the

chemiluminescent substrate, CDP-Star. Band intensity was measured with a densitometer (UN-SCAN-IT, Silk Scientific).

RESULTS AND DISCUSSION

Studies of invertases concentrating on the gene or sequence started in the 1990s. An organ-specific expression of invertase genes, comparing an insoluble acid and soluble acid has been reported in broad bean (Weber *et al.* 1995) and in carrot (Sturm *et al.* 1995). These research groups suggested that a differently localized expression of invertase genes reflects a different role for each enzyme. In broad bean, three distinct cDNAs were cloned using a PCR-based approach, and a nucleotide sequence comparison indicated one soluble and two insoluble acid invertases. One of the insoluble acid invertase mRNAs was detected by *in situ* hybridization to be expressed in the chalazal vein and the inner rows of the thin-walled parenchyma of the seed coat, whereas the gene expression of the other insoluble invertase was confirmed in both roots and seed coats. The soluble acid invertase was expressed in sink organs such as roots, stems, flowers, seed coats, and sink leaves (Weber *et al.* 1995). In carrot seedlings, transcripts of three genes, one insoluble and two soluble acid invertases, were investigated in sink and source organs. High transcript levels of the insoluble invertase were only found in primary roots. The two soluble invertase gene transcripts were accumulated mainly in roots, one predominately in primary roots and the other in developing tap-roots (Sturm *et al.* 1995). Northern blot analysis in the current study showed that the soluble acid invertase gene of *P. sativum* was strongly expressed in sink organs such as young stems and root tips but very weakly in leaves, roots and mature stems. This pattern is very similar to the report that the *V. faba* vacuolar invertase gene is expressed in sink organs such as roots, stems, flower, pods, seed coats, and sink leaves (Weber *et al.* 1995). Apparently PsI-1 was not expressed in some specific tissues, but rather in almost all sink organs. However, the insoluble acid invertase mRNA from pea identified by Zhang *et al.* (1997) was apparently accumulated only in roots. Wu *et al.* (1993) reported that an insoluble acid invertase mRNA was accumulated in pea stem tissues in response to treatment with the growth regulator, gibberellin, whereas Zhang *et al.* (1996) showed that the mRNA of a pea insoluble acid invertase was virtually absent in shoot tissue and it was not detected even after gibberellin treatment on seedlings. The insoluble acid invertase transcript was increased dramatically in shoots in response to wounding (Zhang *et al.* 1996).

1. Identification of cDNA sequence for soluble acid invertase

In previous work (Zhang *et al.* 1997), three PCR products were isolated with identical primers and sequenced. Two clones were proved to be identical to a published sequence (DDBJ/EMBL/GenBank accession No. X85328) and the nucleotide sequence of the other clone was substantially different. The 560 bp long cDNA fragment (PsI-P) was sequenced and the deduced amino acid sequence was compared with a protein database (data not shown). From the comparison data, PsI-P was indicated to have high homology to soluble acid invertases from members of the same family (Fabaceae). As results of RACE, the 5′ RACE product was about 0.9 kb and the 3′ RACE product was about 1.9 kb. The products were cloned into a pCRII-TOPO vector and sequenced. The two sequences overlapped over approximately 300 base pairs. They were merged into one cDNA sequence identified as PsI-1 with a total length of 2237 base pairs (AY112702). The PsI-1 insert had a single open reading frame of 1941 bp, encoding 647 amino acids residues (Fig. 1). A potential poly-adenylation signal 'AANAAT' was identified, starting at position 2191, 13 nucleotides upstream from the poly-A tail. The comparison of the PsI-1 cDNA nucleotide sequence with the GenBank database indicated 92% homology to a vacuolar invertase of *Vicia faba* (Z49831), 83% to a *Vigna radiata* invertase (D10265), and 82% to a *Phaseolus vulgaris* soluble acid invertase (U92438). Based on the homology searches using BLASTX, the deduced amino acid sequence of PsI-1 shared substantial identity with soluble acid invertases from various organisms (Fig. 2). In alignment with amino acid sequences of invertases previously reported, PsI-1 had 84% identity and 87% positives to *V. faba* (CAA89992), 82% identity and 90% positives to *P. vulgaris* (AAB68679), and 72% identity and 80% positives to *V. radiata* (BAA01-107).

2. Tissue specific gene expression

A soluble invertase can be regulated by a variety of external and internal signals. In tomato, the expression of a soluble invertase gene (TIV1) can be repressed by glucose and

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Psa   M-RNDSPYTPLLNGSANNHRR-DLLLVLSGLLLLASIIAFSGYRGGFINSREPHADVS--
Vfa   M-RNDSPYTPLLNASHNNHRRRELLLLFSGLLLLASIIAFS------AYIAQPHADAD--
Vra   MEHHKPLLPTSSHAAPTSSTRKDLLFVLCGLLFLSSLVAYGGYRASGVPHAHLSSPTSNH
pvu   MEHHKPLLPTSSHAAPNPRTRKDLLLLLCALLFLSSLVAFGRNRASNVPHDHVSSSASNH

Psa   SSSSSSASSDEAAKPSAVSRGVSSGVSEKSNTFLSG-KVVGEAESFPWDNTMLSWQRTAF
Vfa   V*****IL****TR*TTL***************LSGNL***GG****N**********
Vra   QQDHQ*PT*LPSS*WYP************SNL*FA-GEG*AS*A*****S*******S*
Pvu   QQEHQ*PT*LPSS*WHA************SSM*FS-GEG*AS*A*****S*******S*

Psa   HFQPEKNWMNDPNGPLYYKGWYHFFYQYNPNGAVWGDIVWGHAVSRDIIHWLHLPLAMVA
Vfa   ************************************************************
Vra   ***************M*********************************M***********
Pvu   ***************M*********************************M***********

Psa   DQWYDSNGVWTGSATILPDGQVIMLYTGSTNESVQVQNLAYPADLNDPLLVDWIKYPSNP
Vfa   *******************************F**************************
Vra   *****KQ***********NGEI**********************PS****L****HTG**
Pvu   *****KQ***********NGEI**********************PS*********HPG**

Psa   VLVPPKGILPKDFRDPTTAWLTTEGKWRITIGSKINKTGVALVYDTVDFKTYERKDVLLN
Vfa   *****P************************************************
Vra   *****P**GA***********S***************I******E******L*EG**R
Pvu   *****P**GA***********S***************I******D******L*NGH*R

Psa   AVPGTGMWECVDFFPVSKKSENGSDT--SINGVEVKHVMKVSLDDDRHDYYSLGTYDEKK
Vfa   *****************M*****L**--*FT*D*****************A********
Vra   ******************NG**L**--*V*A******************AI****DN*
Pvu   ******************N***L**SL*****A*Y***************TI*****N*

Psa   VKFIADDFKNDVGVGLRYDYGIFYASKTFYDQKKNRRVLWGWIGESDSEYADVAKGWASV
Vfa   ********E****I*******************D************************
Vra   *L*TP*V************************N*D*I***************T******
Pvu   *L*TP*V************************NMD*I***************T******

Psa   QSIPRIVKLDKKTGSNLLQWPVAEVESLRLKSDEFKNLKVKPGAVVSLDIETATQLDIDA
Vfa   *****************************R****Q**********V**********V*
Vra   *******R*T***********D*******R*****S*A****S*************VV*
Pvu   *******R**********************R*****S*A****S*************VV*

Psa   EFEIDKEALEKTAQSNVEYECSTSGGASRRGALGPFGLYVLADKGLSEYTPVYFYVVKGS
Vfa   ********************N*********************N***************I
Vra   *****T*S*****E*E*FT*S******AQ*********L****E************I**R
Pvu   *****A*S*Q******E*FT*******AQ*********L****E************I**R

Psa   NGKLNTPFCSDQSRSSLANDVHKQIYGSVVPVLEGEKLSLRILVDHSIVESFAQGGRTCI
Vfa   ****H*S*************************************************
Vra   **N*R*S*********Q****R***F*******K***F***M****************V
Pvu   **N*K*S*********Q****R***F*NI****F***M********************V

Psa   TSRVYPTRAIYGAARLFLFNHAIETNVTASLKVWANEFCIYTPIPPCSKGVRRN
Vfa   *******************N*************QMNSAFIR*YHPDQKRQTS-
Vra   *******K***********N*T*AT********QMNSAFIR*F*FNPDQKS--
Pvu   *******K***********N*T*AT********QMNSAFIR*F*FNPDQKN--
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Fig. 2. Amino acid sequence alignment of a *P. sativum* soluble acid invertase with other known invertases from various plants. The soluble acid invertase sequences from *P. sativum* (Psa), *V. fava* (Vfa, CAA89992), *V. radiata* (Vra, BAA01107) and *P. vulgaris* (Pvu, AAB68679) are aligned. The identical sequence is marked with an asterisk.

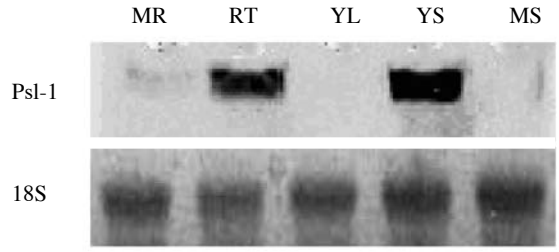

Fig. 3. Tissue-specific gene expression of PsI-1. Total RNAs isolated from mature roots (MR), root tips (RT), young leaves (YL), young stems (YS) and mature stem (MS) were blotted onto a nylon membrane and hybridized with DIG-labeled PsI-P. For an internal control, 18S ribosomal RNA was used.

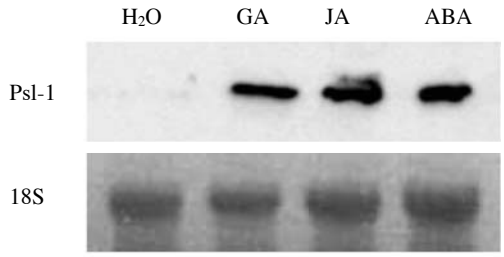

Fig. 4. Effect of ABA, GA and JA on the expression of PsI-1. The whole plant was moistened by ABA, GA and JA solutions for 24 hr. Shoot tissues were obtained for RNA extraction after each treatment and RNA from water (with 2 drops Tween-20/L) sprayed tissue was used as a control. For the internal control, 18S ribosomal RNA was used.

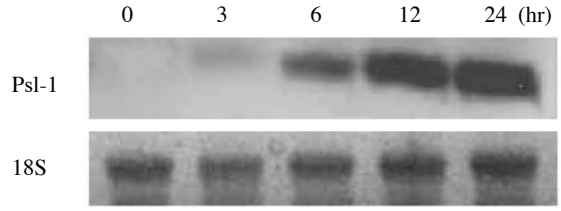

Fig. 5. Time-course accumulation of PsI-1 mRNA induced by GA in shoots. Shoot tissues were obtained from 12-day-old seedlings. The times 0 to 24 hr refer to the times after GA treatment when RNA was extracted. For the internal control, 18S ribosomal RNA was used.

the expression of LIN6 (extracellular invertase) can be stimulated by glucose (Godt and Roitsch 1997). In maize, it was found that two classes of invertase can be identified according to the sensitivity to glucose (Xu *et al*. 1996). The expression of an enzyme is up-regulated by glucose and thus is proposed to function in cell activity at a high glucose concentration while the other enzyme is repressed by glucose and is proposed to work at a low glucose concentration. In the case of pea ovaries, it was found that gibberellin can increase the activity of a soluble neutral invertase, decreasing the soluble acid invertase activity (Estruch and Beltran 1991).

To gain some insight into the function of PsI-1, the tissue expression pattern was analyzed by northern blotting (Fig. 3). Total RNA from mature roots, root tips, young leaves, young stems and mature stems were prepared and subjected to RNA blotting with PsI-P as the probe. A transcript of approximately 2.3 kb was detected and this transcript size was consistent with the cloned cDNA size. PsI-1 was strongly expressed in young stems and root tips. The gene expression in mature roots was very weak and barely detectable in young leaves and mature stems.

2. Regulation of PsI-l expression by ABA, GA and JA

Invertases are regulated by phytohormones such as auxin, cytokinin, gibberic acid (GA), absisic Acid (ABA), brassinosteroid and jasmonic acid (JA). Due to demand of carbohydrate for responses by homones, plant invertases are related to increase enzyme activity for hydrolyzing sucrose to glucose and fructose (Roitsch and Gonzalez 2004). To study whether the expression of the PsI-1 gene was inducible by ABA, GA or JA, total RNA was prepared from tissue samples treated by ABA, GA or JA for 24 hr (Fig. 4) The RNA treated by water (with 2 drops Tween-20/L) was used as a control. PsI-1 was detected in ABA, GA or JA treated shoot tissues and not detected in water treated tissue. To investigate the gene expression profile of the PsI-1 gene by GA, total RNA was prepared from shoot apex tissues collected at 3, 6, 12, and 24 hours after GA treatment. RNA extracted from non-treated tissue was used as a control (Fig. 5). After electrophoresis, blots were hybridized with the PsI-P cDNA probe. As an internal control for equal quantities of total RNA, 18S ribosomal RNA was used. Each band intensity was normalized with that of the corresponding band of 18S ribosomal RNA in duplicate experiments. The mean density was 0.064 at 0 hr, 0.071 at 3 hr, 0.759 at 6 hr, 0.877 at 12 hr and 2.415 at 24 hr post-GA treatment. The PsI-1 gene expression in shoot apexes at 24 hr post-GA treatment was increased approximately 30 fold than that of the non-treated control or at 3hr post-GA treatment. In this current study, northern blotting data showed that the soluble acid invertase transcript of *P. sativum* in shoots was induced by ABA, GA and JA

treatment, and especially the gene expression with a pronounced increase was observed starting from 12 hr post-GA treatment. These data suggests that the PsI-1 expression is up-regulated in response to ABA, GA and JA treatment and these gene expression patterns are strongly consistent with the suggestion that an individual invertase gene product has different functions in a growing plant (Sturm *et al*. 1995; Weber *et al*. 1995).

It is obvious from the extensive list of references that the enzyme invertase has attracted a great deal of attention and has been studied from a physiological, molecular and biochemical/biophysical standpoint. Characterization of genomic DNA from Arabidopsis showed that there may be at least three different isozymes, acid invertases, soluble and insoluble, but with the purification of an insoluble alkaline invertase in broad bean (Ross *et al*. 1996), the four different classes of invertase emerged. Some reports have added to the complexity, showing that several isozymes may exist within each class (Tymowska-LaLanne and Kreis 1998b). Most of the studies have concentrated on the purification, properties and localization of invertases with a focus of interest on their physiological role. A start has been made on the isolation of a gene or genes for a pea soluble acid invertase with the identification of a cDNA sequence that is apparently specific for a soluble acid invertase.

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