

Immunolocalization of Wound-Inducible Insoluble Acid Invertases in Pea (*Pisum sativum L.*)

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완두콩(*Pisum sativum L.*) 상처에서 유도되는 불용성 산성 인버타제의 면역조직화

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Abstract Invertase, that hydrolyzes sucrose into glucose and fructose, plays a great role in carbohydrate reallocation between the photosynthetic source tissue and various sink tissues. Invertase also occurs in a variety of isoforms for various functions in plants. Insoluble invertases were extracted only in buffer solutions containing high concentrations of salt. Within these classes, acid invertase has an optimum activity at acidic pH (pH 4-5). Induction of insoluble acid invertase (INAC-INV) in leaf, stem, and root tissues in response to physical wounding has been investigated. To detect the localization of INAC-INV within the plant, immunolocalization has been performed. In this study, the accumulation of INAC-INV was noticeable to reach maximum levels on 72 hr after mechanical injuries. INAC-INV was induced in wounded leaves 3 times more than control leaves. Immunolocalization results showed that INAC-INV accumulated in wall appositions and intercellular spaces. INAC-INV was also localized at sieve cell walls in phloem tissues close to the site of wounding. Taken together, this study suggested that INAC-INV induction upon wounding injuries can play a role on responses to the high energy demand for wound healing process.

요약 인버타제는 설탕을 포도당과 과당으로 가수분해하며, 광합성 조직과 다양한 수용체 조직 사이의 탄수화물 재배치에서 중요한 역할을 수행한다. 인버타제는 식물에서 다양한 기능을 수행하기 위하여 여러가지 isoforms으로 존재한다. 불용성 인버타제는 고농도의 염을 포함하는 버퍼용액에서만 추출되며, 이 종류의 인버타제 중 산성 인버타제는 산성 pH(pH 4-5)에서 최적의 활성을 갖는다. 물리적인 상처에 반응하여 잎, 줄기 및 뿌리에서 불용성 산성 인버타제(INAC-INV)가 유도되는 것이 연구되어 왔다. 식물에서 효소의 localization을 검출하기 위한 면역조직화 연구를 수행하였다. 이 연구에서 인버타제의 축적은 기계적 손상 후 72시간에 최고수준에 도달하였다. INAC-INV의 활성은 대조군 일보다 상처받은 조직에서 3배까지 증가하였다. 면역조직화 결과는 INAC-INV가 세포벽과 세포간극에 축적되어 있음을 보여주었다. INAC-INV는 또한 상처와 가까운 사부조직의 체관세포벽에 위치하였다. 종합해 볼 때, 이 연구는 상처에 의한 INAC-INV 유도는 상처치료 과정에서 필요한 높은 에너지 요구에 대한 반응에 역할을 할 수 있음을 추측케 한다.

Keywords : Immunocytochemistry, Insoluble acid invertase, Localization, *Pisum sativum L.*, Wounding

본 논문은 한국해양과학기술원 연구과제(PE99315)로 수행되었음.

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Received August 20, 2015

Revised September 1, 2015

Accepted September 11, 2015

Published September 30, 2015

1. Introduction

Carbohydrate reallocation between the photosynthetic source tissue and a various sink tissues competing for limited resources of carbohydrates is a highly dynamic and complex processes that conduct all stage of plant life cycle. For these reasons, the invertase, which hydrolyzes sucrose into glucose and fructose, responsible for the first metabolic reaction of sucrose are critical role for plant development. For the importance of its function in plant metabolism, to date there are more than 300 invertases sequences in the databases and more than 200 different isozymes from 50 plant species. Current knowledge of invertase can propose their different functions. Firstly invertases cleave sucrose to provide growing tissues with hexoses as a source of energy and carbon backbone. Secondly invertases also generate a sucrose gradient between sources and sink tissues to aid sucrose transport. Thirdly invertases can regulate cell turgor for cell expansion and finally invertases control sugar composition in storage organs [1-3].

In addition, some invertases seem to be involved in the responses against environmental factors such as biotic and abiotic stresses [4]. However, the specific roles of the invertase isoforms in the different subcellular compartments are largely unknown. Angiosperm plants have several invertase isoenzymes, which can be subgrouped by their subcellular localization, solubility in low- ionic-strength buffer, optimum pH and isoelectric point [5]. The cellular and physiological functions of each invertase isoform are very complicated and depend on tissue, cellular or subcellular location and developmental stages. Therefore, the expressions of various invertase isoforms seem to be modulated during plant development and under different environmental conditions. The presence of multiple isoforms of invertase might confer a great plasticity to the control of sugar metabolism, reallocation and storage [6].

Invertase activity may also be expected to change as

plants react to stress and infection. When plants are subject to stresses such as contamination by pathogen or wounding, they respond through a broad range of complex defense mechanisms. Many research groups have reported changes in invertase activity under stress, particularly stress induced by infection [7]. Sturm and Chrispeels [8] showed invertase mRNA to be induced by wounding and bacterial infection of carrot tissues. Maturing storage roots contained barely detectable levels of insoluble invertase mRNA. Levels rose slowly but dramatically after physical wounding with maximal expression after 12 hours. Infection of roots and leaves of carrot plants with *Erwinia carotovora* resulted in a rapid increase in the mRNA levels with maximum expression after 1 hour. In studies reported from my colleagues' laboratory, Zhang *et al.* [9] described the induction of insoluble invertase mRNA in leaf, stem, and root tissues in response to physical wounding.

To detect the expression of the protein, encoded for by the gene, enzyme activities are routinely determined in extracts, but by this method no information on the localization of the enzyme within the plant is obtained. Nowadays immunolocalization was a very powerful tool for study the expression as well as localization of protein itself in plant tissues. However, for doing this approach of immunocytochemistry in plant tissues, researchers must have certain protein specific antibody in advance. Unfortunately not many antibodies, which are specific to certain proteins, are available in commercial company or in research groups. Therefore the application of immunocytochemistry work is very limited to research areas which certain antibody are commercially available or customized-made.

In this paper, it is demonstrated that immunocytochemistry is widely applicable to study and understand the localization of an insoluble invertase isozyme. Here, we report the wound- specific localization of insoluble acid invertase(INAC-INV).

2. Materials and Methods

2-1 Wounding treatments and measurement of enzyme activities

Seeds of the garden pea, *Pisum sativum* L. cv. Alaska were planted and grown in the greenhouse at Silla University. To obtain etiolated tissue, pea seeds were surface-sterilized by 10% Clorox solution for 10 min, washed in sterilized water and planted in autoclaved vermiculite. Wounding treatment was performed: one of the upper leaves in each recovered seedling was quickly wounded twice at the central zone perpendicular to the main vein with a pair of forceps. After the treatment was finished, sample leaves and stems were fixed with pre-fixation solution for cellular and subcellular localization assay of invertase isoforms. And other samples were frozen in liquid nitrogen and stored at -80 °C until further use. The leaves and stems from non-treated seedlings were used as the control, which were harvested at the same period as the treated ones. At least three independent repetitions were conducted for each treatment. The measurements of enzyme activities were performed according to the methods of Kim *et. al.* [10,11].

2-2 Light microscopy and immunocytochemistry

Immunocytochemical methods combine the sensitivity and specificity of the antibody-antigen interaction with the ability to visualize immunoreactivity in tissues by light- or electron-microscopy. Immunolocalization involves tissue fixation, dehydration, infiltration, embedding, sectioning, dewaxing, dehydration, blocking with BSA or gelatin solutions, reaction with anti-carrot insoluble invertase antibodies (1/1000 dilution) and secondary antibody conjugated with alkaline phosphatase, and detection of any positive reaction by staining for alkaline phosphatase activity. Dehydrated sections were mounted under a cover-slip with Permount and photographed with a Microlumina slow speed digital

camera on a Zeiss Ultraphot light microscope [12,13]. The images were printed with Shinko CHC-S446I dye sublimation printer. Small pieces (2-5 mm) of tissue were placed in pre-chilled fixative containing 2% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 50 mM citrate/phosphate buffer (pH 7.4) for 2 hr at 4 °C before transferring to 500 W microwaves at high power for 10 sec [14]. Fixed tissue was washed 3×10 min in 50 mM citrate/phosphate buffer, pH 7.4, and 2×10 min washes in double-distilled water. Embedded samples were removed in 1×1 cm wax blocks. Sections (15 mm thick) were cut using a rotary microtome (Spencer Lens Co., Buffalo, NY). Ribbons of 5-7 serial sections were placed on poly-L-lysine-coated microscope slides. The slides were dried in a 37 °C oven overnight before placing in a 60 °C oven for 1-2 min until the wax melted. The cooled slides were stored at room temperature. The rehydrated sections on slides were incubated with 3% BSA in TBS buffer for 2 hr to block any nonspecific protein-binding sites on the sections or the glass. The slides were incubated with selected anti-invertase antibodies and pre-immune serum as a control at room temperature for 20 hr (We give thanks to Dr Sturm.A who donated carrot INAC-INV antiserum.). The antibody was diluted in the blocking solution (1:500). The slides were washed with TBS buffer 5×10 min with constant shaking. The slides were incubated for 1 hr at room temperature in secondary antibody (1:1000 goat anti-rabbit IgG conjugated with alkaline phosphatase also diluted in blocking solution). The washed slides were treated with the alkaline phosphatase assay solution for 30 min at room temperature. The reaction was stopped by washing with tapwater. Slides were dehydrated by passing them through the series of solutions for 10 min each: 50%, 70%, 90%, 100%, 100% of ethanol and then 25% xylene/ethanol, 50% xylene/ethanol, 75% xylene/ethanol, 100% xylene, 100% xylene. The sections were covered with a few drops of very dilute and degassed Permount and cover glasses applied.

2–3 Electron microscopy and immune–gold localization

Using a secondary antibody labelled with colloidal gold particles to replace the alkaline phosphatase label used above, localization of invertase can be achieved at the level of the electron microscope. Tissue pieces are fixed and rinsed as described above in Light microscopy section. For electron microscopy, dehydration was completed in a graded series of ethanol to acetone at room temperature with each change for 10 min. Infiltration with Araldite-Epon mixture (Epon 812 12.5 g, Araldite 502 10 g, dodecylsuccinic anhydride 30 g, N,N-benzyl-dimethylamine 1.5 g) was begun at room temperature by gradually replacing acetone with the mixture for 2 hr, while the bottle was kept open. The molds were left in the fume-hood for one day and transferred to a 60 °C oven overnight. Embedded samples were removed from the mold. The blocks were trimmed for ultramicrotomy and thick sections (0.5 mm) were checked for interesting tissue after staining with toluidine blue solution. Thin sections (70-90 nm) were cut with glass knives on a Reichert OM-U2 Ultramicrotome. Serial thin sections were mounted on clean copper grids coated with a Formvar film, stained, and examined with a Hitachi HS-8 transmission electron microscope. All of the following procedures were performed at room temperature by placing the grids on 50 ml drops of solution in clean Petri dishes with sample-face down. Then the sample was etched with a saturated solution of sodium-meta-periodate for 30 min and rinsed with distilled water several times, 5 min each change [13]. The etched sections were transferred to 1% BSA in TBS buffer for 30 min to block any nonspecific protein-binding sites. The grids were incubated for 2 hr with anti-carrot insoluble invertase antibodies (1/1000 dilution) and pre-immune serum as a control. The antibody was diluted in the blocking solution (1:500). The slides were washed with TBS buffer 5×10 min. The grids were incubated for 1 hr at room temperature in secondary antibody (1:1000

goat anti-rabbit IgG conjugated with 10 nm gold particle diluted in blocking solution). The grids were rinsed in distilled water and post-stained with either 2.65% (W/V) lead citrate for 2min or with 2% (W/V) uranyl acetate for 5 min. The stained grids were air-dried for 2 days.

3. Results

3–1 Invertase assay on wound tissues

Plant invertase gene expression and enzyme activity are known to be influenced by many intracellular and extracellular factors. INAC-INV genes are upregulated by wounding in carrot [8] and in pea [9]. Zhang *et al.* [9] identified a wound-induced mRNA for cell wall INAC-INV in pea. In this study we have examined the

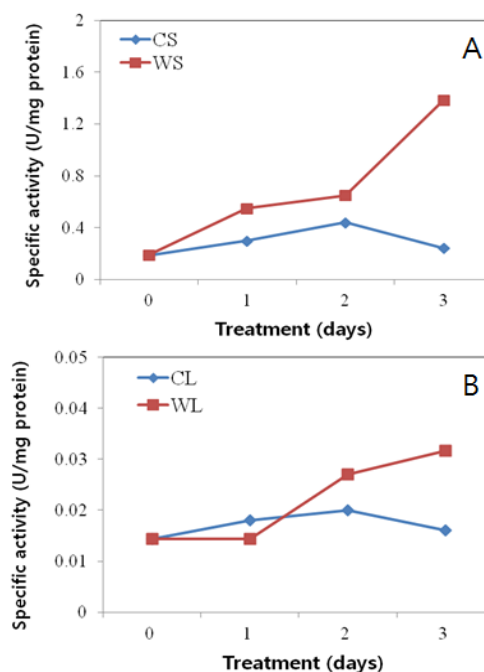


Fig. 1. INAC-INV activity of pea stem (A) and leaf (B) tissue following wounding. INAC-INV activity. Total activity (Y axis) per gram fresh weight (U/gfw) for 3 days (X axis) was measured at intervals following wounding. CS, control, unwounded stem; WS, wounded stem; CL, control, unwounded leaf; WL, wounded leaf.

effect of wounding on the accumulation and localization of the INAC-INV activity. Pea leaf and stem tissues of twelve-day-old tall pea (cv. Alaska) seedlings were selected. The 4th and 5th internodes were wounded using a nail file. Fully expanded mature leaves (source leaves) were wounded using a nail file. After 1-3 days of further growth, wounded tissues were removed for invertase activity assay. All soluble activity was removed from the homogenized tissue by repeated washing and centrifugation. Insoluble invertase activity was assayed, without extraction, in the washed residue at pH 4.0. The assay was controlled for the production of reducing sugar from the tissue in the absence of added substrate. The data in Figs 1A and 1B show a continuous increase of INAC-INV activity in both leaves and stems during the three days after wounding.

3-2 Immunolocalization of invertase on wound tissues

To understand the spatial relationship between INAC-INV protein and the wounding response in pea tissues and cells, the antibody preparation against carrot INAC-INV was used in immunolocalization. This antibody is particularly appropriate because the carrot INAC-INV is known to accumulate in response to wounding [8]. In an effort to determine the immunoreactivity, and cross-reactivity of the available antibodies, samples of the invertase preparations were separated by SDS-PAGE and subjected to immuno-blotting [11]. The antibodies against carrot INAC-INV reacted with INAC-INV.

Immunolocalization studies showed that the pea invertase accumulated in response to stem tissue wounding 3 days after wounding [Fig. 2]. The wounded surface stained very strongly [Fig. 2C, 2D].

Immuno-gold localization by electron microscopy in wounded stem tissue was accomplished using the same primary antibody as in the light microscopic study but using a secondary antibody labeled with colloidal gold particles [Fig. 3]. Thick sections examined by light

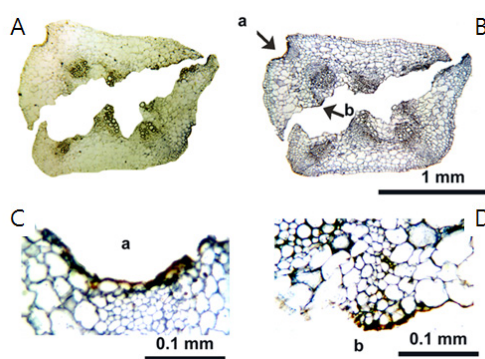


Fig. 2. Immunolocalization of insoluble invertase in wounded stem tissue. Light microscopy of tissue prepared for localization of INAC-INV. The primary antibody was that against carrot INAC-INV. The secondary antibody was alkaline phosphatase-labeled. Pre-immune serum, negative control. Yellow-green coloration reflects no immunoreaction. Invertase localization in a section adjacent to that in 1. Areas a. and b. are enlarged in C and D. A positive blue-purple precipitate is superimposed on a yellow-brown discoloration visible on unstained sections.

microscopy were used to identify the location and types of cells that show localized labeling. Thin sections from specific areas were used for electron-microscopy [Fig. 3A]. As a map for cell identification Fig. 3B is a relatively low magnification (3000 X) of the rectangular area W in Fig. 3A. The localization of gold particles identifying invertase protein indicated that distribution of the insoluble invertase increased in specific tissue and cell types after wounding. Invertase protein accumulated in the cell wall of a phloem sieve tube near the wound area [Fig. 3]. Selected wall regions in this section are seen in Figs 3C and 3D. Figs 3C is an enlargement of the area indicated by the left arrow in Fig. 3B. Fig. 3D are an enlargement of the area by the right arrow. The gold particles are distributed throughout the wall. Control sections using pre-immune serum were negative.

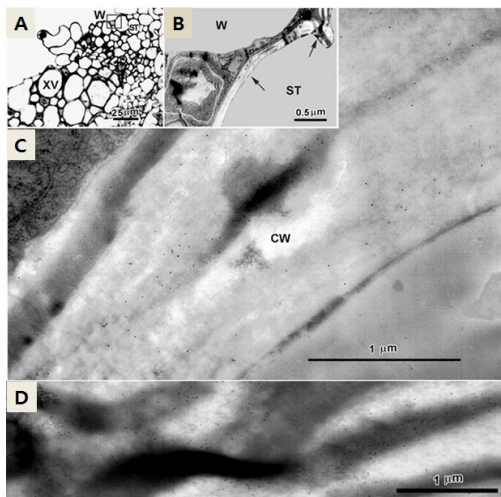


Fig. 3. Electron microscopy of tissue prepared for immunogold localization of INAC-INV. A, Light micrograph of a young internode of pea wounded in vascular tissue. Wounded surface (W), Xylem Vessel (XV), Phloem Sieve Tube (ST); B,. Low magnification electron micrograph of boxed area in A. Arrows indicate areas shown at high magnification in C, and D. Gold particle localization is concentrated in sieve tube walls. C and D antibody against carrot insoluble invertase

4. Discussion

Much of the published information on invertase localization has been derived from activity measurements on tissue extracts from a broad range of plant organs and developmental stages of several plants. Invertase activity in plants has been linked to mechanical injury and pathogen infection [8,9,15-17]. INAC-INV plays a critical role [8,9,16]. In wounded pea tissue, insoluble invertase activity gradually increased over time [Figs. 1A, 1B]. The data in these figures were determined concurrently with the tracking of pea INAC-INV mRNA expression after wounding [9]. Insoluble invertase mRNA appeared from 3 hr after wounding and gradually increased, reaching maximum levels 12 hr after wounding in both stem and leaf tissues [9]. Enzyme activity gradually increased about 24 hr after wounding and reached maximum

levels 3 days after wounding in those stems and leaves [Figs. 1A, 1B]. Induced enzyme activity in leaves was 2-3 times higher than in stems. This difference may well be related to the area and/or intensity of wounding. In localization studies of invertase mRNA by *in situ* hybridization the RNA was localized in the phloem tissues [9]. My results showed the accumulation of significant amounts of invertase close to the wounded surface [Fig. 2]. Immunocytochemical localization by electron microscopy with 10 nm gold particles as the label on the secondary antibody showed invertase protein to be found in substantial quantity only in the wall of a phloem sieve tube cell near the wounded area. This accumulation of insoluble invertase suggests an increase in enzyme activity involved in hydrolyzing sucrose into hexoses. Induction and accumulation of invertase activity in this area may be part of the plant's recovery process after wounding. This is consistent with published suggestions that infected organs or tissues become energy sinks, rapidly unloading carbohydrate [15] and showing increased respiratory activity [18-20].

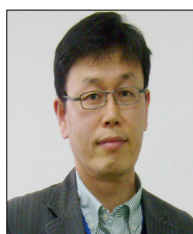
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