Journal of Biochemistry and Molecular Biology, Vol. 31, No. 6, November 1998, pp. 554–559

JOURNAL OF
Biochemistry
and
Molecular Biology

Activities of Sulfhydryl-Related and Phenylpropanoid-Synthesizing Enzymes during Leaf Development of *Arabidopsis thaliana*

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Received 11 June 1998, Accepted 30 July 1998

Activities of glutathione- and thioredoxin-related enzymes and phenylpropanoid-synthesizing enzymes were measured and compared in the developing leaves of *Arabidopsis thaliana*. Phenylalanine ammonia-lyase activity is maximal in the leaves of 2-wk-grown *Arabidopsis*. Tyrosine ammonia-lyase activity is maximal in the leaves of 3-wk-grown and 4-wk-grown *Arabidopsis*. Activity of thioltransferase, an enzyme involved in the reduction of various disulfide compounds, is higher in younger leaves than in older ones. A similar pattern was obtained in the activity of thioredoxin, a small protein known as a cofactor of ribonucleotide reductase and a regulator of photosynthesis. Activity of glutathione reductase is also higher in the younger leaves. Malate dehydrogenase activity remains relatively constant during the development of *Arabidopsis* leaves. The results offer preliminary information for further approach to elucidate the mechanism of growth-dependent variations of these enzymes.

Keywords: *Arabidopsis thaliana*, Glutathione reductase, Phenylalanine ammonia-lyase, Thioltransferase, Thioredoxin.

Introduction

Sulfhydryl biochemistry plays a remarkably broad and important role in cells, because the redox status of cysteine sulfhydryl groups dictates the native structure and/or activity of many enzymes, receptors, protein transcription factors, and transport proteins (Starke *et al.*, 1997). Two well-characterized systems involved in thiol/disulfide interchange exist in the cytosolic fraction of various cells. Thioltransferase obtains reducing equivalents from glutathione, which, in turn, is reduced by NADPH and glutathione reductase, whereas thioredoxin is directly reduced by NADPH via thioredoxin reductase (Holmgren, 1989). Thioltransferase and thioredoxin were reported to serve as regenerative systems for oxidatively damaged proteins *in vivo* as well as *in vitro*, but they contained different substrate specificities (Yoshitake *et al.*, 1994). The thioltransferase-dependent system was found to be more efficient in reducing small molecules and insulin was most effectively reduced by the thioredoxin system (Mannevik *et al.*, 1983).

Thioltransferase and thioredoxin comprise a superfamily of ubiquitous heat-stable and small proteins, containing an active site with the sequence -Cys-X-X-Cys- (Holmgren, 1989). Plant thioredoxin is involved in two enzyme systems such as fructose-1,6-bisphosphatase and $NADP^+$-dependent malate dehydrogenase (Kamo *et al.*, 1989). The $NADP^+$/thioredoxin (h-type) system, which is analogous to bacterial and animal thioredoxins, is widely distributed in plant tissues (Florencio *et al.*, 1988). Five different clones encoding thioredoxin h were isolated from *Arabidopsis thaliana* cDNA libraries (Rivera-Madrid *et al.*, 1995). Thioltransferase has been isolated and characterized from several plant cells such as spinach leaves (Morell *et al.*, 1995), rice (Sha *et al.*, 1997), kale (Sa *et al.*, 1998), *Arabidopsis thaliana* seed (Cho *et al.*, 1998), and Chinease cabbage (Cho *et al.*, 1998). Thioltransferase purified from *Arabidopsis thaliana* seed appeared to have an atypical molecular size of 22 kDa.

Phenylalanine ammonia-lyase and tyrosine ammonia-lyase are involved in the phenylpropanoid metabolism. Phenylalanine ammonia-lyase, usually identified as a tetrameric enzyme, catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid in the first step of the phenylpropanoid pathway, which supplies the precursors for flavonoid pigments, lignins, stilbenes, some alkaloids, and coumarins (Hahlbrock and Scheel, 1989). It also plays

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an important roles in plant development and pathogen defense (Dixon and Lamb, 1990). It is induced by various stress-related stimuli including wounding, heavy-metals, light, phytotoxins, and phytochromes (Smith *et al.*, 1994). In this report, to investigate a possible involvement of glutathione- and thioredoxin-related enzymes and phenylpropanoid-synthesizing enzymes in the leaf development, the enzyme activities were examined and compared during the growing and aging periods.

Materials and Methods

Chemicals Bovine serum albumin (BSA), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (yeast), NADPH, Tris, 5,5′-dithio-2-nitrobenzoic acid (DTNB), acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulfate, and EDTA were obtained from Sigma Chemical Co. (St. Louis, USA). L-Phenylalanine, L-tyrosine, oxaloacetate, and Coomassie Brilliant Blue R-250 were also from Sigma Chemical Co. (St. Louis, USA). 2-Hydroxyethyl disulfide (HED) was purchased from Aldrich Chemical Co. (Milwaukee, USA). Vermiculite, perlite, and peat moss were obtained from a flower shop in Chuncheon, Korea. *E. coli* thioredoxin reductase was kindly provided by Prof. James A. Fuchs, The University of Minnesota, St. Paul, Minnesota, USA. All other chemicals and reagents used were of the highest grade commercially available.

Growth of plant Seeds of *Arabidopsis thaliana* ecotype Columbia were kindly provided by Prof. Hong-Gil Nam, Pohang University of Science and Technology, Korea. Seeds were cultivated in soil, a 1:1:1 mixture of vermiculite, perlite, and peat moss. Cultivation conditions were 26°C (temperature) and 60% (moisture) in a growth chamber.

Preparation of leaf extract *Arabidopsis* leaves were taken out every week after germination and frozen until use. The frozen leaves were ground up in a mortar with buffer A (50 mM Tris·HCl – 1 mM EDTA, pH 7.5) containing sea sand. The mixture was then clarified by centrifugation (10,000 × *g*, 10 min) at 4°C and supernatant was saved for enzyme assays. Seed extract was prepared in a similar procedure.

Thioltransferase activity Thioltransferase catalyzes the reduction of certain disulfides in the presence of glutathione and thus has GSH-disulfide-transhydrogenase. In the present study, 2-hydroxyethyl disulfide was used as a substrate (Holmgren, 1985). Two quartz semimicro cuvettes with 1-cm light path contained 500 μl of mixture at room temperature. To both cuvettes were added 50 μl of 15 mM 2-hydroxyethyl disulfide, 100 μg/ml bovine serum albumin, 1 mM GSH, 6 μg/ml yeast glutathione reductase, 0.4 mM NADPH, and 0.1 M Tris·HCl (pH 8.0)-2 mM EDTA. The absorbance at 340 nm was recorded for 2 min to ensure that both cuvettes were balanced with respect to the nonenzymatic spontaneous reaction between glutathione and 2-hydroxyethyl disulfide. Leaf extract was added to the sample cuvette and an equal volume of buffer A was added to the reference cuvette. The decrease in absorbance was then recorded for a few minutes. The result was calculated as ΔA_{340}/min.

Phenylalanine ammonia-lyase activity Phenylalanine ammonia-lyase activity was measured by a modification of the spectrophotometric assay described previously (Lim *et al.*, 1998). The reaction mixture contained 50 mM Tris·HCl (pH 9.0), 2 mM L-phenylalanine, and leaf extract in a total volume of 1.5 ml. The assay was carried out at 30°C and the reaction was stopped by the addition of 1 ml 2 N HCl. The *t*-cinnamic acid formed was extracted into 2 ml of toluene by vortexing for 10 sec and centrifuging at 1500 × *g* for 10 min. The absorbance at 290 nm of *t*-cinnamic acid recovered in the toluene phase was measured using toluene as a blank.

Glutathione reductase activity The oxidation of NADPH was followed spectrophotometrically at 340 nm (Carlberg and Mannervik, 1985). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0)-1 mM EDTA, 0.1 mM NADPH, and 1 mM GSSG in a total volume of 1 ml. The reaction was initiated by the addition of the leaf extract to the cuvette and the decrease in absorbance at 340 nm was followed. The glutathione reductase activity was expressed as ΔA_{340}/min.

Thioredoxin activity Thioredoxin catalyzes NADPH-dependent reduction of the disulfide bond in DTNB (Luthman and Holmgren, 1982). The assay mixture contained 100 mM Tris·HCl (pH 8.0), 2 mM EDTA, 0.1μg/ml BSA, 0.5 mM DTNB, and 0.24 mM NADPH in a volume of 500 μl. Leaf extract was added into the sample cuvette, whereas buffer A was added into the reference cuvette. The reaction was initiated by adding thioredoxin reductase. An increase in absorbance at 412 nm was directly monitored using the spectrophotometer. Thioredoxin activity was expressed as ΔA_{412}/min.

Tyrosine ammonia-lyase activity The deamination of tyrosine by tyrosine ammonia-lyase was monitored as described previously (Abell and Shen, 1987). For tyrosine ammonia-lyase activity, 2.5 ml of 12 mM L-tyrosine in 0.1 M Tris·HCl buffer (pH 8.5) was added as a substrate to leaf extract. The blank did not contain L-tyrosine. Increase in absorbance at 333 nm was recorded spectrophotometrically after incubation. The activity was expressed as ΔA_{333}/min.

Malate dehydrogenase activity $NADP^+$-malate dehydrogenase activity was measured as described previously (Jacquot *et al.*, 1995). The reaction mixture contained 0.1 M Tris·HCl buffer (pH 8.0), 0.75 mM oxaloacetate, and 0.15 mM NADPH in a volume of 1 ml. The reaction was initiated by the addition of leaf extract and followed spectrophotometrically by the decrease of absorbance at 340 nm. The activity was expressed as ΔA_{340}/min.

Protein determination The protein content in leaf extract was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Results and Discussion

The development and growth of plants is directly related with several metabolic pathways, which are governed by various enzymes. In some cases, the enzyme activities are modulated by light in morphogenetic responses and

regulated at a gene level. Whether it is the enzyme activities that are modulated or their syntheses that are regulated, it is important to understand the variations in enzyme activities during the development and growth of plants. *Arabidopsis thaliana*, a small weed in the mustard family, is a well-known model plant, especially for plant molecular biologists. However, insufficient works have been performed on *Arabidopsis thaliana* on a protein or enzyme level. This article describes the variation in several enzyme activities during the leaf development of *Arabidopsis thaliana*, which would be very important for further approach. For each enzyme activity detected in this article, the appropriate amounts of protein in the extracts were used. It appeared that the repeated experiments gave the same trend of variation in each enzyme activity.

Growth patterns of *Arabidopsis thaliana* Seeds of *Arabidopsis thaliana* ecotype Columbia were cultivated in soil, in a mixture of vermiculite, perlite, and peat moss. Then, the number, width, and length of the *Arabidopsis* leaves were measured during growth (data not shown). The leaves reached a number of eight two weeks after the seeds were sown, and remained constant for up to six weeks. The widths of the leaves gradually increased in three weeks and remained constant thereafter. On the other hand, the length of the leaves appeared to increase continually during the leaf development.

Enzyme levels in *Arabidopsis* seeds Plant seeds harbor various kinds of enzymes in addition to storage proteins. Some of them are necessary for seed germination. Enzyme activities were measured in *Arabidopsis* seeds and compared with those in young leaves (Table 1). Phenylalanine ammonia-lyase activity appeared to be absent in *Arabidopsis* seeds. However, tyrosine ammonia-lyase activity in the seeds was found to be higher than that of the young leaves. Activity of malate dehydrogenase in the seeds was significantly lower than in the young leaves. The activities of thioltransferase, thioredoxin, and glutathione reductase in the seeds were similar to those in the young leaves. Disulfide bonds are one of the main stabilizing forces in the storage proteins of the seeds. The formation of disulfide bonds is believed to function to provide increased structural stability on the one hand and decreased solubility on the other, providing protection against proteolysis. Since the disulfide proteins of seeds are degraded during germination, thioredoxin and thioltransferase may play roles in the reduction of critical disulfide groups of seed proteins and, thereby, trigger germination.

Enzymes involved in the phenylpropanoid pathway During normal plant development, internal and external factors influencing cellular differentiation cause cell groups to become distinct tissue types and organs. During differentiation, specific metabolic branches of the phenylpropanoid pathway are initiated and activated according to environmental and hormonal stimuli (Liang *et al.*, 1989). The two enzymes which head phenylpropanoid metabolism, phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL), are highly regulated by events taking place during various plant developmental stages (Wiermann, 1981). PAL and TAL convert L-phenylalanine and L-tyrosine to ammonia and *t*-cinnamic acid and *p*-coumaric acid, respectively, which are further modified in phenylpropanoid metabolism to precursors used in secondary pathways producing lignin, flavonoids and anthocyanins, phytoalexins, and tannins. The time course for changes in the levels of PAL and TAL were measured in the leaves of *Arabidopsis thaliana*. PAL

Table 1. Comparisons in various enzyme activities between *Arabidopsis thaliana* seeds and young leaves.

Enzyme Activities	Specific Activities	
	Seeds	Young Leaves[1]
Tyrosine ammonia-lyase[2]	$10.3 \times 10^{-4} \pm 0.4 \times 10^{-4}$	$4.4 \times 10^{-4} \pm 0.2 \times 10^{-4}$
Phenylalanine ammonia-lyase[3]	0	$7.7 \times 10^{-4} \pm 0.5 \times 10^{-4}$
Thioltransferase (glutaredoxin)[4]	1.2 ± 0.04	1.3 ± 0.03
Thioredoxin[5]	$5.7 \times 10^{-2} \pm 0.2 \times 10^{-2}$	$6.3 \times 10^{-2} \pm 0.3 \times 10^{-2}$
Glutathione reductase[6]	$1.3 \times 10^{-1} \pm 0.05 \times 10^{-1}$	$1.9 \times 10^{-1} \pm 0.07 \times 10^{-1}$
Malate dehydrogenease[7]	1.9 ± 0.02	5.6 ± 0.04

[1] The 1-wk-grown leaves were used as a source of young leaves.
[2] Specific activities of tyrosine ammonia-lyase were expressed as ΔA_{333}/min/mg protein.
[3] Specific activities of phenylalanine ammonia-lyase were expressed as ΔA_{290}/min/mg protein.
[4] Specific activities of thioltransferase were expressed as ΔA_{340}/min/mg protein.
[5] Specific activities of thioredoxin were expressed as ΔA_{412}/min/mg protein.
[6] Specific activities of glutathione reductase were expressed as ΔA_{340}/min/mg protein.
[7] Specific activities of $NADP^+$-specific malate dehydrogenease were expressed a ΔA_{340}/min/mg protein.

activity was maximal in 2-wk-grown leaves (Fig. 1), whereas TAL activity appeared to be maximal in 3-wk- and 4-wk-grown leaves (Fig. 2). PAL activity was shown to be almost absent in 4-wk- and 5-wk-grown leaves (Fig. 1). They indicate that PAL and TAL activities are regulated in a different way. For example, PAL activity is highly stimulated in tobacco (*Nicotiana tabacum L.*) leaves reacting hypersensitively to tobacco mosaic virus (Fritig *et al.*, 1973). Genomic clones for three *Arabidopsis thaliana* PAL genes containing the entire protein-coding region and upstream and downstream sequences have been obtained and completely sequenced (Wanner *et al.*, 1995). Two *A. thaliana* PAL genes are structurally similar to PAL genes that have been cloned from other plant species. However, PAL has never been purified from *A. thaliana* so far.

Thioredoxin, thioltransferase, and glutathione reductase In the chloroplasts of plant cells, the activities of several enzymes of the Calvin cycle are regulated by light (Pradel *et al.*, 1981). This control is affected through light-induced modifications of the levels of substrates and cofactors (Leegood and Walker, 1980). Besides this type of control, activation of chloroplast enzymes by light also involves the reduction of enzyme disulfide bridges, in a process that is thought to be mediated by thioredoxins (Buchanan, 1980). In this system, the disulfide bridge of thioredoxin is reduced by a specific ferredoxin-thioredoxin reductase in the presence of photoreduced ferredoxin (Droux *et al.*, 1987). Time course changes in thioredoxin activity were measured in the leaves of *Arabidopsis thaliana* (Fig. 3). Thioredoxin activity was found to decrease gradually as the leaves got older. This indicates

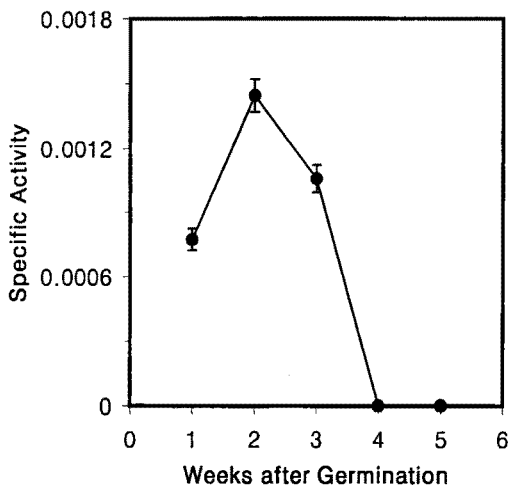

Fig. 1. Variation in specific activities of L-phenylalanine ammonia-lyase during *Arabidopsis* leaf development. Activity of L-phenylalanine ammonia-lyase was assayed as described in Materials and Methods. Its specific activity was expressed as ΔA_{290}/min/mg protein. Each point indicates mean ± SD.

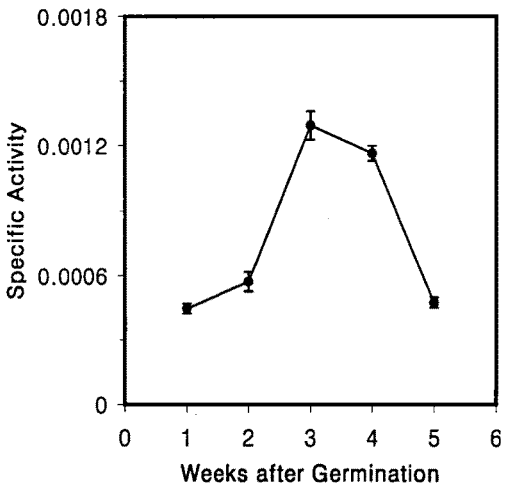

Fig. 2. Variation in specific activities of L-tyrosine ammonia-lyase during *Arabidopsis* leaf development. Activity of L-tyrosine ammonia-lyase was assayed as described in Materials and Methods. The specific activity was expressed as ΔA_{333}/min/mg protein.

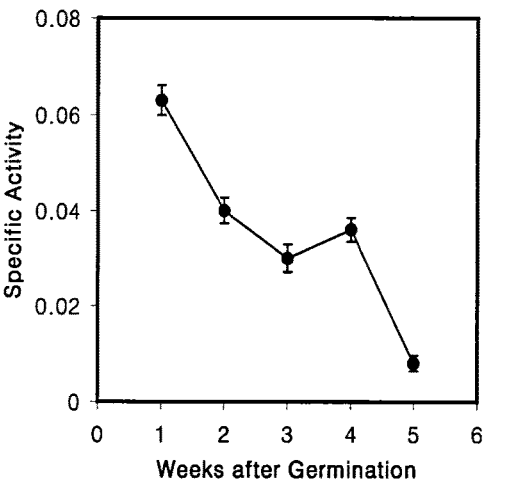

Fig. 3. Variation in specific activities of thioredoxin during *Arabidopsis* leaf development. Thioredoxin activity was assayed as described. The specific activity was expressed as ΔA_{412}/min/mg protein.

that the requirement of thioredoxin is maximal in young leaves of *Arabidopsis thaliana*. It may correspond with a high rate of photosynthesis. Thioredoxin activity showed a linear relationship with the amount of leaf extract used (data not shown).

Thioltransferase catalyzes the reversible thiol-disulfide interchange reactions. The enzyme has a major role in maintaining intracellular thiols in the reduced state and functions in this capacity by coupling to glutathione and glutathione reductase. Thioltransferase also has a role in

cellular regulation by catalyzing the reversible modification of proteins by thiol-disulfide interchange. As a result, thioltransferase is able to control the activity, stability, and correct folding of enzymes through disulfide/dithiol isomerization reactions. Thioltransferase activity was found to be maximal in the 1-wk-grown leaves of *Arabidopsis thaliana* (Fig. 4). After 1-wk-growth, the activity decreased and then remained constant until 5 wk. This indicates that some kind of regulation may be involved in the expression of the thioltransferase gene in *Arabidopsis* leaves. Thioltransferase activity shows a linear relationship with the amount of leaf extract used (data not shown). There is only one example of plant thioltransferase purified from spinach leaves (Morell *et al.*, 1995). Recently, we characterized thioltransferase purified from kale (Sa *et al.*, 1998), Chinese cabbage (Cho *et al.*, 1998), and *Arabidopsis* seed (Cho *et al.*, 1998).

Glutathione reductase is a flavoprotein catalyzing the NADPH-dependent reduction of glutathione disulfide to glutathione. The reaction is essential for the maintenance of glutathione levels. Glutathione has a major role as a reductant in oxidation-reduction processes, and also serves in detoxification and several other cellular functions of great importance. Glutathione reductase activity showed higher values in relatively young leaves than in older leaves of *Arabidopsis thaliana* (Fig. 5). This may imply that the reduced state is required more in young leaves.

$NADP^+$-malate dehydrogenase $NADP^+$-specific malate dehydrogenase catalyzes the reduction of oxaloacetate using NADPH as reductant. It occurs in high activity in C_4 variants in which malate is the principal short-term product of CO_2 assimilation and is located in the chloroplasts of mesophyll cells. The time course for change in $NADP^+$-malate dehydrogenase activity was measured in the leaves of *Arabidopsis thaliana*. The activity did not give a large variation during leaf growth (data not shown).

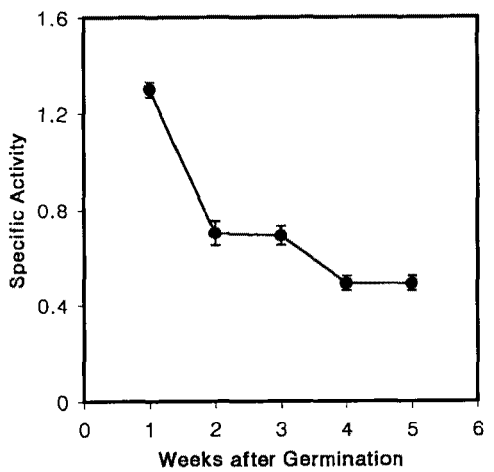

Fig. 4. Variation in specific activities of thioltransferase during *Arabidopsis* leaf development. Activity of thioltransferase was assayed as described in Materials and Methods.

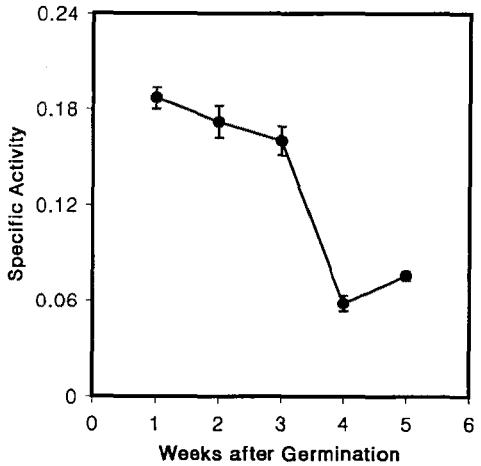

Fig. 5. Variation in specific activities of glutathione reductase during *Arabidopsis* leaf development. Activity of glutathione reductase was assayed as described in Material and Methods.

In this article, variations in sulfhydryl-related and phenylpropanoid-synthesizing enzyme activities of the *Arabidopsis* leaves during development were studied. Activities of phenylalanine ammonia-lyase and tyrosine ammonia-lyase involved in the synthesis of phenylpropanoid increased in the early stages of leaf development and decreased in the late stages. However, activities of sulfhydryl-related enzymes such as thioredoxin, thioltransferase, and glutathione reductase appeared to gradually decrease. The data indicate that sulfhydryl-related enzymes and phenylpropanoid-synthesizing enzymes detected in this study may be closely linked to the leaf development. The sulfhydryl-related enzymes may play more important roles in young leaves. They might be involved in the regulation of various enzymes including those in photosynthesis. However, their precise physiological roles remain to be elucidated. The results obtained may be very useful for protein regulation studies. A further approach should be required for elucidating the mechanism of variations in the enzyme activities during leaf development.

Acknowledgement This work was supported by the Basic Science Research Institute Program (BSRI-96-4439), the Ministry of Education, Republic of Korea.

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