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An Elicitor-Inducible NADP-Malic Enzyme in *Lithospermum erythrorhizon* Cultured Cells: cDNA Cloning and Characterization

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Abstract

The differential display technique was used to isolate genes expressed during yeast extract-induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures, and a cDNA clone whose deduced amino acid sequence is similar to plant cytosolic NADP-malic enzymes was identified and designated *LeME*. The heterologously-expressed LeME oxidized L-malate in presence of NADP, but it did not catalyze formation of 4-hydroxyphenyllactic acid from 4-hydroxyphenylpyruvate in presence of NADPH, one of the crucial biosynthetic steps leading to rosmarinic acid. The potential role of LeME in elicitor-inducible rosmarinic acid biosynthesis is discussed.

Keywords: elicitation, *Lithospermum erythrorhizon*, NADP-malic enzyme, rosmarinic acid biosynthesis.

Accession number: AB078329.

Abbreviations

HPR, 4-hydroxyphenylpyruvate reductase; MJ, methyl jasmonate; PAL, phenylalanine ammonia-lyase; RA, rosmarinic acid; YE, yeast extract.

Introduction

The co-evolution of plants with their pathogens and herbivores has resulted in a remarkably wide array of plant biochemical reactions leading to defense-related compounds. Herbivore- or plant cell wall-derived elicitors often mediate such defense responses. Cell suspension cultures provide us with a convenient model to investigate the effects of elicitors on plant secondary metabolism and establish the underlying mechanisms that regulate these metabolic responses. Rosmarinic acid (α-*O*-caffeoyl-3, 4-dihydroxyphenyllactic acid, RA) is a common hydroxycinnamoyl ester accumulating in Boraginaceae and Lamiaceae plants. In *Lithospermum erythrorhizon* (Boraginaceae) cell suspension cultures, synthesis of rosmarinic acid is rapidly and transiently stimulated by addition of yeast extract (YE) or methyl jasmonate (MJ) (Mizukami *et al.*, 1992, 1993). Elicitation of RA biosynthesis has also been reported in cell suspension and organ cultures of some Lamiaceae species, including *Orthosiphon aristotus* (Sumaryono *et al.*, 1991), *Coleus blumei* (Szabo *et al.*, 1999) and *Salvia miltiorrhiza* (Chen *et al.*, 2001).

During the course of differential display analysis to identify the genes induced during YE elicitation of RA biosynthesis in *L. erythrorhizon* cultured cells, we have isolated a cDNA encoding a NADP-malic enzyme. Here, we show that the mRNA level and activity of the NADP-malic enzyme are strongly up-regulated by addition of YE and MJ to *L. erythrorhizon* cell cultures, and discuss the possible role of the malic enzyme in the cellular response to the elicitor treatment.

Materials and Methods

Plant cell culture and elicitor treatment

Suspension cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. were established from seedling-derived callus tissues and have been maintained in LS liquid medium (Linsmaier and Skoog, 1965) supplemented with 1 μM 2,4-dichlorophenoxy-

acetic acid and 1 μM kinetin. Cell suspension (5 ml) was transferred into 25 ml fresh medium in a 100 ml Erlenmeyer flask at 14-day intervals and cultured on a rotary shaker at 25 ℃ in the dark. Yeast extract (Difco) was dissolved in water, autoclaved at 120 ℃ for 20 min, and aseptically added to the cell suspension at a final concentration of 5 g l^{-1}. Methyl jasmonate (Tokyo Kasei) was dissolved in dimethylsulfoxide and added to the cultures through a membrane filter to give a final concentration of 100 μM. These elicitors were added to the cultures 7 days after cell transfer and the cells were collected by vacuum filtration at defined times, immediately frozen in liquid nitrogen, and stored at −80 ℃.

Differential display

Differential display analysis was performed as described elsewhere (Matsuno *et al.*, 2002), essentially based on the method of Yoshida *et al.* (1994).

Isolation and analysis of nucleic acids

Total RNA was extracted from the frozen cells using TRIzol (Invitrogen) according to a protocol supplied by the manufacturer. Poly(A)$^+$RNA was prepared from the YE-treated cells 8 h after YE addition, using a Quick Prep mRNA Purification Kit (Amersham Biosciences). The cDNA library was constructed using a λZAP-cDNA Synthesis/Gigapack Gold Packaging kit (Stratagene). For northern hybridization, total RNA (20 μg) was fractionated in a formaldehyde-1.25% agarose gel and transferred to a Hybond N+ membrane (Amersham Biosciences). Total DNA was prepared according to the method described by Rogers and Bendich (1994). For Southern hybridization, total DNA (10 μg) was digested with *Bam*HI or *Eco*RI at 37 ℃ for 3 h, fractionated in a 1% agarose gel and transferred to a Hybond N+ membrane. The membrane was hybridized with a digoxigenin-labeled probe overnight and washed in the 2 x SSC buffer containing 0.1% SDS twice for 5 min each at room temperature and then in the 0.1 x SSC buffer containing 0.1% SDS twice for 15 min each at 68 ℃ . The digoxigenin-labeling of the probes using the PCR DIG Probe Synthesis Kit (Roche Biochemicals) and chemiluminescent detection of the hybridization signals were carried out according to a protocol supplied by Roche Biochemicals.

Heterologus expression of LeME

For construction of the pMAL-LeME expression vector, the open reading frame of *LeME* was amplified by PCR using *Pfu* DNA polymerase (Promega) with primers 5'-CGCGAATTCATGGAGAGTAGTAATTTGAAGGATGTTACAGG-3' and 5'-GCGGATCCTCAACGATAAGTGCGGTAGATAGGCGAATAC-3'. These primers correspond to the 5'-and 3'-ends of the open reading frame and introduce an *Eco*RI site upstream of the start codon and a *Bam*HI site downstream of the stop codon. The 1.7 kb PCR product was subcloned into *Eco*RI-*Bam*HI digested pMAL-c2 (RIKEN DNA Bank, Japan) and sequenced to ensure that no mutation was incorporated. The resulting expression vector was transformed into *E. coli* JM109. Protein expression and purification of the recombinant protein was performed as described according to a protocol supplied by New England Biolabs, Inc.

Assay for enzyme activity

The frozen plant cells (0.2 g) were homogenized in 2 ml of 50 mM tris-HCl buffer, pH 7.6, containing 5 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5% (w/v) glycerol. The homogenate was centrifuged at 12000g for 20 min at 4 ℃ and the supernatant was used as an enzyme preparation. Protein concentration was estimated by the method of Bradford (1976).

NADP-malic enzyme activity was measured spectrophotometrically by monitoring NADPH formation at 340 nm as decribed by Maurino *et al.* (2001). 4-Hydroxyphenylpyruvate reductase activity was assayed by detecting formation of 4-hydroxyphenyllactic acid by HPLC as described previously (Mizukami *et al.*, 1993).

Quantitative determination of RA

The frozen cells (about 0.2 g) were extracted with 2 ml methanol at 65 ℃ for 60 min with vigorous shaking. The slurry was centrifuged at 12000g for 5 min and the supernatant was subjected to HPLC. The conditions for HPLC analysis were described previously (Mizukami *et al.*, 1992)

Results

Isolation and identification of LeME cDNA as a YE-inducible gene by differential display

By a simplified differential display approach, three YE-inducible cDNA clones (F86A, F86B and A07A) were obtained and sequenced. Characterization of the A07A (Matsuno *et al.*, 2002) and F86B cDNA fragments will be reported elsewhere. A BLAST search using about 315 bp of the F86A sequence showed that the nucleotide sequence is similar to those of various plant NADP-malic enzymes. The F86A fragment was digoxigenin-labeled and used as a probe to screen about 30000 plaques in a cDNA library constructed from the YE

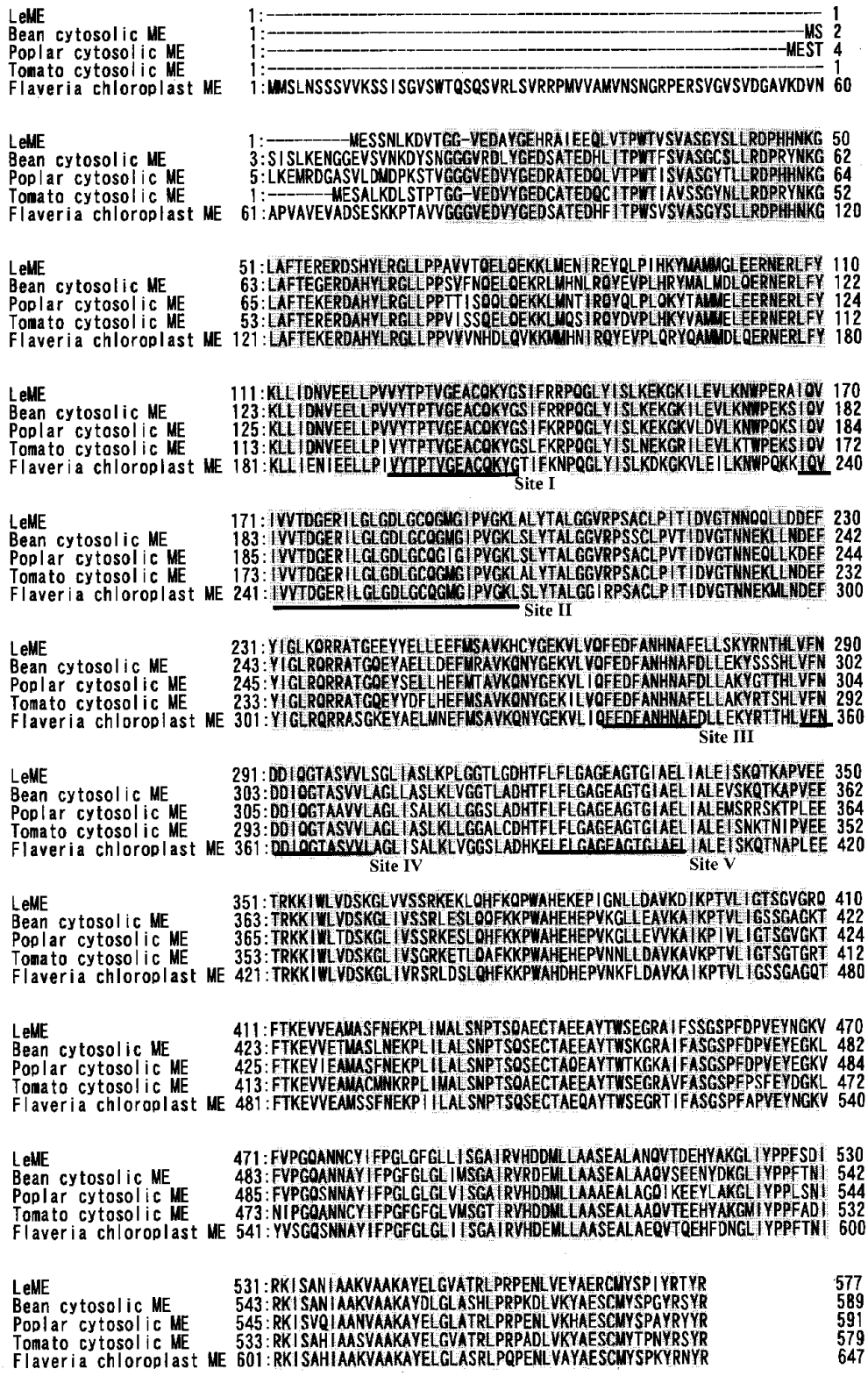

Fig. 1 Multiple alignment of the deduced amino acid sequences of four cytosolic NADP-malic enzymes from C_3 species, *Lithospermum erythrorhizon* (LeME), bean, poplar tomato, and a chloroplast enzyme from the C_3 species *Flaveria pringlei*. Sites I - V are the conserved regions among NADP-malic enzymes (Drincovich *et al.*, 2001). Site IV is named a malic enzyme signature motif. The nucleotide sequence of *LeME* will appear in DDBJ/EMBL/GenBank DNA databases with the accession number AB078329. The accession numbers of bean, poplar, tomato and *Flaveria pringlei* sequences are X80051, X56233, AF001270 and P36444, respectively.

-treated *L. erythrorhizon* cells. After three rounds of plaque hybridization, a cDNA clone (*LeME, L. erythrorhizon* NADP-malic enzyme) covering the entire protein coding region was isolated and sequenced. The cDNA clone was 2064-bp long with an open reading frame corresponding to 577 amino acids, a 49-bp 5' non-coding sequence and a 281-bp 3' non-coding region. The deduced amino acid sequence displayed the presence of a malic enzyme signature motif. A search of DDBJ/EMBL/GenBank databases revealed that the encoded protein shared about 80% amino acid sequence identity with NADP-malic enzymes from various higher plants, especially with cytosolic NADP-malic enzymes from C_3 plants (**Fig. 1**). Analysis of the N-terminal sequence by the algorithm described by Nielsen *et al.* (1997) at the SignalP website (www.cbs.dtu.dk/services/SignalP) indicated the absence of a signal peptide as typically shown in the chloroplast NADP-malic enzyme of *Flaveria pringlei* in **Fig. 1**.

Enzyme activity of the recombinant LeME protein

To examine the catalytic function of the gene product, *LeME* was heterologously expressed in *E. coli* as a fusion protein with maltose binding protein (MBP). The resulting recombinant fusion protein (MBP-LeME) was purified by affinity chromatography on an amylose-resin column and assayed for malic enzyme activity. As shown in **Fig. 2**, the recombinant fusion protein catalyzed formation of NADPH from sodium malate and NADP in a dose-dependent manner. No significant change was observed in the specific activity between the fusion protein and the factor Xa-cleaved protein. The activity was very low when NAD was added in the reaction mixture instead of NADP. The recombinant protein did not catalyze the formation of 4-hydroxyphenyllactic acid from 4-hydroxyphenylpyruvic acid in the presence of NADPH.

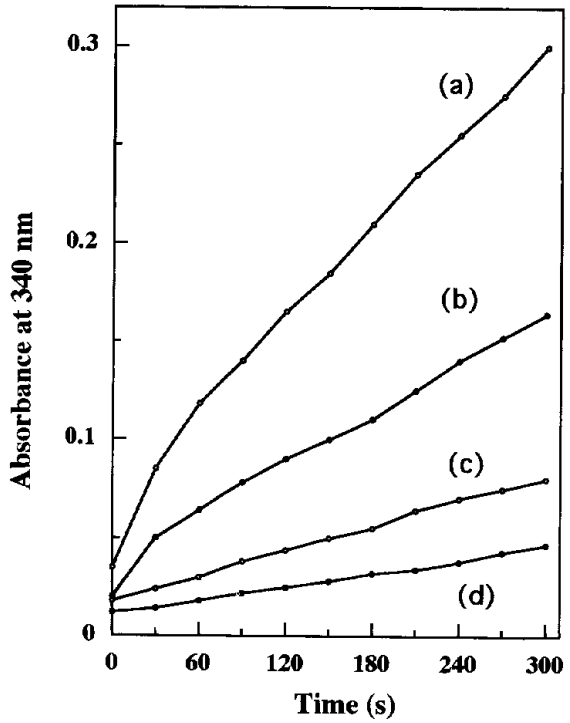

Fig. 2 Formation of NADPH from malic acid and NADP by the recombinant LeME protein. Assays were performed at 30 ℃, in 1 ml 50 mM tris-HCl buffer, pH 7.6, 10 mM MgCl2, 0.5 mM NADP, 4 mM L-malate and (a) 360 μg, (b) 180 μg, (c) 90 μg, or (d) 45 μg recombinant protein.

Changes in the LeME expression and malic enzyme activity during the course of RA induction by elicitor in L. erythrorhizon *cell suspension cultures*

In order to characterize the relationship between *LeME* expression and elicitor-induced RA biosynthesis, we analyzed the changes in LeME mRNA level, in malic enzyme activity and in RA accumulation following different elicitor treatments (**Fig. 3**).

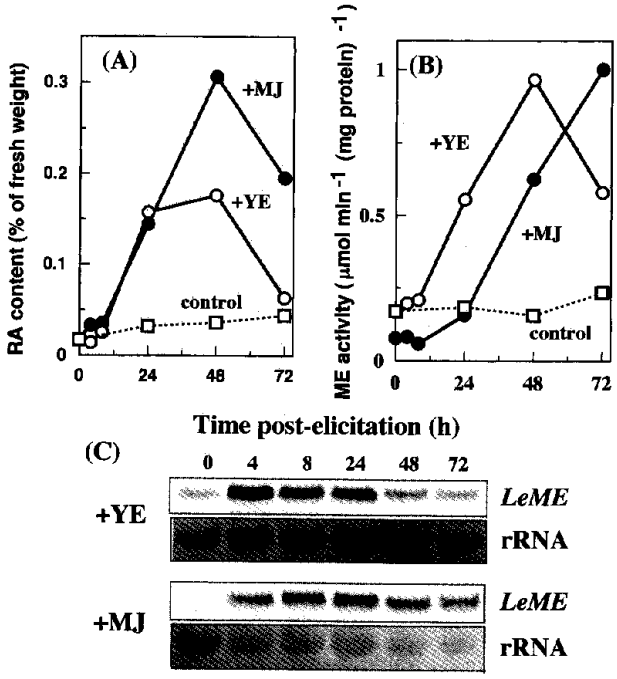

Fig. 3 Changes in rosmarinic acid accumulation (A), NADP-malic enzyme activity (B) and LeME mRNA level (C) in *Lithospermum erythrorhizon* cultured cells treated with yeast extract (YE) or methyl jasmonate (MJ). Yeast extract and methyl jasmonate was added at final concentrations of 5 g l^{-1} and 100 μM, respectively, to the 7-day-old suspension cultures. For northern hybridization, total RNA (20 μg) from the cells were electrophoresed on a formadehyde-agarose gel, transferred to a nylon membrane and hybridized with digoxigenin-labeled probe covering a whole coding region of *LeME*.

RA accumulation in *L. erythrorhizon* cells started to increase gradually within 4–8 h after YE addition, followed by a rapid increase until 24 h. Expression of *LeME* rose within 4 h after YE treatment, reached a peak between 4 and 24 h and thereafter decreased, consistent with the YE-induced changes in RA accumulation. NADP-malic enzyme activity in the soluble fraction of the cultured cells increased drastically after a lag phase of 4 h and reached a peak 48 h after YE addition. MJ treatment, on the other hand, induced a larger increase in RA accumulation than did YE treatment, but the response of the cells to MJ was slower. RA production started to increase 8 h after MJ addition, and rose rapidly until 48 h after MJ treatment. A similar delay was observed in *LeME* expression. *LeME* mRNA levels started to increase within 4 h after MJ treatment and reached a maximum at 24 h. NADP-malic enzyme activity in MJ-treated cells increased after a lag of 8 h and continued to increase linearly up to 72 h after MJ addition. Although RA content was about two-fold higher in the MJ-treated cells than in the YE-treated cells, the peak activity of NADP-malic enzyme induced in the MJ-treated cells was similar to that in the YE-treated cells.

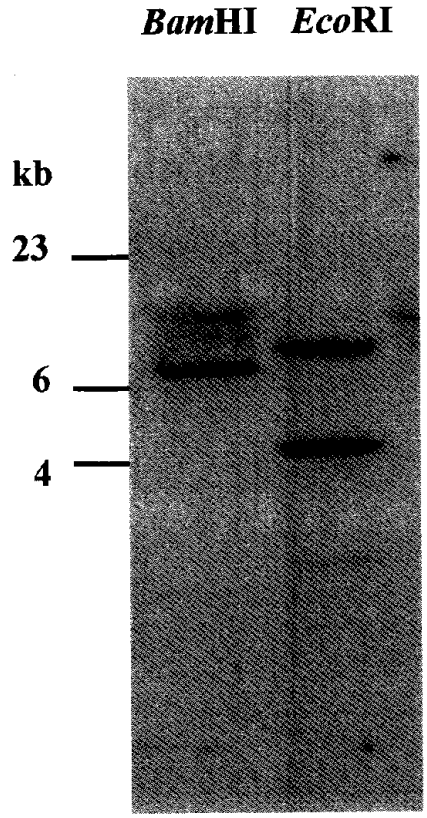

Fig. 4 Southern blot analysis of the *LeME* gene from *Lithospermum erythrorhizon*. Genomic DNA (10 μg) prepared from the cultured cells was digested with *Bam*HI or *Eco*RI, fractionated on an agarose gel, transferred to a nylon membrane and hybridized with digoxigenin-labeled probe covering the entire coding region of *LeME*.

Estimation of gene copy number

To assess the number of NADP-malic enzyme genes in *L. erythrorhizon*, total DNA from the cultured cells was digested with two different restriction enzymes (*Bam*HI and *Eco*RI), neither of which has a recognition site within the cloned cDNA sequence. Under the high stringency conditions, both *Bam*HI and *Eco*RI digests gave three signals, two strong and one weak. This hybridization pattern suggests either that three copies of the NADP-malic enzyme gene are present in *L. erythrorhizon* genome, or that *LeME* is a single-copy gene and the multiple bands are generated due to restriction sites in the introns.

Discussion

In this study we cloned a malic enzyme cDNA, *LeME*, from elicited *L. erythrorhizon* suspension cultures using the differential display technique. The recombinant LeME protein catalyzes formation of NADPH from malic acid and NADP but not from NAD. The deduced amino acid sequence lacks a chloroplast targeting signal peptide, indicating that *LeME* encodes a cytosolic (non-photosynthetic) NADP-malic enzyme.

NADP-malic enzyme is widely distributed in prokaryotes and eukaryotes. It catalyzes the oxidative decarboxylation of L-malate to yield pyruvate, CO_2 and NADPH in the presence of a bivalent cation. In C_4 photosynthesis plants such as maize, chloroplast NADP-malic enzyme plays a key role in photosynthetic metabolism where it generates CO_2 and reducing power for Rubisco and for the photosynthetic carbon reduction cycle, respectively. Cytosolic NADP-malic enzyme in certain crassulacean acid metabolism (CAM) plants performs an analogous role. In contrast, functional roles for cytosolic NADP-malic enzymes in C_3 plants have not been fully clarified. Various cytosolic NADP-malic enzymes lacking transit peptides have been characterized by isolation of their respective cDNA clones from C_3 plants such as bean (Walter *et al.*, 1990), poplar (van Doorsseleare *et al.*, 1991) and grape (Franke *et al.*, 1995). In bean, the malic enzyme mRNA was also induced by fungal elicitor (Walter *et al.*, 1988), and characterization of the bean NADP-malic enzyme gene revealed that fungal elicitor- and UV-inducible *cis*-regulatory elements are present in the promoter region (Walter *et al.*, 1994). Fusion of this promoter to the β-glucuronidase reporter gene confirmed that the promoter was activated by different effectors related to plant

defense responses (Schaaf *et al.*, 1995). Thus, it was concluded that the cytosolic NADP-malic enzymes in C_3 plants may be involved in plant defense responses, possibly by providing NADPH for biosynthesis of defense compounds (Drincovich *et al.*, 2001).

The expression of the *LeME* gene was elevated by addition of YE or MJ *to L. erythrorhizon* cultured cells, and the expression profile was consistent with the changes in enzyme activity of soluble NADP-malic enzyme and RA production in the cells. Elicitation by YE or MJ induced increased activity of both phenylalanine ammonia-lyase (PAL) and 4-hydroxyphenylpyruvate reductase (HPR), entry-point enzymes for the phenylpropanoid pathway and the tyrosine-derived pathway, respectively (Mizukami *et al.*, 1993). Based on this correlation, and the observation that pigeon malic enzyme is known to also catalyze reduction of certain α-keto acids such as pyruvic acid to α-hydroxy acids (Tang and Hsu, 1974), we hypothesized that LeME might represent the gene encoding the HPR activity. However, although it catalyzed oxidation of malic acid, recombinant LeME protein failed to catalyze formation of 4-hydroxyphenyllactic acid from 4-hydroxyphenylpyruvic acid in the presence of NADPH. It is therefore likely that LeME is not directly involved in RA biosynthesis but is probably indirectly contributing to elicitor-induced RA accumulation by providing the NADPH needed at several steps in RA biosynthesis. A cytosolic and non-photosynthetic NADP-malic enzyme of a C_4 plant, maize, was reported to be up-regulated in response to fungal elicitor or jasmonate treatment (Maurino *et al.*, 2001). Recently, it was suggested that cytosolic NADP-malic enzymes from both C_3 *Falveria* species (*F. pringlei*) and C_4 species (*F. trinervia*) have several distinct roles including the supplying NADPH for cytosolic metabolism and the supporting of wound response (Lai *et al.*, 2002). Defense-related inducibility may be a universal feature of non-photosynthetic NADP-malic enzymes in higher plants.

In summary, by using a differential display technique we have cloned from *L. erythrorhizon* suspension cultures a cDNA encoding a cytosolic NADP-malic enzyme (LeME). The heterologously expressed protein catalyzed oxidative decarboxylation of malic acid to yield NADPH, as expected. However, it did not reduce 4-hydroxyphenylpyruvic acid to form 4-hydroxyphenyllactic acid, one of the critical steps in RA biosynthesis, suggesting that LeME is more indirectly involved in RA formation by providing NADPH.

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