Subcellular localization of PAL genes in *Citrus limon*

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Plant defense against pathogens involves mechanisms that include the expression of several genes, which could be located in any of the three compartments that contain genomes in plant cells: nuclei, mitochondria and chloroplasts. To transfer defense genes against pathogen attack from resistant to sensitive plants, it is desirable to know in which genomic context these defense genes are located before they are introduced in the appropriate compartment within the recipient cell. Previous work has suggested that the ***Citrus limon*** *defense response against* ***Alternaria alternata*** *requires functional chloroplasts (Chiong* ***et al****, 1992). Phenylalanine ammonia-lyase (PAL, E.C.4.3.1.5.) is one of the enzymes involved in early events of this defense. To determine whether the involvement of organelles was due to the presence of PAL genes in more than one genome or to the participation of other cellular signals, the subcellular localization of PAL genes in* ***C. limon*** *was examined. Chloroplasts and mitochondria were purified in order to prepare the organelle-derived genomes. Total DNA was also prepared to represent the nuclear genome. Dot blot hybridizations of organellar and total DNA preparations, using a heterologous cDNA PAL probe (Edwards* ***et al****, 1985), showed that PAL genes are located in the nuclear genome.*

Key terms*:* ***Citrus limon****, PAL genes, phenylalanine ammonia-lyase (PAL), subcellular localization.*

INTRODUCTION

Different environmental, developmental and genetic factors interact to control the expression of genes in plants, including those related to their defense against pathogens, which are expressed during incompatible interactions (Agrios, 1988). The elucidation of the mechanisms by which gene expression is controlled is of fundamental interest, due to the advance of genetic engineering and plant transformation. The study of these mechanisms must take into account that plant cells contain three genomes localized in nuclei, mitochondria and chloroplast, that could be expressed independently or interacting among them (Taylor, 1989).

Phenylalanine ammonia lyase (PAL, E.C.4.3.1.5.) catalyzes the conversion of L-phenylalanine to transcinnamic acid, and is the first committed step of the biosynthetic pathway of phenylpropanoid compounds. This enzyme holds a key position in the secondary metabolism of higher plants and is highly regulated during development and lignification (Jones, 1984). PAL levels also

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Abbreviations: BSA: bovine serum albumin; **CTAB**: cetyltrimethylammonium bromide; **EDTA**: ethylenediamine-tetraacetic acid; **GDH**: glutamate dehydrogenase; **HEPES**: N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; **PAL**: phenylalanine ammonia-lyase.

fluctuate in response to environmental stimuli, such as exposure to UV light (Kuhn *et al*, 1984), fungal infection (Loschke *et al*, 1981) and treatment with elicitors (Lawton *et al*, 1983; Dalkin *et al*, 1990). These effectors, among others, induce *de novo* synthesis of PAL.

Several metabolic forms or isoenzymes of PAL have been described in bean (Bolwell *et al*, 1985; Cramer *et al*, 1989) and alfalfa (Jorrin and Dixon, 1990). These polymorphic forms are differentially regulated by developmental cues and environmental factors (Gowri *et al*, 1991; Liang *et al*, 1989). Furthermore, experimental evidence suggests that the expression of PAL in different tissues and cell types is also finely regulated (Ohl *et al*, 1990). Other authors have proposed that the various forms of PAL could be related to differences in subcellular location (Jones, 1984). In fact, PAL is usually detected in the supernatant of whole tissue homogenates (Zimmermann and Hahlbrock, 1975), but it has also been found in organelles, such as chloroplasts from petunia (Ranjeva *et al*, 1977) and spinach (Nishizawa *et al*, 1979).

Fluctuations in PAL levels have been observed in *Citrus* species after injuries of orange flavedo (Ismail and Brown, 1979) and fungal infection of lemon peel (Baudoin and Eckert, 1985). Lemon seedlings inoculated with *Trichoderma harzianum* (Quaas *et al*, 1993) or with *Alternaria alternata* (Roco *et al*, 1993) also exhibit an increase in the expression of the PAL gene(s). Evidence obtained in our laboratory suggests that the defense response of this species against pathogens involves both the nuclear as well as organellar compartments. In fact, this response: a) is suppressed both by prokaryotic and eukaryotic inhibitors of transcription and translation maintaining a basal PAL activity of 5 pkat/mg proteins (Quaas *et al*, 1993; Pérez *et al*, 1994), and b) shows a 90% decrease of PAL induction (12 fold over basal activity) in etiolated seedlings (Chiong *et al*, 1992). These results suggest the presence of a PAL gene outside the nuclear compartment or an interplay of regulatory signals between the nuclear and organellar genomes. None of these possibilities have been explored in the PAL system of *Citrus*.

PAL genes have been analyzed in several species and are generally encoded by a small family of three or four genes in bean (Cramer *et al*, 1989), parsley (Lois *et al*, 1989) and sweet potato (Tanaka *et al*, 1989), 6 genes in rice (Minami *et al*, 1989) and 4 or 5 genes in *Arabidopsis* (Ohl *et al*, 1990). However, in potato at least 40-50 genes coding for PAL have been detected (Joos and Hahlbrock, 1992), while only a single copy of the PAL gene is present in loblolly pine (Whetten and Sederoff, 1992). PAL genes from the yeasts *Rhodotorula toruloides* (Anson *et al*, 1987) and *Rhodotorula rubra* (Filpula *et al*, 1988) have also been reported. Several of the genes described so far from plant species contain a single intron (Joos and Hahlbrock, 1992; Liang *et al*, 1989; Cramer *et al*, 1989), although the PAL gene from loblolly pine does not contain introns (Whetten and Sederoff, 1992).

Most of these reported results suggest a nuclear localization of the gene, although in all cases total DNA has been analyzed. As a first approach to explain the observed requirement of functional organelles for the induction of PAL in the defense response, we explored the localization of the PAL gene(s) in *Citrus limon* seedlings.

MATERIALS AND METHODS

All reagents were of analytical grade and purchased from Sigma, Merck and Gibco-BRL. Restriction enzymes were from Gibco-BRL. (^{35}S)-α-dCTP was obtained from New England Nuclear, Biolabs, USA.

Organelle isolation

C. limon seedlings were obtained from seeds as described (Roco *et al*, 1993) and used for the purification of organelles. They were placed in total darkness at 28°C during 4 days prior to their preparation, to deplete the cells of starch. In some preparations, cotyledons were also removed from the rest of the seedling to avoid the mobilization of starch to other organs.

Organelles were purified by modified protocols from Leegood and Walker (1983) and Jackson and Moore (1979). Seedlings

(100 - 200 g) were washed, their seedcoats removed, surface sterilized in 1% (v/v) commercial NaOCl and chopped manually in successive batches of cold homogenization buffer (0.35 M mannitol, 50 mM HEPES-KOH (pH 7.8), 1 mM EDTA, 0.2% BSA, 4 mM L-cysteine, 0.5% (w/v) PVP 40,000 and 1 mM ß-mercaptoethanol). The homogenate (250-500 ml) was filtered through 6 layers of cheese cloth and centrifuged for 8 min at 1,500 x *g* (chloroplast fraction). The supernatant was centrifuged at 12,000 x *g* for 20 min (mitochondrial fraction). Both pellets were resuspended in a small volume of homogenization buffer and then incubated with DNAse I (5 µg/ml) and 10 mM $MgCl_2$ at 4°C. After an incubation of 30 min, the enzyme was inactivated adding EDTA to a final concentration of 20 mM. The chloroplast fraction was then centrifuged first at low speed (450 x *g* for 2 min) to eliminate debris, and afterwards at 1,500 x *g* for 15 min ($P_{1,500}$). Similarly, the mitochondrial preparation was centrifuged at 1,500 x *g* for 3 min and then at 12,000 x *g* for 20 min ($P_{12,000}$). Both pellets were carefully resuspended with a soft brush in 5 ml gradient buffer (0.3 M mannitol, 10 mM HEPES-KOH pH 7.2, 0.1% (w/v) BSA, 1 mM KH_2PO_4) and loaded on a discontinuous 27%/30%/60% Percoll gradient (5 ml / 6 ml / 5 ml, respectively). The gradients were centrifuged at 9,500 x *g* for 30 min in a fixed angle rotor.

The chloroplast preparations showed a dark green band immediately below the 27%/30% interphase of the Percoll gradient and a green smear in the 30% phase. The dark band was collected, diluted in gradient buffer and centrifuged at 1,500 x *g* for 15 min. The mitochondrial preparations exhibited a thin white band at the 30%/60% interphase and a green smear in the upper region of the Percoll gradient. The white band was collected, diluted in gradient buffer and centrifuged at 12,000 x *g* for 20 min.

Isolation of plant DNA

DNA from purified organelles and from total *C.limon* and 6 day old *Phaseolus vulgaris* seedlings was prepared as described by Doyle and Doyle (1990) with some modifications. Briefly, for the preparation of total DNA, 1.5-2.0 g of fresh seedlings were homogenized at 60°C in CTAB buffer (100 mM Tris-HCl pH 8.0, 0.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 0.2% (v/v) ß-mercaptoethanol), incubated for 45 min at 60°C, extracted twice with chloroform/isoamyl alcohol (24:1) and precipitated overnight at -20°C with 0.6 volumes of cold isopropanol. The DNA was then spun out, the pellet washed in 70% (v/v) ethanol, resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and incubated with RNAse (10 µg/ml) and extracted once with chloroform/isoamyl alcohol (24:1) before precipitating with 1/5 volume of 10 M ammonium acetate and two volumes of ethanol. Yields were approximately 20 µg DNA / g fresh tissue of *C. limon* and ranged between 50 - 100 µg DNA/ g of fresh tissue of *P. vulgaris*. Preparations were pooled for further purification in CsCl/ethidium bromide gradients and centrifuged at 60,000 rpm during 24 h at 20°C in a Ti-80 rotor. All experiments with total DNA were performed using material purified with the CsCl gradient.

For the preparation of organellar DNA, mitochondrial and chloroplast pellets from the Percoll gradient were resuspended in CTAB buffer and processed as described above. Approximate yields were 1 µg of chloroplast DNA and 0.5 µg of mitochondrial DNA per 100 g fresh tissue of *C. limon* seedlings.

Marker enzyme, chlorophyll and protein assays

Glutamate dehydrogenase activity (marker enzyme for mitochondria) was assayed using the procedure described by Leighton *et al* (1968). Chlorophyll was measured in 80% (v/v) acetone extracts of homogenates and subcellular fractions of *Citrus* seedlings. The concentration of chlorophyll was calculated after measuring the absorbance at 652 nm of the organic extracts (Arnon, 1949). Protein concentration was determined by the method of Bradford (1976).

Microscopy

For transmission electron microscopy, mitochondrial and chloroplast pellets were fixed

for 24 h in 2% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.4 and post-fixed for 1 h in 1% (w/v) OsO4. Preparations were dehydrated in a gradient of acetone and embedded in epoxy resin. Ultrathin sections were stained in uranyl acetate and lead citrate and viewed with a Phillips EM 300 electron microscope.

Preparation of pPAL5 probe

Escherichia coli DH5α was used as host for plasmids. Standard recombinant DNA techniques were used to prepare plasmids and obtain restriction fragments (Sambrook *et al*, 1989). After purification of the plasmid DNAs with a CsCl gradient, the *pal* insert was excised from pPAL5 with Pst I (Edwards *et al*, 1985). The insert was subsequently electrophoresed in 1.2% (w/v) agarose gels, stained with ethidium bromide and electroeluted using NA-45 membranes (Schleicher and Schuell, Dassel, Germany). The NA-45 membranes were washed with NET solution (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl pH 8.0) and the DNA recovered with two washes with 250 µl High Salt NET (NET, 1 M NaCl). Ethidium bromide was eliminated with three extractions with water-saturated n-butanol and the DNA was precipitated twice with ethanol and resuspended in 100 µl of sterile water or 20 µl of TE (20 mM Tris-HCl pH 8.0, 1 mM EDTA).

Dot blot hybridizations

For dot blot experiments, DNA samples were sheared through extrusion in a 50 µl Hamilton syringe, denatured at 100°C, applied with a BIO-DOT manifold (Bio-Rad) and drawn on nitrocellulose membranes using vacuum. The *pal* probe was labelled with (^{35}S)-α-dCTP by nick-translation according to the manufacturer's instructions.

Membranes were pre-hybridized for 2 h in 250 µl/cm^2 of pre-hybridization solution (6x SSPE, 5x Denhardt's, 0.5% (w/v) SDS, 50% (v/v) formamide and 200 µg/ml denatured salmon sperm DNA) and then hybridized for 24 h at 42°C in 0.1 ml fresh prehybridization solution containing 400,000 cpm/ml of the labelled *pal* probe and 50% (v/v) formamide in 20% (w/v) dextran sulphate. The blots were washed 5 min in 2x SSC, 0.5% (w/v) SDS and 15 min in 2x SSC, 0.1% (w/v) SDS at room temperature; 30 min in 0.1x SSC, 0.5% (w/v) SDS at 60°C and 30 min in the same solution at room temperature. Autoradiograms were exposed for 24-72 h at -20 or -70°C using XAR 5 film.

RESULTS AND DISCUSSION

Purification of chloroplast and mitochondrial DNA from ***C. limon***

Mitochondria and chloroplast fractions were initially prepared by differential centrifugation which allowed a partial separation of both organelles, as determined by chlorophyll and glutamate dehydrogenase distribution (Table I). Further purification of mitochondria and chloroplasts was achieved using a discontinuous step Percoll gradient, as assessed by electron microscopy (Fig 1). The distribution of chlorophyll and glutamate dehydrogenase activity in the purified fractions confirmed this finding, since in the purified mitochondrial fraction specific activity of the marker enzyme increased 11.3

TABLE I

Distribution of chloroplast and mitochondrial markers in fractions obtained after differential centrifugation

Fraction	% Protein [a]	% GDH [b]	% Chlorophyll [c]
$P_{1,500}$ (chloroplasts)	1.32	27.5	89.8
$P_{12,000}$ (mitochondria)	1.40	61.9	10.2
$S_{12,000}$ (supernatant)	95.15	10.6	0.0

[a] % protein calculated from total proteins (related to all fractions). Recovery of total proteins in all fractions with respect to the homogenate was 75 % (average of 10 fractionations that did not exceed 5% SD).

[b] % GDH calculated from total units (related to all fractions). Recovery of total units in all the fractions with respect to the homogenate was 80 % (average of 5 fractionations that did not exceed 5% SD).

[c] % chlorophyll calculated from total amount (related to all fractions). Recovery of total chlorophyll in all the fractions with respect to homogenate was 78 % (average of 5 fractionations that did not exceed 5% SD).

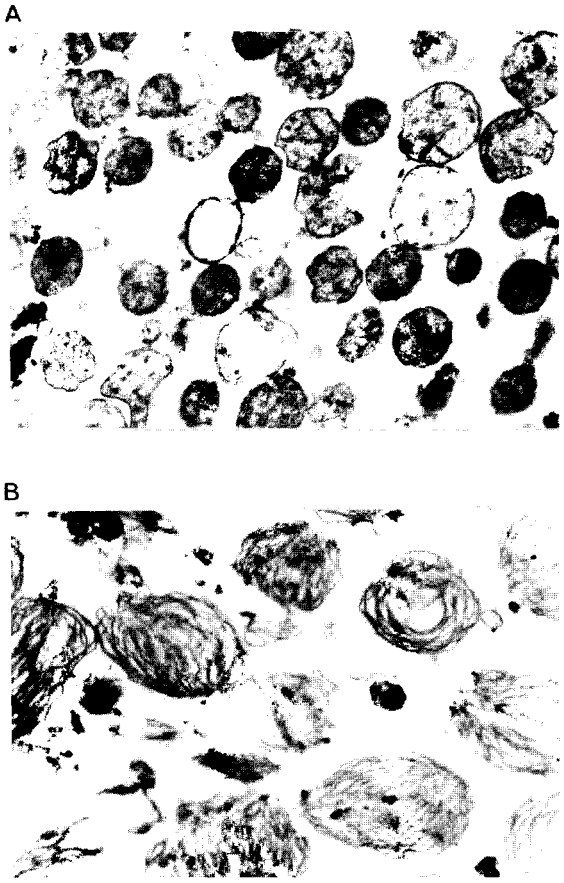

Fig 1. Electron microscopy of chloroplast and mitochondrial fractions purified by a discontinuous Percoll step (21/30/60 %) gradient. **A**: Mitochondrial preparation. Magnification 20,000 x. **B**: Chloroplast preparation. Magnification 10,000 x.

fold and the relative chlorophyll concentration decreased to undetectable levels. Also, in the purified chloroplast fraction the relative concentration of chlorophyll increased 6.3 fold, while the specific activity of glutamate dehydrogenase decreased by 40%.

DNA was extracted from the purified organelles and from seedlings. Their analysis showed sharp bands of high molecular weight with absence of DNA degradation (Fig 2). Further digestion with Pst I showed a different digestion pattern of discrete bands for chloroplast and mitochondrial DNA and a homogeneous smear for total DNA. In spite of the very low yield of mitochondrial DNA, a visual comparison of the patterns of organellar DNA showed that most bands differed in their relative migration, suggesting low contamination with nuclear DNA or cross-contamination.

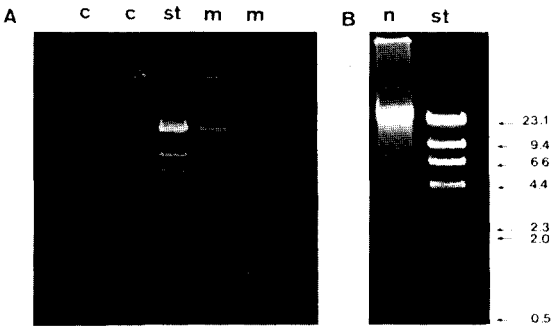

Fig 2. Electrophoresis of DNA preparations from chloroplasts, mitochondria and total DNA on a 1% agarose gel. **1**: DNA from the purified chloroplast fraction. **2**: DNA from the purified mitochondrial fraction. **3**: Lambda DNA digested with Hind III. **4, 5**: Total DNA from lemon seedlings.

Subcellular localization of PAL genes

Figure 3 shows that the PAL gene is present exclusively in nuclear DNA of *C. limon*, as seen by dot-blot hybridizations of total, mitochondrial and chloroplast DNA with a heterologous PAL probe (*pal*) from *Phaseolus vulgaris* (Edwards *et al*, 1985). Therefore, the involvement of organelles in the defense response is not due to the presence of extra-nuclear PAL gene(s). This question had not been addressed directly by other investigators.

This finding reveals that the localization of PAL genes is confined to the nuclear compartment, and that transformation mechanisms have to include only this alternative: to direct this gene to the nucleus.

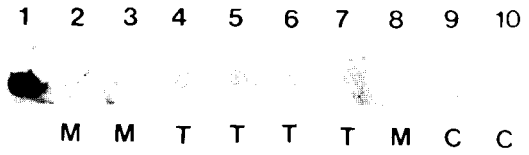

Fig 3. Dot-blot hybridizations of (^{35}S)-α-dCTP *pal* probe to sheared mitochondrial, chloroplast and total DNA preparations. **C**: chloroplast DNA; **M**: mitochondrial DNA; **T**: total DNA. **1**: 10 ng purified *pal* probe (positive control). **2**: 200 ng mitochondrial DNA from *S. tuberosum*. **3**: 400 ng mitochondrial DNA from *S. tuberosum*. **4**: 1 μg total DNA from *P. vulgaris*. **5**: 2 μg total DNA from *P. vulgaris*. **6**: 1 μg total DNA from *C. limon*. **7**: 2 μg total DNA from *C. limon*. **8**: 100 ng mitochondrial DNA from *C. limon*. **9**: 250 ng chloroplast DNA from *C. limon*. **10**: 550 ng chloroplast DNA from *C. limon*.

In consequence, the involvement of organelles in the defense response of *Citrus* seedlings, as evidenced by the effect of antibiotics and the failure to respond of etiolated seedlings, is not related to the localization of PAL genes but to metabolic interaction between organelles. Several interactions are possible: for example, the observed phenomena could be due to the participation of the shikimic pathway which is located within the chloroplast and provides the substrate for the phenylpropanoid metabolism, L-phenylalanine. This amino-acid induces the expression of PAL gene(s) in the yeast *Rhodospiridium toruloides* (Gilbert *et al*, 1983) and increases PAL activity in *Citrus* calli (Thorpe *et al*, 1971), although it has no effect on PAL expression in bean cell suspensions (Jones, 1984). Alternatively, organelles might be involved in the defense response at the level of removal of cinnamic acid, since hydroxylations of this compound occur in plastidic and microsomal fractions (Czichi and Kindl, 1977). Cinnamic acid is known to inhibit transcription of PAL genes (Mavandad *et al*, 1990) and may act as a regulator of the phenylpropanoid pathway (Jones, 1984).

A different explanation of the participation of organelles might involve specific signals from chloroplasts that modulate nuclear gene expression. Such non-proteinaceous signals have been postulated in the regulation of proteins encoded by nuclear genes, but localized in chloroplasts, such as nitrate reductase and the small subunit from RUBISCO, among others (Taylor, 1989). It has been suggested that the concentration of dissolved CO_2 could be the critical parameter involved in PAL induction (Jones, 1984). Then, it can be speculated that if transcription and translation inhibitors are suppressing the synthesis of the large subunit of RUBISCO along with other chloroplastic proteins, the levels of CO_2 should increase, thus repressing the expression of the PAL gene(s). Taken together, the results of this work suggest that the involvement of organelles in the defense response of *C. limon* seedlings is due to metabolic interactions, and now the proposed alternatives that might explain their participation, will have to be explored.

The establishment of the subcellular localization of a gene is important for its targeting to a specific compartment, but does not ensure its expression, particularly in a regulated and complex enzyme such as PAL.

ACKNOWLEDGEMENTS

We are indebted to Dr Christopher J Lamb for his gift of the PAL cDNA clone, and helpful discussions and advice. We thank Drs L Marechal-Drouard and J Weil (Institut de Biologie Moléculaire des Plantes, CNRS, Strasbourg) and Dr Alejandra Moenne (Pontificia Universidad Católica de Chile) for their generous gifts of mitochondrial DNA from *Solanum tuberosum*. We are grateful to Dr Jorge Garrido and Mrs Lucy Messen for their work of electron microscopy and thank Jorge Fernández for his help and advice in some of the early stages of this work. This research was funded by grants FONDECYT 91-0886 and 1940441.

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