Differential Wound Activation of Members of the Phenylalanine Ammonia-Lyase and 4-Coumarate: CoA Ligase Gene Families in Various Organs of Parsley Plants

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We analyzed the developmental regulation and the activation by wounding of several stress-related genes in various parsley organs. The genes encode phenylalanine ammonia-lyase (PAL) and 4-coumarate: CoA ligase (4CL), two enzymes of general phenylpropanoid metabolism; a flavonoid specific enzyme, chalcone synthase (CHS); a furanocoumarin specific enzyme, bergaptol O-methyltransferase (BMT); and a pathogenesis-related protein (PR 1). All genes or gene families exhibited high levels of expression in roots and during certain stages of leaf development. PAL, 4CL and CHS were preferentially expressed in young leaves, BMT and PR 1 in old leaves. An appreciable increase in CHS mRNA levels was observed in wounded leaves. By contrast, root wounding led to a decrease in the existing CHS mRNA levels. A biphasic response (a decrease followed by an increase) to wounding was seen for BMT and PR 1 mRNAs in roots and for BMT mRNA in attached leaves. Using gene-specific oligonucleotide probes to measure the expression rates of three of the four PAL genes and of the two 4CL genes separately we observed a differential behavior of the individual family members under many of the conditions tested. While PAL-3 was preferentially activated in wounded leaves and 4CL-1 in wounded roots, PAL-2 and 4CL-2 were primarily responsible for the high constitutive expression levels in roots and flowering stems respectively. Despite the differential expression of their individual members, the PAL and 4CL gene families displayed very similar changes in the overall patterns of expression, reflecting their closely related functions in phenylpropanoid metabolism.

Introduction

Several genes have been studied with respect to their organ and cell specific or stress related expression in parsley leaves [1–3] or cell suspension cultures [4–7]. Among these genes are those encoding phenylalanine ammonia-lyase (PAL) and 4-coumarate: CoA ligase (4CL), two enzymes catalyzing key reactions of phenylpropanoid metabolism [8]. PAL converts phenylalanine to cinnamic acid, thus connecting primary with secondary plant metabolism, and 4CL generates the common phenolic substrate for numerous developmentally regulated and stress responsive branch pathways.

In parsley, PAL is encoded by a small family of four genes [6, 9] and 4CL by two genes [5, 10]. Gene-specific probes have been used to demonstrate that at least three of the four PAL genes and both 4CL genes are activated in cultured parsley cells upon irradiation with UV light or treatment with fungal elicitor [5, 6]. In an initial experiment with intact plants, evidence was obtained for a differential response of the PAL gene family in wounded roots [6].

In view of the many important functions of PAL and 4CL in plant development, tissue differentiation and stress resistance [8], and with the aim of understanding the role of multiple PAL and 4CL gene copies, we have now further analyzed the expression patterns of the individual genes in different organs and in response to wounding.

Included in these studies were three stress responsive genes whose transcriptional regulation and tissue specific expression have also been investigated to various extents: a single-copy gene encoding chalcone synthase (CHS), the key enzyme of the flavonoid branch pathway [1, 11]; a gene probably also occurring in only one copy [12] that

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encodes bergaptol O-methyltransferase (BMT), a late enzyme of the furanocoumarin branch pathway [2]; and a gene present in three copies encoding a pathogenesis-related protein (PR 1) of unknown function [3].

Materials and Methods

Plant material

Parsley plants were grown in a greenhouse under a 16 h/8 h light/dark cycle and were at least 6 months old. Leaves from different developmental stages were defined as young (all leaves that were not yet fully expanded), intermediate (all fully expanded leaves excluding old leaves) and old (largest leaves not showing signs of senescence). Stems were defined according to the age of the leaves they supported.

Wounding of attached leaves

Intermediate leaves were cut in half with sharp scissors. After the indicated times, slices (~3 mm) parallel to the original cut were removed from the previously wounded leaves and immediately frozen in liquid nitrogen. Intact leaves served as controls.

Wounding of detached leaves and roots

Leaves (~3 mm segments) or roots (~5 mm segments) were sliced with sharp scissors and the segments incubated at room temperature over a moist filter paper in a large tray covered with a damp cloth and then frozen in liquid nitrogen.

Cell cultures

Diploid parsley cell cultures [5, 11] were grown in continuous darkness for 6 days and then exposed to UV-containing white light [13] or treated with 50 µg/ml elicitor derived from the fungus *Phytophthora megasperma* f. sp. *glycinea* [14, 15], or with a combination of UV light and elicitor.

RNA isolation and blot hybridization

RNA was isolated, separated by formaldehyde-agarose gel electrophoresis, blotted and hybridized to ^{32}P-labeled cDNA probes as described previously [6].

Run-on transcription

Isolation of nuclei, transcription and transcript isolation were performed as described in [15]. Blot preparation and hybridization have been described in [6].

Hybridization with gene-specific oligonucleotides

Three gene specific oligonucleotides, GCAGTAAGAAGCCATTGC, GCAGTAACTAGCCATCGC and GCAGTATGATGCCATGGC, corresponding to nucleotide sequences ~920 bp upstream of the polyadenylation site of PAL-1, -2 and -3, respectively [6], and two oligonucleotides, CCGGAGGTTGTGATCAAT and CCCGAGGTAGTGATCGAT, corresponding to a DNA stretch ~520 bp downstream of the translation start site of 4CL-1 and -2, respectively [10], were chemically synthesized (Applied Biosystems model 380 B). These oligonucleotides were end-labeled and used to probe RNA blots as described in [6]. The specific conditions of high stringency hybridization and washing were determined from [17].

Results

Developmental changes

As an indication of gene expression rates for PAL, 4CL, CHS, BMT and PR 1, RNA was extracted from different tissues at different stages of development, blotted, and hybridized with the corresponding cDNA probe. A more or less constitutively expressed gene of unknown function, previously designated as CON 2 [16], was included as a control. The results are shown in Fig. 1.

All tested mRNA levels were high in roots and at some stage during leaf development. An obvious differential behavior was observed in aging leaves for PAL, 4CL and CHS on the one hand and BMT and PR 1 on the other hand. While the former decreased greatly, the latter increased with leaf age. All mRNAs tested were considerably more abundant in the flowering stem than in the vegetative stems. Comparable mRNA levels were always found for PAL and 4CL.

Changes upon wounding

The response to wounding was analyzed in roots and leaves by measuring RNA levels at various

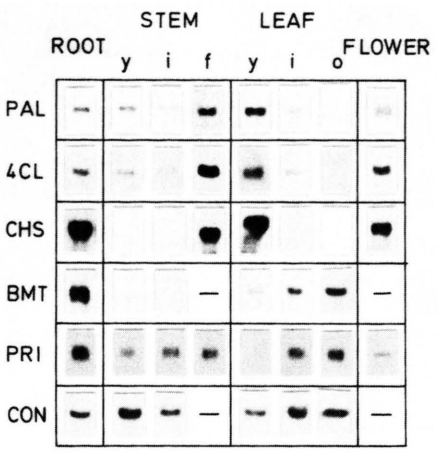

Fig. 1. Developmental changes in the expression patterns of defense-related genes in different parsley organs. Total RNA (10 μg) isolated from the indicated organs was separated on formaldehyde-agarose gels, blotted and hybridized with ^{32}P-labeled cDNA as indicated. Young (y), intermediate (i), flowering (f) and old (o) correspond to the various developmental stages analyzed. The data from each row were obtained by hybridization and exposure of a single blot and thus represent relative levels of expression of each gene in different organs. Two exceptions are the flower and flowering stem data for PAL.

time points. The wound response in leaves differed between the halves remaining attached to the plant and the detached, cut pieces that were incubated on a moist surface. Both sets of data are presented in Fig. 2, together with the results obtained for wounded, detached roots.

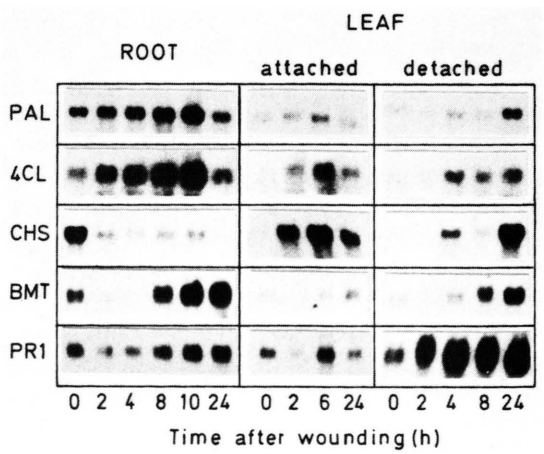

Fig. 2. Wound-induced expression of defense-related genes in parsley roots and leaves. See legend of Fig. 1 for further explanation.

In wounded roots, where all five tested mRNAs were rather abundant, we observed a transient, coordinated increase in the PAL and 4CL mRNA levels. The levels of the other three mRNAs decreased at least transiently. By contrast, the mRNAs of PAL, 4CL and CHS, which were low in mature leaves, increased transiently upon wounding. This increase was very rapid and occurred much earlier in attached than in detached parts of the leaves.

Two of the genes, 4CL and PR1, as well as the reference gene CON2, were tested for transcriptional activity in nuclei isolated from wounded, attached leaves. In agreement with the results shown in Fig. 2, *in vitro* "run on" transcription rates increased rapidly for 4CL and more slowly for PR1. The transcriptional activity of CON2 remained approximately constant (data not shown).

Differential responses within PAL and 4CL gene families

Three synthetic oligonucleotides, specifically detecting PAL-1, PAL-2 and PAL-3 mRNAs, respectively, were available for these studies [6]. Recently, the nucleotide sequence of a fourth, near full length cDNA complementary to PAL-4 mRNA has been described [9]. The portion of the PAL-4 sequence corresponding to the three oligonucleotides turned out to contain several mismatches with respect to PAL-2 and PAL-3, but only one mismatch with respect to PAL-1. Although our experimental conditions were likely to exclude cross-hybridization even between the PAL-1 and PAL-4 probes, this cannot be assumed with certainty. Thus, all of the following results clearly distinguish PAL-1, PAL-2 and PAL-3 from one another, whereas PAL-4 may or may not have been detected to some extent together with PAL-1. The oligonucleotide probes used for measuring 4CL-1 and 4CL-2 mRNAs, respectively, contain three mismatches and thus were highly specific under the conditions used. The first set of columns in Fig. 3 confirms these presumptions by demonstrating the complete lack of cross hybridization among the three PAL or the two 4CL probes.

The second set of results in Fig. 3 indicates a marked differential response in wounded roots, with the 4CL-1 gene being strongly activated and 4CL-2 either inactivated or not affected. By con-

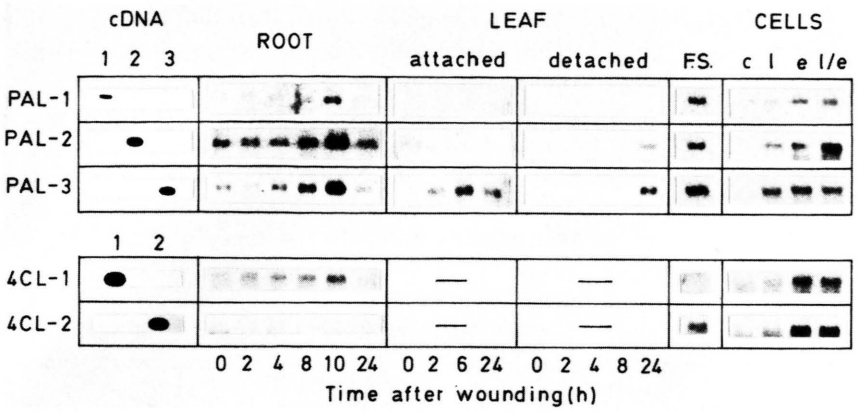

Fig. 3. Differential expression of individual members of PAL and 4CL gene families. Blots were hybridized with gene-specific end-labeled oligonucleotides corresponding to three of the four PAL and both 4CL genes as indicated. In the first column (cDNA), equivalent amounts of respective cDNAs were blotted onto nylon membranes and hybridized in the same solutions as used for the corresponding mRNA blots. F.S., flowering stem. Treatments of cultured cells: c, control; l, 6 h light; e, 3 h elicitor; l/e, 3 h light and elicitor.

trast, all three PAL genes tested were activated to a similar extent, although a high basal level (see also Fig. 2) was mostly due to PAL-2. Only PAL and not 4CL was measured in wounded leaves. In accord with earlier results, PAL-3 but not PAL-1 and PAL-2 was rapidly activated under these conditions.

A different situation was observed in flowering stems and cell suspension cultures, as shown in the last two sets of columns in Fig. 3. The high basal levels in flowering stems were due only to expression of 4CL-2 in the 4CL family, and to expression of all three genes in the PAL family. In cell suspension cultures, however, the strongly induced levels in irradiated or elicitor treated cells were due to high rates of expression of all three PAL genes and both 4CL genes.

Discussion

We have extended earlier observations that the amounts of PAL, 4CL, CHS, BMT and PR 1 proteins and mRNAs vary greatly among different tissues and during development of parsley plants [18, 19]. Particularly striking are the high levels of all five mRNAs in roots, where the metabolic functions of the corresponding proteins have not been investigated.

The wound response seems to differ considerably between roots and leaves. This applies to both the induction or repression of pathways and the differential activation of PAL and 4CL gene family members. With respect to individual pathway activities, the results obtained for CHS and BMT allow to draw some general conclusions. Each of the two enzymes is specific for one particular phenylpropanoid branch pathway, CHS for flavonoid and BMT for furanocoumarin biosynthesis. Hence it seems justified to conclude that both pathways were at least temporarily repressed in wounded roots, where they were rather active prior to wounding, and temporarily induced in wounded leaves, where their activities had previously been low. The response was particularly rapid for CHS induction in attached wounded leaves and coincided with the induction of PAL and 4CL.

This coincidence of PAL and 4CL with CHS induction may have the same causal connection in wounded leaves as in UV-irradiated parsley cell cultures [4] or epidermal cells in light-grown leaves [1, 2]. In both cases, all three enzymes probably act in concert in the production of various flavonoid glycosides [8]. Although a similar relationship of these genes in these two forms of stress is possible, this is not conclusive for two reasons. First, in contrast to the above-mentioned cases, the phenolic compounds derived from the products of PAL and 4CL in wounded leaves have not been identified; they may, perhaps even to a large extent, be different from flavonoids. And second, there are obviously major phenylpropanoid pathways other

than flavonoid and furanocoumarin synthesis operating in parsley, as suggested in wounded roots by the strong induction of PAL and 4CL concomitant with the repression of CHS and BMT (Fig. 2 of this study), as well as in wounded or non-wounded leaves by the *in situ* localization of PAL and 4CL in areas where CHS and BMT are absent [2].

Perhaps the most striking of our results is the high degree of overall coordination of PAL and 4CL mRNA levels that were always maintained despite the apparent differential regulation of individual gene family members, and despite very little structural similarity between the PAL and 4CL promoters analyzed so far ([5, 6] and E. Logemann, unpublished results). On the other hand, the clear-cut differential activation of 4CL-1 in wounded roots is surprising in view of the large structural similarity between the two 4CL genes. These genes differ in only one position within the first 250 nucleotides immediately upstream of the transcriptional start sites, in only 10 out of approximately 1600 nucleotides comprising the coding regions, and in 12 out of 300 nucleotides forming the 3′-untranslated region. Taken together, these results suggest a very complex interplay of *cis*-acting elements, *trans*-acting factors and other signals mediating the coordinated responses of PAL and 4CL to the various developmental and environmental stimuli.

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[1] E. Schmelzer, W. Jahnen, and K. Hahlbrock, Proc. Natl. Acad. Sci. U.S.A. **85,** 2989–2993 (1988).
[2] E. Schmelzer, S. Kruger-Lebus, and K. Hahlbrock, The Plant Cell **1,** 993–1001 (1989).
[3] I. E. Somssich, E. Schmelzer, P. Kawalleck, and K. Hahlbrock, Mol. Gen. Genet. **213,** 93–98 (1988).
[4] J. Chapell and K. Hahlbrock, Nature **311,** 76–78 (1984).
[5] C. Douglas, H. Hoffman, W. Schulz, and K. Hahlbrock, EMBO J. **6,** 1189–1195 (1987).
[6] R. Lois, A. Dietrich, K. Hahlbrock, and W. Schulz, EMBO J. **8,** 1641–1648 (1989).
[7] I. Somssich, E. Schmelzer, J. Bollmann, and K. Hahlbrock, Proc. Natl. Acad. Sci. U.S.A. **83,** 2427–2430 (1986).
[8] K. Hahlbrock and D. Scheel, Annu. Rev. Plant Physiol. Plant Mol. Biol. **40,** 347–369 (1989).
[9] W. Schulz, H.-G. Eiben, and K. Hahlbrock, FEBS Lett. **2,** 335–338 (1989).
[10] E. Lozoya, H. Hoffman, C. Douglas, W. Schulz, D. Scheel, and K. Hahlbrock, Eur. J. Biochem. **176,** 661–667 (1988).
[11] A. Herrmann, W. Schulz, and K. Hahlbrock, Mol. Gen. Genet. **212,** 93–98 (1988).
[12] K. D. Hauffe, K. Hahlbrock, and D. Scheel, Z. Naturforsch. **41c,** 228–239 (1986).
[13] H. Ragg, D. N. Kuhn, and K. Hahlbrock, J. Biol. Chem. **256,** 10061–10065 (1981).
[14] A. R. Ayers, J. Ebel, F. Finelli, N. Berger, and P. Albersheim, Annu. Rev. Plant Physiol. **57,** 751–759 (1976).
[15] D. N. Kuhn, J. Chapell, A. Boudet, and K. Hahlbrock, Proc. Natl. Acad. Sci. U.S.A. **81,** 1102–1106 (1984).
[16] I. E. Somssich, J. Bollmann, K. Hahlbrock, E. Kombrink, and W. Schulz, Plant Mol. Biol. **12,** 227–234 (1989).
[17] W. I. Wood, J. Gitschier, L. A. Lasky, and R. M. Lawn, Proc. Natl. Acad. Sci. U.S.A. **82,** 1585–1588 (1985).
[18] K. Hahlbrock, A. Sutter, E. Wellmann, R. Ortmann, and H. Grisebach, Phytochemistry **10,** 109–116 (1971).
[19] W. Jahnen and K. Hahlbrock, Planta **173,** 453–458 (1988).