In situ Localization of Phenylpropanoid-Related Gene Expression in Different Tissues of Light- and Dark-Grown Parsley Seedlings

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Using *in situ* RNA hybridization and immunohistochemical techniques, we investigated the tissue-specific and light-dependent expression of four genes involved in phenylpropanoid metabolism in various parts of parsley *(Petroselinum crispum)* seedlings. The genes encode phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4CL), two enzymes of general phenylpropanoid metabolism; chalcone synthase (CHS), the key enzyme of flavonoid biosynthesis; and bergaptol O-methyltransferase (BMT), a late enzyme of the furanocoumarin pathway. In young leaves, PAL and 4CL genes were expressed predominantly and in a coordinated fashion in epidermal cells, young xylem cells of vascular bundles, and epithelial cells of oil ducts. The gene(s) encoding BMT was active exclusively in the epithelial cells of oil ducts, whereas CHS gene expression was largely confined to the epidermis. In shoot and root apices, PAL, 4CL and CHS mRNAs were detectable at low levels without distinct patterns. The corresponding proteins, however, accumulated preferentially in the protoderm and pith meristem of the shoot apex and in root-cap cells. A gene encoding pathogenesis-related protein 1 (PR 1) was analyzed in roots for comparison and was found to be expressed predominantly in the cortical cells of root tips. The expression of all genes investigated was, to a greater or lesser extent, dependent on light conditions and tissue age, with highest levels occurring in newly differentiated, light-exposed tissue. In the leaf epidermis of seedlings illuminated for 20 h, PAL and CHS mRNAs and proteins, as well as 4CL protein, were particularly abundant.

Introduction

Phenylpropanoid derivatives are secondary plant metabolites with a wide range of functions, such as flower pigments, structure-related lignins, UV protectants and antimicrobial phytoalexins. The biosynthesis of these compounds is strictly controlled by developmental cues and environmental stimuli, including UV irradiation, pathogen attack, and mechanical wounding [1, 2]. Because of these particular features, many enzymes of phenylpropanoid metabolism and some of the corresponding genes or gene families have been studied extensively [3].

In parsley, particular emphasis has been placed on structural and regulatory aspects as well as the molecular genetic background of four characteristic enzymes: phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4CL), the first and last enzymes of general phenylpropanoid metabolism; chalcone synthase (CHS), the key enzyme of flavonoid biosynthesis; and bergaptol O-methyltransferase (BMT), the enzyme catalyzing the final methylation step in the furanocoumarin phytoalexin pathway [4–8]. PAL, 4CL and BMT have been shown to be developmentally regulated in cotyledons [9], and PAL activity [10] as well as the amount of CHS protein [11, 12] vary greatly with age and light conditions in young leaves. While CHS protein and mRNA are detectable only in epidermal cells of light-grown tissue [11, 12], BMT protein and mRNA are confined to oil-duct epithelial cells in uninfected tissue and, in addition, accumulate locally and transiently around fungal infection sites [13, 14]. In young leaves, PAL and 4CL are more ubiquitous than CHS and BMT, consistent with their more general function, and are most abundant in three types of tissue – epidermal and oil-duct epithelial cells and vascular bundles – as well as in cells surrounding infection sites [13, 14].

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Beyond these scattered pieces of information, however, a systematic picture of gene expression patterns in relation to phenylpropanoid metabolism in parsley has not been established. In this study, we therefore analyze the spatial patterns of PAL, 4CL, CHS and BMT mRNA distribution in cross-sections from various parts of parsley seedlings, as well as the dependence of these patterns on light conditions.

Results

Cross-sections

In extension of previous studies [11, 12, 14] we first examined the spatial patterns of mRNA accumulation in serial cross-sections of an 18-day-old parsley seedling. Positions of sectioning are indicated in Fig. 1, and the results of *in situ* RNA hybridization experiments with PAL, 4CL, BMT and CHS antisense RNA probes are shown in Fig. 2. In the unfolding primary leaf and its petiole (sections 1 and 2), PAL and 4CL were expressed predominantly in three types of tissue – upper and lower epidermis, vascular bundles and oil ducts –, although the hybridization signals for 4CL were relatively weak in the epidermis. In immediately adjacent sections, BMT mRNA was detectable only in the oil ducts, and CHS mRNA only in the epidermis. At the leaf base (section 3), in addition to PAL, 4CL and BMT mRNAs in the oil ducts, high levels of PAL and 4CL mRNAs were present in the developing vascular bundles of the primary petiole. Farther down in the hypocotyl (section 5), the intensity of hybridization signals in oil ducts was drastically reduced, and no hybridization at all occurred in vascular bundles.

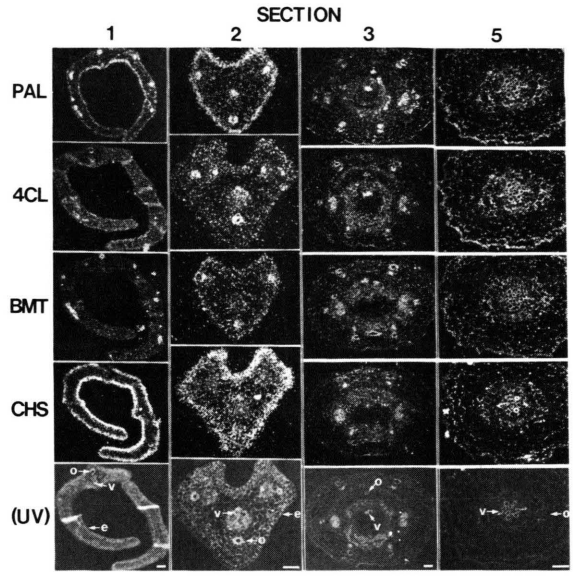

Fig. 2. Spatial patterns of PAL, 4CL, BMT and CHS mRNA accumulation in an 18-day-old parsley seedling. Cross-sections from positions 1–3 and 5 (see Fig. 1) were hybridized with the respective antisense RNA probes. (UV), autofluorescence under UV light; e, epidermis; o, oil duct; v, vascular bundle. Bar: 50 µm.

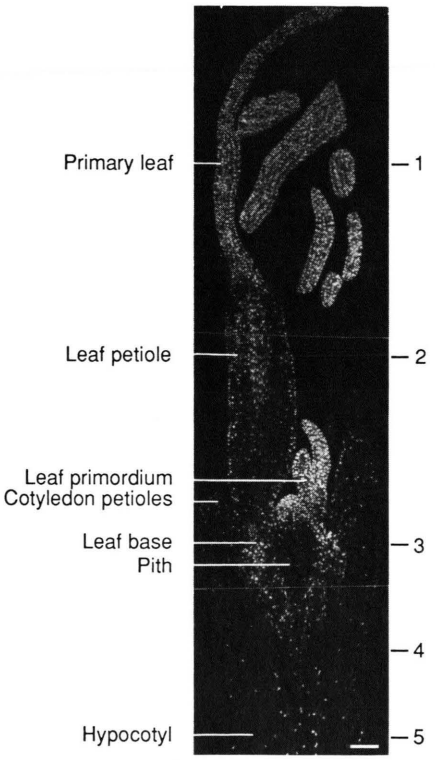

Fig. 1. Longitudinal section of an 18-day-old parsley seedling. The section was stained with DAPI. Cotyledons were excised prior to embedding. Numbers indicate the positions of cross-sections used in Fig. 2. Bar: 100 µm.

Between sections 3 and 5, where immature, lignifying xylem cells were clearly distinguishable from lignified vessels and tracheids either by autofluorescence (Fig. 3a) or by Toluidine Blue O staining (not shown; [15]), hybridization with PAL (Fig. 3b) or 4CL probes was obvious only in developing xylem cells (arrowheads in Fig. 3a). In the uppermost young xylem cell, where the nucleus

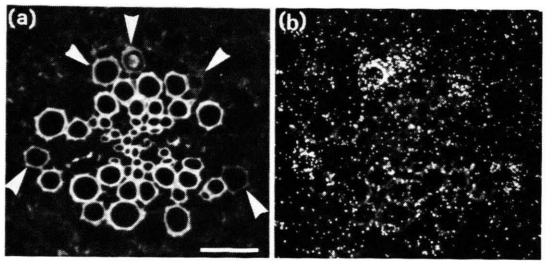

Fig. 3. *In situ* localization of PAL mRNA in xylem cells. (a) UV autofluorescence of a cross-section at position 4 (see Fig. 1) showing the differential brightness of young, developing (arrowheads) and mature xylem cells in the stele of a hypocotyl. (b) The same section hybridized with PAL antisense RNA. Bar: 50 μm.

was still visible (Fig. 3a), hybridization signals were scattered throughout the whole cell (Fig. 3b), whereas in the other cases, signals were confined to a narrow ring adjacent to the cell wall, probably due to the loss of cytoplasm and the gradual vacuolization with xylem cell maturation.

Longitudinal sections

To reveal the three-dimensional patterns of gene expression in a parsley seedling, longitudinal sections were analyzed in the same manner as shown above for cross-sections. Fig. 4 shows such a section around the leaf base. 4CL was selected as a representative of general phenylpropanoid metabolism. As expected (see above), 4CL was expressed predominantly in the epithelial cells of the oil duct. A gradual change in 4CL signal strength was evident in the vascular bundle, in agreement with the observed decrease in 4CL mRNA concentration with maturation of vascular bundles from the leaf base to the hypocotyl (Fig. 2). We also noticed some 4CL mRNA in the phloem. In the apex, including the apical meristem, the ground meristem and the emerging leaf primordium, 4CL mRNA was distributed more or less homogeneously at moderate levels with no apparent abundance in any particular cell type of this region (Fig. 4b).

Consistent with these results, 4CL-specific antibodies reacted with cells of the developing vascular bundle and with epithelial cells of the oil duct. In the apex and leaf primordium, moderate staining occurred homogeneously in all areas, whereas the protoderm of the leaf primordium, the first cell layer of the tunica and, surprisingly, the pith meristem were stained predominantly (Fig. 4c).

On the basis of recent studies of transgenic tobacco plants [16–19], we had expected considerable amounts of PAL, 4CL and CHS mRNAs in the root apex. In all *in situ* hybridization experiments with longitutinal or cross-sections of root

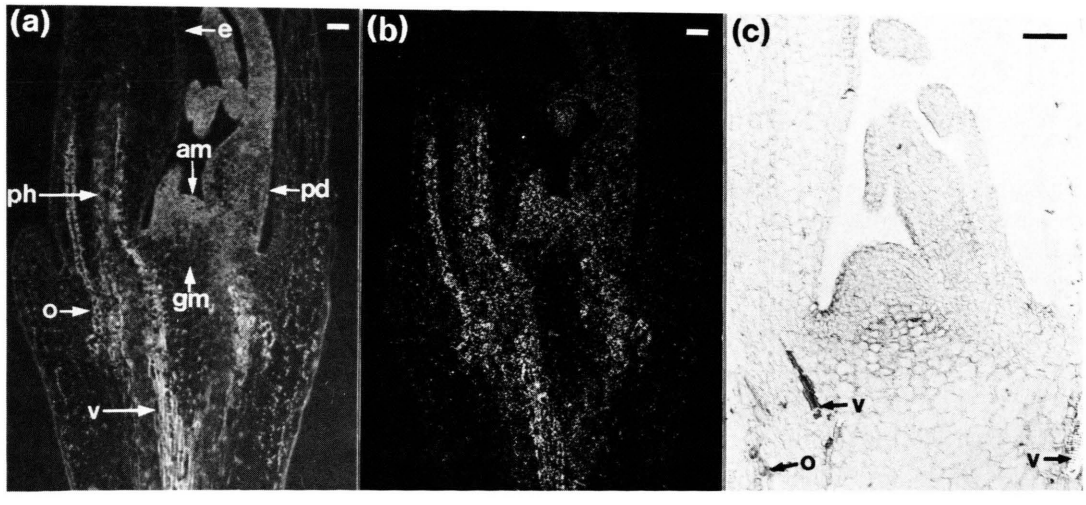

Fig. 4. *In situ* localization of 4CL mRNA and protein in the shoot apex. Two adjacent longitudinal sections of a parsley seedling were either hybridized with 4CL antisense RNA, followed by visual inspection under UV light (a) and autoradiography (b), or stained with 4CL antiserum (c). am, apical meristem; e, epidermis; gm, ground meristem; o, oil duct; pd, protoderm; ph, phloem; v, vascular bundle. Bar: 50 μm.

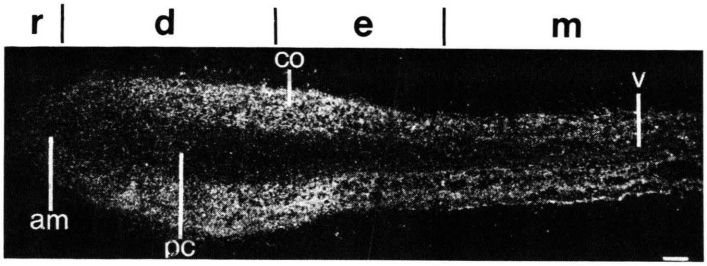

Fig. 5. *In situ* localization of PR 1 mRNA in the root tip. Longitudinal sections of lateral root tips were hybridized with PR 1 antisense RNA. am, apical meristem; co, cortex; d, zone of cell division; e, zone of cell elongation; m, zone of maturation; pc, procambium; r, root cap; v, vascular bundle. Bar: 100 μm.

tips, however, we failed to detect the respective signals, although homogeneous signal distributions slightly above background were consistently observed in all cell types from the cell division zone to the center of the elongation zone (data not shown). As a positive control, we used an additional probe, specific for pathogenesis-related protein 1 (PR 1; [20]), in all experiments with root-tip sections. Fig. 5 demonstrates that PR 1 mRNA accumulated exclusively in the cortical cells of the root tip. Within this area, most of the PR 1 mRNA was localized between the late cell division and the early cell elongation zones. The mRNA level seemed to decline rapidly with root development.

Since *in situ* hybridization did not give the expected mRNA distribution patterns for PAL, 4CL and CHS, we also stained root-tip sections with the respective antisera. In all three cases, the only cells that reacted were root cap cells, as shown in Fig. 6 for one selected enzyme, 4CL. The presence of immunoreactive BMT was also tested, but the result was negative.

Dependence of epidermal gene expression on tissue age

We have previously observed that CHS protein preferentially accumulates in epidermal cells in the aerial parts of young parsley seedlings, and rapidly decreases with further plant development [11]. In agreement with this finding, we have now demonstrated that CHS mRNA is most abundant in the epidermis of young leaves and petioles but not of leaf bases or hypocotyls (Fig. 2). To further investigate this behavior at the mRNA level, we examined the spatial pattern of CHS mRNA distribution in a 25-day-old parsley seedling. At this stage, the seedling has developed a completely unfolded primary leaf (not shown), an unfolding secondary leaf (Fig. 7a–e), and an emerging leaf primordium (Fig. 7g). CHS mRNA was clearly detectable in the leaf primordium (Fig. 7g) and particularly in the emerging upper epidermis of the young leaf (facing inward, Fig. 7a–e) and its petiole (Fig. 7f),

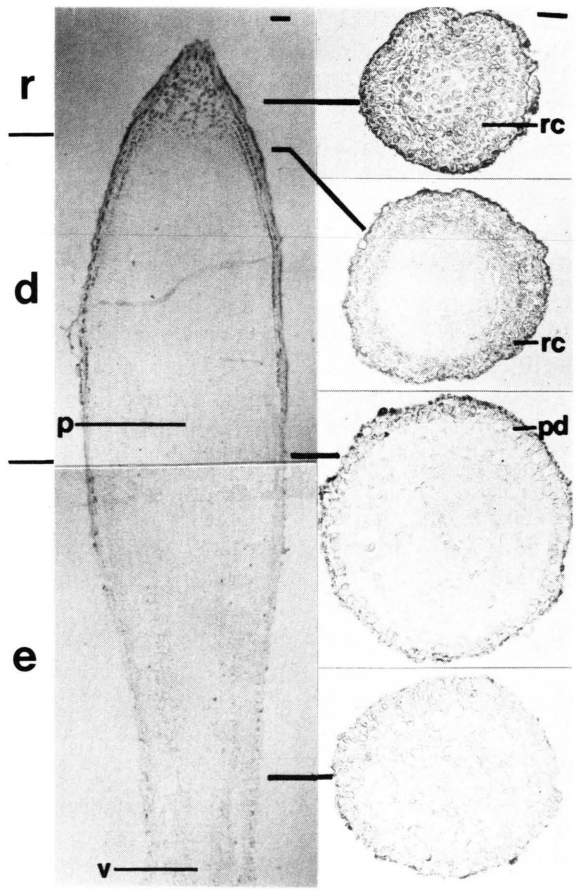

Fig. 6. Immunolocalization of 4CL protein in the root tip. Lateral root tips were sectioned longitudinally (left) or transversely (right) into slices of 5 μm and stained with 4CL antiserum. d, zone of cell division; e, zone of cell elongation; p, pith meristem; pd, protoderm; r, root cap; rc, root cap cells; v, developing vascular bundle. Bar: 50 μm.

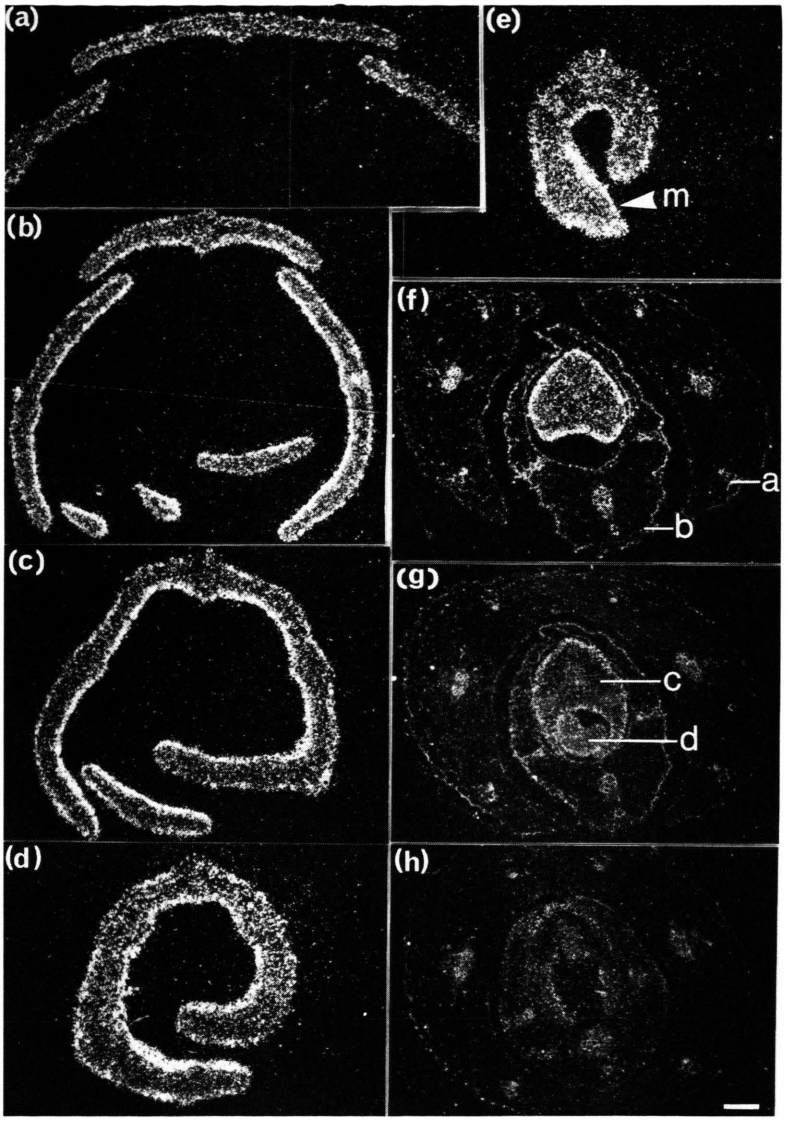

Fig. 7. Distribution patterns of CHS mRNA *in various* aerial parts of a 25-day-old parsley seedling. The seedling was harvested in the late evening and thus had received a full day of illumination (16 h). Serial cross-sections from the top of the secondary leaf (a–e) to the petiole (f) and leaf base (g, h) were hybridized with CHS antisense RNA. a, cotyledon; b, petiole of secondary leaf; c, petiole of primary leaf; d, leaf primordium, m, marginal meristem. Bar: 50 μm.

but not in the epidermis of senescing cotyledon petioles, the primary leaf petiole and the leaf base (Fig. 7f–h), nor in the unfolded primary leaf (not shown) and the uppermost part of the unfolding secondary leaf (Fig. 7a). The highest CHS mRNA levels seemed to accumulate near the edges of the lower parts of the leaf around the marginal meristems (Fig. 7e). Staining of an adjacent section with Acridine Orange [21] revealed particularly high concentrations of cytoplasmic RNA in this leaf area (not shown).

Light dependence

We have previously shown that expression of the PAL, 4CL and CHS genes is strongly induced by light in cultured parsley cells [5, 6, 8, 22], and that the same applies to CHS gene expression in the leaf epidermis [12]. We therefore assumed that PAL and 4CL gene expression in epidermal cells is also light dependent. Fig. 8a–d shows the results obtained for PAL and 4CL mRNAs in cross-sections of leaves that had been either irradiated for 20 h with UV-containing white light or maintained

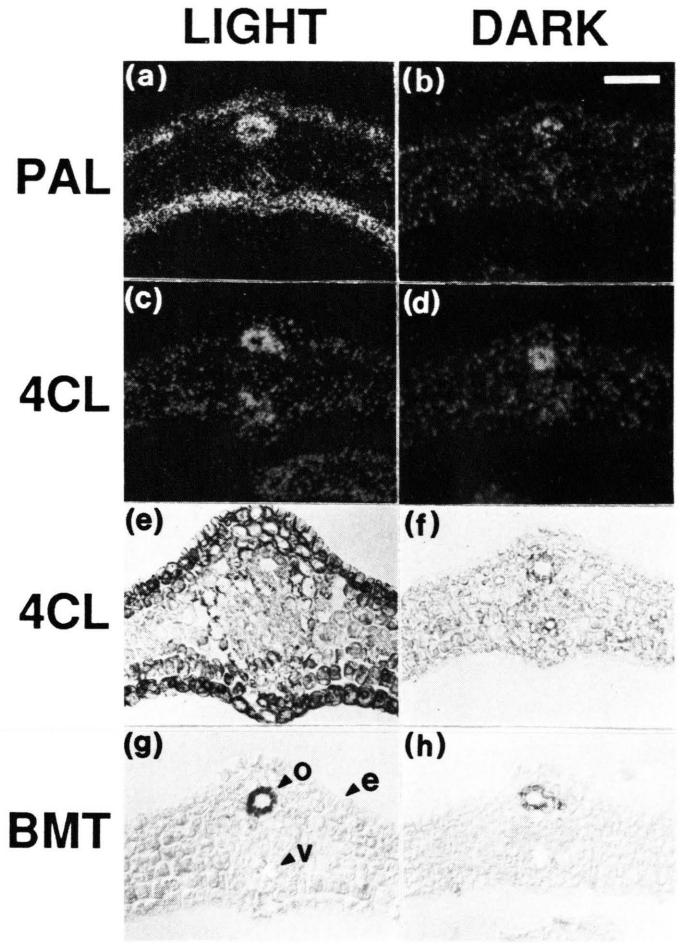

Fig. 8. Light dependence of PAL and 4CL mRNA as well as 4CL and BMT protein accumulation in different types of leaf cell. Serial cross-sections of primary leaves from light-grown (a, c, e, g) or dark-grown (b, d, f, h) parsley seedlings were hybridized with PAL (a, b) or 4CL (c, d) antisense RNA, or stained with 4CL (e, f) or BMT (g, h) antisera. Not shown are positive (light) and negative (dark) controls with CHS antisense RNA and antiserum which were included in each set of experiments and gave the same results as reported previously [11, 12]. Abbreviations are the same as in Fig. 2. Bar: 50 μm.

in the dark. Again, the hybridization signals for PAL mRNA in the epidermis were much stronger than those for 4CL mRNA. To demonstrate that 4CL had responded at all, adjacent cross-sections were analyzed with 4CL-specific antiserum (Fig. 8e,f), and the amounts of total extractable PAL and 4CL mRNAs were compared in dark-grown and irradiated leaves, using CHS mRNA as an internal control (Fig. 9).

Surprisingly, all of the results shown in Fig. 8a–f indicated increased PAL and 4CL mRNA or protein levels in irradiated tissue not only in the upper and lower epidermis (as expected), but also in the oil duct and the vascular bundle. Immunostaining of an additional set of cross-sections with BMT-

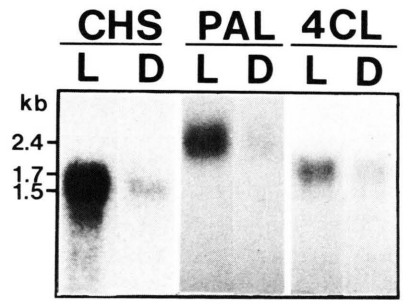

Fig. 9. Light effects on total CHS, PAL and 4CL mRNAs in a parsley seedling. Total RNA was extracted from 16-day-old, light- (L) or dark-grown (D) parsley seedlings. Ten μg each were separated on an agarose gel, blotted and hybridized with labeled antisense RNA as indicated.

specific antiserum (Fig. 8g,h) demonstrated that this applies to the furanocoumarin pathway proper in the oil duct as well.

Discussion

In all aerial parts investigated in young parsley seedlings, phenylpropanoid metabolism seems to be related predominantly to the formation of three major classes of compounds: flavonoids in the epidermis, furanocoumarins in oil ducts, and wall-bound phenolics in vascular tissue. While the chemical nature of wall-bound phenolics is largely unknown, most of the flavonoids [23] and furanocoumarins [24] in parsley have been structurally identified. It is primarily for this reason that enzymes and mRNAs specific for the common supply pathway (general phenylpropanoid metabolism: PAL, 4CL), and for the flavonoid (CHS) and furanocoumarin (BMT) branch pathways, but not for the less well defined, cell wall-related reactions, were analyzed in this study.

Relatively clear-cut results were obtained for CHS and BMT. In parsley, CHS is encoded by a single-copy gene [5] whose expression appears to be largely confined to epidermal cells (Fig. 2 and 7), even during very early stages of organ formation (Fig. 7f and g). The tissue distribution of CHS mRNA observed here is in close agreement with previous findings at the level of immunoreactive CHS protein [11], a coincidence that also applies to BMT in oil ducts (Fig. 2; [13]). That this is not always the case follows from the results discussed below.

The PAL and 4CL expression patterns are much more complex than those of CHS and BMT. In agreement with their common role in the provision of substrates for various phenylpropanoid branch pathways, PAL and 4CL always appear together and the ratio of their total enzymatic activities seems to remain constant throughout plant development and tissue differentiation [3, 25]. PAL is encoded by 4 [8, 26] and 4CL by 2 [6] genes per haploid parsley genome, and initial indications of differential gene regulation within each family [25] have recently been further substantiated (Logemann and Hahlbrock, unpublished results). Although the encoded isoenzymes are rather similar in primary structure and substrate specificity [26, 27], their turnover numbers *in vivo* may well differ. Hence, the observed differences in the ratios of PAL and 4CL mRNA amounts, *e.g.*, in epidermal *versus* oil-duct epithelial cells (Fig. 2 and 8), may be compensated by differential isoenzyme activities. Work is in progress on the functional assignment of individual PAL and 4CL genes to specific phenylpropanoid branch pathways to clarify this point. In any event, the varying PAL/4CL mRNA ratios are unlikely to be due to differential hybridization efficiencies of the two probes, because the respective nucleotide sequences, particularly those of the 4CL-1 and 4CL-2 mRNAs [27], but also those of the 4 PAL mRNAs [26], are very similar. In several independent experiments, signal intensities have been consistently lower for 4CL than for PAL (Fig. 2, 8 and 9; [8, 14]), and a generally higher catalytic rate of the 4CL than the PAL isoenzymes in a hypothetical product/substrate-channelling complex would be the most obvious explanation.

A surprising result of unknown significance is the presence of PAL, 4CL and CHS in root-cap cells, but not in the apical meristem where the promoters of all three types of genes from bean or parsley were found to be very active in transgenic tobacco plants [16–19]. In contrast to these findings in tobacco is a recent report by Ohl *et al.* [28] on transgenic *Arabidopsis* plants. These authors isolated one endogenous PAL promoter, fused it to the β-glucuronidase reporter gene, and found no expression in the root and shoot apices. While their observation was explained by the existence of multiple PAL genes with different metabolic functions and, consequently, with different modes of expression, this interpretation cannot apply to our results. By operational definition, our probes used for *in situ* hybridization and immunostaining detected all members of the PAL and 4CL gene families, respectively, and CHS, as mentioned above, is a single copy gene in parsley. Hence, we consider it more likely that phenylpropanoid metabolism in roots differs between plant species, *e.g.*, between parsley and tobacco. The apparent, low rates of PAL, 4CL and CHS gene expression in root tips of parsley are probably real and not due to technical problems with this tissue, an assumption that is supported by the efficient *in situ* hybridization of PR 1 mRNA in a control experiment using essentially identical conditions.

The results shown in Fig. 8 are also interesting for another reason. They demonstrate not only the induction by light of PAL and 4CL in epidermal cells, as expected from similar findings for CHS [12], but also the presence of considerably higher levels of PAL and 4CL mRNAs in oil ducts and vascular bundles, as well as BMT mRNA in oil ducts, in the light as compared with darkness. Although it is tempting to speculate that this difference is due to an overall stimulation of metabolic activity in light- *versus* dark-grown tissue, a more specific effect cannot be excluded. For example, we have recently shown that BMT mRNA is induced by light in cultured parsley cells, although to a much lesser extent than PAL, 4CL and CHS [29]. In any case, the large net induction of mRNAs in irradiated leaves from low (PAL, 4CL) or undetectable (CHS) to very high levels (Fig. 9) appears to be complemented by additional light effects reaching beyond the immediately affected epidermal cell layers.

Little can be said at this stage about gene transcription rates and mRNA or protein stability *in vivo*. So far, we have used the term "gene expression" in a rather loosely defined way, merely to indicate the fact that an identifyable product of a particular gene or gene family (mRNA or protein) can be localized following its "expression". Too many unknown mechanisms are likely to be involved in the chain of events determining absolute conversion rates of gene activity into end-product accumulation to define "expression" more precisely. The apparent discrepancy between relative 4CL mRNA and protein contents in epidermal *versus* oil-duct epithelial cells in irradiated parsley leaves (Fig. 8c and e), as compared with PAL (Fig. 8a; [13]), may be taken as one example of differential mRNA and protein turnover rates in different cell types. It is likely that this previously neglected area of research will greatly benefit from the sensitive and highly selective *in situ* localization methods that have become available for both mRNAs and proteins.

Experimental procedures

Plant material

For most of the experiments, parsley seedlings (*Petroselinum crispum, cv.* Hamburger Schnitt) were grown for approximately 18 days in a phytochamber under defined conditions, as described by Jahnen and Hahlbrock [13]. Primary roots were obtained from about 25-day-old seedlings grown in the phytochamber; lateral roots were taken from 3-month-old plants grown under greenhouse conditions.

Light treatment

Sixteen-day-old parsley seedlings were transferred to an air-conditioned incubator (20 °C) and illuminated for 20 h with 5 lamps emitting white, UV-containing light (Philips TL 20W/18) at a distance of 35 cm above the seedlings. Control seedlings were kept in the dark at 20 °C for the same period of time.

Histology

Tissue fixation, paraffin embedding and sectioning were performed as described previously [14], except that roots were fixed in 4% paraformaldehyde, 0.25% glutaraldehyde and 100 mM sodium phosphate buffer, pH 7.0, for 2 h, and that the length of the subsequent period of dehydration and paraffin embedding was increased accordingly. For anatomical studies other than UV epifluorescence (excitation filter 365 nm), sections were stained with Toluidine Blue O [15], Acridine Orange [21] or DAPI (4′,6-diamidino-2-phenylindole; [30]). The terminology used for anatomical structures is based on Esau [31] and Raven *et al.* [32].

In situ RNA hybridization

The same procedure was used as described by Schmelzer *et al.* [14], except that the RNA probes were labeled with ^{35}S (from ^{35}S-α-UTP; Amersham, Braunschweig, Germany) instead of ^{3}H, and that the exposure time was reduced to approximately 2 days. Sense and antisense RNA probes were generated from DNA inserts subcloned into either Bluscribe or Bluscript vectors (Vector Cloning Systems, San Diego, U.S.A.), using T3 or T7 RNA polymerases. The cloned DNA inserts were derived from the corresponding cDNAs: a near full-length PAL-1 cDNA (2.4 kb) with a poly(A) tail of seven bases [8]; a 1.5 kb SacI fragment from the 4CL-1 cDNA [6]; an 800 bp BMT cDNA (K. D. Hauffe, K. Hahlbrock, and D. Scheel, manuscript in preparation); a 700 bp PstI fragment from the 5′ half of the CHS cDNA [33]; and a

780 bp PR 1-1 cDNA with a poly(A) tail of 16 bases [20]. Control experiments using sense probes were carried out in all cases presented in this paper.

Immunostaining

Sections of 5 µm from paraffin-embedded seedlings were deparaffinized with xylol. Possible endogenous peroxidase activity was blocked by a 20 min treatment with 3% H_2O_2 in 80% ethanol. Antibodies and staining procedures were essentially the same as those described by Jahnen and Hahlbrock [11]. Crude antisera raised against parsley PAL or 4CL were used without further purification (working solution 200-fold diluted). Pre-immune sera were used in all cases as negative controls.

Microscopy

All data obtained from *in situ* hybridization, immunostaining and histochemical experiments were evaluated under a Zeiss Axiophot microscope. Pictures were taken under dark-field (for *in situ* hybridization), differential interference contrast (for immunostaining) or epifluorescence (for UV autofluorescence) conditions using negative (Agfa PAN 25 and PAN 400) or reversal films (Kodak Ektachrome X-160 and X-200), respectively.

RNA-Blot hybridization

Procedures for RNA isolation and blot hybridization have been described by Wu *et al.* [34]. Probes were the same as those used for *in situ* hybridization, except that they were labeled with ^{32}P instead of ^{35}S.

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