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Subcellular Visualization of Gene Transcripts Encoding Key Proteins of the Chlorophyll Accumulation Process in Developing Chloroplasts[1]

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The coordination of the synthesis of chlorophyll (Chl) and light-harvesting Chl proteins was determined by observing the sequence of appearance of the specific mRNAs for the nuclear genes *CHLH, Por,* and *Lhcb1*2 (AB180). CHLH* encodes a magnesium protoporphyrin chelatase subunit that is involved in the first committed step in Chl biosynthesis; *Por* encodes protochlorophyllide oxidoreductase, which catalyzes the penultimate and only light-dependent step in Chl biosynthesis; and *Lhcb1*2* encodes light-harvesting Chl *a/b* binding protein of the type-1 light-harvesting complex of photosystem II. Using digoxigenin-labeled antisense and sense RNA probes and a highly sensitive in situ hybridization technique, we have visualized the first appearance of the specific mRNAs in postmitotic mesophyll cells of developing 7-d-old wheat leaves (*Triticum aestivum* cv Maris dove). The transcripts for CHLH and POR are detectable in the youngest (18 h postmitotic) leaf tissue containing dividing cells; light-harvesting complex of photosystem II transcripts appear 12 h later. This is consistent with a requirement for accumulation of Chl before synthesis of Chl *a/b* binding protein can proceed at a high rate. All of the transcripts are most abundant in mesophyll cells. In the first leaf the POR message is initially restricted to the palisade, but 12 h later it is also present in the spongy mesophyll cells. All three transcripts aggregated around the surface of the chloroplasts, suggesting that translation may occur preferentially in the vicinity of the target organelle for the primary translation products.

How are the key events in the biogenesis of the photosynthetic apparatus coordinated during normal development of leaf tissue? Until recently, detailed biochemical and molecular characterization of this coordination was limited by the necessity to sample proteins and nucleic acids by tissue homogenization and analysis, resulting in the loss of information about tissue- and cell-specific gene expression and subcellular localization. The availability of specific gene probes and antibodies, combined with the optimization of in situ techniques for subcellular recognition of macromolecules in plant tissues, now enables us to visualize gene action within a single mesophyll cell during development (Marrison and Leech, 1992, 1994).

The developing young wheat leaf (*Triticum aestivum* cv Maris dove) provides an excellent and highly reproducible tissue for investigating photosynthetic development. Since each leaf contains a chronological series of mesophyll cells from immediately postmitotic at the base to fully mature at the tip of the leaf, the complete process of mesophyll cell and chloroplast development can be studied within a single leaf (Boffey et al., 1979; Ellis et al., 1983). Moreover, since cell age can be determined as a function of the cell's position in the leaf, the first appearance of cell constituents can be related directly to cell age (Boffey et al., 1980; Dean and Leech, 1982). There is a further advantage because by simultaneously sampling all the leaves in a young wheat seedling, a freeze-frame of several developmental stages under genetically and environmentally uniform conditions is obtained. To examine the normal sequence of events in the synthesis and assembly of Chl and associated proteins in wheat leaf chloroplasts, we have used RNA probes for three selected nuclear gene transcripts to determine the first appearance and subsequent distribution of the corresponding mRNA species in cells in leaf sections of increasing age and degree of differentiation. The genes chosen were: (a) *CHLH*, encoding a magnesium protoporphyrin chelatase subunit catalyzing the insertion of magnesium into the protoporphyrin ring, a process considered to be the first committed step in Chl biosynthesis; (b) *Por*, encoding POR, which catalyzes the penultimate and only light-dependent step in Chl biosynthesis; and (c) *Lhcb1*2 (AB180)*, encoding type-1 LHCII, a light-harvesting Chl *a/b* binding protein of PSII (Jansson et al., 1992). The details of the probe preparation are given below.

MATERIALS AND METHODS

Plant Material

Young wheat plants (*Triticum aestivum* cv Maris dove) were grown under the same controlled-environment conditions described by Boffey and Leech (1982) except that the light intensity was 60 W m^{-2}. The caryopses (seeds) were always sown at 10:00 AM, and the leaves were har-

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Abbreviations: LHCII, light-harvesting complex of PSII (always refers to LHCII type 1; Jansson et al., 1992); POR, Pchlide oxidoreductase.

vested at 10:00 AM 7 d later. The light period began at 8:00 AM. The plant was cut above the basal node, the coleoptile was removed, and the "shoot" was harvested as a unit consisting of the outer first leaf with the inner second and third leaves rolled inside. Transverse leaf slices 2 mm wide were harvested by cutting at specific distances from the leaf base. The centers of the slices were 25, 125, 225, and 425 mm from the leaf base, i.e. they represent a developmental sequence from young to mature tissue. The basal slice, 25 mm above the node, contained the first, second, and third leaves; in the slice taken 125 mm from the leaf base the first and second leaves were represented, and in the other slices only the first leaf was represented. The leaf slices were fixed and embedded in PEG for in situ hybridization (Marrison and Leech, 1994) or embedded in Spurr's resin for anatomical study as previously described by Marrison and Leech (1992).

In Situ Labeling of Transcripts

Digoxigenin-labeled antisense and sense RNA probes were synthesized by in vitro transcription reactions using 1 μg of linearized DNA template, digoxigenin-11-UTP, and T7 or T3 RNA polymerase according to the manufacturer's protocols (Boehringer Mannheim). All digoxigenin reagents used were purchased from Boehringer Mannheim.

The barley (*Hordeum vulgare* cv Herta) *Por* clone is a 1.3-kb cDNA encoding Pchlide oxidoreductase (P.H.D. Schünmann, unpublished data) in pBS(KS) and has been shown to have sequence identity with the *Por* clone of Schulz et al. (1989). Antisense- and sense-labeled probes were generated using template DNA linearized with *Spe*I or *Eco*RV and transcribed with T3 or T7 polymerase, respectively. The POR-A and POR-B cDNA sequences (accession numbers X15869 and X84738, respectively) show 83% sequence identity over 956 bases, including regions of 100% homology over 71, 32, and multiple tracts of 20 or more bases. Only at the 5′ end, which encodes the putative signal peptide, are the sequences more divergent. Since the riboprobe used in the present study was prepared from a full-length barley cDNA for POR-A, it would hybridize to both the POR-A and the POR-B message under the conditions of stringency applied.

The *CHLH* and *Lhcb1* cDNAs were gifts from Prof. C.N. Hunter (University of Sheffield, Sheffield, UK) and Prof. E. Tobin (University of California, Los Angeles) respectively. The *CHLH* clone was isolated from the *Arabidopsis thaliana* (land race Columbia) leaf library prepared by Raines et al. (1992) and contains a 3.5-kb cDNA insert encoding a magnesium protoporphyrin chelatase subunit in pBS(SK−). Antisense- and sense-labeled probes were generated using template DNA linearized with *Kpn*I or *Spe*I and transcribed with T3 or T7 polymerase, respectively. The *A. thaliana* (land race Columbia) *Lhcb1**2 clone contains a 1.8 kb of *Eco*RI insert encoding the light-harvesting Chl *a/b* binding protein of PSII from pAB180 (Leutweiler et al., 1986) in pBS(SK+). Antisense- and sense-labeled probes were generated using template DNA linearized with *Xba*I or *Sal*I and transcribed with T7 or T3 polymerase, respectively. In situ hybridization was performed on 7-μm-thick transverse sections as described previously by Marrison and Leech (1994). The hybridized probe was detected using anti-digoxigenin-alkaline phosphatase conjugate and overnight color development with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate. Sections were visualized and photographed using a Nikon FXA microscope at 10 V and Kodak Ektachrome 100 Plus Professional color slide film with automatic exposure setting. Four leaf sections from each age zone were hybridized together on a single microscope slide (total of 16 sections) to ensure uniformity of processing. Two slides were processed for the antisense probe and two for the sense probe in each of two separate experiments. The results of probing were completely uniform and reproducible.

RESULTS AND DISCUSSION

Cell age can be determined as a function of the cell's position in the leaf as established by Boffey et al. (1980) and Dean and Leech (1982). To quantify the accumulation of Chl as the wheat leaf cell develops, Chl per plastid was determined for three ages of cell, 30 (125 mm from the leaf base), 36 (225 mm from the leaf base), and 46 h (425 mm from the leaf base), postmitosis. In the youngest cells, 18 h postmitosis (25 mm from the leaf base), the Chl level is too low to be quantified (Boffey et al., 1979). Figure 1a shows the accumulation of Chl in leaf slices from a 7-d-old wheat leaf; the slices sampled are indicated as bold lines in the figure. The values for Chl per plastid were taken from Boffey et al. (1979), in which isolated chloroplasts were counted in a hemocytometer. The wheat leaf is highly heterogeneous, as illustrated in Figure 1b, a stained section of the first wheat leaf taken at 425 mm from the base, which shows the distribution of the principal cell types present at this stage of leaf development when the cells are 46 h postmitotic.

Our aim was to discriminate between the presence and absence of specific transcripts in individual and adjacent cells and to detect discrete patterns of localization associated with particular areas of the leaf or particular cell or tissue types. We reasoned that if the abundance of the cellular messages and the sensitivity of the method were sufficient, then we should also be able to determine the precise intracellular localization of the transcript.

To compare the first leaf with the second and third leaves, cross-sections of the 7-d-old wheat seedling were taken within 0.5 cm of the point of insertion on the vegetative shoot apex. This seedling cross-section contains segments of the first, the second, and the third leaves at different developmental stages. The second and third leaves in this region are shielded from direct light by the sheath formed by the first leaf, but they still receive significant light by funneling and filtering through the surrounding tissue. Using antisense RNA probes, we examined the distribution of selected gene transcripts in the cells of the first and second leaves of this tissue (Fig. 2). POR transcripts are detectable by intense dark-blue staining in both the first and second leaves, and are particularly abundant in the meristematic cells of the second leaf (Fig. 2c). Some transcripts of the *CHLH* gene are also present in the first

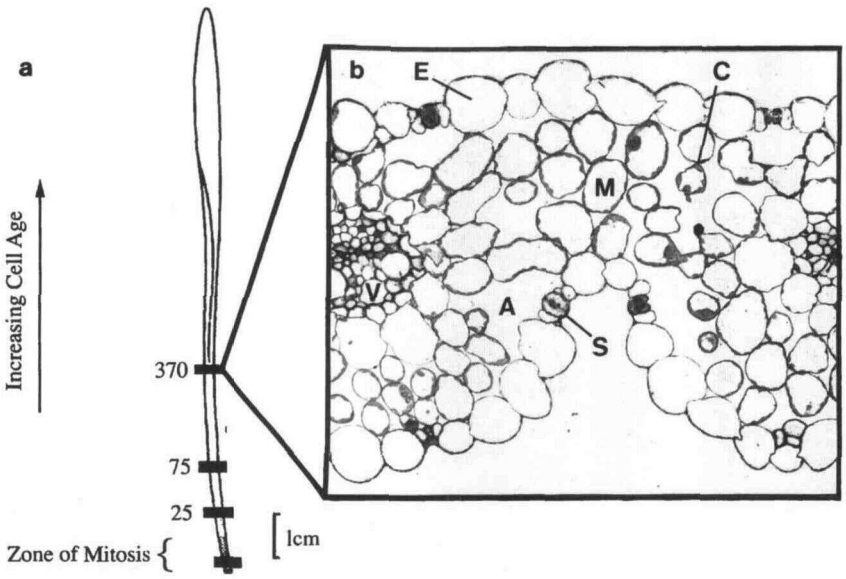

Figure 1. a, Diagram of a 7-d-old wheat leaf showing the plastid Chl content (pg $\times$ 10^{-3}/plastid) and the four regions of sampling (■). b, Transverse section 425 mm from the base of a 7-d-old primary wheat leaf to illustrate the cell anatomy. The section (1 μm thick) was embedded in Spurr's resin and stained with toluidine blue. E, Epidermis; M, mesophyll cell; A, airspace; V, vascular tissue; S, stomate; C, chloroplast.

and second leaves (Fig. 2a) but these appear to be much less abundant than is the message for POR. No *Lhcb1**2 gene transcripts could be detected at this early stage of development (Fig. 2e). Control sections probed with the sense probe gave no signal (Fig. 2, b, d, and f) for any of the transcripts.

Closer examination of the distribution of POR mRNA (Fig. 3) at higher microscopic resolution shows that the transcripts are present in all of the cell types of the second leaf (Fig. 3c), although their abundance is greatest in the cells destined to form the mesophyll parenchyma. Even recently divided cells (Fig. 3c, arrowheads) contain high levels of POR message. A reduced level of POR transcript is also present in the third leaf (Fig. 3d). In contrast, the distribution of the POR message in the first leaf is limited to the two to three layers of cells immediately below the abaxial (outer) epidermis and to the single cell layer (arm palisade) surrounding the vascular tissue (Fig. 3a, arrowheads); there are no POR transcripts in the future spongy mesophyll cell region (Fig. 3b, arrowheads). This distribution of POR mRNA in the first leaf resembles the distribution previously observed (J.L. Marrison and R.M. Leech, unpublished data) for photosynthetic proteins in the youngest cells of the 7-d-old wheat leaf, i.e. the proteins are present only in the outer (abaxial) half of the leaf mesophyll in the cells that will become palisade mesophyll tissue.

As the cells develop, i.e. with increasing distance from the leaf base, the abundances of the three transcripts becomes increasingly more similar (Fig. 4). At 125 mm from the leaf base, where the cells are 30 h old, messages encoding CHLH and POR are of similar abundance in mesophyll cells (Fig. 4, a and c), but LHCII mRNA is just detectable (data not shown). By the time the leaf cells are 36 h old (225 mm from the base), the LHCII mRNA has accumulated to a high level (Fig. 4e). In cells 46 h old (425 mm from the base), all three messages display similar patterns of abundance and distribution in mesophyll cells (Fig. 4, b, d, and f).

In addition to the presence of these nuclear-gene transcripts throughout the cytoplasm, there is clear evidence of aggregation around the developing chloroplasts (Fig. 4, arrowheads). In the high-resolution photomicrographs shown in Figure 4, the clustering of the transcripts for CHLH (Fig. 4b), for POR (Fig. 4d), and for LHCII (Fig. 4f) can be clearly seen around the external surface of the sectioned chloroplasts (arrowheads). In several sections the transcripts are so concentrated that, after staining, continuous blue/black rings are present around the external surfaces of the chloroplasts (e.g., Fig. 4, b and d). To our knowledge, this is the first time that such aggregations have been visualized in wheat leaf mesophyll cells, but we have already described similar aggregations of ch-42 transcripts (Marrison and Leech, 1994) and CHLH transcripts (Gibson et al., 1996) in developing Arabidopsis leaves. We speculated (Gibson et al., 1996) that these aggregations could indicate that translation in the vicinity of the chloroplast envelope is coupled to the import of nascent chloroplast polypeptides by analogy with mitochondria (Verner, 1993). Our present observations of transcript aggregation in another species (wheat) and for another transcript (LHCII) provide strong supporting evidence for this earlier suggestion.

How should these results be interpreted? We have been able to detect CHLH and POR transcripts (Fig. 2, a and c) within the very young meristematic cells at the leaf base (Fig. 1a) when Chl is present in insufficient amounts for quantification per plastid (Boffey et al., 1979). In contrast, LHCII accumulation is known from many previous studies on Gramineae to be developmentally and light regulated (Martineau and Taylor, 1985; Nagy et al., 1988; Ougham and Davies, 1990), and the mRNA is first detectable within 1 to 1.5 cm of the leaf base, depending on species. In these earlier studies the leaf was not dissected into sufficiently fine segments to establish precisely when the LHCII message first appears. Using our high-resolution techniques, we can now show that no LHCII mRNA is detectable until the cells are at least 30 h old, suggesting that the accumulation of Chl precedes the accumulation of the LHCII message. It should be noted that

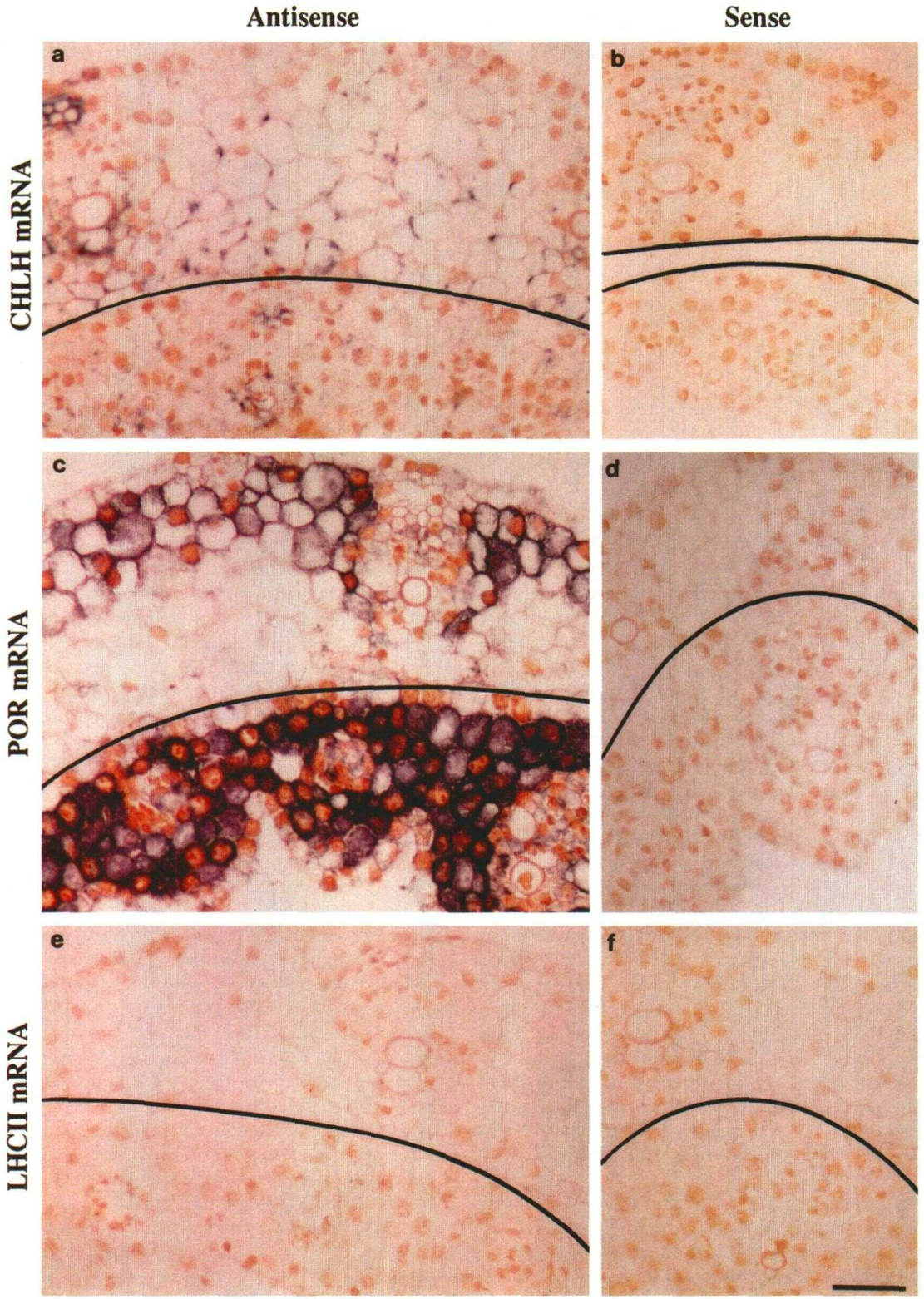

Figure 2. In situ hybridization to sections of a 7-d-old wheat seedling. PEG-embedded transverse sections (7 μm) were taken 25 mm from the base of the wheat seedling. Each area photographed shows a section of both the first and second leaves. The adaxial epidermis of the first leaf and the abaxial epidermis of the second leaf are outlined. The first leaf is above the second leaf in each photograph. Tissue hybridized to the antisense strands of the digoxigenin-labeled RNA probe for CHLH (a), POR (c), and LHCII (e). Tissue hybridized to the sense strands of the digoxigenin-labeled RNA probe for CHLH (b), POR (d), and LHCII (f). The mRNA detected is for type-1 LHCII (Jansson et al., 1992). The transcript is indicated by dark blue staining. Nuclei appear pink with both the antisense and sense probes. Scale bar = 50 μm.

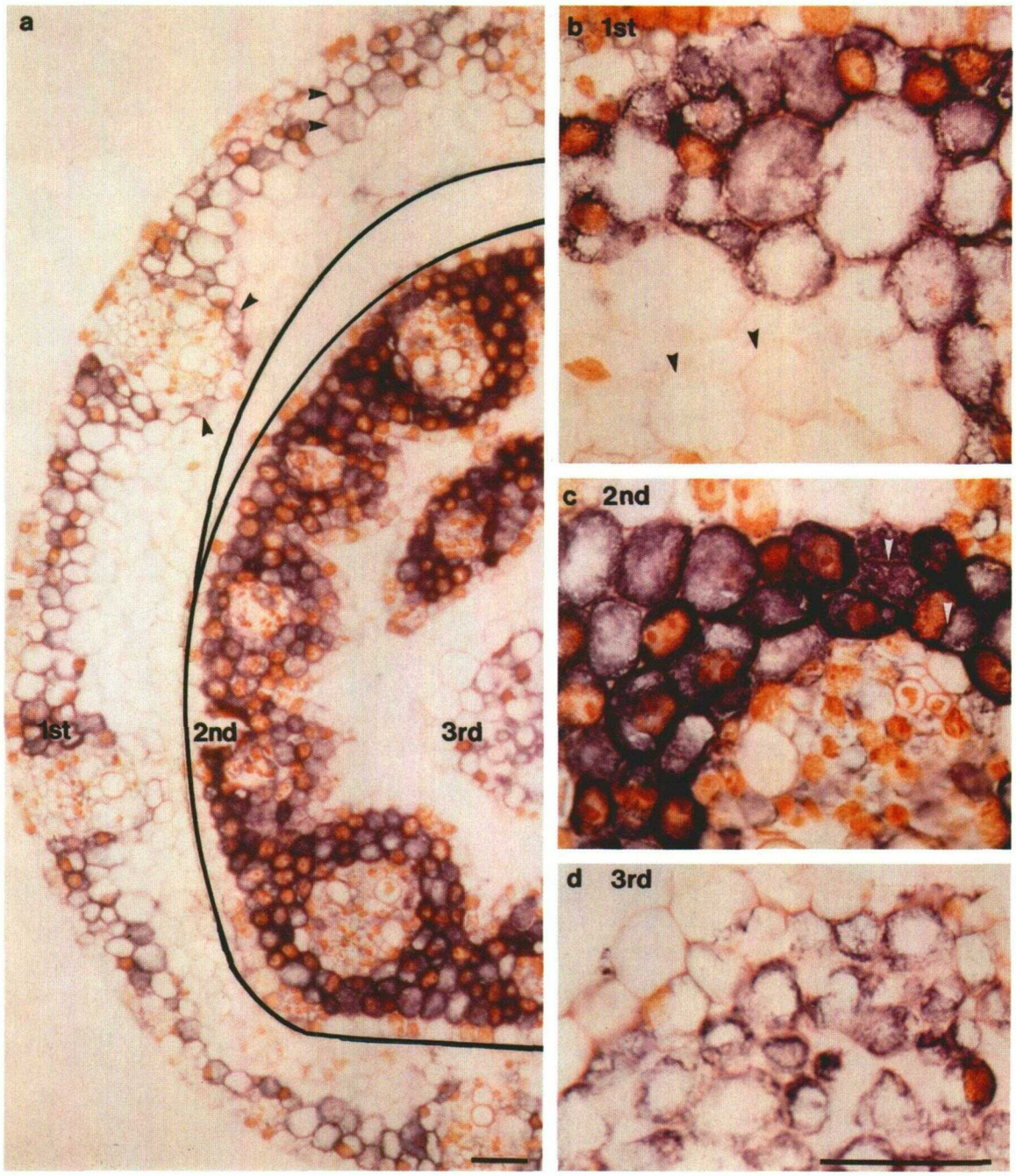

Figure 3. Localization of POR mRNA in sections of a 7-d-old wheat seedling. PEG-embedded transverse sections (7 μm) were taken 25 mm from the base of the wheat seedling and hybridized to the antisense strand of the digoxigenin-labeled RNA probe for POR (blue color reaction). a, All three leaves can be seen at the base of the seedling and show the relative levels of POR mRNA. b to d, POR mRNA localization in the first leaf (b), second leaf (c), and third leaf (d). The adaxial epidermis of the first leaf and the abaxial epidermis of the second leaf are outlined. Scale bar = 50 μm.

LHCII type 1 in wheat is encoded by the *Lhcb1* multigene family (Jansson et al., 1992) and that, since we are using a full-length genomic clone, we will simultaneously detect all type-1 LHCII messages present in the wheat leaf tissue.

The distribution of POR message within the developing wheat leaf tissue is of particular interest. The enzyme POR (EC 1.6.99.1) catalyzes the conversion of Pchlide into Chlide, the penultimate and only light-dependent enzymatic step in the biosynthesis of Chl *a* in angiosperms (Griffiths, 1978; Apel et al., 1980). The enzyme is nuclear encoded as a precursor with a signal peptide, and the mature, processed form catalyzes Chlide biogenesis in the plastids, where it is found primarily in association with the thylakoid outer membrane (Teakle and Griffiths, 1993).

Very high levels of the mRNA for POR, and of the protein, are found in dark-grown tissue of cereals (Apel,

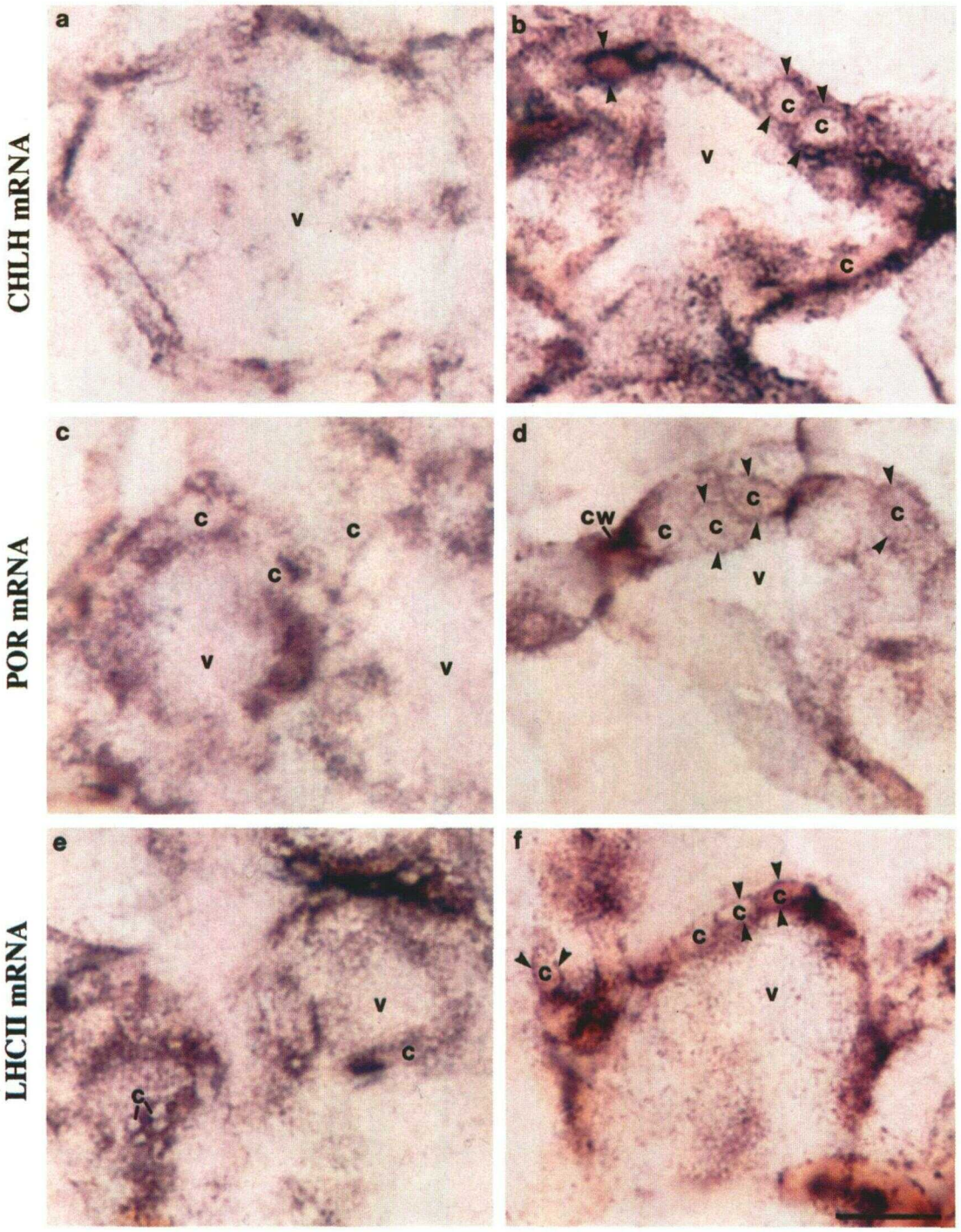

Figure 4. *In situ hybridization to sections of a 7-d-old wheat seedling.* Tissue hybridized to the antisense strand of the digoxigenin-labeled RNA probe for CHLH (a and b), POR (c and d), and LHCII (e and f) proteins. All PEG-embedded transverse sections (7 μm) are taken from the first leaf: a and c, 125 mm; e, 225 mm; b, d, and f, 425 mm from the leaf base. Arrowheads indicate the concentration of the transcripts around the chloroplast periphery. c, Chloroplast; v, vacuole; cw, cell wall. Scale bar = 10 μm.

1981), but there is a very rapid drop in the abundance of both mRNA and protein soon after transfer to white light. Of more relevance, light-grown green tissue of both oats and barley contains enough enzyme activity (Griffiths et al., 1985) to account for the observed rate of Chl biosynthesis in these tissues.

Recent work has provided strong evidence for the existence of two forms of the POR enzyme, designated POR-A and POR-B, with 75% amino acid sequence identity (Holtorf et al., 1995). In barley, each POR is encoded by a single gene. Both POR enzymes require light for the reduction of Pchlide to Chlide, but whereas POR-A is highly abundant

in etiolated tissue and drops below detectable limits in illuminated seedlings when Chl accumulation reaches its maximum rate, POR-B is present at an approximately constant level (much lower than the maximum for POR-A) in both dark-grown and green seedlings. Although the mature form of POR-B has a slightly higher molecular weight than does POR-A, there is little difference in the size of the two mRNA species, making them difficult to resolve by northern blot analysis unless gene-specific probes are used (Holtorf et al., 1995). For the same reason, in situ hybridization based on the full-length cDNA clone used in the present study will reveal the presence of both mRNA species, but it will not provide further information about their relative locations. Where clustering of the POR message is observed, it is predominantly around the plastids (Fig. 4d). A high concentration of POR mRNA is consistently detected in the cytosol, particularly at the leaf base (Figs. 2c and 3), and is detectable in epidermal and vascular cells, but in considerably lower abundance than in leaf mesophyll cells. Dehesh et al. (1986a, 1986b), using both subcellular fractionation and electron microscope immunogold localization, observed POR protein in the cytosol adjacent to the plasmalemma in etiolated barley seedlings. It is not clear whether this protein was POR-A, POR-B, or a mixture of both, or what function, if any, it might serve in this location. The high concentration of POR message in mesophyll cell cytosol detected in the present study parallels the location of the immunolabeled POR in Dehesh's earlier work.

Recent work by Reinbothe et al. (1995a, 1995b) has shown that uptake of the POR precursor peptide into isolated plastids is dependent on the intraplastidic availability of the substrate Pchlide and is slowed down by the presence of the product Chlide. Thus, adequate activities of the enzymes catalyzing earlier stages in the Chl biosynthesis pathway will be a prerequisite for POR uptake into the plastids.

CONCLUSIONS

Using a very sensitive in situ hybridization technique, we have been able to determine the intracellular location of the transcripts for three proteins essential for Chl and for LHCII synthesis during plastid development in wheat leaves.

POR transcript can be detected in the very young tissue containing dividing cells of the second and third leaves of the 7-d-old wheat plant. The message for the magnesium protoporphyrin chelatase enzyme is also present in this young tissue, consistent with the requirement for the substrate Pchlide before POR protein can be taken up by plastids. In the first leaf the POR message is initially restricted to the palisade layers and arm palisade layer of mesophyll cells, but later it is also found in the spongy mesophyll cells.

LHCII message accumulation lags 12 h behind the accumulation of the transcripts of the enzymes required for Chl biosynthesis.

Although CHLH, POR, and LHCII transcripts are most abundant in mesophyll cells, POR message is also observed in other cell types, including epidermal cells. In the first leaf there is a sharp delineation of the zone in which POR message can be detected (Fig. 3). The regulation of the accumulation of the POR protein may involve one or more posttranscriptional changes to ensure that the protein is present only in mesophyll cells, or one or both forms of the POR protein may have a different function within the other cell types.

Some aggregation of all three transcripts (CHLH, POR, and LHCII) can be seen around the plastids (Fig. 4). This suggests that translation of the messages may occur preferentially in the vicinity of the target organelle for the primary translation products. It is interesting to speculate that this very concentrated accumulation of message adjacent to the chloroplast envelope reflects limited transcription and co-translational transport of polypeptides into the plastids. The very rapid uptake of the large gene product of CHLH into isolated chloroplasts has recently been clearly demonstrated by Gibson et al. (1996).

We have shown that it is possible to detect changes in transcript accumulation over very short time periods in leaf development. As more gene probes become available, we anticipate that we will be able to obtain a more comprehensive genetic and biochemical description of the control of chloroplast development within a very precise time frame.

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