Nuclear Localization of Flavonoid Enzymes in Arabidopsis*

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Flavonoids represent one of the oldest, largest, and most diverse families of plant secondary metabolites. These compounds serve a wide range of functions in plants, from pigmentation and UV protection to the regulation of hormone transport. Flavonoids also have interesting pharmacological activities in animals that are increasingly being characterized in terms of effects on specific proteins or other macromolecules. Although flavonoids are found in many different locations both inside and outside the cell, biosynthesis has long been believed to take place exclusively in the cytoplasm. Recent reports from a number of different plant species have documented the presence of flavonoids in nuclei, raising the possibility of novel mechanisms of action for these compounds. Here we present evidence that not only flavonoids, but also at least two of the biosynthetic enzymes, are located in the nucleus in several cell types in Arabidopsis. This is the first indication that differential targeting of the biosynthetic machinery may be used to regulate the deposition of plant secondary products at diverse sites of action within the cell.

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MATERIALS AND METHODS

Plant Material—The Landsberg erecta (La-er) ecotype was used as the wild type in all experiments. Seedlings were grown on the surface of agar medium containing Murashige and Skoog salts and sucrose under continuous white light (120 microeinsteins m$^{-2}$ s$^{-1}$) for 4 days at 21 $\degree$C as described previously (27).

Construction of Transgenic Arabidopsis Plants Expressing Green Fluorescent Protein (GFP) Fusion Proteins—The AtCHS and AtCHI coding regions (34) were amplified using Pfu polymerase (Stratagene)

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and primers that added NcoI sites for fusion to the 5’ end of mGFP5 and either BglII (AtCHS) or BamHI (AtCHI) sites for fusion to the 3’ end. The fragments were then inserted into the NcoI or BglII site in pAVA393 (35). For mGFP5-CHI, the resulting expression vector, consisting of the double-enhanced CaMV 35S promoter, Tobacco Etch Virus translational leader, mGFP5-CHI coding region, and nopaline synthase terminator, was then excised using XmaI and SalI and subcloned into corresponding sites in pBIB-KAN (36). The corresponding region was also subcloned from the empty pAVA393 vector into pBIB-KAN to serve as a control for localization experiments. For the other three fusions, constructs in pAVA393 were digested with KpnI, filled in, digested with SacI, and then cloned into the XmaI and SacI sites in pCAMBIA3300 (Cambia, Canberra, Australia). The five constructs were used to transform *Agrobacterium tumefaciens* strain GV3101 using a freeze-thaw method (37) and then introduced into *Arabidopsis* La-er plants using the floral dip method (38). For the constructs in pBIB-KAN, selection of transformants was on medium containing 0.5× Murashige-Skoog salts and 50 µg/ml kanamycin. For constructs in pCAMBIA3300, selection was carried out by spraying soil-grown plants at 10, 11, 12, and 16 days with a 1:10,000 dilution of glufosinate ammonium concentrate (Basta; AgrEvo Co.), 0.005% Silwet L-77 (Lehle) per the manufacturer’s instructions.

**Immunolocalization, Flavonoid Staining, and Imaging—**Methods were as described previously (27) for immunolocalization using affinity-purified rabbit anti-CHS and chicken anti-CHI antibody preparations, flavonoid staining with diphenylboric acid 2-aminoethyl ester (DPBA), and imaging by electron microscopy or confocal laser scanning microscopy (CLSM). mGFP5 fluorescence was visualized using a LSM510 confocal microscope (Carl Zeiss) using a 1.2 numerical aperture 40× C-Apo water-immersion objective lens, 488 nm argon laser line, and 565–550 nm band pass filter.

**Nuclear Protein Preparations—**Seedlings were immersed in liquid nitrogen in a pre-chilled ceramic mortar and ground with a pestle to a fine powder. The powder (~1.5 g) was transferred to a Dounce homogenizer (Kontes Glass) containing 10 ml of cold nuclei isolation buffer (10 mM MES-KOH, pH 6.3, 330 mM d-sorbitol, 0.1 mM spermine, 0.5 mM spermidine, 2.5 mM EDTA, pH 6.3, 2.5 mM dithiothreitol, 10 mM KCl, 10 mM NaCl, 1 mM MgCl₂, and 1× Protease Complete (EDTA) protease inhibitor mixture (Roche Applied Science)). The suspension was allowed to thaw on ice, and then 10 passes with a loose (0.16 mm) clearance and 10 passes with a tight (0.07 mm) clearance pestle were performed. The resulting homogenate was centrifuged at 380 × g for 6 min at 4 °C to sediment large cellular debris and then filtered through 75 µm NITEX nylon mesh (Precision Vented Screening Media, Briarcliff Manor, NY) to remove smaller cell fragments. The homogenate was centrifuged for 6 min at 500 × g once at 600 × g and then two more times at 700 × g, with the pellet being discarded between each step. This served to remove any large cell wall fragments as well as non-lysed cells as determined by light microscopy. Nuclei were pelleted at 2750 × g for 10 min at 4 °C. The remaining supernatant was centrifuged at 16,000 × g for 10 min at 4 °C to produce the “lysed cell supernatant.” The nuclei-enriched pellet was resuspended in 1 ml of nuclei isolation buffer. To selectively lyse any remaining chloroplasts or mitochondria, 10% Triton X-100 was slowly added to a final concentration of 0.2%. After 5 min of incubation on ice, the suspension was centrifuged at 2750 × g for 10 min at 4 °C. The resulting pellet was washed by resuspension in 3 ml of nuclei isolation buffer and centrifuged at 2750 × g for 10 min at 4 °C. This produced a “nuclei-enriched pellet” with a volume of ~30 µl. Qualitative purity was gauged by resuspending this pellet in 200 µl of nuclei isolation buffer, counterstaining with 1 µg ml⁻¹ 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes) for 10 min on ice, and visualizing by differential interference contrast and fluorescence microscopy (Zeiss Axioskop 2 equipped with a standard DAPI filter and visualizing by differential interference contrast and fluorescence microscope (Zeiss Axioskop 2 equipped with a standard DAPI filter and visualizing by differential interference contrast and fluorescence microscopy studies have provided evidence for the accumulation of flavanoids in the nuclei of diverse plant species, including *Arabidopsis* (18, 23, 28–31). To confirm this findings in *Arabidopsis* seedlings under the growth conditions used in our laboratory, intact 4-day-old La-er plants were stained with the flavonoid-specific dye DPBA, which displays an emission intensity 1–2 orders of magnitude greater when bound to flavonoids or dihydroflavonols than to other flavanoids (39, 40). Optical sectioning by CLSM revealed intense staining in the elongation zone and root tip of wild-type seedlings (Fig. 1a), as previously reported (27, 39), and detected only autofluorescence in *tt5* (86) seedlings, which carry a mutation in the second enzyme of the flavonoid pathway (Fig. 1b) (41). Optical sections through DPBA-stained wild-type epidermal (Fig. 1c) and cortex cells (Fig. 1d) showed fluorescence emanating from nuclei throughout the elongation zone and root tip. Fig. 1e illustrates, at higher magnification, DPBA fluorescence in nuclei as well as cell walls and/or plasmalemma and intracellular membranes, similar to previous reports (28, 31). No obvious nuclear-specific fluorescence was observed in the hypocotyl/root transition zone, although this is likely to be masked by the high levels of endogenous fluorescence in this region (data not shown). It is not possible to distinguish fluorescence from kaempferol versus quercetin (520 and 543 nm emission, respectively) using CLSM; however, it was previously shown thatDefining localization of flavonoids in the nuclei of wild-type and transgenic *Arabidopsis* seedlings using DPBA

**RESULTS**

**Localization of Flavonoid End-Products—**A number of fluorescence microscopy studies have provided evidence for the accumulation of flavanoids in the nuclei of diverse plant species, including *Arabidopsis* (18, 23, 28–31). To confirm these findings in *Arabidopsis* seedlings under the growth conditions used in our laboratory, intact 4-day-old La-er plants were stained with the flavonoid-specific dye DPBA, which displays an emission intensity 1–2 orders of magnitude greater when bound to flavonoids or dihydroflavonols than to other flavanoids (39, 40). Optical sectioning by CLSM revealed intense staining in the elongation zone and root tip of wild-type seedlings (Fig. 1a), as previously reported (27, 39), and detected only autofluorescence in *tt5* (86) seedlings, which carry a mutation in the second enzyme of the flavonoid pathway (Fig. 1b) (41). Optical sections through DPBA-stained wild-type epidermal (Fig. 1c) and cortex cells (Fig. 1d) showed fluorescence emanating from nuclei throughout the elongation zone and root tip. Fig. 1e illustrates, at higher magnification, DPBA fluorescence in nuclei as well as cell walls and/or plasmalemma and intracellular membranes, similar to previous reports (28, 31). No obvious nuclear-specific fluorescence was observed in the hypocotyl/root transition zone, although this is likely to be masked by the high levels of endogenous fluorescence in this region (data not shown). It is not possible to distinguish fluorescence from kaempferol versus quercetin (520 and 543 nm emission, respectively) using CLSM; however, it was previously shown that there is a higher kaempferol/quercetin ratio in the elongation zone and root tip as compared with the hypocotyl/root transition zone (27, 31). DPBA staining in the absence of 0.005% Triton X-100 yielded similar but less intense results, and no fluorescence was detected in this region in *tt5* (86) seedlings when imaged with the parameters used to acquire Fig. 1e (data not shown). It should also be noted that the optical thickness of the images shown in Fig. 1e is 1.5 µm, well below the ~10-µm...
diameter of nuclei in these cells; thus, the fluorescence is clearly intranuclear, not perinuclear.

Immunolocalization of CHS and CHI in Root Cells—To determine whether a link could be established between the nuclear localization of flavonoid end products and the distribution of the flavonoid biosynthetic machinery, co-immunolocalization of CHS and CHI was performed. These enzymes catalyze the first and second committed reactions in the flavonoid pathway, respectively (for a schematic of the pathway, see Ref. 1).

Immunofluorescence CLSM with affinity-purified rabbit anti-CHS and chicken anti-CHI preparations (Fig. 2a) showed co-localization of these enzymes within nuclei of epidermal and cortex cells of the wild-type root tip, as well as in the cytoplasm as previously reported (27). As with DPBA staining (Fig. 1), the fluorescence emanated from within the nucleus and not from perinuclear ER. These signals were highly specific for CHS and CHI because no fluorescence was detected in CHS (tt4(UV118a)) or CHI (tt5(86)) mutants immunolabeled with anti-CHS or anti-CHI antibodies, respectively (27). The signals were also not due to bleed-through from the DAPI channel because no fluorescence was detected in the fluorescein isothiocyanate or rhodamine channels for the nuclei of tt4(UV118a) control samples (Fig. 2b).

Wild-type seedlings co-labeled with antibodies against CHI and the ER-resident protein BiP (an HSP70) showed that, unlike CHI, BiP is excluded from elongation zone nuclei and exhibits a subcellular distribution consistent with ER localization (Fig. 2c). Together, these results point to specific localization not only of flavonoid end products but also of at least part of the biosynthetic machinery to nuclei of root tip epidermal and cortex cells.

To examine this phenomenon at higher resolution, immuno-transmission electron microscopy was performed. Employing an indirect immunogold detection methodology, CHS and CHI were again found to co-localize within nuclei of root tip cells (Fig. 3). Although some of the cellular ultrastructure was sacrificed to favor epitope preservation, it is still possible to detect concentric sheets of perinuclear ER as well as the grainy, electron-dense nucleus. Consistent with the immunofluorescence experiments, labeling for both CHS and CHI was observed throughout the cytoplasm and also within the nucleus and at perinuclear ER. Once again, tt4(UV118a) and tt5(86) mutants failed to exhibit any labeling with affinity-purified anti-CHS or anti-CHI antibodies, respectively, and no labeling was observed in other organelles such as mitochondria (27).

In Vivo Localization of CHS and CHI—To examine the subcellular distribution of CHS and CHI in living seedlings, transgenic plants expressing these enzymes fused to the N or C terminus of mGFP5 were produced. Plants harboring a control
The centriole is comprised mainly of microtubules into tubulin monomers (71). Additionally, the protocol were on ice or at 4 °C, conditions that depolymerize proteins because most of the steps in the nuclear enrichment were performed with nuclei (Fig. 5, top two panels). We were purifying with the enriched nuclear fraction (Fig. 5, top two panels, CHS-mGFP5, CHI-mGFP5, CHS-CHI) and for lines expressing CHI-mGFP5 or CHS-mGFP5-CHI, although fluorescence was less intense (data not shown). The combined size of CHI and mGFP5 is 54 kDa, larger than what is believed to pass the nuclear pore complex by passive diffusion (42), whereas for CHS and mGFP5, it is even larger (70 kDa). Therefore, this experiment provides further evidence that CHS and CHI are actively sequestered in nuclei of the root tip and elongation zone cells.

Detection of CHS and CHI in Isolated Nuclei—As a biochemical complement to the fluorescence microscopy data, we enriched for nuclei from macerated seedlings and assessed the presence of CHS and CHI by immunoblot analysis. Although high levels of CHS and CHI protein were found in the crude cellular lysate, a substantial proportion of each enzyme copurified with the enriched nuclear fraction (Fig. 5, top two panels). The nuclear marker protein histone H1 was detected exclusively in the nuclei-enriched fraction, whereas the cytosolic marker α-tubulin showed only a very small proportion co-fractionating with nuclei (Fig. 5, bottom two panels), much less than the amount seen for CHS and CHI. α-Tubulin served as a suitable “soluble” cytosolic protein marker for these experiments because most of the steps in the nuclear enrichment protocol were on ice or at 4 °C, conditions that depolymerize microtubules into tubulin monomers (71). Additionally, the centriole is comprised mainly of γ-tubulin, which is not immunoreactive with the monoclonal α-tubulin antibody used in this study. It is unlikely that the CHS and CHI in the nuclei-enriched fraction are due to contamination from other organelles or membrane fragments due to the detergent treatment step employed in these experiments, which serves to selectively lyse chloroplasts and mitochondria. It should be noted that this analysis is not quantitative because it is not possible to recover all nuclei (or all protein, for that matter) from intact organisms.

DISCUSSION

Our finding that at least two of the enzymes of flavonoid metabolism are located not only in the cytoplasm but also in the nuclei of some cells, while surprising, is not without precedent. There are, in fact, a growing number of metabolic enzymes for which dual cytoplasmic/nuclear localization has been observed, both in plants and in other organisms. A number of these enzymes are proposed to have distinct functions in these two locations. For example, mammalian Wiskott-Aldrich syndrome protein controls actin polymerization at the cell periphery but acts as a transcriptional regulator in the nucleus (43). In yeast, glucokinase has been shown move to the nucleus and alter gene expression in response to different environmental conditions (44). Mammalian phospholipid scramblase 1 has a postulated role in the redistribution of phospholipids at the plasma membrane and an undetermined function in the nucleus involving interaction with DNA (45). Similarly, the glycolytic enzymes phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase have been proposed to function as the primer recognition protein for DNA polymerase-a and as a uracil glycosylase, respectively, in plant and animal nuclei (46–49). Interestingly, both the cytoplasmic and plastid forms of pea phosphoglycerate kinase are found in the nucleus, indicating that dual distribution can also involve other organelles (50). In other cases, such as for glucokinase in liver cells (51, 52) and phosphatidylinositol-4-phosphate 5-kinase in yeast (53), sequestration in the nucleus may provide a supply of enzyme that can be rapidly mobilized to increase cytoplasmic activity in response to changes in glucose levels or requirements for second messengers. For several other well-known enzymes that clearly exhibit this type of dual localization, including plant and animal aldolase and animal 5-lipoygenase, the reason behind localization of a proportion of these enzymes to the nucleus remains to be established (54–56). Our finding that both flavonoid enzymes and end products are present in different cellular locations appears to represent yet another paradigm, in which differential localization of the biosynthetic machinery provides a mechanism by which to direct the deposition of specific products in the nucleus versus other cellular locations.

The mechanisms underlying the nuclear/cytoplasmic distribution of metabolic enzymes have been studied in a few cases, and it appears that these can be quite diverse. For example, nuclear targeting of glucokinase appears to involve assembly of a nuclear localization signal (NLS) by interaction with the glucokinase regulatory protein and masking of a nuclear export signal by leptomycin (57). Shuttling of PI4P 5-kinase between the cytoplasm and nucleus in yeast may be regulated by palmitoylation and involve both a classical NLS and the product of a
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![Figure 6. Homology model of Arabidopsis CHS (72) showing the predicted nuclear localization domain. Residues corresponding to the predicted NLS (Arg^{21}, Lys^{22}, Arg^{23}, and His^{74}) are in dark gray, and a methionine (Met^{142}) at the dimerization interface is shown in black. The image was produced using Swiss-Pdb viewer, version 3.7 (70).](image)

previously uncharacterized essential gene, BCP1, postulated to function in general nuclear export (53). In the vast majority of these cases, however, nothing is yet known about the determinants of intracellular distribution.

For the enzymes of the flavonoid pathway, several mechanisms may be involved. In the cytoplasm, flavonoid enzyme complexes are believed to assemble at the ER and in electron-dense particles through the association of operationally soluble enzymes such as CHS and CHI with the membrane-bound P450 hydroxylase, flavonoid 3′-hydroxylase (27, 58), CHI, at 27 kDa, is by far the smallest of the flavonoid enzymes and could move through the nuclear pore complex by passive diffusion. However, this is unlikely for the CHI-mGFP5 fusion protein, at 54 kDa, which also localizes to nuclei. Intriguingly, we previously showed that CHI is present in two forms in plant cells that appear to differ by a thiol-sensitive linkage to a moiety that reduces the mobility of the enzyme during SDS-PAGE (59). The immunoblot experiment shown in Fig. 5 suggests that it is primarily the slower-migrating form that accumulates in the nucleus, thus raising the possibility that post-translational modification controls the differential localization of CHI. On the other hand, CHS, at 47 kDa, is the one enzyme of the central flavonoid pathway that possesses sequences resembling a classic NLS. This signal is located on the surface, on the opposite side of the protein from the dimerization interface (Fig. 6), and could function to direct CHS and perhaps other associated enzymes into the nucleus. The localization of end products such as flavanol sulfate esters and flavan-3-ols to the nucleus suggests mechanisms for regulating gene expression. The fact that flavonoid biosynthesis represents an ancient pathway that was apparently derived from primary metabolism suggests that differential localization of enzyme systems may be a much more general mechanism for controlling the site-specific accumulation of end products than has previously been considered.

Flavonoids clearly have the potential to directly affect signaling and gene transcription through interaction with cytoplasmic and nuclear proteins. Our finding that not only the end products but also the components of the biosynthetic machinery are located in the nucleus suggests mechanisms for regulating flavonoid action that have not previously been considered. Determining how the dual localization of flavonoid enzymes is controlled in different compartments, different cell types, and over the course of development, as well as identifying the specific proteins to which flavonoids bind, is now clearly essential in order to fully understand the physiological functions of these compounds, including a possible role in regulating gene expression. The fact that flavonoid biosynthesis represents an ancient pathway that was apparently derived from primary metabolism suggests that differential localization of enzyme systems may be a much more general mechanism for controlling the site-specific accumulation of end products than has previously been considered.

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