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TISSUE LOCALIZATION OF PHYTOCHROME IN DARK-GROWN BARLEY LEAVES

STEPHEN O. DUKE, DAVID H. NETZLEY* and JERRY W. MCCLURE*

Southern Weed Science Laboratory, USDA, SEA, AR, P.O. Box 225, Stoneville, MS 38776, U.S.A.; * Department of Botany, Miami University, Oxford, OH 45056, U.S.A.

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Abstract—Phytochrome was spectrophotometrically determined to be differentially concentrated among separated tissues of dark-grown, norflurazon-treated barley leaves. The relative concentrations in vascular, mesophyll protoplast and epidermal tissues approximated to a ratio of 7:3:1, respectively. Of the total phytochrome about 75% was in the protoplasts with the remainder divided equally between vascular and epidermal tissues.

INTRODUCTION

Although phytochrome has been immunohistochemically localized in plant tissues [1], no direct spectrophotometric assays of phytochrome have been reported from isolated tissues. In this work we report the first spectrophotometric measurements of phytochrome levels in isolated plant tissues.

RESULTS AND DISCUSSION

Phytochrome was found to be concentrated in vascular, photosynthetic (mesophyll plus bundle sheath), and epidermal cells in a ratio of 7:3:1, respectively (Table 1). When corrected for the volume of each tissue type in the leaf, about 75% of the recovered leaf phytochrome was found to be in photosynthetic cells with the remainder divided equally between epidermal and vascular tissue.

The only previous study in which the internal distribution of phytochrome in a plant organ has been examined spectrophotometrically was that of Duke and Williams [2], who found phytochrome to be more concentrated in the internal (vascular plus cortical tissue) than external (epidermal plus cortical tissue) regions of Johnsongrass [(*Sorghum halapense* (L.) Pers.] rhizomes.

Phytochrome concentrations calculated for the entire leaf from individual tissue measurements, however, totalled only about 20% of that measured in intact leaves. Apparently 80% of the phytochrome was lost during incubation. Incubation of barley leaves under the same conditions used to isolate protoplasts, except without cellulase, resulted in a 60% loss of spectrophotometrically assayable phytochrome. This loss during incubation could have been due to decay of P_{fr} formed under green light or P_r loss due to general stress. The latter possibility seems most probable in that phytochrome-saturating green light transforms only 65% of phytochrome to P_{fr} [3] and the green light exposure received in these studies was considerably less than a saturating fluence. Apparent phytochrome loss could also be due to a greater phytochrome concentration in the leaf area measured in the intact leaf (2.5 cm below the apex) than in the entire 3-cm leaf apex used for tissue isolation.

The distribution of phytochrome found by our technique is in partial agreement with immunohisto-

Table 1. Phytochrome distribution and concentration in the tissues of 6-day-old, dark-grown, norflurazon-treated barley primary leaves

Tissue	Relative concentration ($\Delta A_{730-660}/A_{660}$) × 10^{-5}	Relative tissue volume (%)	Relative distribution (%)
Epidermis	18	29	12
Vascular	126	4	12
Photosynthetic (mesophyll plus bundle sheath)	49	67*	76
Intact leaf (actual)	232		
(calculated)	43		

* Includes intercellular space.

chemical evidence of Pratt and Coleman [1], who found phytochrome to be most concentrated in the vascular and epidermal tissue of the primary leaf of 3-day-old barley seedlings. In their study, the pigment was most concentrated about 2 cm below the leaf apex, with most of the stain located in the lower epidermis and the periphery of vascular bundles. This pattern was more clear in barley than in other grass seedlings. The relatively low phytochrome levels that we found in the lower epidermis could be due to differences in tissue age. The upper 3 cm of leaf tissue in a 6-day-old seedling may be equivalent to the upper 1 or 2 cm leaf tissue in a 3-day-old seedling, which was relatively devoid of immunocytochemically detectable phytochrome(s) in Pratt and Coleman's study [1].

Levels of phytochrome in light-grown barley leaves were too low to measure after separation of the tissues. In norflurazon-treated seedlings grown on a 16 hr light (200 $\mu E/m^2/sec$): 8 hr dark cycle, we found about 15% of the phytochrome of intact, dark-grown leaves in intact light-exposed leaves at the end of the dark period.

The physiological significance of our findings are not yet clear, as there is no general agreement on whether or not phytochrome concentration influences the physiological responses controlled by phytochrome. Phytochrome concentration is generally considered to be unimportant [4] but Duke *et al.* [5] and Beggs *et al.* [6] have correlated total phytochrome (i.e. phytochrome concentration) with various physiological responses to light.

EXPERIMENTAL

Plant tissue. Caryopses of *Hordeum vulgare* L. var. Atlas 68 were placed in 9 cm Petri dishes containing 10 ml H_2O and a 9 cm filter paper impregnated with 1 μmol of norflurazon [4-chloro-5-(methyl amino)-2-(α,α,α-trifluoro-*m*-tolyl)-3-(2*H*)-pyridazinone]. Norflurazon is a potent inhibitor of carotenoid synthesis [7] with no significant demonstrable effects on phytochrome functioning [8–10] or dark-growth [9, 10]. The compound was used to eliminate chloroplast development in light-grown seedlings, thus minimizing chlorophyll interference with phytochrome measurements. After 24 hr imbibition, the seeds were placed in 7.5 cm^2 plastic pots, 2 cm below the surface of 6 cm of vermiculite moistened with 2 mM $CaSO_4$. The plants were grown at 23° in complete darkness, except when briefly exposed to dim green light [5] during watering. All manipulations for tissue separations were performed under dim green light.

After 6 days of dark growth, a lateral cut was made through the lower epidermis of the primary leaves, 3 cm below the leaf apex, and the epidermis was peeled off and placed in 25 mM citrate–phosphate buffer (pH 6.6), 0.15 M mannitol, and 0.05 M sucrose at 4° for 4 hr. The remaining leaf was floated, peeled side down in a protoplast isolating medium of 25 mM citrate–phosphate (pH 5.55), 0.45 M mannitol, 0.05 M sucrose, and 1% (w/v) cellulase R-10 for 4 hr at 30°. The protoplasts were poured through a 60 μm stainless steel mesh, centrifuged at 180 *g* for 1 min. and resuspended in a medium of 80 mM Tris–HCl (pH 7.5), 0.45 M mannitol, and 0.05 M sucrose. The protoplasts were centrifuged again at 180 *g* and the pellet was used for the spectrophotometric assay. After the protoplasts were removed from the leaf, vascular strands were teased from the remaining upper epidermis and collected on a 60 μm stainless steel mesh. Light microscopy revealed that each tissue type was essentially homogeneous.

Phytochrome assay. Phytochrome was measured as before [2, 5], except that the tissue was placed in a 5 mm path length aluminum cuvette and an Aminco microbeam condenser was used to focus measuring and reference beams on the samples. Samples were gently packed in the cuvette in incubation buffer except for the intact leaves which were placed back-to-back in the cuvette with their planes perpendicular to the measuring beam. The measuring beam was focused about 2.5 cm below the leaf apices of intact sections. After phytochrome assay the absorbance of the samples at 660 nm was taken to estimate relative light pathlength. No absorbtion peaks were measured in any of the samples at 660 nm and only a small amount of the sample absorption at this wavelength was due to chlorophyll(ide). All prochlorophyllide was transformed before measurements were made. The A_{660} of the samples ranged from 2 to 3. Relative concentration of phytochrome, corrected for different path lengths, was expressed as $\Delta A_{660-730}/A_{660}$. These values were transformed to relative distribution percentages by multiplying each value by the relative tissue volumes (determined from fresh vibratome sections) and dividing each result by the total of all products [i.e. rel. conc × rel. vol./Σ(rel. conc × rel. vol.)]

Results are averages of four different experiments conducted at different times. Phytochrome measurement standard errors averaged 12% of means and the standard errors of correction factors (A_{660}) averaged 4% of means.

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