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THE OCCURRENCE AND PHOTOREGULATION OF FLAVONOIDS IN BARLEY PLASTIDS*

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Abstract—Whole-leaf extracts of etiolated or light-grown barley shoots contain the *C*-glycosylflavones saponarin, lutonarin and lutonarin 3′-methyl ether. Plastids isolated by aqueous techniques contain only saponarin. Contamination experiments using foreign flavonoids indicate that saponarin recovered from plastids is not a contaminant from other cellular fractions. In response to brief red light treatment 24 hr before harvest, saponarin levels are approximately doubled in whole-shoot extracts, but increased about 3.5 fold in plastids. This photocontrolled increase is far-red reversible. Thus saponarin is selectively accumulated in barley plastids and this accumulation is controlled by phytochrome.

INTRODUCTION

In 1966 Kefeli and Tureskaya [1] reported catechin and quercetin glycosides in chloroplasts isolated from willow leaves and in the following year Zaprometov and Buklaeva [2] found that incubation of tea or willow leaves with $^{14}CO_2$ for 30 min yielded phenolics in the chloroplasts with from 7 to 10 times the specific activity as those found in the cytoplasmic fraction. Flavonoids have since been reported in chloroplasts isolated from a variety of plants such as *Impatiens* [3], pea [4], melon and tomato [5], spinach [6] and oats [7–9]. Extensive work on oats indicates that the *C*-glycosylflavones found in these plastids are not contaminants from other cellular fractions and shows that the distribution of specific flavonoids between the plastid and non-plastid compartments varies with developmental stages and light conditions [7–9]. We have recently reported that the enzyme phenylalanine ammonia-lyase is associated with plastids in Atlas 68 barley [10] and both red far-red photo-reversibility studies [11] and detailed action spectra [12] show that the *C*-glycosylflavone saponarin is photocontrolled in whole-shoot extracts of this plant. Thus barley plastids were investigated to determine if they, too, contained flavonoids and if the accumulation of flavonoids in this organelle responds to phytochrome activation as does the level in whole-shoot extracts.

RESULTS

Whole-shoot methanolic extracts from five-day-old etiolated barley seedlings contain about 200 nmol saponarin per gram fresh weight [11]. This level is increased by approximately ten fold when the plants are grown in white light and lutonarin and its 3′-methyl ether are present in approximately 1/3 this amount in the dark or under a range of light conditions [11, 12].

Chloroplasts isolated from 5 day old barley shoots contain saponarin. When the chlorophyll:saponarin ratio is calculated for whole-shoot extracts and for isolated chloroplasts (0.45 mg total chlorophyll per 1 g shoot or 5.88×10^9 chloroplasts [10]), about 21% of the saponarin can be assigned to the chloroplast preparation. However, neither lutonarin nor its 3′-methyl ether can be detected in barley chloroplasts, even when the sample size is increased to 1.2×10^{10} plastids which is equivalent to the number of plastids found in slightly over 2 g of whole shoot samples [10]. Whole shoot samples of this weight contain approximately 1.3 nmol of lutonarin and its 3′-methyl ether [11, 12] and if these compounds are present in chloroplasts preparations to the same degree as is saponarin (viz. *ca* 20%) then one would expect to find about 250 nmol of these flavonoids, an amount about ten times more than is required for detection and quantitation by our techniques [12].

One series of contamination experiments was conducted by adding 75 ml of plastid isolating medium saturated with rutin (*ca* 10 mg) to each 25 ml of normal medium. Chloroplasts were isolated in this solution and the resulting chromatograms examined in UV light with and without ammonia and after spraying with Benedict's reagent. Saponarin was found on the chromatograms as expected but there were no traces of rutin. Other experiments were done by dissolving into 100 ml of the isolating medium a dried total methanolic extract from 30 g of fresh weight *Lemna gibba* plants which produce *ca* 1 mmol/g fr. wt of several flavonoids including cyanidin—3-glucoside, vitexin and orientin [13]. Again, only saponarin was detected in the methanolic extracts prepared from the isolated barley chloroplasts.

As increased saponarin accumulation in whole-shoot extracts of barley is under phytochrome control [11], we investigated this parameter in plastids isolated from

* Part 6 in the series "Phenolic Biosynthesis in Barley Seedlings." For Part 5 see Ref. [10]. This work forms part of a dissertation by the senior author.

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Table 1. Saponarin levels in plastids and in whole shoot extracts from Atlas 68 barley

Light treatment*	Saponarin (nmol/mg protein)	Standard error	% of dark control
	Whole shoot samples		
Dark control	5620	272	100%
2 min FR	6160	280	104
4 min R + 2 min FR	6400	235	108
4 min R	11 600†	256	196
	Plastid fraction		
Dark control	1007	122	100%
2 min FR	1133	17	112
4 min R + 2 min FR	1017	102	101
4 min R	3549†	140	352

* Etiolated plants were grown for 4 days in a darkroom and, when appropriate, given 4 min red (1.1 kerg cm^{-2} sec^{-1}) or 2 min far-red (34 kerg cm^{-2} sec^{-1}) light 24 hr before harvest ($n = 3$). † Significantly different at the 0.01 probability level.

dark grown five-day-old plants that received short-term light treatments 24 hr before harvest. The results are shown in Table 1. The level of saponarin in the plastids is significantly increased by red light and reversed by a subsequent far-red light treatment. Thus saponarin levels in the plastids are controlled by phytochrome and the response is considerably more pronounced in plastids than in whole-shoot preparations.

DISCUSSION

Weissenböck and Schneider [9] contaminated isolated oat chloroplasts with saponaretin and several other flavonoids which are present in oat seedlings. Saponaretin is a minor constituent of whole-leaf oat extracts but enriched in the chloroplasts. After washing the contaminated chloroplasts in isolating medium the exogenous flavonoids were recovered quantitatively leaving the characteristic pattern of flavonoids associated with the plastids. Similar results were obtained by Montes who carried out contamination experiments with flavonoids added to isolated tomato and melon chloroplasts [5]. We conclude that saponarin recovered from barley plastids is not a contaminant of isolation for several reasons: First, contamination experiments show that exogenously supplied flavonoids, in amounts far in excess of levels present in whole shoot homogenates, are not recovered from the isolated chloroplasts. Secondly, only saponarin is recovered from the chloroplasts while whole-shoot extracts contain this flavonoid plus lutonarin and lutonarin 3′-methyl ether. Thirdly, red far-red photoreversibility experiments show that saponarin accumulation in response to phytochrome activation is much more pronounced in plastids than in whole-shoot extracts, not equilibrated between the plastids and the other cellular compartments (Table 1). Finally, other work in this laboratory has shown that flavonoids are not invariable constituents of chloroplast preparations since at least two species of angiosperms are known which contain high levels of flavonoids in whole-leaf methanolic extracts but whose chloroplast appear to be free of flavonoids when prepared by the procedures used in barley plastid isolation [14].

The enrichment of specific flavonoids in chloroplasts at certain developmental stages [7–9] and in response to light (Table 1) could influence the photosynthetic capability of the plant. For example, low concentrations of kaempferol glycosides inhibit ATP formation in chloroplasts isolated from peas [4] or spinach [16] while the *p*-coumarate esters of the glycosides are stimulatory. In the case of oats, saponaretin is enriched in chloroplasts while three other apigenin-based glycosides are associated primarily with the non-plastid fraction [9]. When added to oat chloroplasts lamellar systems at low (5 ng flavonoid/100 μg chlorophyll) levels, the non-plastid flavonoids are considerably more inhibitory to photophosphorylation than is saponaretin [9]. There are also suggestions that flavonoids may play a role as electron acceptors in photosynthesis [6].

Following osmotic shock and washings, about 10% of the flavonoids associated with oat chloroplasts are retained in the lamellar fraction [9]. These results suggest that flavonoids are accumulated within the stroma but do not measure the degree to which non-specific flavonoid–protein binding would occur, or to which functional water soluble constituents would be removed from the lamellae by such treatments. It is also apparent that the addition of exogenous flavonoids to purified chloroplasts could participate artifactually by serving as antioxidants, as enzyme inhibitors, or as substrates for bypass electron transport systems [15].

EXPERIMENTAL

Plant material and light treatments. Culture conditions and light treatments for Atlas 68 barley have been described [11].

Plastid isolation. Chloroplasts were isolated under laboratory lighting conditions. All manipulations of etiolated plants or etioplasts were in the dark or under a dim green safelight [11]. Plastids were isolated from whole shoots excised at the point of emergence from the caryopsis. The shoots were cut into *ca* 1 cm segments and homogenized in a Virtis model 23 homogenizer by 3 successive 2-sec treatments at maximum velocity in 4 vols of cold isolating medium per g fr. wt. The isolating medium consisted of 0.33 M sorbitol, 1 mM $MgCl_2$, 2 mM disodium EDTA, and 0.15 M Pi buffer pH 6.8. Homogenate was filtered through 2 layers of cheesecloth and 2 layers of Miracloth. The filtrate was centrifuged for 1 min at 200 *g*, the pellet discarded, and the supernatant recentrifuged at 2000 *g* for 1 min. The plastid-rich pellet was resuspended in 25 ml of isolating medium, a 0.5 ml aliquot taken for protein and/or chlorophyll determination, and the plastids collected by recentrifugation at 2000 *g* for 2 min. All isolation steps were carried out at 4° in a Sorvall RC2-B refrigerated centrifuge using a SS-34 rotor. The plastid preparations were monitored for intactness using phase contrast and Nomarski optics microscopy, as well as by scanning and transmission electron microscopy [16]. By these criteria, the pellet consisted of a mixture of about 75% class I and 25% class II chloroplast. Pooled plastids from *ca* 250 g of fresh shoots were used for each flavonoid assay.

Extraction and chromatography of plastid flavonoids. The plastid pellet was homogenized with 50% aq. MeOH containing 0.25% HCl and the debris removed by centrifugation. The supernatant was reduced in volume *in vacuo* and applied to a sheet (46 × 57 cm) of Whatman No. 3 MM paper, which was developed in *t*-BuOH–HOAc–H_2O (3:1:1), and in the second dimension in 5% HOAc. Quantitation of saponarin was by published techniques [12].

Whole shoot samples. Four shoots were thoroughly ground in a Ten Broeck homogenizer with 8 ml MeOH containing 0.25% HCl. Cellular debris were removed by centrifugation and the supernatant treated as described for the plastid extracts.

Protein and chlorophyll determinations. Protein was determined by the technique of Schacterle and Pollack [17], chlorophyll by Arnon's technique [18].

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