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ACTION SPECTRA FOR *C*-GLUCOSYLFLAVONE ACCUMULATION IN *HORDEUM VULGARE* PLUMULES*

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Abstract—Five-day-old etiolated barley plumules contain the *C*-glucosylflavones saponarin, lutonarin, and lutonarin 3′-methyl ether. When harvested 24 hr after illumination, increased flavonoid levels were essentially linear with increased energies of monochromatic light at seven wavelengths between 450 and 750 nm. Action spectra for saponarin and for a mixture of lutonarin and its 3′-methyl ether were determined between 380 and 760 nm at 6·6 kerg·cm^{-2}. The saponarin action spectrum showed distinct peaks at 620 and at 660 nm. These two peaks were similar in their photoreversibility when followed by either 6·6 or 34 kerg·cm^{-2} of far-red light. Phytochrome is apparently the photoreceptor for the saponarin action spectrum. Lutonarin and its 3′-methyl ether showed peaks at 520, 580, 620 and near 660 nm. The 660 nm peak was not photoreversible by 6·6 kerg·cm^{-2}, but was by 34 kerg·cm^{-2}, of far-red light. Phytochrome and protochlorophyll are the likely photoreceptors for these 3′-substituted flavonoids.

INTRODUCTION

IN A previous paper[1] increased saponarin (6-*C*-glucosyl-7-*O*-glucosylapigenin) production in 4-day-old barley plumules was found to be under a typical low-energy phytochrome control and either white or blue light was required for the appearance of lutonarin (6-*C*-glucosyl-7-*O*-glucosylluteolin) or its 3′-methyl ether. In order to understand the degree to which photoreceptors other than phytochrome might be involved, we undertook action spectra for increased flavonoid levels. It was through action spectra that phytochrome responses were first discovered[2] and in simple cases the action spectrum for a response can be fitted to the absorption spectrum of the light absorbing pigment.[3]

Action spectra have been reported for anthocyanin production in a number of plants[4] but, insofar as we can determine, not for other classes of flavonoids. The anthocyanin action spectra are often quite perplexing. For example, Ng *et al.*[5] compared action spectra for anthocyanin production in several plants and concluded that their most evident characteristic was their almost total lack of disagreement. One of the major problems in determining anthocyanin action spectra is the usual requirement for preconditioning with either white or blue light.[4,5] Under such conditions many low energy photoresponses would surely be

* Part II in the series "Phenolic Biosynthesis in Barley Seedlings". For Part I see Ref. 1.

[1] MCCLURE, J. W. and WILSON, K. G. (1970) *Phytochemistry* **9**, 763.
[2] PARKER, M. W., HENDRICKS, S. B., BORTHWICK, H. S. and SCULLY, N. J. (1946) *Bot. Gaz.* **108**, 1.
[3] ALLEN, B. B. (1964) in *Photophysiology* (GIESE, A. C., ed.), Vol. 1, p. 83, Academic Press, New York.
[4] HENDRICKS, S. B. and BORTHWICK, H. S. (1965) in *Chemistry and Biochemistry of Plant Pigments* (GOODWIN, T. W., ed.), p. 405, Academic Press, New York.
[5] NG, Y. L., THIMANN, K. V. and GORDON, S. A. (1964) *Arch. Biochem. Biophys.* **107**, 550.

saturated and action spectra are usually meaningful only when the light intensity is such that light limits the reaction.[3] Aothocyanin synthesis is also an extremely complex physiological response. Grill and Vince[6] present evidence that at least two light steps are involved in anthocyanin synthesis in turnip (*Brassica rapa*) and three light reactions seem to be involved in cyanidin 3-glucoside production in the duckweed *Spirodela intermedia*; one for increased C_{15} precursor, another for substitution at the 3′-position, and a high energy requiring step for conversion from dihydroflavonol to anthocyanidin.[7]

In contrast, flavones, and presumably *C*-glucosylflavones, are derived at an earlier flavanone stage[8] and low levels are found in dark-grown plants.[1,9] In short, biosynthetic and physiological controls of synthesis are apparently more complex for anthocyanins than for flavone glycosides.

Since flavonoids of different B-ring substitution patterns are found in barley[1] action spectra should contribute to our understanding of substitution at the 3′-position. By using 5-day-old-plants, rather than 4-day-old plants as in the previous paper,[1] we found all three flavonoids and were able to determine action spectra for saponarin and for a mixture of lutonarin and its 3′-methyl ether.

RESULTS

Preliminary experiments with 5-day-old etiolated plants showed saponarin as well as small amounts of lutonarin and its 3′-methyl ether. These last two compounds do not separate readily on paper chromatograms and in early stages we separated them by pro-

TABLE 1. *C*-GLUCOSYLFLAVONE LEVELS IN ETIOLATED BARLEY 24 hr AFTER EXPOSURE TO 10 min OF MONOCHROMATIC LIGHT

Wavelength (nm)	Total energy (kerg·cm^{-2})	Saponarin (nM/g fr. wt)	Lutonarin + 3′OMe (nM/g fr. wt)
400	11·8	288	70
450	13·4	228	71
500	14·4	263	96
550	12·6	366	119
600	10·7	676	192
640	11·0	670	150
650	11·1	675	165
660	10·4	470	151
675	9·8	475	152
700	9·1	440	197
750	7·1	246	87
Dark control	—	167	47

Plants were harvested 24 hr after illumination. Lutonarin and its 3′-methyl ether were measured as a mixture.

[6] GRILL, R. and VINCE, D. (1964) *Planta* **63**, 1.
[7] MCCLURE, J. W. (1970) in *Phylogenetic Phytochemistry* (HARBORNE, J. B., ed.), p. 233, Academic Press, New York.
[8] GRISEBACH, H. (1968) in *Recent Advances in Phytochemistry* (MABRY, T. J., ALSTON, R. E. and RUNECKLES, V. C., eds.), Vol. 1, p. 379, Appleton-Century-Crofts, New York.
[9] MCCLURE, J. W. (1968) *Plant Physiol.* **43**, 193.

longed development in the aqueous solvent system.[1] Under many conditions, light as well as darkness, the amount of lutonarin equalled that of its 3′-methyl ether as might be expected from previous work on photocontrol of flavone and flavonol glycosides of equivalent B-ring substitution patterns.[9] In subsequent work, for ease of quantitation and considering the relatively low accumulation of these two compounds, we isolated a mixture of lutonarin and its 3′-methyl ether and accepted it as an indication of the level of 3′-*O*-substituted flavonoids present in the plant at harvest.

Linearity of Flavonoid Production with Increasing Energies of Light

For an action spectrum to accurately distinguish wavelengths most effective in inducing a response, a region of sensitivity must first be determined and linearity of responses proven. A preliminary experiment was carried out by exposing etiolated plants to monochromatic light for 10 min and then returning them to the dark for 24 hr. The results are shown in Table 1. In order to be assured that the system was not being saturated at any wavelength, the responses to increasing energies were examined for linearity at seven wavelengths and at various incident energies. The results of this experiment are shown in Fig. 1. On the basis of these experiments, an energy of 6·6 kerg·cm^{-2} was chosen as a non-saturating yet effective level for the action spectra.

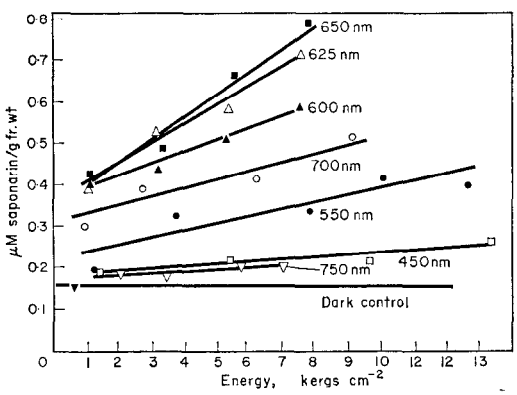

FIG. 1. SAPONARIN LEVELS IN ETIOLATED ATLAS 46 BARLEY IN RESPONSE TO DIFFERENT ENERGIES AND WAVELENGTHS OF LIGHT.

Plants were harvested 24 hr after illumination. Each point is the average of two replications.

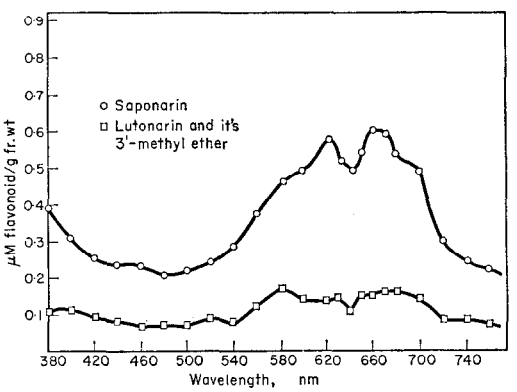

FIG. 2. A 6·6 kerg·cm^{-2} ACTION SPECTRUM OF THE INCREASED PRODUCTION OF SAPONARIN AND A MIXTURE OF LUTONARIN AND ITS 3′-METHYL ETHER BETWEEN 380 AND 760 nm.

Plants were harvested 24 hr after illumination. Each point is the average of 6–12 determinations. The standard deviation calculated was less than the range of values that may be indicated on the figure.

The Action Spectra

The action of spectra were determined within the range 380–760 nm (Fig. 2). The monochrometer would not provide shorter wavelengths and as the investigation progressed it became apparent that wavelengths longer than about 740 nm were essentially ineffective. Response was determined at 10 nm intervals for the critical region from 620 to 680 nm and at 20 nm intervals for other wavelengths. Each interval from 620 to 680 was determined in triplicate on four different occasions. Other wavelengths were examined in triplicate on at

least two different occasions. The calculated error term was small and not significantly different from that previously reported.[1]

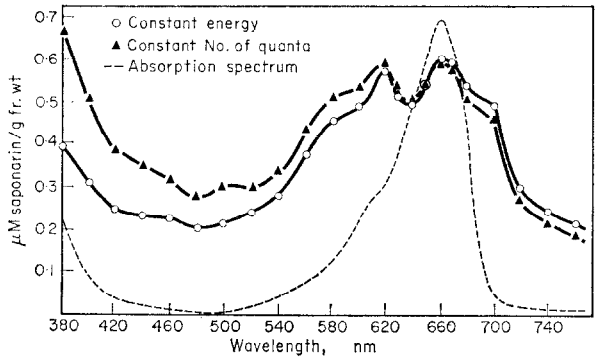

FIG. 3. ACTION SPECTRUM OF INCREASED SAPONARIN LEVELS IN ETIOLATED ATLAS 46 BARLEY PLUMULES MODIFIED TO SHOW RESPONSE AT CONSTANT ENERGY (6·6 kerg·cm^{-2}) AND CONSTANT NUMBER OF QUANTA (3·62 × 10^9 ein). FOR PURPOSES OF THE DISCUSSION, THE ABSORPTION SPECTRUM OF PURIFIED PHYTOCHROME HAS BEEN SUPERIMPOSED.
Plants were harvested 24 hr after illumination.

It is often informative to present spectra in terms of einsteins to demonstrate quantum efficiency. With linearity demonstrated (Fig. 1), the results can be converted to quantum yield. The action spectrum for saponarin modified to show responses in both kerg·cm^{-2} and einsteins is presented in Fig. 3. For comparison and discussion, the absorbance spectrum of purified oat phytochrome in solution[10] is also shown.

Interpretation of the Action Spectra

The action spectrum for saponarin has peaks near 620 nm and in the 660–670 nm region. Green light was essentially ineffective but increased response was observed in the near UV. Lutonarin and its 3′-methyl ether were found in all experimental plants and there are several similarities between the spectra for saponarin and for these two 3′-substituted flavonoids; little response in the region below 540 nm, a minimum near 640 nm, and responses in the 660–700 nm range.

One difference in the two action spectra is near 580 nm where lutonarin and its 3′-methyl ether show a definite response while the curve for saponarin has only a slight shoulder. Also, there is no peak in the 620 nm region for lutonarin and its 3′-methyl ether while the saponarin action spectrum has a well-defined peak in this region. Finally, the responses to shorter wavelengths were very slight for the 3′-substituted flavonoids.

Similarities in the action spectra are to be expected if a part of the spectra can be attributed to increased production of common precursors; differences should be anticipated if there are specific photocontrols for substitution at the 3′-position.

The two peaks for saponarin at 620 and at 660 nm might be explained in several ways. For example; two different photoreceptors could be involved, a 'shadow' pigment might be removing a significant amount of light at 640 nm, or energy could be absorbed by a photoreceptor at 620 nm and transferred to a 660 nm photoreceptor. If phytochrome is

[10] Adapted from MUNFORD, F. E. and JENNER, E. K. L. (1966) *Biochemistry* **51**, 3675.

involved, two additional and complicating factors must be considered. First, long-lived transitional intermediates in phytochrome transformation may be involved in the action spectrum and their absorbance characteristics may differ from that of stabilized phytochrome *in vitro*.[11] Secondly, when one measures physiological responses to red and far-red light treatments, against spectrophotometrically detectable phytochrome ratios in the tissues, there is usually no correlation.[12] Thus our understanding of phytochrome within the living tissue depends primarily on the spectrophotometric method which does not provide information on the active fraction of the total phytochrome population.[11]

Still, most workers assume that red activation and far-red reversibility are characteristics associated only with phytochrome and thus proof of phytochrome involvement in a process.[4] We attempted to test the involvement of phytochrome in both the 620 and 660 nm peaks of the action spectra by assuming that if phytochrome were the sole photoreceptor involved then the degree of far-red photoreversibility of 620 and 660 nm treated plants should be proportional at different far-red energies.

TABLE 2. EFFECTS OF TWO ENERGIES OF FAR-RED LGIHT ON FLAVONOID LEVELS IN ETIOLATED BARLEY PLUMULES PREILLUMINATED WITH 620 OR 630 nm LIGHT

Light treatment*	Saponarin (nM/g fr. wt)	Lutonarin + 3′-OMe (nM/g fr. wt)
	Individual light effects	
Dark control	213	32
660 nm	631	129
620 nm	606	142
730 nm	273	83
Broad band far-red	260	37
	Combined light effects	
660 + 730 nm	509 (−21·4%)†	151 (+17·1%)
620 + 730 nm	451 (−25·6%)	144 (+1·1%)
660 + broad band far-red	348 (−44·9%)	78 (−59·6%)
620 + broad band far-red	347 (−42·7%)	68 (−52·1%)

* Etiolated plants were given light on the fourth day after planting and harvested 24 hr later. Monochromatic light at 6·6 kerg·cm^{-2}, broad band far-red at 34·kerg·cm^{-2}. Lutonarin and its 3′-methyl ether were measured as a mixture.

† Percentage reversal (−) or promotion (+) attributable to following the 660 or 620 nm treatments with far-red light from the monochrometer or from a broad far-red source.

Photoreversibility of 620 *and* 660 *nm points of the action spectra*

Etiolated plants were given 6·6 kerg·cm^{-2} of 620, 660 or 730 nm light or 34 kerg·cm^{-2} from a broad-band far-red light source, alone or in various combinations (Table 2). We conclude that since saponarin production at either 620 or 660 nm is proportionally reversible by two different energies of far-red light, the peaks at both 620 and 660 nm in this action spectrum are due to phytochrome.

Photoreversibility of lutonarin and its 3′-methyl ether did not follow the same pattern as saponarin (Table 2). Light of 730 nm alone gave marked increases of these 3′-substituted

[11] BRIGGS, W. R. and FORK, D. C. (1969) *Plant Physiol.* **44**, 1089.
[12] BELLINI, E. and HILLMAN, W. S. (1971) *Plant Physiol.* **47**, 667.

flavonoids while saponarin was only slightly increased in the same plants. When 6·6 kerg. cm^{-2} of either 620 or 660 nm light was followed by 6·6 kerg·cm^{-2} of 730 nm light, there was a slight increase in lutonarin and its 3′-methyl either but photoreversibility of approx. 23% of the saponarin. When monochromatic red light was followed by 34 kerg·cm^{-2} from a broad-band far-red light source, there was an even higher degree of photoreversibility of these 3′-substituted flavonoids (*ca.* 55%) than of saponarin (*ca.* 43%).

These results suggest that phytochrome controls increased saponarin production while phytochrome and some additional system, perhaps in the plastids, controls substitution of *C*-glucosylflavones at the 3′-position.

DISCUSSION

Nothing is known of enzymes in barley plumules that degrade *C*-glucosylflavones, or whether light causes an increase in flavonoid synthesis or simply stops degradation and allows synthesis to continue and the flavonoids to accumulate. However, if the apparent lack of turnover of *C*-glucosylflavones in *Spirodela*[13] is applicable to barley, then the levels of flavonoid measured in this work probably reflect synthesis. If there is turnover of barley flavonoids, then our data report control of accumulation.

The action spectra are in agreement with our earlier work on phytochrome control of barley flavonoids, especially the spectrum for saponarin. The peaks at both 620 and 660 nm seem both to be attributable to phytochrome since different levels of far-red light result in similar degrees of photoreversibility for each of these red wavelengths. The 640 nm depression in the saponarin action spectrum is possibly due to absorbance by protochlorophillide a acting as a shadow pigment. Koski *et al.*[14] determined very accurate action spectra for the photoconversion of protochlorophillide in etiolated corn leaves and they show a major peak at 640 nm which falls off rapidly at 620 and 660 nm.

It is likely that higher energy action spectra for barley flavonoids would saturate the photoconversion of protochlorophyllide and yield action spectra with some differences in the red region. We have not attempted such spectra since our monochrometer system produces relatively low energies of irradiation and complicated photomorphogenetic effects are expected if illumination times are hours instead of minutes.[3] Koski *et al.*[15] determined energy requirements for protochlorophyll conversion and saturation was accomplished in about 60 sec from a 150 lm daylight fluorescent light source. This would be roughly equivalent to about 250 kerg·cm^{-2} of total radiant energy and would require, with our system, approx. 3 hr of illumination at the less energetic red end of the spectrum (Table 1).

The action spectrum of the 3′-*O*-substituted flavonoids is more complex than for saponarin, and has peaks at around 520, 580, 630 and in the 660–690 nm region. The lack of photoreversibility of the 660 nm peak with 6·6 kerg·cm^{-2} of 730 nm light, in contrast to efficient photoreversibility of saponarin in the same experimental plants, is taken as evidence for the involvement of an additional photoreceptor for the 3′-substituted flavonoids. This photoreceptor is possibly in the plastids. Several enzymes of phenolic biosynthetics have been isolated from chloroplasts,[16] flavonoids have been conclusively identified in spinach

[13] WALLACE, J. W., MABRY, T. J. and ALSTON, R. E. (1969) *Phytochemistry* **8**, 95.
[14] KOSKI, V. M., FRENCH, C. S. and SMITH, J. H. C. (1951) *Arch. Biochem. Biophys.* **31**, 1.
[15] KOSKI, V. M. (1950) *Arch. Biochem. Biophys.* **29**, 339.
[16] SATO, M. (1967) *Phytochemistry* **6**, 1363.

chloroplast,[17] and we[18] have found flavonoids, substituted cinnamic acid esters, and PAL activity in chloroplast of several plants including both etioplast and chloroplast of Atlas 46 barley.

It is suggested that the action spectrum of lutonarin and its 3′-methyl ether reflect the requirement for organization of the chloroplast and that the 520, 580 and 620 nm peaks in the action spectra can be explained by the absorption peaks at these wavelengths in protochlorophyll.[14,15] Peaks in the 660 nm region probably reflect phytochrome participation since these are photoreversible in part by 34 kerg·cm^{-2} of far red light.

The differences in action spectra of saponarin and the 3′-substituted flavonoids are consistent with our earlier work[1,7,9] on specific light and organelle development requirements for 3′-substitution.

The degree to which precursors of these flavonoids are controlled by light is under investigation and subsequent papers in this series will report action spectra for PAL and photocontrol of the major substituted cinnamic acids of barley plumules.

EXPERIMENTAL

Plant material and light treatments. Atlas 46 barley (*Hordeum vulgare* L.), a gift of G. Wiebe, USDA, Beltsville, Maryland, was grown in glass beakers on water-saturated vermiculite.[1] All manipulations and harvesting steps from soaking the seeds through homogenizing the tissues in acidified MeOH were carried out in the darkroom under the dim green safelight.[1] A Bausch & Lomb high-intensity grating monochrometer was used for the action spectra with a bandpass of 10 nm at 500 nm. Second order diffraction was removed by Corning glass filters 0–52, 0–53 or 3–69. Light energies were measured with an ISCO spectroradiometer calibrated against a tungsten iodine lamp certified by the Bureau of Standards (Washington, D.C.) and operated in an ISCO model SRC regulated power supply designed for these purposes. The monochrometer assembly emits a cone of light 12 cm in dia. at the working distance of 30 cm. To equalize light falling on each plant, the beakers were rotated at 30 rpm. Exposure times for the 6·6 kerg·cm^{-2} treatments varied from a minimum of 4 min 18 sec at 480 nm to 9 min 6 sec at 780 nm. O_3 generated by the xenon lamp was removed by pulling air across the lamp and through a bed of charcoal.

Extraction and determination of C-glucosylflavones. Techniques and problems of isolating and quantitating barley flavonoids have been described.[1] Saponarin was determined by its absorbance at 333 nm (ϵ 1·75 × 10^4) and the mixture of lutonarin and its 3′-methyl ether at 349 nm (ϵ 1·88 × 10^4). Quantitative values in figures and tables have been corrected for loss during processing.[1]

Acknowledgements—Ths work was supported by the National Science Foundation (Grant GB 16636 to J. W. McC.) and by funds from the Miami University Research Committee and Instrumentation Laboratory. The technical assistance of Mary M. Saunders during the photoreversibility studies is gratefully acknowledged.

[17] OETTMEIER, W. and HEUPEL, A. (1972) *Z. Naturforsch.* **27b,** 177.
[18] SAUNDERS, J. A. and MCCLURE, J. W. (1972) *Am. J. Botany* **59,** 673 (abstract only).