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Ultraviolet Action Spectrum for Anthocyanin Formation in Broom Sorghum First Internodes[1]

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ABSTRACT

An action spectrum for anthocyanin formation in dark-grown broom sorghum (*Sorghum bicolor* Moench, cv Acme Broomcorn and cv Sekisho-kuzairai Fukuyama Broomcorn) seedlings was determined over the wavelength range from 260 to 735 nanometers. The action peaks were at 290, 650, 385, and 480 nanometers in descending order of height. The action of the 290-nanometer peak was not affected by subsequently given far red light, whereas those of the other three action peaks were nullified completely. The nullification of the 385-nanometer peak action by far red light was reversible. When an irradiation at these action peaks was followed by a phytochrome-saturating fluence of red light irradiation, the action of the 290-nanometer peak remained, whereas that of the 385-nanometer peak as well as those of the 650- and 480-nanometer peaks was masked by the action of the second irradiation. These findings suggested that the 290- and 385-nanometer action peaks involved different photoreceptors, the latter being phytochrome. The blue light-absorbing photoreceptor as reported to be a prerequisite for phytochrome action in milo sorghum was not found to exist in the broom sorghums.

The action spectrum deprived of the involvement of phytochrome was determined in the ultraviolet region by irradiating with far red light following monochromatic ultraviolet light. The spectrum had a single intense peak at 290 nanometers and no action at all at wavelengths longer than 350 nanometers.

Detailed studies have been devoted to the action of the visible light in anthocyanin formation, and phytochrome being involved as a photoreceptor has been established in red cabbage (22) and mustard (17) seedlings as well as apple skin (23). Phytochrome has been shown to modulate anthocyanin synthesis in sorghum (4, 5), maize (8), and tomato (7) seedlings and synthesis of flavone glycoside in parsley suspension cultures (26) when a preirradiation of blue (4, 5, 8) or UV (5, 26) light is given.

An intense action of UV on anthocyanin formation in apple skin has been known for many years (1, 16, 19). The effective wavelengths in the UV and blue region have been reported to be 360 and 450 nm (19), and 290 to 312 nm (1) with apple skin, 300 to 338 nm in eggplant and soybean hypocotyls (11), 372 and 438 nm in a tissue culture of *Haplopappus gracillis* (14), and shorter wavelengths than 300 nm in flavone glycoside synthesis in a parsley suspension culture (26). In *Spirodela oligorhiza* (18) the action peaks have been shown to be 300 and 705 nm. These data on the efficiency of UV wavelengths are still fragmentary, and even contradictory in some respects.

An analysis of the findings reported thus far suggests that there seem to be two types of UV action on anthocyanin formation. One is to exert the action independently of phytochrome (14, 15, 18), and the other is a requirement for phytochome-stimulated anthocyanin formation (4, 5, 7, 26). Moreover, UV may exert an action on phytochrome itself through the absorption band in the UV region (3, 20). To clarify the complicated mechanism of UV action, determination of a precise action spectrum and analysis of UV action would be required using sharp monochromatic UV and as short an irradiation period as possible.

In the present study, cultivars of broom sorghum that form anthocyanin in response to UV as well as to R[4] light without any preirradiation were used.

In this study we present an action spectrum from the UV through the visible region using irradiation periods as short as 1 or 2 min, clarify the presence of phytochrome-mediated and nonphytochrome-mediated action of UV, and present the action spectrum for the latter.

MATERIALS AND METHODS

Plant Materials. The cultivars of sorghum (*Sorghum bicolor* Moench) used were Horikawazairai, Kokkaku-2, Sudax, Sekisho-kuzairai Fukuyama Broomcorn, and Acme Broomcorn. Seeds of the first four cultivars were supplied from Tokyo University Experimental Farm, Tanashi, Tokyo, and those of the last cultivar from National Grassland Research Institute, Nishinasuno, Tochigi, Japan.

Seeds were soaked in a Uspulun solution (42 μg/ml aqueous solution of methoxyethylmercury chloride, Merck) for 48 h at 12°C (the low temperature treatment improved germination), and sown, 2 g (dry weight) seeds each, on several layers of moistened Kleenex tissue in 9-cm Petri dishes, and germinated at 23 or 25°C in the dark for 30 to 48 h. Seedlings at a uniform developmental stage were selected and transplanted lengthwise in a row in a Seedling Case, plastic case of 15 (length) × 5 (width) × 10 (height) cm, filled with vermiculite. Plants were grown in the dark at 20 to 25°C for 48 h and irradiated. The temperature was controlled to obtain 5 to 7 cm tall seedlings when irradiated. After irradiation, seedlings were kept for 24 h at 25°C in the dark until harvest except for a time-course study.

In the time-course study, seedlings were transplanted in small pots (6 cm in diameter) filled with 0.7% agar.

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[4] Abbreviations: R, red light; FR, far red light.

Light Sources and Irradiation. For determining action spectra and some other experiments, monochromatic lights were supplied by a large spectrograph equipped with a 6 or 30 kw xenon arc discharge lamp of National Institute for Basic Biology at Okazaki (25). Seedling Cases were placed perpendicularly to the beam direction along or slightly behind the focal plain. Any possible exposure to scattered lights of other wavelengths was avoided by a folding screen placed on the both sides and back of the Case. Seedlings were harvested, being divided into halves on the shorter and longer wavelength sides. A Case spanned 12 nm of wavelengths. Description of the wavelength and fluence rate of each Case was representatively made with the value at the middle of the Case.

In the other experiments, the following lights from 20-w fluorescent tubes with or without filters were used: UV310 was a UV of half width 290 to 338 nm (emission peak 310 nm) obtained from 310 UV tubes without filter; UV310-0, a UV of 300 to 338 nm (310 nm) obtained through a layer of polyvinyl chloride film (UVC-0, Mitsuitoatsu, Ltd.) from the same tubes; UV310-2, a light of wavelength longer than 380 nm emitted from the same tubes. This light was filtered through another kind of film (UVC-2, Mitsuitoatsu, Ltd.). UV310 and UV310-0 contained the light corresponding to UV310-2 besides their main band of emission. Detailed description of these irradiation systems was presented in a previous paper (11). Red-1 was a R of 645 to 667 nm (657 nm) from red tubes (FL20S Re-66, Toshiba, Ltd.) with a red acrylic resin filter (Acrylite 102, Mitsubishi Rayon, Ltd.); Red-2 was obtained from three purple tubes (Fishlux, FL20S BRF, Toshiba, Ltd.) through two layers of red acrylic resin sheet (CF109-3, Mitsubishi Rayon, Ltd.), and its emission spectrum was virtually the same as that of Red-1; and FR760 was a FR of 736 to 807 nm (760 nm) obtained through a layer of acrylic resin sheet (IRP-1, NEC) from a FR tube (FL20S FR-74, Toshiba, Ltd.).

Plants were irradiated unilaterally unless otherwise stated. All handling of plants except irradiation was done under dim green safelight.

Determination of Anthocyanin Content. Since anthocyanin was formed solely in the irradiated part of first internodes, which was equal in length in every treatment, the irradiated part was excised and soaked in 1% HCl-methanol overnight at 5°C in the dark. The volume of HCl-methanol used was 0.2 ml × the number of internode segments. The methanol extracts were decanted and their absorption spectra were recorded from 740 to 400 nm, and A at 528 or 530 nm was read from the charts. In fluence-response curves for determining action spectra, A at 528 nm of the skirt of UV absorption was subtracted to secure the precision of small amount of anthocyanin.

Paper Chromatography. HCl-methanol extracts of anthocyanin were chromatographed on Toyo filter paper #51 and developed ascendingly with 1-butanol:2 M HCl (1:1, v/v, upper layer), HCl:acetic acid:H_2O (3:30:10, v/v), and HCl:HCO_2H:H_2O (2:5:3, v/v) as solvent according to Harborne (10). To minimize the possibility of hydrolysis of anthocyanin, fresh extracts were spotted without concentration.

Table I. *Different Responses to UV and R Light in Anthocyanin Formation of Some Sorghum Cultivars*

Seedlings were irradiated for 90 min vertically with Red-1 (290 μw cm^{-2}), UV310-2 (65 μw cm^{-2}), UV310-0 (120 μw cm^{-2}), and UV310 (200 μw cm^{-2}).

Cultivars	Dark	Red-1	UV310-2	UV310-0	UV310
			A_{528}		
Horikawazairai	0.005	0.007	0.050	0.613	0.241
Kokkaku-2	0.001	0.001	0.002	0.580	0.246
Sudax	0.001	0.010	0.141	0.655	0.284
Sekishokuzairai Fukuyama Broomcorn	0.002	0.541	1.760	3.014	0.868

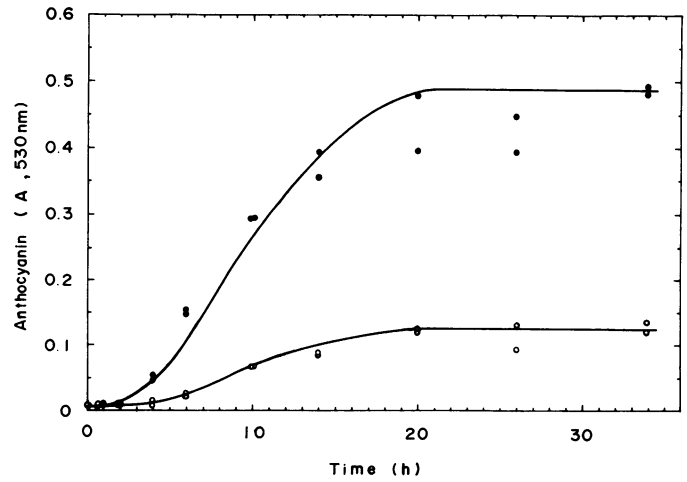

FIG. 1. Time course of anthocyanin formation in sorghum first internodes. Seedlings were irradiated vertically with UV310-0 of 190 μw cm^{-2} (●) and Red-2 of 280 μw cm^{-2} (○) for 10 min, then incubated in dark for various periods of time until harvest. Abscissa indicates periods of time after the onset of irradiation (cv Acme Broomcorn).

RESULTS

Different Responses to UV and R of Sorghum Cultivars. First, the responsiveness of some cultivars of sorghum to UV and R was tested, and it was found that cultivars Harikawazairai, Kokkaku-2, and Sudax formed anthocyanin in response only to UV but not to R, and cv Sekishokuzairai Fukuyama Broomcorn responded to R as well, and was sensitive to UV (Table I). It seems that there are two types of sorghum, R-responsive and nonresponsive, whereas both types respond to UV. In further experiments in this paper, cv Broomcorn was exclusively used.

Time Course of Anthocyanin Formation. When either 10 min of 310-0 or Red-2 was given, anthocyanin formation began after a 2-h latent period and reached a plateau after 20 h (Fig. 1). This pattern of pigment accumulation was not different with the wavelengths of light given. Therefore, the amount of the pigment 24 h after the irradiation was determined as a measure of the average rate of pigment formation.

Fluence-Response Curves. When the photon fluence of irradiation and the amount of the pigment formed were plotted on logarithmic scales, a linear curve was obtained before reaching a plateau. Some representative fluence-response curves are shown in Figure 2. The slope of the curves varied with the wavelengths of light as reported previously (2, 12, 24), and was steep in the neighborhood of 263 and 333 nm. The plateaus of the curves were also different with the wavelengths of light (data not shown).

Action Spectrum. Since the fluence-response curves are not parallel, the shape of the resulting action spectrum varies naturally. Therefore, the anthocyanin level for reading the photon fluence required was selected as low as possible. The action spectrum in Figure 3 (solid line) was obtained by plotting the reciprocals of the photon fluences required for anthocyanin formation of 0.02. The sensitivity of plants to monochromatic irradiation varied from experiment to experiment, but numerous experiments gave virtually the same shape of action spectrum. In Figure 3, the data of two typical experiments were used. The reciprocals of fluences obtained from one experiment fitted those from the other experiment when multiplied by a factor 2.0. This action spectrum had the peaks at 290, 650, 385, and 480 nm with the height in this descending order.

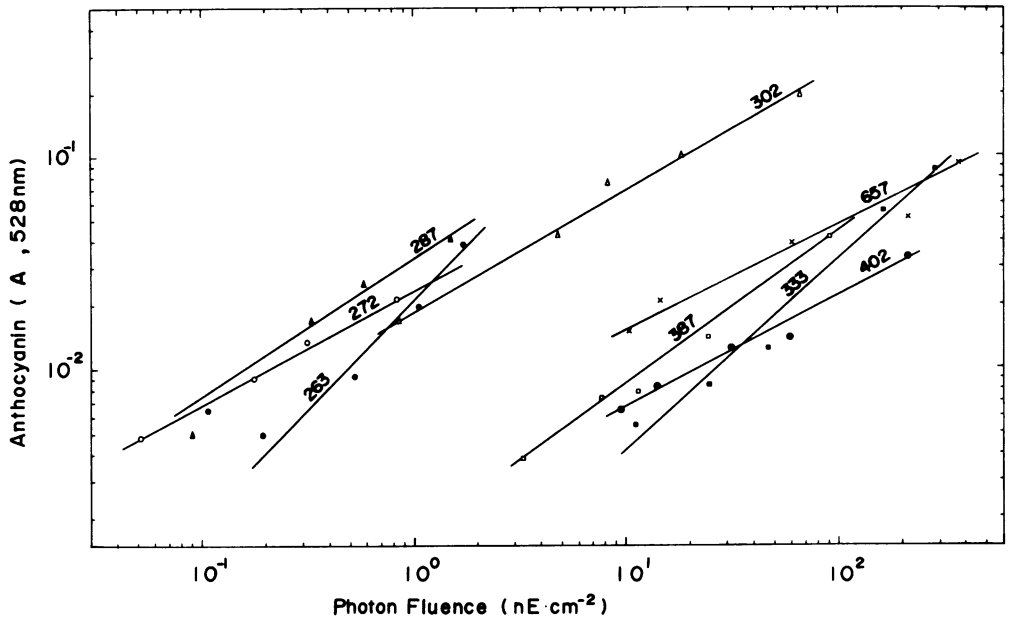

FIG. 2. Representative fluence-response curves for anthocyanin formation with monochromatic lights. Irradiation period was 1 or 2 min. Symbols used are: ●, 263 nm; ○, 272 nm; ▲, 287 nm; △, 302 nm; ■, 333 nm; □, 387 nm; , 402 nm; ×, 657 nm. Absorbance of nonirradiated control was 0.0004 (cv Acme Broomcorn).

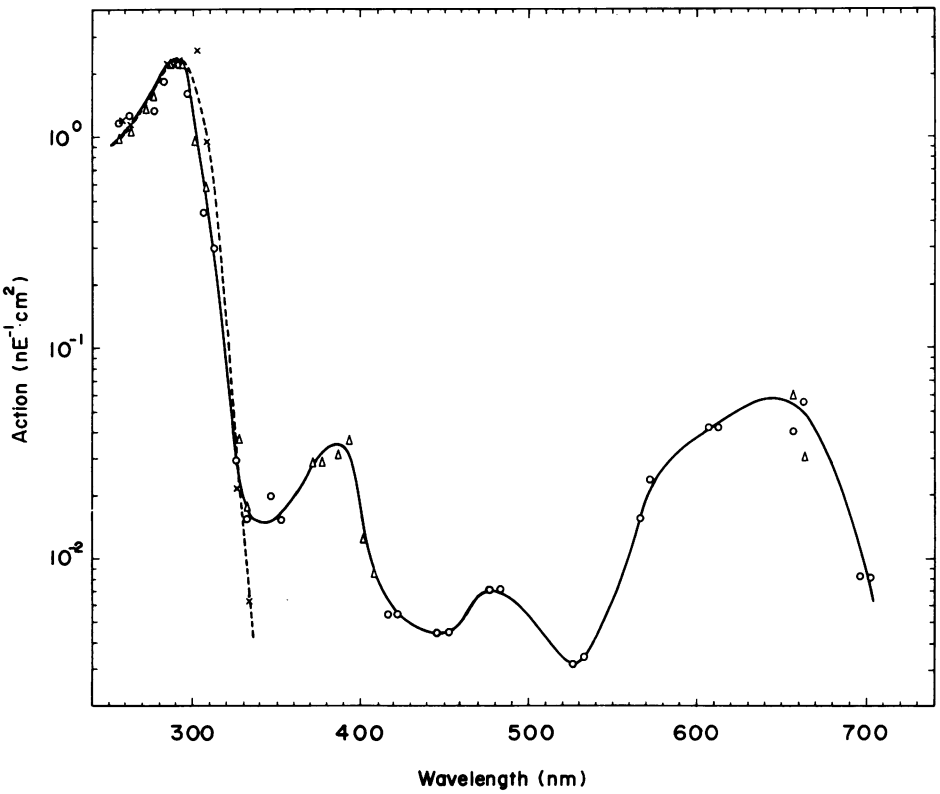

FIG. 3. Action spectrum for anthocyanin formation (——). Action is expressed by reciprocals of photon fluences required for anthocyanin formation of A 0.02 were plotted against wavelengths. ○ and △ represent, respectively, data obtained from two typical experiments. Data of ○ were multiplied by a factor 2.0 to normalize to value at 290 nm of other experiment. - - - Shows an action spectrum deprived of phytochrome involvement. In this experiment, monochromatic light irradiations were immediately followed by a phytochrome-saturating fluence of FR irradiation (260 μw cm^{-2}, 5 min, FR760).

Effect of FR on the Action of UV. In order to examine whether the action spectrum involves the mediation of phytochrome, an irradiation with a monochromatic light at various wavelengths was given for 2 or 10 min, then immediately (within 1 min) irradiation with FR was done for 1 and 3 min. FR (735-nm light) given solely for up to 13 min did not cause formation of anthocyanin (Fig. 4, first figure). The action of 660-, 610-, and 480-nm lights were completely nullified by FR. In the case of the 10-min irradiation with 390-nm light, the reversing effect of FR was weaker than in the case of 660- and 610-nm lights, but sufficiently

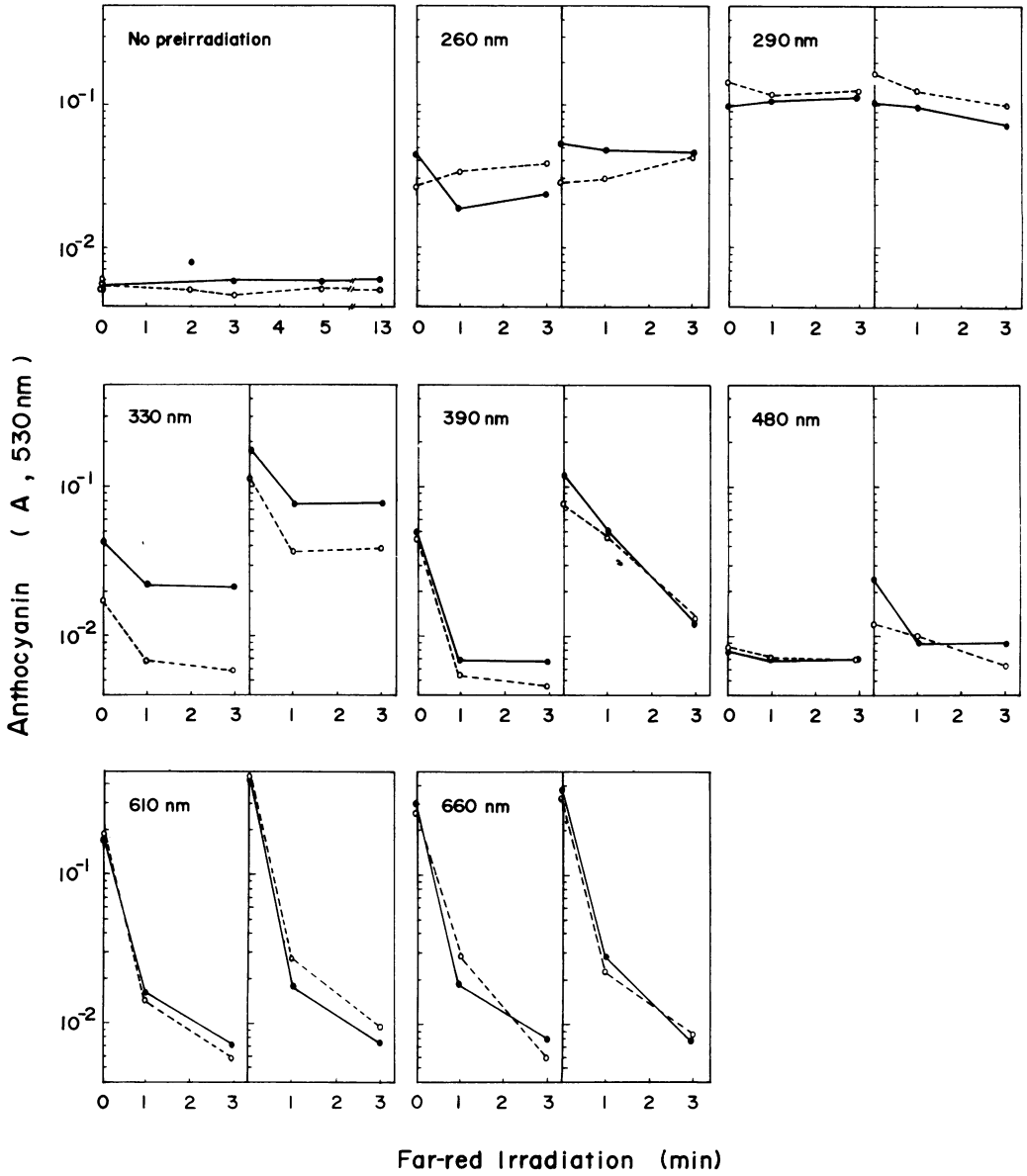

FIG. 4. Effect of FR on actions of monochromatic lights. Seedlings were irradiated first with a monochromatic light for 2 min (left figure) or 10 min (right figure), and then with FR (735 nm) for periods of time indicated on abscissa. Wavelength shown in each figure is middle wavelength of light given, and —— and - - - represent, respectively, 3 nm shorter and longer wavelengths than indicated (see "Materials and Methods"). Fluence rates measured at middle wavelengths were 9.7 μw cm^{-2} (260 nm), 34 (290 nm), 150 (330 nm), 340 (390 nm), 560 (480 nm), 460 (610 nm), 270 (660 nm), and 285 (735 nm) (cv Sekishokuzairai Fukuyama Broomcorn).

large fluence of FR caused a full nullification (see below). In contrast, the action of 330-nm light was only partially suppressed by a 1-min exposure to FR, and the suppression was not intensified by a prolonged (3 min) exposure (Fig. 5), indicating that the partial suppression was not due to an insufficient FR fluence. The actions of 290- and 260-nm lights were not affected by any fluence of FR. On the shorter-wavelength side of 260 nm, a slight suppression was observed when the irradiation was 2 min as well as 10 min, but this may presumably be ascribed to some stray light.[5]

The action of 390-nm light was repeatedly reversed by FR just like that of 660-nm light (Table II). When alternate exposures to 390-nm UV and FR were repeated, the efficiency of the UV increased significantly, whereas the reversion by FR was repeatedly complete. The increase in the efficiency of the last actinic irradiation was also the case with 660 nm. Grill and Vince (9) reported that with turnip hypocotyls a FR irradiation preceding a R irradiation increased the efficiency of the latter, however, this was not the case with our plant materials. Irradiations with FR followed by 390-nm light gave no difference from the effect of a sole irradiation with 390-nm light (data not shown).

Effect of a Saturating Fluence of R on the Action of UV. Another experiment was performed to examine an involvement of phytochrome. Irradiation with monochromatic lights at various fluences was given first. This was followed by a phytochrome-saturating fluence of R (Red-1, 360 μw cm^{-2} for 10 min, see Fig. 5, last figure). Wavelengths of 350 to 735 nm caused no additional anthocyanin production over that caused by R. Wavelengths from 260 to 330 nm caused additional anthocyanin formation when given before R.

[5] In the rest of the experiments except for this and the R saturation experiments, installation of a filter to cut off visible light removed this possibility.

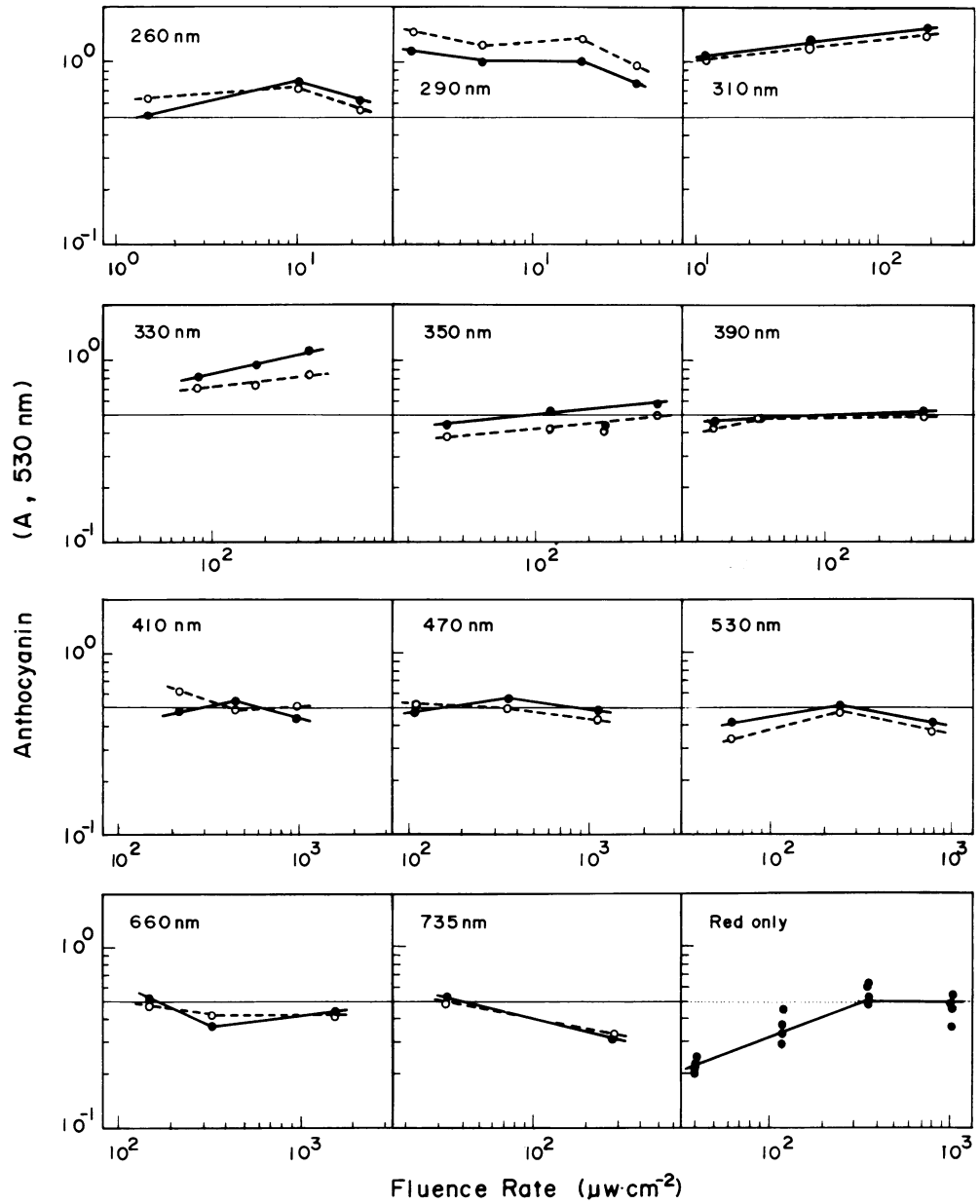

FIG. 5. Effect of R saturation on action of monochromatic lights. Plants were irradiated first with a monochromatic light of fluence rate indicated on abscissa for 10 min and then immediately with a saturating fluence of R (360 μw cm^{-2}, 10 min, Red-1). Wavelength shown in each figure is middle wavelength of light given, and —— and - - - represent, respectively, 3 nm shorter and longer wavelengths than indicated (see "Materials and Methods"). Absorbance of nonirradiated control was 0.0066 (cv Sekishokuzairai Fukuyama Broomcorn).

These findings, *i.e.* those of the FR nullification and of the R saturation, indicate that the action of 385-nm peak as well as of the 480- and 650-nm peaks is mediated by phytochrome, whereas the action of the 290-nm peak does not involve phytochrome but is mediated by another photoreceptor.

UV Action Spectrum Deprived of Phytochrome Involvement. To present the spectral property of the unidentified photoreceptor, an action spectrum was determined in the following way: after irradiation of monochromatic lights from 260 through 400 nm at various fluences, sufficient fluence of FR irradiation (FR760, 260 μw cm^{-2} for 5 min) was given, and the resulting responses were plotted against the fluences of the UV. From these fluence-response curves (not shown) the photon fluences required for a definite amount of response (A, 0.02) were read and their reciprocals were plotted against wavelengths (Fig. 3, dashed line). This experiment was done at the same time as that which gave data points shown as triangles in Figure 3 and therefore, no correction of the data was required.

Identity of Anthocyanin Formed by UV and R. Paper chromatography of HCl-methanol extracts from sorghum seedlings irradiated with UV (UV310-0), R (Red-2), or combination of the two gave only one anthocyanin spot with all the solvent systems used except HCl-acetic acid-H_2O. When the samples from these sources were stored in a refrigerator for a long time, an accompanying small pink spot appeared just above (R_F 0.9) the main spot of about R_F0.8 on development with HCl-acetic acid-H_2O, but a freshly extracted sample did not show the small spot. These results indicate that both 290-nm UV and R induced the formation of an identical anthocyanin.

DISCUSSION

The action spectrum presented in this paper (Fig. 3) has action peaks at 260, 650, 385, and 480 nm, their height being in this

Table II. *Reversible Action of 390-nm Light/FR and 660-nm Light/FR in Anthocyanin Formation*

All irradiations were done with the spectrograph equipped with a 30-kw xenon lamp. In A and B, respectively, 735- and 750-nm lights were used for FR irradiation. The fluence rate was 390, 320, 286, and 228 $\mu w\ cm^{-2}$ for 390-, 660-, 735-, and 750-nm lights; the irradiation period, 5 min each. The actions of the last irradiations with 390- or 660-nm light are the difference between (3) and (1), (5) and (4), (7) and (6), (9) and (1), (11) and (10), (13) and (12).

Irradiations	Anthocyanin			
	A	B	Mean	Action of the last actinic irradiation
			A_{528}	
(1) Dark control			0.003	
(2) FR	0.006	0.005	0.006	
(3) 390	0.249	0.140	0.195	0.192
(4) 390-FR	0.015	0.014	0.015	
(5) 390-FR-390	0.258	0.270	0.264	0.249
(6) 390-FR-390-FR	0.022	0.019	0.021	
(7) 390-FR-390-FR-390	0.323	0.355	0.339	0.318
(8) 390-FR-390-FR-390-FR	0.020	0.017	0.019	
(9) 660	0.315	0.398	0.357	0.354
(10) 660-FR	0.012	0.024	0.018	
(11) 660-FR-660	0.406	0.453	0.430	0.417
(12) 660-FR-660-FR	0.013	0.037	0.025	
(13) 660-FR-660-FR-660	0.488	0.494	0.491	0.466
(14) 660-FR-660-FR-660-FR	0.018	0.037	0.028	

descending order. The peak at 290 nm is close to that at 300 nm of the action spectrum for *Spirodela* (18), but the peaks at 650 and 385 nm are absent in *Spirodela*. The latter two peaks agree well with the absorption spectrum of isolated phytochrome (Pr form) and also with the action spectrum for an *in vitro* conversion from Pr to Pfr (3, 20). The weak action in the blue region and complete absence of action in the FR region (see also Fig. 4) noticed in our action spectrum are also similar to the isolated phytochrome, and different from the action spectra obtained with turnip (9, 22) and red cabbage hypocotyls (22).

The action at the 290-nm peak was not affected by subsequently given FR (Fig. 4), and was not masked by the action of a phytochrome-saturating fluence of R (Fig. 5). These findings indicate that this action peak involves a photoreceptor other than phytochrome. The action spectrum in which the unknown photoreceptor is solely involved was presented (Fig. 3, dashed line). Phytochrome has an absorption at 280 nm due to its protein moiety and energy absorbed has been shown to be transferred for conversion of the chromophore (20), but the above stated findings exclude the possibility of phytochrome being involved.

Ng *et al.* (18) and Lindoo and Caldwell (15) with anthocyanin formation in *Spirodela oligorhiza* fronds and *Rumex patientia* leaf discs, respectively, have reported that the action of this wavelength region does not involve phytochrome. Drumm-Herrel and Mohr (6) have reported that a UV-B photoreceptor obviously different from the blue/UV-A photoreceptor (cryptochrome) is involved in anthocyanin formation of milo sorghum first internodes. The 290-nm photoreceptor described in the present paper may be identical with photoreceptors that have been claimed by these workers.

The nature of the 385-nm peak is in contrast to that of the 290-nm peak. Its action was completely reversed by FR (Fig. 4) and the FR reversion was repeatable (Table II). When a saturating fluence of R was given and the remaining Pr which escaped the conversion to Pfr by 390-nm light (representing the 385-nm peak) was converted to Pfr, the effect of the preceding 390-nm irradiation was masked. These findings, together with the peak wavelength, indicate that the action peak involves phytochrome.

The action of 390-nm light was slightly less susceptible to the FR reversal than that of 610- or 660-nm light which represents the 650-nm peak (Fig. 4). This phenomenon may raise questions that the Pfr due to 390-nm light might not be completely identical with the Pfr due to 610- to 660-nm light, or that 390-nm light might produce another FR-absorbing pigment, which exerts a screening effect on reversion of Pfr to Pr, or that the 390-nm light may stabilize the Pfr which is produced by this light (refer to Ref. 21 for stabilization effect of UV). These questions are retained unanswered in the present study..

R was effective without preirradiation with blue light. The action peak at 480 nm is extremely low, and the action was masked by the effect of phytochrome-saturating R. These features of photoresponse do not correspond to the blue light effect described in milo sorghum by Downs and Siegelman (4).

Thus, it may be concluded that in the UV-induced anthocyanin formation, two photoreceptors are involved: the one having an absorption peak at 290 nm and phytochrome. The blue or UV-A-absorbing photoreceptor described as a prerequisite for phytochrome action (4, 5, 7) is absent in the broom sorghum cultivars used in this study. Some papers have described that the actions of UV in inducing the formation of anthocyanin (5, 7) and flavone glycoside (26, 27), and epinasty of wheat first leaf (21) were reduced by subsequently given FR. The UV used were of a broad band and the effects of FR, only partial. The view that two photoreceptors are involved in the UV action, one of which is susceptible and the other resistant to FR, may provide an explanation to these reported effects of FR. Recently Ohtani and Kumagai (13) have reported that in the floral induction of *Lemna paucicostata* only one action peak at 380 nm in the near UV region is assigned to phytochrome.

The photoresponse of the broom sorghum used in the present study is different from milo sorghum used by other workers (4–6), and resembles that of apple skin in that R is effective without a preirradiation of blue or UV-A (23). It can be seen also in Table I that there are two types of sorghums different in photoresponse for anthocyanin formation. But the presence of some synergistic action of UV-B and UV-A as reported with milo sorghum is not negated with the broom sorghum in the present study.

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