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PHENOLIC METABOLISM, GROWTH, AND UV–B TOLERANCE IN PHENYLALANINE AMMONIA-LYASE-INHIBITED RED CABBAGE SEEDLINGS

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Brassica oleraceae*; cruciferae; red cabbage; sinapoyl esters; anthocyanin; 2-aminoindan-2-phosphonic acid (AIP); phenylalanine ammonia-lyase (PAL); UV–B; photosynthetic photosystem II (PSII); hypocotyl elongation.

Abstract—Red cabbage seedlings were grown with or without the phenylalanine ammonia-lyase (PAL) inhibitor, 2-amino-indan-2-phosphonic acid (AIP), at concentrations ranging from 0.5 to 50 μM. The I_{50} for anthocyanin accumulation was <0.1 μM, with $>99\%$ inhibition at 10 μM, but levels of sinapic acid esters were essentially unchanged by AIP. When grown with 50 μM AIP, fresh and dry weights were increased slightly over controls, total chlorophylls were unchanged, and microscopic examination revealed no apparent effect of AIP on plant architecture. This suggests no toxic effect of AIP in red cabbage seedlings at levels highly effective in inhibiting PAL. At 50 μM AIP, the cotyledon area was slightly increased but hypocotyls were significantly reduced in length, perhaps the result of enhanced blue light sensitivity in the absence of anthocyanins. Negative phloroglucinol reactions in AIP-grown plants are consistent with AIP inhibition of lignification. Plants grown with 50 μM AIP were about twice as sensitive as control plant to UV–B damage of photosystem II, suggesting that phenylpropanoids carried over from the seed, as well as flavonoids, serve as UV screens in young red cabbage seedlings.

INTRODUCTION

Stratospheric ozone is largely responsible for attenuation of solar UV radiation between 280 and 315 nm (UV–B) before it reaches the earth's surface. There is a strong likelihood that stratospheric ozone depletion from halogens of anthropogenic [1] and [2] natural origins will continue and result in significant increases in the proportion of UV–B in terrestrial solar spectral distribution. Since UV–B radiation can affect the physiology and development, and thus biomass and seed yield, of plants [3], understanding plant responses to UV–B may be directly applicable to problems of growing crop plants and potentially important to long-term changes in ecosystem composition [4, 5].

Flavonoids and hydroxycinnamic acids exhibit high UV absorptivities, are expressed to a great extent in the epidermis, and are virtually ubiquitous in land plants [6]. This has led to a long standing hypothesis that one of their primary adaptive advantages is absorption of harmful UV–B and protection of underlying photosynthetic tissues [7].

Most evidence for a role of flavonoids and other phenolics as UV screens is correlative [8]. Generally, when plants are grown under enhanced UV–B, soluble phenolics and photosynthetic tolerance to UV increase simultaneously [3, 4, 9]. However, other presumably adaptive responses are also elicited by UV radiation. These include increased leaf thickness [10], DNA photolyase expression [11], increased carotenoid content [12], and increased rates of PS II protein synthesis [13]. Increases in leaf thickness are thought to protect photosynthetic tissues by increasing the pathlength which light must take to reach potential damage target sites [10]. Cyclobutane dimers formed by UV radiation are repaired by photolyases which are clearly not produced in response to damage but are under photocontrol [11, 14]. Carotenoids scavenge free radicals formed by UV radiation [15] and may provide UV screening within chloroplasts.

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Table 1. Effect of growth with 50 μM AIP on selected morphological characters, and weights, of 7-day-old red cabbage seedlings

	Control		50 μM AIP		
	Mean	s.e.	Mean	s.e.	*p*
Cotyledon Area (cm^2)	0.150	0.0036	0.159	0.0037	0.062
Midrib Length (cm)	0.271	0.0031	0.279	0.0035	0.10
Perimeter (cm)	1.607	0.0205	1.663	0.0199	0.05
Petiole Length (cm)	0.150	0.0038	0.114	0.0032	<0.001
Hypocotyl Length (cm)	0.614	0.130	0.562	0.0792	<0.001
Fresh Weight (mg)	15.157	0.0135	16.142	0.0082	0.029
Dry Weight (mg)	1.806	0.0770	2.072	0.0753	0.03

Morphological values are from 93 plants, dry weights from four samples of 100 plants. Results of Student's *t*-test are shown.

For such reasons it has not been possible to factor out, from a wide range of biochemical and structural responses to UV, the importance of secondary phenolics as UV screens since they themselves are induced or quantitatively regulated by UV.

The primary goal of this work was to directly examine the role of secondary phenols in conferring UV-B tolerance in red cabbage. By growing the plants in the presence of 2-aminoindan-2-phosphonic acid (AIP) we were able to produce plants with reduced secondary phenolic metabolism. AIP is a recently developed [16] highly-specific inhibitor of L-phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5.) which initiates flavonoid and hydroxycinnamic acid biosynthesis.

Red cabbage seedlings develop rapidly and produce relatively large amounts of cyanidin glycosides exclusively by *de novo* synthesis through PAL [17]. Since anthocyanins are easily quantified by simple spectrophotometric techniques this allowed rapid characterization of the extent of *in vivo* PAL inhibition. Although hydroxycinnamic acid metabolism has not been fully characterized in the red cabbage system, some aspects of its hydroxycinnamate metabolism [18, 19] have been shown to be similar to that of radish whose soluble sinapoyl chemistry and biochemistry has been well characterized [20, 21, 22].

RESULTS

Growth and development

Effects of 50 μM AIP on cotyledon areas, fresh weight, dry weight, and length of hypocotyls and petioles, are shown in Table 1. AIP caused an increase in cotyledon area of about 5% and slightly increased both fresh and dry weight. Hypocotyls and petioles of AIP-grown plants were consistently significantly shorter than those of controls while midrib length of the expanding cotyledon was relatively unchanged (Table 1).

Microscopy

As seen in Fig. 1, bright field microscopy of cotyledon cross sections showed anthocyanins of control plants mostly in mesophyll cells immediately adjacent to the epidermis and in scattered epidermal cells. In plants grown on 50 μM AIP anthocyanins were not detected and the anatomy of the cotyledon appeared unchanged. The vascular tissue appeared more dense in control plants than in plants grown with AIP but close examination revealed that the xylem elements of AIP-grown plants were well formed and had conspicuous annular tracheary thickenings.

When cotyledon cross sections were fumed with ammonia and viewed under UV (not shown) both control and AIP-grown plants exhibited yellow green fluorescence typical of sinapic acid esters, as might be expected for cotyledons recently expanded from seeds rich in these compounds [22]. When ammonia-fumed cotyledons were examined by UV epifluorescence, anthocyanins appeared dark and epidermal cells fluoresced blue in both control and AIP-treated plants (Fig. 1). Xylem cell walls exhibited intense blue fluorescence associated with wall-bound phenolics in both treated and untreated plants. When cotyledon or hypocotyl sections were treated with phloroglucinol to detect lignin, a positive color reaction was observed only in cotyledons and hypocotyls of control plants. This is seen best in cross sections of hypocotyls (Fig. 1). Scanning electron microscopy of freeze-fractured cotyledons (not shown) revealed no apparent difference in tissue or cellular structure attributable to AIP.

Total soluble phenolics

UV–vis spectra of 50% MeOH extracts of hypocotyls and cotyledons from plants grown on water or on 50 μM AIP are shown in Fig. 2. Hypocotyls and cotyledons in both treatment groups had absorbance maxima at 330 nm typical of soluble sinapoyl esters, and hypocotyls from control plant, displayed an additional peak around 280 nm which might derive

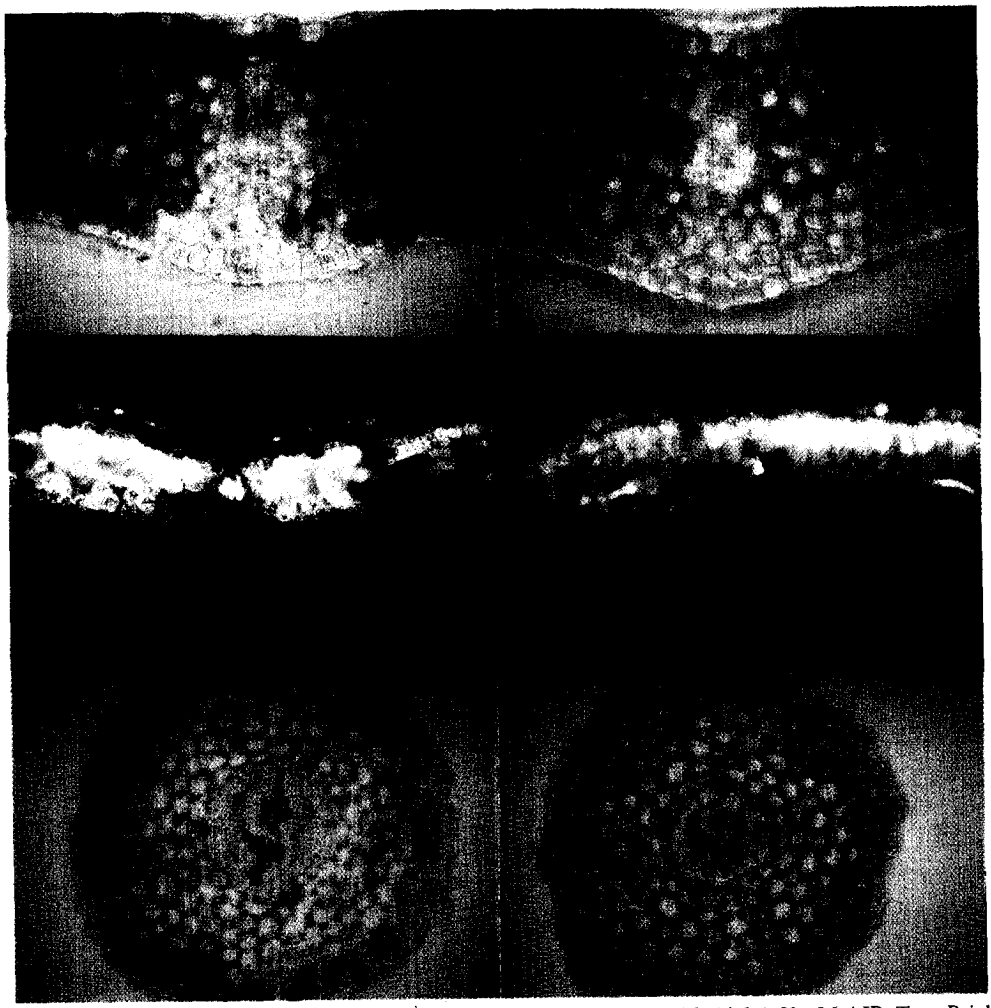

Fig. 1. Optical microscopy of 7 day old red cabbage grown without (left) and with (right) 50 μM AIP. Top: Bright fieled microscopy of transversely sectioned red cabbage cotyledons. Middle: UV-epifluorescence of ammonia-fumed cotyledon cross sections. Bottom: Phloroglucinol reaction in cross-sectioned hypocotyls.

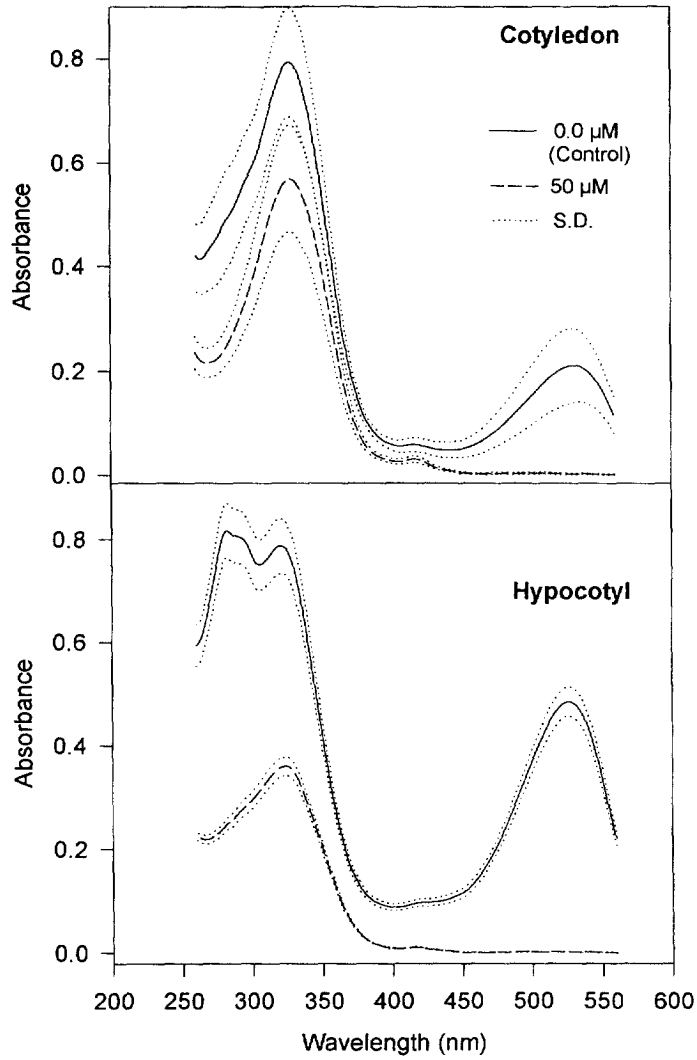

Fig. 2. Average absorbance (±s.d.) of cotyledon and hypocotyl extracts between 560 and 260 nm from 7 day old red cabbage seedlings grown with or without 50 μM AIP. $n = 30$ samples of 4 cotyledons and 7 samples of 8 hypocotyls.

from Band I absorbance of acylated cyanidins of red cabbage [17] or from soluble phenylpropanoid esters. The isolated peak at 525 nm is also characteristic for cyanidin derivatives found in red cabbage [17] and is absent in plants grown on 50 μM AIP solution. In cotyledons the reduction in absorbance at 330 nm in AIP treated plants is almost exactly the same as the absolute reduction at 525 nm, about 0.2 A.U. This relationship was observed in plants grown at all AIP levels investigated.

The effect of various levels of AIP on absorbance at 330 and 525 nm of extracts from both hypocotyls and cotyledons is shown in Fig. 3. For clarity, data from plants grown on 10 and 20 μM AIP were omitted for presentation to allow expansion of the ordinates. Fifty percent reduction in anthocyanin accumulation (I_{50}) was achieved in both hypocotyls and cotyledons of plants grown on 0.1 μM AIP solutions in this system. A reduction of ca 80% and 90% was elicited by treatment with 1.0 μM AIP in hypocotyls and cotyledons, respectively.

Absorbance at 330 nm of 50% MeOH extracts was variable from seedling to seedling and not completely eliminated even at the highest AIP levels. Similar absorbance at this wavelength was found in extracts from seeds (from which the seedcoat had been removed) or in plants which had been grown on 50 μM AIP (not shown). We conclude that this absorbance was due to constitutive, preformed, sinapoyl choline and other aromatic choline esters characteristic of seeds from all cruciferous species [19] and that variation was attributable to seed-to-seed variation in stored soluble sinapoyl complexes.

HPLC

Sinapoyl choline, sinapoyl glucose, and two putative sinapoyl esters which we call Unk1 and Unk2,

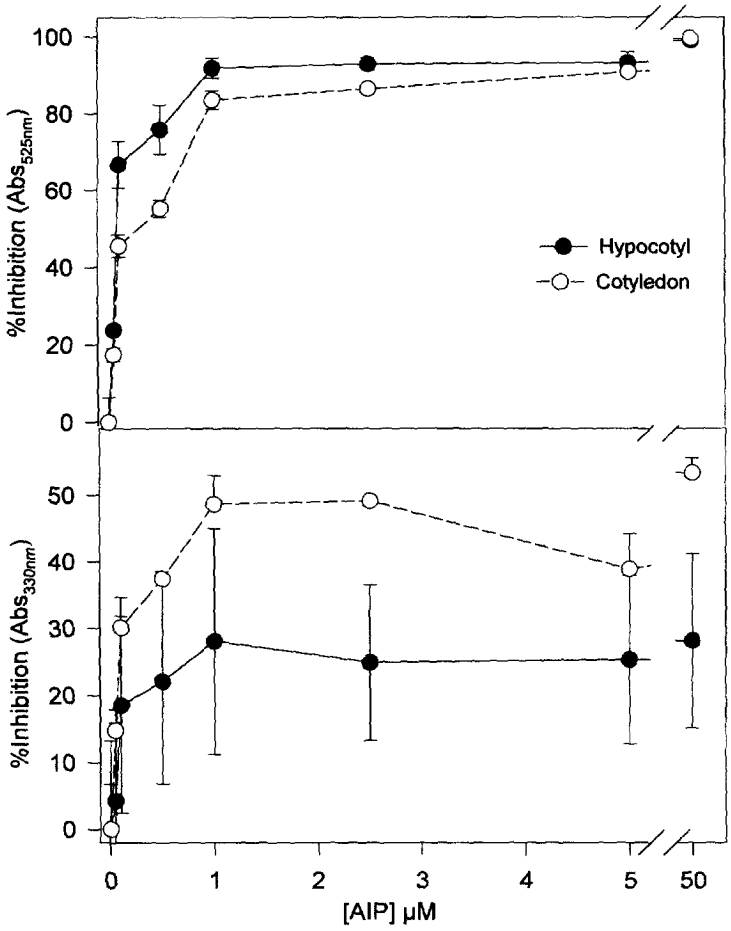

Fig. 3. Reduction of absorbance at 525 nm (inhibition of anthocyanin accumulation, top) and reduction of absorbance at 330 nm (bottom) vs inhibitor concentration of extracts (10 ml) from 7 day old red cabbage seedlings grown at various concentrations of AIP. Bars are ±s.d.

were the major soluble phenolics found in 7 day old cabbage plants. When these compounds were assayed over a 12 day period, growth with AIP had little effect on rates of change of any of these compounds (Fig. 4).

Photosynthetic fluorescence kinetics

As shown in Fig. 5, plants grown with 50 μM AIP exhibited significantly increased sensitivity to UV-B-decreased PSII potential photochemical efficiency. This can be correlated to Fv/Fm, the ratio of variable chlorophyll fluorescence to maximal chlorophyll fluorescence in dark-adapted leaves [23]. Fluorescence induction curves provide information about the activity of PSII, its interaction with PS I and CO_2 fixation, and Fv/Fm is a useful and widely-accepted indicator of UV stress and UV-penetration into photosynthetic tissues [24]. Fv/Fm decreased in response to UV-B treatments in controls as well, but those grown with AIP were more than twice as sensitive (by linear regression, control plants: $d(Fv/Fm)/dt = -2.13e\text{-}4$ ($SE = 8.07e\text{-}5$), treated plants: $d(Fv/Fm)/dt = -5.74e\text{-}4$ ($SE = 9.07e\text{-}5$).

DISCUSSION

Anthocyanin production in red cabbage seedlings proceeds exclusively by *de novo* synthesis via PAL [17]. Based on this and results from the present work, AIP inhibited PAL activity in a dose dependent manner (Fig. 3). The I_{50} of anthocyanin synthesis determined in the present work (0.05 to 0.1 μM) was an order of magnitude less than reported for buckwheat hypocotyls [16] which have higher specific PAL activity than most dicot seedlings [6].

When phloroglucinol was applied to sectioned hypocotyls and cotyledons of AIP-grown plants, no color reaction was observed (Fig. 1) suggesting that lignin synthesis was blocked. However, when freshly sectioned tissues were fumed with ammonia and viewed by UV-epifluorescence microscopy (Fig. 1) the veins exhibited intense fluorescence consistent with either lignification or some other class of phenolics associated with the cell walls [25]. The fluorescence technique may be more sensitive in detecting trace lignin-like depositions than the histochemical techniques used. Alternatively, this fluorescence may

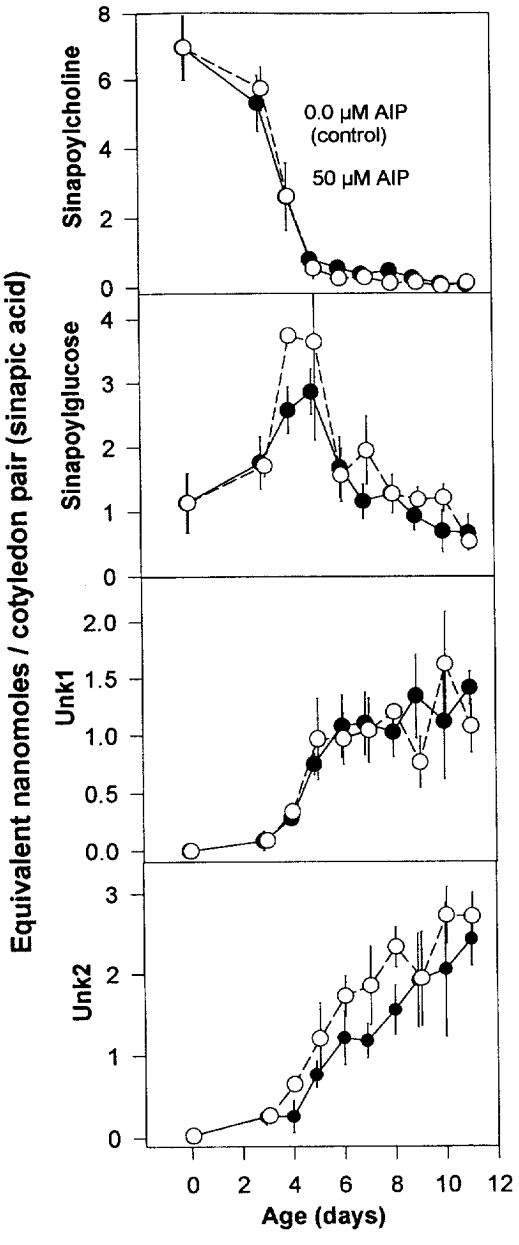

Fig. 4. Developmental changes in soluble sinapoyl ester content in red cabbage cotyledons from plants grown with (open symbols) and without (filled symbols) 50 μM AIP. Mean values $\pm$ s.d. ($n = 3$) are shown.

derive from wall-bound phenolic esters [26]. Another possibility, earlier suggested by Strack [27] and Amrhein *et al.* [28], is that sinapoyl esters may be translocated to developing xylem vessel cell walls during development of cruciferous seedlings.

Since AIP is a competitive inhibitor, PAL activity cannot be completely eliminated. Although bulk PAL activity was apparently almost completely inhibited in plants grown on 50 μM AIP as evidenced by its effect on blocking anthocyanins (Fig. 2), HPLC disclosed barely detectable traces of anthocyanin in plants grown with 50 μM AIP (data not shown).

Plants grown on 50 μM AIP had slightly higher shoot fresh and dry weights than did controls (Table 1). This might result from a lack of anthocyanin screening pigment in treated plants which allowed increased levels of photosynthetically active radiation to reach the mesophyll of developing plants [29]. Regardless of the mechanism, these results suggest that AIP was not toxic to developing red cabbage seedlings at levels applied.

A slight increase in average cotyledon area was observed in plants grown with 50 μM AIP (Table 1) but this did not approach statistically significant levels until rather large numbers of cotyledons were measured. This seems consistent with a slight increase in the blue component of photosynthetically apparent radiation (PAR) being delivered to the photosynthetically active tissues within the cotyledons. In contrast, petiole and hypocotyl lengths were significantly reduced by AIP (Table 1). That is, a stem-specific decrease in organ expansion was observed.

Phenolic cross linking is known to reduce cell wall extensibility [26, 30] and cell wall extensibility has been proposed as a determining factor in final cell and organ size [31].

Alteration of stem expansion, but not cotyledon expansion, by AIP may relate to blue and near UV photomorphogenic responses associated with reduced anthocyanin pigmentation. Tomato, radish, arabidopsis, cucumber and cabbage all exhibit reduced hypocotyl expansion rates when exposed to blue or UV radiation [32, 33 and references therein]. When red and white cabbage were exposed to blue and UV light, white cabbage hypocotyl expansion was inhibited to a greater extent than red cabbage [34]. Likewise, AIP treated plants, ("white" cabbages), exhibited shorter hypocotyls than control plants ("red" cabbages). The cool white sources used in these experiments deliver appreciable UV–A and blue light. It seems plausible that inhibition of anthocyanins enhanced the level of blue light within the hypocotyls, resulting in stem shortening.

It should be noted that prior to the current investigation, Arabidopsis mutants were the only other system in which blue/UV photomorphogenic hypocotyl responses and phenolic expression had been successfully manipulated [32, 33].

In 7-day old cabbage plants two groups of phenolic compounds are normally found in cotyledon extracts, constitutive compounds carried over from the seed and those formed by *de novo* synthesis through PAL. Sinapoyl choline, sinapoyl glucose and the putative sinapoyl esters Unk1 and Unk2, are relatively unaffected by AIP (Fig. 4) *vis à vis* cyanidin derivatives. Anthocyanins comprise a major portion of UV absorbing compounds (Fig. 2). By HPLC these anthocyanins elicited nearly identical detector response at both 330 and 525 nm (not shown) as previously

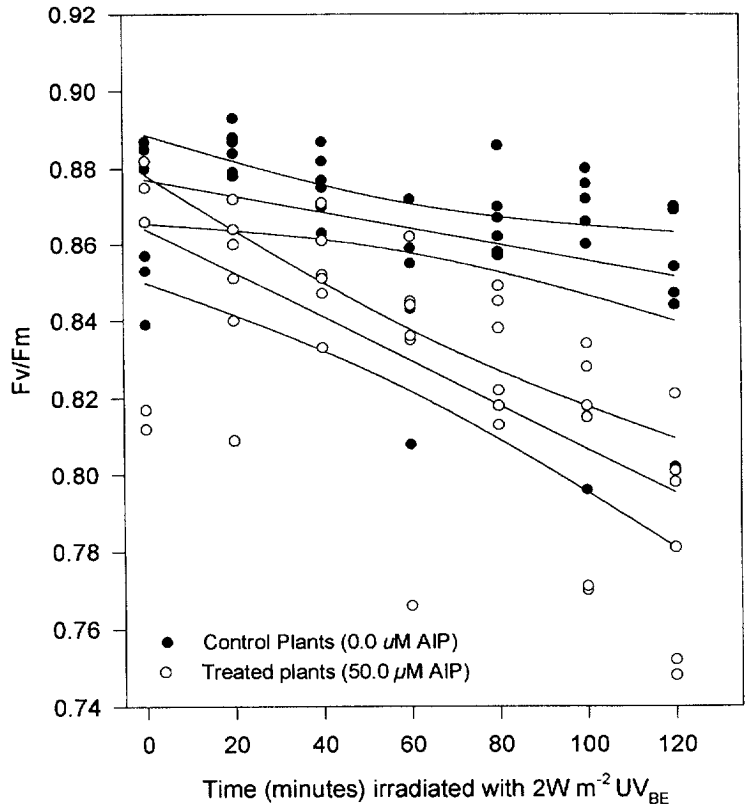

Fig. 5. Ratio of variable chlorophyll fluorescence to maximal chlorophyll fluorescence (Fv/Fm) of dark-adapted cabbage cotyledons grown with (open symbols) or without (closed symbols) 50 μM AIP, as a function of time exposed to 2 W m^{-2} $UV\text{-}B_{BE}$ immediately before a 2 h dark adaption period. Results of least squares linear regression with 95% confidence interval are depicted. Fv/Fm of non-irradiated cotyledons (time = 0) did not significantly differ between treatments ($p = 0.43$, t-test).

reported for acylated anthocyanins of red cabbage [35].

Our results show that acylated anthocyanins are almost exclusively responsible for the differences in soluble UV absorbing compounds between control and AIP-grown cotyledons, and the Fv/Fm responses of red cabbage seedlings are similar to those of the related species *Arabidopsis thaliana* where hydroxycinnamic acids are the primary UV–B screens (36). For such reasons we attribute the increased sensitivity to UV–B radiation (Fig. 5) in AIP treated plants to an absence of anthocyanin production (Fig. 2) within rather clearly defined regions (Fig. 1) of cotyledons which are architecturally unchanged by growth with AIP.

EXPERIMENTAL

Synthesis of 2-aminoindan-2-phosphonic acid (AIP)

AIP was synthesized (by DCG) by procedures in Zon and Amrhein [16]. Its identity was confirmed by comparison of IR spectroscopic and MP of the synthesized compound against published values [16] and an authentic sample provided by Professor Amrhein.

Plant material

Red cabbage (*Brassica oleraceae rubrum.* cv Red Acre) seeds were from the Holmes Seed Company, Canton OH. Two seeds were placed in each well of multiwell tissue culture plates, on two 1.25 cm diameter disks of Whatman 3 mm filter paper. Growth was initiated by adding 0.5 ml of water or water plus 50 μM (unless otherwise indicated) AIP. Seedlings were grown for 7 days, unless otherwise indicated, in a plant growth chamber (23°/19° day/night temperatures, 16 h photoperiod, 300 μE m^{-2} s^{-1} PAR, cool white fluorescent lamps).

Morphometric parameters

Cotyledons, petioles and hypocotyls were excised and arranged on the adhesive surface of clear polyurethane packing tape, the tape attached to Whatman 3 mm filter paper, and the specimens placed in a press to air dry. Digital images were scanned at 100 pixels

cm^{-1} and SigmaScan software used to determine cotyledon area and the length of hypocotyls, petioles, and cotyledon midribs. Weights were determined to the nearest mg, dry weights from samples dried at 60°.

Optical microscopy

Fresh 100 μM sections were prepared on a Vibratome Series 100 vibrating microtome, mounted in water, viewed with and without NH_3 and with and without fluorescence excitation from an Hg-Xe arc lamp, and images photographed onto Ektachrome 200 ASA film. Exposure times were not held constant between treatments. For lignin detection, sections were mounted in water and a saturated solution of phloroglucinol in 20% HCl drawn across and under the coverslip.

UV treatments and photosynthetic fluorescence kinetics

Seedlings were transferred to 12 cm disposable polystyrene hexagonal dishes and covered with solarized cellulose diacetate film to cut off wavelengths <290 nm and increase humidity. The dishes were placed under fluorescent sunlamps delivering a biologically effective ultraviolet flux [37] of 2 W m^{-2}, and a total photosynthetically active irradiance between 400 and 800 nm of 20 μM m^{-2} s^{-1}, for the times indicated in Fig. 5. The spectral distribution of these sources has been described elsewhere [38]. UV–B sources and quantitation were as in Liu *et al.* [30]. Immediately after irradiation, cotyledons were excised and placed in chambers allowing gas exchange while maintaining an atmosphere saturated with water vapor. After a 2 h dark adaptation period, the photochemical efficiency of PSII, which can be correlated with Fv/Fm, the ratio of variable chlorophyll fluorescence to maximal chlorophyll fluorescence, was determined using a CF-1000 Morgan Scientific Inc. steady state chlorophyll fluorescence kinetic measurement system.

Characterization and quantitation of phenolics

To determine total soluble phenolics which might contribute to UV–B screening, two cotyledon paris or eight hypocotyls were extracted with 10 ml of MeOH:H_2O:HcL (50:50:1 *v/v*) in the dark at room temperature for 3–5 days. Absorbance of the extracts was determined at 1 nm intervals between 560 to 260 nm on a Varian-Cary 210 UV–vis spectrophotometer modified for serial data transfer to an IBM AT computer.

For HPLC of sinapoyl esters, five peeled seeds or cotyledon paris were placed in 2 ml flat bottom microcentrifuge tubes with three carbon steel ball bearings (diameters of 3.17, 3.97 and 4.76 mm) purchased from a local bicycle shop. Tubes were placed in a flotation collar in a liquid nitrogen bath and repeatedly agitated on a Vortex mixer until the plant material was commuted to a fine powder. The bearings were removed with a ceramic magnet which was rinsed while near the lip of the tube with 50% aqueous MeOH containing 0.1% glacial acetic acid. Tubes were capped, returned to the flotation collar, sonicated for 15 min, centrifuged at 14000 *g* for 10 min, and the supernatant poured into 5 ml volumetric flasks. Pellets were resuspended and extracted 2×, pooled supernatants brought to volume and passed through a 0.22 micron nylon microcentrifuge filter, and used directly for HPLC. Sinapoyl glucose and sinapoyl choline from red cabbage were identified by paper chromatography [39] and HPLC [40].

Reverse phase HPLC was modified from Strack and Klug [40]. 20 μl of filtered extract was autoinjected onto an Absorbosphere HS (Alltech) 250×4.6 mm column containing 7 μM spherical particles with a 20% carbon load of C18 and protected by a 7.5×4 mm guard column packed with 5 μM C18. Compounds were eluted from the column using a linear gradient of from 15% solvent B (20% glacial HOAc plus 0.1% orthophosphoric acid in acetonitrile) in solvent A (0.1% orthophosphoric acid) increased to 40% B over 25 min at a flow rate of 1.5 ml min^{-1} with detection at 528, 330 and 300 nm using a Waters 490E programmable multiwavelength detector. Gradient control and data acquisition was accomplished using Interactive Microware Inc. software and hardware and an IBM PS-II/386 personal computer. Data for each wavelength monitored was converted to ASCII files and analyzed using PeakFit software (Jandel Scientific) employing an exponentially modified Gaussian model fitted to the chromatogram peaks [41] and compared to sinapic acid standards coinjected with each run.

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