Metabolic Reduction of Phenylpropanoid Compounds in Primary Leaves of Rye (*Secale cereale* L.) Leads to Increased UV-B Sensitivity of Photosynthesis

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In the epidermal layers of rye primary leaves two flavone glycosides and several hydroxycinnamoyl esters are localized, whereas the mesophyll contains two flavone glucuronides and two anthocyanins. The concentrations of all these potential UV-B protective phenylpropanoid compounds could be reduced by application of 2-aminoindan-2-phosphonic acid (AIP), an efficient inhibitor of phenylalanine ammonia-lyase (EC 4.1.3.5). Photosystem II in the primary leaves of seven-days-old plants, grown in the presence of 20 µM AIP up to an age of 80 h, was more severely affected by UV-B than in control plants with the normal concentration of phenylpropanoid compounds. Damage of photosystem II *in vivo* was estimated by measuring the chlorophyll *a* fluorescence (parameter F_v/F_m) of PS II. The results indicate an essential role of phenylpropanoid compounds as UV-B protectants in rye primary leaves.

Introduction

The destruction of the stratospheric ozone layer, first predicted 1974 [1], will result in increasing UV-B levels in the sunlight spectrum on the earth's surface. Because of the high biological activity of this radiation, it will be of fundamental interest to understand UV-B-protective mechanisms realized in plants. A more extensive knowledge of the basic mechanisms of UV-B-sensitivity and -resistance, respectively, will be helpful to classify plant species in general, as well as for breeding new plant cultivars. Establishing reliable UV-B stress parameters in plants will be one step, screening for UV-B resistant varieties adapted to high UV-B levels [2, 3] another step in reaching this goal.

Flavonoids are supposed to be potential UV protective compounds, due to their strong selective absorption in the UV range [4]. Further indications supporting this assumption are their predominant epidermal localization in leaves [5, 6] as well as induction or stimulation of phenylpropanoid metabolism by UV-B light [7, 8]. UV-B-induced pyrimidine dimer formation in the DNA and damage of the photosynthetic apparatus are two commonly used parameters to estimate UV-B sensitivity of plants [9–11]. Especially photosystem II has been found to be particularly sensitive to UV-B irradiation. The ratio of variable to maximum fluorescence ($F_v/F_m = F_m - F_o/F_m$) serves as an indicator of photochemical efficiency of PS II [12] and characteristically decreases after irradiation with UV-B light [13, 14]. Strong evidence for a correlation between flavonoid content and extent of UV-stress was provided by Tevini *et al.* [15]. UV-B pre-irradiated primary leaves of rye, which reacted with an increase of epidermal flavonoids, showed less UV-B damaging effects on PS II compared to non-preirradiated plants with lower concentration of epidermal flavonoids.

Apart from flavonoids, plants generally synthesize and accumulate various phenylpropanoid compounds, *e.g.* soluble as well as insoluble hydroxycinnamic acid (HCA) conjugates ([16, 17] and literature cited) in high amounts, which show maximum absorption in the UV range. Two fur-

Abbreviations: AIP, 2-aminoindan-2-phosphonic acid; AOA, aminooxy acetic acid; L-AOPP, L-2-aminooxy-3-phenylpropanoic acid; Chl *a*, chlorophyll *a*; F_o, initial fluorescence; F_m, maximum fluorescence; F_v/F_m, ratio of variable to maximum fluorescence; HCA, hydroxycinnamic acid; PAL, phenylalanine ammonia-lyase; PAR, photosynthetically active radiation; PS II, photosystem II; TAL, tyrosine ammonia-lyase; UV-B, ultraviolet-B.

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ther approaches may be useful to elucidate the possible UV-B protective role of flavonoids and other phenylpropanoid compounds:

1) Mutants with a genetic block in an early step of phenylpropanoid and/or flavonoid biosynthesis could serve as useful UV-B sensitive model systems. Recently, mutants of *Arabidopsis thaliana,* deficient in different steps of phenylpropanoid synthesis have been described [18] which appeared to be more sensitive to UV-B light than wild type plants.

2) The second approach is based on the reduction of compounds of general phenylpropanoid metabolism by application of an efficient inhibitor of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), the key enzyme of general phenylpropanoid metabolism. In recent years several inhibitors of PAL have been investigated [19, 20]. Anthocyanin biosynthesis in buckwheat (*Fagopyrum esculentum* L.) hypocotyls served as a model system to determine the *in vivo* efficiency of various inhibitory analogues. Recently, the synthesis of a new compound, 2-aminoindan-2-phosphonic acid (Fig. 1), was described [21], which seems to be the strongest inhibitor of PAL *in vivo* known so far. Thus, AIP could serve as a powerful tool to study functional aspects and the biological importance of phenylpropanoid compounds.

In rye primary leaves, a series of HCA esters, primarily of *p*-coumaric and ferulic acid, were found to be localized specifically in the epidermal layers [22–24]. These compounds may serve as additional major UV-screens together with epidermal flavonoids and flavonoids located in subepidermal layers of the photosynthetically active mesophyll [24, 25].

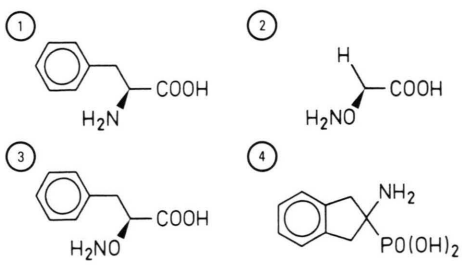

Fig. 1. Structure of PAL-inhibitors AOA (**2**), L-AOPP (**3**) and AIP (**4**) compared to the *in vivo* PAL-substrate phenylalanine (**1**).

In the present work, we were able to reduce the concentrations of phenylpropanoid compounds in rye primary leaves by application of AIP to young seedlings. Analysis of Chl *a* fluorescence of PS II after UV-B irradiation of the plants revealed higher UV-B sensitivity as compared to leaves of control plants with normal phenylpropanoid levels, indicating UV-B protective functions of these compounds.

Materials and Methods

Chemicals

AIP and L-AOPP were kindly provided by N. Amrhein, Zürich, CH. AOA, L-phenylalanine, L-tyrosine and *o*-phthaldialdehyde reagent solution were obtained from Sigma, Deisenhofen, F.R.G., *p*-coumaric acid and ferulic acid from Roth, Karlsruhe, F.R.G., and Miracloth from Calbiochem, La Jolla, U.S.A. Methanol was purchased from Schindler, Cologne, F.R.G., and destilled before use on HPLC. Tetrahydrofuran and *o*-phosphoric acid were obtained from Baker, Deventer, NL, and all standard chemicals and salts from Merck, Darmstadt, F.R.G.

Plant material and growth conditions

Caryopses of rye (*Secale cereale* L. *cv.* Kustro; von Lochow Petkus, Bergen, F.R.G.) were soaked under gentle shaking in Knop's solution, plus 0.1% Hoagland's solution with or without 10, 20 or 40 μM AIP for 8 h. Soaked caryopses were planted on a 1 cm high layer of quartz sand (grain size 1–2 mm, Steine und Erden, Weilerswist, F.R.G.) in Petri dishes (Falcon, 15 cm diameter). The quartz sand was extensively washed with MeOH and double destilled water and dried at 100 °C before use. To this substrate the corresponding solution was added (50 ml) and dishes were covered with transparent plastic lids. Seedlings were grown under standardized conditions in a phytotron as described earlier [22] for 72 h. Subsequently, the seedlings were transplanted to a water soaked peat/soil mixture and cultivated until an age of 7 days.

UV-B irradiation

The 7-day-old seedlings were irradiated with two UV-B lamps (TL 12/20 W, Philips, Hamburg,

F.R.G.). Light was filtered by cut off filters (series WG 295, Schott, Mainz, F.R.G.) to eliminate the small UV-C part emitted by the lamps. For our experiments we chose an UV-B source of high irradiance without any portion of PAR to maximize damaging effects for a more distinct expression of UV-B stress. Light from UV-B lamps used here also contains UV-A light (see [26] for spectral energy distribution), which may cause additive damaging effects, although UV action spectra recorded with plants indicate less contribution of UV-A compared to UV-B [27, 28]. For this reason, we ascribe the observed effects in the present experiments solely to UV-B light. Distance between lamps and leaf-tips was 7 cm. The fluence rate received by the leaf tips, measured with a spectroradiometer (model DM 150 BC, Bentham Instruments, Reading, Berkshire, U.K.), was 6.07 $W \cdot m^{-2}$. The corresponding spectral energy distribution will be published elsewhere.

Chlorophyll fluorescence

Recording of Chl *a* fluorescence was performed immediately after UV-B irradiation of the seedlings. Samples each of six primary leaves were harvested in time intervals of 30 min over a period of 5 h of UV-B irradiation. Chlorophyll fluorescence was measured using a pulse amplitude modulation fluorometer (PAM 101; H. Walz, Effeltrich, F.R.G.). The apical segments of the leaves (3.5 cm) were clamped side by side in a custom made plexiglass cuvette, with their adaxial sides orientated towards the fiber-optic, which was fixed close to the chamber lid. After dark adaption of leaves for 10 min, the initial fluorescence level F_o was determined by excitation with weak light (1.6 kHz, 0.05 $\mu mol \cdot m^{-2} \cdot sec^{-1}$). After reaching a stable F_o-level, maximum fluorescence F_m was induced by application of a 2 sec pulse of actinic white light (1000 kHz, 9000 $\mu mol \cdot m^{-2} \cdot sec^{-1}$).

Extraction and quantification of phenylpropanoid compounds

Whole primary leaves and leaf segments from fluorescence measurements were homogenized and extracted with 40% MeOH in a mortar at room temperature. After centrifugation and two additional washing steps of the pellet, the clear supernatants were analyzed by high-performance-liquid-chromatography (HPLC). The HPLC system LC-10A of Shimadzu (Tokyo, J) was used in combination with the Shimadzu C-R5A integrator. Separation of the compounds was performed on a Nucleosil RP-8 column (125 mm/4.6 mm, 5 µm grain size; CS Chromatographie, Langerwehe, F.R.G.) at a constant flow rate of 1 $ml \cdot min^{-1}$ using a linear gradient from 6% to 16% MeOH, 10% to 18% tetrahydrofuran and 84% to 66% water containing 1% *o*-phosphoric acid within 15 min. Re-equilibration was achieved by a 10 min isocratic rinse with the initial solvent composition. Phenylpropanoid compounds were detected at 340 nm (flavonoids), 300 nm (HCA esters) and 520 nm (anthocyanins), respectively. Calibration for quantification of flavonoids was performed with authentic compounds from our laboratory collection. For HCA esters, ferulic acid and *p*-coumaric acid (Roth) served as standards and the mean value of both compounds was used for quantification.

Extraction of amino acids and quantification of phenylalanine and tyrosine

Primary leaves of 80-h-old AIP-treated and non-treated seedlings were extracted in cold 4.5% trichloroacetic acid with mortar and pestle for 5 min. The crude extracts were filtered through miracloth and centrifuged for 15 min at 4000 × *g*. 17 µl of the clear supernatants were subjected to HPLC after neutralization with 3 µl 1 M KOH. Immediately before analysis samples were derivatized with 15 µl *o*-phthaldialdehyde reagent (Sigma). After 3 min incubation, 20 µl of the sample were injected onto a Hypersil RP-18 column (125 mm/4.6 mm, 5 µm grain size; CS Chromatographie, Langerwehe, F.R.G.) and eluted at a flow rate of 1 $ml \cdot min^{-1}$ in the following gradient system (solvent A: 0.1 M potassium acetate, pH 5.5; solvent B: MeOH): 20 min from 85% to 55% A, 10 min from 55% to 25% A, 4 min isocratic with 100% B and 5 min with 85% A for re-equilibration. Detection was performed with a fluorometer (excitation wavelength 340 nm, detection wavelength 450 nm). Identification and quantification of phenylalanine and tyrosine was achieved by co-chromatography with phenylalanine and tyrosine and using both as external standards.

Results

Phenylpropanoid and flavonoid biosynthesis in rye primary leaves is characterized by a strict tissue specific compartmentation [22, 24, 25]. Cells of both, the abaxial and adaxial epidermal layer accumulate two glycosides of the flavone isovitexin, R_3 and R_4, and a number of hydroxycinnamoyl esters [23], whereas the mesophyll contains two flavone glucuronides of the luteolin type, R_1 and R_2, and two anthocyanins, the cyanidin glucosides R_I and R_{II} [24, 29]. Cultivation of seedlings in the presence of AIP for 80 h leads to a significant reduction of all these phenylpropanoid compounds in 7-day-old leaves (Fig. 2). At the tissue level, epidermal flavonoids and hydroxycinnamoyl esters were reduced by 65%, after cultivation in 10 µM AIP while flavonoids of the mesophyll tissue were reduced by 43%, as compared to control plants.

Fifty percent inhibition of flavonoid biosynthesis could be achieved with approximately 10 µM AIP (I_{50}-value). Reduction of flavone glycosides and HCA esters is correlated with the decrease of anthocyanins (Fig. 2), which makes the anthocyanin a reliable visible marker for the extent of phenylpropanoid and flavonoid inhibition in this system.

With increasing concentration of AIP the inhibition of phenylpropanoid metabolism became proportionally less efficient (Fig. 2). Moreover, negative effects on growth, fresh weight and root development were more pronounced (data not shown). Concentrations of AIP up to 40 µM caused linear decreases of fresh weight (41%) and leaf length (33%) compared to control plants, whereas the area of the leaf tips was not significantly reduced. Hence, as a compromise, in all following experiments a concentration of 20 µM AIP was chosen to obtain a drastic reduction in HCA ester and flavonoid content with only relatively small effects on leaf and seedling development.

In addition, two further PAL-inhibitors, AOA and L-AOPP (Fig. 1), were investigated for their inhibitory function on phenylpropanoid metabolism in rye seedlings. Although L-AOPP is a strong, well established PAL-inhibitor in dicotyledonous plants, no inhibitory effect was detectable in the monocotyledonous plant rye up to concentrations of 0.2 mM (data not shown). AOA, the first PAL inhibitor established, with less *in vivo* efficiency compared to L-AOPP [20], was not inhibitory up to concentrations of 4 mM (data not shown).

An increase in the content of soluble phenylalanine, the substrate of the PAL catalyzed reaction, in AIP-incubated plants is regarded as an indication for the inhibition of PAL *in vivo* [21]. Accordingly, when 80-h-old rye seedlings were incubated in 20 µM AIP solution, the concentration of soluble phenylalanine was increased 3- to 4-fold as compared to control plants (Fig. 3).

In many plant systems, notably grasses, tyrosine ammonia-lyase (TAL)-activity is detectable *in vitro* [30]. Since AIP was found to be an effective inhibitor also of TAL from several grasses (N. Amrhein, personal communication), we investigated the extractable tyrosine-pool from rye leaves. No increase of tyrosine was found in leaves cultivated in AIP compared to the controls (data

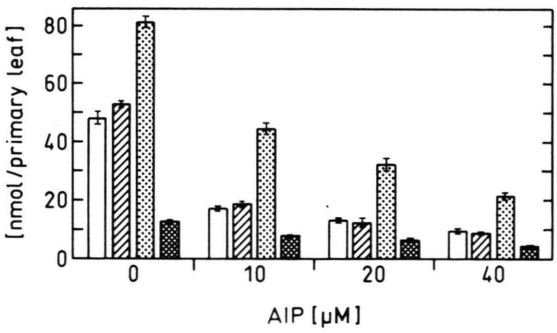

Fig. 2. Reduction of the content of phenylpropanoid compounds in 7-day-old rye primary leaves cultivated in 0 up to 40 µM AIP. □: HCA esters, ▨: flavonoids R_3, R_4, ▩: flavonoids R_1, R_2, ■: anthocyanins R_I, R_{II}. Bars indicate standard deviation of 3 independent experiments.

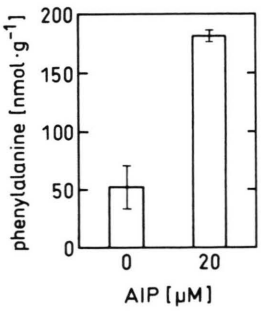

Fig. 3. Content of soluble phenylalanine in 80-h-old rye primary leaves cultivated with or without 20 µM AIP. Bars represent standard deviation of 4 independent experiments.

not shown), suggesting, that no significant TAL activity is involved in phenylpropanoid synthesis *in vivo*. The quantitative pattern of the other amino acids remained unchanged, too.

Measurement of Chl *a* fluorescence was performed with unfolded apical leaf segments of 7-day-old plants, irradiated with UV-B light for up to 5 h. Values of the F_v/F_m-ratio were identical (0.81) in non-irradiated control or AIP-treated plants, indicating no effect of the inhibitor on the activity of PS II (Fig. 4). Irradiation with UV-B of 20 µM AIP treated leaves caused an almost linear decrease of the F_v/F_m value from 0.81 to approximately 0.65 during the irradiation time of 5 h (Fig. 4b). In contrast, only a slight decline from 0.81 to 0.78 was observed in irradiated leaves cultivated in absence of the inhibitor (Fig. 4a).

Corresponding concentrations of flavonoids and HCA esters, extracted from the same irradiated apical leaf segments subsequent to fluorescence recording, are shown in Fig. 5. Epidermal and mesophyll flavonoids as well as HCA esters are significantly reduced in these leaf segments. Thus, decreased HCA ester and flavonoid content coincides with increased damaging effects of UV-B radiation on photosynthesis, estimated from changes in Chl *a* fluorescence of PS II.

The effects of UV-B irradiation on the two basic fluorescence parameters F_o and F_m are shown in Fig. 6. Initial fluorescence F_o seemed to be unaffected in UV-B irradiated segments of control plants and remained constant over the irradiation time of 5 h. Thus, the small decrease of F_v/F_m (Fig. 4a) is caused only by the slight decrease of the maximum fluorescence F_m (Fig. 6a). In contrast, leaf segments with reduced flavonoid and HCA ester levels showed, apart from a more drastic decrease in F_m, a slight increase of F_o (Fig. 6b), which contributes to the stronger decline of F_v/F_m (Fig. 4b) during UV-B irradiation.

Discussion

The aim of this work was to investigate the possible protective function of phenylpropanoid compounds against UV-B radiation, by reducing their content in rye primary leaves and measuring the damaging effects of UV-B radiation on photosynthesis. The new efficient inhibitor of PAL (EC 4.3.1.5), 2-aminoindan-2-phosphonic acid, seems to be an excellent tool for studying biological functions of phenylpropanoid compounds in plants. Using this inhibitor, a strong reduction of both, hydroxycinnamoyl esters and flavonoids, could be

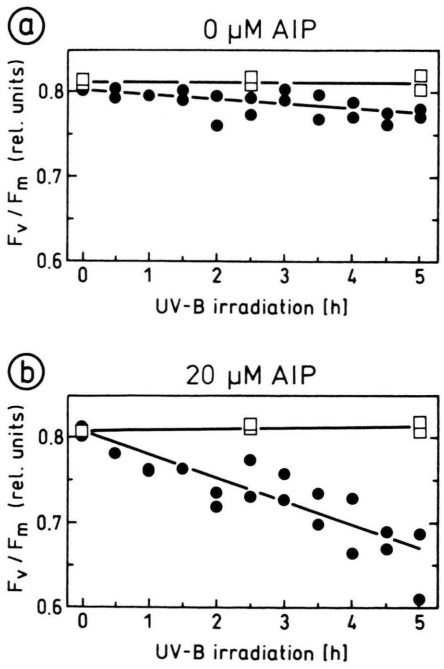

Fig. 4. F_v/F_m-ratio in apical primary leaf segments of 7-day-old plants irradiated with UV-B light up to 5 h (circles). a: control plants; b: plants cultivated in 20 µM AIP-solution. Squares indicate F_v/F_m values in non-irradiated plants. Results of two independent series are shown.

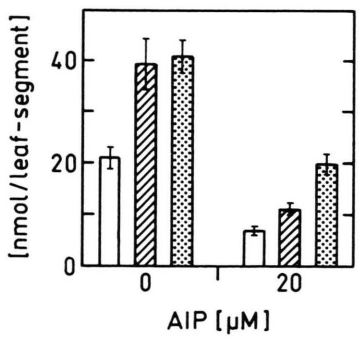

Fig. 5. Concentration of flavonoids and hydroxycinnamoyl esters in apical primary leaf segments of plants cultivated with or without AIP. Each sample consists of the 6 leaf segments used for fluorescence measurements. □: HCA esters, ▨: flavonoids R_3, R_4, ▩: flavonoids R_1, R_2. Bars indicate standard deviation of 6 samples taken during the whole irradiation period.

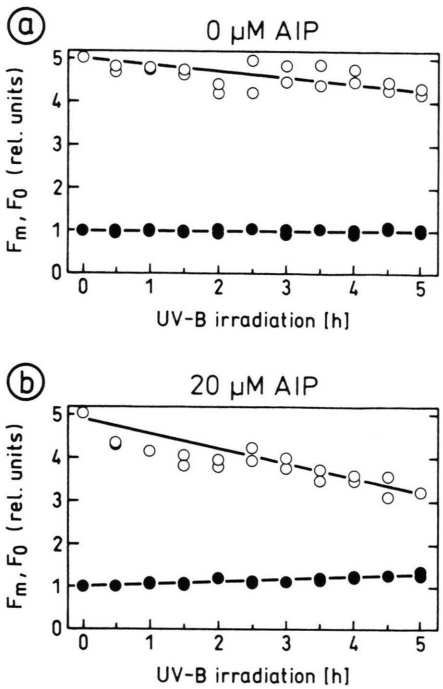

Fig. 6. Initial fluorescence F_o (closed circles) and maximum fluorescence F_m (open circles) in apical primary leaf segments of 7-day-old plants irradiated with UV-B light up to 5 h. a: control plants; b: plants cultivated in 20 µM AIP. To demonstrate the development of F_m and F_o and their absolute relation, values at irradiation time 0 are set to 1 for F_o and 5 for F_m, respectively.

achieved in rye primary leaves, although generally gramineous plants seem to be less sensitive to the knwon PAL-inhibitors (N. Amrhein, personal communication).

Transplanting AIP-cultivated seedlings on AIP-free substrate after an incubation time of 80 h did not lead to an increase in phenylpropanoid biosynthesis up to an age of 7 days compared to control plants. This may be due to the strong dependence of phenylpropanoid metabolism on leaf-age and -development in rye primary leaves [22, 25], which possibly prevents stimulation of the pathway in plants older than 80 h. An endogenous AIP-pool, remaining high enough to maintain PAL inhibition after transplanting, could be an alternative explanation.

In spite of the inhibition of phenylpropanoid synthesis produced by AIP in the rye system, the observed effect is much smaller (I_{50}-value for rye primary leaves is about 10 µM) than in buckweat hypocotyls, where an AIP concentration of 1.5 µM resulted in a 50% inhibition of anthocyanin synthesis [21]. Buckwheat hypocotyls were, however, cultivated in direct contact with inhibitor solution, whereas in our system AIP was mainly taken up by the roots. The observed stronger inhibition of phenylpropanoid biosynthesis in the outer epidermal layers compared to the inner mesophyll tissue in rye (Fig. 2) could, to some extent, indicate direct uptake of AIP *via* the leaf surface.

L-AOPP at 0.2 mM hardly affected the level of phenylpropanoid metabolites in rye, whereas it has been found to be a powerful PAL-inhibitor *in vivo* in various dicotyledonous plants [31, 32]. This is confirmed by earlier results, where 0.3 mM L-AOPP caused no increase of the phenylalanine level in 5-day-old rye seedlings compared to L-AOPP-free control plants [20].

Inhibition of PAL using 20 µM AIP leads to a strong increase, 3- to 4-fold, of the soluble phenylalanine pool in the rye leaf within 80 h. However, this increase, compared to control plants, is much more distinct in buckwheat hypocotyls, where a 10-fold amount on a fresh weight basis was measured after cultivation in 1.5 µM AIP [21], a further evidence for less inhibitory efficiency of AIP in the monocotyledonous rye seedling.

Apart from inhibition of phenylpropanoid synthesis AIP reduces fresh weight, leaf length and root development as a function of its concentration. It is yet unclear whether these effects are caused by the reduction of phenylpropanoid metabolism (*e.g.* of lignin biosynthesis), or result from unspecific side effects of AIP. However, for our studies on the UV-B protective importance of phenylpropanoid compounds, slight inhibitory effects can be tolerated. Requirement of intact, unfolded leaves for fluorescence measurement and the need of comparison with control plants allows to apply AIP concentrations up to 20 µM in this system.

Fluorescence of Chl *a* in PS II is a frequently used parameter to determine the effects of environmental stresses on photosynthesis. Irradiation of leaves with UV-B light led to a loss of photochemical efficiency, estimated as decline in the F_v/F_m-ratio in various plant species [13, 14].

A continuous and strong decrease of F_v/F_m was observed during UV-B irradiation in rye primary leaves with reduced HCA ester and flavonoid con-

tent, but only a slight decline was observed in leaves of control plants. Diminished F_v/F_m values reflect a decrease of potential photochemical efficiency in PS II reaction centres. This fluorescence parameter, however, permits no further conclusions concerning the targets of UV-B damage. The observed F_0 increase is difficult to interprete. Increases in F_0 are sometimes observed in conjunction with photoinhibition of PS II by visible light [*e.g.* 33]. As described in a model [34], an F_0 increase can result from the inactivation of reaction centres, but it may have other reasons, too. Increasing F_0 values seem to be a characteristic result of high levels of UV-B radiation [9], which may be intensified in our experiments because of the lack of any white light sources in the irradiation system [35].

The stable F_0 value in leaf segments of control plants may therefore be caused by the higher amounts of HCA esters and flavonoids and the more effective shielding of the photosynthetically active mesophyll cells. Anthocyanins may play a minor role as UV-B protecting compounds in rye primary leaves, due to their low absorption in the UV-B range as measured by HPLC (data not shown) and their low concentration in the mesophyll tissue.

The strong and almost linear decrease in fluorescence F_v/F_m over the irradiation period points to a relationship between UV-B dose and damage of PS II. This is supported by the effect of inhibited synthesis of phenylpropanoid compounds. Reduction of these metabolites causes a diminished absorption of harmful UV-B radiation in the epidermal layers as well as in the mesophyll and therefore leads to increased damaging effects in the UV-B sensitive photosynthetically active mesophyll tissue, resulting in an F_0 increase and a stronger F_v/F_m decrease, respectively.

The role of the mesophyll flavonoids R_1 and R_2 as UV-B protecting compounds remains as yet unclear. However, there is some evidence for a predominant localization of these compounds in the subepidermal mesophyll layers (Reuber and Weissenböck, unpublished results), which could contribute to an effective protection of the mesophyll cells as well. The possible shielding function of these flavonoids will be investigated with AIP-treated leaves with their epidermal layer removed by mechanical peeling prior to UV-B irradiation.

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