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Light-induced betacyanin and flavonol accumulation in bladder cells of *Mesembryanthemum crystallinum*

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Abstract

Treatment of the halophyte *Mesembryanthemum crystallinum* L. (ice plant) (Aizoaceae) with high intensities of white light resulted in a rapid cell-specific accumulation of betacyanins and flavonoids with 6-methoxyisorhamnetin 3-*O*-{[(2‴-*E*-feruloyl)-3‴-*O*-(β-D-glucopyranosyl)](2″-*O*-β-D-xylopyranosyl)}-β-D-glucopyranoside (mesembryanthin) as the predominant component, within bladder cells of the leaf epidermis. Induced accumulation of these metabolites was first detected 18 h after the initiation of light treatment in bladder cells located at the tip of young leaves followed by the bladder cells located on the epidermis of fully expanded leaves. UV-A light apparently is sufficient to induce accumulation of betacyanins and flavonoids. Application of 2-aminoindan 2-phosphonic acid, a specific inhibitor of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), not only inhibited the accumulation of flavonoids but also reduced betacyanin formation. Based on these observations we suggest these bladder cells as a model system to study regulation of betacyanin and flavonoid biosyntheses.

Keywords: *Mesembryanthemum crystallinum*; Aizoaceae; Bladder cells; Betacyanins; Flavonoids; Light induction

1. Introduction

The halophyte *Mesembryanthemum crystallinum* L. (ice plant) (Aizoaceae) has been established as a model system to study osmotic stress effects (Bohnert et al., 1988; Bohnert & Jensen, 1996). Salinity and drought, but also low temperatures, initiate a complex network of hormonal and transcriptional responses leading to induction and/or repression of gene expression (Cushman, Vernon, & Bohnert, 1993). One of the most dramatic responses is the transition from C_3 photosynthesis to the Crassulacean acid metabolism (Winter, 1973).

Up to now, little attention has been paid to stress-induced changes of the ice plant's secondary metabolism, which shares a unique feature with members of most families of the plant order Caryophyllales with regard to tissue pigmentation: the accumulation of betalains instead of anthocyanins (Stafford, 1994). Betalain-producing plants are unable to convert flavan-3,4-diols to anthocyanidins. Instead, they convert tyrosine via Dopa to the building blocks of the red–violet betacyanins and yellow betaxanthins (Steglich & Strack, 1990). While the function of these pigments in flower and fruit coloration might be obvious, their role in pigmentation of vegetative tissues is unknown. Fig. 1 shows the structure of betanidin and a typical anthocyanidin, cyanidin. Both are aglycones of various glycosylated structures and their acylated forms.

While there is extensive research on flavonoid biosynthesis, there is limited information on betalain biosynthesis in higher plants. Joy IV, Sugiyama, Fukuda, & Komamine (1995) describe polyphenol oxidase cDNAs of *Phytolacca americana* coding for tyrosinases and speculated on its possible involvement in betalain biosynthesis. A tyrosinase as part of the betalain bio-

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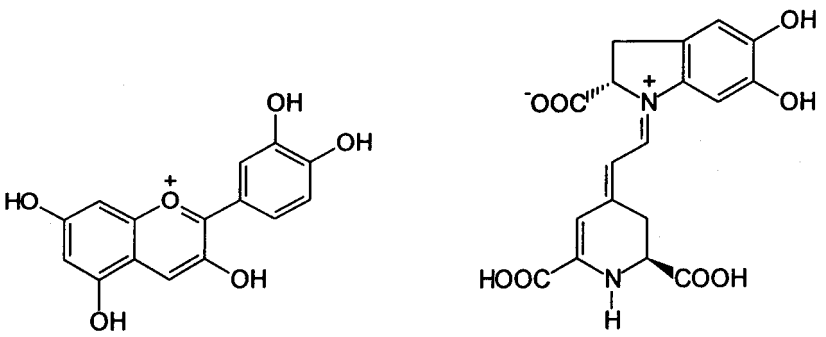

Fig. 1. Structures of a typical anthocyanidin, cyanidin, and betanidin, accumulating as various glycosylated structures and their acylated forms. Both structures exhibit similar λ_{max} values.

synthetic pathway of *Portulaca grandiflora* has recently been characterized (Steiner, Schliemann, Böhm, & Strack, 1999). By using the technique of particle bombardment, Mueller, Hinz, Uzé, Sautter, & Zryd (1997) were able to show expression of a cDNA, encoding *Amanita muscaria* Dopa dioxygenase, in white petals of *Portulaca grandiflora*. Vogt, Zimmermann, Grimm, Meyer, & Strack (1997) purified betanidin glucosyltransferases from *Dorotheanthus bellidiformis* and discussed on the basis of substrate specificity their phylogenetic relationship with flavonoid glucosyltransferases. Finally, the formation of betacyanins acylated with hydroxycinnamic acids has been reported to be catalyzed by 1-*O*-hydroxycinnamoyl-β-glucose-dependent hydroxycinnamoyltransferases (Bokern, Heuer, & Strack, 1992).

The occurrence of flavonoids and betalains within the same tissue requires a close coordination of the arogenate-derived phenylalanine and tyrosine pathway branches. This should preferably be studied in intact plant systems exhibiting simultaneous induction of flavonoids and betalains in synchronously responding cells. In this respect the epidermal bladder cells of *M. crystallinum* are an ideal system.

With this report we introduce *M. crystallinum* as a model system for molecular studies on betalain biosynthesis. We describe the effects of high intensities of white light, the effect of UV-light and the application of 2-aminoindan 2-phosphonic acid (AIP) (Zon & Amrhein, 1992), an effective inhibitor of phenylalanine ammonia-lyase (PAL; EC 4.1.3.5).

2. Results and discussion

2.1. Induction of betacyanin and flavonoid accumulation

In initial experiments with adult plants of the halophyte *M. crystallinum*, betacyanins, mainly betanin (betanidin 5-*O*-glucoside), were detected in ripening fruits, as has been shown for other *Mesembryanthemum* species (Piattelli & Minale, 1964). When the plants were grown in the presence of 1 M NaCl or exposed to drought, fruit ripening and pigmentation of the fruits was markedly stimulated (data not shown). No betacyanin accumulated in non-flowering young plants (6- to 10-week-old) in response to salt stress (data not shown), but increased light intensity was positively correlated with betacyanin formation in the epidermal bladder cells of the leaves. Irradiation with light intensities of 500 μE initiated betacyanin accumulation in bladder cells of young leaf tips and those of fully expanded leaves of 10-week-old *M. crystallinum* plants. The accumulation started 18 h after light treatment (trace amounts detected by HPLC) and was visible to the naked eye by approximately 48 h. Fig. 2 displays bladder cells from a leaf exposed to light intensities of 100–200 μE (control plants) compared to those treated with 500 μE. Fig. 3 shows bladder cells located at the leaf tips from control and light-treated plants. HPLC analyses revealed that besides betacyanins the light-treated bladder cells accumulated appreciable amounts of phenylpropanoids, especially complex substituted flavonoids.

For routine quantitative analysis we were unable to completely separate the bladder cells from other leaf cells and a portion of the flavonoids may be derived from non-bladder cells. However, puncturing of individual bladder cells with a stainless steel needle and injection of the leakage demonstrated that the major induced flavonoid compound (mesembryanthin) was nearly exclusively located in the bladder cells and not in the epidermal layer or in the leaf mesophyll. Fig. 4 shows HPLC profiles of the betacyanin (A) and flavonoid patterns (B) obtained from extracts of bladder cells at the leaf tip seven days after high light treat-

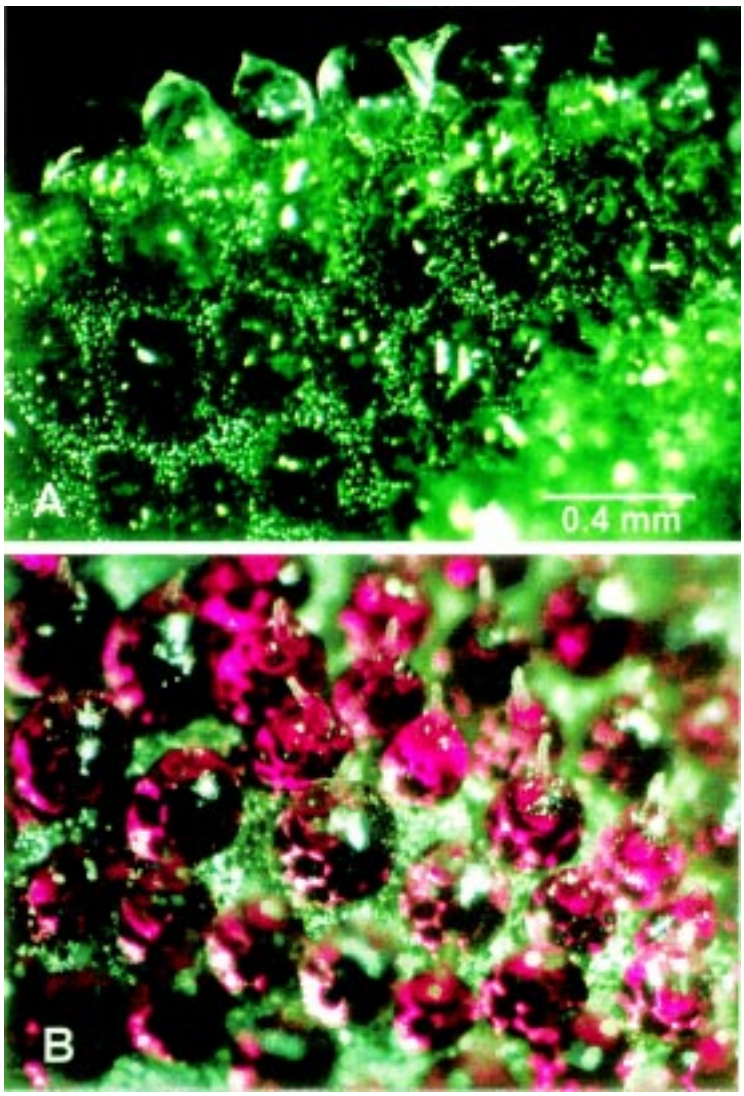

Fig. 2. Light micrograph of bladder cells located on fully expanded 10-week-old *M. crystallinum* leaves. A: Irradiation with light intensities of 100–150 μE (control plants); B: irradiation with 500 μE.

ment. Comparison of the flavonoid patterns from individual bladder cells and an epidermal tissue of a corresponding fresh mass including some of these cells (inset in Fig. 4B) demonstrates the predominant location of mesembryanthin in the bladder cells.

Fig. 5 shows one-week time courses of light-induced betacyanin and flavonoid formation in the leaf tips of 10-week-old *M. crystallinum* plants. Mesembryanthin reached two- to three-fold higher concentrations than the amounts of betacyanins. Together with several other conjugated flavonoids accumulating in the leaf epidermis and the leaf mesophyll, the flavonoid concentration was five- to ten-fold higher compared to the betacyanin concentration (data not shown). The strongest increase in betacyanin and flavonoid concentrations was usually observed from 72 to 120 h and from 24 to 96 h, respectively, after initiation of light treatment and apparently ceased at about 120 h. Betacyanin and flavonoid accumulation in bladder cells located on fully expanded leaves started approximately 12 h later and was not as uniform as in young leaf tips (data not shown). By calculating number and average volume of individual bladder cells, betacyanin concentration was estimated to 5 mM (bladder cells located at fully expanded leaves) and 8 to 10 mM (bladder cells of leaf tips). Seedlings of plants younger than six weeks did not respond or showed a very weak response to light treatments.

In contrast to betacyanin accumulation, only traces of one betaxanthin, indicaxanthin (condensation product of betalamic acid with proline), were detected in light-treated bladder cells. It is uncertain whether this betaxanthin is formed in situ or artificially during bladder cell extraction. Betalamic acid and amino acids can react spontaneously to form betaxanthins (Terradas & Wyler, 1991; Hempel & Böhm, 1997;

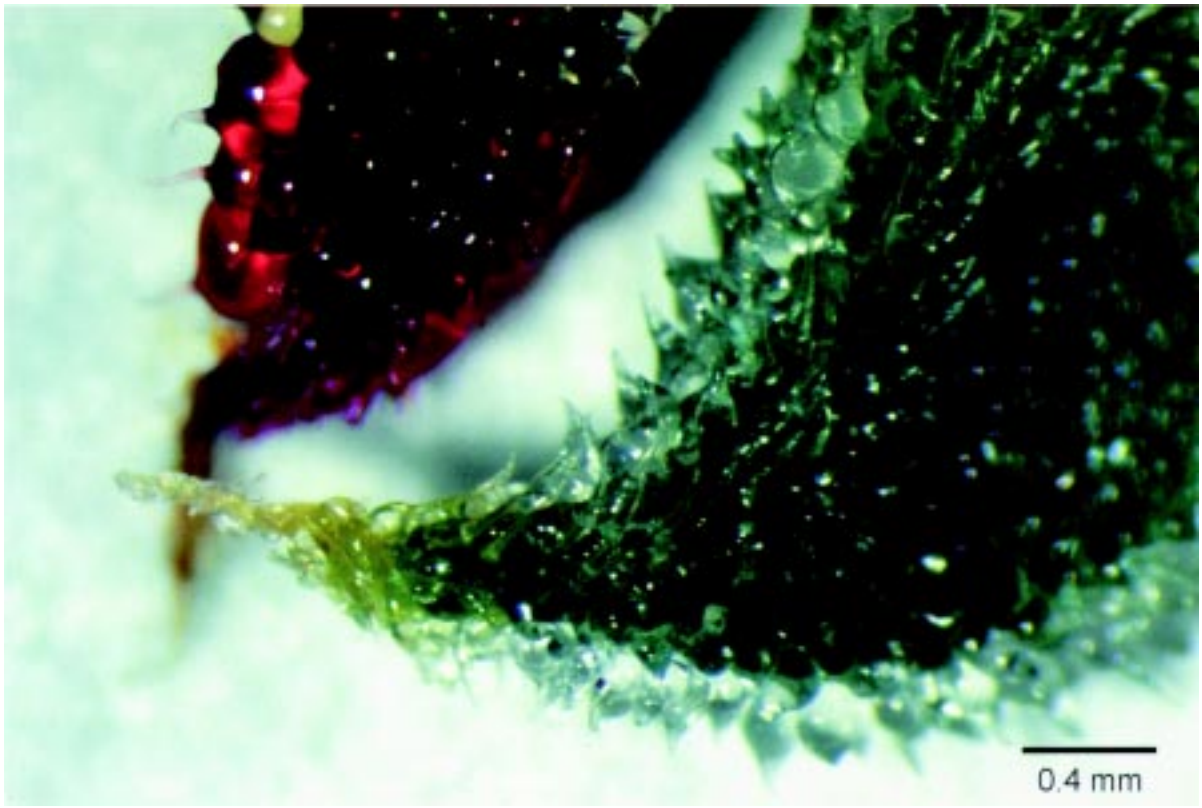

Fig. 3. Light micrograph of bladder cells located at the leaf tips. The bladder cells treated with light intensity of 500 μE are red in color due to betacyanin accumulation, those treated with 100–150 μE remained colorless.

Schliemann, Kobayashi, & Strack, 1999) or with *cyclo*-Dopa to form betanidin (Schliemann, Steiner, & Strack, 1998; Schliemann et al., 1999). The possible precursors of indicaxanthin, betalamic acid and proline, accumulate in light-treated bladder cells to about 5 and 750 μM, respectively.

2.2. Betacyanin and flavonoid analyses

Fig. 4 shows an HPLC trace of betacyanins from bladder cells located at the leaf tips, almost identical to traces obtained from bladder cells located on the fully expanded leaves. Besides betanin as one of the major betacyanins a set of complex structures were tentatively identified as mono- and diferuloylated di- and triglucosylated betanidin. Betanin (**2**) and lampranthin II (**4**, monoferuloylbetanin) were identified by comparison with reference compounds from our betacyanin collection. The feruloyl moieties of the complex structures were identified from alkaline hydrolysates by HPLC with authentic hydroxycinnamic acids. The numbers of hexoses, most likely glucoses and ferulic acids were determined by mass spectrometry (details not shown). The molecular peak $[M+H]^+$ of the diglucoside (compound **1**) was at *m*/*z* 713, those of the feruloylated betacyanins at *m*/*z* 889, 727, 1228 and 1065 for compounds **3**, **4**, **5**, **6**, respectively (for compound identification see Fig. 4A).

The major induced flavonoid from bladder cells of the leaf tips (Fig. 4B, peak **7**), which is mesembryanthin, was characterized by HPLC, retention time and online UV–VIS spectroscopy (λ_{max} 335, 254). The structure of this flavonoid was elucidated from ESI-MS as well as 1D and 2D ^{1}H and ^{13}C NMR data. To the best of our knowledge mesembryanthin is a new natural flavonol conjugate.

The molecular weight is indicated by an $[M+H]^+$ ion at *m*/*z* 979. While the mass of the flavonoid aglycone is corroborated by a peak at *m*/*z* 347 $[A+H]^+$, the presence of the ferulic acid moiety is consistent with the ion at *m*/*z* 177. The main fragments are the monoglycosylated flavonoid (*m*/*z* 509 $[A+H+162]^+$) and the complementary feruloyl disaccaride moiety (*m*/*z* 471). The 2D ^{1}H COSY spectrum showed the presence of two three-spin 1,3,4-trisubstituted aromatic systems and a double bond with the *E*-configuration (J = 15.9 Hz). These systems were readily identified from the ^{13}C data as belonging to a terminal feruloyl system and the B-ring of a flavonoid system. Only one singlet in the region 6–7 ppm, belonging to H-6 or H-8 of the latter system, immediately indicated a further substitutent in the A-ring in addition to the usual two hydroxy groups at C-5 and C-7. Three sugar moieties

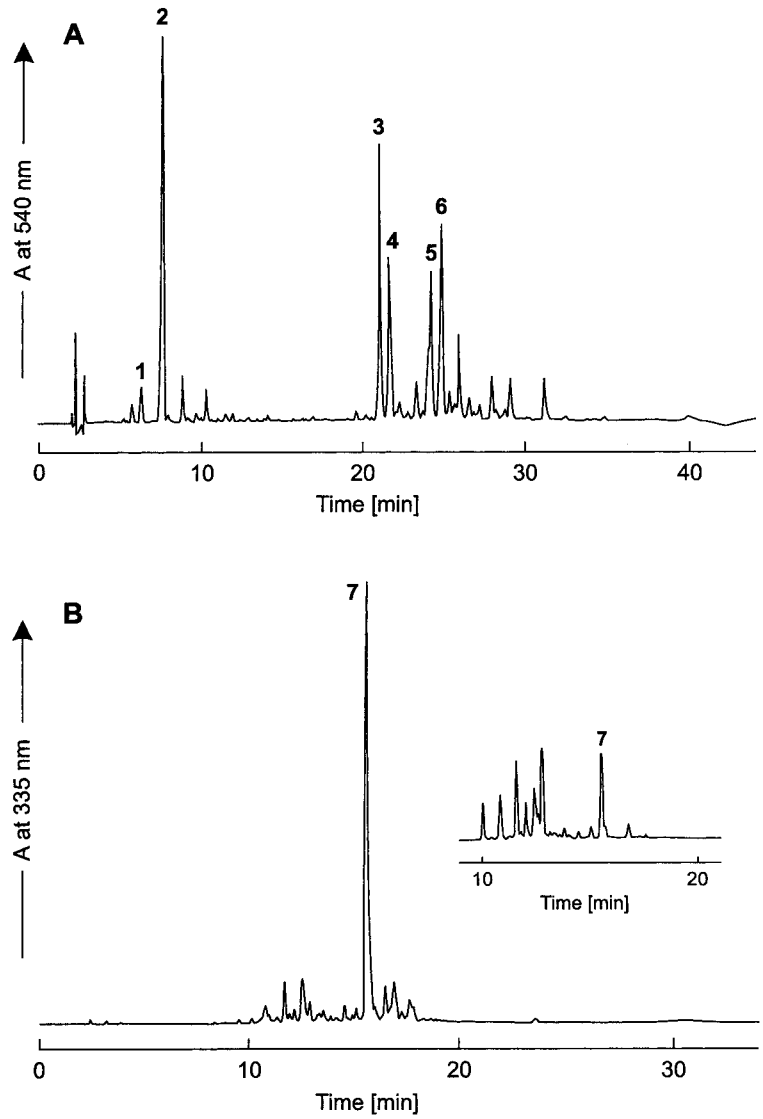

Fig. 4. HPLC profile from extracts of bladder cells located at the leaf tip seven days after high light treatment; betacyanins at 540 nm (A) and mesembryanthin at 335 nm (B), 0.40 full scale absorbance units. Peak identification: **1**, betanidin 5-*O*-diglucoside; **2**, betanin (betanidin 5-*O*-glucoside); **3**, monoferuloyl betanidin 5-*O*-diglucoside; **4**, lampranthin II [betanidin 5-*O*-(6′-feruloylglucoside)]; **5**, diferuloyl betanidin 5-*O*-triglucoside; **6**, diferuloyl betanidin 5-*O*-diglucoside; **7** mesembryanthin. The inset in (B) shows the profile of the flavonoid pattern from epidermal tissue of a leaf area including some bladder cells corresponding to the fresh mass from which only the bladder cells of the leaf tip were extracted with a stainless steel needle.

were apparent from the three anomeric protons characteristic of β-pyranose systems. Only one of these, corresponding to the anomeric proton at 5.25 ppm, could readily be identified from the 2D COSY spectrum and magnitudes of the associated coupling constants as a C-2-acylated β-xylopyranose moiety. The overlap of several signals for the two remaining systems did not allow distinction between β-glucopyranose and β-galactopyranose systems from the coupling constants, although the ^{1}H chemical shifts of H-4 and the ^{13}C chemical shifts were only compatible with the former system in both cases. Finally three aromatic methoxy groups were present.

The ^{13}C signal assignments and linkages between the various fragments followed from the correlations in the HMBC spectrum (^{1}H-detected long-range ^{13}C–^{1}H correlation). In this spectrum all three-bond correlations were observed and permitted the unambiguous sequence to be determined. For the sugar sequence two such correlations for each inter-unit linkage identified the sequence while sequences involving a quaternary carbon afforded only one correlation. The

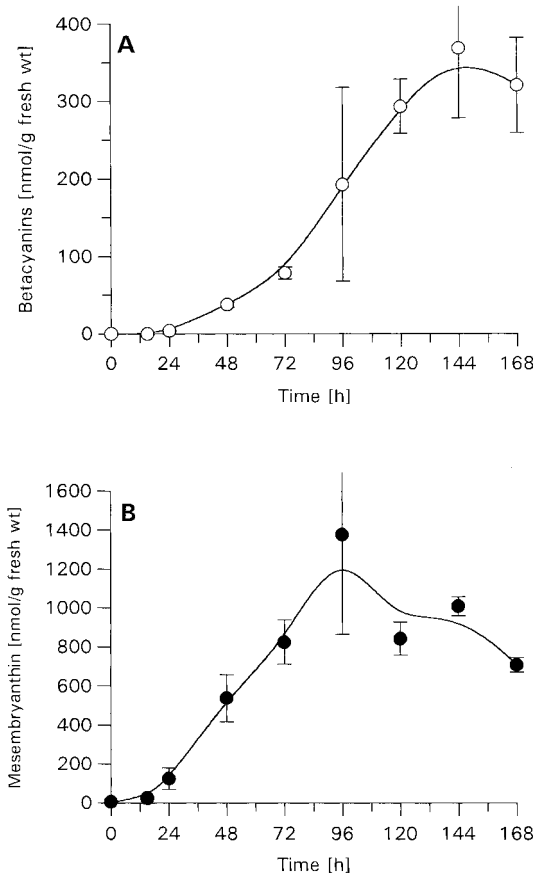

Fig. 5. Time courses of betacyanin (A) and mesembryanthin (B) accumulation in bladder cells located at the leaf tip treated with light intensity of 500 μE.

methoxyl groups of the B-ring and feruloyl moiety showed correlations to the respective substituted carbons (C-3) which had been previously assigned from the internal couplings within the aromatic ring systems.

Although the third methoxyl group must be attached to the A-ring of the flavonoid, its position at C-6 or C-8 could not be unambiguously established from the long-range ^{13}C–^{1}H correlations. However, since the introduction of a methoxyl group has little effect upon the chemical shift of a proton or carbon in a *meta* position to the substituent, the shifts of the free position in the A-ring afford an indirect method for determining the methoxyl group position. Thus, ^{1}H and ^{13}C shifts of 6.38 and 94.8 ppm, respectively, correspond well with methoxyl group substitution at C-6 (expected shifts of H-8: ~6.4 and C-8: 95 ppm, respectively) but not with the alternative at C-8 (expected shifts of H-6: ~6.2 and C-6: 100 ppm, respectively) (Strack, Heilemann, Mömken, & Wray, 1988).

This new structure, 6-methoxyisorhamnetin 3-*O*-{[(2‴-*E*-feruloyl)-3‴-*O*-(β-D-glucopyranosyl)](2″-β-D-xylopyranosyl)}-β-D-glucopyranoside (mesembryanthin) (Fig. 6), is predominant among a set of related minor flavonols with similar spectral and structural properties. One minor compound, also identified by mass spectrometry, 1D and 2D ^{1}H COSY, differs from mesembryanthin by the absence of the 6-methoxyl group and is therefore isorhamnetin 3-*O*-{[(2‴-*E*-feruloyl)-3‴-*O*-(β-D-glucopyranosyl)](2″-β-D-

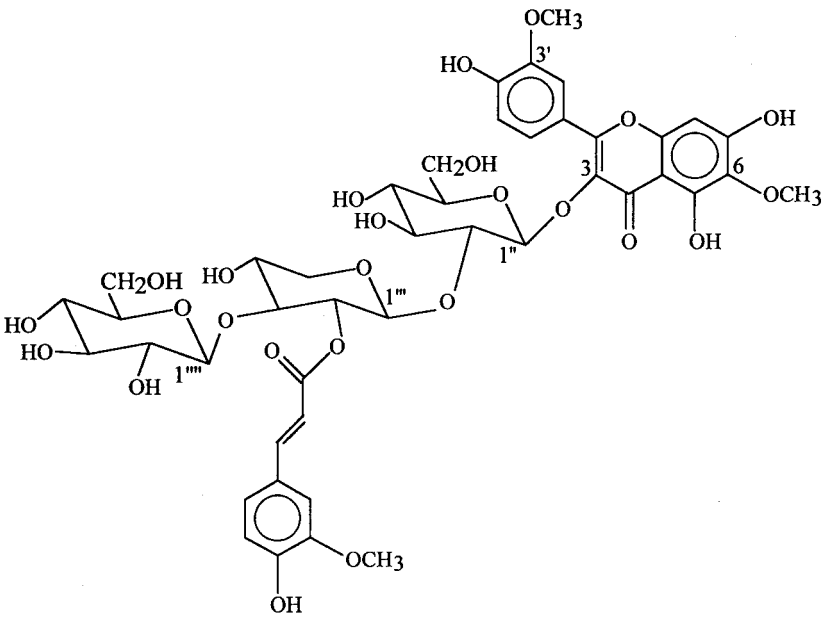

Fig. 6. Structure of 6-methoxyisorhamnetin 3-*O*-{[(2‴-*E*-feruloyl)-3‴-*O*-(β-D-glucopyranosyl)](2″-β-D-xylopyranosyl)}-β-D-glucopyranoside (mesembryanthin).

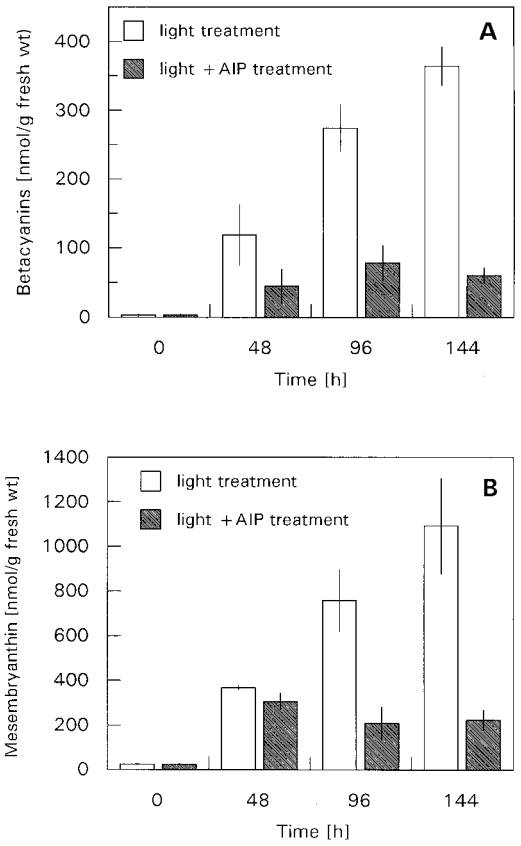

Fig. 7. Accumulation of betacyanins (A) and mesembryanthin (B) in leaf tips after spraying *M. crystallinum* for one week with 10 μM AIP twice a day during irradiation of the leaf tips with high intensities of light (1100 μE).

xylopyranosyl)}-β-D-glucopyranoside (data not shown).

6-Methoxyl substitutions of flavonols are rather rare except for their widespread occurrence in the Asteraceae (Wollenweber, 1994). Mesembryanthin is similar to highly glycosylated 6-methoxyquercetin derivatives identified from leaves of *Spinacia oleracea* (Aritomi, Komori, & Kawasaki, 1986) which is also a member of the Caryophyllales.

2.3. Repression of betacyanin and flavonoid accumulation by 2-aminoindan 2-phosphonic acid (AIP)

Flavonoids and hydroxycinnamic acid conjugates have been suggested to be effective protectors against UV-light (Li, Ou-Lee, Raba, Amundson, & Last, 1993) and antioxidants (Castelluccio et al., 1995). Upon reducing the amount of light-induced phenylpropanoids by spraying the plants with a solution of the PAL inhibitor, (AIP) (Zon & Amrhein, 1992), we expected a compensating increase of the betacyanin levels at high light intensities. We observed, however, that accumulation of the betacyanins was as strongly repressed as the accumulation of flavonoids (Fig. 7). At the same time, the pivotal precursors phenylalanine and tyrosine accumulated from trace amounts up to 300 nmol and from about 250 to 500 nmol mg^{-1} fresh weight, respectively. One possibility to explain the unexpected AIP-dependent repression of betacyanin accumulation is the reduction of possible intermolecular stabilization of the betacyanins by high concentrations of phenylpropanoids. Such stabilization mechanisms have been studied extensively with anthocyanins (Mistry, Cai, Lilley, & Haslam, 1991; Bloor, 1997). Interactions of betacyanins with flavonoids or hydroxycinnamic acid conjugates may also protect betacyanins from bleaching by superoxide radicals (Yamasaki, Uefuji, & Sakihama, 1996). Spraying the AIP-treated leaves with a 1 mM solution of 4-coumaric acid led to a slight complementation (10–20%) of the phenylpropanoid pathway, but the level of betacyanins remained strongly repressed. These unexpected results await further studies.

2.4. Light quality versus light quantity

Besides phytomorphogenesis (Neuhaus, Bowler, Kern, & Chua, 1993), the stimulation of the general phenylpropanoid and flavonoid pathways are characteristic features of photosensory perception (Chory, 1993; Quail, 1994). Responses to UV-light are mediated by specific transcription factors (Quattrocchio, Wing, Leppen, Mol, & Koes, 1993) and they rapidly promote enhanced transcription levels of PAL and chalcone synthase (CHS; EC 2.3.1.74) (Chappell & Hahlbrock, 1984). A tissue-specific response to light treatment has been shown in parsley leaves. Schmelzer, Jahnen, & Hahlbrock (1988) demonstrated by in situ localization that epidermal cells accumulate light-induced CHS mRNA, CHS protein and the flavonoid end products.

In *M. crystallinum* the amounts of accumulating betacyanins and flavonoids were not only correlated with the quantity of white light, but also depended on the irradiation spectrum of the light sources. When Osram mercury lamps were used as the light source at 500 μE, the betacyanin pattern and quantity was higher compared to plants grown in a phytotron, where the light source (Philips Powerstar) was covered with a 3 mm shield of Plexiglas, which efficiently blocks UV- and blue light up to 380 nm (Philips Manual, 1984). The overall betacyanin and flavonoid accumulation of these plants was reduced (even at 1100 μE) to 40%, whereas additional UV-light (Osram Eversun) could restore pigmentation. When the additional UV-source was covered with the UV-B blocking transparency Folanorm™, accumulation of flavonoids and betacyanins after seven days of irradiation was not reduced as compared to control plants exposed to UV-A and UV-B irradiation. The

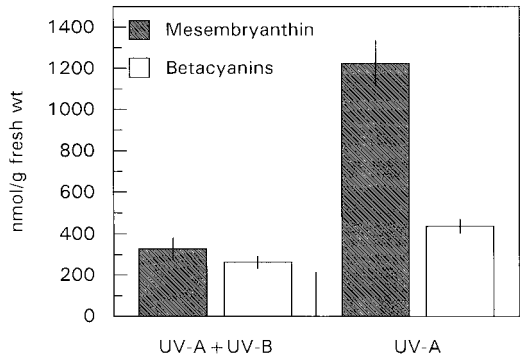

Fig. 8. UV-light dependent accumulation of betacyanins and mesembryanthin seven days after high light irradiation.

actual concentrations even increased 3-fold (flavonoids) and 1.5-fold (betacyanins), respectively (Fig. 8). This clearly demonstrates that UV-A is sufficient to induce flavonoid and betacyanin formation. In addition, the data indicate that UV-B somehow suppresses flavonoid formation by an as yet unknown mechanism. It is interesting to note that despite a 50% increase in total betacyanins, the total amount of one major component, betanin, remained constant, regardless of UV-B exposure. The lower amount of accumulated betacyanins with UV-B is only due to a decrease in the feruloylated betacyanins. This may be in correspondence with strong UV-absorbing properties of the feruloylated compounds between 280 and 320 nm (data not shown). Kishima, Shimaya, & Adachi (1995) suggested a correlation between blue light irradiation and betalain accumulation in callus cultures of *Portulaca grandiflora*. However, UV- and blue light might operate synergistically through separate receptors, as has been shown for the regulation of CHS gene expression in *Arabidopsis* (Fuglevand, Jackson, & Jenkins, 1996). In conclusion, the light-induction system described in this communication, bladder cells of *M. crystallinum*, appears to be superior to previously described ones, whole plants and cell cultures, e.g. *Amaranthus tricolor* (Piattelli, 1981) and *Portulaca grandiflora* (Kishima, Nozaki, Akashi, & Adachi, 1991). Although detailed enzymatic analyses are limited by the small amounts of inducible tissue, the bladder cells are an ideal light-controlled model system for molecular studies on betalain biosynthesis and on the regulation of the arogenate-derived phenylalanine and tyrosine pathway branches, leading to flavonoids and betalains, respectively.

3. Experimental

3.1. Plant material and growth conditions

Mesembryanthemum crystallinum L. (ice plant) was grown from seeds for 10 weeks in the greenhouse at a light intensity of 100–200 μE (sodium vapor lights) for a 16/8-h 25–30/21–22°C day/night cycle. Treatment of these plants with high intensities of white light was performed in a phytotron for a 18/6-h 30/20°C day/night cycle. Light sources were Osram mercury lamps (KHD 250 HD) with 500 μE or Philips Powerstar (HQI T 250/D) with 1100 μE supplemented with an additional UV-source [Osram Eversun L 100/79 Super, UV-A (75 W/m^2) + UV-B (1.5 W/m^2)]. Control plants were grown at 150 μE (Powerstar HQI T 250/D). To specifically block UV-B irradiation, Osram Eversun L 100/79 Super were covered with Folanorm® (100 μm) transparencies (Dr. Schleussner GmbH, Dreieich, Germany). The experiments were started after an initial dark period of 6 h. Leaf tissues were harvested starting 9–18 h after initiation of light exposure.

3.2. Application of compounds

Salt stress was induced by daily watering with 1 M NaCl in Hoagland's solution. 2-Aminoindan 2-phosphonic acid (AIP) and 4-coumarate (Serva, Heidelberg, Germany) were applied by spraying the plants twice a day with 10 μM aq. AIP (provided by N. Amrhein, Zürich, Switzerland) with or without 1 mM 4-coumarate (in 2 mM KPi buffer, pH 7.0). The first treatment was carried out 2 h before initiation of the light treatments.

3.3. Extraction and high-performance liquid chromatography (HPLC) analysis

Five-leaf-tip samples were harvested and crushed in an Eppendorf tube in the presence of 50% aq. MeOH (100 μl per 10 mg tissue). The homogenates were kept at −20°C and were centrifuged before HPLC analysis. Discs from fully grown leaves were obtained with a corkborer (0.4 cm i.d.) and extracted likewise. Each value is the mean of three replicates. Localization of mesembryanthin was performed by puncturing bladder cells of the leaf tips with a stainless steel needle and crushing adjacent epidermis tissue (30 mg each) with 50% aq. MeOH. Both extracts were adjusted to 300 μl.

Reversed phase liquid chromatography (HPLC) [Waters Millipore (Eschborn, Germany)] was performed essentially as described previously (Vogt et al., 1997) with a Nucleosil 5 μm C_{18} column (25 cm length, 4 mm i.d.; Macherey & Nagel, Düren, Germany). Compounds were analyzed with a linear gradient: within 20 min and/or 30 min from 10% B (80 % aq. MeCN) in A (1.5% aq. H_3PO_4) to 45 % B at a flow rate of 1 ml min^{-1}; detection of betacyanins at 540 nm, betaxanthins at 470 nm, betalamic acid at 408 nm, mesembryanthin at 335 nm and maxplot detection of

flavonoids and aromatic amino acids between 260 and 400 nm. Identification and quantification was achieved with reference compounds. Betanin isolated from lyophylized red beet juice (Roth, Germany) was used as standard for the analysis of betacyanins, ferulic acid, tyrosine and phenylalanine (Serva, Heidelberg, Germany) for hydroxycinnamic acid conjugates and the respective amino acids, and quercetin 3-*O*-glucoside (provided by E. Wollenweber, Darmstadt, Germany) for the flavonoid conjugates. Isolation of the acylated betacyanins for mass spectrometry (MS) was achieved by HPLC as decribed above, except that 1.5% phosphoric acid was replaced by 1% formic acid.

The induced major flavonoid compound was purified from 10 g of leaf tips harvested 5 to 7 days after high light treatment, extracted with 50% aq. MeOH and separated by preparative reversed-phase liquid chromatoghraphy on a RCM 10 μm C_{18} column (40 cm length, 25 mm i.d.; Waters Millipore, Eschborn, Germany). Compounds were eluted with a 30-min linear gradient from 10% MeOH in 1 % aq. formic acid to 50 % MeOH at a flow rate of 10 ml min^{-1}, followed by chromatography on Sephadex LH-20 (Pharmacia, Freiburg, Germany) with 80% aq. MeOH prior to MS and NMR analysis.

Alkaline hydrolyses of betacyanins and mesembryanthin were performed by incubation of individual compounds for 20 min in 0.5 N NaOH at 50°C and the liberated acyl moieties (ferulic acid) were identified by HPLC with reference hydroxycinnamic acids. Acidic hydrolyses were carried out by treatment for 1 h with 1 N aq. HCl at 80°C.

3.4. MS and NMR analysis

The new flavonol conjugate, 6-methoxyisorhamnetin 3-*O*-{[(2‴-*E*-feruloyl)-3‴-*O*-(β-D-glucopyranosyl)](2″-β-D-xylopyranosyl)}-β-D-glucopyranoside (*mesembryanthin*), was identified by MS and NMR spectroscopy. The positive ion electrospray ionization (ESI) mass spectra were obtained from a Finnigan MAT TSQ 7000 (electrospray voltage 4.5 kV, heated capillary temperature 220°C; sheath gas: nitrogen) coupled with a Micro-Tech Ultra-Plus Micro LC system equipped with a Ultrasep 4 μm C_{18} column (100 mm length, 1 mm i.d; Sepserv, Berlin, Germany). Compounds were separated with a 10-min linear gradient from 10% MeCN in 0.2 aq. acetic acid to 50% MeCN at a flow rate of 70 μl min^{-1}. ESI-MS (*m*/*z*), 979 $[M+H]^+$, 509, 471, 347, 177.

^1H (1D, 2D COSY) and ^{13}C (1D, 2D HMBC (Bax & Summers, 1986)) NMR spectra were recorded on a Bruker DMX 600 NMR spectrometer at 300 K locked to the major deuterium resonance of the solvent, CD_3OD. Chemical shifts are given in ppm relative to the relevant signals of the solvent (^1H: residual proton signals at 3.35 ppm, ^{13}C: 49.0 ppm) and coupling constants in Hz (F, feruloyl moiety). The multiplicities of the ^{13}C signals were deduced from the correlations in the HMBC spectrum.

^1H NMR (CD_3OD): δ 7.85 [d, H-2′, *J*(2′– 6′) 2.0], 7.49 [dd, H-6′, *J*(6′– 5′) 8.5], 7.47 [d, H-F7, *J*(F7–F8) 15.9], 6.93 [d, H-5′], 6.90 [d, H-F2, *J*(F2–F6) 1.8], 6.77 [dd, H-F6, *J*(F6–F5) 8.2], 6.65 [d, H-F5], 6.38 [s, H-8], 6.25 [d, H-F8], 6.01 [d, H-1″, *J*(1″– 2″) 7.9], 5.25 [d, H-1‴, *J*(1‴– 2‴) 6.7), 5.08 [dd, H-2‴, *J*(2‴– 3‴) 8.3], 4.50 [d, H-1⁗, *J*(1⁗– 2⁗) 7.8], 4.14 [dd, H-5‴A, *J*(5‴A–4‴) 5.1, *J*(5‴A–5‴B) 11.8], 3.99 [s, H-3′-OMe], 3.93 [s, H-6-OMe], 3.93 [m, H-6A*, H-3‴], 3.80 (s, H-F3-OMe), 3.77–3.73 [m, H-3″, H-4‴], 3.75 [dd, H-6A**, *J*(6A**–5**) 2.1, *J*(6A**–6B**) 12.4], 3.69 [dd, H-6B*, *J*(6B*–5*) 6.0, *J*(6B*–6A*) 11.9], 3.64 [dd, H-2″, *J*(2″– 3″) 9.5], 3.57 [dd, H-6B**, *J*(6B**–5**) 5.3], 3.44 [dd, H-5‴B, *J*(5‴B–4‴)9.1], 3.39–3.31 [m, H-4⁗], 3.38 [m, H-5*], 3.35 [m, H-4″, H-3⁗], 3.31 [m, H-5**], 3.26 [dd, H-2⁗, *J*(2⁗– 3⁗) 8.8]. The similarity of the shifts of H-4″ and H-4⁗ of the two glucose systems did not allow an unambiguous assignment of H-5, H-6A and H-6B of these systems (denoted by * and **) to be made from the 2D COSY spectrum.

^{13}C NMR (CD_3OD): δ 179.5 (s, C-4), 168.3 (s, C-F9), 158.4 (s, C-9), 157.7 (s, C-2), 153.7, 153.5 (s ×2, C-5, C-7), 150.7 (s, C-4′), 150.4 (s, C-F4), 149.1 (s, C-F3), 148.4 (s, C-3′), 146.8 (s, C-F7), 134.5 (s, C-3), 132.5 (s, C-6), 127.5 (s, C-F1), 123.7 (d ×2, C-6′, C-F6), 123.4 (s, C-1′), 116.2 (d, C-5′), 116.0 (d, C-F5), 115.5 (d, C-F8), 114.3 (d, C-2′), 111.2 (d, C-F2), 106.4 (s, C-10), 104.4 (d, C-1⁗), 100.1 (d, C-1‴), 98.6 (d, C-1″), 94.8 (d, C-8), 83.0 (d, C-3‴), 81.5 (d, C-2″), 78.5, 78.2, 77.7 (d ×3, C-3⁗, C-5″, C-5⁗), 76.6 (d, C-3″), 74.9 (d, C-2⁗), 73.1 (d, C-2‴), 71.7, 71.5 (d ×2, C-4″, C-4⁗), 69.5 (d, C-4‴), 65.8 (t, C-5‴), 62.6, 62.3 (t ×2, C-6″, C-6⁗), 61.0 (q, C-6-OMe), 56.8 (t, C-3′-OMe), 56.3 (t, C-F3-OMe).

HMBC correlations (sequential correlations are shown in bold type for the relevant carbon): H-6-OMe: **C-6**; H-8: C-4/C-6/C-7/C-9/C-10; H-2′: **C-2**/C-3′/C-4′/C-6′; H-3′OMe: **C-3′**; H-5′: C-1′/C-3′/C-4′,/C-6′; H-6′: **C-2**/C-1′/C-2′/C-4′/C-5′; H-1″: **C-3**/C-3″; H-2″: C-1″/**C-1‴**; H-1‴: **C-2″**/C-2‴/C-3‴/C-5‴; H-2‴: **C-F9**; H-3‴: C-2‴/**C-1⁗**; H-5‴A: C-1‴/C-3‴/C-4‴; H-5‴B: C-1‴/C-3‴/C-4‴; H-1⁗: **C-3⁗**; H-F2: C-F1/C-F3/C-F4/C-F6/C-F7; H-F3-OMe: **C-F3**; H-F5: C-F1/C-F3/C-F4/C-F6; H-F6: C-F1/C-F2/C-F4/C-F5/C-F7; H-F7: C-F1/C-F2/C-F6/C-F8/C-F9; H-F8: C-F1/C-F7/C-F9.

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