

# Temporal synthesis and radiolabelling of the sorghum 3-deoxyanthocyanidin phytoalexins and the anthocyanin, cyanidin 3-dimalonyl glucoside

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## SUMMARY

Sorghum (*Sorghum bicolor*) synthesizes a complex mixture of 3-deoxyanthocyanidin phytoalexins in response to inoculation with the non-pathogenic fungus *Bipolaris maydis*. The anthocyanin cyanidin 3-dimalonyl glucoside, is also synthesized naturally in response to light. To determine the order and time of appearance of these compounds, etiolated sorghum mesocotyls were inoculated with *B. maydis* and tissue extracts were analysed by photodiode array-HPLC every 2 h for the first 24 h and at 48 h post inoculation (hpi). Uninoculated and inoculated etiolated mesocotyls were also allowed to incorporate L-[U-<sup>14</sup>C] phenylalanine. Apigeninidin appeared at 10 hpi, followed by luteolinidin and apigeninidin 5-O-arabinoside at 14 hpi. Luteolinidin 5-methylether was not detected until 18 hpi and apigeninidin 7-methylether not until 20 hpi. The concentrations of the primary phytoalexins, apigeninidin, luteolinidin and apigeninidin 5-O-arabinoside, rose steadily between 12 and 24 hpi, and the levels of apigeninidin and luteolinidin were approximately equivalent by 24 hpi. However, between 24 and 48 hpi luteolinidin and luteolinidin 5-methylether accumulated rapidly so that by 48 hpi the amounts of luteolinidin and luteolinidin 5-methylether had increased approximately twofold. Radiolabelling also showed that <sup>14</sup>C was incorporated into the 3-deoxyanthocyanidins and cyanidin 3-dimalonyl glucoside. Several other unidentified phenolic compounds also accumulated radioactivity.

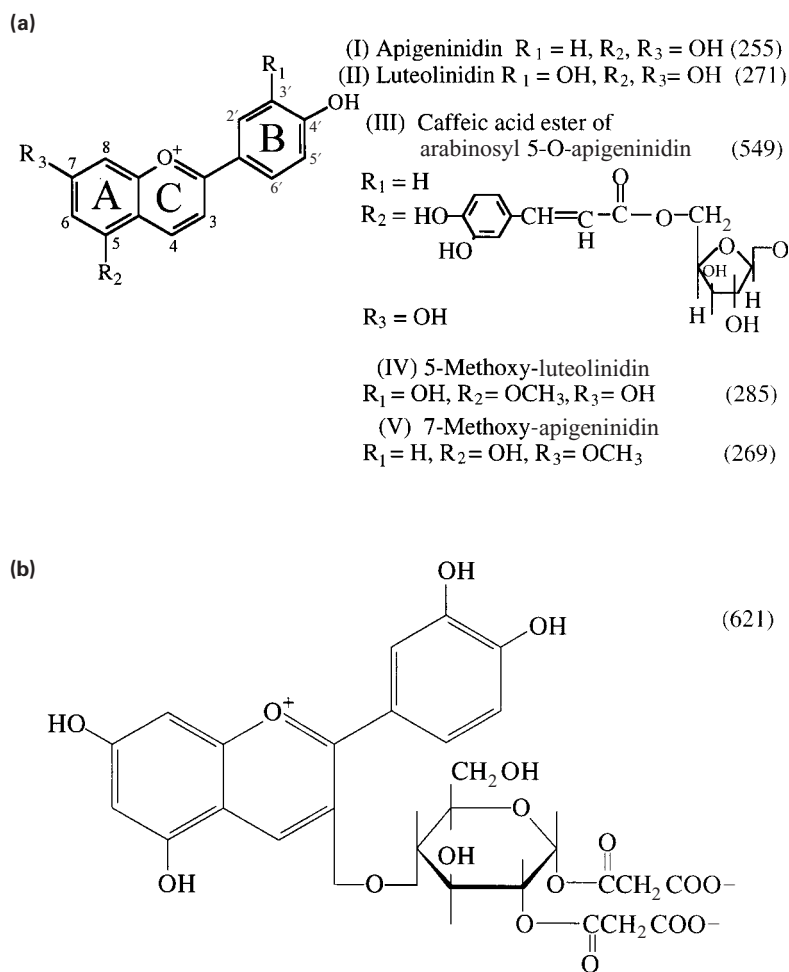
Key words: anthocyanin, apigeninidin, luteolinidin, MALDI-TOF, phytoalexin biosynthesis.

## INTRODUCTION

Plants synthesize a variety of secondary metabolites in response to a wide range of stimuli. In some cultivars of sorghum (*Sorghum bicolor* (L.) Moench), anthocyanin pigments are produced in response to light (Orczyk *et al.*, 1996; Weiergang *et al.*, 1996). Anthocyanins constitute a large class of flavonoid compounds and are one of the most important and widespread groups of plant pigments. They serve to attract animals for pollination and seed dispersal and are also believed to be important as protectants against UV irradiation (Stapleton & Walbot, 1994; Holton & Cornish, 1995). In response to attempted fungal infection by both pathogenic and non-pathogenic fungi, sorghum produces a complex mixture of flavonoid secondary metabolites. The major components of this mixture are a group of

structurally related compounds, the 3-deoxyanthocyanidins, apigeninidin, luteolinidin, luteolinidin 5-methylether, apigeninidin 7-methylether and the caffeic acid ester of arabinosyl 5-O-apigeninidin. This family of compounds is common only in the tropical New World angiosperm families Gesneriaceae and Bignoniaceae, where they function as flower pigments (Harborne, 1993). In the genus *Sorghum*, a member of the Poaceae, the 3-deoxyanthocyanidins serve as phytoalexins (Nicholson *et al.*, 1988; Hipskind *et al.*, 1990; Aida *et al.*, 1996; Lo *et al.*, 1996). Phytoalexins are low-molecular-weight antimicrobial compounds produced by plants in response to infection or stress (Nicholson & Hammerschmidt, 1992; VanEtten *et al.*, 1994; Smith 1996). In sorghum leaf tissue, these phytoalexins first appear in the cells which are being invaded, where they accumulate in inclusions in the cytoplasm (Snyder & Nicholson, 1990; Snyder *et al.*, 1991). The inclusions migrate to the site of attempted

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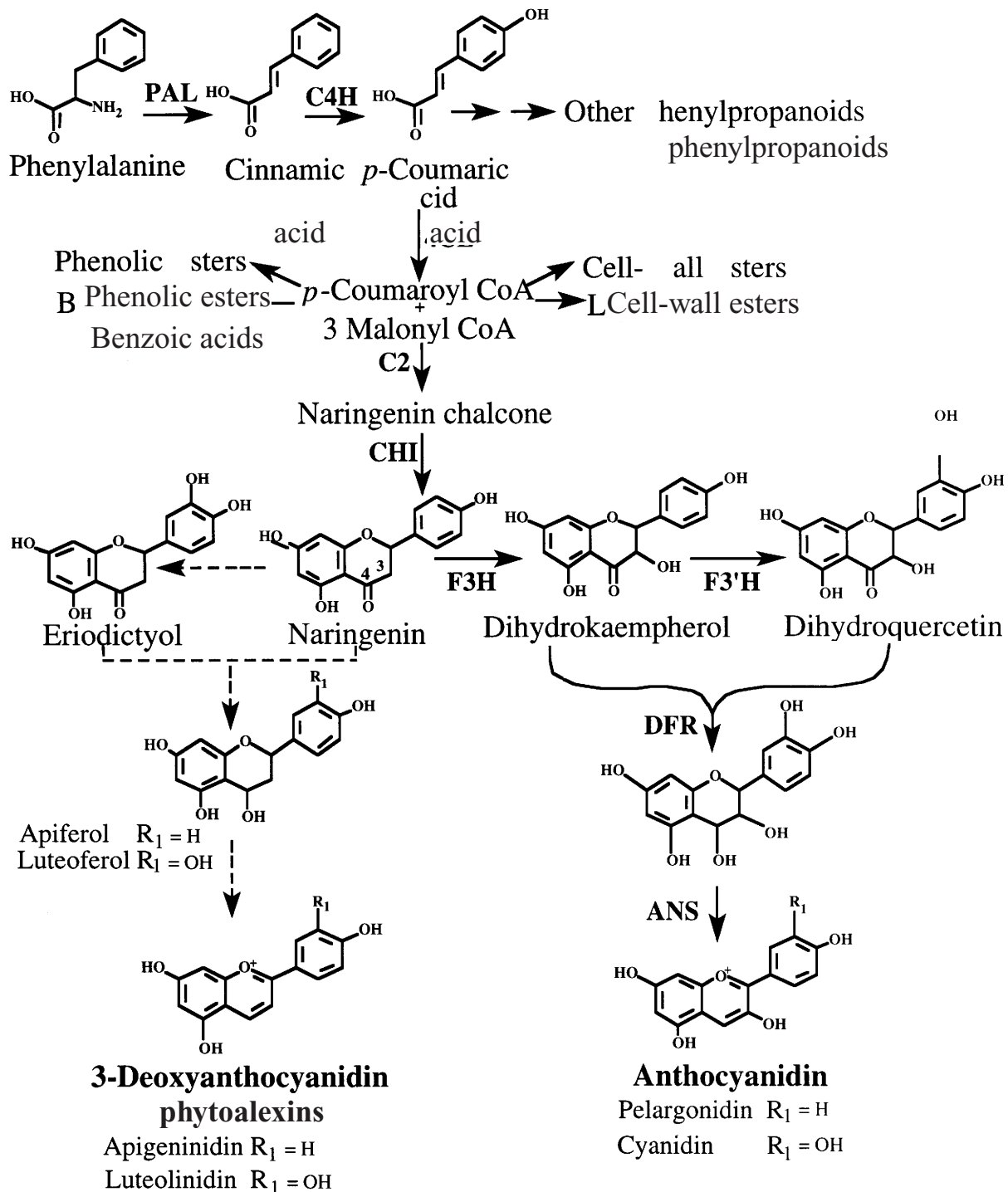


**Fig. 1.** (a) The 3-deoxyanthocyanidin phytoalexins and (b) cyanidin-3-dimalonyl glucoside. Molecular weight ( $M_r$ ) shown in brackets.

penetration, become pigmented, lose their spherical shape and ultimately release their contents into the cytoplasm, killing the cell and restricting further development of the pathogen.

The accumulation of 3-deoxyanthocyanidin phytoalexins is a site-specific response localized around the site of attempted fungal penetration (Nicholson *et al.*, 1987, 1988; Snyder & Nicholson, 1990). The accumulation of the phytoalexins occurs much more rapidly in infected cells of resistant cultivars than in susceptible cultivars, preventing the proliferation of fungal hyphae throughout the tissue (Wharton & Julian, 1996). However, most of the previous studies have been carried out at 24 h intervals after inoculation when the phytoalexins are present in easily detectable quantities. At present, no information is available on the temporal sequence of accumulation of these phytoalexins within the first 24 h after inoculation with either non-pathogenic or pathogenic fungi. It is not known whether the phytoalexins all appear at the same time or whether they appear in sequence. This information is important in order to obtain an understanding of the biochemical pathways involved in the synthesis of these compounds.

The 3-deoxyanthocyanidins are structurally very similar to the common 3-hydroxylated anthocyanidins (e.g. cyanidin) which are produced in response to light, but they lack the hydroxyl moiety at the number 3 carbon of the flavonoid oxygen heterocycle (Fig. 1). However, neither the exact route of biosynthesis nor intermediary compounds in the synthesis of these phytoalexins has been identified. Unlike the phytoalexin response, genetic studies have established the route of biosynthesis for the common anthocyanidins and anthocyanins in maize and numerous other plant species (Harborne & Grayer, 1988; Jayaran & Peterson, 1990). However, the enzyme activities corresponding to all of these genes have not been demonstrated (Heller & Forkmann, 1988; Ruhnau & Forkmann, 1988; Sitch & Forkmann, 1988). Anthocyanidins originate from the phenylpropanoid and flavonoid pathways via the flavanone naringenin (Fig. 2). Synthesis of 3-deoxyanthocyanidins has been suggested to involve reduction of naringenin to the flavan-4-ol apiferol (Fig. 2) on the basis that soluble protein extracts from flower petals of *Sinningia cardinalis* could reduce naringenin or eriodictyol to the flavan-4-ols, apiferol and luteoferol, respectively, in an NADPH-



**Fig. 2.** Biosynthetic pathways for anthocyanidins and 3-deoxyanthocyanidins. Both compounds are flavonoids and are derived from the phenylpropanoid pathway. The biosynthetic routes for 3-deoxyanthocyanidins and anthocyanidins are proposed to diverge from one another after the synthesis of naringenin. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-hydroxycinnamic acid:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; DFR, dihydroflavanone 4-reductase; ANS, anthocyanidin synthase. Dashed arrows indicate proposed metabolic steps.

dependent reaction (Stich & Forkmann, 1988). However, although this enzyme activity was presumed to represent a dihydroflavanone-4-reductase (DFR), no specific enzyme was isolated or characterized from the crude protein extract (Stich & Forkmann, 1988). On the basis of the identification of 3-deoxyanthocyanidin compounds following acid

hydrolysis of phlobaphenes from maize pericarp (Styles & Ceska, 1977), DFR is believed to catalyse the reduction of the 4-carbon on the oxygen heterocycle in both the anthocyanidin and the deoxyanthocyanidin pathways. However, biochemical analysis of extracts from the same maize lines by plasma desorption mass spectrometry (PDMS),

which is capable of detecting compounds present at the picogram level, could not detect any 3-deoxyanthocyanidins (Hipskind *et al.*, 1996). Furthermore, flavanone-3-hydroxylase (F3H) must be either absent or bypassed, because hydroxylation at the 3-carbon of the oxygen heterocycle would give rise to the normal 3-hydroxylated anthocyanidins. Thus, the biosynthesis of anthocyanidins and 3-deoxyanthocyanidin phytoalexins might represent two partially overlapping pathways (Fig. 2), with the conversion of naringenin to either a dihydroflavonol (e.g. dihydrokaempferol) or to a flavan-4-ol (e.g. apiferol) representing the branch point in the pathways (Dixon & Pavia, 1995; Holton & Cornish, 1995). This is consistent with the model proposed by Stafford (1990), on the basis of genetic and biochemical studies in maize which suggested that the enzymes encoded by the *A1* and *A2* genes, DFR and anthocyanin synthase (ANS), respectively, have broad substrate specificity that allow for the synthesis of both flavan-4-ols and flavan-3,4-diols and the subsequent formation of flavylum cations (Fig. 2). However, previous attempts to isolate DFR and ANS (*A1* and *A2*, respectively) in enzyme assays or corresponding cDNAs from crude sorghum plant extracts have been unsuccessful (Nicholson & Hipskind, 1996). Furthermore, recent studies have strongly suggested that neither the *A1* nor the *A2* gene is involved in 3-deoxyanthocyanidin synthesis and that synthesis of 3-deoxyanthocyanidins and 3-hydroxylated anthocyanidins in sorghum occurs by two distinct but competing biosynthetic and regulatory pathways (Hipskind *et al.*, 1996; Weiergang *et al.*, 1996; Lo & Nicholson, 1998). Moreover, it was shown that 3-deoxyanthocyanidins are not actually produced in maize, nor are they present naturally in maize pericarp (Hipskind *et al.*, 1996).

On the basis of our inability to assay for DFR or to demonstrate the involvement of *A1* and *A2* genes in plants synthesizing the 3-deoxyanthocyanidin phytoalexins (Hipskind *et al.*, 1996; Nicholson & Hipskind, 1996; Weiergang *et al.*, 1996; Lo & Nicholson, 1998), we believe that the identification of metabolic intermediates in the synthesis of the phytoalexins is critical to the elucidation of the route of 3-deoxyanthocyanidin synthesis.

There are no known reports of constitutive accumulation of naringenin or eriodictyol in sorghum (Stafford, 1998). Thus, the accumulation of the 3-deoxyanthocyanidin phytoalexins is likely to proceed through *de novo* synthesis, and the 3-deoxyanthocyanidin precursor compounds at any one time are likely to be present at extremely low concentrations or have a rapid turnover. In the present investigation, we have used L-[U-<sup>14</sup>C]-phenylalanine to trace intermediates in the phytoalexin pathway. A similar approach has been used previously by Preisig *et al.* (1990) for the characterization of pisatin isoflavonoid intermediates from

pea. In the experiments reported here, the order and time of appearance of the 3-deoxyanthocyanidin phytoalexins and the 3-hydroxylated anthocyanin cyanidin 3-dimalonyl glucoside within the first 24 h after inoculation of sorghum mesocotyls have been determined. Second, we have shown that <sup>14</sup>C is incorporated into both the 3-deoxyanthocyanidins and the 3-hydroxylated anthocyanin. In addition to incorporation into the 3-deoxyanthocyanidins and the 3-hydroxylated anthocyanin, radioactivity was also shown to accumulate in several, as yet unidentified, phenolic compounds. Knowledge of which 3-deoxyanthocyanidin is synthesized first may assist in the identification of the precursors and their temporal relationship in the biosynthesis of these compounds.

## MATERIALS AND METHODS

### *Plant material*

Seeds of sorghum (*Sorghum bicolor* L. Moench cv. DK46, Dekalb Pfizer Genetics, Lubbock, TX, USA) were imbibed in water for 12 h. The seeds were then planted in rolls of germination paper and incubated in darkness for 4 d at room temperature. This treatment produced seedlings with uniformly etiolated mesocotyls. For time course studies, the seedlings were inoculated as described later. For radioisotope tracer experiments, etiolated seedlings were excised 5 mm above the point of attachment to the seed and then trimmed another 5 mm from the cut end to remove vascular tissue containing air embolisms. The seedlings were then placed (two per well) in every other well of 96-well micro-titre plates containing either 250 µl L-[U-<sup>14</sup>C] phenylalanine (approx. 4 µCi ml<sup>-1</sup>; Amersham, Piscataway, NJ, USA) or distilled water (as a control). To prevent accidental spillage of the test solutions and to hold the mesocotyls upright, the micro-titre plates were covered with Parafilm and the mesocotyls were placed in the wells through holes poked in the Parafilm. This technique also produced an effective seal around the base of the mesocotyls preventing evaporation of the test solutions from the wells. The seedlings in the micro-titre plates were then inoculated as described in the following section.

### *Fungal culture and inoculation*

Phytoalexin biosynthesis was induced by inoculation of cv. DK46 with the fungus *Bipolar maydis* (Nisik. and Miy.) (perfect state = *Cochliobolus heterostrophus* (Drechs.) Drechs.), a non-pathogen of sorghum. This fungus was chosen because previous studies had shown its attempted penetration elicits an extremely rapid phytoalexin response (Nicholson *et al.*, 1987). Fungal cultures were maintained on

PDA (potato dextrose agar) under constant illumination at 25°C. For inoculation, conidial suspensions were prepared by flooding the surface of 7–14-d-old cultures with distilled water and then gently scraping the surface with a microscope slide to dislodge the conidia. The solution was filtered through muslin cloth to remove hyphal fragments, leaving the conidial suspension which was adjusted to approx. 50000 spores ml<sup>-1</sup> with distilled water containing Tween 20 at *c.* 0.01% (v/v). The resulting suspension was then misted onto etiolated mesocotyls with an atomizer, and the plants were incubated under constant illumination (60 μmol m<sup>-2</sup> s<sup>-1</sup>) at 25°C in a plastic bag to maintain 100% rh. For controls, plants were sprayed with distilled water (also containing Tween) rather than the conidial suspension.

#### *Temporal synthesis of anthocyanins and phytoalexins*

Triplicate samples of mesocotyl tissue were collected at 0 h, 10 h and then at 2 h intervals up to 24 h post inoculation (hpi). At each time interval, one sample was also collected from uninoculated tissue. The mesocotyls were excised 5 mm above the point of attachment to the seed and 5 mm below the coleoptile, and the coleoptile tissue was discarded. The remaining mesocotyl tissue was then weighed (approx. 20 mg), cut into 5 mm segments, and immediately placed in 1 ml of HPLC grade methanol contained in a micro-centrifuge tube. Anthocyanins and phytoalexins were allowed to leach from the tissue at 4°C for 24 h in the dark, after which time the extracts were pipetted into a fresh micro-centrifuge tube, evaporated down to 100 μl under a stream of N<sub>2</sub>, and analysed by HPLC and mass spectrometry.

#### *Extraction of radiolabelled plant tissue*

Triplicate samples of radiolabelled mesocotyl tissue were collected at 24 and 48 hpi. The mesocotyls were excised and the coleoptile tissue was discarded. The tissue was then weighed (approx. 20 mg), extracted into HPLC grade methanol and processed for analysis as already described.

#### *HPLC-radioisotope analysis*

The quantification and composition of plant extracts was determined by HPLC on two reverse-phase C-18 Beckman Ultrasphere columns (25 and 15 cm, respectively; Beckman, Fullerton, CA, USA) connected in tandem. Samples (20 μl) were eluted isocratically with 60% solvent A (0.6% perchloric acid) and 40% solvent B (methanol) at 0.8 ml min<sup>-1</sup>. Compounds absorbing in the range 200–600 nm were detected with a Beckman 168-photodiode-array detector connected to an IBM PC running the

Beckman Gold Nouveau® HPLC analysis software. Anthocyanin concentrations were estimated based on absorption coefficients of 34700 M<sup>-1</sup> cm<sup>-1</sup> for cyanidin chloride (Fuleki & Francis, 1968), and phytoalexin concentrations were determined based on absorption coefficients of 13 800 M<sup>-1</sup> cm<sup>-1</sup> for luteolinidin, 18000 M<sup>-1</sup> cm<sup>-1</sup> for apigeninidin (Stafford, 1965), and 12700 M<sup>-1</sup> cm<sup>-1</sup> for the caffeic acid ester of arabinosyl-5-O-apigeninidin (Hipskind *et al.*, 1990). The extinction coefficients of luteolinidin and apigeninidin were also used to estimate the concentrations of their methylated derivatives (luteolinidin 5-methylether and apigeninidin 7-methylether, respectively) (Lo *et al.*, 1996). Retention times and UV and visible-spectra were used to identify the flavonoid compounds in test samples by comparison with those of anthocyanin and phytoalexin standards. Standards included luteolinidin, apigeninidin, the caffeic acid ester of arabinosyl-5-O-apigeninidin, each of which had been isolated or synthesized previously in this laboratory (Hipskind *et al.*, 1990), and commercially available cyanidin chloride (ICN, Irvine, CA, USA).

Chromatographic separation of radiolabelled plant extracts was achieved by HPLC as already described, and the <sup>14</sup>C radiolabelled compounds in the plant extracts were detected with a Beckman 171-radioisotope detector coupled to the Beckman 168 photodiode-array detector. Data from the radioisotope detector were also collected and analysed with the Gold Nouveau HPLC analysis software.

#### *Thin layer chromatography (TLC)-autoradiography*

Plant extracts, dissolved in approx. 30 μl of HPLC grade methanol, were subjected to TLC on 20 × 20 cm silica gel 60 F254 TLC plates (Alltech Associates, Inc., Deerfield, IL, USA) with a solvent system of ethyl acetate:formic acid:water:concentrated HCl (85:6:8:1). This solvent system is most suitable for the separation of the 3-deoxyanthocyanidins (Hipskind *et al.*, 1990). The separated bands were visualized with UV (longwave and shortwave) and visible light and circled with a pencil. The plates were then placed in a Packard electronic autoradiographic imager (Packard, Downer Grove, IL, USA) to produce an image of any radioactive bands present. The positions of the radioactive bands were then compared with those visible under UV and/or visible light to determine which of the separated plant extracts were radiolabelled. The identity of those radioactive bands presumed to be deoxyanthocyanidin phytoalexins was determined by comparison of R<sub>F</sub> values with available standards. The bands were extracted into HPLC grade methanol, and analysed by HPLC. Bands corresponding to labelled bands, but derived from extracts of plants that had not received L-[U-<sup>14</sup>C]-phenylalanine, were also

extracted into methanol and analysed by Matrix-assisted-laser-desorption-ionization coupled with time-of-flight mass analysis (MALDI-TOF) mass spectrometry.

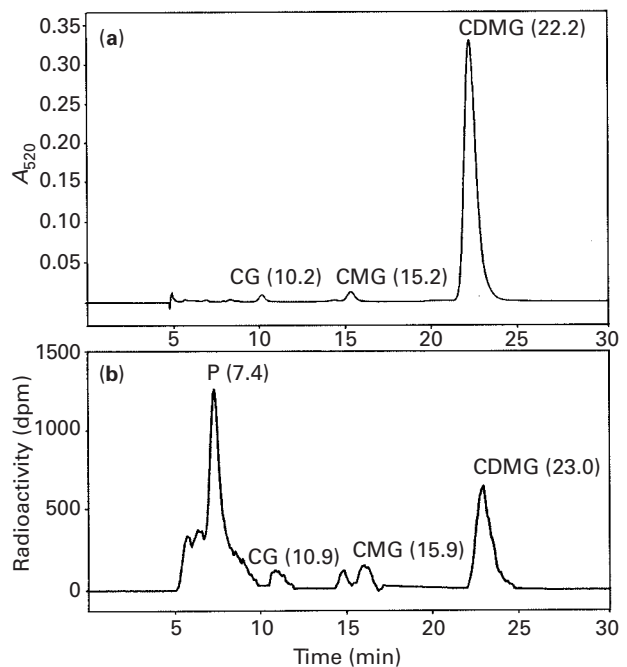
*Matrix-assisted-laser-desorption-ionization coupled with time-of-flight mass (MALDI-TOF) analysis*

MALDI-TOF has been used for routine mass spectrometric analysis of a wide range of biomolecules, including peptides and proteins, oligonucleotides, oligosaccharides, glycoproteins, and flavonoids (Edmondson & Russell, 1996; Sugui *et al.*, 1998). MALDI is considered a soft desorption ionization technique that enables molecular weight ( $M_r$ ) information to be obtained on both fragile and non-volatile high-mass molecules. This occurs because samples are included in crystals of a matrix material, usually an organic acid, which absorbs a substantial amount of the laser energy and allows for a soft desorption ionization of the sample. Plant extracts were analysed at the Purdue Mass Spectrometry Center with a PerSeptive Biosystems (Framingham, MA, USA) Voyager MALDI-TOF instrument operating in a positive ion linear mode with a nitrogen laser (387 nm) at an accelerating voltage of 28 kV. For sample preparation, the matrix,  $\alpha$ -cyano-4-hydroxycinnamic acid (10 mg), was solubilized in 1 ml of a mixture of water, 1% trifluoroacetic acid and acetonitrile (4:1:5). The compounds of interest were then mixed with the matrix for 1 min (in a ratio of 2 parts matrix:1 part sample), after which 2  $\mu$ l of the mixture were applied to the sample plate and the solvents allowed to evaporate at 25°C. If crystals did not form, then the ratio of matrix to sample was adjusted until the resulting mixture crystallized.

RESULTS

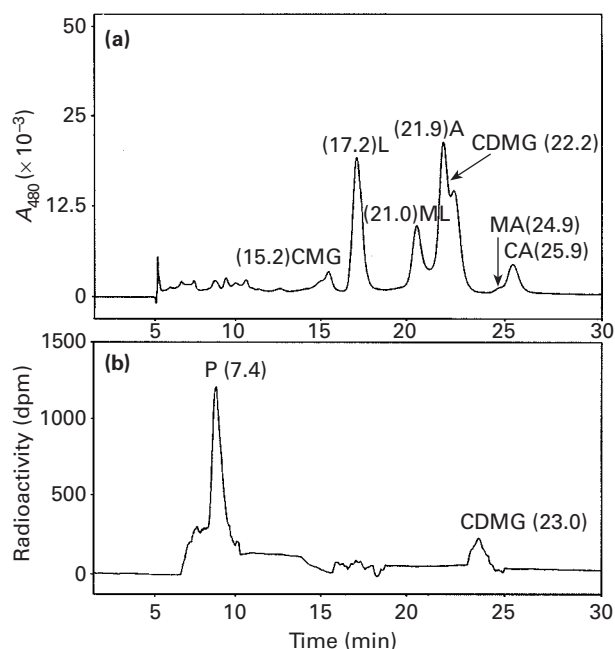
*Analysis of anthocyanin and phytoalexin pigments*

Analysis by HPLC photodiode array of extracts from uninoculated sorghum cv. DK46 sampled 24 h after exposure to light, revealed three main compounds absorbing in the visible range 400–600 nm. These three compounds had retention times of 10.2, 15.2 and 22.2 min respectively, and their UV spectra, generated by the photodiode array, showed that they absorbed most strongly at 520 nm (Fig. 3a). The relatively high retention times of 15.2 and 22.2 min are indicative of acylated anthocyanins (Harborne & Grayer, 1988). Comparison of the UV and/or visible spectrum of the peak at 22.2 min to that of cyanidin chloride revealed a 90% similarity. MALDI-TOF analysis of these extracts using the method of Sugui *et al.* (1998) showed three major ions with molecular masses of 621, 535 and 287 (Fig. 5a). Previous results



**Fig. 3.** Biochemical analysis of extracts from uninoculated sorghum mesocotyls of cv. DK46, 24 h after exposure to light. HPLC photodiode array analysis (a) revealed three main compounds absorbing in the visible range 400–600 nm but most strongly at 520 nm. A major peak (22.2 min), corresponding to cyanidin 3-dimalonyl-glucoside (CDMG) was observed, along with two minor peaks (10.2 and 15.2 min) corresponding to cyanidin 3-O-glucoside (CG) and cyanidin 3-malonyl-glucoside (CMG), respectively. Radioisotope analysis (b) showed four peaks (7.4, 10.9, 15.9 and 23 min) which were detected *c.* 0.7 min after detection of the anthocyanins by photodiode array. This time delay was approximately equivalent to the lag time between the two detectors. The major peak, P, corresponds to phenylalanine.

have shown that the 535 mass to charge ratio ( $m/z$ ) and 621  $m/z$  ions correspond to mono- and dimalonyl derivatives of cyanidin 3-O-glucoside, previously identified in sorghum and maize (Hipskind *et al.*, 1996; Lo & Nicholson, 1998). The 287  $m/z$  ion is the aglycone cyanidin, which is generated by MALDI. The two minor peaks in Fig. 3a at 10.2 and 15.2 min are thought to represent cyanidin 3-O-glucoside and cyanidin 3-malonyl-glucoside, respectively. The presence of the non-acylated glucose moiety would make this compound less hydrophobic and decrease its retention time. The peaks of cyanidin 3-O-glucoside and cyanidin-3-monomalonyl glucoside, seen in Fig. 3a, are thought to be artefacts, formed from cyanidin-3-dimalonyl glucoside as a result of hydrolysis by the highly acidic HPLC solvent system a single peak splits into two because the anthocyanins were extracted into 100% methanol, which is a stronger solvent than that of the isocratic HPLC-column solvent condition (Hipskind *et al.*, 1996). Samples taken at 48 hpi revealed identical results to those observed at 24 hpi, except that the amount of the compounds was greater.



**Fig. 4.** Biochemical analysis of extracts from inoculated sorghum mesocotyls of cv. DK46, 24 h after inoculation with *B. maydis*. HPLC photodiode array analysis (a) revealed four main compounds, along with the anthocyanins absorbing in the visible range 400–600 nm but most strongly at 480 nm. These peaks corresponded to the 3-deoxyanthocyanidin phytoalexins, luteolinidin (L, 17.2 min), luteolinidin 5-methyl ether (ML, 21.0 min), apigeninidin (A, 21.9 min), apigeninidin 7-methyl ether (MA, 24.9 min), and the caffeic acid ester of arabinosyl-5-O-apigeninidin (CA, 25.9 min). Cyanidin mono- (CMG) and dimalonil-glucoside (CDMG) had retention times of 15.1 and 22.1 min, respectively. Radioisotope analysis (b) showed two peaks (7.4 and 23 min) which were detected *c.* 0.8 min after detection of cyanidin 3-dimalonyl glucoside by photodiode array. This time delay was approximately equivalent to the lag time between the two detectors. The major peak, P, corresponds to phenylalanine.

HPLC photodiode array analysis of extracts taken from inoculated mesocotyls of cv. DK46 24 hpi revealed five compounds in addition to the anthocyanins (Fig. 4a). These compounds all absorbed most strongly *c.* 480 nm and were identified as the 3-deoxyanthocyanidins (Fig. 4a), luteolinidin (17.2 min), luteolinidin 5-methylether (21.0 min), apigeninidin (21.9 min), apigeninidin 7-methylether (24.9 min), and the caffeic acid ester of arabinosyl 5-O-apigeninidin (25.9 min). MALDI-TOF analysis of the plant extracts from the inoculated mesocotyls (Fig. 5b) also revealed the anthocyanin ions of 621 *m/z*, 535 *m/z* and 287 *m/z*, as well as the 3-deoxyanthocyanidin ions of apigeninidin (255 *m/z*), apigeninidin 7-methylether (269 *m/z*), luteolinidin (271 *m/z*), and luteolinidin 5-methylether (285 *m/z*). Although HPLC analysis revealed the presence of the caffeic acid ester of arabinosyl 5-O-apigeninidin, this compound was not detected by MALDI-TOF analysis. Samples taken at 48 hpi revealed identical results to those observed at 24 hpi, except that the compounds were in greater amounts.

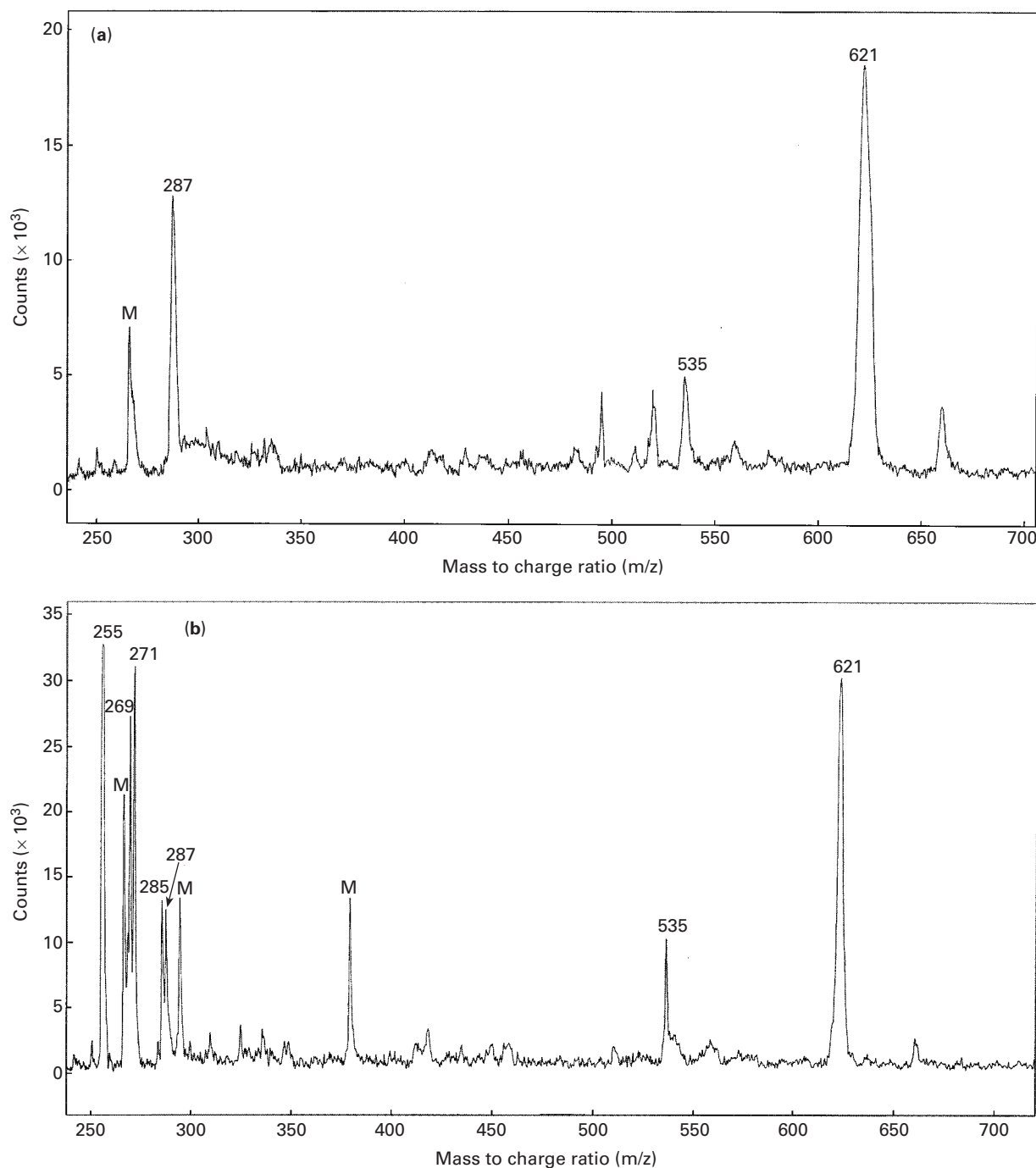
#### *Quantification of anthocyanin and 3-deoxyanthocyanidin production*

Anthocyanins in plant extracts are usually measured quantitatively by their absorbance between 500–550 nm (Strack & Wray, 1989). However, because the pigmented 3-deoxyanthocyanidins in extracts from inoculated tissue would have interfered with the spectrophotometric measurement of anthocyanins, the anthocyanins were quantified by HPLC using the absorption coefficient of cyanidin chloride (Fuleki & Francis, 1968). Accumulation of the anthocyanin, cyanidin 3-dimalonyl glucoside, in uninoculated and inoculated DK46 mesocotyls is shown in Fig. 6. The anthocyanin was first detected in uninoculated tissue 10 h after exposure to light and accumulated through 48 h post-illumination. Anthocyanin accumulation was first detected 14 hpi in inoculated tissue and the amount and rate of accumulation was much lower compared with that in uninoculated tissue (Fig. 6). By 24 hpi, the total amount of anthocyanin that had accumulated was *c.* eight times less than that in uninoculated control plants (Fig. 6).

Accumulation of the 3-deoxyanthocyanidin phytoalexins in inoculated plant mesocotyls is shown in the inset of Fig. 6. None of the 3-deoxyanthocyanidins accumulated in uninoculated tissue. In inoculated tissue, apigeninidin was the first 3-deoxyanthocyanidin detected (at 10 hpi) followed by luteolinidin and the caffeic acid ester of arabinosyl 5-O-apigeninidin, which were both first detected at 14 hpi. Luteolinidin 5-methylether was not detected until 18 hpi and apigeninidin 7-methylether not until 20 hpi. The concentrations of the three major phytoalexins (apigeninidin, luteolinidin and the caffeic acid ester of arabinosyl 5-O-apigeninidin) rose steadily between 12 and 24 hpi with those of apigeninidin and its caffeic acid ester being higher than that of luteolinidin. However, the concentration of luteolinidin started to increase more rapidly by 18 hpi so that by 24 hpi the amounts of apigeninidin and luteolinidin were about the same, and by 48 hpi the concentration of luteolinidin was approximately twice that of apigeninidin. The concentrations of luteolinidin 5-methylether and apigeninidin 7-methylether remained very low for the first 24 h. However, the accumulation of luteolinidin 5-methylether increased rapidly between 24 and 48 hpi so that, by 48 hpi, the concentrations of luteolinidin 5-methylether and the caffeic acid ester of 5-O-arabinosyl apigeninidin were approximately the same.

#### *Radioisotope analysis*

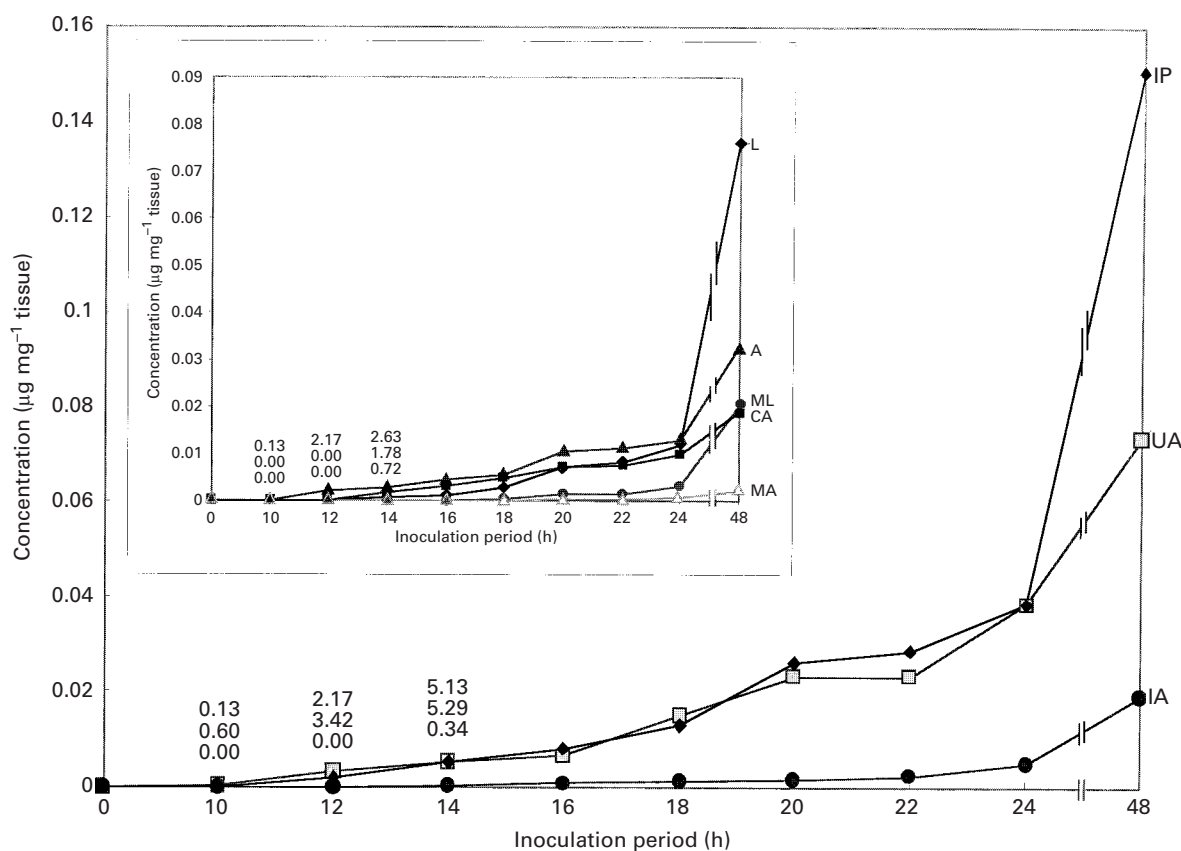
Radiolabelled plant extracts were separated by HPLC. Radioisotope analysis of extracts from uninoculated sorghum cv. DK46 at 24 h after



**Fig. 5.** Matrix-assisted-laser-desorption-ionization coupled with time-of-flight mass (MALDI-TOF) analysis of sorghum cv. DK46 mesocotyl extracts. (a) Pigments extracted from uninoculated plants 24 h after exposure to light. A major ion at 621  $m/z$  (mass to charge ratio) and two minor ions at 287 and 535  $m/z$  were detected; 621 and 535  $m/z$  correspond to the  $M_r$  of dimalonyl and monomalonyl derivatives of cyanidin 3-glucoside respectively, where 287  $m/z$  corresponds to the aglycone cyanidin. (b) Pigment extracted from plants 24 h after inoculation with *B. maydis* and exposure to light. Major ions corresponding to the molecular masses of luteolinidin (271  $m/z$ ), luteolinidin 5-methylether (285  $m/z$ ), apigeninidin (255  $m/z$ ) and apigeninidin 7-methylether (269  $m/z$ ), were detected in addition to the anthocyanin ions. M, Matrix peaks.

exposure to light showed four main peaks at 7.4, 10.9, 15.9 and 23 min, respectively (Fig. 3b). The peak at 7.4 min was shown to co-chromatograph with authentic  $^{14}\text{C}$ -phenylalanine, and the lag between the HPLC photodiode array detector and the radioisotope detector was established at approx. 0.7 min. The other three peaks detected were shown

to correspond to the anthocyanin, cyanidin 3-dimalonyl glucoside (23 min), and its derivatives cyanidin 3-monomalonyl glucoside (15.9 min) and cyanidin 3-O-glucoside (10.9 min). It should be noted that the cyanidin 3-O-glucoside and the cyanidin monomalonyl glucoside are artefacts as already described in the 'Analysis of anthocyanin



**Fig. 6.** Accumulation of anthocyanins and total phytoalexins in etiolated mesocotyls of sorghum cv. DK46 between 0 and 24 h after exposure to light or after inoculation with *Bipolaris maydis*. UA, total anthocyanins from uninoculated plants (grey square); IA, total anthocyanins from inoculated plants (closed circle); IP, total 3-deoxyanthocyanidins from inoculated plants (closed diamond). The values for phytoalexins are the sum of the mean values for individual phytoalexins at each time. The values above time points 10, 12 and 14 represent the mean concentrations for IP, UA and IA in  $\text{ng mg}^{-1}$  tissue, respectively. Inset: accumulation of individual 3-deoxyanthocyanidin phytoalexins between 0 and 24 h after inoculation. A, apigeninidin (closed triangle); CA, caffeic acid ester of arabinosyl-5-O-apigeninidin (closed square); L, luteolinidin (closed diamond); ML, luteolinidin 5-methylether (closed circle); MA, apigeninidin 7-methylether (open triangle).

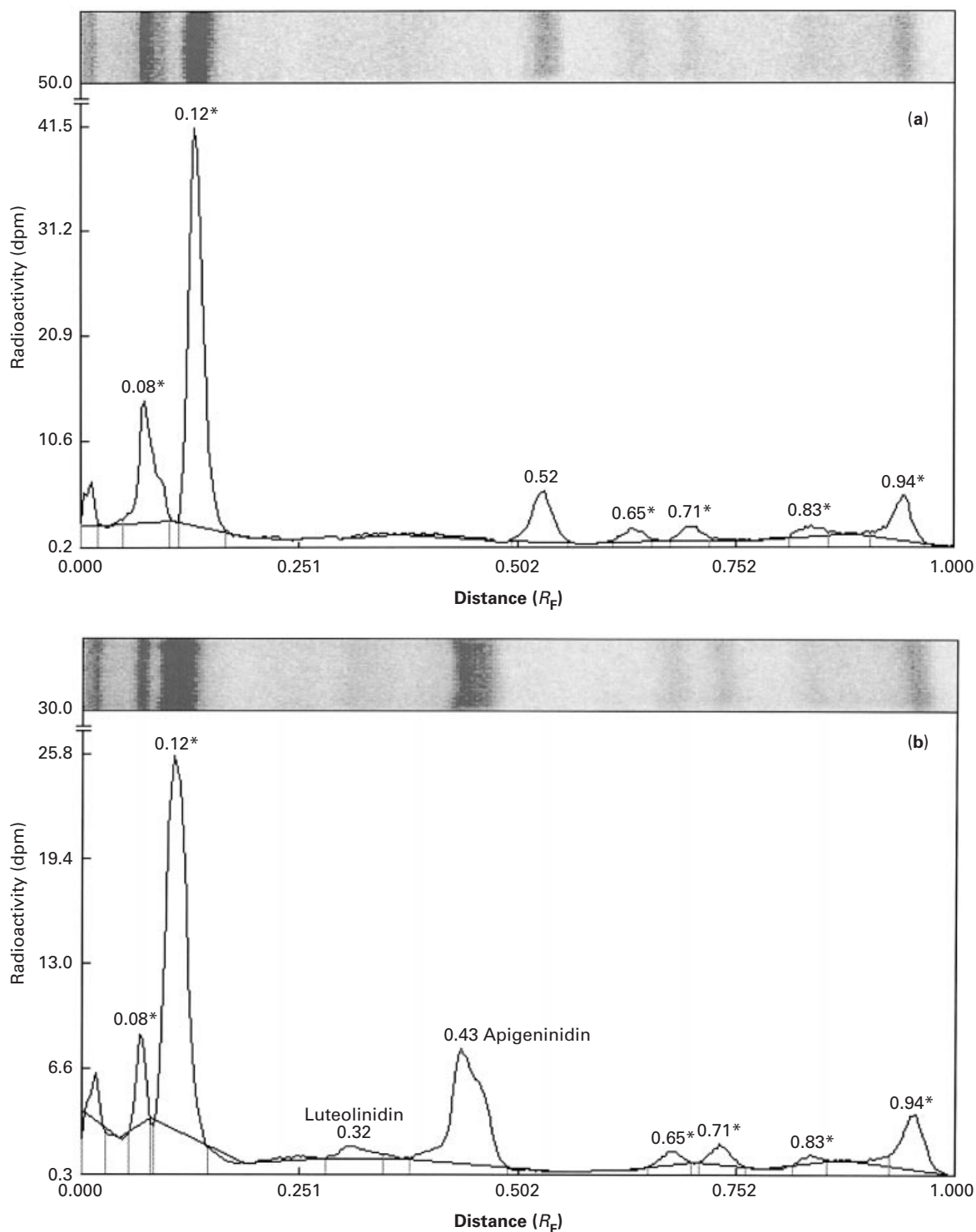
and phytoalexin pigments' section. These radio-labelled anthocyanins were also detected at 48 h after exposure to light. The 24 and 48 h samples differed only in that the amount of  $^{14}\text{C}$  incorporated into the anthocyanin increased twofold.

In plant extracts from inoculated mesocotyls of cv. DK46 at 24 hpi, cyanidin 3-dimalonyl glucoside and the known 3-deoxyanthocyanidin phytoalexins were easily detected by HPLC photodiode array analysis (Fig. 4a). However, simultaneous radioisotope analysis of these samples showed only two radioactive peaks, at 7.4 and 23 min (Fig. 4b) which were identified as phenylalanine and cyanidin 3-dimalonyl glucoside as already described. No radiolabelled phytoalexins were detected. However, when extracts from inoculated plants were subjected to TLC and assayed by electronic autoradiography, the 3-deoxyanthocyanidin phytoalexins, as well as a number of unidentified compounds, were clearly evident (Fig. 7b). This suggests that the  $^{14}\text{C}$  must have been incorporated into other compounds that were present at concentrations too low to be detected by the radioisotope detector connected to the HPLC.

Furthermore, the amounts of  $^{14}\text{C}$ -labelled cyanidin 3-dimalonyl glucoside detected by the radioisotope detector were also much lower in inoculated mesocotyls than in uninoculated controls (cf. Figs 3b and 4b), suggesting that  $^{14}\text{C}$  had been diverted into other compounds. As in the extracts from uninoculated plants, radiolabelled anthocyanins were also detected at 48 hpi. Again, the only difference between the 24 and 48 h samples was that the amount of  $^{14}\text{C}$  incorporated into the anthocyanin increased twofold.

#### *Thin layer chromatography (TLC) autoradiography*

Analysis of autoradiographs of TLC plates on which extracts from uninoculated plants had been separated revealed the presence of seven distinct bands at 24 (Fig 7a) and 48 h after the plants were exposed to light. Autoradiography of TLC plates on which extracts from inoculated plants sampled at 24 h had been separated, revealed the presence of eight distinct bands (Fig. 7b). In extracts from both uninoculated and inoculated plants, the pattern of



**Fig. 7.** Thin layer chromatography (TLC)-autoradiography of plant extracts from (a) uninoculated and (b) inoculated sorghum plants 24 h after exposure to light. Six radiolabelled bands (\*) were common to both uninoculated and inoculated extracts. Of these six bands, bands with  $R_F$  values of 0.08 and 0.12 were identified as containing mono- and di-malonyl derivatives of cyanidin 3-O-glucoside. Values above the peaks are  $R_F$  values.

radioactive bands at 48 h was identical to that at 24 h except that the radioactivity in the bands was significantly greater. Comparison of the autoradiographs revealed that six bands were common to samples from both uninoculated and inoculated plants. These bands had  $R_F$  values of *c.* 0.08, 0.12, 0.65, 0.71, 0.83 and 0.94, respectively. Of the bands

common to both uninoculated and inoculated plants, those with  $R_F = 0.08$  and  $R_F = 0.12$  were identified by HPLC analysis as containing the mono- and di-malonyl derivatives of cyanidin 3-O-glucoside, respectively. This was confirmed by MALDI-TOF analysis of the equivalent bands from unlabelled plant extracts. None of the other common bands was

detected by MALDI-TOF mass spectrometry. The phytoalexins, luteolinidin and apigeninidin, with  $R_F$  values of 0.32 and 0.43, respectively, were found to be present only in extracts from inoculated tissue (Fig. 7b); their identity was confirmed by both HPLC and MALDI-TOF analysis.

#### DISCUSSION

The purpose of the present investigation was threefold: to determine the order and time of the first appearance of the 3-deoxyanthocyanidin phytoalexins and the anthocyanin cyanidin 3-dimalonyl glucoside; to determine whether L-[U- $^{14}$ C]-phenylalanine is an effective precursor; and, then, to determine whether HPLC/radioisotope analysis or TLC/autoradiography would be sufficiently sensitive to detect radioactivity either in the phytoalexins or in potential metabolic intermediates. The latter was important because earlier attempts to elucidate the biochemical route of synthesis have been unsuccessful, including investigations at the molecular and enzymic levels (Hipskind *et al.*, 1996; Nicholson & Hipskind, 1996; Lo & Nicholson, 1998; Weiergang *et al.*, 1996; Orczyk *et al.*, 1996). In spite of these problems, we recently presented evidence strongly suggesting that, when exposed to an inducer, sorghum 'shuts down' the synthesis of anthocyanin pigments, in favour of 3-deoxyanthocyanidin phytoalexin synthesis (Lo & Nicholson, 1998). By contrast, synthesis of anthocyanidins and/or anthocyanins occurs naturally in sorghum as a response to light (Weiergang *et al.*, 1996; Lo & Nicholson, 1998). These observations allowed us to distinguish the branch points of the syntheses of the normal 3-hydroxylated anthocyanins and the unusual 3-deoxyanthocyanidins.

In the present study, we have documented the sequence of appearance of the 3-deoxyanthocyanidin phytoalexins following inoculation and have demonstrated that radiolabelling facilitates compound detection and isolation. These experiments also demonstrated that the radioisotope was incorporated in other, as yet unidentified, phenolic compounds (Fig. 7). That these compounds are phenols was ascertained by comparison of their absorption spectra with those published for various flavonoids and phenylpropanoids (Markham, 1982; Harborne & Grayer, 1988). It is not known whether these compounds represent metabolic intermediates.

The first phytoalexin to appear after inoculation was apigeninidin, the simplest and most hydrophilic of the phytoalexins. The next compound to appear was the caffeic acid ester of arabinosyl 5-O-apigeninidin, followed closely by increasing levels of luteolinidin. The methoxylated derivatives of apigeninidin and luteolinidin, apigeninidin 7-methylether and luteolinidin 5-methylether, respectively, did not accumulate to detectable levels until *c.*

18 hpi. The concentrations of these two compounds remained very low ( $<0.2$  ng  $\text{mg}^{-1}$  tissue) for the first 24 h. The concentrations of both luteolinidin and luteolinidin 5-methylether rose dramatically between 24 and 48 hpi. Thus, the 3-deoxyanthocyanidins accumulate sequentially, with the less toxic apigeninidin-based compounds appearing as early as 10 hpi (at  $<1$  ng  $\text{mg}^{-1}$  tissue).

Luteolinidin and luteolinidin 5-methylether are significantly more fungitoxic than the other 3-deoxyanthocyanidin phytoalexins (Lo *et al.*, 1996; Nicholson *et al.*, 1987). The rapid increase in accumulation of the two luteolinidin compounds between 24 and 48 hpi is significant, as it corresponds to the time of attempted penetration and intracellular growth of the fungal pathogen (Wharton & Julian, 1996). These authors showed that in the resistant cv. SC748-5, concentrations of the phytoalexins increased rapidly between 18 and 42 hpi, whereas in the susceptible cv. KAD 332, phytoalexin accumulation did not begin until 66 hpi. Moreover, the phytoalexin inclusions in the resistant cultivar were more darkly pigmented than those in the susceptible strain. Recent studies on the resistant cv. SC748-5 and the susceptible cv. BTx623 have shown that the luteolinidin compounds were present only in the resistant cultivar (Lo *et al.*, 1999). The presence of luteolinidin compounds in the resistant cultivar could therefore account for the much darker pigmented inclusions, since luteolinidin has a higher absorption maximum (495 nm in methanol) than apigeninidin and its compounds (477-480 nm in methanol), and appears a dark rose colour under visible light (Harborne, 1967; Nicholson *et al.*, 1987).

In sorghum, resistance may thus be correlated to the more complex phytoalexin compounds, in particular luteolinidin and luteolinidin 5-methylether, which appear to be synthesized only in incompatible interactions. This is consistent with previous studies which have shown that methoxylated flavonoid compounds are more fungitoxic than their non-methoxylated precursors (VanEtten *et al.*, 1980; Kodama *et al.*, 1992; Aida *et al.*, 1996).

Luteolinidin differs from apigeninidin by *o*-dihydroxylation of the B ring (Fig. 1a), mediated by a flavonoid 3'-hydroxylase. In maize, this enzyme has been identified as a cytochrome P-450 monooxygenase and was shown to have a wide range of flavonoid substrates, including naringenin, apigenin and kaempferol (Larson & Bussard, 1986). Therefore, 3'-hydroxylation could occur at any point during the synthesis of 3-deoxyanthocyanidins. However, since *o*-methylation generally takes place near the end of biosynthetic pathways (Tobias & Larson, 1991; Reinecke & Kindl, 1994), luteolinidin 5-methylether is more likely to be synthesized from luteolinidin. This could explain the low levels of accumulation of methoxylated phytoalexins during

the first 24 hpi. Knowing the sequence of accumulation of the 3-deoxyanthocyanidins allows speculation about the biosynthetic pathway leading to phytoalexin formation. This may, in the future, enable the production of sorghum cultivars with enhanced levels of the more active phytoalexin constituents.

In addition to providing insights into the timing of synthesis of the various 3-deoxyanthocyanidin phytoalexins, the results presented also confirm our previous suggestion that sorghum 'shuts down' the synthesis of anthocyanin pigments in favour of phytoalexin synthesis (Lo & Nicholson, 1998). Concentrations of anthocyanin decrease significantly in inoculated plants but not in uninoculated plants (Fig. 6). These observations were further supported by radioisotope analysis, which showed that the concentration of  $^{14}\text{C}$ -labelled anthocyanins in inoculated plants was approximately half that in uninoculated plants (cf. Figs 3 and 4, and cf. Fig. 7a and b).

Analysis of  $^{14}\text{C}$ -labelled extracts from uninoculated and inoculated plants by TLC and autoradiography confirmed the results of the HPLC-based radioisotope analysis. Furthermore, TLC revealed several unknown phenolic compounds that were not present at sufficiently high concentrations for detection by the HPLC-radioisotope detector. However, even though the 3-deoxyanthocyanidins were not detected by the radioisotope detector, the main phytoalexins, apigeninidin and luteolinidin, were easily detectable by autoradiography. The identity of the radioactive bands corresponding to these compounds was confirmed by MALDI-TOF analysis of equivalent bands from TLC of unlabelled extracts. TLC and/or autoradiography, combined with MALDI-TOF analysis, therefore provides a rapid, reliable, and highly sensitive means of detection and identification of the 3-deoxyanthocyanidin phytoalexins.

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