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Occurrence and formation of indole-3-acetamide in *Arabidopsis thaliana*

Abstract An HPLC/GC–MS/MS technique (high-pressure liquid chromatography in combination with gas chromatography–tandem mass spectrometry) has been worked out to analyze indole-3-acetamide (IAM) with very high sensitivity, using isotopically labelled IAM as an internal standard. Using this technique, the occurrence of IAM in sterile-grown *Arabidopsis thaliana* (L.) Heynh. was demonstrated unequivocally. In comparison, plants grown under non-sterile conditions in soil in a greenhouse showed approximately 50% higher average levels of IAM, but the differences were not statistically significant. Thus, microbial contributions to the IAM extracted from the tissue are likely to be minor. Levels of IAM in sterile-grown seedlings were highest in imbibed seeds and then sharply declined during the first 24 h of germination and further during early seedling development to remain below 20–30 pmol g^{-1} fresh weight throughout the rosette stage. The decline in indole-3-aetic acid (IAA) levels during germination was paralleled by a similar decline in IAM levels. Recombinant nitrilase isoforms 1, 2 and 3, known to synthesize IAA from indole-3-acetonitrile, were shown to produce significant amounts of IAM in vitro as a second end product of the reaction besides IAA. *NIT*2 was earlier shown to be highly expressed in developing and in mature *A. thaliana* embryos, and *NIT*3 is the dominantly active gene in the hypocotyl and the cotyledons of young, germinating seedlings. Collectively, these data suggest that the elevated levels of IAM in seeds and germinating seedlings result from nitrilase action on indole-3-acetonitrile, a metabolite produced in the plants presumably from glucobrassicin turnover.

Dedicated to Prof. Dr. Nikolaus Amrhein, Zurich, on the occasion of his 60th birthday.

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Keywords *Arabidopsis* · $[^2H]_5$-Labelled indole-3-acetamide · Indole-3-acetamide · Indole-3-acetic acid · Indole-3-acetonitrile · Nitrilase

Abbreviations GC–MS/MS: gas chromatography–tandem mass spectrometry · FW: fresh weight · IAA: indole-3-acetic acid · IAM: indole-3-acetamide · IAN: indole-3-acetonitrile · TFA(A): trifluoroacetic acid (anhydride) · TMO: tryptophan-2-monooxygenase

Introduction

Despite decades of work, it is still uncertain how plants synthesize their major growth hormone, indole-3-acetic acid (IAA). The only biosynthetic route to IAA that has been worked out definitely is the bacterial indole-3-acetamide (IAM) pathway catalyzed by tryptophan-2-monooxygenase (L-tryptophan → IAM) and indole-3-acetamide hydrolase (IAM → IAA) operating in a number of bacteria including the genera *Agrobacterium* (for review: Weiler and Schröder 1987), *Azospirillum* (Bar and Okon 1993), *Pseudomonas* (Magie et al. 1963) and *Streptomyces* (Manulis et al. 1994). It is widely held that this pathway is not operative in higher plants, despite reports of IAM-hydrolyzing activity in some species including rice (Kawaguchi et al. 1991) and *Poncirus trifoliata* (Kawaguchi et al. 1993), and reports on the occurrence of IAM in *Prunus jamasakura* (Saotome et al. 1993) and *Citrus unshiu* (Igoshi et al. 1971; Takahashi et al. 1975).

The powerful tools of molecular biology and genetics, and the availability of the complete genome sequence have made *Arabidopsis thaliana* the model organism of choice in plant biology, and there is currently a renewed interest in elucidating the biosynthesis, metabolism and transport of IAA in this species (for reviews: Eckhardt 2001; and Muday and DeLong 2001).

As part of a survey of indolic constituents in *A. thaliana*, we report here the presence of IAM in this species. We furthermore show that the production of

IAM from indole-3-acetonitrile (IAN) is an intrinsic property of the IAN-converting nitrilases and provide indications that nitrilase can be involved in generating IAM in the plant. During all steps in our procedures, we took great care to avoid conditions known to artificially produce IAM from IAA-conjugates (Zenk 1961).

Materials and methods

Plant material

All experiments were carried out with *Arabidopsis thaliana* (L.) Heynh. C24 (seed stock originally provided by L. Willmitzer, MPI-PP Golm, Germany). Plants were grown, if not stated otherwise, in a greenhouse in a 2:1 (v/v) mixture of standard soil and sand for 4–5 weeks in short days (6 h photoperiod). They were then transferred to long days (16 h) until harvest. The average temperature during nights was 18–20 °C, during days 22–24 °C, and photosynthetically active radiation (PAR) was no less than 150 µmol photons m^{-2} s^{-1} (supplementary light, if required, from sodium-vapor lamps).

For growth under sterile conditions, surface-sterilized seeds [2 min 70% (v/v) ethanol, 5 min 5% NaOCl, 5× sterile water] were suspended in 0.1% (w/v) agar and spread on the surface of solidified MS-medium in petri dishes (Murashige and Skoog salts and vitamins, supplemented with 2% (w/v) sucrose; Murashige and Skoog 1962). To release the seeds from dormancy, the plates were incubated at 4 °C for 2 days and then transferred to phytotron chambers (8 h photoperiod at 24 °C, 16 h darkness at 20 °C, PAR 105 µmol photons m^{-2} s^{-1} from standard white fluorescent tubes). At the age of 4 weeks, plants were transferred to sterile glass jars and grown under otherwise the same conditions, for a further 4–6 weeks.

Synthesis of $[^2H]_5$-IAM

$[^2H]_5$-Indole-3-acetamide ($[^2H]_5$-IAM) was prepared enzymatically from $[^2H]_5$-L-tryptophan using tryptophan-2-monooxygenase (TMO). Recombinant TMO was produced from *Escherichia coli* harboring the plasmid pLUT5, kindly provided by Dr. Paul F. Fitzpatrick (Department of Biochemistry and Biophysics, Texas A&M University, College Station, Tex., USA), as described by Emanuele et al. (1995).

For the synthesis of $[^2H]_5$-IAM, 1 mmol $[^2H]_5$-L-tryptophan (isotopic abundance 98%; Cambridge Isotope Laboratories, Andover, Miss., USA), dissolved in distilled water, was mixed with 2 ml TMO preparation (14 mg of protein ml^{-1} in 0.1 M Tris–HCl, pH 8.3, containing 12 mM β-mercaptoethanol and 1 mM EDTA) and incubated for 3 h at 37 °C. The reaction mixture was then acidified (pH 2.0) and extracted twice with 5 ml each of ethyl acetate. The combined organic phases were reduced to dryness, and the crude reaction product was dissolved in 1 ml chloroform. $[^2H]_5$-IAM was purified from the mixture by thin-layer chromatography (TLC) on silica gel. The solvent system was prepared by saturating a mixture of 300 ml iso-hexane and 200 ml ethyl acetate with 2.5% (v/v) aqueous formic acid. To the organic phase of this system was finally added 10% (v/v) methanol. The reaction product ($R_f = 0.19$) was eluted with methanol and was recovered in 91% yield. Its identity and purity were checked by gas chromatography–mass spectrometry (GC–MS) as will be described below.

Extraction and pre-purification of indolic compounds from plant tissue

Plants grown either in the greenhouse under non-sterile conditions or in the phytotron under sterile conditions were used to harvest, per datum point, 0.5 g of imbibed seeds or whole plantlets (leaf and root tissue). Immediately after harvest, the tissue was immersed in boiling methanol containing, as an internal standard, 10–50 pmol of $[^2H]_5$-IAM (depending on the amount of IAM expected, which was determined in preliminary experiments), and boiled for 10 min. The tissue was then re-extracted twice for 20 min each with 20 ml methanol at ambient temperature. The combined extracts were filtered and reduced to dryness in a rotary evaporator. The residue was taken up in 2 ml chloroform. This extract was applied onto a silica gel thin-layer plate, which was developed in the solvent system described above. The IAM zone was eluted with 20 ml of methanol and the eluted material was, after removal of the solvent under reduced pressure, re-dissolved in 0.2–0.3 ml of HPLC solvent (see below), followed by further purification by high-pressure liquid chromatography (HPLC). In cases when both IAM and IAA were to be analyzed from the same sample, the TLC step was omitted.

Expression and purification of recombinant nitrilases

Cloning of the *A. thaliana* nitrilase isoforms into the pET21-b(+) vector (Novagen, Madison, Wis., USA) was as described earlier (Vorwerk et al. 2001). For the expression of nitrilase enzymes, the *E. coli* strain BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, Calif., USA) was used. Purification of the recombinant nitrilases was done as described by Piotrowski et al. (2001). The purified nitrilase fraction showed homogeneity of at least 95%, as judged by Coomassie-blue stained SDS–polyacrylamide gels.

Enzymatic analysis of nitrilase activity

Nitrilase activity was determined by analyzing the amount of reaction products by HPLC as described below. The enzymatic conversion of IAN was carried out in 1 ml 50 mM Tris–HCl (pH 8.0) containing 5 mM IAN as the substrate and 1 µg of the purified enzyme. The samples were incubated overnight at 30 °C and thereafter acidified (pH > 2.0) with 1 N HCl. The organic compounds were extracted with 1 ml ethyl acetate, dried under vacuum and resuspended in the mobile HPLC phase described below. The amounts of IAA and IAM, respectively, were calculated by comparison of their peak areas with those of reference compounds representing known concentrations of IAM or IAA. All values were corrected for recovery.

HPLC of indolic constituents

HPLC of indolic compounds was carried out on a stationary silica phase (ZorbaxSil 5 µm, 250 mm×4 mm i.d.; Knauer, Bad Homburg, Germany) using the same mobile phase as in the case of TLC (see above) and a flow rate of 1.5 ml min^{-1} (retention times: IAN = 2.3 min, IAA = 2.7 min, IAM = 4.7 min). The material eluting between 2.5 and 3.5 min and between 4 and 5 min was collected and analyzed further by GC–MS.

GC–MS of IAM

All samples to be analyzed by GC–MS were vacuum-dried, re-dissolved in 20 µl methanol and treated with 100 µl ethereal diazomethane. For the analysis of IAM, samples were again vacuum-dried and then treated with 50 µl trifluoroacetic acid anhydride (TFAA) for 1 min, then dried in a stream of nitrogen and re-dissolved in 10 µl of chloroform. Aliquots (1 µl) of these samples were injected into the GC–MS system for separation and mass-fragment analysis.

All spectra were recorded on a Varian Saturn 2000 ion-trap mass spectrometer using a Varian CP-3800 gas chromatograph fitted with a CombiPal autoinjector (Varian, Walnut Creek, Calif., USA). The mass spectrometer was operated in the CI–MRM mode with methanol as the reactant gas and positive ion detection. Instrument settings were:

- GC – splitless injection (1 µl), injector temperature 260 °C; ZB-50 fused silica open tubular column (30 m, 0.25 mm i.d., 0.25 µm film thickness; Phenomenex, Aschaffenburg, Germany),

He carrier gas 1 ml min^{-1}; temperature program: 1 min isothermically at 50 °C, linear ramp at 20 °C min^{-1} to 250 °C, transfer line temperature 260 °C.

- MS – mass range 100–380 amu at 3 scans s^{-1}, parent ion ($[M+H]^+$) selection for trifluoroacetylated IAM set at m/z 367 ($[^1H]$-species) and m/z 372 ($[^2H]_5$-species), for IAA methyl ester at m/z 190 ($[^1H]$-species) and m/z 192 ($[^2H]_2$-species).

Unlabelled IAM ($R_t = 8.75$ min) was characterized by the fragment ions (m/z): 339, 323, 242 and 226, and the pentadeuterated species ($[^2H]_5$-IAM: $R_t = 8.70$ min) by the fragment ions (m/z): 344, 328, 247 and 231 (resonant wave form, excitation amplitude: 0.7 V). IAA-species were identified by the fragment ions m/z 130 ($R_t = 11.85$ min, unlabelled IAA) and m/z 132 ($R_t = 11.83$ min, $[^2H]_2$-IAA) applying an excitation amplitude of 0.5 V. The amount of endogenous compound was calculated from the signal ratios of the unlabelled over the deuterated mass fragments.

Results

Purification of IAM and analysis by gas chromatography–tandem mass spectrometry (GC–MS/MS)

For all experiments described here, samples to be analyzed for the presence of IAM were pre-purified by two sequential chromatographic steps, TLC followed by HPLC, before derivatization with TFAA and finally GC–MS or GC–MS/MS analysis. As our HPLC method separated IAM from IAA and IAN completely (see Fig. 1), the two pre-purification steps effectively removed all IAN and IAA from the samples to be analyzed for IAM.

Under our GC–MS conditions, it was found that derivatization with TFAA resulted in higher sensitivity of detection of IAM than conversion of IAM to the trimethylsilyl derivative (not shown). In chemical ionization mode with positive ion detection and methanol as reactant gas, the TFA derivative of IAM gave a molecular ion $[M+H]^+$ of m/z = 367 and the TFA derivative of $[^2H]_5$-IAM gave the expected molecular ion $[M+H]^+$ of m/z = 372. In order to increase the selectivity and sensitivity of the mass-spectrometric analysis, all mass spectra were recorded in MS/MS mode with parent-ion selection at m/z = 367 and 372 and monitoring of the complete daughter-ion spectra. Model spectra of the TFA derivatives of IAM and $[^2H]_5$-IAM are given in Fig. 2, together with the proposed structure of the derivatives and their fragmentation pattern (Fig. 2c). Per injection, as little as 10 pg of the IAM derivative can be detected.

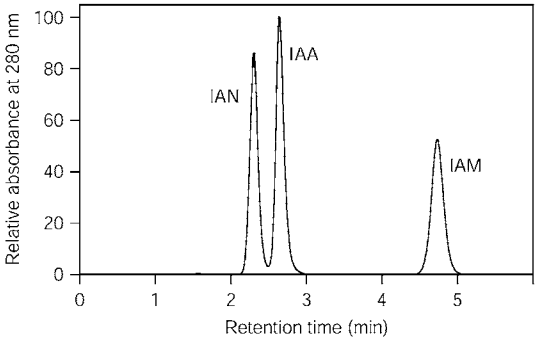

Fig. 1. Separation of indolic compounds. The elution behaviour of the three analysed indolic substances (100 ng of each injected) on HPLC is shown using relative absorbance units (100% = peak maximum)

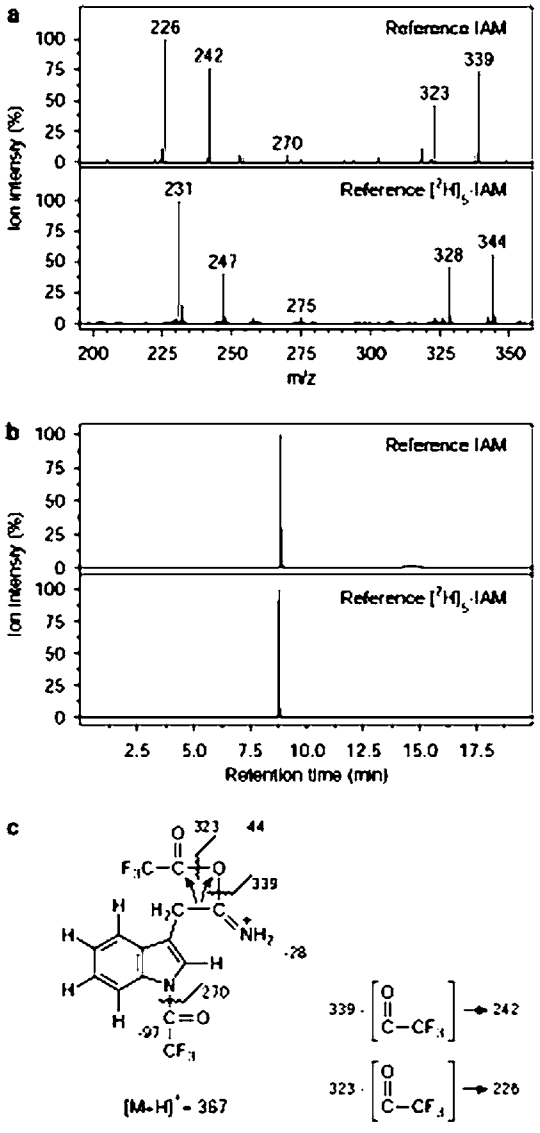

Fig. 2a–c. Preparation and mass-spectrometric analysis of $[^2H]_5$-IAM. Pentadeuterated L-tryptophan was converted to $[^2H]_5$-IAM at high levels by using heterologously expressed tryptophan-2-monooxygenase from *Pseudomonas savastanoi*. **a** MS/MS analysis of the characteristic parent ions (m/z = 367 for unlabelled IAM or m/z = 372 for $[^2H]_5$-IAM) gave the specific fragment patterns shown. **b** The reaction product $[^2H]_5$-IAM elutes at $R_t = 525$ s, unlabelled IAM at $R_t = 527$ s. **c** Proposed fragmentation and structure of the TFA-derivative of IAM. As all five deuterium atoms in $[^2H]_5$-IAM are attached to the indolic ring system, the masses of all fragments shown here for unlabelled IAM have to be shifted + 5 atomic mass units to give the observed fragment masses for the pentadeuterated internal standard

Occurrence of IAM in *A. thaliana*

Using the combination of two-stage TLC–HPLC prepurification, TFAA derivatization and GC–MS/MS analysis (all stages in the presence of 10–50 pmol $[^2H]_5$-IAM as an internal standard, depending on the expected amount of endogenous IAM), the occurrence of IAM in shoots of sterile-grown, 2- to 3-week-old rosette plants was proven (Fig. 3). Using the MS/MS technique, clear daughter-ion spectra could be recorded from the molecular ions (m/z 367 or 372) with little background noise. The absolute amounts of IAM extracted from vegetative rosette plants, 2–3 weeks old, were very low. There was a tendency towards slightly higher IAM levels in non-sterile-grown plants (30.9 ± 5.0 pmol g^{-1} FW, mean ± SD, $n = 3$) compared to sterile-grown plants (20.5 ± 9.1 pmol g^{-1} FW, $n = 3$) at the same growth stage, but the differences were marginal. Thus, it can be excluded that the IAM extracted from the plants is of microbial origin. In line with this argument is the observation that in adult rosettes (ca. 5–7 cm diameter) of greenhouse-grown plants, IAM levels dropped further (0.74 ± 0.03 pmol g^{-1} FW, $n = 4$) and were fairly constant among different batches of plants.

The sensitivity of the analytical technique allowed us to record in parallel the levels of IAM and IAA in imbibed seeds and during the early stages of seedling development. In this case, extracts from imbibed, surface-sterilized seeds or sterile-grown seedlings at the ages indicated (Fig. 4) were extracted, and extracts processed with two internal standards, $[^2H]_5$-IAM and $[^2H]_2$-IAA, simultaneously as detailed in *Material and methods*. Compared with 2-week-old seedlings, imbibed, ungerminated seeds contained 40-fold (IAM) and 270-fold (IAA) higher levels of the two indolic compounds. Both the levels of IAM and those of IAA decreased sharply and in parallel during the first 3–5 days, and in particular during the first 24 h, of germination (Fig. 4).

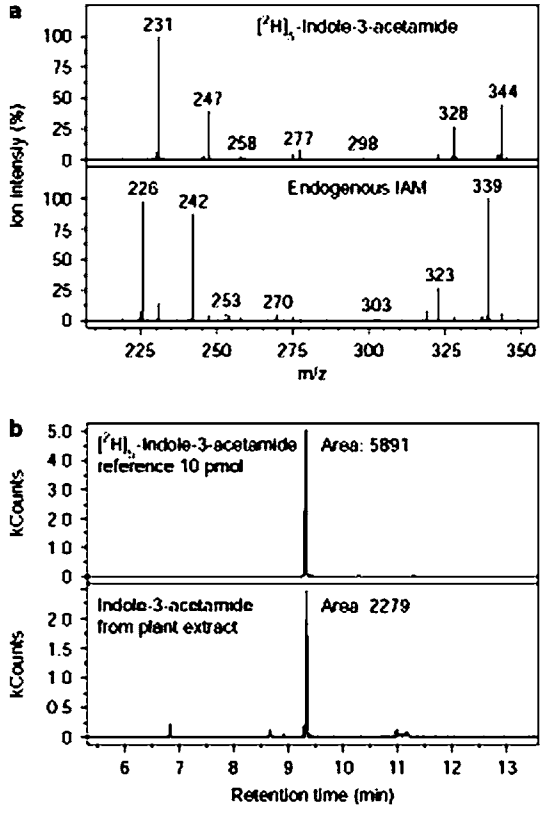

Fig. 3a, b. Detection of endogenous IAM. Endogenous IAM in sterile-grown *Arabidopsis thaliana* was analysed by extracting the organic compounds from rosettes of 6-week-old plants with boiling methanol in the presence of 10 pmol internal $[^2H]_5$-IAM standard. The extract was purified by HPLC, and the IAM fraction Rt = 4.2–5.2 min was collected and analyzed by GC–MS/MS. **a** Characteristic MS/MS fragment patterns for endogenous IAM (m/z = 226, 242, 323, 339) and $[^2H]_5$-IAM (m/z = 231, 247, 328, 344). **b** Retention of endogenous and reference IAM on the GC column. The standard $[^2H]_5$-IAM elutes at Rt = 557 s, endogenous IAM at Rt = 559 s

IAM is a reaction product of *A. thaliana* nitrilases

Analysis of the reaction products of β-cyanoalanine hydrolase/nitrilase encoded by the *A. thaliana NIT4* gene (Piotrowski et al. 2001) has revealed that this enzyme makes two true end products: the amide (asparagine) and the acid (aspartate) from the substrate, β-cyanoalanine. In a preliminary earlier report, it was stated that all four *A. thaliana* nitrilases would convert IAN to IAA with a side-product of IAM noted on thin-layer

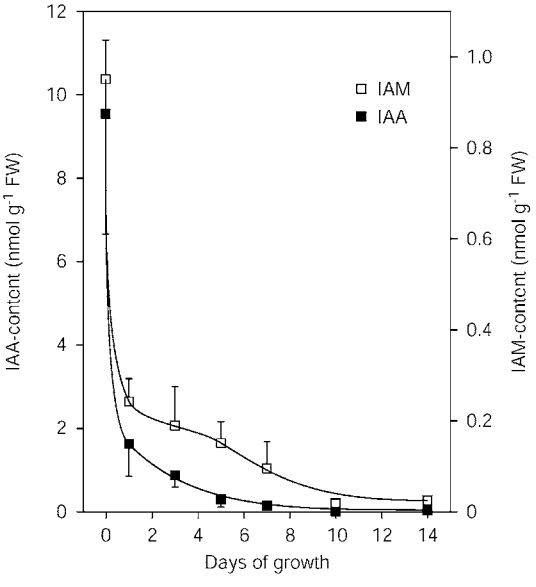

Fig. 4. IAA and IAM in sterile-grown seedlings of *A. thaliana*. The time axis gives days of growth under short-day conditions. The amount of IAA and IAM at the first time point (0 days) was measured in imbibed seeds after stratification. The indolic compounds were extracted with methanol and separated by HPLC. Fractions at $R_t = 2.2$–3.2 min (IAA) and $R_t = 4.2$–5.2 min (IAM) were collected and further analysed by GC–MS/MS. The data are averages ± SD of at least $n = 5$ independent experiments

chromatograms (Bartel and Fink 1994). However, this reaction product was neither identified unambiguously in that study nor has it been confirmed in later studies that NIT4 uses IAN as a substrate (Vorwerk et al. 2001). Thus, a re-examination was carried out using recombinant NIT1, NIT2 and NIT3 in highly purified form. To ease purification, all nitrilases were expressed from their cDNAs as C-terminally hexahistidine-tagged fusion proteins (Vorwerk et al. 2001) and were purified to apparent homogeneity as judged by SDS–PAGE (data not shown). Enzyme assays with the purified nitrilases gave proof of the formation of both IAM and IAA as end products of the reactions of each of the three nitrilase isoforms (Fig. 5). As IAM was not converted further to IAA by the nitrilases (data not shown), IAM must be considered as a true end product, and not an intermediate, of enzyme catalysis, as in the case of formation of asparagine and aspartate from β-cyanoalanine in NIT4 catalysis (Piotrowski et al. 2001). The three nitrilases produced a fairly constant ratio of IAM:IAA with IAA being in each case the dominant reaction product (IAM:IAA ratios for the purified, recombinant nitrilases were: NIT1, 1:5.4; NIT2, 1:3.2; NIT3, 1:1.9).

Discussion

Arabidopsis thaliana, the only plant whose genome has been sequenced completely to date, is currently under scrutiny in a world-wide effort to understand the functions and regulation of all of its gene products, its full complement of metabolites, the pathways involved in their formation and their further metabolism, and the molecular basis of development. In this context, efforts have been intensified during recent years to understand how the plant makes its auxin growth-hormone, indole-3-acetic acid (IAA), and several pathways and possible intermediates have been proposed (for review: Eckhardt 2001), starting from the precursor L-tryptophan.

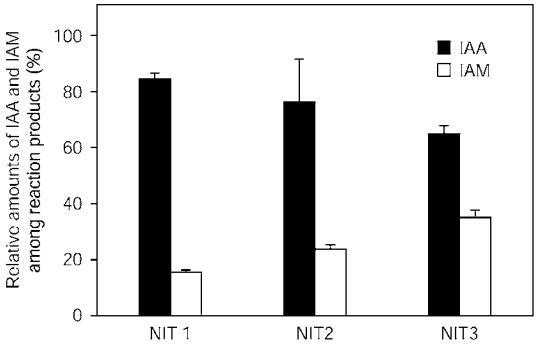

Fig. 5. Conversion of IAN by *A. thaliana* NIT1–3. Purified protein (1 µg) was incubated at 30 °C for 20 h with 5 mM IAN as substrate. The reaction products, IAA (*black bars*) and IAM (*white bars*), were detected and analyzed photometrically ($\lambda = 280$ nm) after HPLC separation. The data shown represent means ± SD derived from $n = 3$ experiments

A potential IAA precursor is IAM. A route to IAA from L-tryptophan via IAM has been established definitely in several bacteria including *Agrobacterium* species (see Weiler and Schröder 1987, for review, and literature cited in the introduction). Doubts have been cast on early reports on the occurrence of IAM in higher plants due to the propensity of IAA-glycosyl esters to form IAM in solutions containing ammonia (Zenk 1961). By avoiding such steps in the workup procedures, IAM was subsequently detected in young fruits of *Citrus unshiu* (Takahashi et al. 1975) and hypocotyls of Japanese cherry (Saotome et al. 1993) using mass-spectrometric techniques, but the significance of this metabolite remained in question, mainly because in these studies, non-sterile tissue had been used (with the chance of microbial contamination as a source of IAM).

Using an improved, highly sensitive method to quantitate IAM by GC–MS/MS in the presence of a deuterated internal standard, we were able to show the presence of IAM in *A. thaliana* plants grown from seeds under strictly sterile conditions. The levels of IAM were very low in rosette plants and declined from ≈ 3.5 ng g^{-1} FW in plants 2 weeks old to ≈ 130 pg g^{-1} FW in fully grown rosettes. Such low levels likely would have gone undetected with earlier technology. We have detected no evidence for a microbial contribution to extractable IAM in our plant material; thus, IAM is a genuine metabolite of *A. thaliana*. Highest levels of IAM were recorded in imbibed seeds, and levels fell rapidly and sharply during seedling development (cf. Fig. 4).

As it is known that (i) the genes for the IAN-converting isoforms 2 and 3 of *A. thaliana* nitrilase are active specifically during embryo development (*NIT2*, Vorwerk et al. 2001) and early germination (*NIT3*, Kutz et al. 2002) and (ii) amides may be side-products of nitrilase activity (Bartel and Fink 1994; Piotrowski et al. 2001), we examined the conversion of IAN by purified, recombinant NIT1, NIT2 and NIT3 (Fig. 5). Indeed, it was shown conclusively by GC–MS that, besides IAA, IAM is a true reaction product of all three nitrilase isoenzymes, when IAN is used as substrate. This observation was independently verified for NIT1 in a recent study appearing in print during the preparation of this manuscript (Osswald et al. 2002). IAM went undetected by the GC–MS technique used in our previous work which included methylation of samples and focussed on acidic metabolites (Vorwerk et al. 2001).

We propose that IAM in seeds and germinating plantlets of *A. thaliana* is produced from IAN by nitrilases. This conclusion is supported by the following:

i. Those stages of development characterized by high levels of IAM are also characterized by a high state of activity of the genes encoding nitrilases 2 and 3 (Vorwerk et al. 2001; Kutz et al. 2002).
ii. IAN is abundant in seeds and very young plants (Müller et al. 1998), as is the IAN precursor, glucobrassicin (Petersen et al. 2002).

iii. Seed glucosinolates are rapidly metabolized during the onset of germination (Elliot and Stowe 1971; Bodnaryk and Palaniswamy 1990) including glucobrassicin, a major seed glucosinolate in *A. thaliana* (Petersen et al. 2002).
iv. The breakdown of glucosinolates predominantly yields nitriles, and only small amounts of isothiocyanates, in the *Arabidopsis* strain used in our study (Lambrix et al. 2001).

A further, strong indication for our conclusion stems from the observation that each of the nitrilases, similar to what has been observed for NIT4 in the case of β-cyanoalanine conversion (Piotrowski et al. 2001), produces the amide and the carboxylic acid at a relatively constant ratio (cf. Fig. 5). This finding agrees with the observation that IAM and IAA levels in plants during the first few days of development declined with a similar time course, and that the IAM:IAA ratios in the plant tissues are generally in the same range as those observed in vitro for the recombinant enzymes (cf. Fig. 4). From the above hypothesis, it also follows that the IAA found in imbibed seeds and during early seedling growth should be derived – at least to some extent – from IAN through nitrilase catalysis. NIT2 seems to be the candidate isoenzyme for laying down IAA and IAM during seed development and NIT3 could play a role during the early stages of germination. An argument against a potential involvement of the nitrilases in the formation of IAM (and, by the same reasoning, also of IAA) from IAN present in the plant is the very high K_m value (in the mM range) of the recombinant isoenzymes for the substrate IAN, which contrasts with much lower K_m values of the same enzymes for other substrates (e.g., 200–400 μM for the preferred substrate phenylpropionitrile; Vorwerk et al. 2001). These data would indicate that, when confronted with a mixture of endogenous nitriles, IAN might not be the preferred, but rather a very poor, substrate. The situation would be different if the enzymes were challenged with IAN as the predominant substrate. Recently, this was shown to be the case under particular circumstances. In sulphur-starved *A. thaliana* plants, the *NIT3* gene is strongly induced and concomitantly, the turnover of glucobrassicin is intensified over that of the other glucosinolates (Kutz et al. 2002). On the other hand, there is strong evidence that, in planta, nitrilases are much more effective compared to the recombinant enzymes in vitro. E.g., Schmidt et al. (1996) have expressed NIT2 from its cDNA in tobacco (devoid of any endogenous IAN-hydrolyzing activity) and found very pronounced auxin-overproduction phenotypes as well as elevated IAA levels in root systems given as little as 10 μM IAN. This concentration is in the range of the endogenous IAN level in *A. thaliana* roots (13 μM, Schmidt et al. 1996). Thus, from the enzymatic in vitro data, conclusions about the situation in vivo might be misleading because as yet unknown mechanisms of regulation at the enzyme level might drastically alter the performance of nitrilases in vivo. Further studies employing nitrilase antisense plants or knock-out mutants and precursor feeding experiments should shed light on the sources and fate of IAM and its relation to auxin metabolism in *A. thaliana* and the role of nitrilases in these processes, particularly during seed formation and early seedling development. It is clear, though, that seed IAM shows further metabolism during germination, as the total quantity of IAM drops from initially 11.8 pg per imbibed seed at day 0 to 2.8 pg per seedling at days 1–3 of germination. Thereafter, the total amount of IAM per plant increases again to 27.9 pg between days 5 and 14 (recalculated from data in Fig. 4).

As glucosinolates and nitriles, as well as the nitrilases homologous to the *A. thaliana* NIT1/NIT2/NIT3 family are restricted in occurrence to the Brassicaceae, it will also be important to determine the occurrence of IAM among members of this as well as other families.

Acknowledgements This work was funded by the Deutsche Forschungsgemeinschaft, Bonn (SPP 1067) and Fonds der Chemischen Industrie, Frankfurt/M. (literature provision). The authors thank Dr. Paul Fitzpatrick, Texas A&M University, for providing the TMO-plasmid pLUT5.

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