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Tryptophan-dependent indole-3-acetic acid biosynthesis by 'IAA-synthase' proceeds via indole-3-acetamide

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ABSTRACT

Plants are suggested to produce their major growth promoting phytohormone, indole-3-acetic acid (IAA), via multiple redundantly operating pathways. Although great effort has been made and plenty of possible routes have been proposed based on experimental evidence, a complete pathway for IAA production has yet to be demonstrated. In this study, an *in-vitro* approach was taken to examine the conversion of L-tryptophan (L-trp) to IAA by gas chromatography-mass spectrometry (GC–MS). Especially the influence of putative reaction intermediates on the enzymatic conversion of L-trp to IAA was analyzed. Among the substances tested only indole-3-acetamide (IAM) showed a pronounced effect on the L-trp conversion. We additionally report that IAM is synthesized from L-trp and that it is further converted to IAA by the utilized cell free *Arabidopsis* extract. Together, our results underscore the functionality of an IAM-dependent auxin biosynthesis pathway in *Arabidopsis thaliana*.

1. Introduction

Plants regulate their growth and development with the help of small signaling molecules, collectively termed phytohormones. Among these compounds the auxin group is of major importance. Virtually every process which is associated with the promotion of plant growth is to some extent affected by members of this group. The causal relationships of auxin action and physiological functions have been examined in great detail in the past decades by various laboratories. Even the molecular mode of action has just recently been unveiled (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). In this respect, it seems very astonishing that the biosynthesis of indole-3-acetic acid (IAA), the predominant auxin *in planta*, is still only partially understood. Mainly owing to the fact that no auxin auxotroph mutants are available, the current working hypothesis is that redundant pathways (Fig. 1) operate in parallel with each other (Woodward and Bartel, 2005; Pollmann et al., 2006a). Basically, two precursors of IAA formation are presumed, either tryptophan or a tryptophan precursor, i.e. indole or indole-glycerophosphate. Whereas for the tryptophan-independent pathway so far neither intermediates nor participating enzymes have been presented, the routes of tryptophan-dependent IAA production proceed via several probable intermediates, e.g. indole-3-pyruvate, indole-3-acetaldoxime, indole-3-acetaldehyde (IAAld), indole-3-acetonitrile (IAN), indole-3-acetamide (IAM), or tryptamine (TAM), respectively. All of these compounds are proposed to participate in IAA biosynthesis because they were either found to occur *in planta* or because enzymes have been isolated, capable of either producing these substances or converting them to IAA or an appropriate precursor. Until today, however, none of the proposed biosynthetic pathways for the synthesis of IAA is completely uncovered within one species, since most of the suggested biosynthetic pathways are proposed on the basis of combined results obtained from different plant species. In 2000, Müller and Weiler described an enzyme complex to be involved in auxin biosynthesis. This soluble enzyme complex, termed 'IAA-synthase', is estimated to have a size of approximately 160–180 kDa and to convert L-tryptophan (L-trp) to IAA without the production of free intermediates. By assessing co-factor dependencies and the influence of putative intermediates on IAA formation, the authors were able to exclude several enzyme classes from the complex. For example, neither NADH nor NADPH seemed to play an important role, thus, ruling out most P450 enzymes, in particular CYP79B2, CYP79B3 (Hull et al., 2000; Mikkelsen et al., 2000), CYP83B1 (Bak et al., 2001; Bak and Feyereisen, 2001), and CYP79A13 (Nafisi et al., 2007). Furthermore, it has been shown that neither IAN nor IAAld interfered with IAA formation in the conducted experiments, thus suggesting that neither aldehyde oxidases nor nitrilases participate in the assembly of the 'IAA-synthase' complex. Nevertheless, due to a strict oxygen-dependency, the incorporation of two molecules of oxygen derived from water, and the co-purification of a nitrilase it was suggested

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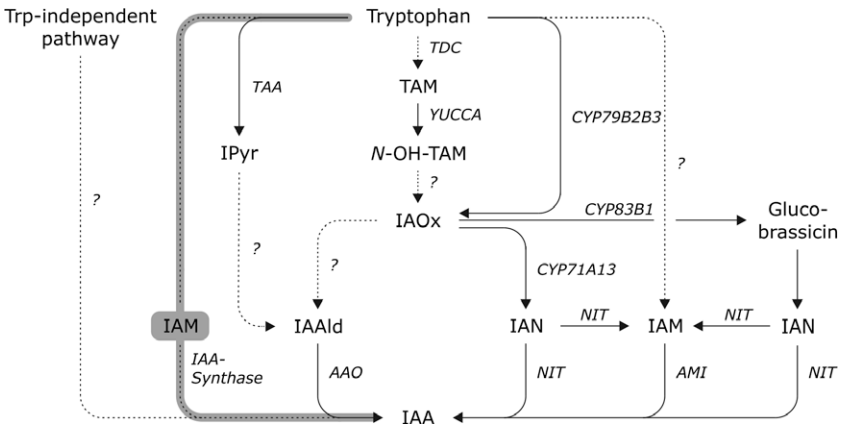

Fig. 1. Proposed pathways of IAA biosynthesis in *Arabidopsis thaliana*. Solid arrows indicate reaction steps for which the corresponding genes/gene products are known, dashed lines indicate assumed reaction steps for which the predicted genes/enzymes are yet to be identified. The actual contribution of this study to auxin biosynthesis is indicated by the light grey background. AAO, aldehyde oxidase; AMI, amidase; CYP, cytochrome P450; IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; IPyr, indole-3-pyruvate; *N*-OH–TAM, *N*-hydroxyltryptamine; TAA, tryptophan aminotransferase; TAM, tryptamine; TDC, tryptophan decarboxylase; YUCCA, flavin-dependent monooxygenase.

that the terminal enzymatic reaction in the 'IAA-synthase' complex is most likely catalyzed by a nitrilase. Thereafter, in a gain-of-function screen (Zhao et al., 2001), an auxin overproduction mutant, *yucca*, was identified, which was characterized by an approximately 50% increased level of free IAA as well as by corresponding phenotypic alterations of the mutant, resembling known auxin overproducing mutants such as *sur1* (King et al., 1995; Boerjan et al., 1995; Celenza et al., 1995; Lehman et al., 1996; Mikkelsen et al., 2004), *sur2* (Delarue et al., 1998), and plants overexpressing the *iaaM* gene from *Agrobacterium tumefaciens* (Romano et al., 1995). By plasmid rescue the gene responsible for the auxin phenotype was isolated and described to code for a flavin-dependent monooxygenase-like protein. Though the *in-vivo* activity of YUCCA and its homologues is yet to be uncovered, it has been shown that YUCCA is able to *N*-hydroxylate TAM *in vitro* (Zhao et al., 2001). Moreover, by using a toxic tryptophan derivative, 5-methyltryptophan, a tryptophan-dependency of the underlying TAM-dependent pathway has been emphasized. Today, 11 YUCCA-homologues have been identified in *Arabidopsis* and mutant analysis has implicated specific members of this family to be involved in IAA synthesis. In addition, in 2003, an amidase catalyzing the conversion of IAM to IAA, and herewith an alternative terminal reaction step of IAA synthesis, has been detected in *Arabidopsis* (Pollmann et al., 2003) by query of the *Arabidopsis* proteome with the sequence of known IAM-hydrolases from plant pathogenic bacteria (Thomashow et al., 1984). Together with the finding that IAM is an endogenous constituent of sterilely raised *Arabidopsis* plantlets (Pollmann et al., 2002) as well as of other plant species (Takahashi et al., 1975; Saotome et al., 1993; Rajagopal et al., 1994), it may be concluded that an IAM-dependent pathway for the production of IAA is operative *in planta*. With regard to these novel findings, suggesting IAA biosynthesis to proceed via TAM and IAM, respectively, the two compounds appeared to be favorable candidates for intermediates of 'IAA-synthase' catalyzed IAA formation. Hence, it seemed rewarding to extend previous work on the 'IAA-synthase' and to assess if either TAM or IAM interfere with IAA production in the previously described *in-vitro* system (Müller and Weiler, 2000). Here, we report the identification of an 'IAA-synthase'-like enzyme activity in a variety of different plant species deriving from nine independent families. In addition, we show that IAA formation in the underlying experimental system is highly interfered by addition of IAM to the reaction mixture. Moreover, we provide evidence that IAM is both a true intermediate of tryptophan-dependent IAA formation and a precursor of IAA *in vitro*.

2. Results

2.1. 'IAA-synthase' activity in various plant species

In a previous study, Müller and Weiler (2000) presented a not yet entirely characterized soluble protein complex, referred to as 'IAA-synthase', capable of converting L-trp to IAA without the detectable release of any intermediate. However, this study was restricted to *Arabidopsis thaliana*. To assess if comparable enzymatic activities are detectable in other plant species, or if this pathway of IAA biosynthesis is a special trait of *Arabidopsis*, we analyzed cell free protein extracts from 14 different species deriving from nine families for their ability to convert $[^2H]_5$-L-trp to $[^2H]_5$-IAA *in vitro*. To do so, protein extracts were aliquoted after sedimentation of cell wall fragments and cell debris by centrifugation. The first half was directly analyzed whereas the second half was concentrated by an ammonium sulphate precipitation (0–70% saturation) and subsequently desalted on PD-10 columns (Amersham Biosciences) prior to GC–MS analysis. All of the examined protein extracts were capable of converting $[^2H]_5$-L-trp to $[^2H]_5$-IAA (Fig. 2), even though at different specific activities. Apparently, not all protein extracts withstood the concentration procedure without loss of activity. Especially in mustard, corn, and tomato the specific enzyme activity dropped remarkably. This might be indicative for a weaker interaction of the components of the proposed 'IAA-synthase', or its generally lower stability. In contrast, the parsley, thale cress, cress, buckwheat, and spinach extracts showed an enhanced specific activity after the precipitation procedure, most likely emphasizing a stronger interaction of the complex partners and, thus, a possible feasibility of this plant species for further protein purification approaches. Owing to the excellent genetic accessibility with a complete sequence coverage and good availability of mutants (Bevan and Walsh, 2005; Alonso et al., 2003) as well as the relatively high enzymatic activity, *Arabidopsis thaliana* was chosen for further assessments.

2.2. IAA production in a NIT1 knockout

Deduced from the point that, except of oxygen, in none of the studied plant species co-factors, like for instance NADH or NADPH, were needed to preserve the enzymatic activity it can be concluded that an enzyme complex comparable with the 'IAA-synthase' may be functioning in those plants. This finding is, however, at clear variance with the previously drawn conclusions by Müller and

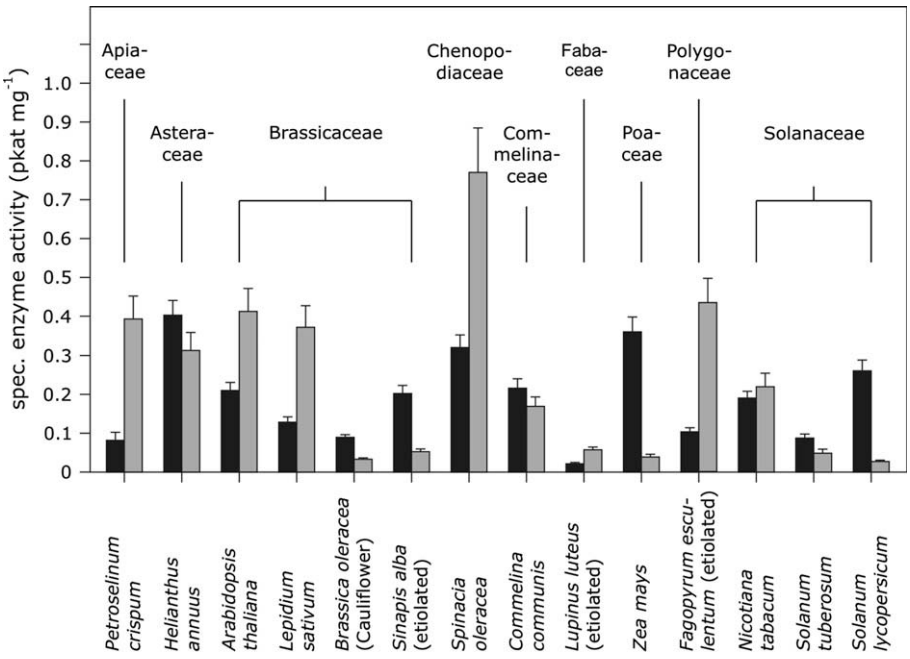

Fig. 2. Specific activities of tryptophan-dependent IAA formation in different plant species. IAA production in either crude protein extracts (black bars) or in 0–70% (saturation) ammonium sulphate precipitated samples (grey bars) has been determined by GC–MS analysis. All activities were calculated on an internal $[^2H]_2$-IAA standard, added prior to incubation. The families of the individual species are indicated in the figure.

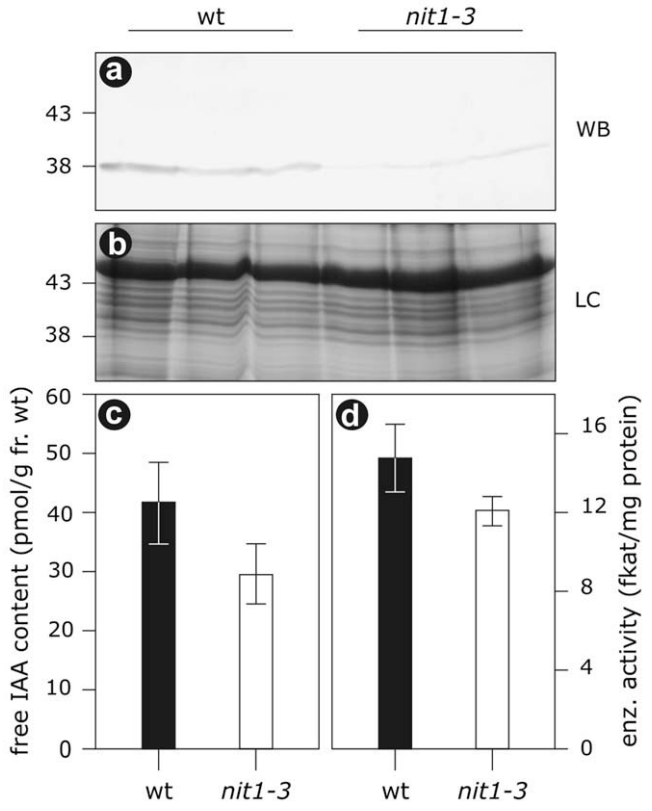

Fig. 3. Free IAA content and *in vitro* IAA formation in the *nit1-3* background. In order to verify the reduced nitrilase content in *nit1-3*, 50 μg of crude protein extracts of either *nit1-3* or wild type, taken from three independent preparations, were separated by SDS–PAGE on a 12.5% resolving gel. (a) Shows the corresponding western blot (WB) of the region around 43 kDa. In (b), a coomassie stained gel as a loading control (LC) is depicted. For immunological detection a NIT1 antiserum was used. The lower panel shows the content of free IAA (c) as well as the specific IAA-forming activity (d) in *nit1-3* relative to wild type. The shown data represent means ± SD of $n \geqslant 3$ separate experiments.

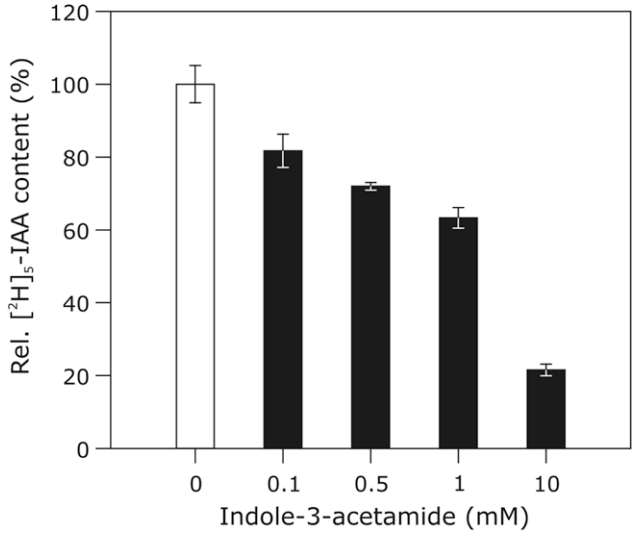

Fig. 4. Impact of indole-3-acetamide on the *in-vitro* conversion of $[^2H]_5$-L-tryptophan to $[^2H]_5$-IAA. Plant extracts containing 1 mM $[^2H]_5$-L-trp as substrate and increasing amounts of IAM were incubated for 6 h at 37 °C. Thereafter, samples were acidified, extracted with equal volumes of ethyl acetate, and the contained carboxylic acids derivatized by using ethereal diazomethane. Finally, IAA contents were estimated by GC–MS measurements. Produced amounts of IAA were calculated on an internal $[^2H]_2$-IAA standard, which was added before the incubation was performed. To be able to compare $[^2H]_2$-IAA production in the different samples, the amount of produced $[^2H]_2$-IAA in wild type extracts was set to 100%. The absolute amount of $[^2H]_2$-IAA in control extracts was 0.81 ± 0.03 nmol sample^{-1}. Depicted results are means ± SD derived from $n \geqslant 3$ experiments.

Weiler (2000) who suggested a nitrilase to be a favorable candidate for the catalysis of the final reaction step in 'IAA-synthase'-assisted IAA formation. Based on our data this seems no longer tenable, as appropriate enzymes, homologous to the members of the NIT1 group of *Arabidopsis* (Vorwerk et al., 2001), are restricted to the families of the Musaceae, Poaceae, and Brassicaceae. To further corroborate this finding, we compared the 'IAA-synthase' activity

in *nit1-3* seedlings (Normanly et al., 1997) with that of sterilely grown wild type plantlets. For this purpose, we firstly analyzed the nitrilase content in the knockout background, to exclude the possibility that the NIT1 deficiency is restored by one of the other isoenzymes. As determined by western blotting and subsequent immunological detection, the *nit1-3* mutant shows a reduction in its nitrilase content (Fig. 3a). Thus, neither NIT2, which is almost not expressed in two weeks old plants (Vorwerk et al., 2001), nor NIT3 seem to be capable of taking over the NIT1 function. Secondly, we determined both free IAA contents and specific 'IAA-synthase' activity in *nit1-3* and wild type, respectively. In contrast to previous studies (Normanly et al., 1997), we found the free IAA content in the knockout line to be reduced by about 29% (Fig. 3c), which is most likely due to a different developmental stage of the utilized seedlings. In addition, the formation of IAA from L-trp is reduced by approximately 18% in *nit1-3* (Fig. 3d).

2.3. IAM, but not TAM, interferes with IAA production in vitro

Besides the above mentioned nitrilase-dependent IAA biosynthetic route, a tryptamine-dependent pathway has recently been suggested to operate in *Arabidopsis* (Zhao et al., 2001). With respect to this finding, emphasizing tryptophan-dependent IAA formation to proceed via tryptamine (TAM), we analyzed the effect of TAM (given in 10-fold molecular excess over $[^2H]_5$-L-trp) on labeled IAA formation. In the experiment, however, TAM showed no effect on $[^2H]_5$-IAA production (data not shown), thus, TAM is most probably also no intermediate of the examined *in-vitro* reaction.

The possibly easiest way to produce IAA from L-trp proceeds via the IAM-pathway, which is used by several plant pathogenic bacteria. Here, after invasion of the pathogens, a linearized T-DNA is used to transform host cell genomes. On these T-DNA fragments, among others, genes for a tryptophan 2-monooxygenase (*iaaM*) and an IAM-hydrolase (*iaaH*) are encoded. After expression in the host cells, the two gene products are capable of efficiently producing IAA from L-trp via an intermediate compound, IAM.

In a continuative approach, we tested the effect of indole-3-acetamide (IAM) on the *in-vitro* formation of IAA. For this, concentrated protein preparations were incubated with 1 mM $[^2H]_5$-L-trp as substrate and increasing amounts of IAM for 6 h. The reaction mixtures were acidified and the carboxylic acids extracted into ethyl acetate. Finally, the produced $[^2H]_5$-IAA was quantified by

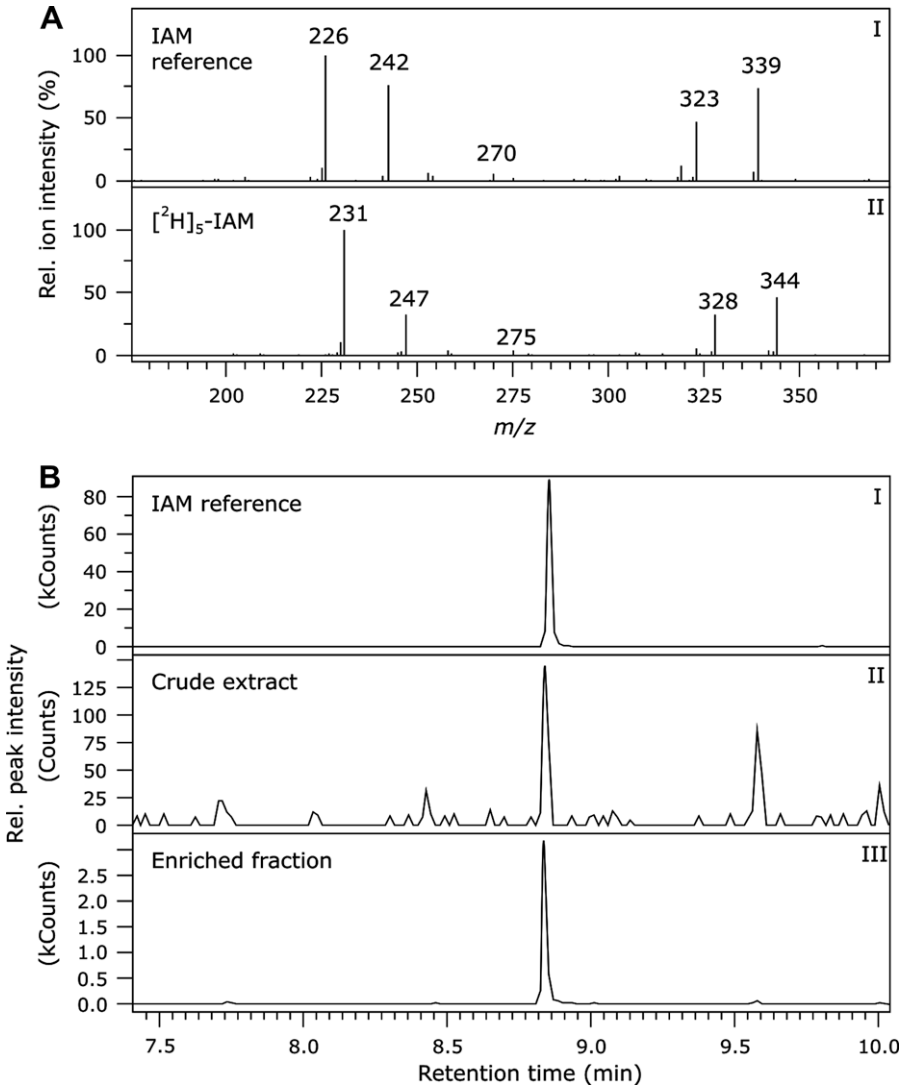

Fig. 5. Qualitative mass spectrometric detection of $[^2H]_5$-indole-3-acetamide derived from the precursor $[^2H]_5$-L-tryptophan. Pentadeuterated L-trp has been converted to $[^2H]_5$-IAM by either crude extracts (B II) or enriched protein fractions (B III) of *Arabidopsis thaliana*. MS/MS detection of the parent ions *m/z* 367 for unlabeled IAM and *m/z* 372 for pentadeuterated IAM, respectively, gave rise to the characteristic fragmentation pattern shown in A. In B chromatograms of either an unlabeled IAM reference (B I) or of enzymatically produced $[^2H]_5$-IAM are illustrated.

using GC–MS means. Fig. 4 illustrates the dose-dependent effect of IAM on the $[^2H]_5$-L-trp to $[^2H]_5$-IAA conversion. IAM, added in a molar 1:1 ratio relative to $[^2H]_5$-L-trp, reduced the isotopic abundance of ^2H in the sample in $[^2H]_5$-IAA to about 63% compared to the control reaction without IAM. When adding IAM in a 10-fold molecular excess over $[^2H]_5$-L-trp, the dilution was even more pronounced, showing an isotopic abundance of ^2H of only 21% relative to the control.

The dilution of the isotopic abundance can be explained by two conceivable reasons: firstly, IAM may have an inhibitory function or, secondly, it may be an intermediate of the 'IAA-synthase'-dependent biosynthetic route, competing with endogenous IAM for free catalytic binding sites. Accordingly, in the latter case, there should $[^2H]_5$-IAM be detectable in the reaction mixture. To explore this working hypothesis, mass spectrometric analyses were performed using a HPLC/GC–MS/MS technique previously established in our laboratory (Pollmann et al., 2002). Reaction mixtures containing 0.5 mM $[^2H]_5$-L-trp as substrate were incubated over a time period of 6 h, before the indolic compounds were extracted into ethyl acetate. Prior to GC–MS/MS analysis, the indolic constituents were separated by HPLC and the corresponding peaks individually collected. After taking samples to dryness and derivatizing of the IAM fraction by using trifluoroacetic anhydride, it was possible to identify $[^2H]_5$-IAM as a component of the reaction mixture by SRM-mass spectra (Fig. 5). The spectrum of trifluoroacetylated IAM showed the characteristic fragment ions for $[^2H]_5$-IAM at *m/z* 344, *m/z* 328, *m/z* 247, and *m/z* 231 (Pollmann et al., 2002). Whereas heat and protease treated samples showed no formation of $[^2H]_5$-IAM (data not shown), in further chromatographically purified protein preparations (see Supplementary data 1 and 2) an increase of the $[^2H]_5$-IAM content of approximately 120-fold was detectable (Fig. 5B panel III vs. panel II), which virtually correlates with the achieved enrichment of 'IAA-synthase' activity in the samples. The missing activity in inactivated samples as well as the increased $[^2H]_5$-IAM content in enriched protein preparations demonstrate the enzymatic formation of IAM from the precursor $[^2H]_5$-L-trp in the analyzed extracts. The presented results led to the assumption that IAM is a true intermediate of tryptophan-dependent IAA biosynthesis in the examined *in-vitro* system. Hence, IAM is supposed to be further converted to IAA.

To test whether $[^2H]_5$-IAM is, in a final reaction step, converted to $[^2H]_5$-IAA in the applied *in-vitro* system, we monitored this particular reaction step by GC–MS analysis. In this context, we firstly prepared labeled $[^2H]_5$-IAM from $[^2H]_5$-L-trp by the use of a bacterial tryptophan 2-monooxygenase from *Pseudomonas savastanoi*. The purified $[^2H]_5$-IAM was then used as substrate for the 'IAA-synthase' in the purified protein preparations. As expected, in these samples $[^2H]_5$-IAA formation was detectable (Fig. 6). To further corroborate that tryptophan and IAM-dependent IAA formation are tightly connected to each other, we performed isotope dilution experiments in a reversed manner, trying to affect the $[^2H]_5$-IAM-dependent $[^2H]_5$-IAA synthesis by adding increasing amounts of unlabeled L-trp. Fig. 6 depicts the L-trp concentration-dependent reduction of $[^2H]_5$-IAA production from $[^2H]_5$-IAM.

2.4. AMI1 as a possible component of 'IAA-synthase'

To determine whether AMI1 is possibly part of the 'IAA-synthase' complex, we undertook an immunological approach. For this purpose, enriched protein preparations were preincubated with various volumes of either preimmunserum or AMI1 antiserum. Thereafter, the samples were centrifuged and the remaining 'IAA-synthase' activity in the supernatants was analyzed. Whereas with the preimmunserum no precipitation of the 'IAA-synthase' activity was achieved, utilization of the AMI1 antiserum led to a decrease of the IAA-forming activity in the supernatant by about 52% (see

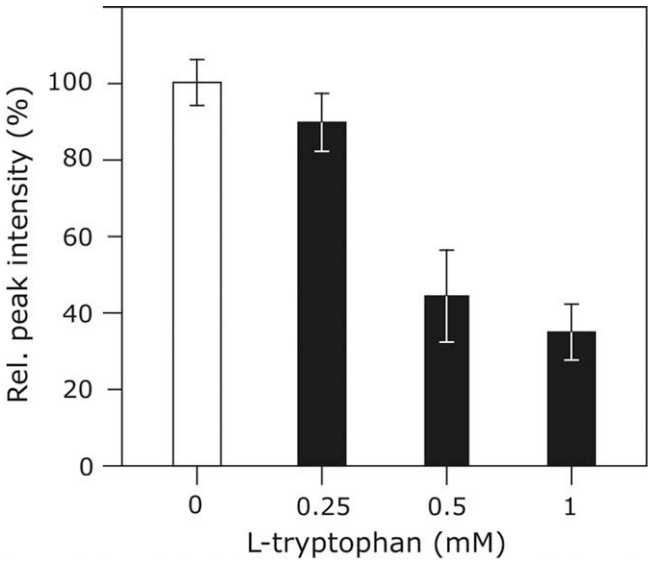

Fig. 6. Influence of L-tryptophan on the conversion of $[^2H]_5$-IAM to $[^2H]_5$-IAA. For the analysis of the interfering properties of unlabeled L-trp on the formation of $[^2H]_5$-IAA from $[^2H]_5$-IAM, enriched protein fractions have been co-incubated with 1 mM $[^2H]_5$-IAM as substrate and indicated amounts of L-trp. After incubation for 6 h at 37 °C, synthesized IAA has been assayed by GC–MS means. For normalization, the maximum amount of produced $[^2H]_5$-IAA (0.54 ± 0.03 nmol sample^{-1}) was set to 100%. The figure shows means ± SD derived from $n \geqslant 3$ individually performed experiments.

Fig. 7). Hence, it can be concluded that AMI1 can contribute to IAA formation *in vitro*, and that AMI1 or a closely related amidase-like protein may be part of the 'IAA-synthase'. Unfortunately, neither attempts to directly analyze the precipitated protein fraction after SDS–PAGE nor ongoing experiments making use of serum-free, immunospecific α-AMI1 antibodies were successful in identifying components of the 'IAA-synthase' complex. In the first case, the high load of interfering albumines and globulines among

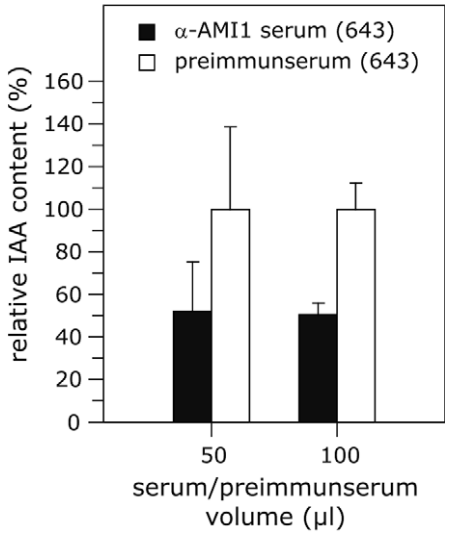

Fig. 7. Immunoprecipitation of 'IAA-synthase' activity by an AMI1 antiserum. Enriched protein fractions have been preincubated with indicated volumes of either AMI1 antiserum or the corresponding preimmunserum as an appropriate control. Subsequently the supernatant was transferred into fresh tubes and the remaining IAA-forming activity was analyzed by GC–MS analysis of the enzymatically produced IAA (for details see experimental procedures). The absolute amount of IAA produced in the samples containing the preimmunserum (1.01 ± 0.3 nmol sample^{-1}) was set to 100%. The results comprise means ± SD from $n \geqslant 2$ independently performed experiments.

the separated proteins made protein mass spectrometrical assessments impossible. In the latter case, it seemed as if the remaining antibody/antigene concentration was not high enough to facilitate cross-linking and thus precipitation of components of the 'IAA-synthase' complex. In fact, using the purified immunospecific α-AMI1 antibodies rather led to an increase of IAA production in the supernatant, which is most probably caused by the stabilization of the enzyme complex. However, this observation at least underscores the finding that the α-AMI1 antibody interacts with the L-trp converting enzymatic activity *in vitro*.

3. Conclusion

In the present study, evidence is provided for the occurrence of 'IAA-synthase' activity in a variety of different plant species. Furthermore, we provide indication of an involvement of an IAM-hydrolase rather than a nitrilase in the 'IAA-synthase' enzyme complex. Based on our data the participation of a nitrilase seems no longer tenable, as appropriate enzymes, homologous to the members of the NIT1 group of *Arabidopsis* (Vorwerk et al., 2001), are restricted to the families of the Musaceae, Poaceae, and Brassicaceae. In addition, we analyzed IAA contents and IAA production in *nit1-3* mutant background relative to Col-0 wild type. This experiment revealed only modest alterations in IAA contents and in IAA-forming enzyme activity. In summary, these finding likewise underscore two different aspects: on the one hand NIT1 is capable of contributing to auxin biosynthesis *in vitro*; on the other hand this contribution is only of minor importance, as the knockout line remains more than 80% of its ability to produce IAA. Thus, it is appreciably improbable that a nitrilase is catalyzing the last step in an 'IAA-synthase' complex. This conclusion has previously been corroborated with the evidence that neither indole-3-acetonitrile (IAN) nor indole-3-acetaldehyde (IAAld) had an influence on the conversion of $[^2H]_5$-L-trp to $[^2H]_5$-IAA (Müller and Weiler, 2000). Additionally, the same result was obtained when TAM was added. Moreover, no labeled $[^2H]_5$-IAN and $[^2H]_5$-IAAld were detected in the reaction mixture when $[^2H]_5$-L-trp was used as substrate. Accordingly, IAA production catalyzed by the 'IAA-synthase' most likely makes no use of these compounds.

It is yet too early to assume that IAA is formed via a bacteria-like two step reaction pathway in *Arabidopsis*, as nothing is known about the initial reaction step so far. Nevertheless, the functional occurrence of such a pathway in plants has been suggested by a couple of independent laboratories who either found IAM to be an endogenous constituent in plants (Takahashi et al., 1975; Saotome et al., 1993; Rajagopal et al., 1994; Pollmann et al., 2002) or who observed the enzymatic conversion of IAM to IAA in extracts of various plant species (Kawaguchi et al., 1993; Arai et al., 2004). In this regard, the identification and characterization of an IAM-hydrolyzing amidase (AMI1) from *Arabidopsis thaliana* is noteworthy (Pollmann et al., 2003, 2006b), as it further confirms the possible operational capability of an IAM-pathway in the plant kingdom. However, considering the presented analytical details, it may be suggested that the final reaction step in the 'IAA-synthase' is seemingly catalyzed by AMI1, or a close homolog, possibly from the same enzyme family (Neu et al., 2007).

To further enrich the 'IAA-synthase' activity will be a challenging task for the future, as it will help to perform convincing protein mass spectrometric analyses which hopefully allow the unequivocal identification of components of this complex. Unraveling of the initial reaction step of L-trp conversion to IAM is likewise an exciting challenge for upcoming studies. Samples containing both L-trp and $[^2H]_5$-IAM as substrates showed a higher combined production of IAA (unlabeled + labeled IAA) than comparable control samples, containing only L-trp in vast excess. In the substrate saturated reaction mixtures, the L-trp content cannot be a limiting factor, which may point out that the formation of IAM rather than its conversion to IAA is the rate-limiting step in this biosynthetic pathway.

From the current point of view, a couple of possible enzymatic reactions can be assumed to be able to contribute to this reaction. Firstly, a bacteria-like tryptophan 2-monooxygenase could be responsible for the production of IAM. Unfortunately, such a protein cannot be identified with certainty only based on *in-silico* analysis, as this class of oxygenases carries several conserved domain structures which hamper the attempt to cut down the number of putative candidate proteins. Secondly, L-trp can initially be deaminated by the action of an appropriate transaminase. The occurrence of such an enzyme has just recently been described (Tao et al., 2008; Stepanova et al., 2008). This reaction sequence presumably proceeds with the decarboxylation of the intermediate, indole-3-pyruvate, leading directly to indole-3-acetaldehyde, thus surpassing IAM and making L-trp-transaminase not to a favorable candidate for the initial L-trp conversion in an 'IAA-synthase'-dependent reaction. Additionally, previous studies excluded aldehyde oxidases from the 'IAA-synthase' complex (Müller and Weiler, 2000).

Alternatively, L-trp could initially be decarboxylated by suitable tryptophan decarboxylases leading to the intermediate reaction product, TAM. Such aromatic L-amino acid decarboxylases have already been described to occur in plants, e.g. *Catharanthus roseus* (De Luca et al., 1989), *Camptotheca acuminata* (Lopez-Meyer and Nessler, 1997), while the occurrence of TAM as well as the existence of corresponding genes and proteins, respectively, are yet to be characterized in *Arabidopsis*. However, several publications suggest TAM to be the substrate of YUCCA family members from *Arabidopsis* (Zhao et al., 2001; Kim et al., 2007) and tomato (Expósito-Rodríguez et al., 2007), which is in clear variance with earlier $[^2H]_2O$-feeding studies pointing out that TAM is, though endogenous to tomato, most likely no precursor of IAA biosynthesis (Cooney and Nonhebel, 1991; Nonhebel et al., 1993). Anyhow, based on the results described here and those described earlier by Müller and Weiler (2000), a participation of a tryptophan decarboxylase as well as of a P450 enzyme such as, for instance, CYP71A13 (Nafisi et al., 2007) in the 'IAA-synthase' complex can be excluded, because neither TAM was able to interfere with IAA formation nor is the enzymatic reaction NADH or NADPH-dependent. This is further corroborated by the fact that no production of $[^2H]_5$-indole-3-acetaldoxime in the cell free extracts was detectable. Summing up, an initial oxidative decarboxylation as, for example, accomplished by bacterial tryptophan 2-monooxygenases seems to be the most probable starting point of the 'IAA-synthase' catalyzed L-trp conversion. A so far enzymatically uncharacterized member of the YUCCA family could be a potential candidate for this particular task. Work on the restoration of higher order *yuc* mutants point towards a so far undiscovered connection of IAM formation and YUCCA enzymes. Infertile YUCCA double and triple mutants were rescued by tissue-specific IAM production facilitated by co-expression of a bacterial tryptophan 2-monooxygenase. In these experiments, the expression of a tryptophan 2-monooxygenase, that exclusively catalyzes the conversion of L-trp to IAM (Emanuele and Fitzpatrick, 1995a,b), under the control of several different *YUCCA* promoters (Cheng et al., 2006), but not the supply with exogenous IAA helped to restore a wild type-like phenotype.

In conclusion, this could possibly indicate that IAM is an intermediate in YUCCA-dependent IAA production. Assuming, that TAM is probably not the preferred *in-vivo* substrate of YUCCA, which is in fact still an open question, a YUCCA isoenzyme could operate upstream of an IAM-specific amidase. In this, it could be responsible for the rate-limiting production of IAM, perhaps also in a bigger metabolic complex as e.g. the 'IAA-synthase'.

4. Experimental

4.1. Plant material

Apart from the experiments on the distribution of the 'IAA-synthase' activity in the plant kingdom and the comparison of *nit1-3* with Col-0 (NASC, stock N1092) seedlings, all experiments made use of *Arabidopsis thaliana* (L.) Heynh., ecotype C24. The original seed stock was kindly provided by L. Willmitzer, Max Planck Institute of Molecular Plant Physiology, Golm, Germany. The *nit1-3* line, containing a premature stop codon, was provided by the Nottingham Arabidopsis Stock Center (NASC), stock N3738. Plants were grown under greenhouse conditions in a 2:1 (v/v) mixture of standard soil and sand for 4 to 5 weeks in short days (8 h photoperiod). The plants were then transferred to long days (16 h photoperiod) until they were harvested. Average temperature during night times was maintained at 18–20 °C, during days at 22–24 °C. The photosynthetically active radiation was no less than 150 $\mu E\ m^{-2}\ s^{-1}$. The other plant species indicated in Fig. 2 were either obtained from the botanical garden of the Ruhr-University Bochum or freshly bought on a market.

4.2. Preparation of enzyme extracts

Freshly harvested leaf material was grounded in liquid nitrogen and then further homogenized in 50 mM HEPES buffer pH 8.5 (1 ml (g fr. wt)$^{-1}$), containing 200 mM sucrose, 3 mM EDTA, 3 mM DTT, and 5% (w/v) insoluble polyvinylpyrrolidone (Polyclar AT, Serva, Heidelberg, Germany). The solution was filtered through two layers of gauze to retain coarse ingredients, before the flow through was centrifuged at 10,000*g* and 4 °C for 30 min. The obtained supernatant was then either taken for direct analyses or precipitated by ammonium sulphate (70% saturation). The precipitate was either (in case of volumes <10 ml) re-suspended in 100 mM potassium phosphate buffer pH 8 and desalted by gelfiltration on PD-10 columns (Amersham Biosciences) or alternatively re-suspended in 10 mM Tris/HCl pH 8 and dialyzed for 16 h (4 °C) against circa 100 volumes of 10 mM Tris/HCl pH 8. Thereafter, the solution was centrifuged (100,000*g*, 4 °C, 45 min) to pellet insoluble components, cell debris, and membranes. In this concentrated form, the supernatants were then used for subsequent assays.

4.3. Enrichment of the 'IAA-synthase' activity

To further enrich the 'IAA-synthase' activity in the 100,000*g* centrifuged supernatant, we conducted purification with fast liquid protein chromatography on a hydroxyapatite matrix. A column (16 × 1.6 cm i.d.), loaded with hydroxyapatite (MacroPrep Ceramic Hydroxyapatite, Bio-Rad), was at first equilibrated with 10 mM Tris/HCl pH 8. Then the column was loaded with the protein solution, before unspecifically bound polypeptides were washed off the column with 70 ml of 5 mM potassium phosphate buffer pH 8. Bound proteins were thereafter eluted with a step gradient of increasing potassium phosphate buffer concentrations (60 ml 50 mM K-Pi buffer, pH 8; 60 ml 200 mM K-Pi buffer, pH 8; 60 ml 400 mM K-Pi buffer, pH 8). Chromatography was carried out at 4 °C with a constant flow rate of 0.5 ml min^{-1}. The major part of the activity eluted with the 50 mM K-Pi step (80–120 ml) (see Supplementary data 1). The corresponding fractions were pooled and used in the following purification step. In this second chromatographic step, the combined fractions, showing highest L-trp to IAA conversion, were separated on an ion exchange chromatography resin (MacroPrep High Q, Bio-Rad). In this case, the column (15 × 1 cm i.d.) was equilibrated with 50 mM Tris/HCl pH 8 prior to loading of the sample. Non-bound proteins were washed off the column with 70 ml 50 mM Tris/HCl pH 8. Afterwards, the elution of specifically bound proteins was performed using again a step gradient, this time with increasing concentrations of chloride ions in the buffer (60 ml 50 mM Tris/HCl, pH 8, 50 mM NaCl; 60 ml 50 mM Tris/HCl, pH 8, 200 mM NaCl; 60 ml 50 mM Tris/HCl, pH 8, 400 mM NaCl). As before, chromatography was conducted at 4 °C and a constant flow rate of 0.5 ml min^{-1}. Here, most activity eluted with the 200 mM NaCl step (Supplementary data 2). All fractions comprised in the respective peak were combined and used in subsequent experiments.

4.4. Enzymatic assays

To assess putative intermediates of the 'IAA-synthase' catalyzed reaction, 500 µl reaction mixtures were prepared containing 400 µl of an appropriate protein fraction together with 1 mM of $[^2H]_5$-L-trp (Cambridge Isotope Laboratories, Andover, USA) as substrate in 100 mM potassium phosphate buffer pH 8. In addition, these samples were supplemented, at the concentrations indicated, with indolic compounds, in particular tryptamine (TAM) and indole-3-acetamide (IAM), both obtained from Sigma–Aldrich, Munich, Germany. The reaction mixtures were incubated at 37 °C for at least 6 h, before the amount of produced $[^2H]_5$-IAA was determined by GC–MS analysis. To study the effect of the added indolic compounds on the activity of the 'IAA-synthase', the isotopic abundance of deuterium in $[^2H]_5$-IAA in the samples containing either TAM or IAM was compared to that in control reactions (set as 100%) lacking these additional substances. In order to be able to quantify the produced IAA and $[^2H]_5$-IAA, all samples were internally standardized with $[^2H]_2$-IAA (Campro Scientific, Berlin, Germany) in concentrations depending on the estimated content of formed IAA in the reaction mixtures. In order to identify $[^2H]_5$-IAM, liberated from the 'IAA-synthase' complex, reaction mixtures as described above were incubated with 0.5 mM $[^2H]_5$-L-trp as substrate. After an incubation of 6 h at 37 °C released $[^2H]_5$-IAM was extracted and analyzed qualitatively using a combined HPLC/GC–MS/MS technique (*vide infra*). To enable the detection of both $[^2H]_5$-IAA derived from $[^2H]_5$-IAM and the effect of unlabeled L-trp on this particular enzymatic conversion, samples of 500 µl, comparable to those described above, were incubated with 1 mM $[^2H]_5$-IAM and additionally with the indicated amounts of L-trp. They were as well kept at 37 °C for 6 h, before they were acidified (pH 2) and extracted with equal volumes of ethyl acetate. The content of $[^2H]_5$-IAA derived from $[^2H]_5$-IAM as well as unlabeled IAA produced from L-trp was determined, and the ratio of labeled over unlabeled IAA calculated, to assess the general formation of $[^2H]_5$-IAA from the precursor $[^2H]_5$-IAM and the effect of unlabeled L-trp onto this conversion, respectively. In all experiments, samples treated with trypsin for 30 min at 37 °C, followed by 20 min of heat inactivation (95 °C) were analyzed in conjunction with the samples specified above to ensure that the retrieved reaction products were in fact due to enzymatic conversions and not obtained by spontaneous autocatalytic turnover. For the purpose of calculating the specific activities of the 'IAA-synthase' in the corresponding reaction mixtures, protein concentrations were detected according to Bradford (1976) using serum albumin as a protein standard.

4.5. Immunoprecipitation

To analyze the influence of α-AMI1 antibodies (Pollmann et al., 2003) on the *in-vitro* conversion of L-trp to IAA, 200 µl of enriched protein preparations were preincubated (overnight, 4 °C) with various volumes of either preimmunserum or AMI1 antiserum in an overhead shaker. Then the samples were centrifuged (14,000*g*, 4 °C, 30 min) and the resulting supernatants transferred to fresh

tubes. Thereafter, these protein samples were used in subsequent enzymatic assays.

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

Pentadeuterated IAM was enzymatically prepared using a bacterial tryptophan 2-monooxygenase from *Pseudomonas savastanoi*, according to Pollmann et al. (2002). Recombinant protein, produced as described by Emanuele et al. (1995), was used to convert $[^2H]_5$-L-trp (Cambridge Isotope Laboratories, Andover, USA) to $[^2H]_5$-IAM. The reaction product was then purified by HPLC, using a ZorbaxSil 5 μm column (250 mm × 4 mm i.d.; Knauer, Bad Homburg, Germany) and a solvent system prepared by saturating a mixture of 300 ml *iso*-hexane and 200 ml ethyl acetate with 2.5% (v/v) aqueous formic acid. Finally, to the organic phase of this system 10% (v/v) methanol was added.

4.7. Quantification of IAA

Produced IAA was detected according to the procedure described by Müller and Weiler (2000). In summary, the samples were initially acidified to pH 2 by adding concentrated HCl, then IAA was extracted into ethyl acetate, and the organic phase concentrated to dryness. Hereafter, the residue was derivatized with ethereal diazomethane, taken to dryness again, before being re-dissolved in 35 μl of chloroform. All spectra were recorded on a Varian Saturn 2000 mass spectrometer coupled to a Varian GC 3800 gas chromatograph. The mass spectrometer worked in CI–MS/MS mode, as the reactant gas methanol was used. The GC settings were as follows: splitless injection (1 μl), injector temperature 260 °C; as column a ZB50 (30 m, 0.25 mm i.d., 0.25 μm film thickness; Phenomenex, Aschaffenburg, Germany) was utilized with helium as the carrier gas at a flow rate of 1 ml min^{-1}. Temperature program was: 1 min 80 °C, linear ramp at 30 °C min^{-1} to 200 °C, linear ramp at 5 °C min^{-1} to 250 °C. Mass spectrometry conditions were: transfer line 260 °C; detector: mass range of 100–380 amu with 3 scans s^{-1}, positive ion mode. Unlabeled IAA was detected by collecting the parent ion *m/z* 190, $[M+H]^+$ of the IAA methyl ester and observing the characteristic fragment ion *m/z* 130 (quinolinium-ion). Pentadeuterated IAA was analyzed by collecting *m/z* 195, $[M+H]^+$ of the $[^2H]_5$-IAA methyl ester and scrutinizing the fragment ions *m/z* 134 + 135 (quinolinium-ion). To allow calculation of produced IAA and $[^2H]_5$-IAA, the parent ion *m/z* 192, $[M+H]^+$ of the $[^2H]_2$-IAA methyl ester, which was used as an internal standard, was as well collected and the fragment ion *m/z* 132 (quinolinium-ion) was analyzed.

4.8. Analysis of IAM formation

The examination of IAM in the reaction mixtures was carried out as previously described (Pollmann et al., 2002). Briefly, indolic compounds were extracted into ethyl acetate, taken to dryness, and re-dissolved in an appropriate volume of the HPLC solvent. Subsequently the sample was subjected to HPLC separation on a silica based column (ZorbaxSil 5 μm, 250 mm × 4 mm i.d.; Knauer, Bad Homburg, Germany). The peak referring to IAM was collected, the contained substances dried under a stream of nitrogen, followed by a derivatization with trifluoroacetic anhydride. Thereafter, 1 μl of the trifluoroacetylated sample was injected into a Varian GC 3800 gas chromatograph coupled to a Varian Saturn 2000 mass spectrometer. The mass spectrometer was operated in CI–MS/MS mode with methanol as the reactant gas and positive ion detection mode. Further settings were: splitless injection (1 μl), injector temperature 260 °C; ZB50 fused silica column (30 m, 0.25 mm i.d., 0.25 μm film thickness; Phenomenex, Aschaffenburg, Germany), helium 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. The mass spectrometer operated in a mass range of 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).

4.9. Gel electrophoresis and protein immunoblotting

Denaturing gel electrophoresis was performed according to Laemmli (1970). The discontinuous systems consisted of 4% stacking gels and 12.5% resolving gels. Protein blotting onto nitrocellulose was carried out electrophoretically overnight (4 °C, 60 mA) as described by Towbin et al. (1979). Immunodetection followed standard procedures (Parets-Soler et al., 1990) with goat anti-rabbit IgG-conjugated alkaline phosphatase as the secondary antibody and 4-nitrotetrazolium blue and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Preparation and reactivity of the α-NIT1-antibody towards NIT1, but also NIT2 and NIT3, has previously been described by Schmidt et al. (1996) and Vorwerk et al. (2001).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.01.021.

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