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Review Article

Synthesis and hydrolysis of auxins and their conjugates with different side-chain lengths: are all products active auxins?

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Abstract

Plants need hormone substances to regulate a plethora of responses during their life cycle. One major hormone class is called auxin, which is involved in many developmental processes. Besides the major auxin indole-3-acetic acid, there are other auxin-like molecules present in some, but not in all plants, an example would be chlorinated IAA in legumes. Among these are also the auxins with longer chains, indole-3-propionic acid and indole-3-butyric acid. The auxin-dependent growth response is dependent on the concentration of the compound. While lower concentrations are mainly growth promoting, high concentrations are actually inhibiting some developmental processes. Therefore, tight control of the auxin concentration is essential for proper growth and development. This can be achieved by altering the amount of active auxin via transport, biosynthesis, degradation or reversible conjugation to small molecules. In addition, plants use auxin during their interaction with the environment, for example during abiotic stresses such as salt, temperature or water stress to adapt the growth responses specifically. Furthermore, auxin is involved in the development of plant disease symptoms, such as tumor growth or aberrant tissue formation. However, together with other plant hormones such as salicylic acid auxin can also modulate disease progression or resistance in different plant – microbe combinations.

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INTRODUCTION

Auxins are involved in a plethora of developmental processes and control organ formation as well as cellular differentiations *(1)*. In addition, auxins seem to be involved in abiotic *(2)* as well as biotic stress reactions *(3)*. The major auxin in plants is indole-3-acetic acid (IAA), but there are other natural derivatives with similar, lower or even higher bioactivity *(4)*. In any case, the response is strongly dependent on the actual concentration of a given auxin in the plant, since high concentrations of auxin might be inhibiting growth responses *(5)*. Therefore, the tight control of auxin levels is essential, wich can be achieved by either regulating biosynthesis, conjugation to inactive compounds, degradation and transport *(6, 7)*. While the other components of the homeostatic system are also important, this review will concentrate mainly on the formation of auxin conjugates and their hydrolysis to the free hormone (Figure 1), but will touch other regulatory aspects such as transport when appropriate (Figure 2). An additional aspect will be the formation of IAA from another auxin with a longer side chain,

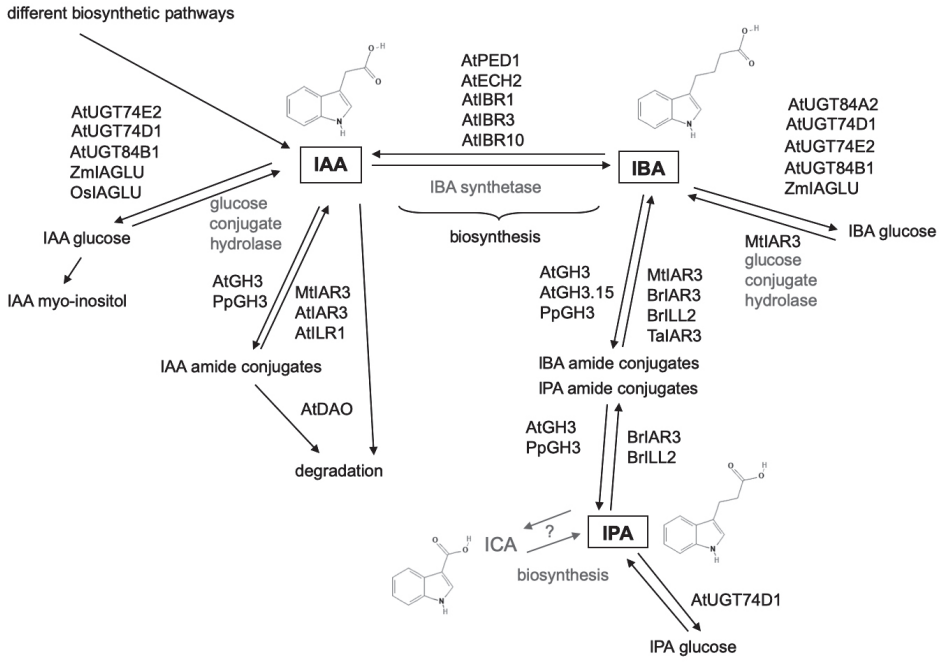

Figure 1. Biosynthesis and metabolism of indole-3-acetic acid (IAA), indole-3-propionic acid (IPA) and indole-3-butyric acid (IBA). *High molecular weight conjugates are not shown. Known genes or genes where a function has been assumed in IBA metabolism and which are discussed throughout the review are given. The prefix At stands for Arabiopsis thaliana, Br for Brassica rapa, Mt for Medicago truncatula, Pp for Physcomitrella patens, Ta for Triticum aestivum and Zm for Zea mays. For IBA synthetase only an enzymatic activity is known, therefore given in grey. Whether IPA can be converted to the two unit shorter side-chain derivative indole-3-carboxylic acid (ICA) and vice versa, as IBA and IAA interconversions, has not been shown as yet, therefore is indicated by a question mark. All structures were taken from PubChem (https://pubchem.ncbi.nlm.nih.gov; accessed 17.1.2020).*

indole-3-butyric acid (IBA) *(8)*. The question whether the longer-chain auxins that are conjugated and hydrolyzed with high efficiency are auxins per se or rather inactive compounds that need conversion to the active auxin IAA will be discussed.

The auxin IBA has been used for decades in the rooting of cuttings and has thus become of economical importance in horticulture *(9)*. Since its discovery as endogenous compound, its use has been widely accepted in a variety of formulations for many different plant species. In contrast to indole-3-acetic acid (IAA), the major auxin in most plants, it was believed that IBA has a better stability, different metabolism and transport and would thus serve as better source for auxin *(10, 11)*. Many studies using so called easy- and difficult-to-root species have been conducted to find a common theme *(12, 13)*, but so far no clear-cut evidence has emerged that would allow a conclusion why IBA is sometimes superior to IAA.

Within the last decades it has been accepted that IBA is an endogenous compound in a variety of plant species. The occurrence as well as endogenous levels have been determined by mass spectrometry methods *(10, 14)*. The biosynthesis and metabolism of IBA has been investigated by biochemical, molecular and genetic tools *(8, 11)*. It has been shown in the model plant *Arabidopsis thaliana* that the conversion of IBA to IAA most likely excerts its function, but additional data point to a role for IBA independently of IAA. In addition, IBA can be converted to IAA by steps similar to ß-oxidation in peroxisomes *(6, 8)*. The biosynthesis of IBA from IAA has been described on an enzymatic level *(15)*, but so far no genes have been cloned. Evidence is emerging for other plant species such as *Oryza sativa* (rice), *Zea mays* (maize) or the model legume *Medicago truncatula* that IBA acts as plant hormone.

More recently, for two longer side-chain auxins, IBA and indole-3-propionic acid (IPA) the hydrolysis from amino acid conjugates has been described *(16, 17)*, but the significance of this reaction is not yet clear and will be discussed below. The structures of all three auxins and their possible conversion to other compounds can be found in Figure 1. Since the identification of these compounds with auxin activity there is a matter of debate as to whether they act as auxin molecules themselves or through the interconversion to IAA, as was described for IBA in Arabidopsis *(18, 19)*. However, signaling components specific for IBA have been identified *(20)* and there is evidence that IBA is transported differently from IAA and this could add to its specific function *(21, 22)*. Evidence for both assumptions are presented in one chapter. However, since also the long-chain auxins are conjugated and hydrolysed like IAA, it could well be that there are

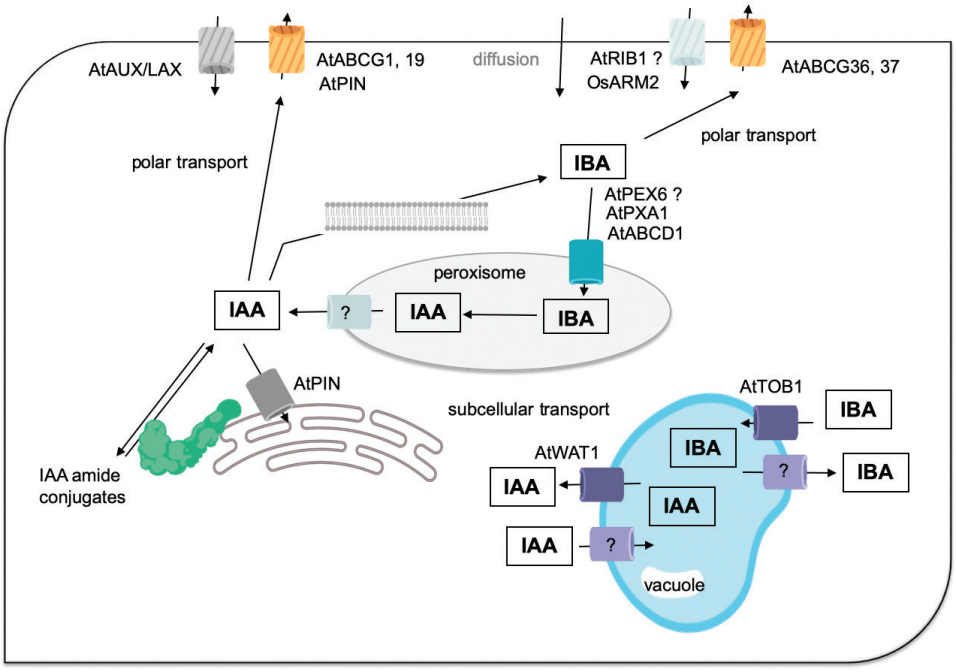

Figure 2. Subcellular compartimentation and intracellular and inter-tissue transport of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). *IAA and IBA are taken up into the cell as well as exported by different carrier proteins. For IBA the influx carrier has not yet been identified in Arabidopsis thaliana, but there is a candidate in Oryza sativa. IAA efflux is determined by the presence of PIN proteins, but also some ABC-type transporters, whereas IBA seems to be exclusively exported by ABCG efflux carriers, albeit different ones than IAA. IAA conjugates are probably synthesized in the cytosol, but their hydrolysis might take place, at least partially in or at the ER, where some hydrolases are sopposed to reside due to their ER retention sequences. Further, IAA con be transported intot the ER by a specific PIN protein. The vacuolar transport is also indicated, but much less is known here about its possible function for subcellular auxin homeostasis as well as the transporters located in the tonoplast. Some symbols were taken from the free version of biorender (https://app.biorender.com; accessed 4.2.2020). The prefix At stands for Arabidopsis thaliana and Os for Oryza sativa.*

functions of these two hormones that have not yet been discovered. For example, IBA is metabolized similar to IAA to form conjugates with amino acids or sugars *(7)*, but in contrast to IAA nothing is so far known about its degradation. In addition, IPA conjugates are strongly cleaved in *Brassica rapa* (Chinese cabbage) by several hydrolases *(17)*.

I. FUNCTIONS FOR AUXIN CONJUGATES

The role of auxin conjugates has been attributed mainly as a storage for the homeostasis of free auxin compounds in normal growth and development *(7)*, but more recently, additional functions in abiotic and biotic stress situation have been found. This is mainly due to the discovery of new enzyme activities for gene families mainly in Arabidopsis, but also in other plant species. Both, the formation of conjugates as well as their hydrolysis has to be taken into account here. Two major classes of auxin conjugates are known, ester and amide bonds with either glucose or amino acids, respectively *(7)*. The formation of glucose conjugates had been attributed mainly to monocot family members, and consequently, the first ester conjugate synthase was found in maize *(23)*. However, similar enzymes were found in dicots, so that characteristic does not seem to fit any more *(24)*. Both classes of conjugates will be described here, but amide conjugates with peptides or proteins will not be included *(25–27)*. Furthermore, the focus will be on the enzymatic activities related to IAA (see Figure 3 for other potential substrates in conjugation).

AMIDE CONJUGATES

The conjugate formation has been attributed to be a consequence of high auxin levels, since genes encoding members of the GH3 family of auxin conjugate synthetases belong to the auxin-inducible gene families *(28)*. Their active hydrolysis contributes to the levels of free IAA and thus growing tissues, *e.g.* of *Arabidopsis* show high expression of such conjugate hydrolases *(29)*. However, at least two amino acid conjugates have been found to be involved in the irreversible degradation pathway *(30)*. Since the conjugate forming and hydrolysing proteins are encoded by large gene families *(29, 31–33)* the study of their role in development is difficult unless multiple mutants are generated in such studies *(29, 34)*. The overexpression leads often to stronger phenotypes and was there-

Table 1. *Using TILLING (Targeting Induced Local Lesions IN Genomes) to isolate mutants of three Brassica rapa conjugate hydrolases with the wild type rapid cycling B. rapa R-o-18 line. The Reverse Genetics facility RevGenUK at John Innes Centre, was used as a service (Colbert et al.). The technique enables high-throughput screening for induced point mutations, where induction is done by EMS and screening by selectively pooling the DNA and amplifying with fluorescently labelled primers, of which mismatched heteroduplexes were generated between wild type and mutant DNA and these were further identified by sequencing. Using this approach a set of putative mutants was generated for each hydrolase gene; n – total number of mutations.*

Type of mutation		Hydrolase		
		BrIAR3	BrILL2	BrILL6
Amino acid change	missense	12	5	13
	neutral	6	4	3
Premature stop		1	2	1
Splice site		1	0	0
Non-coding region		3	2	5
n		23	13	22

fore also used *(35)*. Also, genes isolated from monocots have been expressed in dicots to study their function *(36, 37)*. In many cases the "typical" auxin deficiency or "high" auxin phenotypes have been described *(7, 35)*. However, sometimes the phenotype can only be seen under high auxin conditions *(29, 38)*.

Some enzymatic activities have been related to individual auxins, *i.e.* mainly/only IAA or IBA is conjugated. However, there are also enzymes with a more promiscuous substrate preference towards the carboxylic acid. Some IAA conjugates seem to be involved in tolerance against abiotic stress *(33, 39–42)* while others specifically seem to be important in plant – microbe interactions *(43–48)*. A *Populus tremula x tremuloides* (poplar) IAA conjugate hydrolase specifically identified undere salt stress conditions conferred salt tolerance to Arabidopsis when heterologously overexpressed *(49)*. In Chinese cabbage the expression of a set of auxin amino acid conjugate hydrolase genes responded to stress hormones, salicylic acid (SA) and jasmonic acid (JA) *(50)*. To study the complex function of this family their activities were identified and shown to be rather specific for longer-chain auxin conjugates with alanine *(17)*. All alanine conjugates could be hydrolysed *in planta* when incubated with high concentrations of the respective conjugate, but with higher affinity for IPA-Ala, followed by IBA-Ala and last IAA-Ala *(17)* and these were confirmed by Molecuar Dynamics simulations where IPA-Ala showed the highst affinity within the binding pocket *(51)*. Furthermore, in a TILLING population *(52)* several mutations were found that are going to be analysed in the future (Table 1; for details of the methodology see legend). Some mutations leading to a premature stop codon might be the promising ones for future studies unless it was shown that the single amino acid changes could contribute to alterations in enzymatic activities. This might be possible to predict due to previous modelling studies *(17)*. However, it should be noted that even truncation of a *Medicago* hydrolase gene family only led to changes in enzyme activity and not to complete loss *(53)*.

Some interesting features were attributed during plant defense to amino acid conjugates. On the one hand, their formation can be indirectly linked to defense since overexpression lowers IAA levels and thereby also reduces for example cell wall weakening enzymes, the expansins. With a stronger cell wall the pathogens cannot enter as easily and a basal resistance was conferred *(44)*. On the other hand, a direct effect of the amino acid conjugate of IAA with aspartate (IAA-Asp), that is thought to be part of the degradation route *(30)* was shown to act as a pathogenicity signal for two different phytopathogens *(43)*. The expression of pathogen-associated genes of *Pseudomonas syringae* were induced by IAA-Asp and consequently, the knockdown of the responsible GH3 gene resulted in more resistant plants, whereas addition of IAA-Asp increased the susceptibility *(43)*. Furthermore, the Arabidopsis GH3.5 protein is at the crossroad of IAA and the defense hormone SA, since it can conjugate both plant hormones to amino acids *(45)*. In addition, differential regulation for conjugate hydrolase as well as for synthetase genes has been reported in the interaction of plants with symbionts such as arbuscular mycorriza and rhizobia *(47, 48)*. Highlighting only a few examples for the involvement of GH3 proteins in plant – pathogen interaction already show the diversity of possible functions.

ESTER CONJUGATES

The upper paragraphs described some examples for the involvement of amino acid conjugates in the stress response of plants, while the next will deal with the formation of ester conjugates (for their possible involvement see Figure 1). The large family of UDP-glucose dependent glycosylases contain several members that are capable to form auxin conjugates with glucose *(24)* similar to the IAGLU protein from maize, the first in its class to be isolated *(23)*. In addition to the glucose ester, also the formation of conjugates with *myo*-inositol were found *(54)*. A UDP-glucose transferase from rice altered shoot architecture and root gravitropism when overexpressed *(55)*. The identification of an Arabidopsis homolog UGT-84A1 catalyzing the conjugation of IAA to glucose showed the first time the existence of this reaction on a molecular level in dicot plants *(56)*. Overexpression in the homologous system led to altered phenotypes typical to auxin deficiency *(57)*. While for the formation of amide conjugates IAA as a substrate was in the focus, for the formation of ester conjugates also an important role for IBA

conjugates with glucose were detected in Arabidopsis *(58–60)*. The closest homolog to the Arabidopsis UDP-glucose transferase specific for IAA, UGT84A2, showed high specificity for IBA, but only IAA was tested as other substrate *(60)*, so additional activites could not be found if present. A role for this gene product in the flowering time was proposed bysed on extopic expression *(60)*. In the work using a different glucosyltransferase from Arabidopsis (UGT74E2) it was shown that overexpression led to an altered stress response, i.e. the plants were more tolerant to water stress *(58)*. Furthermore, additional enzymes have been characterized from that large superfamily that catalyse not only the conjugation of IBA and IAA to glucose, but use also IPA as substrate *(59)*. Overexpression of the gene resulted in an accumulation of IBA-glucose as well as a curling leaf phenotype in Arabidopsis, suggesting a physiological role of the encoded enzyme UGT74D1 in affecting the activity of auxins as well *(59)*. Additionally, glycosyltransferases might be involved in the modification of glycoproteins with IAA as shown for *Pisum sativum* (pea) *(61)*.

While the synthesis of auxin ester conjugates, especially with glucose, is now quite well investigated, the hydrolysis of such conjugates is much less clear. The reaction to conjugate IAA with glucose seems to be an equilibrium reaction and only the formation of IAA *myo*-inositol then shifts the ratio of free to conjugated IAA in favour of the conjugate *(62)*. Therefore, it was long thought that for the first reaction there is no specific enzymatic activity necessary. However, on the enzymatic level, there are reports on the hydrolysis of IAA and IBA glucose described *(63)*. Furthermore, there are reports on the hydrolysis of ester conjugates by the ILR family of auxin amino acid conjugate hydrolases *(48)*.

II. EVOLUTION OF AUXIN CONJUGATE SYNTHESIS AND HYDROLYSIS

The development of land plants might be tightly connected with auxin function, but also the specific regulation. Even in algae there might be a regulation because in

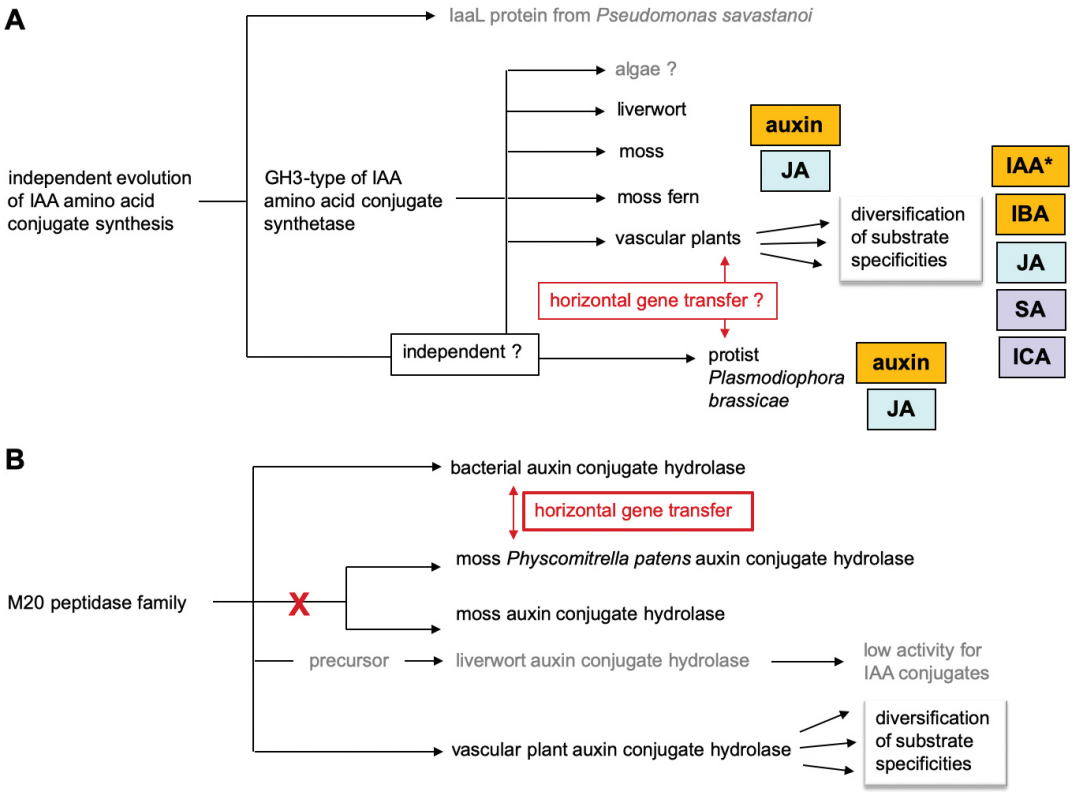

Figure 3. Possible evolution of (A) the auxin amino acid conjugate synthesis and (B) the hydrolysis of auxin amino acid conjugates, both in microbes and plants. *For the conjugation at least two completely independent events have led to the evolution of two completely different protein types in a plant pathogenic bacterium, Pseudomonas savastanoi, and in all other organisms to the GH3 family. Whether in the plant pathogenic protist Plasmodiophora brassicae the GH3 activity was independently evolved or acquired by a horizontal gene transfer event, is not clear. The auxin conjugate hydrolases have evolved from the M20 peptidase family, which is present in microbes and plants. Also here, there seem to be two different events in evolution that led to the differentiation of bacterial and plant enzymes since they cluster very differently in phylogenetic analyses (see text). Algae must have conjugate synthesis since in some species auxin conjugates were found. The events shown are not to scale in evolutionary terms; auxin means IAA, IPA, IBA; *makes probably also IPA and in some cases IBA conjugates.*

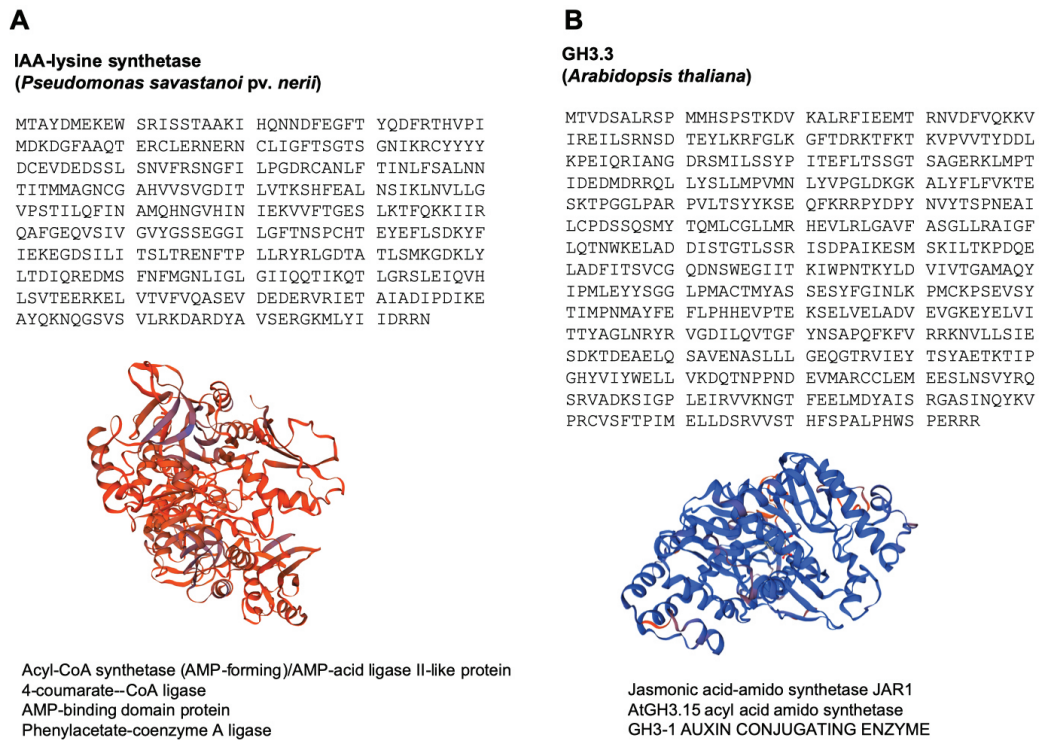

Figure 4. (A) The amino acid sequence of IaaL of *Pseudomonas savastanoi* (accession KU351686.1) and (B) of *Arabidopsis thaliana* GH3.3 protein (accession LUHQ0100000) of auxin amino acid conjugate synthetases. *The protein from P. savastanoi is 395 amino acids in size while the GH3.3 as one example has 595 amino acids. Below the protein sequences are protein models created in SWISS MODEL (https://swissmodel.expasy.org; accessed on 04.03.2020) for the respective sequence and the three or four closest hits used for the model are shown.*

some species auxin conjugates were found *(64)*. A body plan obviously needed the development of a system to control auxin-dependent growth *(65)*. Therefore, not only the biosynthesis and transport of auxin is needed, but also the possibility to control homeostasis on a more specific level. Next to degradation the synthesis of auxin conjugates with amino acids (and sugars?) has been found in many plant species (Figure 3). The biosynthesis, conjugation and degradation were determined *(65–68)* and it was found that amide conjugate formation seems to be present in the land plant lineage at least with the presence of liverworts *(67, 69)*, where the GH3 proteins are involved in thallus development and seem to be essential for auxin function *(69)*. In moss, specifically *Physcomitrella patens* the conjugation of IAA, but also IPA and IBA by the same enzymes has been found *(38)*. Interestingly, the two moss GH3 enzymes are also able to conjugate the hormone jasmonic acid to amino acids *(38)*, a characteristic that later in vascular plants has evolved in a larger number of enzymes with higher substrate specificities for the carboxy substrate *(32, 70)*. For example, the large GH3 protein family in Arabidopsis has substrate specificities for JA only in one enzyme (JAR1) *(70)*. IAA can be conjugated by 6 family members *(32)* and there is a specific enzyme (GH3.15) for IBA amino acid conjugate formation *(71)*. Whether IPA is conjugated by other family members similar with IAA has not been extensively studied. In addition to IAA, salicylic acid can be conjugated by one GH3 family member of Arabidopsis (GH3.5) that can also conjugate IAA *(45)* and it was shown that a specific conjugate with isochorismic acid and glutamate is a precursor in the SA biosynthesis pathway *(72)*.

Microbes are also able to conjugate IAA. The conjugation of all three auxins (IAA, IPA and IBA) to amino acids has also been found – at least – under *in vitro* conditions for the pathogenic protist *Plasmodiophora brassicae (73)* that causes the clubroot disease of *Brassica* crops. During disease progression the growth promoting plant hormones play a role in forming a hypertrophied root tissue *(74)*. The role for conjugate synthesis in the disease development is as yet unclear, even though one possibility is that JA can also be conjugated to amino acids, except the conjugate with isoleucine which is the active ligand of the plant JA receptor in the defense reponse *(75)*. There are two possibilities how the protist might have acquired the gene, one is via horizontal gene transfer from the host and the other is by independent evolution (Figure 3A). For the horizontal gene transfer scenario via host plants there is some evidence since it was shown that *Plasmodiophora* can take up and integrate DNA from its host *(76)*. Another, seemingly independent event, has led to the conjugate synthase IaaL from the phytopathogenic bac-

terium *Pseudomonas savastanoi (77).* In contrast to the GH3-type the bacterial enzyme conjugates specifically lysine to IAA (Figure 3A). The size of the two proteins differ considerably, while the IaaL protein consists of 395 amino acids, the GH3 family is much larger, *i.e.* GH3.3 consists of 595 amino acids (Figure 4). Modeling both proteins reveals for the IaaL protein similarities to Acyl-CoA synthetase (AMP-forming)/AMP-acid ligase II-like protein, 4-coumarate-CoA ligase, AMP-binding domain protein, and Phenylacetate-coenzyme A ligase as the first four hits, in contrast to the GH3 protein (Figure 4).

While the conjugation was unequivocally been shown also in non-vascular land plants, the situation where hydrolysis of conjugates has occurred is not yet clear *(78).* The amino acid conjugates with IAA and other auxins were found to be hydrolysed back to free auxin by a member of the M20 family of peptidases *(7, 79).* However, it is thought, at least for Arabidopsis this seems confirmed *(30)* that two amino acid conjugates of IAA with aspartate and glutamate are substrates for the degradation pathway. In contrast to this species, for example a family of conjugate hydrolases from the model legume plant *Medicago* is able to partially hydrolyze also IAA-Asp *(48).* Unlike for conjugate synthesis, the hydrolysis is not yet present in liverworts or mosses *(80, 81)* (Figure 3B). In *Marchantia polymorpha* (liverworts) the presence of a putative precursor of the M20 peptidase family was detected that had a very low activity for IAA amino acid conjugates *(80).* However, it could be mutated into a more active variant *in vitro*. In moss genome sequences no plant type amino acid hydrolase was found *(81).* However, in moss the presence of conjugate hydrolase sequences was found which had high homology to bacterial type IAA conjugate hydrolases *(79, 81)*, which were active. Therefore, it was hypothesized that mosses lost the hydrolases and some (at least *Physcomitrella*) re-acquired bacterial sequences by horizontal gene transfer *(81).* As already mentioned, there is an independent evolutionary pathway that occurred in some bacteria since the bacterial sequences cluster far from the typical plant ones even though their activity seems similar (Figure 3B) *(82–84).* Such bacteria might be associated in the rhizosphere in plants.

III. THE ROLE OF LONG CHAIN AUXINS

As pointed out above, there are additional auxin-like molecules with longer side chain, such as IPA and IBA which are also strongly conjugated and hydrolysed with enzymes that have similar or even higher activities than with IAA or IAA conjugates *(16, 17).* Therefore, the possible roles of these auxin derivatives will be discussed below. Details on the biosynthesis, metabolism and transport of IAA, IPA and IBA are summarized in Figures 1 and 2, which are often related on *in vitro* evidence by heterologous expression of the genes encoding the enzymes involved in the metabolism. Other results are based on mutant analyses or overexpression of genes *in planta*.

INDOLE-3-BUTYRIC ACID: AN AUXIN TO REGULATE PLANT GROWTH OR ONLY ANOTHER SOURCE OF FREE IAA IN ARABIDOPSIS?

The reports on the natural occurrence of IPA are scarce and some authors even refer to IPA as an artificial auxin compound *(59)*, but IBA has been reported and quantified in many plant species *(11, 14).* Nevertheless, there are early reports on the bioactivity of IPA and IBA as biocontrol agents in plant – pathogen interactions, but they were added to the plants, so it is not clear whether these two auxins would exert this effect also naturally *(85).* Even though the existence of IBA in many plant species, including Arabidopsis, has been confirmed *(14, 86–89)*, there are recent reports that are not able to identify IBA in Arabidopsis and other plant species *(90).* This enigma has been attributed to different growth conditions as well as different methodologies involved in the detection. In addition, radioactively labeled IBA was available which was extensively used for studies on IBA metabolism and transport *(21, 86, 91–94).* Newer studies on IBA transport and uptake on the cellular level use also heavy isotope labeled IBA combined with GC-MS or LC-MS *(95, 96).*

ß-Oxidation to IAA. IBA was thought to be more efficient in comparison to IAA in root formation and this was explained by its higher stability against degradation *(97).* Next, it was suggested that IBA is a precursor of IAA and could be converted to IAA by ß-oxidation, where two carbons are released during each cycle, to have an auxin effect *(8, 98).* A search for "classical" IBA deficient mutants so far not possible due to missing genes for biosynthesis, so the mutants that are available for Arabidopsis are deficient in conversion of IBA to IAA and IBA transport *(8, 95).* Next to the conversion of IBA to IAA it was reported that IAA is also precursor for IBA, not only *in vivo (86, 90, 91)* but also *in vitro (15).* The enzyme was termed IBA synthetase, found in maize and Arabidopsis, so a search was conducted on different Arabidopsis mutants and ecotypes for altered patterns in IAA, IBA and also IBA synthesis determined *in vitro* using the IBA synthetase assay *(99).* However, this work did not identify respective mutants that could be used to further elucidate functions.

In Arabidopsis the function of IBA has mainly been attributed to its fuction as precursor for IAA *(8, 98, 100).* A summary of the different possibilities of IBA function vs. IBA-to-IAA conversion in Arabidopsis and other plant species during development and biotic interactions is shown in Figure 5. The mutant *aim1* defective in ß-oxidation showed resistance to 2,4-dichlorophenoxybutyric acid, which is converted to 2,4-dichlorophenoxyacetic acid (2,4-D), a compound with herbicide characteristics, by the ß-oxidation pathway and provided thus first indications for this pathway in the conversion of butyric acid side chains *(101).* The compelling evidence came from long-lasting work in different laboratories showing that

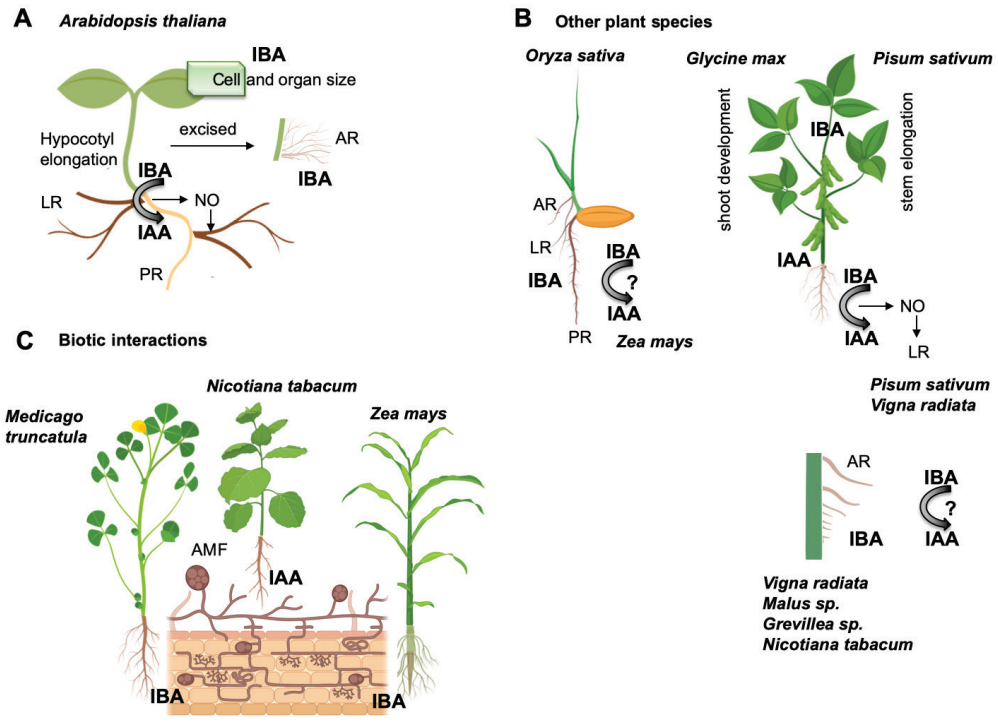

Figure 5. A summary of the function of IBA vs IBA-to-IAA conversion in Arabidopsis thaliana (A) and in other plant species during development (B) and interaction with arbuscular mycorrhizal fungi (C). *AMF = arbuscular mycorrhizal fungi; AR = adventitious roots; LR = lateral roots; NO = nitric oxide; PR = primary roots. Plant species and AMF cartoon pictures were taken from the free version of biorender (https://app.biorender.com; accessed 4.2.2020).*

most traits associated with IBA are reduced in plants with altered conversion of IBA to IAA *(8, 18, 89, 95, 98)*. Steps alongside ß-oxidation reactions in the peroxisome were found to be responsible for this conversion *(8, 95, 98, 102)*. Among the functions discussed for IBA-to-IAA conversion in Arabidopsis are lateral and adventitious root formation as well as light regulated hypocotyl elongation *(18, 89, 103)*, but is also important for cell expansion *(104)* (Figure 5A).

A different concept has been brought up by Schlicht *et al. (105)* who proposed that the rooting effect of IBA in Arabidopsis is due to nitric oxide (NO) as signal which is produced during ß-oxidation of IBA to IAA. Rooting was inhibited by an inhibitor of NO production *(105)*. Similarly, in pea root development there was an IBA induced NO burst that led to the induction of lateral roots under osmotic stress *(106)*. These findings are supported by work showing that NO mediates the auxin response, leading to adventitious root formation. Treatment of mung bean explants with IBA plus an NO donor together resulted in an increased number of adventitious roots compared with explants treated with the NO donor or IBA alone. On the contrary, the action of IBA was significantly reduced by the specific NO scavenger *(107)*.

Signaling pathways differ? However, there are other parts of the IBA vs. IAA reactions involved in signaling and transport that hint towards a separate role of IBA as well, since the two auxin molecules behave differently. While some transport data could be explained by uptake of IBA and then conversion to IAA, other data such as mutant phenotypes that can be rescued by IBA, but not IAA indicate a separate role in other plant species (see below). Furthermore, even in Arabidopsis there are reports where IAA and IBA seem to differ in their responses. For adventitious root formation on Arabidopsis stem segments a role for IBA, but not IAA was demonstrated since IBA induced the response at concentrations where IAA was uneffective *(19)*. If IBA needs to be converted to IAA, it should be active at higher concentrations than IAA. Using known IBA response mutants it was also reported that IBA induced the ectopic formation of metaxylem in the hypocotyl of Arabidopsis without conversion to IAA, but IAA favoured protoxylem formation *(108)*.

The isolation of *ibr5* as an Arabidopsis indole-3-butyric acid–response mutant gave new insights in possible signal transduction components for IBA signaling *(20)*. Although this mutant is also less responsive to indole-3-acetic acid, synthetic auxins, auxin transport inhibitors, and the phytohormone abscisic acid, it is a promising specific candidate for the IBA signaling pathway. In accordance with other IBA-response mutants, *ibr5* has a long root and a short hypocotyl when grown in the light. In addition, *ibr5* displays aberrant vascular patterning and increased leaf serration. IBR5 was identified as dual-

specificity phosphatase linking auxin and abscisic acid signaling pathways *(20)*. Analysis of an independent mutation in the *IBR5* gene called *TINKERBELL* associated IBR5 negatively with cell and organ size *(109)*. An Arabidopsis MAP kinase (MPK12) interacts with and is dephosphorylated by the phosphatase IBR5 *(110)*. Mutants in the *MPK12* gene have altered root phenotypes in response to auxin, so the authors hypothesized that MPK12 is a negative regulator of auxin-induced root formation which is regulated by IBR5 *(110)*. During experiments searching for components of adventitious root formation in Arabidopsis an IBA-inducible phosphatase 2A regulatory subunit was identified that could be part of this signaling chain *(19)*. Other candidates were identified through microarray analysis of adventitious root formation revealed upregulation of PINHEAD/ZWILLE-like proteins after IBA treatment *(111)*. PINHEAD is a member of a family of proteins in Arabidopsis that includes the translation factor eIF2C.

What about differences in transport? Many cellular transporters of IAA do not transport IBA (100). There are three types of transport for IAA and IBA that need to be considered, one is the long distance transport, the second is uptake into the cell and the third uptake into subcellular compartments (see Figure 2) where the auxins can be metabolized *(95, 100)*. IBA is converted to IAA in the peroxisome, so for this function the transport across the peroxisome membrane is important. Where the conversion of IAA to IBA takes place is not clear at the moment, but the IBA synthetase enzyme was found to be associated with a microsomal fraction and not with organelles, both in maize and Arabidopsis *(15, 99)*. In addition, transport of IAA and IBA into and out of the vacuole has to be considered since recently putative transport molecules have been identified that could be involved in this subcellular transport *(95)*.

Long distance transport differed between IAA and IBA in Arabidopsis stem segments *(21, 93)*. In a forward genetic screen a mutant that discriminates between the two known endogenous auxins in Arabidopsis, IAA and IBA, was identified in a screen for root gravitropic mutants and was coined *rib1* (resistant to IBA) *(94)*. *rib1* was resistant to IBA and 2,4-D, but had a wild-type response to IAA and NAA, therefore, the *rib1* mutation specifically affects IBA response in mutant plants *(94)*. The *rib1* mutants also showed an increase in hypocotyl length and number of lateral roots but mature plants were phenotypically similar to wild type *(112)*. In addition, it has different transport properties for IAA and IBA *(94, 112)* and is therefore also a good candidate for an IBA influx carrier *(95)*, albeit the respective gene has yet to be identified. Further evidence for differences between IBA and IAA transport in Arabidopsis came from studies using auxin inducible promoter-reporter constructs to monitor the effect of flavonoids on the distribution of exogenously applied IBA or IAA *(22)*. In this work the structural features of flavonoids which are involved in the modulation of auxin distribution or transport *(113, 114)* in Arabidopsis were evaluated. Differences of the same flavonoids on IAA and IBA distribution in leaves and roots were established *(22)*. While IAA is taken up by the AUX/LAX family an IBA uptake carrier has not been identified (see above). Uptake by diffusion is a possibility that cannot be ruled out and work from the 1990s suggest that IBA uptake is higher at low pH *(115)* which would be in line with the model for chemiosmotic polar transport of auxins *(116)*. For the efflux of IAA the PIN transporter family is involved whereas IBA seems to be exclusively exported by ABC transporter types *(95, 100)*. However, there are also ABCG type transporters for the efflux of IAA, albeit different ones than for IBA *(96, 117)*.

Transport of IBA intracellulary is also necessary to bring IBA into the peroxisomes for ß-oxidation and thus conversion to IAA. Mutant screens have identified possible proteins which could have this function for IBA *(95, 100)*. For instance, an *ibr* mutant defective in the peroxisome transporter PXA1 was identified *(118)*, which is implicated in importing IBA, the jasmonic acid precursor 12-oxophytodienoic acid, and fatty acids (or the corresponding CoA derivatives) into peroxisomes for ß-oxidation. *pex6* was also identified in an IBA resistance screen. *AtPEX6* encodes an apparent ATPase similar to yeast and human proteins required for peroxisomal biogenesis, which might be also involved in transport processes. PEX1 and PEX6 are interacting ATPases required for matrix protein import *(119)*. While the differences in uptake and efflux as well as intracellular distribution could be signs of the importance of both auxins as individual compounds, there is also the interpretation possible that the specific transport is important to regulate precursor and active auxin *(95)*.

EVIDENCE FOR IBA ACTION IN OTHER PLANT SPECIES

While in Arabidopsis there is strong genetic evidence that IBA functions as source for IAA, in other plant species the situation can be different. First, there are developmental steps that give indications for IBA as an auxin per se, such as lateral and / or adventitious roots *(120–122)*. Second, a variety of stresses induced the synthesis of IBA and consequently the endogenous content of IBA was also increased, whereas IAA was less affected *(123)*. Third, after inoculation of maize roots with an arbuscular mycorrhizal fungus (AMF), the concentrations of IBA, but not IAA, were elevated *(124, 125)* and gene expression was differentially affected when *Medicago* plants were treated with IBA and AMF *(126)*.

Organ formation. Most investigations have focussed on roots, namely adventitious and lateral root development. In rice a lot of research was focussed on the identification of mutants in lateral root formation *(127–130)*.

Among these were the mutants *arm1, 2 (127, 128)* and *lrt1, 2 (129, 130)*. The identification and characterization of such mutants revealed for example the *arm2* gene to encode for a distinct uptake carrier for IBA *(128).* It was shown that in the mutant *arm2* the acropetal and basipetal transport of IBA but not IAA was defective in roots. In consequence, IBA but not IAA was able to rescue the mutant phenotype of lesser lateral roots *(128).* The effects of auxins on lateral root initiation and root gravity response in rice were investigated using the mutant *lrt1*, which fails to form lateral roots and shows a reduced root gravity response *(129).* Application of IBA to the *lrt1* mutant restored both lateral root initiation and root gravitropism. However, application of IAA restored only root gravitropic response. These results suggest that IBA is more effective than IAA in lateral root formation and that IBA function can be clearly separated from IAA, suggesting that IBA may promote lateral root formation in rice without conversion to IAA *(130).* A second lateral rootless mutant *lrt2* was isolated in screening for 2,4-D resistance in rice *(131). lrt2* also failed to form lateral roots and exhibited altered root response to gravity. The effects of the two auxins IAA and IBA on lateral root development were totally different from each other depending on the application method. When the roots were incubated with an auxin solution, IAA inhibited lateral root development, while IBA was stimulatory. In contrast, when auxin was applied to the shoot, IAA promoted lateral root formation, while IBA did not *(131).* These data again demonstrate that IAA and IBA can be separated in their effects. However, maize seedlings showed similar patterns of primary root elongation and lateral root formation when IAA and IBA were compared *(122),* even though in other work maize has been shown to alter the root system specifically after increasing IBA when the roots were inoculated with AMF (*(124, 125)*; see also below).

Most studies within the last decades have focussed on using IBA in the induction of adventitious rooting, but only a few studies so far have tried to elucidate the molecular background of adventitious root formation. Application of IBA to cuttings of many plant species results in the induction of adventitious roots and in many cases more efficiently than IAA *(9).* The induction of adventitious roots was observed after IBA, but much less after IAA application in *Vigna radiata* (mung bean) *(97, 121)* and *Malus* sp. (apple) cuttings *(132).* In the latter plant species IBA could be converted to IAA, but only at a very low percentage of ca 1% which led the authors to suggest that either IBA itself is active or that it modulates the activity of IAA. In line with these findings, for *Grevillea sp.* it was shown that IBA did not alter the endogenous levels of IAA during the induction of adventitious rooting *(13).* The *Nicotiana tabacum* (tobacco) mutant *rac* did not respond to IBA treatment with the induction of adventitious roots, although cell division was normal *(133).* Since *rac* plantlets were not impaired in auxin transport and did not contain altered levels of auxin conjugates, it was assumed that the *rac* mutantion blocks an essential process for auxin induced adventitious root formation. Later experiments showed that the IBA-dependent induction process of adventitious roots occurred in *rac* shoots, although at a lower rate than in wild type *(134, 135).*

During shoot development of *Glycine max* (soybean) IBA was found to accumulate in the apical zone, whereas IAA accumulated in the basal zone *(136).* While there could be the possibility that IBA poses the inactive precursor in the apex, it is more likely that the two auxins have different functions along the shoot. In pea IBA was better in promoting stem elongation of intact plants than IAA *(137).*

Abiotic and biotic interactions. In maize, IBA biosynthesis and endogenous IBA levels were differentially influenced by abiotic stress factors *(123).* Therefore, IBA may act as a hormone involved in stress adaptation during growth responses mediated in specific plant species. When seedlings were exposed to drought stress, IBA synthetase activity strongly increased, whereas high amounts of water inhibited IBA synthesis. Consequently, the enzyme activity was enhanced by osmotic stress factors such as NaCl and sorbitol *(123).* The maize seedlings showed prominent changes in their root morphology when cultivated in different humidity conditions, *i.e.* growth under mild drought stress showed the most developed root systems with respect to the number of roots and root hairs, whereas those seedlings cultured on higher amounts of water showed fewer lateral roots, although the main roots were longer *(123).* Therefore, a correlation between the type of root system and IBA was hypothesized. Similarly, in Arabidopsis IBA synthetase was inducible by drought stress conditions in the two ecotypes Columbia and Landsberg erecta which showed different IBA synthetase activity when cultivated with various degrees of drought stress *(99).* In this context it might be interesting to note that two mutants *aba1* and *aba3* had higher IBA synthetase activity than wild type *(99).*

Auxin effects have been associated with development of root architecture (see above). Changes in root morphology after inoculation with AM fungi have been reported by several groups, especially induction of lateral root formation *(138, 139).* Therefore, in many laboratories the effect of mycorrization on IAA levels in host roots was investigated *(140).* In various investigations e.g. with tomato, no clear effect on IAA levels could be observed *(141),* but in soybean the IAA levels increased during AMF colonization *(142).* However, when IBA was measured, the picture was more uniform and there were more distinct differences between colonized and uncolonized roots in different plant species such as maize *(124, 125)* and *Medicago (48, 126).* The increase in endogenous IBA in maize was accompanied by an increase of IBA synthetase activity and treatment of control roots with IBA showed that the phenotype of mycorrhizal maize roots could be mimicked by this auxin at low concentrations *(125).* The functional role of

IBA in AMF of maize roots was further corroborated by empoying a halogenated IBA analogue *(143).* A synthetic IBA analogue, 4,4,4-trifluoro-3-(indole-3)butyric acid *(144)* had an inhibitory effect on IBA- and AMF-induced root development which coincides with a decrease in the endogenous free IBA and the AM infection rate *(143).* These results suggested that IBA, but not IAA plays a role in initial processes during AM colonization of maize roots. To analyze the effect of IBA during AM symbiosis further, the model legume *Medicago* was used *(48).* As with maize, IBA was increased throughout AMF colonization, whereas IAA was only at a very late time point. Two transcripts for auxin conjugate hydrolases, which were able to cleave also IBA conjugates, were increased by about 2-fold in AMF inoculated roots compared to controls *(48).* Gene expression between AMF colonized and IBA treated plants was investigated using microarray analysis and it was shown that several transcripts were induced both by IBA and AMF colonization *(126).*

CONCLUSION

While undoubtly the mentioned compounds exert auxin activites, the precise role for these longer chain auxins has not yet been fully elucidated. In Arabidopsis the major function of IBA seems being a precursor of IAA, albeit there is some evidence for IBA function without having to be converted. In other plant species, the situation is not as clear yet since the function is often more difficult to elucidate on the molecular level. However, the evolution of auxin compounds has resulted in several active species and their role will be subject to future studies.

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