Darwin Review

Auxin conjugates: their role for plant development and in the evolution of land plants

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Abstract

Auxin conjugates are thought to play important roles as storage forms for the active plant hormone indole-3-acetic acid (IAA). In its free form, IAA comprises only up to 25% of the total amount of IAA, depending on the tissue and the plant species studied. The major forms of IAA conjugate are low molecular weight ester or amide forms, but there is increasing evidence of the occurrence of peptides and proteins modified by IAA. Since the discovery of genes and enzymes involved in synthesis and hydrolysis of auxin conjugates, much knowledge has been gained on the biochemistry and function of these compounds, but there is still much to discover. For example, recent work has shown that some auxin conjugate hydrolases prefer conjugates with longer-chain auxins such as indole-3-propionic acid and indole-3-butyric acid as substrate. Also, the compartmentation of these reactions in the cell or in tissues has not been resolved in great detail. The function of auxin conjugates has been mainly elucidated by mutant analysis in genes for synthesis or hydrolysis and a possible function for conjugates inferred from these results. In the evolution of land plants auxin conjugates seem to be connected with the development of certain traits such as embryo, shoot, and vasculature. Most likely, the synthesis of auxin conjugates was developed first, since it has been already detected in moss, whereas sequences typical of auxin conjugate hydrolases were found according to database entries first in moss ferns. The implications for the regulation of auxin levels in different species will be discussed.

Key words: amide conjugates, amidohydrolases, development, ester conjugates, evolution, GH3 proteins, indole-3-acetic acid, indole-3-butyric acid, indole-3-propionic acid, protein conjugates, stress.

Introduction

The plant hormone auxin is involved in a plethora of different developmental processes during the life cycle of a plant (Davies, 2010). In most tissues the auxin responses are concentration dependent and different tissues respond in a distinct manner to varying amounts of exogenous auxins (Thimann, 1937). Higher auxin concentrations might often be inhibitory, so the optimum endogenous level must be tightly controlled. For the regulation of auxin homeostasis various mechanisms exist, such as biosynthesis, degradation, transport, and conjugate formation; the latter can be hydrolysed to the active auxin (Fig. 1). The major auxin found in plants is indole-3-acetic acid (IAA). IAA (for all abbreviations see Supplementary Table S1 at JXB online) can be converted to ester conjugates with sugars involving UDP-glucose transferases, amide conjugates with amino acids by IAA amino acid conjugate synthetases. Only a fraction of IAA conjugates such as IAA–Ala, IAA–Leu, IAA–Phe, and others are hydrolysed back to free IAA via auxin amino acid conjugate hydrolases, whereas amino acid conjugates with IAA–Asp and IAA–Glu are thought to be precursors for a degradation pathway. Finally, IAA–Trp is thought to be an inhibitor of auxin action. Additionally, there are protein conjugates with IAA, but it is not clear which function they might have and how they are made. Besides IAA there are...
several other molecules with auxin activity such as indole-3-butyrionic acid (IBA) (Fig. 1), 4-Cl-IAA, and indole-3-propionic acid (IPA). They can all be conjugated or their conjugates with amino acids hydrolysed at least in vitro. This review will discuss the biochemistry, the possible roles, and the evolution of auxin conjugate formation and hydrolysis.

**What kind of auxin conjugates are present in plants?**

**Low molecular weight conjugates**

Auxin conjugates can be divided into three main groups (for recent review see Bajguz and Piotrowska, 2009): (i) low molecular weight ester conjugates with sugar moieties, (ii) low molecular weight amide conjugates with amino acids, and (iii) high molecular weight conjugates with peptides and proteins also via an amide bond. Typically, the amount of conjugate present is analysed by mild (for ester conjugates) or strong (for amide conjugates) alkaline hydrolysis (e.g. Chen et al., 1988; Sztein et al., 1999). The indole moiety (e.g. IAA, IBA, IPA, 4-Cl-IAA) as well as the conjugate partner can vary, so that the plant can produce many different combinations of conjugates (Bajguz and Piotrowska, 2009). Also, other auxin-type molecules such as phenylacetic acid (PAA; Ludwig-Müller and Cohen, 2002) can be conjugated (Jentschel et al., 2007). These do not occur in all tissues at the same time, but up to now only little is known about the spectrum of auxin conjugates present in a given tissue. This is mostly due to the still difficult analysis of individual conjugates. Consequently, what we know about the conjugates present is mostly based on total conjugates determined after hydrolysis, or by in vivo feeding experiments as well as enzymatic studies and the substrates the respective enzymes are able to convert. However, whether this is ultimately the same spectrum as the one present in plants is not yet known.

Ester conjugates have been identified from a variety of plant species, mainly by mild alkaline hydrolysis. Even though the majority are linked via an ester bond, some are actually not ester but acyl alkyl acetal bonds (Normandy et al., 2010). The presence of IAA-glucose and IAA-myoinositol has been confirmed for monocots as well as dicots. The ester conjugates constitute the major portion of IAA in maize kernels (Bandurski et al., 1995), where the enzymatic synthesis of IAA-glucose and IAA-myoinositol has been described (Michalczuk and Bandurski, 1982). Ester conjugates are also present in seeds of other plant species in high amounts, such as Scots pine seeds and contribute to the increase in free IAA during germination (Ljung et al., 2001). In addition, there is evidence that IAA-glucose is present in Arabidopsis (Tam et al., 2000; Jackson et al., 2002; Ludwig-Müller et al., 2005) and tomato (Iyer et al., 2005). In seeds of Scots pine and vegetative tissues of rice, N-glucoses of IAA amides with aspartate (IAA–Asp–N–Glc) and glutamate (IAA–Glu–N–Glc) were reported which yielded IAA–N–glucose (Ljung et al., 2001; Kai et al., 2007b).

Amide conjugates occur in most plant species so far investigated. Individual IAA amide conjugates have been identified, e.g. IAA–Asp in Scots pine (Anderson and Sandberg, 1982) and Douglas fir (Chiwoha and von Aderkas, 2002), IAA–Glu and IAA–Asp in cucumber (Sonner and Purves, 1985) and soybean (Cohen, 1982; Epstein et al., 1986), and IAA–Ala in spruce (Ostin et al., 1992b). A broader range of IAA conjugated to amino acids has been investigated and identified in Arabidopsis (Tam et al., 2000; Kowalczyk and Sandberg, 2001) and Helleborus niger (Pencík et al., 2009). The development of novel techniques for the enrichment of auxin conjugates before analysis has helped to elucidate more details about auxin conjugates present. A combination of immunopurification with the synthesis of labelled standards for IAA amide conjugates and sensitive liquid chromatography–mass spectrometry (LC–MS) identified a range of amide conjugates from Helleborus (Pencík et al., 2009). In addition, high-throughput methods for more than one auxin type have been established (Barkawi et al., 2008) and will be useful not only for quantification but also for identification of auxin metabolites. Kai et al. (2007a) postulated the occurrence of IAA–Val and IAA–Phe in Arabidopsis based on the identification of the oxidative metabolites 6-OH-IAA–Val and 6-OH-IAA–Phe (see below, ‘Degradation’). From other plant species the possible range of IAA conjugates with amino acids has been deduced from feeding experiments with IAA; so it was shown that the moss Physcomitrella patens formed IAA conjugates in vivo with valine and leucine/isoleucine (Ludwig-Müller et al., 2009b), whereas feeding of high IAA levels in Arabidopsis led to the formation of IAA–Asp and IAA–Glu (Barratt et al., 1999). Since the occurrence of both simple ester and amide conjugates has been extensively reviewed by Bajguz and Piotrowska (2009), the overview here has been kept
relatively brief and not all compounds possibly present in an individual plant species have been discussed. However, a novel class of IAA conjugates recently found will be described in more detail below. These conjugates constitute protein(s) that have IAA covalently attached.

High molecular weight conjugates

The need to search for novel types of conjugate came from the work of Jerry Cohen’s group who showed that the sum of conjugates after hydrolysis did not fit with the amount of individual compounds. They were able to extract peptides from bean that were bound to IAA (Bialek and Cohen, 1986). Later, a 42-kDa protein was isolated from bean seeds and the respective gene cloned (Walz et al., 2002). Also, IAA was found to be attached to this protein called PvIAP1. Interestingly, the attachment of IAA to PvIAP1 was plant species specific, because heterologous expression of the bean gene PvIAP1 in Arabidopsis and Medicago truncatula did not result in the attachment of IAA to the protein (Walz et al., 2008). PvIAP1 has high homology to other plant seed storage proteins such as some late embryogenesis abundant (LEA) proteins in Arabidopsis. The attachment site for IAA could be identified as a lysine residue at position 245 in PvIAP1 (discussed in Walz et al., 2002). Later, a 42-kDa protein was isolated from bean seeds and the respective gene cloned (Walz et al., 2002). Also, IAA was found to be attached to this protein called PvIAP1. Interestingly, the attachment of IAA to PvIAP1 was plant species specific, because heterologous expression of the bean gene PvIAP1 in Arabidopsis and Medicago truncatula did not result in the attachment of IAA to the protein (Walz et al., 2008). PvIAP1 has high homology to other plant seed storage proteins such as some late embryogenesis abundant (LEA) proteins in Arabidopsis. The attachment site for IAA could be identified as a lysine residue at position 245 in PvIAP1 (discussed in Walz et al., 2008). Meanwhile IAA conjugates with proteins were tentatively identified in Arabidopsis (Seidel et al., 2006), but the nature of these proteins is still under investigation. In strawberry IAA was attached to a protein with known function, i.e. an ATPase (Park et al., 2006). Additionally, protein conjugates with IAA and 4-Cl-IAA were reported from pea, but the protein moiety has not been identified so far (Park et al., 2010). It seems from an increasing number of experiments that (i) these IAA proteins occur in a variety of plant species, and (ii) that different tissues might contain different proteins bound to IAA. Whether this phenomenon constitutes a mechanism to inactivate IAA or whether IAA modifies the protein(s) has to be elucidated in future work.

In addition to the low molecular weight ester conjugates with glucose, myo-inositol, and other minor disaccharide conjugates such as IAA-inositol-galactose, high molecular weight ester conjugates, such as IAA-glucan, are also discussed (reviewed in Normanly et al., 2010). Percival and Bandurski (1976) described an IAA ester glycoprotein fraction isolated from oat seeds. IAA-glucose may also be used to modify higher molecular weight conjugates in legume seeds, because more label after feeding of radioactive IAA was found in an insoluble fraction compared with the soluble fraction (Jakubowska and Kowalczyk, 2004). Enzymatic hydrolysis of IAA-labelled high molecular weight compounds gave rise to free IAA and compound(s) corresponding to IAA-glucose isomers.

The biochemistry of auxin conjugate synthesis and hydrolysis

Hydrolysis

The hydrolysis of auxin conjugates with amino acids was discovered by mutant analysis of Arabidopsis in the 1990s (e.g. Bartel and Fink, 1995). Since then the activities of so-called auxin amino acid conjugate hydrolases (also termed amidohydrolases) have been reported from several plant species, including dicots and monocots. Due to the emerging sequences for complete genomes the occurrence of putative hydrolyses in land plants can be assumed (see below, ‘Evolution’). While for many plant taxa amidohydrolase sequences are predicted, only for a few has the activity been studied; those will be reviewed below. The higher free IAA in the tissue when a certain hydrolyase is either present or mutated was used for mutant screens: mutants are more resistant to an amino acid conjugate of IAA if the hydrolyase involved in the metabolism is not functional (Bartel and Fink, 1995; Davies et al., 1999; Rampey et al., 2004). This assay can be also used to test for the in vivo hydrolysis of IAA amino acid conjugates (Savić et al., 2009). In Arabidopsis so far seven genes encoding homologous family members have been identified and for four the activity as a GST fusion protein has been determined (LeClerc et al., 2002). It was shown that the family in Arabidopsis has distinct, yet overlapping substrate specificity for different IAA conjugates tested. From Arabidopsis suecica, a close relative of Arabidopsis thaliana (Fig. 2A), a homologue of the amidohydrolase AtILR1 was isolated, which is also active on IAA amino acid conjugates (Campanella et al., 2003b).

Indirect evidence for functional auxin amino acid conjugate hydrolases of the ILL family came from transformation experiments (Junghans et al., 2006), in which an ILL homologue from poplar, called PcILL3, was isolated. Transformation into Arabidopsis rendered the transgenic lines more sensitive to the IAA conjugate with leucine, but not alanine and aspartate, indicating that IAA-Leu is cleaved by the poplar enzyme.

Further studies on auxin conjugate hydrolases from other plant species revealed that some are more specific for longer-chain auxin conjugates (Campanella et al., 2004; Savić et al., 2009). In wheat an amidohydrolase was characterized that preferred IBA-Ala over IPA-Ala and IAA-Ala (Campanella et al., 2004), whereas two enzymes from Chinese cabbage had higher preference for IPA-Ala (Savić et al., 2009). From M. truncatula a family of amidohydrolases was cloned that converted a rather broad range of auxin conjugates that could be cleaved by an individual enzyme (Campanella et al., 2010).

The hydrolysis of the second group of major auxin conjugates with sugars is less well understood. Since the synthesis of IAA-glucose is an equilibrium, it was thought that with low IAA-glucose synthesis (see ‘Synthesis’), the equilibrium would be on the side of the non-specific hydrolysis reaction. However, this would not fit into the concept of controlled auxin levels. Indeed, some of the amidohydrolases from M. truncatula are able to cleave IAA-glucose in vitro as well (Campanella et al., 2008). Whether this is also their function in vivo is not clear. Work from
of oat, potato, and bean, as well as in maize kernels. Kowalczyk et al. (2003) described a bifunctional system for the ester conjugate hydrolyses. However, molecular data are still missing on ester conjugate hydrolysis. Kowalczyk et al. (2003) described a bifunctional system for the synthesis and hydrolysis of IAA–myo-inositol from immature endosperm of maize kernels (see below, ‘Synthesis’).

Synthesis

The synthesis of auxin conjugates has remained an enigma for a long time. Although the enzymatic synthesis of ester conjugates was described several decades ago (e.g. Michalczuk and Bandurski, 1982) and the first gene for an IAA glucose synthase (iaglu) was cloned in a pioneering work by Szerszen et al. (1994) from maize, understanding of the enzymatic synthesis of amide conjugates remained unclear until the work of Paul Staswick’s group (Staswick et al., 2002, 2005). The cloning of UDP-glucose transferase genes and subsequent biochemical characterization has led to the identification of an iaglu homologue from Arabidopsis (AtUGT84B1; Jackson et al., 2001), although its presence in Arabidopsis was suggested earlier by in vivo experiments (Ludwig-Müller and Epstein, 1993). There is no distinct signature to the IAA-specific transferase, even though this is suggested in annotations when comparing genes from other plants with the Arabidopsis UDP-glucosyltransferases (UGTs). Because there are too many UDP-glucose genes present in plant genomes, the biochemical characterization is difficult and might be the reason why no further IAA glucose synthase gene with an assigned function has been reported so far. Recent work reported that a hydrogen peroxide-responsive UGT (UGT74E2) from Arabidopsis converted specifically IBA to its conjugate with glucose (Tognetti et al., 2010). The ester conjugate IAA–myo-inositol is thought to be synthesized from IAA–glucose (Kowalczyk et al., 2003). An enzyme for the transfer of IAA from IAA-glucose to myo-inositol from maize kernels was described by this group. Amino acid sequencing of the protein showed similarity to serine carboxypeptidase-like acyltransferases (Kowalczyk et al., 2003). Interestingly, the enzyme could also catalyse the reverse reaction, i.e. the hydrolysis to free IAA (see ‘Hydrolysis’).

Amide conjugates were thought to be synthetized upon treatment of plant tissue with high IAA concentrations (Venis, 1972; Hangarter and Good, 1981; Barratt et al., 1999). Interestingly, several of the GH3 genes were isolated as auxin-inducible genes (Hagen and Guilfoyle, 1985). This makes sense because at high auxin levels conjugation would be increased and the plant could not suffer from high auxin (see ‘Function’). The family of GH3 genes in Arabidopsis consists of 19 family members (Staswick et al., 2005), of which at least seven are able to catalyse the synthesis of IAA amide conjugates. These belong to the so-called group II genes (Staswick et al., 2005). It should be noted that the synthesis of the IAA conjugate with tryptophan has a special role since it is not an inactive auxin conjugate but rather has activity as a growth inhibitor (Staswick, 2009). The first member of the auxin-inducible GH3 gene family was isolated from soybean (Hagen and Guilfoyle, 1985). Up to now GH3 family members involved most likely in IAA conjugation have been reported from a variety of plant species (for more details see the recent review on GH3s by Wang et al., 2008), among them the moss P. patens (Bierfreund et al., 2004; Ludwig-Müller et al., 2009a), tobacco (Roux and Perrot-Rechenmann, 1997), rice (Jain et al., 2006), and pungent pepper (Liu et al., 2005), the latter regulated by auxin and ethylene. Despite this, only for a few plant species, in addition to Arabidopsis, has the activity of GH3 proteins as adenylating enzymes been demonstrated, e.g. for P. patens (Ludwig-Müller et al., 2009b), and rice (Chen et al., 2009). The possible functions of GH3 proteins for auxin homeostasis will be discussed below in more detail.

Fig. 2. (A). Phylogenetic relationship between 5 of 19 representative tribes of the Brassicaceae including the Arabididae with A. thaliana and A. suecica, and the Brassicae with the genus Brassica (adapted from Price et al., 1994). (B) Sequence comparison between the putative hydrolase domains of ILR1 and IAR3 from A. thaliana and sILR1 from A. suecica and the consequences for substrate specificity. Green labelled amino acids: similar between ILR1 and sILR1, but different in IAR3; red labelled amino acids: similar between sILR1 and IAR3, but different in ILR1; blue labelled amino acid: different in all three hydrolases. Activity data from LeClerc et al. (2002) and Campanella et al. (2003b). Red histograms: ILR1; yellow histograms: IAR3; blue histograms: sILR1.

Jakubowska et al. (1993) showed enzymatic hydrolysis of the ester conjugates 4-O- and 6-O-IAA–glucose in storage tissues of oat, potato, and bean, as well as in maize kernels (Jakubowska and Kowalczyk, 2005). However, molecular data are still missing on ester conjugate hydrolysis. Kowalczyk et al. (2003) described a bifunctional system for the synthesis and hydrolysis of IAA–myo-inositol from immature endosperm of maize kernels (see below, ‘Synthesis’).
Degradation

There is evidence that some of the auxin conjugates, i.e., those formed with aspartate and glutamate, might not be storage conjugates, but rather a form of IAA that is subject to degradation. The results described rely entirely on the identification of the respective metabolites since so far no gene involved in the oxidative metabolism of IAA conjugates has been cloned. Tuominen et al. (1994) demonstrated the conversion of IAA–Asp to oxindole-3-acetyl-N-Asp and oxindole-3-acetic acid following initial conjugate formation in Populus hybrids, suggesting that this may be a normal route for the breakdown of endogenous IAA. Sasaki et al. (1994) reported the accumulation of IAA–Asp in embryogenic and non-embryogenic cells of carrot, whereas degradation to Ox-IAA–Asp was only found in non-embryogenic cells, indicating cell type differences in auxin amino acid conjugate accumulation and further degradation. In Arabidopsis it was shown that IAA–Asp and IAA–Glu accumulated after treatment with high IAA levels (Barratt et al., 1999). Östlin et al. (1998) analysed the oxidative Arabidopsis metabolites in more detail. After feeding high levels of IAA, IAA–Asp was further oxidized to Ox-IAA–Asp and OH-IAA–Asp. This was in contrast to the metabolic fate of IAA–Glu, since that conjugate was not further metabolized under these experimental conditions. However, in M. truncatula several conjugate hydrolyses were described that could also hydrolyse IAA–Asp (Campanella et al., 2008). In legumes (Vicia faba and Dalbergia dolichepetala) the main oxidative metabolites of IAA–Asp and IAA–Glu were DiOx-IAA–Asp and (tentatively identified) DiOx-IAA–Glu (Östlin et al., 1992a).

Induction of catabolism to yield 2-oxindole-3-acetic acid and irreversible conjugation to indole-3-acetyl-N-aspartic acid was noticed at the same time as de novo synthesis of IAA was first detected in Scots pine seeds (Ljung et al., 2001). As part of the homeostatic regulation IAA was further metabolized to the two conjugates glucopranosyl-1-N-indole-3-acetyl-N-aspartic acid and glucopranosyl-1-N-indole-3-acetic acid, reported in this work for the first time. These metabolites have also been found in rice (Kai et al., 2007b). In addition, from rice the oxidative metabolites 6-OH-IAA–Val and 6-OH-IAA–Phe have been isolated (Kai et al., 2007a), which do not derive from IAA–Asp or IAA–Glu, but from IAA conjugates with amino acids, which can be hydrolysed to free IAA. Kai et al. (2007a) also found oxidation products of ester conjugates in Arabidopsis. They identified Ox-IAA–glucose as a major IAA metabolite. Therefore, it is not possible to strictly separate between IAA amino acid conjugates destined for catabolism or hydrolysis. In addition, the degradation/hydrolysis patterns are also partially plant specific.

Structural features

The auxin amidohydrolases belong structurally to the M20 peptidase family, members of which typically have two cations in the active centre. While for bacterial dipeptidases this is Zn$^{2+}$ (Rawlings and Barrett, 1995), in the auxin conjugate hydrodrolases it is most likely Mn$^{2+}$ (Bitto et al., 2009); although Cu$^{2+}$ can substitute for Mn$^{2+}$ in enzymatic assays for some hydrodrolases, but Zn$^{2+}$ could not (LeClerg et al., 2002).

Already small amino acid changes in the active site of these amidohydrolases result in different substrate preferences. From the close relative of A. thaliana, A. suecica, a hydrolase gene was cloned based on its sequence homology (Campanella et al., 2003b). The gene was heterologously expressed in Escherichia coli and it was found that a set of different substrates was converted to free IAA by the respective protein, even though amino acid changes were small (Fig. 2). It can be seen that AsILR1 has more identical amino acids in the putative hydrolase domain with AtILR1 (in green) than AtIAR3 (in red), but the substrates converted resembled that of AtIAR3. Site-directed mutagenesis experiments could lead to further clarification of which amino acids are important for which substrate cleavage.

The Arabidopsis IAA amino acid conjugate hydrolase AtIILL2 has been crystallized. This has shed light on the active site as well as other amino acids that might be necessary for proper function. Bitto et al. (2009) have proposed several amino acid residues in the active centre different in various hydrodrolases from different species which could be important for substrate specificity. For example, Leu175 was hypothesized to be responsible for selectivity against IAA–amino acid conjugates with amino acid side-chains bulkier than alanine or serine (Bitto et al., 2009). However, Leu175 is not conserved in the Arabidopsis amidohydrolases, it is only present in AtIILL2 and its closest homologue AtIILL1. In AtIILL1, this residue is replaced by Tyr176, which could stabilize the aromatic side-chains of the preferred substrates of this amidohydrolase isofrom (IAA–Phe) by a stacking interaction (Bitto et al., 2009). The wheat IAR3 homologue (TaIAR3; Campanella et al., 2004) contains Gly168 in the corresponding position of the putative selectivity filter residue Leu175 in AtIILL2. The only other notable difference is a single-residue insertion (Thr375 of TaIAR3) located in the vicinity of the residues forming the hydrophobic cavity for the indole ring. It is possible that these two modifications within the active site of TaIAR3 contribute to the ability of this enzyme to productively bind and hydrolyse auxin derivatives with longer side-chains (Bitto et al., 2009). Savić et al. (2009) have extended these suggestions to a Brassica rapa hydrolase BrIILL2 with preference for amino acid conjugates with IPA using a modelling approach based on the crystal structure of AtIILL2. The best fit for the hypothetical substrate pocket was indeed found for IPA–Ala. However, several binding modes were suggested, so that the amino acid residues responsible for the substrate specificity have yet to be identified (Savić et al., 2009).

Previously, it was shown for a bacterial hydrolase from Enterobacter agglomerans, also based on modelling using bacterial peptidases as template (Chou et al., 2004), that the amino acids His-404 and His-405 are essential for enzyme
catalytic activities of the bacterial IAA–Asp hydrolase. The authors suggested that the two amino acid residues might be involved in metal binding.

Such data are not available for the enzymes involved in conjugation. Only Staswick et al. (2002) showed that the three-dimensional putative structure of the AtGH3 family proteins resembles that of firefly luciferase, which helped to elucidate the function of these adenylating enzymes.

**Subcellular compartmentation of auxin conjugate synthesis and hydrolysis**

Based on the result obtained so far, the synthesis of auxin amino acid conjugates most likely takes place in the cytosol (Fig. 3). In *P. patens* the cytosolic localization of one GH3 protein (PpGH3-1) was shown as a green fluorescent protein (GFP) fusion (Ludwig-Mueller et al., 2009b) and for the second (PpGH3-2) also a cytosolic localization was predicted based on the amino acid sequence. Similarly, the *Arabidopsis* GH3 family does not give sequence evidence for a different localization, but further experimental data are needed.

Most of the auxin conjugate hydrolases have endoplasmic reticulum (ER) retention sequences attached, but not all of them (Campanella et al., 2003a). The phylogenetic trees of 66 orthologues across the plant kingdom resolved as two separate monocot clades, one with members possessing ER retention sequences and one lacking them (Campanella et al., 2003a). The *Arabidopsis* isoforms AtIAR1, AtIAR3, and AtIAR5 meet stringent bioinformatics criteria for localization in the ER (Bitto et al., 2009) including a predicted amino-terminal signal sequence and the carboxy-terminal HDEL/KDEL sequence. However, the remaining *Arabidopsis* isoforms either lack ER retention sequence motifs (AtIAR3) or have variant motifs found so far in fungi and animals (Derks and Madrid, 2001; Raykhel et al., 2007) but not yet in plants (AtILR1, KSEL; AtII L2, HEEL). The ER retention signal is not a prerequisite for activity, because two out of the five amidohydrolases characterized so far from *M. truncatula* lack such a tetrapeptide, but show activity (Campanella et al., 2008) and TaIAR3 has the unusual sequence motif RDEL (Campanella et al., 2004). On the other hand, the two *B. rapa* hydrolases BrIAR2 and BrIAR3 both have the typical ER retrieval signal (Schuller and Ludwig-Mueller, 2006).

An interesting hypothesis is that IAA conjugates may be recognized at the ER (or recruited to it) by the auxin binding protein 1 (ABP1), which also has an ER retention signal (Anai et al., 1997). The auxin conjugates could then be hydrolysed by the enzymes also localized in the ER (Fig. 3). However, the subcellular localization could not be demonstrated for any of the known proteins. Interestingly, the bean protein PvIAP1 heterologously expressed in *Arabidopsis* and *M. truncatula* also co-localized to some extent with the endomembrane system (Walz et al., 2008). The co-localization of an auxin receptor, auxin amino acid conjugate hydrolases, and a protein with IAA attached suggest a possible, not yet identified function of the ER in auxin homeostasis/biology.

The fact that synthesis and conjugation/hydrolysis are taking place in different compartments demands that IAA and maybe also IAA conjugates are transported (Fig. 3). While import into a cell and export from a cell have been quite well characterized, the intracellular transport mechanisms have not been so well elucidated. Work from auxin transport gave additional indications that the ER might be an important compartment, at least in *Arabidopsis*, for auxin conjugates (Mravec et al., 2009). The IAA transport protein PIN5 is localized to the ER and mutants displayed altered auxin conjugate patterns (Mravec et al., 2009).

**Transcriptional control of auxin homeostasis**

The genes for auxin conjugate synthesis and hydrolysis are differentially regulated in various tissues and during development (Supplementary Fig. S1 at JXB online), and upon abiotic stress, pathogen infection (see below), and also hormone treatment (Supplementary Fig. S2 at JXB online) as analysed by Genevestigator (Zimmermann et al., 2004). However, our knowledge of the transcriptional control of genes involved in auxin homeostasis is still scarce.

The GH3 family has been isolated as part of the auxin-inducible genes also including Aux/IAA genes (Delker et al., 2008). The control of at least part of the GH3 gene family in
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Arabidopsis is via the auxin-responsive (ARF) transcription factors. ARFs are regulated by proteolytic inactivation of the Aux/IAA repressors via the TIR signalling pathway (e.g. Santner and Estelle, 2009). High auxin levels result in inactivation of Aux/IAA repressors, while the ARF proteins can act as transcriptional activators or repressors depending on the type of ARF (Santner and Estelle, 2009). ARFs are positive regulators of transcription in the case of GH3 genes of Arabidopsis, because these are up-regulated after auxin treatment and increase the level of auxin amide conjugates. To bind ARFs, the auxin-responsive gene needs an auxin-responsive element (AuxRE) in its promoter (Umasov et al., 1997). There are 23 ARF genes in Arabidopsis, and some bind to GH3 promoters to regulate gene expression. ARF8 was the first ARF for which it was demonstrated that it is capable of regulating the expression of three AtGH3 genes involved in the adenylation of IAA (Tian et al., 2004). The three GH3 genes were down-regulated in the arf8-1 mutant and up-regulated in ARF8 overexpression lines. Concomitantly, the free auxin level was decreased in the latter. These results suggest that ARF8 might positively regulate the expression of GH3, which results in the formation of IAA amino acid conjugates. Unfortunately, conjugated levels of auxin were not determined in this study (Tian et al., 2004). Several ARFs are targets for regulation by microRNAs (Bartel and Bartel, 2003). It was demonstrated that the microRNA167, which targets ARF8, consequently influenced GH3 expression in rice (Yang et al., 2006). Furthermore, it was shown that disrupting miR160 regulation of ARF17 increases ARF17 mRNA levels, which leads to altered GH3 gene expression (Mallory et al., 2005). These results indicated that ARF17 is another possible transcriptional regulator of GH3 genes. Also, a mutation in ARF7 causes reduced expression of some GH3 genes including YDK1, suggesting that ARF7 positively regulates YDK1 expression (Stowe-Evans et al., 1998; Takase et al., 2004).

While this regulation of transcript abundance of GH3 family members is quite well studied, the transcriptional control of other factors affecting auxin homeostasis has not yet been investigated. The Arabidopsis transcription factor STYLISH1 (STY1) (Sohlberg et al., 2006) might regulate GH3 gene expression in a more indirect fashion. The prime target might be the YUCCA (YUC) genes involved in auxin biosynthesis and subsequently high IAA levels would induce GH3 gene expression, which was also altered in STY1-overexpressing plants. However, evidence for this scenario comes only from the time course of gene expression analysis after induction of STY1 where YUC4 was up-regulated already after 30 min but GH3-2 (YDK1) only after 3 h. In accordance with this, decreased expression of GH3 genes and YUC4 was detected in mutant sty plants (Sohlberg et al., 2006).

Recent research identified a transcription factor of Arabidopsis UPRIGHT ROSETTE (URO), whose over-expression (uro-D) disturbed auxin homeostasis (Sun et al., 2010). Both free IAA and ester conjugates of IAA were increased in seedlings and older rosette leaves of uro mutants compared with wild type, whereas amide bound IAA was decreased. According to microarray data, a member of the GH3 gene family was up-regulated. The mechanism of auxin homeostatic control by URO seems to be conserved since overexpression of URO in P. patens altered the response to an auxin-inducible promoter-reporter fusion (Sun et al., 2010). Although it has not been shown whether URO controls auxin homeostasis in a direct or indirect fashion, this novel transcription factor will provide a valuable tool to unravel parts of the control of auxin conjugate formation.

Other effects may be more indirect. Screens for IAA amino acid conjugate-resistant mutants yielded not only amidohydrolases, but also other proteins involved in, e.g. metal homeostasis (Rampey et al., 2006). The ILR3 gene encodes an Arabidopsis basic helix–loop–helix leucine zipper protein, which is thought to modulate both auxin responsiveness and metal homeostasis by regulating metal transporters needed to supply the metal ions essential for amidohydrolase activity.

Possible functions for auxin conjugates

Development

Some functions of auxin conjugates have been elucidated using mutants of Arabidopsis. However, since there are gene families for each amidohydrolase and auxin conjugate synthetase as well as UDP-glucose transferases, the phenotypes are usually not very clear. In Arabidopsis single mutants of various auxin conjugate hydrolase genes did not show a phenotype apart from lower sensitivity to defined auxin conjugates (Bartel and Fink, 1995; Davies et al., 1999). If the respective hydrolase is no longer present, then less IAA is formed and the inhibitory effect of the free IAA should also be lower. Only triple mutants of Arabidopsis hydrolases began to show phenotypes even when not cultivated on auxin conjugates (Rampey et al., 2004). Overexpression of auxin conjugate hydrolases (from E. agglomerans) also did not result in an apparent phenotype (Tam and Normanly, 2002). Expression patterns of auxin conjugate hydrolases from A. thaliana, A. suecica, M. truncatula, and Triticum aestivum suggest their presence in growing tissues, specifically in seedlings (Campanella et al., 2003b, 2004, 2008). Promoter–reporter lines for Arabidopsis amidohydrolases revealed a complex spatial expression pattern (Rampey et al., 2004).

The GH3 family of auxin conjugate synthetases also plays many roles during development. While single mutants of GH3 genes in Arabidopsis do not show apparent phenotypes, the overexpression of GH3-2 (ydk1-D) and GH3-6 (df1-D) caused strong developmental phenotypes such as short hypocotyls (Nakazawa et al., 2001; Takase et al., 2004). Mutants in GH3-9, also belonging to class II enzymes of auxin conjugate synthetases, displayed a shorter root phenotype and higher sensitivity to auxin-regulated root growth, indicating a role for GH3-9 in root development (Khan and Stone, 2007). Also, for an overexpressing
line of GH3-5 (gh3.5-1D) an altered root phenotype was described in addition to a curled leaf phenotype (Zhang et al., 2007). Despite their probable overlapping functions, individual members of the GH3 family might have tissue-specific roles. In P. patens, there are only two GH3 genes instead of 19 as in Arabidopsis or even more in rice (Terol et al., 2006). In the moss, the single knockout mutants also did not show a phenotype; only when they were grown on high levels of IAA was growth retarded (Ludwig-Müller et al., 2009b). However, the double knockout mutants grow somewhat more slowly even without addition of IAA. In rice several insertion mutants in GH3 genes (GH3-5, GH3-7) were investigated using rice retrotransposon insertion mutants and the phenotypes of the insertion mutants of these genes showed dwarfism, sterility, vivipary, and leaf withering (Jain et al., 2006). However, it needs to be demonstrated experimentally that the phenotypic changes are specific to insertional mutagenesis of OsGH3 genes (Jain et al., 2006).

Overexpression of rice GH3-8 displayed retarded growth phenotypes (Ding et al., 2008). A GH3 gene from Capsicum chinense (CcGH3) was expressed in fruit when auxin levels were decreasing, but further experiments demonstrated that it was induced by endogenous ethylene (Liu et al., 2005). When overexpressed in tomato, CcGH3 promoted ripening of ethylene-treated fruit. So for CcGH3 it has been suggested that it might be the intersection between auxin and ethylene signalling in ripening fruit.

GH3 genes are thought to have a function in light-regulated development of Arabidopsis. Analyses of YDK1 (GH3-2) expression has shown that this gene is inhibited by blue and far-red light (Takase et al., 2004). DFL2 (GH3-10) gene expression was induced transiently after a red light pulse (Takase et al., 2003). Overexpression of DFL2 caused a short hypocotyl phenotype when grown under red and blue light. Conversely, antisense plants had a long hypocotyl under red light. The results suggested that DFL2 is acting downstream of red light signal transduction (Takase et al., 2003). However, for DFL2 no adenylation of IAA has been demonstrated (Staswick et al., 2005), so it is not clear whether the observed phenotypes are connected to auxin homeostasis. Furthermore, GH3-5 (WES1) was found to be involved in shade-avoidance responses and acts downstream of phytochrome B (Park et al., 2007b; Tanaka et al., 2002). For the two P. patens GH3 genes no involvement in light-regulated growth was reported (Bierfreund et al., 2004).

An Arabidopsis allele of HLS1, UNUSUAL SUGAR RESPONSE2 (UNS2), linked GH3 gene expression to apical hook formation in the darkness and sugar signalling (Ohto et al., 2006). In the uns2 mutant sugar-responsive gene expression was altered. Also, the expression of a GH3 gene was up-regulated in wild type and the mutant in response to IAA; however, transcript levels were higher in the mutant than in the control plants and mutants contained higher transcript levels even without auxin (Ohto et al., 2006). It was proposed that ARF2 negatively regulated apical hook formation in an HLS1-dependent manner (Li et al., 2004). ARFs are involved in transcriptional control of some GH3 genes (see ‘Transcriptional control’). HLS1 encodes a protein homologous to N-acetyltransferases (Lehman et al., 1996), which led to a model in which the acetylation of a protein, here ARF2, could result in altered protein stability, which in turn could alter GH3 transcription (Li et al., 2004).

The role of the IAA–Trp conjugate is different from that of other IAA conjugates (Staswick, 2009). In contrast to being a storage form for free IAA, IAA–Trp was shown to be an inhibitor of several IAA-induced growth responses. Unexpectedly, IAA–Trp caused agravitropic root growth in seedlings, while Trp alone did not. In addition, IAA–Trp nearly eliminated seedling root inhibition caused by high concentrations of IAA and inhibited IAA-dependent stimulation of lateral root growth. IAA–Trp was found to be present as endogenous compound (Staswick, 2009). These results showed that IAA–Trp constitutes a previously unrecognized mechanism to regulate auxin action.

Overexpression of maize iaglu (Ludwig-Müller et al., 2005) and the homologue from Arabidopsis, UGT84B1, in Arabidopsis (Jackson et al., 2002) resulted in elevated levels of IAA–glucose, but not IAA. Transgenic plants harbouring the iaglu gene had smaller roots and showed less inhibition of root growth when cultivated on increasing concentrations of IAA, but not IBA and 2,4-dichlorophenoxyacetic acid (2,4-D) (Ludwig-Müller et al., 2005). However, they did not show any hypocotyl growth defect and only slight growth retardation of the inflorescence. However, leaf shape was altered in transgenic lines (Ludwig-Müller et al., 2005). Overexpression of the homologous Arabidopsis IAA glucose synthase resulted also in higher IAA sensitivity as shown by root growth inhibition and in addition the leaves looked typically rounded, wrinkly, curling, and often had a disrupted midrib, indicating that vascular development was disturbed (Jackson et al., 2002). Comparison of the dataset obtained in those two experiments suggested that both enzymes from maize and Arabidopsis caused similar phenotypes after overexpression indicating similar roles in planta. Overexpression of maize iaglu in tomato resulted in an almost complete lack of root initiation and development (Iyer et al., 2005). On the other hand, antisense transgenic plants, had unusually well-developed root systems at early developmental stages. Mature plants were indistinguishable from the wild type and fruit set as well as ripening also appeared to be unaffected by the presence of the antisense transgene. IAA–glucose was reduced and free IAA increased in antisense seedlings (Iyer et al., 2005).

**Abiotic stress**

Evidence has been presented that auxin conjugates could be involved in abiotic stress tolerance. Junghans et al. (2006) found an auxin conjugate hydrolase from poplar in salt-stressed tissue. Overexpression of PcILL3 in Arabidopsis also rendered these transgenic lines more salt tolerant. Gray et al. (1998) showed that IAA levels increased at higher
temperatures. Furthermore, temperature-sensitive cells of henbane had altered auxin conjugate levels (Oetiker and Aeschbacher, 1997). There seemed to be involvement of auxin conjugation formation at higher temperatures. Interestingly, not only Arabidopsis but also P. patens showed an increased auxin response in auxin-responsive promoter::GUS lines (DR5::GUS and GH3::GUS, respectively; Fig. 4), indicating that this response is conserved in evolution. Available mutants will help to elucidate the possible role of auxin conjugates during temperature stress.

GH3 genes are also directly involved in stress tolerance. WES1 for example was also induced by various stress conditions such as cold, drought, and heat treatment as well as by the stress hormones salicylic acid (SA) and abscisic acid (ABA). It is interesting to note that WES1 (GH3-5) can adenylate not only IAA but also SA (Staswick et al., 2005). A WES1-overproducing line (wes1-D) was resistant to abiotic stresses such as drought, freezing, and salt, but also high temperatures (Park et al., 2007a), whereas a T-DNA insertional mutant showed reduced stress resistance. In addition, stress-responsive genes were up-regulated in the wes1-D mutant. Interestingly, C-repeat/dehydration-responsive element-binding factor (CBF) genes were directly regulated by auxin (repressed by IAA), and the repression was attenuated in line with higher auxin conjugate formation (Park et al., 2007a).

It should be noted that GH3 genes could be involved in detoxification of auxinic herbicides, because it was reported that transcription of an auxin-inducible GH3 gene from soybean could be induced by dicamba very rapidly, but to a lesser extent by 2,4-D (Kelley et al., 2004). Non-auxinic herbicides did not induce this GH3 gene.

Finally, formation of IBA–glucose was recently implicated in stress responses in Arabidopsis (Tognetti et al., 2010). In wild-type plants the expression of UGT74E2 (see ‘Synthesis’) was inducible after application of a wide variety of different stresses. Ectopic expression of the gene resulted in increased concentrations of IBA–glucose and free IBA, as well as a modified IAA conjugation pattern. This perturbation of IBA and IAA homeostasis resulted in significantly improved survival during drought and salt stress treatments, indicating a role of IBA ester conjugates in stress adaptation mechanisms (Tognetti et al., 2010).

Biotic interactions
In M. truncatula, several hydrolase genes were dramatically up-regulated after inoculation with Sinorhizobium meliloti and somewhat less after inoculation with Glomus intraradices, indicating a possible role for symbiosis with rhizobia and arbuscular mycorrhiza (Campanella et al., 2008). Also, in Chinese cabbage (B. rapa) the enzymatic activity of IAA–Asp hydrolysis increased in infected root galls compared with control roots (Ludwig-Müller et al., 1996). Since the cloning of several B. rapa hydrolases (Schuller and Ludwig-Müller, 2006) the expression of these hydrolase genes has been investigated during root gall formation, but it was only slightly altered. Interestingly, the

Fig. 4. Induction of auxin-responsive promoter–reporter constructs in A. thaliana (DR5::GUS) (At) and P. patens (GH3::GUS) (Pp) at 23 °C and 28 °C. The GH3 promoter was from soybean. The pictures were taken by Assia Gabriellian, Technische Universität Dresden, Germany.
B. rapa hydrolases convert longer-chain auxin conjugates to free auxins to a much greater extent than IAA conjugates (Savić et al., 2009). The observation that several auxin conjugate hydrolases, e.g. from wheat and Chinese cabbage, do not prefer IAA amino acid conjugates, but conjugates with IBA (TaIAR3; Campanella et al., 2004) or IPA (BrI2L2, BrIAR3; Savić et al., 2009) points to additional, not yet discovered, roles for auxin conjugates. IBA is another naturally occurring auxin with activities especially during adventitious and lateral root formation but also arbuscular mycorrhiza establishment (Ludwig-Müller, 2000, 2009, 2010). There is some discussion about whether IBA should be regarded as a precursor of IAA since several of its functions can be explained only by its conversion to IAA via β-oxidation (Bartel et al., 2001). On the other hand, IBA itself is converted to conjugates (Ludwig-Müller and Epstein, 1993), which can be hydrolysed with high specificity, e.g. in wheat (Campanella et al., 2004) or Chinese cabbage (Savić et al., 2009). Despite the fact that IPA has been identified as an endogenous compound in some plant species (Segal and Wightman, 1982; Schneider et al., 1985), up to now no indication of a possible role has been described. Since Brassica hydrolases prefer conjugates with IBA above IAA as substrate an additional role must be postulated (Savić et al., 2009). One hypothesis comes from work by Matsuda et al. (1998), who found that IPA derivatives displayed antimicrobial activity against Pseudomonas.

An increase in GH3 genes has been reported during plant–pathogen interactions where elevated auxin levels were found. For example, during the clubroot disease of Arabidopsis caused by the protist Plasmodiophora brassicae, root galls develop concommitantly with high auxin levels (Ludwig-Müller et al., 2009c). In these galls various GH3 genes were up-regulated (Siemens et al., 2006). In Agrobacterium tumefaciens tumours on Arabidopsis stems similar observations concerning the up-regulation of GH3 genes have been made (Deeken et al., 2006). In both cases the up-regulation of GH3 genes might simply be the answer of the host plant to increasing auxin levels; however, a more direct role for auxin conjugates in pathogenesis cannot be ruled out. On the other hand, upon ectomycorrhizal establishment in Pinus pinaster, a GH3 homologue, the first identified from gymnosperms, was down-regulated in root systems (Reddy et al., 2006), indicating probably the need for more active auxin in root growth. The Pinus GH3 gene was not down-regulated after inoculation with a non-mycorrhizal fungal mutant strain (Reddy et al., 2006).

Arabidopsis WES1 (GH3-5) was induced by SA and Pseudomonas syringae infection (Park et al., 2007a; Zhang et al., 2007). As already mentioned above WES1 possesses dual substrate specificity for both IAA and SA (Staswick et al., 2005). It was therefore postulated that WES1 might play a role in SA-mediated biotic stress responses because WES1 expression was induced after SA but not jasmonic acid (JA) application (Park et al., 2007a). In wes1-D, a line overexpressing GH3-5, the transcript level of PR-1 was high even before infection and it increased even further after inoculation (Park et al., 2007a). In agreement with gene expression patterns wes1-D was more resistant to pathogenic infection. Interestingly, auxin also regulates PR-1 expression (Park et al., 2007a). Zhang et al. (2007) demonstrated the presence of SA-Asp in P. syringae-infected Arabidopsis leaves, even though free SA levels were also higher. In addition, dfl1-D (GH3-6-overexpressor) impairs the same set of R-gene-mediated resistance as gh3.5-1D (Zhang et al., 2007), indicating also a role of GH3-6 in resistance. These data point to a role for the auxin pathway in both cases. It should be noted that at least two other GH3 genes from Arabidopsis are involved in plant defence (GH3-11 and GH3-12), but they do not adenylate IAA (Staswick et al., 2005). GH3-11 (JAR1) is responsible for the adenylation of JA to form the active isoleucine conjugate (Staswick et al., 2002), whereas GH3-12 might act in the metabolism of SA (Jagadeeswaran et al., 2007; Nobuta et al., 2007). The rice GH3-8 gene acts in plant defence via changes in expansin gene expression (Ding et al., 2008). The overexpression line of GH3-8 displayed enhanced resistance to the rice pathogen Xanthomonas oryzae pv. oryzae. This enhanced resistance may be due to more rigid cell walls in response to lower levels of expansins (Ding et al., 2008). It should be noted that several expansins are auxin inducible and thus might be repressed in plants where GH3 proteins act to synthesize more conjugates. Since not all GH3 proteins are simply acting through the increased or decreased levels of free auxin, further elucidation of their more direct role for plant–pathogen interaction will be necessary.

**Auxin conjugation during evolution**

Sequences for auxin amino acid conjugate hydrolases have been identified in many plant species including gymnosperms, monocots, and dicots (Campanella et al., 2003a), and phylogenetic analyses indicated that GH3 proteins are also highly conserved all over the plant kingdom (Staswick et al., 2005; Terol et al., 2006; Paponov et al., 2009). Experimental evidence connects auxin conjugates to the development of a body plan, vasculature, and embryo development (Sztein et al., 1999, 2000; Cooke et al., 2002). Work from Sztein et al. (1999, 2000) has revealed evidence for auxin conjugate formation in mosses (e.g. Funaria, Polytrichum, Sphagnum) and hornworts (Phaeoceros), whereas in liverworts (e.g. Marchantia, Pallavicinia, Sphaerocarpus) the auxin conjugate levels/synthesis of conjugates was very low and occurred at a slow rate. This is consistent with the idea that liverworts retained many primitive structures and therefore would also employ a less sophisticated mechanism to control auxin homeostasis (Sztein et al., 1999). The hornwort Phaeoceros possesses high IAA amino acid conjugates, close to concentrations found in vascular plants (Sztein et al., 2000). Furthermore, in the moss fern Selaginella kraussiana and the fern Ceratopteris richardii increasing levels of amide-conjugated IAA were found after feeding of labelled IAA, the latter
accumulated high amounts of IAA–Asp and IAA–Glu (Sztein et al., 1999). In other ferns, Gingko, and gymnosperms auxin conjugate synthesis was also found to a high extent (Sztein et al., 1995). They hypothesized based on feeding experiments with labelled IAA that auxin conjugation started to play a role in the homeostasis of auxin most likely in the group of mosses. Corroborating evidence is the different conjugation pattern found in protonema and gametophores of the moss P. patens after feeding of IAA (Ludwig-Müller et al., 2009b). Only gametophores conjugated IAA extensively to amino acid conjugates and this was connected to the presence of GH3 proteins. In the P. patens genome only two GH3 genes are present (Bierfreund et al., 2004). Both can synthesize a spectrum of amino acid conjugates with IAA and IBA, although one of the proteins (PpGH3-2) is more active with IAA than the other (Ludwig-Müller et al., 2009b). Whether IBA is present in P. patens is not yet known. It should be noted that both proteins were also able to convert JA to amino acid conjugates, which resembles the activity of JAR1 from Arabidopsis (Staswick et al., 2002; Staswick and Tiryaki, 2004). Mutant analysis of single and double knockout lines showed phenotypes similar to those in Arabidopsis, higher sensitivity to free auxin and loss of the ability to form IAA amino acid conjugates in vivo (Ludwig-Müller et al., 2009b).

However, in the double knockout mutants the presence of IAA ester conjugates in moss was confirmed (Ludwig-Müller et al., 2009b). Only gametophores conjugated IAA extensively to amino acid conjugates and this was connected to the presence of GH3 proteins. In the P. patens genome only two GH3 genes are present (Bierfreund et al., 2004). Both can synthesize a spectrum of amino acid conjugates with IAA and IBA, although one of the proteins (PpGH3-2) is more active with IAA than the other (Ludwig-Müller et al., 2009b). Whether IBA is present in P. patens is not yet known. It should be noted that both proteins were also able to convert JA to amino acid conjugates, which resembles the activity of JAR1 from Arabidopsis (Staswick et al., 2002; Staswick and Tiryaki, 2004). Mutant analysis of single and double knockout lines showed phenotypes similar to those in Arabidopsis, higher sensitivity to free auxin and loss of the ability to form IAA amino acid conjugates in vivo (Ludwig-Müller et al., 2009b).

Interestingly, recent work shows that IAA, as well as a few other indolic compounds, but no IAA conjugates, were present in the brown alga Ectocarpus siliculosus (Le Bail et al., 2010). An in silico survey of auxin biosynthesis, conjugation, perception, and transport genes showed that mainly homologues for IAA biosynthesis genes from land plants were found in the E. siliculosus genome. However, work from Stirk et al. (2004) showed that in growth-promoting extracts of the kelps Ecklonia maxima and Macrocystis pyrifera auxin conjugates, i.e. IAA–Asp, IAA–Ala, IAA–Gly, and IAA–Leu, were found, IAA–Asp being the IAA conjugate with the highest concentration. The authors also looked for IAA–Val and IAA–Phe, which could not be detected by high-performance (HP)LC–MS. Despite the lack of IAA conjugates in other multicellular algae, it should be noted that IAA was present (i.e. Sztein et al., 2000; Basu et al., 2002; Le Bail et al., 2010). In Nitella spp., a member of the Charophyta, the appearance of IAA could probably be linked to the development of land plants (Ross and Reid, 2010). Also, growing tips of Nitella thalli have low levels of IAA conjugates comprising only 30% of the total IAA pool (Sztein et al., 2000). In algae, which are surrounded by water, the secretion of unwanted compounds would be much easier to accomplish. For the control of free IAA levels in land plants who cannot easily secret

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**Fig. 5.** Model for the evolution of the GH3 family from a hypothetical ancestor to the GH3 family in A. thaliana. It is hypothesized that the ancestor could synthesize conjugates with JA and IAA. After gene duplication further specialization occurred as in the moss P. patens, where one GH3 protein (PpGH3-1) already has reduced activity towards IAA, but not JA, whereas the other (PpGH3-2) has the capability to convert both substrates to amino acid conjugates. The activity with JA was probably lost later in evolution in this branch. JAR1 is the GH3 protein from Arabidopsis that converts only JA to its amino acid conjugate with isoleucine. Red, IAA; blue, JA.
molecules, first auxin conjugate synthesis was developed (moss), followed by the usage of conjugates to generate free IAA via hydrolysis (moss fern). In *Chlamydomonas* spp. and other unicellular algae, the evidence for the presence of IAA and genes associated with auxin homeostasis and signalling is not convincing at present (Dutcher et al., 1992; Rensing et al., 2008). Ross and Reid (2010) suggested that unicellular algae may have once had the capacity to synthesise auxin but lost that capacity, or alternatively, the ancestral unicellular algae lacked the ability to synthesize auxin but that capacity has since arisen independently in different multicellular algal groups.

From this it can be concluded that conjugation and hydrolysis of auxin conjugates must have evolved independently, once within the bacteria probably living in close proximity or even a parasitic relationship with their auxin-synthesizing host, and at least once (but maybe also several times) in the algal/plant lineage (Fig. 6). The phytopathogenic bacterium *Pseudomonas savastanoi* conjugates IAA to the amino acid lysine (Romano et al., 1991; Spena et al., 1991), but there is no significant homology of IAA lysine synthase to the plant GH3 family, which most closely resembles the structure of firefly luciferase (Staswick et al., 2002). Also it is interesting that IAA–Lys has so far not been shown to be hydrolysed by a plant amidohydrolase. On the other hand, bacterial hydrolases for IAA–Asp and IAA–Ala have been described (Chou et al., 1998; Chou and Huang, 2005), which also belong to the M20 peptidase family. The biochemistry of the amidohydrolase superfamily has been well characterized. A structural landmark for this group of predominantly hydrolytic enzymes is a mononuclear or binuclear metal centre, whose main role is to activate the substrate for cleavage (Roodveldt and Tawfik, 2005). While in dipeptidases that metal is usually Zn$^{2+}$, it is replaced by Mn$^{2+}$ in the auxin amino acid conjugate hydrolase family (see ‘Structure’), also in the bacterial ones. Maybe dipeptidases were therefore the ancestors of the auxin conjugate hydrolase family in bacteria and plants. It could be assumed that a structurally related dipeptide, e.g. Trp-Asp, is cleaved by a dipeptidase and that could have helped to evolve into an enzyme capable of cleaving IAA–Asp. While phytopathogenic bacteria, which live in close relation to the host plant, might need to control the levels of free IAA by themselves, free-living bacteria might use host-derived IAA conjugates and convert them into free IAA to alter the rhizosphere (Fig. 6). The plant enzymes for controlling auxin homeostasis have evolved during the generation of land plants (Fig. 6), necessary for shoot and vasculature development as well as embryogenesis (Cooke et al., 2002). However, much more sequence information is necessary from complete genomes combined with biochemical data on enzymatic activities within different groups of plants to draw any final conclusions about at which point auxin conjugation became an important feature of controlling auxin levels.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Expression analysis of IAA conjugate synthetases (GH3) and hydrolases in *A. thaliana* during development and in different tissues using Genevestigator (Zimmermann et al., 2004). Left: GH3 gene expression (blue, JA adenylation; red, IAA adenylation; green, IAA and SA adenylation), right: hydrolase gene expression. Note that not all genes of the hydrolase family were on the microarray, so no expression data can be given for those.

**Supplementary Fig. S2.** Expression analysis of IAA conjugate synthetases (GH3) and hydrolases in *A. thaliana* after abiotic and biotic stresses using Genevestigator (Zimmermann et al., 2004). Left: GH3 gene expression, right: hydrolase gene expression. The genes are in the same sequence as given for Fig. S1.

**Supplementary Table S1.** Abbreviations of all conjugates and genes/proteins used.

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