Title: Localisation and interactions between Arabidopsis auxin biosynthetic enzymes in the TAA/YUC-dependent pathway

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We show that a subset of enzymes in the Arabidopsis TAA/YUC route of auxin biosynthesis is localised to the endoplasmic reticulum and that microsomal fractions can produce auxin.

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Summary

The growth regulator auxin is involved in all key developmental processes in plants. A complex network of a multiplicity of potential auxin biosynthetic pathways as well as transport, signalling plus conjugation and deconjugation lead to a complicated system of auxin function. This raises the question how such a complex and multifaceted system producing such a powerful and important molecule as auxin can be effectively organised and controlled. Here we report that a subset of auxin biosynthetic enzymes in the TAA/YUC route of auxin biosynthesis is localised to the endoplasmic reticulum (ER). ER microsomal fractions also contain a significant percentage of auxin biosynthetic activity. This could point toward a model of auxin function using ER membrane location and subcellular compartmentation for supplementary layers of regulation. Additionally we show specific protein-protein interactions between some of the enzymes in the TAA/YUC route of auxin biosynthesis.

Introduction

Auxin is the major plant growth hormone and responsible for important processes including photo- and gravitropism, senescence, responses to pathogens and abiotic stress (Sundberg and Østergaard, 2009; Llavata Peris et al., 2010; Scarpella et al., 2010; Zhao, 2010). At the cellular level auxin controls a broad variety of functions such as cell elongation, endocytosis and cell polarity (Perrot-Rechenmann, 2010, Grunewald and Friml, 2010).

Multiple pathways enhance the complexity of auxin biosynthesis. Parallel tryptophan-dependent and -independent pathways (Woodward and Bartel, 2005; Wang et al., 2015; Pieck et al., 2015; Kasahara 2015) act in different organs, developmental stages and environmental conditions (Normanly and Bartel, 1999; Östin et al., 1999). All these different routes can be independently and differentially regulated to build a metabolic network capable of dynamic changes to keep up auxin homeostasis or supply auxin maxima for local demands. Hence identifying the main or most dominant pathway of auxin biosynthesis and
combining data from various species is rather challenging and problematical (reviewed in Tivendale et al., 2014).

The TAA/YUC route in Arabidopsis auxin biosynthesis

As the first ever reported auxin depletion phenotype in Arabidopsis was published from knockouts of YUC genes, current research has concentrated on the TAA/YUC route of auxin biosynthesis. Multiple loss-of-function yucca mutations result in reduced IAA concentrations and defects in development, including plant height and fertility (Zhao et al., 2001; Cheng et al., 2006). The first step in auxin biosynthesis is catalysed by a protein family represented by Weak Ethylene Insensitive8 (Wei8) / Tryptophan Aminotransferase Of Arabidopsis 1 (TAA1).

TAA1, TAR1 and TAR2 convert the amino acid tryptophan (Trp) to indole-3-pyruvic acid (IPyA) (Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009; Zhou et al., 2011). TAA1 was shown to be responsible for rapid changes in IAA levels in shade avoidance and taa1 mutants displayed reduced auxin levels (Tao et al., 2008). IPyA is then further converted to the auxin indole-3-acetic acid (IAA) by YUC proteins, a family of flavin-dependent monooxygenases. Interestingly even in a quadruple Arabidopsis yucca mutant the IAA levels are still 50% of WT levels (Stepanova et al., 2011). The TAA and YUC protein families jointly form a two-step biosynthetic route and constitute the main auxin biosynthesis pathway in Arabidopsis and maize (Mashiguchi et al., 2011; Phillips et al., 2011; Won et al., 2011, Kriechbaumer et al., 2012; Kriechbaumer et al., 2015).

Subcellular location of auxin biosynthetic enzymes

We previously showed that the Arabidopsis YUCCA gene YUCCA4 exists in two major splice isoforms resulting in YUCCA4.2 featuring a C-terminal hydrophobic transmembrane domain (TMD) and therefore localising to the endoplasmic reticulum (ER) (Kriechbaumer et al., 2012). Additionally it was shown that in maize (Zea mays) roots and coleoptiles auxin biosynthetic activity can be found in microsomal fractions and at least three maize auxin biosynthetic proteins (ZmSPI1, ZmTAR1 and ZmTAR3) show ER-localisation (Kriechbaumer...
et al., 2015). This could indicate a model of auxin function using ER membrane localisation and subcellular compartmentation for additional layers of regulation and raises the question about localisation of all the components of the Arabidopsis TAA/YUC route.

Here we report on the subcellular location of Arabidopsis TAA and YUC enzymes and in vivo interactions between these enzymes.

**Results**

**Bioinformatics analysis of enzymes in the Arabidopsis TAA/YUC pathway**

*In silico* analysis of enzymes suggested being involved in the TAA/YUC route of Arabidopsis auxin biosynthesis was carried out. This analysis predicted potential hydrophobic transmembrane domains (TMD) for YUC3, YUC4.1, YUC5, and TAR2 (Table 1). According to the algorithm TMHMM, YUC3 could feature an N-terminal TMD between the amino acid (aa) 31 and 53 for membrane insertion with the C-terminus facing the cytosol. YUC4.1 was shown to possess a C-terminal TMD with the enzymatic N-terminus facing the cytosol (Kriechbaumer et al., 2012). For YUC5 TMHMM predicts a TMD between aa 248 and 270 with the N-terminus resting in the cytosol (Table 1). TAR2 is suggested to have a TMD between aa 7 and 26 with the N-terminal part of the enzyme facing the ER lumen. Additionally using the prediction algorithm TargetP YUC5, YUC8, YUC9, YUC11, and TAR2 are indicated to possess an N-terminal signal anchor.

Another set of proteins in the TAA/YUC pathway of auxin biosynthesis (YUC1, YUC2, YUC4.1, YUC6, YUC7, YUC10, TAA1, and TAR1) are predicted to be cytosolic and don’t feature any hydrophobic domains. TMHMM indicates weak TMDs for YUC6 and YUC11 but their probability calculations put them far below cut-off threshold (Table 1).

**Subcellular localisation of auxin biosynthetic enzymes**

The subcellular localisation of the proteins in the TAR/YUC auxin biosynthetic pathway in Arabidopsis was tested using Agrobacterium-mediated transient expression in tobacco leaf
epidermal cells (Sparkes *et al.*, 2006). Proteins of interest in this respect were, of course, enzymes with predicted TM domains and therefore with potential membrane localisations (Table 1). We have shown before that YUC4 exists in two splice variants with YUC4.1 being located in the cytoplasm, whereas YUC4.2 gains a C-terminal TMD in the splicing process and is therefore localised to the ER with its enzymatic N-terminal domain facing the cytoplasm (Kriechbaumer *et al.*, 2012). Separately, TAA1 has been shown to be localised in the cytoplasm (Stepanova *et al.*, 2008; Tao *et al.*, 2008).

In the current study we have fused the remaining TAA/TAR and YUC proteins to N- or C-terminal fluorescent tags, respectively, so as not to interfere with the predicted TMDs. To determine their subcellular localisation these fusion proteins were co-expressed with the ER marker GFP-HDEL (Figure 1) and visualised by confocal microscopy. As predicted by their domain structure YUC3, YUC5, YUC8, YUC9, and TAR2 show colocalisation with the ER-marker GFP-HDEL (Figure 1). Interestingly also YUC7 shows ER-membrane localisation (Figure 1). YUC1, YUC2, YUC6, YUC11 and TAA1 are found in the cytosol (Figure 1).

To quantify the co-localization of the auxin constructs and the ER marker GFP-HDEL, Pearson’s correlation coefficients (r) in the co-localized volume were determined using the ImageJ Pearson–Spearman correlation (PSC) colocalisation plug-in (French *et al.*, 2008). Values and representative scatter plots are shown in Supplementary Figure S1. In this analysis, r of 1 indicates a perfect correlation with the ER marker, and a value of 0 shows no correlation. As to be expected, the ER membrane proteins TAR2, YUC5, 7, 8, and 9 show correlation coefficients between 0.31 and 0.4. Cytosolic proteins have significantly lower r values in the range of 0.02 to 0.09 (Figure S1). As a proof-of-concept we also overexpressed TAR2-mCherry in Arabidopsis in a stable manner and could confirm the ER localisation of TAR2 in Arabidopsis (Supplementary Figure S2).

As the auxin biosynthetic enzymes used in this study are tagged with fluorescent proteins it is important to show that the enzymes are still functional and correctly folded. For this we applied a novel leaf curling bioassay (Figure 2). Tobacco leaves expressing a combination of
a tagged TAA/TAR protein and a tagged-YUC protein show extensive leaf curling (Figure 2).

This effect can be mimicked by injecting a 1 mM IAA solution in to the leaves. Interestingly
expression of two TAA/TAR or two YUC constructs does not result in this leaf curling (Fig .2)
indicating that both steps of the pathway are necessary to produce IAA amounts sufficient to
produce the phenotype in tobacco leaves.

**Auxin biosynthetic activity in Arabidopsis microsomes**

Given the presence of at least six auxin biosynthetic enzymes on the ER membrane it was of
interest to find out if auxin activity could also be found linked to the ER. For this ER
microsome fractions were isolated from 5 days old Arabidopsis seedlings using a protocol
modified from soybean and maize extractions (Abell et al., 1997; Kriechbaumer et al.,
2015a).

To establish the purity of the microsomal fraction, immunoblots with three different antibodies
were performed with cytosolic and microsomal fractions or total protein extract and
microsomal fractions, respectively. The cytosolic and microsomal fractions were probed with
antibodies raised against the cytosolic heat shock protein 70 (Hsp70, Figure 3). The
microsomal fraction showed no detectable Hsp70 protein. To account for potential plasma
membrane contamination, the total protein extract and the microsomal fraction and were
blotted with anti-H^{+}ATPase antibodies recognizing the plasma membrane protein H^{+}ATPase
in a variety of plants and fungi including Arabidopsis. An H^{+}ATPase band could be identified
in the total protein extract but not in the microsomal fraction (Figure 3). Contamination of the
microsomal fraction with mitochondria was investigated using antibodies against alternative
oxidases (anti-AOX1/2). These quinol oxidases are located in the plant inner mitochondrial
membrane. This mitochondrial marker could be detected in the total protein extract but not in
the microsomal fraction (Figure 3).

Enzymatic tests using Trp or IPyA as a substrate were carried out using the microsomal
fractions, the cytosolic supernatant as well as total Arabidopsis protein extract (Figure 4).
Boiled protein extracts from each fraction were used as negative controls to deduct unspecific IAA conversion from the enzymatic conversion in the assays. Assays were snap-frozen immediately after incubation time, IAA was extracted by ethyl acetate phase separation and quantified via HPLC and confirmed by GC-MS (Kriechbaumer et al., 2015a). Unspecific conversion was less than 5% of the enzymatic conversion rate for both substrates.

Auxin biosynthetic activity with the substrates Trp and IPyA was found both in microsomal as well as cytosolic fractions of Arabidopsis seedlings (Figure 4). The ER-linked conversion of Trp was about 18% of the total conversion for IPyA which was 25% (Figure 4).

**Protein-protein interactions between auxin biosynthetic enzymes**

The membrane association of auxin biosynthetic enzymes and ER-linked auxin activity raises the intriguing possibility that auxin biosynthesis might be compartmentalised. Additionally, metabolic channelling in an "IAA synthase complex" has been postulated (Müller and Weiler, 2000). The formation of such metabolons characteristically comprises specific interactions between soluble enzymes that might be anchored to a membrane either by membrane-bound structural proteins that serve as nucleation sites for metabolon formation or by membrane-bound proteins involved in the pathway carried out by the metabolon. More evidence comes to light that pathways thought to contain only cytoplasmic enzymes are actually forming metabolons for subcellular structuring (reviewed in Jørgensen et al., 2005). Such a metabolon-based regulatory system could also explain how a single molecule like auxin can be effective in so many different developmental processes (Hawes et al., 2015).

To investigate the involvement of metabolic channelling, protein-protein interactions between TAA/TAR and YUC enzymes were investigated. To test for potential protein-protein interactions between auxin biosynthetic enzymes in the TAA/YUC pathway the methodology of FRET-FLIM was applied. Here the sensitivity and accuracy of Förster resonance energy transfer (FRET) to determine the colocalisation of two colour chromophores can now be improved to determine physical interactions by addition of fluorescence lifetime imaging...
microscopy (FLIM). The technique allows measuring and determination of the space map of picoseconds fluorescence decay at each pixel of the image through confocal single and multiphoton excitation. FRET-FLIM measures the reduction in the excited state lifetime of the donor GFP fluorescence when an acceptor fluorophore (RFP) is within a distance of 1 to 10 nm, thus allowing FRET to occur and indicating a physical interaction between the two proteins of interest (Osterrieder et al., 2009; Sparkes et al., 2010; Schoberer and Botchway, 2014; Kriechbaumer et al., 2015). A reduction of as little as 200 ps in the excited-state lifetime of the GFP-labelled protein can represent quenching and indicates a protein-protein interaction (Stubbs et al., 2005). Due to limitations in the speed of photon counting of the FLIM apparatus, measurements were taken from the ER associated with the nuclear envelope as these areas of the ER are high-expressing with relatively low mobility. This enabled more reliable measurements than the fast-moving cortical ER (Sparkes et al., 2010; Kriechbaumer et al., 2015b).

Protein-protein interactions were first investigated using the ER-localised TAR2 protein as a donor (Figure 1, Table 2, Figure 5A) and both cytosolic as well as ER-localised YUC enzymes, respectively, as acceptors. Cytosolic YUC enzymes were included in this study as this method is sensitive enough to detect interactions between ER-anchored and cytosolic proteins at the interface between cytosol and ER (Kriechbaumer et al., 2015b) which is especially important in the context of metabolon formation between membrane-anchored and non-anchored but nonetheless interacting proteins. For this TAR2 fused to GFP was expressed transiently in tobacco epidermal leaf cells alone or together with YUC-proteins fused to mCherry. At least two biological samples with three different replicas each were used for statistical analysis.

TAR2-GFP alone showed a fluorescence lifetime of 3.1 ± 0.03 ns. Figure 5 shows the FRET-FLIM analysis for TAR2-GFP alone (Figure 5A-E, negative control) and for two interactions with YUC5-mCherry (Figure 5F-J) and YUC9-mCherry (Figure 5K-O), respectively. Raw FRET-FLIM images are shown in Figure 5A, F and K. The following analysis takes into account the lifetime values of each pixel within the region of interest which is visualized by a
pseudo-coloured lifetime map (Figure 5B, G and L). The graphs in Figure 5C, H and M show the distribution of these lifetimes within regions of interest with blue shades representing longer GFP fluorescence lifetimes than green ones. Decay curves (Figure 5D, I and N) of a representative single pixel highlight an optimal single exponential fit, where $\chi^2$ values from 0.9 to 1.2 were considered an excellent fit to the data points (binning factor of 2 is applied). Confocal images showing the GFP construct in green and the mCherry construct in red are shown in Figure 5E, J and O.

This analysis example shows that most likely TAR2 and YUC5 interact as the lifetime values for the GFP/mCherry fusion pair (2.8 ± 0.03 ns; Table 2) are lower than those for TAR2-GFP alone (3.0 ± 0.05 ns). An interaction for TAR2 and YUC9 could not be determined as the lifetime for the fusion pair TAR2/YUC9 (3.0 ± 0.05 ns) is not statistically different from the lifetime of the negative control TAR2-GFP alone. Supplementary Figure S3 shows representative examples for FRET-FLIM data and the analysis steps for each combination tested (Figure S3).

Protein-protein interaction for TAR2 with other YUC proteins were tested and in this analysis TAR2 showed protein-protein interaction with YUC5 and YUC8 but not with YUC1, YUC2, YUC3, YUC6, YUC7, YUC9 and YUC11 (Table 2, Figures 6A and S3). Supplementary Figure S4 shows the colocalisation between TAR2 and YUC5 or YUC8, respectively (Figure S4).

Finally the protein-protein interaction between YUC-proteins was investigated (Table 2, Figure 6B and C and S3). As they interact with TAR2 and are ER-localised the enzymes YUC5 (Figure 6B) and YUC8 (Figure 6C) were chosen for this experiment. Both YUC5 and YUC8 showed interaction with a variety of YUC proteins tested: YUC5 with YUC5, YUC7, YUC9, and YUC11 (Figure 6B, S3 and Table 2) and YUC8 with YUC7 and YUC9 (Figure 6C, S3 and Table 2). Neither YUC5 nor YUC8 showed significant interaction with the cytosolic TAA1 protein.


**Discussion**

**Localisation of auxin biosynthesis**

Localisation studies of proteins involved in auxin function have long suggested the involvement of various sub-cellular compartments; auxin precursor pathways such as the shikimate and Trp biosynthetic pathways are suggested to be localised to plastids (Woodward and Bartel, 2005; Tzin and Gallili, 2010), whereas the further steps are believed to be localised in the cytosol (Woodward and Bartel, 2005; Mano and Nemoto, 2012; reviewed in Ljung, 2013). We have shown here that in transient overexpression in tobacco leaf epidermal cells a subset of auxin biosynthetic enzymes involved in the TAA/YUC route are localised to the ER membrane whilst others are cytosolic. With TAR2 and YUC4.2, 5, 7, 8, and 9 localised on the ER membrane and TAA1 and YUC1, 2, 3, 4.1, 6 and 11 in the cytoplasm (Figure 1; Kriechbaumer et al., 2012) we have a dual localisation for both steps in the TAA/YUC route of Arabidopsis auxin biosynthesis. We have shown a similar scenario before for *Zea mays* with *Zm*TAR1, *Zm*TAR3 and the YUC orthologue *Zm*SPI1 localised at the ER membrane and *Zm*VT2 and *Zm*YUC1 remaining cytoplasmic (Kriechbaumer et al., 2015a). Interestingly three of the ER-localised YUC proteins (YUC7, YUC8 and YUC9) together with the cytosolic YUC1 can suppress the dwarf phenotype of a weak brassinosteroid receptor mutant *bri1-301* (Kang et al., 2010). An auxin characteristic plant phenotype and overlapping expression pattern in the embryo have been shown in the quadruple mutant of yuc1/4/10/11 and YUC1, 2, 4, and 6 redundantly control venation in leaves and flowers (Cheng et al., 2006). It is noted that these YUC proteins are all cytosolic – with the yuc4 insertion not determining between the splice variants. It will be of great interest to create multiple mutants according to the localisation of proteins to evaluate the contribution of membrane anchoring to auxin biosynthetic capacity.

Additionally, in both Arabidopsis seedlings (Figure 4) and maize primary roots and coleoptiles (Kriechbaumer et al., 2015a) a significant percentage of auxin biosynthetic activity can be found in the microsomal fraction which mainly consists of ER. This activity together
with the localisation of enzymes involved raises the intriguing possibility of an additional level of regulation of biosynthesis and potentially also storage of compounds in different subcellular compartments. Trp is involved in a variety of other pathways such as the biosynthesis of proteins and defence compounds and also the size of the Trp pool is about 40 times larger than the pool of IAA and 25 times larger than for IPyA (Novák et al., 2012). This highlights the need for compartmentalisation of precursors and/or enzymes involved as well as pathway regulation to avoid overproduction of the highly active IAA molecules (Sairanen et al., 2012). Fluorescent auxin analogues that do not display auxin activity in planta but have been shown to mimic transport of endogenous IAA are also mainly localized to the endoplasmic reticulum in cultured cells and roots, indicating the possibility of a subcellular compartmentalised auxin gradient in the cells (Hayashi et al., 2014).

Recent data also indicate a regulatory role for the transport into the ER via specific PIN and PILS proteins; localization studies revealed that PIN5, PIN6, and PIN8 mainly localize to the ER (Mravec et al., 2009; Dal Bosco et al., 2012; Ding et al., 2012; Bender et al., 2013; Sawchuk et al., 2013) but PIN5 and PIN8 could also be detected on the plasma membrane (Ganguly et al., 2014). Hereby PIN5 and PILS2 and PILS5 are capable of enhancing auxin compartmentation between ER and cytosol whereas the pollen-specific PIN8 protein is suggested to act antagonistically and decrease compartmentation (Mravec et al., 2009; Barbez et al., 2012). Overexpression of the ER-localised PIN5 results in a decrease of free IAA and increased levels of conjugated IAA possibly suggesting additional levels of auxin regulation in the ER lumen (Mravec et al., 2009). It is suggested that ER-localised PINs function in regulating auxin homeostasis via subcellular auxin compartmentalization, as auxin transported into ER lumen is inaccessible for nuclear signaling (Mravec et al., 2009). In the ER auxin can then be inactivated by ER-localized auxin conjugating enzymes (Mravec et al., 2009) for instance several IAA-amino acid conjugate hydrolases have been shown to be located at the ER (Woodward and Bartel, 2005).
Protein interactions between auxin biosynthetic enzymes raising the possibility of a metabolon?

Precursor channelling via an IAA synthase complex has been suggested as many enzymes potentially involved in auxin biosynthesis have low substrate specificities and turnover rates (Pollmann et al., 2009). The existence of an auxin biosynthetic metabolon (Müller and Weiler, 2000; Kriechbaumer et al., 2006) a functional multienzyme complex tethered together by non-covalent binding typically receiving stabilization from membrane or cytoskeletal anchoring, is also a suggested possibility. Such multi-enzyme complexes have been shown for the Calvin-Benson cycle (Graciet et al., 2004), Arabidopsis dhurrin (Nielsen et al., 2008; Jensen et al., 2011) and sporopollenin biosynthetic pathways (Lallemand et al., 2013) and recently the isoflavonoid pathway in soybean (Dastmalchi et al., 2016). Metabolons allow for direct transport of the product from an enzymatic reaction to act as a substrate for the next enzymatic step thereby enhancing substrate concentrations and turnover rates and protection for unstable or toxic intermediates (Srere, 1985; Ralston and Yu, 2006; Møller, 2010; reviewed in Hawes et al., 2015). For example the intermediate IPyA is highly unstable when dissolved in water and converts to IAA; this is far less the case if IPyA is dissolved in alcohols such as methanol or in plant extracts. Additionally many enzymes suggested to be involved in auxin biosynthesis have low substrate specificities and turnover rates. To exemplify turnover rates, for YUC6 the $k_{cat}$ for oxidation of NADPH was shown to be $0.31 \text{s}^{-1}$ (Dai et al., 2013) whereas RuBisCO which is considered to have a low turnover rate has a $k_{cat}$ of $\sim 3 \text{s}^{-1}$ (Sage, 2002). To compensate for the low turnover kinetics of these enzymes an auxin metabolon has been postulated (Müller and Weiler, 2000). However, purification attempts (Müller and Weiler, 2000; Kriechbaumer et al., 2006) and yeast-2-hybrid approaches have not identified the proteins involved in auxin biosynthesis. A possible explanation is that IAA biosynthesis occurs at membrane surfaces catalysed by membrane anchored enzymes such as YUCCA4.2 or metabolons, which would impede the detection by such approaches due to their membrane binding or nuclear mislocalisation in the conventional yeast-2-hybrid, respectively. Such a metabolon-based regulatory system could
also explain how a single molecule like auxin can be effective and strictly controlled in so
many different developmental processes. The ER-membrane localised proteins YUC4.2
(Kriechbaumer et al., 2012) and YUC5, YUC7, YUC8, YUC9, and TAR2 could well work as
scaffolding protein for such a metabolon complex also allowing for other cytosolic TAA/YUC
enzymes to be part of the complex by protein-protein interactions.

The FRET-FLIM interactions between TAR2 and YUC5 and YUC8 (Figure 6A), respectively,
as well as the interactions between YUC5 (Figure 6B) and YUC8 (Figure 6C) with other YUC
proteins could potentially be the building blocks of larger protein complexes aiding further
regulatory mechanisms. This protein complex can be composed of membrane-bound and
cytosolic enzymes together with scaffolding and regulatory proteins such as P450 enzymes
or chaperone proteins. This raises the question why seemingly in a very specific manner
YUC5 and YUC8 interact with TAR2 but not the other ER-localised proteins YUC7 or YUC9
or even cytosolic YUC proteins (Figure 6A)? YUC5 was first described in the super1-D
mutant that acts as a suppressor of the partial loss-of-function mutant allele er-103 of the
ERECTA gene (Woodward et al., 2005b). ERECTA is involved in inflorescence architecture
(Torii et al., 1996) and overexpression of YUC5 results in elevated free auxin levels and
characteristic phenotypes such as increased hypocotyl length and narrow leaves (Woodward
et al., 2005b). It was concluded that auxin biosynthesis via YUC5 and the ERECTA pathway
work independently but with potential overlaps in determining inflorescence architecture via
cell division and cell expansion (Woodward et al., 2005b). YUC5 is mainly expressed in roots
and young vegetative tissue but not in flowers and during the inflorescence developmental
stage (Woodward et al., 2005b).

YUC8 was recently linked to jasmonic acid (Hentrich et al., 2013a) and ethylene signalling
(Hentrich et al., 2013b) and is furthermore regulated by temperature via the phytochrome-
interacting factor 4 (PIF4; Sun et al., 2012). Both YUC5 and YUC8 together with YUC2 and
YUC9 are transcriptionally up-regulated when plants are under shade, also correlating with
an increase in free auxin levels (Xie et al., 2015); the transcription factor KANADI1 is capable
of transcriptional repression of YUC2, YUC5 and YUC8 and can therefore inhibit shade-
induced auxin biosynthesis (Xie et al., 2015). Furthermore both YUC5 and YUC8 again
interact with a different subset of the YUC proteins tested: YUC5 homodimerizes with YUC5
and interacts with the ER-localised YUC7 and YUC9 as well as with the cytosolic protein
YUC11 (Figure 6B); YUC8 shows interaction with the drought-induced YUC7 and
homodimerizes with YUC8 itself (Figure 6C). Further investigation of these interactions as
well as proteomic immunoprecipitation studies will aim to reveal the composition and
dynamics of such a protein complex.

Auxin biosynthesis responds to a plethora of environmental factors and therefore has to be
rather versatile. It has recently been shown to relate for example, to sugar signalling; via the
Phytochrome Interacting Factor (PIF) transcription factors soluble sugars can upregulate IAA
biosynthesis in Arabidopsis (Sairanen et al., 2012; Lilley et al., 2012) and sugars have also
been shown to influence auxin biosynthesis in developing maize kernels (LeClere et al.,
2010). Another factor is light: IAA biosynthesis via the TAA1/YUC pathway is stimulated in
response to changes in the ratio of red to far-red light in shade conditions (Tao et al., 2008).
Also this response is under the regulation of PIF genes (Hornitschek et al., 2012). PIF-
mediated regulation has also been shown for TAA1 and YUC8 in temperature regulation of
IAA biosynthesis (Franklin et al., 2011; Sun et al., 2012). It is intriguing that all these different
responses are regulated by PIF transcription factors. The potential of a metabolon with
changing compositions and/or numbers of TAA/TAR and YUC proteins would provide
additional regulatory power under changing environmental and developmental situations.

ER lipid subdomains have been described to be capable of supporting metabolon assembly
(Zajchowski and Robbins, 2002) and metabolon localisation on the ER could potentially also
add an aspect of mobility as the ER surfaces has been shown to be mobile (Runions et al.
2006). For instance, ER micro-domains could move metabolons around in an actin-guided
way if under pathogen attack (Chuong et al., 2004). It is a possibility that metabolon
formation allows production of the basic structures and depending on developmental stage,
tissue or stress situation additional or different enzymes could be recruited to the metabolons for specific structural changes, such as in output or to supply additional regulatory aspects during production (Jørgensen et al., 2005).
Experimental procedures

Cloning of expression plasmids
Primers were obtained from Eurofins Genomics. Q5 high-fidelity DNA polymerase (New England Biolabs) was used for all polymerase chain reaction reactions. Genes of interest were cloned into the modified binary vector pB7WGF2 containing an N- or pB7FWG containing a C-terminal GFP fluorescent proteins (Karimi et al., 2005) using Gateway technology (Invitrogen).

Plant material and transient expression in tobacco epidermal leaf cells
For Agrobacterium-mediated transient expression, 5-week-old tobacco (Nicotiana tabacum SR1 cv Petit Havana) plants grown in the greenhouse were used. Transient expression was carried out according to Sparkes et al. (2006). In brief, each expression vector was introduced into Agrobacterium strain GV3101 by heat shock. Transformants were inoculated into 5 ml of YEB medium (per litre: 5 g of beef extract, 1 g of yeast extract, 5 g of sucrose and 0.5 g of MgSO$_4$$\cdot$$7$H$_2$O) with 50 $\mu$g/ml spectinomycin and rifampicin. After overnight shaking at 25°C, 1 ml of the bacterial culture was pelleted by centrifugation at 2,200 \times g for 5 min at room temperature. The pellet was washed twice with 1 ml of infiltration buffer (50 mM MES, 2 mM Na$_3$PO$_4$$\cdot$$12$H$_2$O, 0.1 mM acetosyringone and 5 mg/ml glucose) and then resuspended in 1 ml of infiltration buffer. The bacterial suspension was diluted to a final OD$_{600}$ of 0.1 and carefully pressed through the stomata on the lower epidermal surface using a 1 ml syringe. Transformed plants then were incubated under normal growth conditions for 48 h. Images were taken using a Zeiss 880 laser scanning confocal microscope with 63x oil immersion objective. For imaging of the GFP/RFP combinations, samples were excited using 488 and 543 nm laser lines in multi-track mode with line switching. Images were edited using the ZEN image browser.

Leaf curling bioassay
For the leaf curling assay to assess induced auxin biosynthesis, tobacco plants were infiltrated with TAA, TAR and YUC constructs in varying combinations as described above. 1 mM IAA dissolved in infiltration buffer was infiltrated into the leaf epidermal cells the same way. Plants were kept in growth chambers for 48h before images were taken. Expression was checked using confocal microscopy as described above.

**FRET-FLIM data acquisition**

Epidermal samples of tobacco leaves infiltrated as described above were excised and the GFP and mRFP expression levels in the plant within the region of interest were confirmed using a Nikon EC2 confocal microscope with excitation at 488 and 543 nm, respectively. FRET-FLIM data capture was performed according to Osterrieder et al. (2009) and Kriechbaumer et al. (2015b) using a two-photon microscope at the Central Laser Facility of the Rutherford Appleton Laboratory.

In brief, a two-photon microscope built around a Nikon TE2000-U inverted microscope was used with a modified Nikon EC2 confocal scanning microscope to allow for multiphoton FLIM (Botchway et al., 2015). 920 nm laser light was produced by a mode-locked titanium sapphire laser (Mira; Coherent Lasers), producing 200-fs pulses at 76 MHz, pumped by a solid-state continuous wave 532-nm laser (Verdi V18; Coherent Laser). The laser beam was focused to a diffraction limited spot through a water-immersion objective (Nikon VC; 360, numerical aperture of 1.2) to illuminate specimens on the microscope stage. Fluorescence emission was collected without descanning, bypassing the scanning system, and passed through a BG39 (Comar) filter to block the near-infrared laser light. Line, frame, and pixel clock signals were generated and synchronized with an external detector in the form of a fast microchannel plate photomultiplier tube (Hamamatsu R3809U). Linking these via a time-correlated single-photoncounting PC module SPC830 (Becker and Hickl) generated the raw FLIM data.

Data were analyzed by obtaining excited-state lifetime values of a region of interest on the nucleus, and calculations were made using SPC Image analysis software version 5.1.
(Becker and Hickl). The distribution of lifetime values within the region of interest was generated and displayed as a curve. Only values that had a $\chi^2$ between 0.9 and 1.4 were taken. The median lifetime value and minimum and maximum values for one-quarter of the median lifetime values from the curve were taken to generate the range of lifetimes per sample. At least three nuclei from at least two independent biological samples per protein-protein combination were analyzed, and the average of the ranges was taken.

**ER microsome preparation**

All following steps were performed on ice or 4°C unless indicated otherwise. 5 g of Arabidopsis seedlings (5 days after germination) were ground in liquid nitrogen using a mortar and pestle. The resulting powder was homogenised in approximately 4 ml of buffer A (25 mM TEA-HOAc pH7.5, 50 mM KOAc pH7.5, 5 mM Mg(OAc)$_2$, 0.25 M sucrose, 4 mM DTT). Then 4 ml of buffer B (100 mM TEA-HOAc pH7.5, 20 mM EDTA) were added and the suspension was incubated on ice for 10 min. After centrifugation at 1,000 g for 10 min the resulting supernatant was poured over 2 layers of cheese cloth into a fresh tube. That extract was centrifuged again at 4,500 g for 25 min. In ultracentrifuge tubes the 8 ml suspension were layered on 4 ml of sucrose cushion (Buffer C: 25 mM TEA-HOAc pH7.5, 25 mM KOAc pH7.5, 2 mM Mg(OAc)$_2$, 0.5 M sucrose, 4 mM DTT). Using the swing-out rotor SW41 this was spun for 90 min at 93,000 g. The resulting pellet was resuspended in 200 µl buffer D (25 mM TEA-HOAc pH7.5, 0.25M sucrose, 1 mM DTT) using a glass rod and a 2 ml Potter-Elvehjem homogeniser. Freshly prepared microsomes were used for enzymatic assays straight away.

**IAA quantification**

Enzymatic activity tests with microsomal and cytosolic fractions were carried out in 100 mM TRIS-HCl, pH 8.0, using 20 µl of plant extract, 1 mM NADPH, 100 µM FAD, and 100 µM tryptophan or IPA in a total volume of 100 µl. As an internal standard, for further GC_MS analysis 2,4,5,6,7-pentadeuteriated IAA (Cambridge Isotope Laboratories, UK) was included.
After incubation for 1 h in a 37 °C water bath, the assays were snap-frozen in liquid nitrogen and IAA extracted by ethyl acetate phase separation (Park *et al.*, 2003; Kriechbaumer *et al.*, 2007).

In brief, the pH of the sample was increased over 9.5 with 1 M Na$_2$CO$_3$ and the then extracted with 400 μl of ethyl acetate. The aqueous lower phase was recovered, 200 μl of water were added, the partitioning procedure was repeated, and again the aqueous phase was recovered and combined with the aqueous phase from the previous partitioning step. The collected aqueous phase was acidified with acetic acid to a pH below 2.5 and partitioned twice with addition of 400 μl of ethyl acetate for each step. This time the organic phases were collected and the liquid evaporated using a speed-vac (Centrivap, Labconco). The dried pellets were re-dissolved in 100% methanol and analysed via high-performance liquid chromatography (HPLC) with a reverse phase column (Apollo C18, 250 mm×4.6 mm, 5 μm, Grace). IAA was quantified via a HPLC system (Waters 600E) in isocratic flow of 0.8 ml min$^{-1}$ with a 40:60 mixture of buffer A (10% methanol, 0.3% acetate) and buffer B (90% methanol, 0.3% acetate). Peaks were identified by comparison with the standard substances with respect to retention time and UV spectrum using both a UV monitor (Waters 486) and a fluorescence monitor (Waters 470).

To confirm and quantify IAA GC-MS was applied (Kriechbaumer *et al.*, 2015a). In brief: IAA-containing HPLC fractions were collected, and dried and dissolved in 20 μl of methanol. For derivatization 50 μl of ethereal diazomethane (Sigma-Aldrich) were added to each sample and incubated for 30 min in a fume hood. Tubes were set to dried under vacuum for 10 min and any remaining solution in the tubes was blown off with pure N2 gas. The derivatized samples were dissolved in 10 μl of pure methanol and 1 μl of the solution was injected to gas chromatography–mass spectrometry (GC-MS; CP-3800, Saturn 2200, Varian) in the split-less mode. The identity of derivatized IAA was confirmed by 130 and 189 fragmentation ions and normalized against the internal standard recognized by 135 and 184 fragmentation ions. The signals in the peak area of the 130 fragmentation ion were quantified using external standards.
Western blotting of total protein extract, microsomal and cytosolic fractions

100 μg protein of total protein extract, the cytosolic and microsomal fractions, respectively, was separated on a 12% (v/v) SDS–polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with antiHsp70 antibodies (1:1000, Agrisera), anti-H+ATPase antibodies (1:1000, Agrisera), or anti-AOX1/2 antibodies (1:1000, Agrisera), respectively. The membrane was further incubated with anti-rabbit immunoglobulin G conjugated with Cy5, and the signal was detected with a fluorescence scanner using a red fluorescence filter.

Acknowledgements

The authors thank Dr Joseph McKenna for the gift of the mCherry-vector. This work was supported by the Science and Technology Facilities Council Program (grant no. 14230008) awarded to CH.
References


**Table 1.** In silico prediction of targeting sequences identified by WoLFPSORT or TargetP 1.1. TMDs were predicted using the computational algorithms TMHMM, signal peptides using SignalP4.1 (http://www.expasy.org/tools/).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>TMD predicted by TMHMM (position; N-terminus)</th>
<th>Targeting Sequences by WoLFPSORT (residues) [TargetP 1.1]</th>
<th>Localisation (evidence)</th>
<th>Gene ID, Length [aa] &amp; Expression</th>
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<td>None (evidence of NLS)</td>
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<td>AT4G13260.1, 415 Ubiquitously expressed</td>
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<tr>
<td>YUC3</td>
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Table 2: Fluorescence lifetimes FRET-FLIM analysis.

Donor and acceptor protein constructs are indicated together with the average fluorescence lifetime (in ns) for the donor fluorophore and the standard error for each combination. Δ indicates the change in lifetime in comparison to the donor control without acceptor present.

<table>
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<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>GFP-fluorescence lifetime [ns]</th>
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<td>3.0 ± 0.05</td>
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<td>TAA-mCherry</td>
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Figure legends

Figure 1: Transient expression and localization of auxin biosynthetic proteins in tobacco leaf cells.

Co-expression with the ER luminal marker GFP–HDEL (green) is shown for the TAA/TAR proteins TAR2 and TAA1 (A). YUC5, YUC7, YUC8, and YUC9 fused to mCherry (pink) colocalise with the ER marker GFP HDEL (B). YUC1, YUC2, YUC3, YUC6, and YUC11 show a cytosolic distribution and do not colocalise with GFP-HDEL (C). Red size bar = 5µm.

Figure 2: Leaf curling bioassay to determine the functionality of tagged enzymes. Expression of a combination of TAA/TAR and YUC results in leaf bending; two TAA/TAR proteins or two YUC proteins do not have this effect. Examples shown here are YUC5/TAA1 (A) and YUC2/TAR2 (B) for leaf bending (right hand side of leaves) and YUC2/YUC5 (A) and TAA1/TAR2 (B) combined (left hand side of leaves). An IAA solution was infiltrated as a control (C, right hand side of leaf). Front and side views for each leaf are shown.

Figure 3: Immunoblot analysis of microsomal fractions.

Immunoblot analysis of Hsp70 proteins in the Arabidopsis seedling cytosolic (C) and microsomal (M) fraction and immunoblot analysis of plasma membrane H^+ATPase proteins and mitochondrial alternative oxidases (AOX1/2) in the Arabidopsis total protein extract (T) and microsomal (M) fraction. Western blots of 100 μg of protein from each fraction were probed with diluted (1:1000) antibodies. Anti-Hsp70 recognize the cytosolic Hsp70 protein, anti-H^+ATPase antibodies detect the plasma membrane localised H^+ATPase protein and anti-AOX1/2 antibodies bind the mitochondrial AOX1/2 protein.

Figure 4: Enzymatic conversion of tryptophan (grey bars) and IPyA (white bars) to IAA by microsomal (Micro) fractions, cytosolic (Cyt) fractions, or total plant extract (Total) of Arabidopsis seedlings 5 d after germination. Standard errors and percentages normalized to total plant extract are indicated. n=2 (two biological samples with three replicates each).
Figure 5: FRET-FLIM analysis of TAR2 without an interaction partner (A–E) or with YUC5 (F–J) or YUC9 (K-O), respectively.

A, F and K display the raw FRET-FLIM data. The pseudo-coloured lifetime maps in B, G and L show the lifetime values for each point within the region of interest, whilst the distribution of lifetimes across the image is shown in C, H and M. Blue shades representing longer GFP fluorescence lifetimes than green ones. D, I and N display representative decay curves of a single point with an optimal single exponential fit, where $\chi^2$ values from 0.9 to 1.2 were considered an excellent fit to the data points (binning factor of 2 was applied). The confocal images for the analysis in E, J and O show the GFP-construct in green and the m-Cherry construct in red.

This example of FRET-FLIM analysis shows TAR2-GFP alone as a negative control, YUC5 for protein-protein interaction and YUC9 for no interaction with TAR2. The fluorescence lifetime values for TAR2-GFP+YUC5-mCherry are 2.92 ± 0.03 ns and therefore statistically lower than the lifetime values for the TAR2-GFP fusion alone (3.04 ± 0.03 ns). In contrast the lifetime value for the donor-acceptor combination TAR2-GFP/YUC9-mCherry is with 3.05 ± 0.06 ns not statistically different from the negative control, TAR2-GFP alone, hence indicating that TAR2 and YUC9 do not interact.

Figure 6: Fluorescence lifetimes in FRET-FLIM interactions with TAA/TAR and YUC proteins.

The bar graphs represent average fluorescence lifetimes (ns) and the corresponding SE values for the GFP donors TAR2 (A), YUC5 (B), and YUC8 (C). The data show the candidate interaction proteins (blue bars) compared with TAR2-GFP, GFP-YUC5 or YUC8-GFP without interaction partners (grey bars). Lifetimes significantly lower than those of TAR2-GFP, GFP-YUC5 or YUC8-GFP alone (lower than blue line) indicate protein-protein interactions.
Supplementary data

Supplementary Figure S1: Colocalisation of auxin biosynthetic proteins with the ER-marker GFP-HDEL. Pearson-Spearman coefficients and scatterplots using the ImageJ plug-in PSC (French et al., 2008) are listed and representative scatter plots shown.

Supplementary Figure S2: Stable expression of TAR2-mCherry in Arabidopsis thaliana. TAR2-mCherry labels the ER network in Arabidopsis.

Supplementary Figure S3: Representative FRE-FLIM data for interactions tested with TAR2-GFP, GFP-YUC5, or YUC8-GFP, respectively, as donor proteins. Corresponding confocal images with the GFP constructs in green and tested interacting proteins in red are shown on the right hand side.

Supplementary Figure S4: Transient expression and colocalisation of in tobacco leaf cells. The auxin biosynthetic proteins YUC5-mCherry and YUC8-mCherry are co-expression with TAR2-GFP. Red size bar = 5µm.
Figure 1A

<table>
<thead>
<tr>
<th>TAR2/TAA localisations</th>
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<tbody>
<tr>
<td>TAR2-mCherry</td>
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<tr>
<td>TAA1-mCherry</td>
</tr>
</tbody>
</table>
Figure 1B

YUC localisations – ER membrane

YUC5-mCherry  GFP-HDEL  Merge

YUC7-mCherry  GFP-HDEL  Merge

YUC8-mCherry  GFP-HDEL  Merge

YUC9-mCherry  GFP-HDEL  Merge
Figure 1C

YUC localisations - cytosolic

YUC1-mCherry  GFP-HDEL  Merge

YUC2-mCherry  GFP-HDEL  Merge

YUC3-mCherry  GFP-HDEL  Merge

YUC6-mCherry  GFP-HDEL  Merge

YUC11-mCherry  GFP-HDEL  Merge
Figure 3

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<td>kDa</td>
<td>C</td>
<td>M</td>
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Legend:
- C: Control
- M: Mutant
- T: Treatment
Figure 6C

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YUC8-GFP
### Supplementary Figure S1

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<thead>
<tr>
<th>Protein combination</th>
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<td>TAR2-mCherry + GFP-HDEL</td>
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<td>ER</td>
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<tr>
<td>TAA-mCherry + GFP-HDEL</td>
<td>0.09</td>
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### Representative scatter plots:

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<tr>
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<td><img src="image3.png" alt="YUC9+HDEL" /></td>
<td><img src="image4.png" alt="YUC1+HDEL" /></td>
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</table>
Supplementary Figure S3

clover-TAR2 +

(-)

YUC1

YUC2

YUC3
clover-TAR2 +

YUC6

YUC7

YUC8

YUC11
GFP-YUC5 +
YUC8-GFP +