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REVIEW PAPER

The pathway of auxin biosynthesis in plants

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Abstract

The plant hormone auxin, which is predominantly represented by indole-3-acetic acid (IAA), is involved in the regulation of plant growth and development. Although IAA was the first plant hormone identified, the biosynthetic pathway at the genetic level has remained unclear. Two major pathways for IAA biosynthesis have been proposed: the tryptophan (Trp)-independent and Trp-dependent pathways. In Trp-dependent IAA biosynthesis, four pathways have been postulated in plants: (i) the indole-3-acetamide (IAM) pathway; (ii) the indole-3-pyruvic acid (IPA) pathway; (iii) the tryptamine (TAM) pathway; and (iv) the indole-3-acetaldoxime (IAOX) pathway. Although different plant species may have unique strategies and modifications to optimize their metabolic pathways, plants would be expected to share evolutionarily conserved core mechanisms for auxin biosynthesis because IAA is a fundamental substance in the plant life cycle. In this review, the genes now known to be involved in auxin biosynthesis are summarized and the major IAA biosynthetic pathway distributed widely in the plant kingdom is discussed on the basis of biochemical and molecular biological findings and bioinformatics studies. Based on evolutionarily conserved core mechanisms, it is thought that the pathway via IAM or IPA is the major route(s) to IAA in plants.

Key words: Auxin, auxin biosynthesis, IAA, indole-3-acetaldehyde, indole-3-acetaldoxime, indole-3-acetamide, indole-3-acetic acid, indole-3-pyruvic acid, plant hormone.

1. Introduction

In 1880, Charles Darwin proposed that some plant growth responses are regulated by 'a matter which transmits its effects from one part of the plant to another' (Darwin and Darwin, 1880). Then, half a century later, this substance termed auxin was identified as indole-3-acetic acid (IAA) (Kögl and Kostermans, 1934; Went and Thimann, 1937). This phytohormone auxin is a key regulator of many aspects of plant growth and development, including cell division and elongation, differentiation, tropisms, apical dominance, senescence, abscission, and flowering (Woodward and Bartel, 2005; Teale *et al.*, 2006).

Although much progress has been made in defining IAA signalling pathways, the biosynthesis of IAA and its regulation by environmental and developmental signals remain poorly understood. *De novo* auxin biosynthesis plays an essential role in many developmental processes. Determining the molecular mechanisms of auxin biosynthesis may provide new tools for solving difficult plant development questions, defining the roles of auxin in plant development, understanding auxin transport, and studying the mechanisms of auxin in regulating plant development.

Auxin biosynthesis in plants is fairly complex. Multiple pathways have been postulated that contribute to *de novo* auxin biosynthesis (Fig. 1). Although different plant species may have unique strategies and modifications to optimize their metabolic pathways, it would seem reasonable that plants would share evolutionarily conserved core mechanisms for auxin biosynthesis because IAA is a fundamental substance in the plant life cycle.

In this review, the focus is on assessing the consensus pathway for auxin biosynthesis, and the genes and metabolites

Abbreviations: IAA, indole-3-acetic acid; IAD, indole-3-acetaldehyde; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAOX, indole-3-acetaldoxime; IPA, indole-3-pyruvic acid; TAM, tryptamine; Trp, tryptophan.

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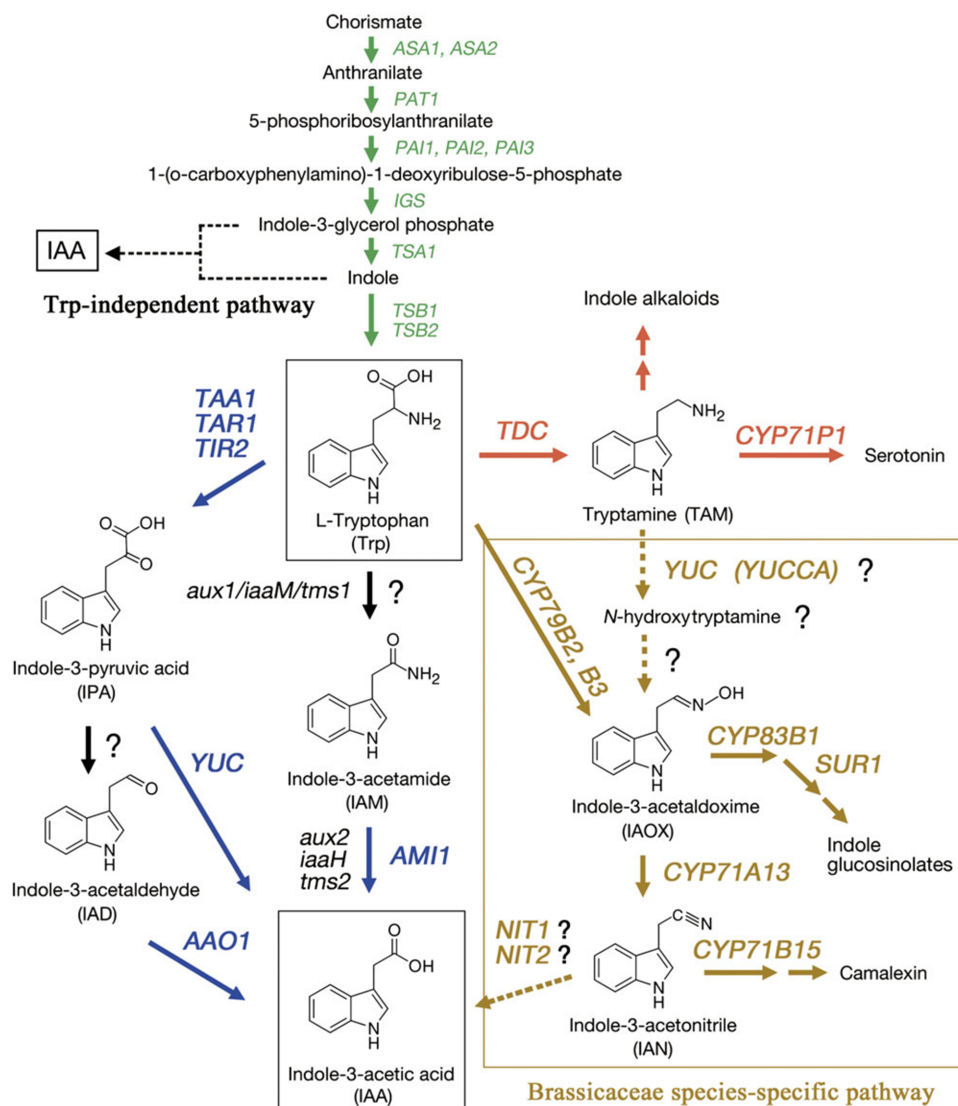

Fig. 1. Presumptive pathways for IAA biosynthesis in plants. Green arrows indicate the tryptophan synthetic pathway in the chloroplast. A thin dashed black arrow denotes the tryptophan-independent IAA biosynthetic pathway. Blue arrows indicate steps for which the gene and enzymatic function are known in the tryptophan-dependent IAA biosynthetic pathway. Red arrows indicate the indole alkaloid and serotonin biosynthetic pathway. Mustard-coloured arrows indicate the Brassicaceae species-specific pathway. Black arrows indicate steps for which the gene(s) and enzymatic function(s) are unknown. Dashed mustard-coloured arrows indicate steps for which the gene and enzymatic function(s) remain poorly understood. Letters in italics show genes involved in the conversion process. Lower case letters in italics indicate bacterial genes.

involved in auxin biosynthesis in the plant kingdom are summarized.

2. Multiple pathways postulated for auxin biosynthesis in plants

Two major pathways for IAA biosynthesis have been proposed in plants: the tryptophan (Trp)-independent and Trp-dependent pathways (Woodward and Bartel, 2005; Chandler, 2009; Normanly, 2010). In Trp-independent IAA biosynthesis, indole-3-glycerol phosphate or indole is the likely precursor, but little is known about the biochemical pathway to IAA (Ouyang *et al.*, 2000; Zhang *et al.*, 2008) (Fig. 1).

In Trp-dependent IAA biosynthesis, several pathways have been postulated (Woodward and Bartel, 2005; Pollmann *et al.*, 2006*a*; Chandler, 2009; Mano *et al.*, 2010; Normanly, 2010; Zhao, 2010): (i) the indole-3-acetamide (IAM) pathway; (ii) the indole-3-pyruvic acid (IPA) pathway; (iii) the tryptamine (TAM) pathway; and (iv) the indole-3-acetaldoxime (IAOX) pathway (Fig. 1). Genes that have been suggested to participate in IAA biosynthesis in plants and their respective functions are summarized in Table 1. Although auxin IAA was the first plant hormone identified, little is known regarding the genetic basis of the key enzymes involved in the IAA biosynthetic pathway(s), and it is unclear whether all pathways exist in all plant species.

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3. Tryptophan is synthesized in the chloroplast

Trp is synthesized from chorismate via indole-3-glycerol phosphate in the chloroplast (Radwanski and Last, 1995) (Fig. 1). The *ASA1* and *ASA2* genes of *Arabidopsis thaliana* encode the α-subunit of anthranilate synthase, the enzyme catalysing the first reaction of the tryptophan biosynthetic pathway. Both predicted proteins have putative chloroplast transit peptides at their N-termini (Niyogi and Fink, 1992; Bohlmann *et al.*, 1996). The *IGS* gene of *A. thaliana* encodes indole-3-glycerol phosphate synthase, which catalyses the conversion of 1-(*O*-carboxyphenylamino)-1-deoxyribulose-5-phosphate to indole-3-glycerol phosphate, a branch point compound in the Trp-independent IAA biosynthetic pathway (Li *et al.*, 1995; Ouyang *et al.*, 2000). The deduced *Arabidopsis* IGS protein also has a putative chloroplast target sequence of ~80 amino acid residues at the N-terminus (Li *et al.*, 1995).

Trp synthase is a typical heterotetrameric $\alpha_2\beta_2$ complex consisting of Trp synthase α (TSA1) and Trp synthase β (TSB) subunits, encoded by *TSA1* and *TSB1, 2* genes, respectively. *Arabidopsis thaliana* has two closely related, non-allelic tryptophan synthase β genes (*TSB1* and *TSB2*), each containing a chloroplast target sequence at the N-terminus (Last *et al.*, 1991). Trp is used to produce many indole-containing substances in plants, such as IAA, indole glucosinolates, phytoalexins, and TAM derivatives.

4. The indole-3-acetamide pathway

4.1. Studies on hairy roots led to the discovery of a new route in IAA biosynthesis

The IAA biosynthetic pathway via IAM was thought to be a bacteria-specific pathway because no evidence for this pathway had been found in plants. The plant pathogen *Agrobacterium rhizogenes* harbours a large root-inducing (Ri) plasmid and generates hairy-root disease, which is characterized by root proliferation from the infection site. A portion of the Ri plasmid, designated the T-DNA, is transferred to the host plant cell, integrated into the plant genome, and expressed in polyadenylated mRNA (Moore *et al.*, 1979; White and Nester, 1980; Chilton *et al.*, 1982). Hairy roots can grow aseptically in phytohormone-free media (Mano *et al.*, 1986, 1989; Mano, 1993).

In hairy roots, IAA is synthesized from Trp by a two-step reaction as a result of the expression of the integrated genes *aux1* (also referred to as *iaaM/tms1*) and *aux2* (also referred to as *iaaH/tms2*) of Ri TR-DNA (Yamada *et al.*, 1985; Camilleri and Jouanin, 1991; Gaudin *et al.*, 1993; Casanova *et al.*, 2005). The auxin biosynthetic pathway catalysed by the *aux1* and *aux2* gene products is similar to that in *Agrobacterium tumefaciens* and *Pseudomonas syringae* (Comai and Kosuge, 1982; Schroder *et al.*, 1984; Thomashow *et al.*, 1984; Yamada *et al.*, 1985; Camilleri and Jouanin, 1991). Trp is first converted to IAM by the enzyme tryptophan-2-monooxygenase encoded by the *aux1/iaaM/tms1* gene (Yamada *et al.*, 1985; Camilleri and Jouanin, 1991; Gaudin *et al.*, 1993) (Fig. 1). Then, IAM is converted to IAA by indole-3-acetamide hydrolase encoded by the *aux2/iaaH/tms2* gene (Yamada *et al.*, 1985; Camilleri and Jouanin, 1991; Gaudin *et al.*, 1993; Nemoto *et al.*, 2009*a*, *b*; Mano *et al.*, 2010).

Tobacco (*Nicotiana tabacum*) Bright Yellow-2 (BY-2) cells proliferate rapidly, exhibiting considerable homogeneity, like meristematic cells (Nagata *et al.*, 1992), and requiring only auxin for culture. Thus, a transgenic BY-2 cell line transformed with the Ri plasmid is an excellent tool for the investigation of the role of auxin in the plant cell division that takes place in the meristem. Based on the finding that the overexpression of the *aux1* gene of the Ri plasmid allowed BY-2 cells to grow rapidly in the absence of auxin (Nemoto *et al.*, 2009*a*, *c*), the indole-3-acetamide hydrolase gene was recently isolated from *Nicotiana* sp. and designated *NtAMI1* (Nemoto *et al.*, 2009*b*). The *NtAMI1* gene consists of 1278 bp, encoding a putative protein of 425 amino acids with a calculated molecular weight of 45052 (GenBank accession number AB457638), and it is the same length as the *AtAMI1* gene (GenBank accession number NM100769) isolated from *A. thaliana* based on the sequence of the *Agrobacterium aux2* gene (Pollmann *et al.*, 2003) (Table 1).

4.2. The AtAMI1 and NtAMI1 genes encode indole-3-acetamide hydrolase, which functions in the cytoplasm

Expression vectors to produce the AtAMI1 or NtAMI1 fusion protein in *Escherichia coli* were constructed to confirm that the gene products have the enzyme activity. Fusion proteins of AtAMI1 (Pollmann *et al.*, 2003) and NtAMI1 (Nemoto *et al.*, 2009b) have the enzyme activity converting IAM to IAA, indicating that these *AMI1* genes encode indole-3-acetamide hydrolase (Fig. 1, Table 1). A specific serine residue, Ser137 of AtAMI1 and Ser136 of NtAMI1, is essential for AMI1 enzymatic activity (Neu *et al.*, 2007; Lehmann *et al.*, 2010; Mano *et al.*, 2010). By experiments using fusion proteins of AMI1–green fluorescent protein (GFP), the AMI1 protein was confirmed to be located in the cytoplasm of plant cells (Pollmann *et al.*, 2006*b*).

4.3. IAM, as a metabolic intermediate of auxin biosynthesis, is detected in every plant species

IAM has been detected in many plants including the fruits of *Citrus unshiu* (Takahashi *et al.*, 1975), the hypocotyls of Japanese cherry (Saotome *et al.*, 1993), aseptically grown squash seedlings (Rajagopal *et al.*, 1994), tobacco (Lemcke *et al.*, 2000), *Arabidopsis* (Pollmann *et al.*, 2002; Sugawara *et al.*, 2009), maize, rice, and tobacco (Sugawara *et al.*, 2009). The levels of IAM are ~10 ng g^{-1} fresh weight (FW) in 2-week-old seedlings (Sugawara *et al.*, 2009) or ~3.5 ng g^{-1} FW in 2-week-old plants to ~130 pg g^{-1} FW in fully grown rosettes (Pollmann *et al.*, 2002) in *Arabidopsis*. The levels of IAM are 156±60 pmol g^{-1} FW in untransformed tobacco (Lemcke *et al.*, 2000), ~3 ng g^{-1} FW in 2-day-old coleoptiles of rice, ~11 ng g^{-1} FW in 3-day-old coleoptile

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Table 1. Plant genes thought to be involved in IAA biosynthesis

Gene	Orthologue	Gene product (localization)	Plant species	Reference
(i) IAM pathway				
AMI1	*AtAMI1*	Indole-3-acetamide hydrolase (cytoplasm)	*Arabidopsis thaliana*	Pollmann *et al.* (2003); Neu *et al.* (2007)
	NtAMI1	Indole-3-acetamide hydrolase (cytoplasm)	*Nicotiana tabacum*	Nemoto *et al.* (2009*b*); Mano *et al.* (2010)
(ii) IPA pathway				
TAA1	*TAA1*	Tryptophan aminotransferase (cytoplasm)	*Arabidopsis thaliana*	Stepanova *et al.* (2008); Tao *et al.* (2008)
	TIR2	Tryptophan aminotransferase (?)	*Arabidopsis thaliana*	Yamada *et al.* (2009)
	TAR1	Tryptophan aminotransferase (?)	*Arabidopsis thaliana*	Stepanova *et al.* (2008)
AAO1	*zmAO-1*	Aldehyde oxidase (cytoplasm)	*Zea mays*	Sekimoto *et al.* (1997)
	atAO-1	Aldehyde oxidase (cytoplasm)	*Arabidopsis thaliana*	Sekimoto *et al.* (1998)
YUC	*AtYUC1,2,4,6*	Flavin monooxygenase-like enzyme (?)	*Arabidopsis thaliana*	Mashiguchi *et al.* (2011); Stepanova *et al.* (2011); Won *et al.* (2011)
(iii) TAM pathway				
TDC	*TDC*	Tryptophan decarboxylase (cytoplasm)	*Catharanthus roseus*	De Luca *et al.* (1989)
	TDC	Tryptophan decarboxylase (?)	*Camptotheca acuminata*	López-Meyse and Nessler (1997)
	OpTDC	Tryptophan decarboxylase (?)	*Ophiorrhiza pumila*	Yamazaki *et al.* (2003)
	TDC	Tryptophan decarboxylase (?)	*Oryza sativa*	Ueno *et al.* (2003)
	TDC	Tryptophan decarboxylase (?)	*Oryza sativa*	Kang *et al.* (2007)
YUC (*YUCCA*) ?	*AtYUC1*	Flavin monooxygenase-like enzyme (?)	*Arabidopsis thaliana*	Zhao *et al.* (2001)
	FZY	Flavin monooxygenase-like protein (?)	*Petunia hybrida*	Tobena-Santamaria *et al.* (2002)
	OsYUC1	Flavin monooxygenase-like protein (?)	*Oryza sativa*	Yamamoto *et al.* (2007)
	spi1	YUCCA-like flavin monooxygenase (?)	*Zea mays*	Gallavotti *et al.* (2008)
	SlFZY	No conversion of TAM to *N*-hydroxyTAM (?)	*Solanum lycopersicum*	Tivendale *et al.* (2010)
	ToFZY	YUCCA-like flavin monooxygenase (?)	*Solanum lycopersicum*	Exposito-Rodriguez *et al.* (2011)
	PsYUC-like	No conversion of TAM to *N*-hydroxyTAM (?)	*Pisum sativum*	Tivendale *et al.* (2010)
(iv) IAOX pathway				
CYP79B2/B3	*CYP79B2/B3*	Trp-specific P450 monooxygenase (chloroplast)	*Arabidopsis thaliana*	Hull *et al.* (2000); Mikkelsen *et al.* (2000)
NIT1/2 ?	*AtNIT1, 2*	Nitrilase (membrane)	*Arabidopsis thaliana*	Bartling *et al.* (1992); Bartel and Fink (1994)
	ZmNIT1, 2	Nitrilase (?)	*Zea mays*	Park *et al.* (2003)

tips of maize, and ~1 ng g^{-1} FW in 2-month-old shoot apexes of tobacco (Sugawara *et al.*, 2009).

Indole-3-acetamide hydrolase activity has been detected in rice cells (Kawaguchi *et al.*, 1991; Arai *et al.*, 2004) and in a crude extract from young fruits of *Poncirus trifoliate* (Kawaguchi *et al.*, 1993). Cell-free extracts from various plant species, including *Arabidopsis*, cauliflower, maize, potato, sunflower, tobacco, tomato, and white mustard, can convert Trp into IAM, which is further converted to IAA (Pollmann *et al.*, 2009). These findings indicate that IAM is a native compound and that an IAM-dependent pathway for IAA biosynthesis is generally operative in plants, regardless of whether they are monocots or dicots.

4.4. IAM is incorporated into plant cells and converted to IAA by the AMI1 *gene product*

To examine which compound(s) is a precursor of IAA biosynthesis for cell division, BY-2 cells were cultured in LS solid medium containing Trp, IAM, indole-3-acetonitrile (IAN), and indole-3-acetaldehyde (IAD). These feeding experiments showed that BY-2 cells can grow in LS media containing 10^{-4} M IAM or 10^{-5} M IAA, but cannot grow in Trp-, IAN-, or IAD-containing media (Nemoto *et al.*, 2009*b*). It was confirmed by chemical analysis that the incorporated IAM was converted into IAA in BY-2 cells (K. Nemoto *et al.*, unpublished data). IAN and IAD may not be efficiently incorporated into plant cells and/or may not be converted into IAA in plant cells.

Overexpression of the *NtAMI1* gene allowed BY-2 cells to proliferate at lower concentrations of IAM, whereas RNA interference (RNAi)-mediated suppression of *NtAMI1* severely inhibited plant cell division in IAM-containing medium. These *in vivo* experiments using transgenic plant cells show that IAM is incorporated into plant cells and converted into IAA by the *AMI1* gene product, and also show that IAM itself does not possess auxin activity (Nemoto *et al.*, 2009*b*).

4.5. The AMI1 family is widely distributed in the plant kingdom

By combining a homology search with a phylogenetic tree analysis, it is possible to clarify relationships between sequences and to determine genetic distances. The nucleotide sequence for the *NtAMI1* coding region was submitted to the DDBJ/GenBank/EMBL databases, and DNA alignment was determined by a FASTA search of all sequences, including expressed sequence tags (ESTs) (Mano *et al.*, 2010). Phylogenetic analysis with the nucleotide sequences using ClustalW and the Neighbor–Joining (NJ) method showed that sequences homologous to the *NtAMI1* gene

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can be classified into two major groups, *AMI1* and *Toc64*, and also showed that the *AMI1* gene is widespread in the plant kingdom in both monocots and dicots (Mano *et al.*, 2010).

For this review, the amino acid sequence of the NtAMI1 protein was newly submitted to DDBJ/GenBank/EMBL, and amino acid sequence alignment was determined by a FASTA search of all sequences in the database (Protein Similarity Search of EMBL-EBI). After excluding redundancy caused by the same proteins having different accession numbers, a phylogenetic tree was newly constructed by ClustalW2 and the NJ method using the tools of the EMBL-EBI program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Fig. 2, Table 2). Additionally, *Arabidopsis lyrata* and *Oryza sativa* subsp. *indica* were excluded because they are the allied species of *A. thaliana* and *O. sativa* subsp. *japonica*, respectively. The moss *Physcomitrella patens* was also excluded because moss is not a higher plant.

The phylogenetic tree shows that the AMI1 protein is evolutionarily different from the 'translocon on the outer membrane of chloroplasts of the 64 kDa' (Toc64) protein (Sohrt and Soll, 2000; Chew *et al.*, 2004; Qbadou *et al.*, 2007; Schlegel *et al.*, 2007) (Fig. 2), which is an integral membrane protein of the outer envelope of chloroplasts and mitochondria that consists of an inactive amidase domain and three tetratricopeptide repeat (TPR) motifs at the C-terminal end (Sohrt and Soll, 2000; Lee *et al.*, 2004; Schlegel *et al.*, 2007; Kalanon and McFadden, 2008).

Plants belonging to the AMI1 family were tobacco, tomato, grape, poplar, and *Arabidopsis* in dicots, and maize, sorghum, rice, and wheat in monocots (Fig. 2). The phylogenetic tree also shows that the AMI1 protein is widespread in the plant kingdom (Mano *et al.*, 2010) (Fig. 2). The IAM pathway can be a consensus pathway for auxin biosynthesis.

4.6. How is IAM synthesized in plants?

IAM is present in many plants, as described above. Cell-free extracts from various plant species can convert Trp into IAM, which is further converted to IAA (Pollmann *et al.*, 2009). Bioinformatics studies also suggest that the IAA biosynthetic pathway via IAM is widespread in the plant kingdom (Mano *et al.*, 2010) (Fig. 2).

So, how is IAM synthesized? Two possibilities are (i) that IAM is synthesized via indole-3-glycerol phosphate in the Trp-independent pathway; and (ii) that IAM is synthesized via Trp by the tryptophan-2-monooxygenase encoded by the gene homologous to the bacterial *aux1* gene. The search for sequences homologous to the *aux1* gene, however, has

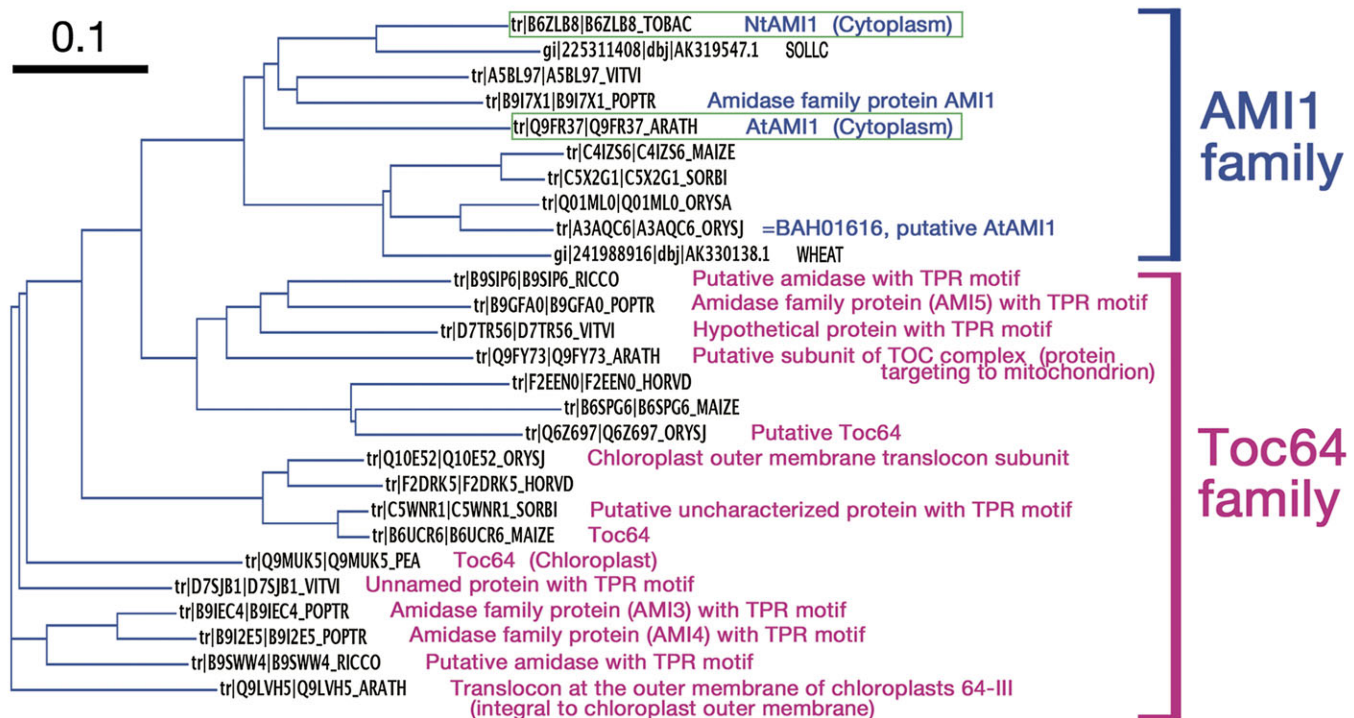

Fig. 2. Phylogenetic analysis of the deduced amino acid sequences of AMI1 proteins. The amino acid sequence of the NtAMI1 protein was submitted to DDBJ/GenBank/EMBL, and an amino acid sequence alignment was determined using a FASTA search of all sequences in the database (Protein Similarity Search of EMBL-EBI). After excluding redundancy caused by the same proteins having different accession numbers, a phylogenetic tree was constructed using ClustalW2 and the NJ method using the tools of the EMBL-EBI program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Additionally, *Arabidopsis lyrata* and *Oryza sativa* subsp. *indica* were excluded because they are the allied species of *Arabidopsis thaliana* and *Oryza sativa* subsp. *japonica*, respectively. The moss *Physcomitrella patens* was excluded because moss is not a higher plant. The scale bar indicates 0.1 substitution per amino acid.

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Table 2. Abbreviations used in the phylogenetic tree

Abbreviation in EMBL-EBI[a]	Scientific name	Common name	Abbreviation in EMBL-EBI[a]	Scientific name	Common name
AEGTA	*Aegilops tauschii*	Tausch's goatgrass	9LILI	*Triglochin maritima*	Seaside arrowgrass
ALLCE	*Allium cepa*	Onion	LOTJA	*Lotus japonicus*	Miyakogusa
ALLCG	*Allium cepa* var. *aggregatum*	Shallot	MAIZE	*Zea mays*	Maize
ALLSA	*Allium sativum*	Garlic	MANES	*Manihot esculenta*	Cassava
ALLTU	*Allium tuberosum*	Garlic chives	ORYSA	*Oryza sativa*	Rice
ARAHY	*Arachis hypogaea*	Peanut	ORYSJ	*Oryza sativa* subsp. *japonica*	Rice
ARATH	*Arabidopsis thaliana*	Mouse-ear cress	PAPSO	*Papaver somniferum*	Opium poppy
BRACM	*Brassica campestris*	Field mustard	PEA	*Pisum sativum*	Garden pea
BRANA	*Brassica napus*	Rape	PETCR	*Petroselinum crispum*	Parsley
BRAOA	*Brassica oleracea* var. *alboglabra*	Chinese kale	PETHY	*Petunia hybrida*	Petunia
BRAOB	*Brassica oleracea* var. *botrytis*	Cauliflower	POPTR	*Populus trichocarpa*	Western balsam poplar
BRAOL	*Brassica oleracea*	Wild cabbage	RAUVE	*Rauvolfia verticillata*	Common devil-pepper
BRARC	*Brassica rapa* subsp. *chinensis*	Pak-choi	RICCO	*Ricinus communis*	Castor bean
BRARP	*Brassica rapa* subsp. *pekinensis*	Chinese cabbage	ROSDA	*Rosa damascena*	Damask rose
CAMAC	*Camptotheca acuminata*	Happy tree	ROSHC	*Rosa hybrid cultivar*	
CAPAN	*Capsicum annuum*	Bell pepper	SINAL	*Sinapis alba*	White mustard
CATRO	*Catharanthus roseus*	Madagascar periwinkle	SOLLC	*Solanum lycopersicum*	Tomato
CIMRA	*Cimicifuga racemosa*	Black cohosh	SORBI	*Sorghum bicolor*	Sorghum
CITSI	*Citrus sinensis*	Sweet orange	SOYBN	*Glycine max*	Soybean
9GENT	*Ophiorrhiza prostrata*		THEHA	*Thellungiella halophila*	Salt cress
HORSP	*Hordeum spontaneum*	Wild Barley	THLFG	*Thalictrum flavum* subsp. *glaucum*	Yellow meadow rue
HORVD	*Hordeum vulgare* var. *distichum*	Two-rowed barley	TOBAC	*Nicotiana tabacum*	Common tobacco
HORVU	*Hordeum vulgare*	Barley	VITVI	*Vitis vinifera*	Grape
LACSA	*Lactuca sativa*	Garden lettuce	WHEAT	*Triticum aestivum*	Wheat

[a] The red letters show monocotyledonous plants and the black letters show dicotyledonous plants.

not found a candidate. The plant indole-3-acetamide hydrolase group is phylogenetically distantly related to the bacterial indole-3-acetamide hydrolase group (Mano *et al.*, 2010). In the case of tryptophan-2-monooxygenase, the plant enzyme may be too divergent from the bacterial counterparts to be recognizable, based on the homology search. It would be desirable to isolate genes related to IAM synthesis in plants, the gene encoding tryptophan-2-monooxygenase, or novel genes in a Trp-independent pathway.

5. The indole-3-pyruvic acid pathway

5.1. The TAA1 *gene encodes an alliinase-related protein with Trp aminotransferase activity functioning in the cytoplasm*

The *TAA1* (*TRYPTOPHAN AMINOTRANSFERASE of ARABIDOPSIS 1*) gene, which encodes an aminotransferase that converts Trp to IPA (Fig. 1), was isolated by two groups based on the characterization of mutants that were defective in shade avoidance (Tao *et al.*, 2008) and in ethylene responses (Stepanova *et al.*, 2008) (Table 1). Mutations in *TAA1* alone lead to a dramatic reduction in free IAA levels, suggesting that IPA-dependent IAA biosynthesis is an important pathway for the biosynthesis of free IAA. Within 1 h after transferring *Arabidopsis* seedlings from white light to shade, the levels of free IAA increased in the wild type due to an increase in the rate of IAA biosynthesis; in contrast, IAA levels were reduced in mutant seedlings lacking the TAA1 protein, and there was no significant change in IAA levels in response to shade (Tao *et al.*, 2008). These findings indicate that *TAA1* is required for the rapid increase in auxin levels through *de novo* IAA biosynthesis upon exposure to shade.

Overexpression of *TAA1* under the control of the cauliflower mosaic virus (CaMV) 35S promoter does not cause auxin overproduction phenotypes and also does not enhance hypocotyl elongation in the shade, suggesting that TAA1 is unlikely to be a rate-limiting enzyme in auxin biosynthesis (Stepanova *et al.*, 2008; Tao *et al.*, 2008). The TAA1 protein was confirmed to be located in the cytoplasm of plant cells by experiments using transgenic lines overexpressing TAA1–yellow fluorescent protein (YFP) and TAA1–GFP fusion proteins (Stepanova *et al.*, 2008; Tao *et al.*, 2008).

5.2. The TIR2 *gene is identical to the* TAA1 *gene*

Molecular characterization revealed that the *TIR2* (*TRANSPORT INHIBITOR RESPONSE 2*) gene was identical to the *TAA1* gene (Yamada *et al.*, 2009) (Fig. 1, Table 1). The *tir2* mutant, which is resistant to the auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA), has a short hypocotyl. This phenotype can be rescued by IPA and IAA, suggesting that *TIR2* may be involved in the auxin biosynthetic pathway. Overexpression of *TIR2* does

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not result in growth defects and the plants display normal sensitivity to exogenous Trp, suggesting that increasing endogenous IPA levels does not result in the synthesis of more IAA (Yamada *et al.*, 2009).

5.3. Trp aminotransferase activity of TAA1 protein in vitro

TAA1 protein, which belongs to the superfamily of the α class of pyridoxal-5'-phosphate (PLP)-dependent enzymes, possesses Trp aminotransferase activity that is stimulated by the presence of PLP in the reaction mixture. Examination of substrate specificity showed that the purified TAA1 protein uses L-Trp, but not D-Trp, as a substrate and also uses L-Phe, Tyr, Leu, Ala, Met, and Gln as substrates. *In vitro* production of IPA by the TAA1 protein was confirmed using liquid chromatography–mass spectrometry (LC/MS) (Tao *et al.*, 2008). Purified recombinant glutathione *S*-transferase (GST)–TAA1 protein also showed aminotransferase activity, and IPA is confirmed as the main product of the GST–TAA1 enzymatic activity by HPLC–MS ion chromatogram and ion mass spectrum (Stepanova *et al.*, 2008).

5.4. Four genes closely related to TAA1 in the Arabidopsis genome

The genes which are closely related to *TAA1* in the *Arabidopsis* genome have been found and referred to as '*TRYPTOPHAN AMINOTRANSFERASE RELATED 1 to 4*' (*TAR1–TAR4*). The *TAR1* gene is expressed in seedlings at very low levels (~500 times lower than *TAA1*). A whole-gene translational fusion of *TAR1* with *GFP* failed to reveal GFP fluorescence in etiolated transgenic seedlings (Stepanova *et al.*, 2008).

5.5. Protein structures of TAA/TIR2 and TAR1–TAR4

The *TAA1/TIR2* gene is one of a five-member gene family in *Arabidopsis*: *TAA1* and *TAR1–TAR4* genes (Stepanova *et al.*, 2008). TAA1/TIR2 protein does not contain the N-terminal extension, consistent with the finding that the TAA1 protein is localized to the cytoplasm (Stepanova *et al.*, 2008; Tao *et al.*, 2008). TAR1 protein also does not contain the N-terminal extension (Stepanova *et al.*, 2008).

On the other hand, TAR2–TAR4 proteins contain an N-terminal extension, predicted to be a signal peptide, suggesting that these proteins may function in the vacuole, similar to the onion or garlic alliinases (Stepanova *et al.*, 2008). These findings show that the function of TAR2–TAR4 proteins is different from that of TAA1/TIR2 protein.

5.6. TAA1/TIR2 has only a small family in the plant kingdom

A phylogenetic tree was constructed by ClustalW2 and the NJ method using the tools of the EMBL-EBI program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) by submitting the amino acid sequence of TAA1 protein, as described in the previous section. The phylogenetic tree shows that TAA1/TIR2 has only a small family in the plant kingdom (Fig. 3, Table 2). TAR1 (accession number Q9LR29) may be a paralogue of TAA1/TIR2. The TAA1/TIR2 protein (391 amino acids), having Trp aminotransferase activity (EC 2.6.1.27), is different from an alliin lyase (EC 4.4.1.4), such as TAR2 (accession number Q94A02, 440 amino acids). Interestingly, *Thellungiella halophila* (salt cress; accession number E4MXR3) is a close relative of *A. thaliana*; analysis of ESTs reveals 90–95% nucleotide identity between *Arabidopsis* and *Thellungiella* in transcripts for well-known housekeeping genes (Zhu, 2001; Taji *et al.*, 2010). Thus, *TAA1/TIR2* may be an *Arabidopsis*-specific gene.

The *vanishing tassel2* (*vt2*) gene has been isolated from maize, but the enzymatic activity of the *vt2* gene product is unknown (Phillips *et al.*, 2011). Although these authors suggested that *vt2* encodes a co-orthologue of the *TAA1/TAR1/TAR2* genes of *Arabidopsis*, based on positional cloning and phylogenetic analyses, the *vt2* gene product (accession number F2FB37, 530 amino acids) contains an N-terminal signal peptide, like the TAR2–TAR4 proteins that function in the vacuole. Accession number F2FB37 was not included in the best-scoring candidates by a FASTA search submitting TAA1 amino acid sequence; thus, the vt2 protein appears different from TAA1/TIR2.

5.7. It is not known whether IPA is converted into IAD in plants

In bacteria, two pathways for IAA biosynthesis are widespread: the IAM pathway (Trp→IAM→IAA) and the IPA pathway (Trp→IPA→IAD→IAA). Most phytopathogens, such as *A. rhizogenes*, *A. tumefaciens*, and *P. syringae* pv. *savastonoi*, use the IAM pathway to synthesize IAA, as described above (Schroder *et al.*, 1984; Thomashow *et al.*, 1984; Yamada *et al.*, 1985; Gaudin and Jouanin, 1995; Casanova *et al.*, 2005), whereas the IPA pathway is found in plant growth-promoting rhizobacteria species, including *Azospirillum brasilense*, *Enterobacter cloacae*, and *Pseudomonas putida* (Koga *et al.*, 1991; Costacurta *et al.*, 1994; Patten and Glick, 2002).

In the bacterial IPA pathway, the precursor Trp is converted to IPA by Trp aminotransferase, and IPA is then converted to indole-3-acetaldehyde (IAD) by indole-3-pyruvate decarboxylase. IAA is produced after oxidation of IAD by indole-3-acetaldehyde oxidase. However, it is unknown whether IPA is converted into IAD in plants, as neither the gene nor the enzyme has yet been isolated (Fig. 1). Is the IPA pathway widespread in the plant kingdom?

There are few reports concerning the metabolic intermediates of the IPA pathway. IPA has been only detected in *Arabidopsis* seedlings; levels varied from 4 ng g^{-1} to 13 ng g^{-1} (Tam and Normanly, 1998). IAD has only been detected in pea (*Pisum sativum*); ~20 ng g^{-1} FW in roots, 4 ng g^{-1} FW in the apical bud, 1.2 ng g^{-1} FW in the leaf, and 0.7 ng g^{-1} FW in the internode (Quittenden *et al.*, 2009).

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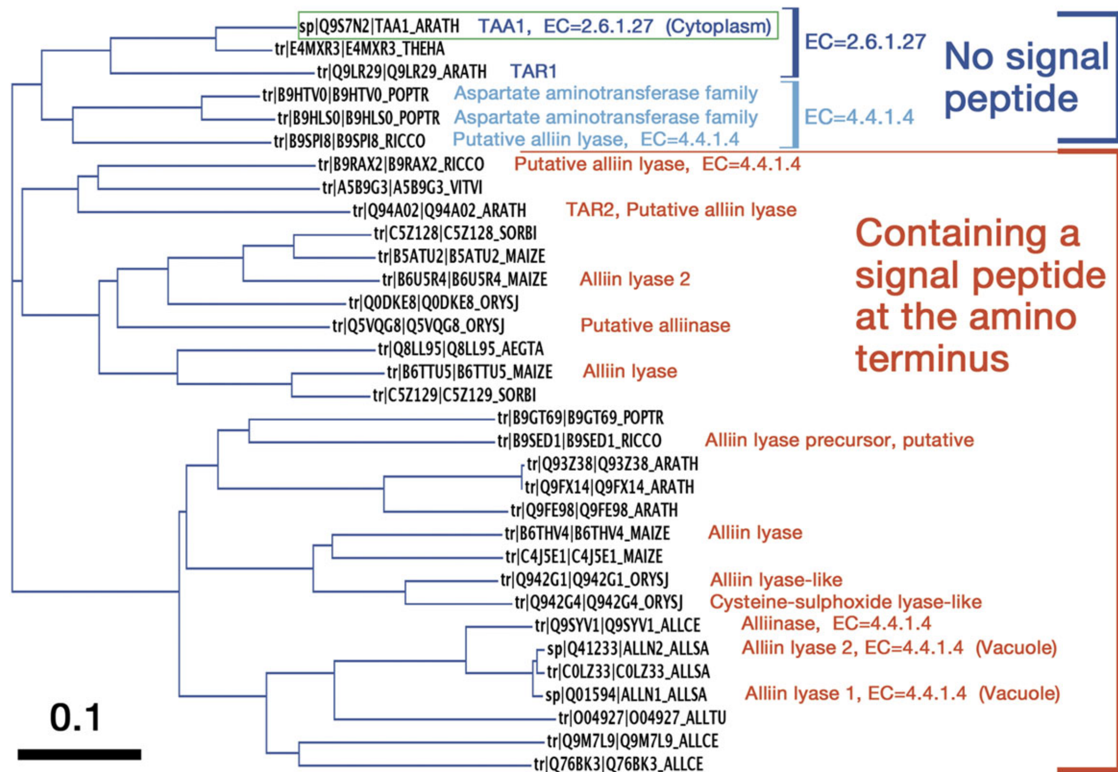

Fig. 3. Phylogenetic analysis of the deduced amino acid sequences of TAA1 proteins. The amino acid sequence of the *Arabidopsis* TAA1 protein was submitted to DDBJ/GenBank/EMBL, and a phylogenetic tree was constructed, as described in the legend of Fig. 2. The scale bar indicates 0.1 substitution per amino acid.

5.8. The aldehyde oxidases have a broad substrate specificity

Aldehyde oxidases have been identified in cucumber (Bower *et al.*, 1978), maize (Koshiba and Matsuyama, 1993; Koshiba *et al.*, 1996), and *Arabidopsis* (Seo *et al.*, 1998). Higher activity of an aldehyde oxidase has also been measured in the auxin-overproducing *superroot1* (*sur1*) mutant of *A. thaliana*. *Arabidopsis* has three isoforms (AO1–AO3), and one of them (AO1) shows a higher substrate preference for indole-3-aldehyde and abscisic aldehyde (Seo *et al.*, 1998). The indole-3-acetaldehyde oxidase activity has also been detected in the AO1 sample (Seo *et al.*, 1998).

Generally, aldehyde oxidases are characterized by broad substrate specificity and play an important role in many developmental processes as well as in a variety of abiotic and biotic stress responses (Mendel and Hansch, 2002; Mauch-Mani and Mauch, 2005; Mendel and Bittner, 2006; Verslues and Bray, 2006; Garattini *et al.*, 2008). It seems likely that plant aldehyde oxidases are also involved in several metabolic reactions as well as the conversions of abscisic aldehyde to abscisic acid, indole-3-acetaldehyde to IAA, and benzaldehyde to benzoic acid, as detected in maize aldehyde oxidase (Koshiba *et al.*, 1996).

Corresponding cDNAs have been isolated from maize (*zmAO-1* and *zmAO-2*) and *Arabidopsis* (*atAO-1*, *-2*, *-3*, and *-4*) (Sekimoto *et al.*, 1997, 1998) (Table 1). It has been suggested that the product of the *AAO3* gene (formerly called *atAO-3*) is abscisic aldehyde oxidase (Seo *et al.*, 2000*a*, *b*). Three cDNAs encoding aldehyde oxidase proteins in *P. sativum* (*PsAO1*, *PsAO2*, and *PsAO3*) have been isolated based on an RT-PCR (reverse transcription-polymerase chain reaction) strategy, although the enzymatic activity of these gene products is unknown (Zdunek-Zastocka, 2008).

Abscisic aldehyde is the native precursor of the plant hormone abscisic acid, which is involved in many aspects of plant growth and development, including adaptation to a variety of environmental stresses (Mauch-Mani and Mauch, 2005; Mendel and Bittner, 2006 Verslues and Bray, 2006). Aldehyde oxidases in plants are essential for many physiological processes that require the involvement of abscisic acid and perhaps also of auxins. It is necessary to determine which gene encodes indole-3-acetaldehyde oxidase.

5.9. The aldehyde oxidase family

Aldehyde oxidases (EC 1.2.3.1) are a group of structurally conserved cytosolic proteins, represented in both the animal and plant kingdoms, and they constitute a subfamily of molybdo-flavoenzymes. These enzymes require a molybdopterin cofactor and flavin adenine dinucleotide for catalytic activity, and they catalyse the hydroxylation of N-heterocycles and the oxidation of aldehydes to the corresponding acid (Mendel, 2007, 2011).

A phylogenetic tree shows that plant aldehyde oxidases belong to a multigene family (Fig. 4, Table 2). These

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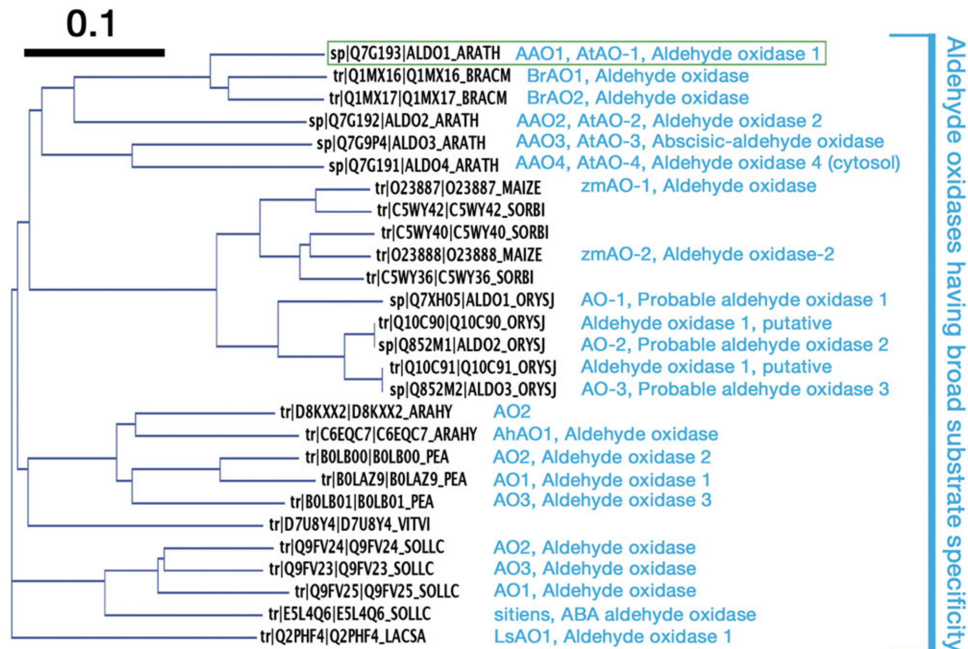

Fig. 4. Phylogenetic analysis of the deduced amino acid sequences of AAO1 proteins. The amino acid sequence of the *Arabidopsis* AAO1 protein was submitted to DDBJ/GenBank/EMBL, and a phylogenetic tree was constructed, as described in the legend of Fig. 2. The scale bar indicates 0.1 substitution per amino acid.

enzymes may possess a broad substrate specificity; thus, it is not easy to estimate which group(s) may be involved in the conversion of IAD to IAA by analysing relationships between sequences.

6. The tryptamine pathway

6.1. The TDC *gene encodes Trp decarboxylase which is widespread in the plant kingdom*

Tryptophan decarboxylase (TDC; EC 4.1.1.28) is a cytosolic enzyme that converts Trp to TAM (Fig. 1), which is a protoalkaloid in an early step of the terpenoid indole alkaloid biosynthetic pathway (Di Fiore *et al.*, 2002). TDC is well characterized at the molecular and biochemical level, and *TDC* genes have been isolated from *Catharanthus roseus* (De Luca *et al.*, 1989), *Camptotheca acuminata* (Lopez-Meyer and Nessler, 1997), *Ophiorrhiza pumila* (Yamazaki *et al.*, 2003), and rice (Ueno *et al.*, 2003; Kang *et al.*, 2007) (Table 1).

A phylogenetic tree was constructed by ClustalW2 and the NJ method using the tools of the EMBL-EBI program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) by submitting the amino acid sequence of TDC protein of *C. roseus*, as described above (Fig. 5, Table 2). Plants belonging to the TDC group were Apocynaceae (*C. roseus* and *Rauvolfia verticillata*), Solanaceae (*Capsicum annuum*), Cornaceae (*Camptotheca acuminata*), Rubiaceae (*Ophiorrhiza pumila* and *Ophiorrhiza prostrata*), and Ranunculaceae (*Cimicifuga racemosa*) in dicots, and rice, wheat, and barley in monocots. The phylogenetic tree shows that the TDC protein is widespread in the plant kingdom (Fig. 5).

6.2. *Tryptamine is regarded as the intermediate in indole alkaloid and serotonin biosynthesis*

These *TDC* genes have been functionally characterized to participate in indole alkaloid and serotonin biosynthesis (Fig. 1). Transgenic tobacco plants overexpressing the *TDC* gene of *C. roseus* accumulated very high levels of TAM, whereas IAA levels were unaffected (Songstad *et al.*, 1990). Transgenic rice plants overexpressing the *TDC* gene showed a normal phenotype and contained 25-fold and 11-fold higher serotonin in the leaves and seeds, respectively, than did wild-type plants (Kang *et al.*, 2007).

TDC activity in rice plants may be implicated in the production of TAM-derived metabolites, resulting in sekiguchi lesions or serotonin derivatives. Rice sekiguchi lesion (*sl*) mutants accumulate TAM, a candidate substrate for serotonin biosynthesis. Serotonin (5-hydroxytryptamine) is a well-known neurotransmitter in mammals and is widely distributed in plants. The product of the *SL* gene, isolated by map-based cloning, was identified as tryptamine 5-hydroxylase for serotonin biosynthesis and designated CYP71P1 in the cytochrome P450 monooxygenase family (Fujiwara *et al.*, 2010) (Fig. 1). It is thought that TDC hardly participates in IAA biosynthesis (Facchini *et al.*, 2000).

6.3. *The* YUCCA *gene family*

It has also been proposed that the TAM pathway is one of the IAA biosynthetic pathways (Fig. 1). The *YUCCA* gene, which encodes a flavin monooxygenase-like enzyme that appears to oxidize TAM to *N*-hydroxytryptamine, has been isolated from *A. thaliana* (Zhao *et al.*, 2001). Orthologous genes of *YUCCA* have been found in other plants, including

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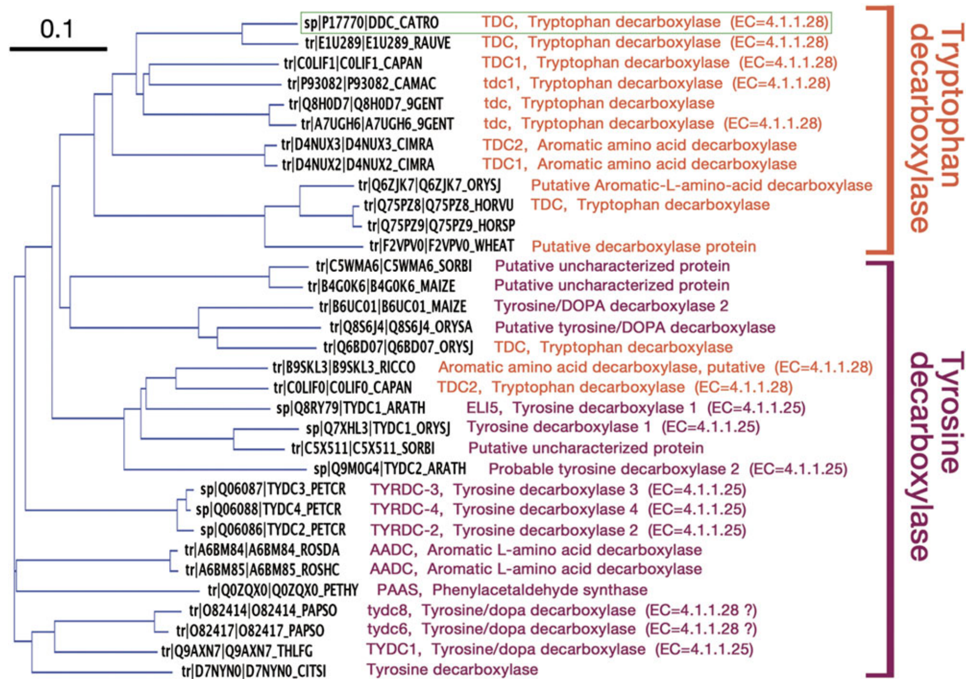

Fig. 5. Phylogenetic analysis of the deduced amino acid sequences of TDC proteins. The amino acid sequence of the *Catharanthus roseus* TDC protein was submitted to DDBJ/GenBank/EMBL, and a phylogenetic tree was constructed, as described in the legend of Fig. 2. The scale bar indicates 0.1 substitution per amino acid.

petunia (*FZY*) (Tobena-Santamaria *et al.*, 2002), rice (*OsYUCCA1–OsYUCCA7*) (Yamamoto *et al.*, 2007), maize (*spi1*) (Gallavotti *et al.*, 2008), pea (*PsYUC*-like) (Tivendale *et al.*, 2010), and tomato (*ToFZY*) (Exposito-Rodriguez *et al.*, 2011) (Table 1).

A phylogenetic tree shows that YUCCA proteins belong to a multigene family (Fig. 6, Table 2). YUC1 is a member of an *Arabidopsis* flavin monooxygenase-like protein clade that includes 11 members (YUC1–YUC11), a subset of which appears to have overlapping functions (Zhao *et al.*, 2001; Cheng *et al.*, 2006, 2007). The enzymes *Arabidopsis* AtYUC1 and AtYUC6, tomato ToFZY1, and maize ZmYUC have been reported to catalyse the conversion of TAM to *N*-hydroxytryptamine (Zhao *et al.*, 2001; LeClere *et al.*, 2010; Exposito-Rodriguez *et al.*, 2011).

6.4. A phantom compound: N-hydroxytryptamine

Transgenic rice plants overexpressing *OsYUCCA1* (=*OsYUC1*) exhibit increased IAA levels and characteristic auxin overproduction phenotypes, whereas plants expressing antisense *OsYUC1* cDNA display defects that are similar to those of rice auxin-insensitive mutants (Yamamoto *et al.*, 2007). Also, *FZY* overexpression results in increased IAA levels and in an auxin overproduction phenotype in transgenic petunia (Tobena-Santamaria *et al.*, 2002). Based on these results, it has been suggested that the TAM pathway to IAA is widespread in both dicots and monocots.

On the other hand, it has recently been reported that YUC (YUCCA) may not catalyse the conversion of TAM to *N*-hydroxytryptamine (Tivendale *et al.*, 2010; Ross *et al.*, 2011). Tivendale *et al.* indicated that there were major inconsistencies between the mass spectra reported for the reaction products, and indicated that the conclusions of Zhao *et al.* (2001), which were based on *in vitro* assays followed by mass spectrometry or HPLC analyses, need to be confirmed (Tivendale *et al.*, 2010). They also point out that Zhao *et al.* made no firmer statement than that their mass spectra were consistent with *N*-hydroxytryptamine.

The compound *N*-hydroxytryptamine is relatively unknown, with no report of its presence in plants to date. Based on the mass spectral data for authentic *N*-hydroxytryptamine, 5-hydroxytryptamine (serotonin), and TAM, Tivendale *et al.* (2010) concluded that at least some of the published mass spectral data for the YUC *in vitro* product are inconsistent with *N*-hydroxytryptamine and that there is now a significant possibility that the product obtained by Zhao *et al.* was not *N*-hydroxytryptamine (Tivendale *et al.*, 2010). Zhao (2010) also has noted that further investigations are needed to determine whether TAM is the *in vivo* substrate for YUCs because flavin monooxygenases are known to have broad substrate specificities *in vitro*.

6.5. Re-examining the function of YUCCA genes

It has been thought that the function of *YUCCA* genes should be re-examined, because there are some inconsistencies in the data. TAM is present in barley (*Hordeum vulgare*) (Schneider and Wightman, 1974), tomato (*Solanum lycopersicum*) (Cooney and Nonhebel, 1991), rice (*O. sativa*) (Ishihara *et al.*, 2008), pea (*P. sativum*) (Quittenden *et al.*, 2009), and *Arabidopsis* (Sugawara *et al.*, 2009). However, *N*-hydroxytryptamine was not detected in pea roots, and ^{14}C-labelled TAM, incorporated rapidly in pea roots, was

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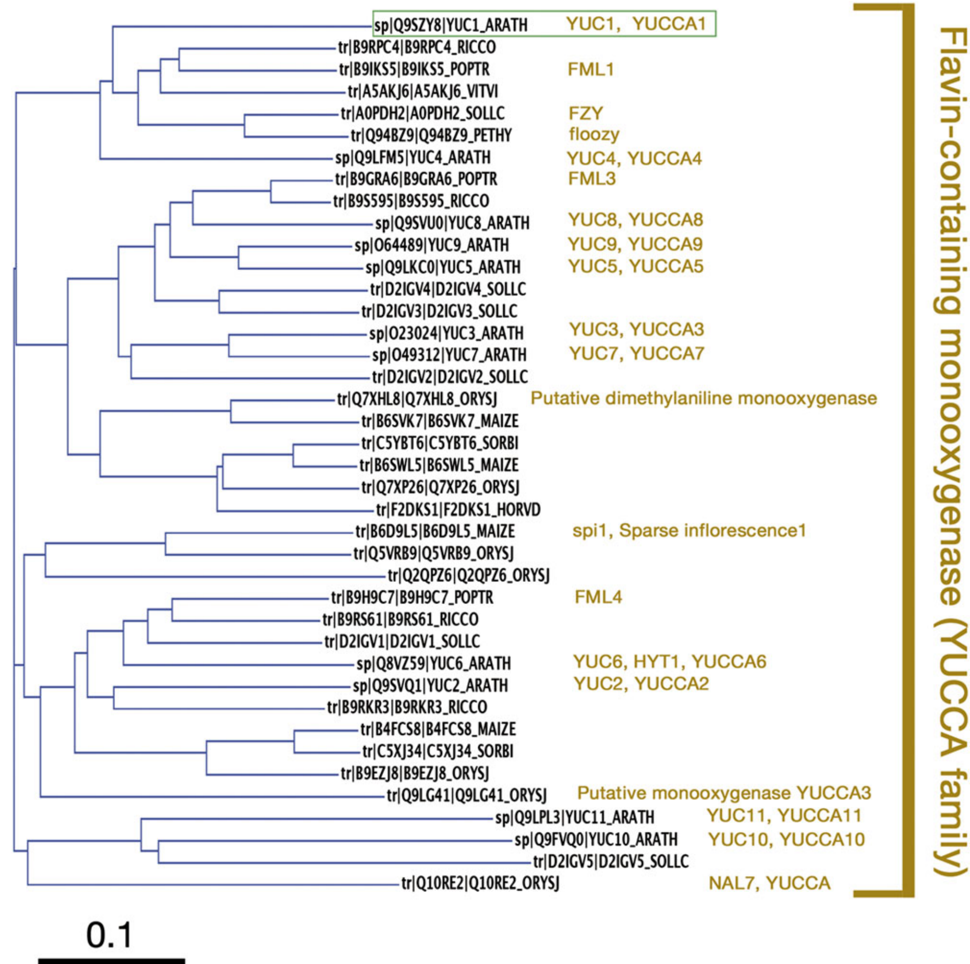

Fig. 6. Phylogenetic analysis of the deduced amino acid sequences of YUC1 proteins. The amino acid sequence of the *Arabidopsis* YUC1 protein was submitted to DDBJ/GenBank/EMBL, and a phylogenetic tree was constructed, as described in the legend of Fig. 2. The scale bar indicates 0.1 substitution per amino acid.

not converted to [^{14}C]*N*-hydroxytryptamine (Quittenden *et al.*, 2009). ^{14}C-labelled TAM was converted to [^{14}C]*N*-acetyltryptamine in pea roots, which is not involved in IAA biosynthesis (Quittenden *et al.*, 2009). TAM is not metabolized to IAA in pea seeds, although a *PsYUC*-like gene is strongly expressed in these organs (Tivendale *et al.*, 2010). Can YUCCA convert TAM to *N*-hydroxytryptamine?

Based on the data showing that the developmental defects of *yuc1yuc4* and *yuc1yuc2yuc6* in *Arabidopsis* were rescued by tissue-specific expression of the bacterial auxin biosynthesis gene *iaaM* but not by application of exogenous auxin, Cheng *et al.* (2006) have suggested that spatially and temporally regulated auxin biosynthesis by the *YUC* genes is essential for the formation of floral organs and vascular tissues. Note that the bacterial *iaaM* gene, which is the same as the *aux1/tms1* gene, encodes the enzyme tryptophan-2-monooxygenase, which converts Trp to IAM (Huffman *et al.*, 1984; Thomashow *et al.*, 1984; Offringa *et al.*, 1986; Nemoto *et al.*, 2009*a*; Mano *et al.*, 2010) but does not convert TAM to *N*-hydroxytryptamine. The substance synthesized by the *iaaM* gene product is IAM; thus, the interpretation by Cheng *et al.* (2006) that loss-of-function *yuc* mutants can be rescued by the *iaaM* gene does not seem to make sense.

Combining these findings, there is currently insufficient evidence to consider *N*-hydroxytryptamine as an intermediate in IAA biosynthesis, and the YUCCA function and the TAM pathway to IAA remain poorly understood.

7. The indole-3-acetaldoxime pathway

7.1. Cytochrome P450 enzymes CYP79B2 and CYP79B3 convert Trp to indole-3-acetaldoxime

IAOX is synthesized from Trp by two homologous cytochrome P450 enzymes, CYP79B2 and CYP79B3 (Fig. 1), which contain a chloroplast transit peptide at the N-terminus. Both enzymes are predicted to be targeted to the chloroplast (Hull and Celenza, 2000; Hull *et al.*, 2000; Mikkelsen *et al.*, 2000). These enzymes for the formation of IAOX have only been conclusively demonstrated in *Arabidopsis* (Hull *et al.*, 2000; Mikkelsen *et al.*, 2000) and *Brassica* (Kindl, 1968; Ludwig-Müller and Hilgenberg, 1988).

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7.2. The CYP79B family is restricted to only Brassicaceae species

Two *Arabidopsis* genes, *CYP79B2* and *CYP79B3*, have been isolated and characterized (Hull and Celenza, 2000; Hull *et al.*, 2000; Mikkelsen *et al.*, 2000) (Table 1). The *CYP79B* gene family has only been identified in *Arabidopsis*, *Brassica napus*, and *Sinapis alba* (Bak *et al.*, 1998). A phylogenetic tree shows that the CYP79B protein family (= Trp-specific P450 enzymes) is also restricted to only Brassicaceae species (Fig. 7, Table 2), indicating that IAOX-dependent IAA biosynthesis is not a common pathway in plants.

7.3. IAOX is found only in the Brassicaceae

IAOX was first isolated from *Brassica oleracea* (Kindl, 1968) and identified by mass spectrometry from extracts of *Brassica campestris* (Ludwig-Müller and Hilgenberg, 1988), and has been recently found in *Arabidopsis* (Sugawara *et al.*, 2009). The level of IAOX in *Arabidopsis* plants was ~1.7 ng g^{-1} FW by liquid chromatography–electrospray ionization-mass/mass spectrometry (LC-ESI-MS/MS) analysis.

However, IAOX has not been found in tomato (Cooney and Nonhebel, 1991) or pea (Quittenden *et al.*, 2009). Similarly, IAOX has not been detected in rice, maize, or tobacco (Sugawara *et al.*, 2009). Although tobacco plants do not have detectable levels of IAOX (limit of detection ~10 pg g^{-1} FW), transgenic tobacco plants expressing either the *CYP79B2* or *CYP79B3* genes from *Arabidopsis* under the CaMV 35S promoter accumulate a large amount of IAOX in the range of 1–9 mg g^{-1} FW (Nonhebel *et al.*, 2011). This biochemical evidence that IAOX is not found in plants other than Brassicaceae also indicates that IAOX-dependent IAA biosynthesis is not a common but rather a species-specific pathway in plants (Sugawara *et al.*, 2009).

7.4. IAOX is a metabolic intermediate in indole glucosinolate and camalexin biosynthesis

IAOX is well known as an intermediate in the synthesis of plant secondary metabolites, such as indole glucosinolates, the alkaloid camalexin, and IAN, in Brassicaceae species including *Arabidopsis* (Hansen and Halkier, 2005; de Vos *et al.*, 2008; Mikkelsen *et al.*, 2009) (Fig. 1). The level of IAOX in wild-type *Arabidopsis* is several orders of magnitude lower than the amounts of the end-products camalexin or indole glucosinolates, as well as IAN. It is also significantly lower than that of IAM and IAA in *Arabidopsis* (Sugawara *et al.*, 2009).

IAOX is channelled into indole glucosinolate biosynthesis by the cytochrome P450 CYP83B1 (Barlier *et al.*, 2000; Bak *et al.*, 2001) and into camalexin biosynthesis by the cytochrome P450 CYP71A13 (Nafisi *et al.*, 2007; Mikkelsen *et al.*, 2009) (Fig. 1). The cytochrome P450 CYP71B15 is essential for the final step in camalexin biosynthesis (Schuhegger *et al.*, 2006; Bottcher *et al.*, 2009). Camalexin is a characteristic phytoalexin of *Arabidopsis* and is synthesized only under inducing conditions such as infection by plant pathogens. Thus, IAOX is presumed to be funnelled into IAN and camalexin under inducing conditions (Glawischnig, 2007) (Fig. 1).

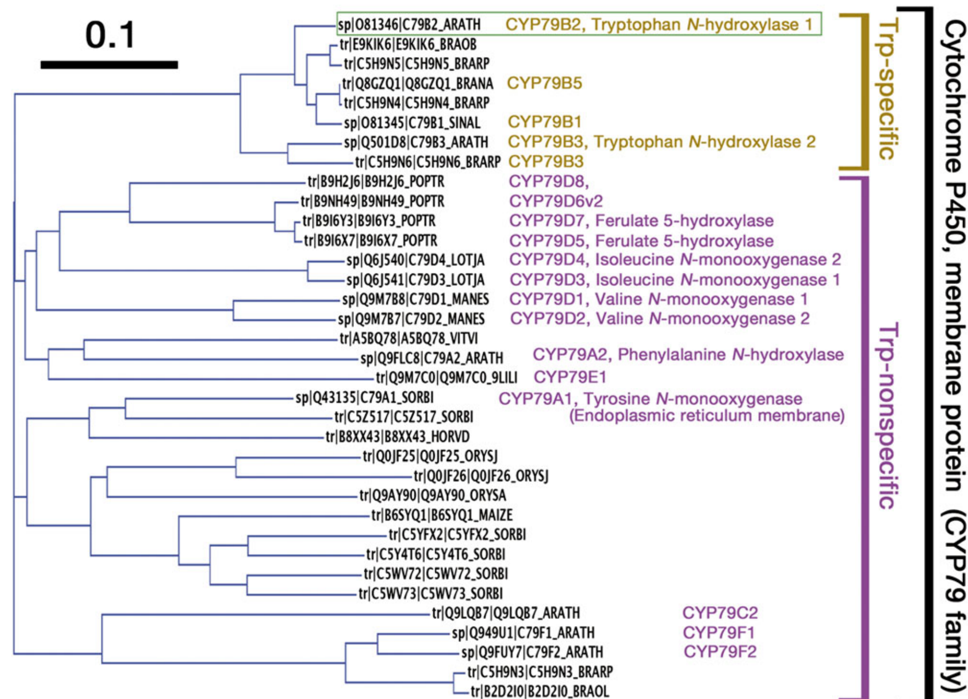

Fig. 7. Phylogenetic analysis of the deduced amino acid sequences of CYP79B2 proteins. The amino acid sequence of the *Arabidopsis* CYP79B2 protein was submitted to DDBJ/GenBank/EMBL, and a phylogenetic tree was constructed, as described in the legend of Fig. 2. The scale bar indicates 0.1 substitution per amino acid.

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7.5. CYP79B is involved primarily in indole glucosinolate and camalexin biosynthesis

CYP79B2 is induced in response to infection by pathogens (Hull *et al.*, 2000), is wound inducible, and is expressed in leaves, stem, flowers, and roots, with the highest expression in roots (Mikkelsen *et al.*, 2000). The *CYP79B2* expression is adapted for camalexin formation, while the main function of *CYP79B3* is in indole glucosinolate biosynthesis (Glawischnig, 2007). Double knock-out *Arabidopsis* mutants of *CYP79B2* and *CYP79B3* genes do not have any detectable indole glucosinolates or camalexin, and also have drastically reduced levels of IAN (Zhao *et al.*, 2002; Glawischnig *et al.*, 2004), whereas the IAA content of the mutants is almost the same as in the wild-type strain under normal conditions (Zhao *et al.*, 2002). On the other hand, the transgenic *Arabidopsis* lines overexpressing the *CYP79B2* gene have significantly elevated levels of indole glucosinolates and IAN (Mikkelsen *et al.*, 2000; Zhao *et al.*, 2002), although the overexpressors show a normal content of IAA (Zhao *et al.*, 2002). These findings indicate that CYP79B is involved in indole glucosinolate and camalexin biosynthesis (Mikkelsen *et al.*, 2000).

7.6. IAN is directly formed by dehydration of IAOX catalysed by CYP71A13

IAN has also been proposed as an intermediate in IAA biosynthesis via indole glucosinolate metabolism. It was thought that IAN was an enzymatic breakdown product of indole glucosinolate, induced upon tissue damage (Halkier and Gershenzon, 2006). However, Nafisi *et al.* (2007) demonstrated that (i) CYP71A13 enzyme converted IAOX to IAN *in vitro*; (ii) exogenously supplied IAN restored camalexin production in *Arabidopsis* CYP71A13-defective mutants; and (iii) expression of *CYP79B2* and *CYP71A13* genes in *Nicotiana benthamiana*, which does not normally produce IAOX or IAN, resulted in the conversion of Trp to IAN. Based on these results, the authors concluded that CYP71A13 catalyses the conversion of IAOX to IAN in camalexin biosynthesis in Brassicaceae species (Nafisi *et al.*, 2007). IAN is subsequently converted to camalexin by the cytochrome P450 CYP71B15 in Brassicaceae species (Bottcher *et al.*, 2009) (Fig. 1). Thus, the IAA biosynthesis pathway via IAOX and IAN is somewhat unclear even in the Brassicaceae species.

7.7. IAOX and IAN in the plant kingdom: 'To be or not to be: that is the question.'

As described above, IAOX is not present in plants other than indole glucosinolate-producing plants such as Brassicaceae species, indicating that IAOX-dependent IAA biosynthesis is not a common pathway in plants. Although the exact mechanism(s) by which IAOX is converted to IAA remains unclear, IAN and/or IAD have been suggested as potential intermediates for IAA biosynthesis (Barlier *et al.*, 2000; Bak *et al.*, 2001). Based on early reports of the cell-free conversion of IAOX to IAA via IAD in extracts of *Brassica* (Mahadevan, 1963; Rajagopal and Larsen, 1972; Helmlinger *et al.*, 1987), IAOX has long been a subject of interest as a possible precursor of IAD. However, no enzyme or gene has yet been identified in the IAOX→IAD pathway. Thus, the IAOX→IAD pathway has been left out of Fig. 1.

IAN has not been detected in rice, maize, tobacco, or pea (Quittenden *et al.*, 2009; Sugawara *et al.*, 2009), although it is present at the level of 9720 ng g^{-1} FW in *Arabidopsis* seedling extracts (Sugawara *et al.*, 2009). IAN has been recognized as a phytoalexin, an inducible metabolite involved in defence responses against fungal attack, in *Brassica juncea* (Pedras *et al.*, 2002). There is no evidence of the conversion of $[^2H_5]$IAOX to $[^2H_5]$IAN in feeding experiments using pea (Quittenden *et al.*, 2009). These findings indicate that IAN is not a common intermediate in IAA biosynthesis in plants.

7.8. *Arabidopsis* nitrilases are involved primarily in the conversion to carboxylic acids of nitriles derived from indole glucosinolates

It has been thought that plant nitrilases convert IAN to IAA. Although not widely recognized, this view has changed considerably in recent years (Piotrowski, 2008). The conclusion is that it seems unlikely that plant nitrilases participate in the conversion of IAN to IAA.

Nitrilase genes (*AtNIT1–AtRNIT4*) have been isolated from *Arabidopsis* (Bartling *et al.*, 1992, 1994; Bartel and Fink, 1994; Hillebrand *et al.*, 1996, 1998) (Fig. 1, Table 1). *AtNIT1*, *2*, and *3* gene products were thought to participate in the conversion of IAN to IAA (Bartling *et al.*, 1992; Bartel and Fink, 1994). However, these three nitrilases have been found to have a strong substrate preference towards phenylpropionitrile, allylcyanide, phenylthio acetonitrile, and methylthio acetonitrile (Vorwerk *et al.*, 2001). IAN hydrolysis by these nitrilases *in vitro* is inefficient. The preferred substrates are either naturally occurring substrates, which may originate from glucosinolate breakdown, or close relatives of these. Thus, a major function of AtNIT1, AtNIT2, and AtNIT3, appears to be the conversion to carboxylic acids of nitriles derived from indole glucosinolate turnover or degradation (Vorwerk *et al.*, 2001).

The *AtNIT4* gene product hydrolyses β-cyanoalanine to aspartic acid and ammonia (Piotrowski *et al.*, 2001; Janowitz *et al.*, 2009). β-Cyanoalanine is synthesized as an intermediate during cyanide detoxification and is also produced as a defensive compound against herbivory (Ressler *et al.*, 1997; Janowitz *et al.*, 2009). AtNIT4 may represent an important detoxification mechanism in *A. thaliana*, hydrolysing β-cyanoalanine to non-toxic products (Howden *et al.*, 2009).

7.9. Maize nitrilases may function in the detoxification of β-cyanoalanine *in vivo*

Although IAOX and IAN have not been detected in rice, maize, or tobacco (Sugawara *et al.*, 2009), an *in vitro* activity of the *ZmNIT2* gene product that converts IAN to IAA has been reported (Park *et al.*, 2003; Kriechbaumer

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et al., 2006; Kriechbaumer *et al.*, 2007). How should this be interpreted?

Maize has two nitrilase genes, *ZmNIT1* and *ZmNIT2* (Park *et al.*, 2003) (Table 1). Expression vectors to produce the ZmNIT1 or ZmNIT2 fusion proteins in *E. coli* were constructed to confirm that the gene products had the enzyme activity *in vitro*. ZmNIT1 did not show the activity toward the tested nitrile compounds including IAN (Park *et al.*, 2003), but only hydrolysed β-cyanoalanine (Kriechbaumer *et al.*, 2007). ZmNIT2 showed high activity toward IAN, 3-phenylpropionitrile, allylcyanide, methylthioacetonitrile, and 4-phenylbutyronitrile, which was hydrolysed most rapidly (Park *et al.*, 2003).

ZmNIT1/ZmNIT2 heteromers have been shown to participate in cyanide detoxification via β-cyanoalanine turnover, although ZmNIT2 homomers can hydrolyse IAN to IAA *in vitro* (Kriechbaumer *et al.*, 2007). ZmNIT1 and ZmNIT2 share 75% sequence identity at the amino acid level, and ZmNIT2 shows relatively high homology to *Arabidopsis* AtNIT4 (Park *et al.*, 2003). A phylogenetic tree showed that ZmNIT1 and ZmNIT2 are nearer to AtNIT4 than to the group of AtNIT1/AtNIT2/AtNIT3 (Fig. 8, Table 2).

Several members of the Gramineae, such as maize ZmNIT1/2 and rice OsNIT4, belong to the nitrilases of the AtNIT4 group (Fig. 8). Nitrilases belonging to the AtNIT4 family may have a different and more general function and are apparently not associated with IAA biosynthesis (Piotrowski *et al.*, 2001). It has recently been shown that the AtNIT4 homologue of *Sorghum bicolor* must form heteromeric complexes in order to have high activity with β-cyanoalanine (Jenrich *et al.*, 2007). OsNIT4 and tobacco TNIT4A/4B, which are homologues of AtNIT4, have high substrate specificity for β-cyanoalanine but do not hydrolyse IAN to IAA (Piotrowski *et al.*, 2001). ZmNIT2 protein may form the heteromer by associating with ZmNIT1 protein and participate in the detoxification of β-cyanoalanine *in vivo*.

Thus, recent works have shown that nitrilases are involved in the process of cyanide detoxification, in the catabolism of cyanogenic glycosides, and presumably in the catabolism of glucosinolates. It seems unlikely that the indole-3-acetaldoxime pathway via IAOX and IAN is involved in IAA biosynthesis.

8. The redefined *TAA1–YUC* pathway

After the submission of this review article, several papers showing that the YUC family functions in the conversion of IPA to IAA in *A. thaliana* were published (Mashiguchi *et al.*, 2011; Won *et al.*, 2011; Stepanova *et al.*, 2011). The genetic, enzymatic, and metabolite-based evidence indicated that TAA and YUC families function in the same auxin biosynthetic pathway in *Arabidopsis*. It was proposed that the *TAA1–YUC* pathway is 'the main auxin biosynthesis pathway in *Arabidopsis*' (Mashiguchi *et al.*, 2011).

8.1. The TAA family mainly produces IPA from Trp in Arabidopsis

In the Trp-auxotroph *trp1-1* mutants of *Arabidopsis* cultured in liquid media containing [$^{13}C_{11}$,$^{15}N_2$]Trp, Trp was efficiently converted to IPA as well as IAD (Mashiguchi *et al.*, 2011). To test which compound is mainly produced from Trp in *Arabidopsis*, estradiol-inducible *TAA1* overexpression (*TAA1ox*) plants were used in feeding experiments. The IPA content was increased drastically in *TAA1ox* plants, whereas the IAD content did not show a significant change relative to that in vector control plants, indicating that IAD is most probably not implicated in the IPA pathway but in another Trp-dependent pathway (Mashiguchi *et al.*, 2011). These results, together with the evidence that TAA1 protein possesses Trp aminotransferase

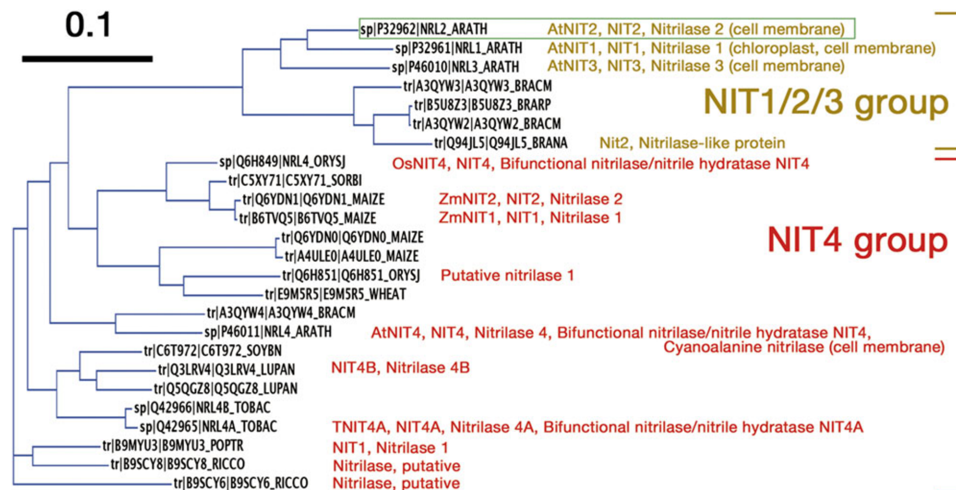

Fig. 8. Phylogenetic analysis of the deduced amino acid sequences of NIT2 proteins. The amino acid sequence of the *Arabidopsis* NIT2 protein was submitted to DDBJ/GenBank/EMBL, and a phylogenetic tree was constructed, as described in the legend of Fig. 2. The scale bar indicates 0.1 substitution per amino acid.

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activity (Tao *et al.*, 2008; Stepanova *et al.*, 2008) as described above, show that the TAA family mainly produces IPA from Trp in *Arabidopsis.*

L-Kynurenine (Kyn), which inhibits ethylene responses by decreasing ethylene-induced auxin biosynthesis in *A. thaliana* root tissues, effectively and selectively bound to the substrate pocket of TAA1/TAR proteins (He *et al.*, 2011). The treatment of this potent inhibitor of *in vivo* TAA1/TAR activity blocked all of the high auxin phenotypes of *YUC1ox* plants (Stepanova *et al.*, 2011). These results suggested that the high auxin phenotypes of *YUC1ox* require the function of TAA1/TARs genes, and also suggested the linear action model for TAA1/TARs and YUCs genes (Stepanova *et al.*, 2011).

8.2. Revision in the function of YUC genes: YUC proteins convert IPA to IAA

Fusion protein of GST–YUC2 heterologously expressed in *E. coli* converted IPA to IAA *in vitro* in an NADPH-dependent manner, but did not convert IPA to IAD (Mashiguchi *et al.*, 2011). The production of IAA was confirmed by LC-ESI-MS/MS. TAM was not a substrate of GST–YUC2 in this assay condition, indicating that the YUC family is implicated in the conversion of IPA to IAA in *Arabidopsis* (Mashiguchi *et al.*, 2011).

8.3. Synergistic interactions between TAA and YUC in IAA biosynthesis of Arabidopsis

The IPA content was 33% reduced in estradiol-inducible *YUC6* overexpression plants (*YUC6ox*) of *Arabidopsis* relative to that in vector control plants, whereas the IAA content was 34–47% increased in *YUC6ox* plants (Mashiguchi *et al.*, 2011). The IPA content in double knock-out *Arabidopsis* mutants of *TAA1* and *TAR2* genes, *wei8 tar2-2*, was reduced dramatically, and the *wei8 tar2-2* mutants showed severe growth. In contrast, the *yuc1 yuc2 yuc6* triple mutants had an elevated content of IPA (Mashiguchi *et al.*, 2011; Won *et al.*, 2011). Interestingly, the *yuc1 yuc4 wei8 tar2* quadruple mutants did not make any hypocotyls and roots, although the juvenile plants of *wei8 tar2-1* were similar to plants of *yuc1 yuc4* mutants (Won *et al.*, 2011). The IAA content in the quadruple *yuc1/2/4/6* mutants was nearly as low as in *wei8-2 tar2-1* mutants, and was much lower compared with that in the wild-type plants (Stepanova *et al.*, 2011). The IAA content was 44% increased in *YUC6ox* plants relative to that in vector control plants, and it was 146% increased in *TAA1 YUC6* co-overexpression plants (*TAA1ox YUC6ox*) of *Arabidopsis* (Mashiguchi *et al.*, 2011).

These results showing that the *taa* mutants were partially IPA deficient and the *yuc* mutants accumulated IPA indicated that TAAs are responsible for converting Trp to IPA, and also indicated that YUC and TAA work in the same pathway in *Arabidopsis*. In future, it must be revealed whether or not the *TAA1–YUC* pathway is widely distributed in the plant kingdom.

8.4. Different mechanisms for auxin biosynthesis in YUC genes and the iaaM gene

In some plant pathogenic bacteria, Trp is first converted to IAM by the enzyme tryptophan-2-monooxygenase encoded by the *aux1/iaaM/tms1* gene (Yamada *et al.*, 1985; Camilleri and Jouanin, 1991; Gaudin *et al.*, 1993), as described above. Cheng *et al.* (2006) showed that expression of the bacterial auxin biosynthesis gene *iaaM* rescued *yuc* mutant phenotypes, as discussed above. Overexpression of the *iaaM* gene led to the typical auxin overproduction phenotypes in both wild-type and *wei8 tar2* mutants of *Arabidopsis* (Won *et al.*, 2011). Together with the results showing that the *iaaM* gene also partially rescued the defects of *wei8 tar2* phenotypes at juvenile and adult stages, the authors indicated that *YUC* genes and *iaaM* genes probably use different mechanisms for auxin biosynthesis in *Arabidopsis* (Won *et al.*, 2011). Overexpression of the *aux1/iaaM/tms1* gene of the Ri plasmid allowed tobacco BY-2 cells to grow in the absence of auxin (Nemoto *et al.*, 2009*a*, *c*), as described earlier in this review. These results indicate that the production of IAM in plant cells is important in IAA biosynthesis.

9. Conclusions and perspectives

Plants would be expected to share evolutionarily conserved core mechanisms for auxin biosynthesis because IAA is a fundamental substance in the plant life cycle, although different plant species may have unique strategies and modifications to optimize their metabolic pathways. Biochemical and molecular biological findings and bioinformatics studies indicate that the best candidate for the major pathway of IAA biosynthesis is the IAM pathway and/or the IPA pathway.

In the IAM pathway, indole-3-acetamide hydrolase, encoded by the *AMI1* gene, is widely distributed in the plant kingdom. On the other hand, in the IPA pathway, TAA1/TIR2, which participates in the conversion of Trp to IPA, may be a Brassicaceae-specific enzyme. In the TAM pathway, the function of YUCs is ambiguous. The IAOX pathway is a Brassicaceae species-specific pathway that may be involved in the synthesis of plant secondary metabolites, such as indole glucosinolates and the alkaloid camalexin.

For the next step in advancing our understanding, it must be revealed whether or not the *TAA1–YUC* pathway is widely distributed in the plant kingdom. Additionally, the gene(s) functioning in IAM biosynthesis must be identified. By analysing the expression of the IAM biosynthesis gene(s), together with the *AMI1* gene, it will be possible finally to determine how, when, and where auxin is synthesized in plants.

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