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DISSECTING THE ROLE OF *Arabidopsis thaliana* AMIDASE 1
IN AUXIN BIOSYNTHESIS.

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DISSECTING THE ROLE OF *Arabidopsis thaliana* AMIDASE 1
IN AUXIN BIOSYNTHESIS.

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EL PRESIDENTE

LOS VOCALES

EL SECRETARIO

A mis padres: Paulino y Alicia

A Manu

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SUMMARY

SUMMARY

Over the course of time plants have evolved sophisticated mechanisms to adjust their growth and development to environmental changes. This adaptive property is attributed to their capacity of detecting environmental signals that are subsequently processed and translated into appropriate physiological changes.

As any other living organism, plants use hormones that, in very little concentrations, are in charge of controlling these physiological changes. From all the phytohormones, auxins are considered as the main group of molecules to regulate plant growth. Within this group, indol-3-acetic acid (IAA) is the most common naturally occurring auxin in plants.

Nowadays, it is known that IAA has essential roles in regulating plant growth, including embryo and root patterning, organ formation, vascular tissue differentiation and growth responses to environmental cues (Davies 2010). However, the current knowledge on how IAA is produced still remains incomplete.

It is assumed that the following enzymatic pathways contribute to the biosynthesis of IAA: the indole-3-pyruvic acid (IPA)-pathway, the indole-3-acetaldoxime (IAOx)-route and the indole-3-acetamide (IAM)-pathway. However, there are many reactions that are still elusive in these pathways. For instance, the monooxygenase involved in the initial reaction step of the IAM-pathway, which catalyses the conversion of tryptophan (Trp) to IAM, remains unidentified, while the second enzyme, AMIDASE1 (AMI1), which transforms IAM into IAA has been identified a number of years ago (Pollmann, Neu and Weiler 2003).

In *Arabidopsis thaliana*, the majority of IAM originates from the IAOx-pathway that is specific to *Brassicaceae* (Sugawara et al. 2009). This possibly restricts AMI1 action to this plant family. The AMI1 activity has neo-functionalized over the course of evolution according to the metabolic properties of *Arabidopsis*. On the other hand, the occurrence of IAM has been detected in a number of different kind of plants, including seaweeds (Pollmann et al. 2002; Stirk et al. 2009; Sugawara et al. 2009).

Hence, with the aim of elucidating some of the open gaps in IAA-biosynthesis, we focused our attention on the analysis and characterization of AMI1-related processes.

Firstly, the phylogenetic analysis and the subsequent enzymatic study of selected plant amidases have demonstrated that the enzymatic function of AMI1 and its homologous enzymes is very well conserved, and seemingly likely essential for plants, which is highlighted by the wide distribution of AMI1-like enzymes in the plant kingdom.

In addition, the use of loss- and gain-of-function lines for *AMI1* has confirmed its participation

in the conversion of IAM to IAA *in vivo*.

On the other hand, qRT-PCR analysis and histochemical assays have broadened our understanding of the relationship of *AMI1* with the rest of the metabolic intermediates and molecular components that are forming part in the complex network of IAA biosynthetic processes. As one of our principal findings, we detected a metabolite-dependent regulation of *AMI1* gene expression, principally driven by IAM and IAA. Here, IAM is repressing *AMI1* gene expression, whereas IAA induces it.

The latter observation has been substantiated by the generation and analysis of novel mutant lines: *ami1-2*/DR5:GUS; pAMI1:GUS/YUC9ox, pAMI1:GUS/*rty1*; *ami1-2/rty1*. All together, the obtained results suggested an important role for AMI1 in auxin homeostasis, embryo development and seed maturation.

Microarray experiments utilizing different mutant lines of *AMI1* (*ami1*; AMI1ind) have furthermore underlined that the main function of AMI1 is rather the control of the endogenous IAM pool of plants, than the production of auxin. In this context, AMI1 could be indirectly related with the regulation of plant stress responses, floral development and sugar sensing.

Finally, striving for the complete elucidation of the IAM-dependent auxin biosynthesis route, we sought in this thesis for a molecular candidate to catalyse the conversion of IAOx or Trp into IAM. Using transcriptomics and forward genetics approaches, we were able to identify a very promising candidate for this task, CYP71B22. It is still to draw a final conclusion on the activity of CYP71B22, but all of our experiments performed thus far propose this enzyme as the best candidate for this reaction.

RESUMEN

Las plantas han ido evolucionando a lo largo del tiempo para sobrevivir y adaptar su crecimiento y desarrollo a los cambios medioambientales. Esta propiedad adaptativa la han llevado a cabo gracias a su capacidad de detectar las señales que les llegan del exterior y que posteriormente procesan y traducen en adecuados cambios fisiológicos.

Como cualquier organismo vivo, las plantas usan hormonas que, en mínimas concentraciones, son las encargadas de controlar esos cambios fisiológicos.

Entre dichas fitohormonas, destacan las auxinas, ya que se las considera las principales moléculas reguladoras del desarrollo vegetal. Y a su vez, dentro de las auxinas destaca el ácido indol-3-acético (IAA), que es la auxina más común que podemos encontrar en la naturaleza.

Hoy día, se conoce prácticamente todo de la función que desempeña el IAA en las plantas: interviene en la regulación del crecimiento vegetal; en el desarrollo de las raíces y del embrión; en la formación de órganos vegetales; en la diferenciación de tejido vascular e influye en el crecimiento de la planta atendiendo a las señales medioambientales recibidas (Davies 2010). Sin embargo, cómo es la biosíntesis del IAA no está del todo esclarecida hoy día.

Actualmente, se conocen diferentes rutas enzimáticas que conducen a su producción: la ruta del ácido indol-3-pirúvico (IPA); la ruta del indol-3-acetaldoxima (IAOx) y la del indol-3-acetamida (IAM). No obstante, actualmente faltan aún por confirmar y conocer varias reacciones de dichas rutas. Por ejemplo, en la ruta del IAM la monooxigenasa involucrada en llevar a cabo la primera reacción enzimática, catalizando la conversión del triptófano (Trp) en IAM, no ha sido aún identificada. Sin embargo, la segunda enzima, AMIDASA 1 (AMI1), que transforma el IAM en IAA, fue identificada hace algunos años (Pollmann, Neu and Weiler 2003).

En *Arabidopsis thaliana*, la mayoría del IAM se origina de la ruta del IAOx, que es específica de la familia de plantas *Brassicaceae* (Sugawara et al. 2009). Esto, posiblemente restringe la actividad de AMI1 a esta familia vegetal. Por otro lado, la presencia de IAM ha sido detectada en un número de plantas diferentes, incluyendo a las algas marinas (Pollmann et al. 2002; Stirk et al. 2009; Sugawara et al. 2009).

Por todo esto y con el objetivo de elucidar algunas de las reacciones desconocidas en la biosíntesis del IAA, hemos centrado nuestra atención en el análisis y caracterización de los procesos relacionados con AMI1.

Primeramente, el estudio filogenético y el subsecuente estudio enzimático de varias amidasas

RESUMEN

vegetales han demostrado que la función enzimática de AMI1 y sus enzimas homólogas está muy bien conservada, y que además, parece ser esencial para las plantas, lo que es confirmado por la amplia distribución que presentan enzimas homólogas a AMI1, en el Reino Vegetal.

Además, el uso de las líneas de ganancia y pérdida de función para *AMI1* ha confirmado que su participación en la conversión de IAM en IAA, previamente detectada *in vitro*, también tiene lugar *in vivo*.

Por otro lado, análisis por qRT-PCR y ensayos histoquímicos han ampliado nuestro conocimiento sobre la relación de *AMI1* y el resto de intermediarios metabólicos y componentes moleculares que constituyen la compleja red de los procesos biosintéticos de IAA. Como uno de nuestros principales resultados, destaca la regulación dependiente de metabolitos de la expresión génica de *AMI1*, principalmente llevada a cabo por IAM e IAA. Donde IAM tiene un efecto represor sobre *AMI1*, mientras que IAA lo induce.

La última observación ha sido sustanciada por la generación y el análisis de nuevas líneas mutantes: *ami1-2/DR5:GUS*; *pAMI1:GUS/YUC9ox*, *pAMI1:GUS/rty1*; *ami1-2/rty1*. Los resultados obtenidos con dichas líneas han sugerido un importante papel de AMI1 en la homeostasis de auxinas, el desarrollo del embrión y maduración de la semilla en Arabidopsis.

También, el uso de experimentos de microarray con las diferentes líneas de *AMI1* (*ami1*; *AMI1ind*) han señalado que la principal función de AMI1 es la de controlar el pool endógeno de IAM más que la de producir auxinas. En este contexto, AMI1 podría estar indirectamente relacionada con la regulación de las respuestas de estrés vegetal, desarrollo floral y percepción de azúcares.

Finalmente, y debido a la importancia de ir completando las rutas de biosíntesis de IAA, en este trabajo se ha buscado a un candidato molecular que pudiera estar catalizando la conversión de IAOx o Trp en IAM. Utilizando los resultados de los experimentos transcriptómicos realizados, pudimos detectar un posible candidato para dicha reacción: CYP71B22. Y aunque aún falta concluir más sobre la actividad de CYP71B22, nuestros primeros estudios han dado a entender que podría ser la enzima idónea para llevar a cabo dicha reacción.

ABBREVIATIONS

ABBREVIATIONS

APS	Ammonium peroxodisulphate
APT1	Adenine Phosphoribosyl Transferase 1,
BAP	Benzylaminopurine
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CaCl₂	Calcium chloride
cDNA	Complementary DNA
CLi	Lithium chloride
DEPC	Di-ethyl-pyro-carbonate
DMSO	Di-methyl-sulfoxide
DNA	<i>De-oxy-ribonucleic acid</i>
DsRed	Red fluorescence protein
EDTA	Ethyl-ene-di-amine-tetra-acetic acid
EGFP	Enhanced green fluorescence protein
EtOH	Ethanol
g	G-force
GFP	Green fluorescence protein
g/L	Gramm/litre
GUS	β-glucuronidase, <i>uidA</i> gene
h	Hours
HPLC-MS	(High-Performance Liquid Chromatography-Mass Spectrometry).
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilobase
KCl	potassium chloride
LB	lysogeny broth
MeOH	Methanol
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MgCl	Magnesium chloride
MgSO₄	Magnesium sulphate
min	minutes
ml	Millilitre
mm	millimetres
MnCl₂	Manganese chloride
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
MS	Murashige and Skoog medium
mRNA	messenger RNA
NaCl	sodium chloride
Na₂HPO₄	Sodium phosphate
ng	Nano grams
o/n	Over-night
PAA	polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
qRT-PCR	<i>quantitative Reverse Transcription Polymerase Chain Reaction</i>
RNA	ribonucleic acid
RNA-Seq	<i>RNA sequencing</i>
RT	Room Temperature
SALK	It is the short name for: Salk Institute Genomic Analysis Laboratory.
SD	Short Day
SDS	Sodium dodecyl sulphate
SOB	Super Optimal Broth

ABBREVIATIONS

SYBR	N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-
Green I	phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
T-DNA	Transfer DNA
TEMED	Tetramethylethylenediamine
TMSDS	Tri-methyl-silyl-different-grades of substitution
T_m	Temperature melting
Tris	Tris-(hydroxyl-methyl)-amino-methane
UBQ10	<i>Ubiquitin 10</i>
UV	Ultra violet
v/v	Volume/volume
w/v	weight/volume
X-Gluc	5-bromo-4-chloro-3-indolyl β-D-glucuronic acid
μg/ml	Micrograms/millilitre
μL	microliter
UTR	Untranslated region

1 INTRODUCTION

1.1 Phytohormones

Plants are sessile individuals that, driven by evolution, have developed the capacity to successfully adapt to continuous changes in their environment. Over time, they have developed sophisticated mechanisms to shape their body plan and optimize their metabolism according to specific environmental demands.

This developmental adaptation that facilitates plants to maintain an appropriate transcriptional reprogramming of tissues answering to external stimuli involves the permanent activity of meristematic tissues, *de novo* organogenesis, a notable capability for regeneration, and direct growth responses to external stimuli (Vanneste and Friml 2009).

It is not very well understood how all these processes are controlled, but as with animals, plants possess a limited number of small signalling molecules, collectively referred to as plant or phytohormones, that are key-players in the regulation of these processes.

In a formerly applied approach, plant hormones have been classified into five 'classical' phytohormone groups, including auxin, abscisic acid (ABA), cytokinin (CK), gibberellin (GA) and ethylene (ET) (Davies, 2010). However, over the last years, a number of additional molecules with hormonal properties have been recognized. Those include, e.g. brassinosteroids (BRs), jasmonates, salicylic acid (SA) and strigolactones (SLs) (Browse 2009; Gomez-Roldan et al. 2008; Loake and Grant 2007; Umehara et al. 2008; Vert et al. 2005; Wasternack 2007).

All the plant hormones can largely be divided into two groups. One group is composed of plant hormones classified as developmental promoters/messengers, such as auxins, CK, and GA, whereas the other group contains stress- and defence-related hormones, including ET, ABA, jasmonic acid (JA), and SA.

Phytohormones influence the developmental program of a given tissue by triggering transcriptional reprogramming of affected cells at sub-micromolar concentrations. They act in a combinatorial manner to determine a multitude of different physiological processes that are not only dependent on the perceived stimulus, but also on the specific properties of the responding tissue (Bennett, Bellini, and Van Der Straeten 2005). Today, much research on plant hormone biology involves the investigation on their molecular mode of action, plant hormone crosstalk, and interactions with other factors, with the focus on elucidating how they orchestrate plant growth and responses to environmental inputs (Eckardt 2015; Murphy 2015).

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The physiological interaction between plant hormones is commonly referred to as crosstalk, a phenomenon that has been put forth by the observation of physiological responses elicited by exogenous applications of plant hormones (Spartz and Gray 2008). Crosstalk is not only occurring at the level of hormone responses, but also at the biosynthesis level, creating a delicate response network (Weiss and Ori 2007).

Plant hormone crosstalk can be classified as: direct, indirect and co-regulation types (Chandler 2009a; Hoffmann, Hentrich, and Pollmann 2011). Direct crosstalk is produced when different hormones are affected by the same stimulus, triggering a common response, in a combinatory way over the same promoters or target proteins. In the case of indirect crosstalk, a hormone-signalling pathway triggers a specific response for one stimulus perceived. At the same time, it will modulate the perception, sensitivity, abundance, transport, etc., of a second hormone. This latter will also induce a response that can be additional or opposite to the response triggered by the first hormone. In some occasions, the first hormone can induce more than one hormone-signalling pathway, increasing the magnitude of the response.

Finally, co-regulation occurs when the same stimulus activates several hormonal-signalling pathways that will affect different targets (genes/proteins) in an additive, synergistic, or antagonistic manner.

On the other hand, hormone crosstalk has also been functionally explained in terms of additivity, antagonism and synergism or cooperativity. The former, additivity, could basically been described as the phenomenon of co-regulation; the second one or antagonism is the result of an opposite effect produced by two hormones over a same target (e.g. the effect of cytokinins vs auxins; gibberellic acid vs. abscisic acid). Eventually, synergism is the enhanced response caused via two or more hormones, regulating common target genes, as for instance, the synergy of auxins and BRs (Chandler 2009a).

1.2 Auxins

Despite the fact that there are many different plant hormones, plant shape is largely controlled by one of them, the auxin. The collective term auxin refers to a class of compounds sharing similar physiological functions in plants, which can also be found in humans, animals and microorganisms (Teale, Paponov, and Palme 2006). In plants, they are known to be essential for the regulation of a wealth of different processes of plant development at all levels, starting at the cellular level, through organs, and ultimately to the whole plant

(Thimann 1977; Davies 2010). As with the other plant hormones, auxin acts at extremely low cellular concentrations. Remarkably, auxin action is dose dependent. Too high cellular auxin levels can exert inhibitory effects on plant growth and development (Hoffmann et al. 2010). Moreover, auxin also acts in short periods of time, being capable of altering gene expression levels of downstream genes within only some minutes (Teale, Paponov, and Palme 2006).

Albeit indole-3-acetic acid (IAA) is considered the major auxin and most important growth promoter found in plants, a number of additional naturally occurring compounds that also possess auxin effects have been reported to date, i.e. 4-Cl-IAA (Slovin et al. 1999), and phenyl-2-acetic acid (PAA) (Ludwig-Müller and Cohen 2002). Moreover, a variety of IAA precursors such as indole-3-acetamide (IAM), indole-3-acetonitrile (IAN), indole-3-pyruvic acid (IPA), and indole-3-butyric acid (IBA) exhibit auxin-like effects, most likely due to their conversion to IAA (Sánchez-Parra et al. 2014). However, these “other auxins” have commonly less pronounced effects than IAA (Abeles and Rubinstein 1964).

Moreover, IAA does not act exclusively alone. Much more, it is participating in a comprehensive regulatory network in which IAA is known to interact with other phytohormones to steer plant growth and development as well as plant stress responses. In many of these processes auxin has a prominent effect, controlling the expression and, in the end, action of other plant hormones, such as CKs, GAs, ET, etc.

1.3 Crosstalk of auxin

As mentioned above, auxin interacts with other phytohormones resulting in biochemical interactions known as "crosstalks".

Most crosstalk has been studied by analysing the interaction of phytohormone pairs in the past, but it has also been demonstrated that interactions can employ three or even more hormones. Some examples are the following: IAA, ET and BRs (De Grauwe et al. 2005); GA, IAA and ET or GA, CK and IAA (Weiss and Ori 2007); IAA, JA and ABA (Hoffmann et al. 2010); JA, IAA and ET (Hentrich et al. 2013) etc.

Regarding the role of auxin in plant hormone crosstalk, much studies focused on the effects exerted on plant development. The arguably best-studied crosstalk of IAA is that with CK. In this case, both hormones have usually an opposite action. For instance, CK and IAA have a role

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on "lateral bud outgrowth" but, where CK promote this developmental process, auxin has an inhibitory effect (Chandler 2009a; Schaller, Bishopp, and Kieber 2015).

Nevertheless, the action of auxin is sometimes enforced through the interaction with other phytohormones. This is the case in the crosstalk between BR and IAA. Both hormones act redundantly in the same developmental processes: cell and hypocotyl elongation, lateral root development, control of flowering time, etc. (Nemhauser, Mockler, and Chory 2004; Vert et al. 2008). Another example is the interaction between SLs and IAA, since these two hormones synergistically stimulate secondary plant growth, regulating cambium activity (Agusti et al. 2011).

The previous examples show the influence, of auxins interacting with other phytohormones, on plant physiology and development. However, IAA also contributes to plant defence responses through its interaction with SA and/or JA, two hormones that are involved in defence against plant pathogens (Truman et al. 2010). While SA is considered the leading signalling molecule in defence against biotrophic pathogens, JA is central in the defence against necrotrophic pathogens and insects (Glazebrook 2005).

Finally, hormonal interaction can occur at the level of biosynthesis. For example, it has been demonstrated that auxin can decrease the concentration of CK in plants, by either decreasing biosynthesis, by oxidative breakdown or by conjugation (Nordström et al. 2004). On the other hand, CK acts as positive regulators of auxin biosynthesis giving rise to a regulatory loop produced by these two hormones (Jones et al. 2010).

Another clear case is the crosstalk between ET and IAA. On the one hand, IAA has been shown to enhance ET formation in *Arabidopsis thaliana*. It seems that auxin stimulates the activity of 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASES (ACSs), in particular by inducing the expression of three specific ACS genes: ACS6, ACS8, and ACS11. The corresponding gene products catalyse the rate-limiting step of ethylene biosynthesis, converting S-ADENOSYL METHIONINE (S-AdoMet) to ACC (Chandler 2009a; Paponov et al. 2008; Wang, Li, and Ecker 2002)(Figure 1.1). Thus, alterations in ethylene production can be correlated with changes in endogenous auxin levels, although the fact that auxin stimulates ethylene biosynthesis does not make the presence of auxin a requirement for the production of ET.

In turn, ethylene is known also influence auxin biosynthesis. It has been described that two genes, *WEAK ETHYLENE INSENSITIVE2/ANTHRANILATE SYNTHASE α 1* (WEI2/ASA1) and *WEAK*

ETHYLENE INSENSITIVE7/ANTHRANILATE SYNTHASE β 1 (WEI7/ASB1), encoding two subunits of anthranilate synthase, are positively transcriptionally regulated by ET. These enzymes catalyse the conversion of chorismate to anthranilate, which is a reaction in the biosynthesis of tryptophan (Trp), the main IAA precursor (Stepanova et al. 2005).

Figure 1.1 Model of interaction between auxin and ethylene.

The biosynthesis of ethylene starts with the formation of S-ADENOSYL METHIONINE (S-AdoMet or SAM) from methionine. The reaction is catalysed by the SAM synthetase. The rate-limiting step of ethylene synthesis is the conversion of S-AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) under most conditions. ACC is the immediate precursor of ethylene. IAA induces the expression of genes encoding for ACS enzymes (horizontal arrow indicates the hormonal interaction). Finally, ACC oxidase (ACO) catalyses the final step of ethylene synthesis using ACC as substrate.

Nevertheless, the link between tissue-specific ET responses and auxin biosynthesis was not fully demonstrated until the *weak ethylene insensitive8 (wei8)* mutant was discovered in *A. thaliana* (Stepanova et al. 2008). This mutant was identified in a screening in which Stepanova *et al.* looked for mutants that displayed ethylene defects in roots. They could map the mutation locus to a gene encoding for a *TRYPTOPHAN AMINOTRANSFERASE OF*

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ARABIDOPSIS 1 (TAA1). Finally, they demonstrated that TAA1 participates in IAA biosynthesis, converting Trp into IPA.

Conclusively, both hormones form a kind of regulatory network in which they are mutually controlling the cellular content of the other.

Nonetheless, it is important to note that the interaction of phytohormones depends on the affected biological process, responding tissue, developmental stage, and/or environmental conditions. And of course, the operability of the perception and signalling transduction machinery has to be guaranteed, to trigger corresponding adequate hormonal responses.

1.4 Auxin signalling

Auxin signalling regulates cell responses to variations in auxin levels that are mediated by a combination of auxin metabolism and transport.

Despite the doubts that still remain with respect to the molecular mechanism of this process, it is accepted knowledge that several signalling molecules actively contribute to the integration and processing of the auxin signalling.

Firstly, the main process that controls the activation or repression of auxin-responsive genes occurs between AUX/IAA proteins (Auxin/Indole-3-acetic acid) and the ARFs (Auxin Response Factors). The former ones, forming a family of 29 members in Arabidopsis (Abel and Theologis, 1996), are transcriptional repressors of ARFs. The ARFs, in turn, are transcriptional activators that regulate the expression of auxin responsive genes. In Arabidopsis, ARFs compose a group of 23 members (Liscum and Reed 2002).

Auxin-responsive genes are generally characterized by a conserved motif in their promoter sequence, called ARE (Auxin Response Elements) (Ulmasov et al. 1995). This motif is recognised by the ARFs by their N-terminal B3-like DNA-binding domain. The resulting binding of an ARF to a corresponding promoter segment activates the response to auxin. However, the C-terminal region of the ARFs is similar to the C-terminal domain of the AUX/IAA repressors, promoting their heterodimerization, thereby causing the transcriptional blockade of responsive genes (Teale, Paponov, and Palme 2006; Ulmasov et al. 1997).

To break the repressor-activator binding, a protein complex known as the auxin receptor ($SCF^{TIR1/AFB}$) marks AUX/IAA proteins with Ubiquitin (Ub) molecules in the presence of auxin,

which directs the Aux/IAA proteins to degradation through the 26S proteasome complex (Mockaitis and Estelle 2008). Selectivity of this system is achieved through the various F-box proteins that are known to be target-specific. In case of the auxin signalling cascade, the corresponding F-box protein found in the SCF (Skp1-Cul1-Fbox) complex is TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (Dharmasiri, Dharmasiri, and Estelle 2005; Kepinski and Leyser 2005). However, TIR1 is not the only auxin-receptor, since there are a few other TIR1-related F-box proteins [AUXIN-BINDING F-BOX (AFB)] that also interact with Aux/IAA in an auxin-dependent manner, mediating auxin responses (Hoffmann, Hentrich, and Pollmann 2011; Vanneste and Friml 2009).

These receptors (TIR1/AFBs) bind auxin molecules in their binding cavity and thereby increase the affinity of the SCF complex for its target, the AUX/IAAs. This finally leads the ubiquitination and subsequent degradation of the AUX/IAA proteins (Tan et al. 2007).

The SCF is an ubiquitin protein ligase E3 complex (Gray et al. 1999), this implies that its constituent proteins participate in the ubiquitination process, also in auxin signalling. Between these proteins there is a highly conserved central scaffold protein, called CULLIN1 (CUL1) that is associated with the adaptor protein S-PHASE KINASE-ASSOCIATED PROTEIN 1 (Skp1; for Arabidopsis Skp1-related1 (ASK1)) (Petroski and Deshaies 2005; Smalle and Vierstra 2004). This adaptor protein provides the binding site for the F-box protein. Another fourth subunit, called RING BOX PROTEIN 1 (RBX1) (Kamura et al. 1999), is the element that binds to the Ub-conjugating enzyme (E2) and promotes transfer of Ub to the F-box-protein bound substrate.

Once targets carry multiple ubiquitinations, they are substrates for degradation by the 26S proteasome or ubiquitin-26S proteasome system (UPS) (Woodward and Bartel 2005).

To conclude, SCF and the associated protein-degradation machinery, together with Aux/IAAs and ARFs, represent the full signal-transduction cascade from the auxin signal to gene expression (Teale, Paponov, and Palme 2006).

Nevertheless, such as signalling machinery must be regulated. This regulation involves the interaction of different proteins to maintain the stability and the normal function of the SCF complex. These protein interactions are carried out by the following: AUXIN RESISTANT 1 (AXR1), a protein which encodes one subunit of a ubiquitin E1-like enzyme complex, that in concert with another activating enzyme, E1 C-TERMINAL RELATED1 (ECR1), and RUB-CONJUGATING ENZYME1, RCE1, conjugate an ubiquitin-related molecule (RUB, also referred to as NEDD8) to CUL1, component of SCF (Dharmasiri et al. 2003; Leyser et al. 1993; del Pozo and

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Estelle 1999). CUL1 is also associated with a protein complex called the CONSTITUTIVE PHOTOMORPHOGENESIS 9 (COP9) SIGNALOSOME (CSN), which removes the conjugated RUB1 from CUL1 (Schwechheimer et al. 2001). The cycle of RUB1 conjugation and its removal appears to be critical for the function of SCF and might limit the capacity of auxin responsiveness in cells.

CSN resembles the 26S proteasome lid (Li and Deng 2003) and could function directly in both RUB removal and ubiquitin-mediated substrate degradation (Woodward and Bartel 2005).

In addition, it seems to exist an alternative, proteasome-independent auxin signalling pathway that appears to act through the AUXIN BINDING PROTEIN1 (ABP1) receptor (Hertel, Thomson, and Russo 1972; Löbner and Klämbt 1985a, 1985b). This receptor binds auxin molecules mediating rapid auxin responses such as cell wall loosening, cytoskeleton rearrangement and regulation of endocytosis, leading to cell expansion (Ljung 2013). Moreover, ABP1 is considered the starting point of the signalling pathway acting as a negative regulator of the SCF pathway (Tomas et al. 2013).

Apart from the characterized complex of proteins controlling the transcriptional response, auxin signalling can occur in a SCF independent manner. One example is the link that indicates a role for Rac-like (ROP) GTPases in auxin action. Auxin activates ROP3 and both auxin and active ROP3 (or RAC1) cause Aux/IAA proteins to aggregate into discrete nuclear bodies (Teale, Paponov, and Palme 2006). These structures are proteolytically active, resulting in the 26S proteasome mediated degradation of Aux/IAA proteins. Another event, also triggered by auxin, is a rapid increase of the MITOGEN-ACTIVATING PROTEIN KINASE (MAPK) activity. The MAPK activity causes a decrease in the transcription of auxin-responsive genes. In this way, MAPK pathway represents a novel route activated by auxin that is potentially TIR1 independent and that negatively affects expression of auxin-responsive genes (Vanneste and Friml 2009).

1.5 Auxin transport

Explanations about how the action and function of auxin takes place in plants involve the understanding of how the phytohormone is distributed and transported in the plant corpus. It is widely accepted that its distribution is realized by two distinct physiological ways that channel auxin into specific plant tissues. First to mention is the rapid translocation of auxin in mature phloem with the mass flow of other metabolites. Secondly, auxin transport occurs in a

cell-to-cell manner basipetally, from the apical aerial tissues towards the root tip, and from the root tip towards the outer lateral root cap and epidermis tissues. The former is called fast and non-polar auxin transport, while the latter and slower process is known as cell-to-cell polar auxin transport (PAT) (Michniewicz, Brewer, and Friml 2007; Rozov et al. 2013). However, there is also mounting evidence for the penetration of auxin from the phloem to the PAT system and vice versa (Rozov et al. 2013).

Due to a lack of knowledge on auxin import proteins, PAT was for a long time explained by the “chemiosmotic hypothesis”. This theory describes that auxin, in its protonated form, can passively enter the cells through the plasma membrane (PM). Since the neutral pH in the cytoplasm leads to a rapid deprotonation of auxin, the hormone is trapped within the cell. The regulated export of auxin from the cells is realized by the virtue of efflux carriers, mainly of the PIN FORMED (PIN) protein family. In this manner, the auxin flux can be driven unidirectionally within a given tissue. Nowadays, the original chemiosmotic hypothesis has been updated and extended. In addition to the PIN IAA-exporter, it has been proposed that the presence of proton pumps in the part of the PM facing the apoplast favour the protonation of IAA outside the cell and, thereby, facilitate its entry (Vanneste and Friml 2009). Nonetheless, today we know that there are auxin influx transporters, too, that directly import auxin into the cells. The best-described cellular auxin influx carrier is the PM spanning AUXIN 1 (AUX1) transporter. *AUX1* is expressed in various tissues, and the corresponding transporter plays important roles in processes such as gravitropism, phototropism, lateral root spacing, phyllotactic patterning, lateral root emergence, and root hair development (Vanneste and Friml 2009). In addition to *AUX1*, the Arabidopsis genome contains a small number of other *Like-AUX1* (*LAX*) genes that presumably also contribute to auxin transport (Tanaka et al. 2006).

The PIN protein family consists of eight different members in Arabidopsis. Their localization is extremely dynamic and it changes rapidly. Nonetheless, they are usually distributed polarly in the PM, determining the direction of auxin flow and the overall distribution of auxin within a given tissue or organ (Figure 1.2, adapted from Garay-Arroyo et al. 2014). The polarity of PIN proteins is regulated by the antagonistic action of the proteins serine/threonine kinase PINOID (PID) and PROTEIN PHOSPHATASE 2A (PP2A) (Benamins and Scheres 2008; Rozov et al. 2013; H. Tanaka et al. 2006; Teale, Paponov, and Palme 2006). In addition, there are reports underlining that some PIN proteins are localized in the endoplasmic reticulum (ER), instead of localizing to the PM (Ding et al. 2012). Nevertheless, they show distinct spatio-temporal expression pattern to always maintain optimum free IAA levels. In this way, PIN proteins are closely related to processes such as tropisms, embryo development, root meristem patterning,

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organogenesis, and vascular tissue differentiation (Vanneste and Friml 2009). Besides PIN proteins, other components exist that also contribute to auxin efflux. The so-called ABCB/PGP (P-glycoproteins of the ABCB transporter family) and the PILS (PIN-LIKES) proteins are believed to transport IAA between cytosol and either the apoplastic space or the endoplasmic reticulum.

Figure 1.2 Transport of auxin (adapted from Garay-Arroyo (2014)).

The differences in pH between the apoplastic space and the cytosol facilitate IAA transport over the plasma membrane, since IAA is a weak acid. In the cytosol (pH≈7) IAA is in its anionic form, while in the apoplast (pH≈5), IAA is protonated as IAAH, and it can pass through the plasma membrane into the cytosol. The PAT system relies on specific transporters localized in the plasma membrane: influx carriers (AUX1/LAX) (purple) and efflux carriers [PIN 1,2,3,4 and 7 (brown) and ABCB (black)]. To be transported to the endoplasmic reticulum, there are other transporters that are also believed to contribute to IAA transport: PIN5 (light blue), PIN6 (dark blue) and PILS (orange).

To guarantee successful plant development, it is extremely important to maintain control of auxin transport elements (De Grauwe et al. 2005). This control is given by two main components: an Aux/IAA-dependent manner and a vesicle trafficking system (Benjamins and Scheres 2008; Tanaka et al. 2006; Teale, Paponov, and Palme 2006). In the first one, the SCF^{TIR1} complex is again involved in sensing auxin to release the ARFs from Aux/IAA repression and, thus, permitting up-regulation of the expression of auxin transport components. In this context, the transcriptional regulation of *PLETHORA1* and 2 (*PLT1* and *PLT2*), both encoding important master transcription factors that orchestrate the expression of *PIN* genes, has to be mentioned (Blilou et al. 2005). In the second layer of control, endosomal cycling is the process by which the localization of PIN proteins is regulated. PIN proteins undergo constitutive

recycling by clathrin-dependent endocytosis between the plasma membrane and endosomes according to the necessities of auxin flow. The more auxin is present in the cell, the less endocytosis of PIN proteins will occur. However, a decrease in auxin levels induces the internalization of PIN auxin efflux carriers, resulting in the regulated canalization of the phytohormone (Vanneste and Friml 2009). As can be taken from these two layers of control through which cellular auxin homeostasis is maintained, the major read-out is always the current cellular auxin content, which in turn affects its own transport, behaving even as the primary signal in many cases (Figure 1.3, adapted from Vanneste and Friml 2009).

Figure 1.3 Feedback regulation of auxin [adapted from Vanneste and Friml (2009)]

Different endogenous or exogenous signals induce the auxin response by modulating its biosynthesis and/or transport. The auxin levels acquired after the initiation of auxin response, controlled by the $SCF^{TIR1/AFB}$ complex, trigger the transcriptional response activating or repressing genes, related with the signal perceived, to carry out different developmental processes. Moreover, auxin signalling provides feedback, regulating the rate and polarity of its own transport.

1.6 Auxin homeostasis

The auxin gradients produced in plant tissues through the PAT system change dynamically during developmental processes and in response to external signals, being crucial for large parts of the effects governed by auxin. Auxin maxima, or in other words, the local accumulation of IAA within a single cell or a small group of cells, contribute to determine auxin action on a tissue or organ-specific level.

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As discussed above, IAA is involved in a plethora of crosstalk events that include plenty of other plant hormones, resulting in multiple combinations of interactions with even more than two hormones in some cases. The enormous number of combinations between the TIR1/AFB receptors and Aux/IAA repressors and Aux/IAA and ARFs elements, respectively, add to the complexity of the system and facilitate multiple physiological effects within the plant (Rigal, Ma, and Robert 2014). The development of such a complex and versatile regulatory system may be taken as a piece of evidence underlining the importance of auxin for successful plant development, adaptation, and in general, to the entire plant life-cycle. This fundamental importance of auxin for the development of plants, not only in dicots as *Arabidopsis*, but also in monocots as for instance maize and rice, has recently been demonstrated (Mcsteen 2010). In brief, and putting it in the words of Benjamins and Scheres (2008), auxin can be considered a star in the field of plant development.

Numerous recent research projects have associated auxin function with important processes in a plant's life cycle that are ultimately involved in regulating plant growth and development in response to changes in their environment. One example is the recently disclosed role of auxin in plant defence responses, e.g. in oxidative stress (Krishnamurthy and Rathinasabapathi 2013), cold stress (Rahman 2013) or pathogen defence (Carná et al. 2014). In all of these examples, alterations of auxin transport elements, i.e. AUX1 and PIN proteins, are mentioned. These alterations underline the role of auxin in the adaption of plant development under different kind of stresses.

Due to these and many other examples, by inference, auxin transport and signalling play an important role in successful plant development and adaptation to environmental cues. However, other mechanisms, like for instance IAA oxidation, conjugation and auxin biosynthesis, are likely to be further molecular processes that represent essential factors contributing to the control of cellular auxin homeostasis, too (Chandler 2009b; Lau, Ju, and Smet 2008).

1.7 Conjugation and oxidation of IAA

Conjugation of IAA is considered to inactivate the active hormone, free IAA. It either marks the first step in IAA degradation or serves the storage of IAA within the cell in its inactive form, from which it can be liberated on demand. Remarkably, approximately 99% of cellular IAA is found in its conjugated form. In general, the conjugation of IAA involves linking of the free acid to either sugars or amino acids, giving rise to various different IAA amino acid amides and IAA sugar ester (Ludwig-Müller 2011). In addition to those amide and ester forms, a high molecular

weight conjugate has been described in bean, in which IAA is supposed to be bound to a 42 kDa polypeptide (Walz et al. 2002). IAA conjugates are suggested to hold several functions ranging from storage, transport, compartmentalization, excess IAA detoxification, and protection against peroxidative degradation (Cohen and Bandurski 1982).

At present, there is only little knowledge on enzymes that are involved in the formation of IAA sugar ester. However, one gene from maize, referred to as *IAGLU*, has been reported to be responsible for the production of IAA-glucose (Szerszen, Szczygłowski and Bandurski 1994). A homologous gene from Arabidopsis, *AtUGT84B1*, has also been discovered and characterized (Jackson et al. 2001). On the other hand, formation of IAA amide conjugates has been demonstrated to proceed through the enzymatic activity of a family of GH3 enzymes. In Arabidopsis, the *GH3* gene family comprises 19 members (Staswick et al. 2005). With respect to the formation of higher molecular weight conjugates, it has to be remarked that they have so far only been detected in very few plant species, including bean, Arabidopsis, strawberry and pea (Park, Cohen and Slovin 2006; Park et al. 2010; Seidel et al. 2006; Walz et al. 2002), which calls their general importance into doubt.

Despite the fact that conjugates are classified by their structure, they can also be differentiated by their function. On the one hand, ester conjugates are supposed to be the first step of IAA degradation, although there are also auxin amino acid conjugates such as IAA-Asp and IAA-Glu, suggested to be intermediates in auxin degradation. On the other hand, some other amide conjugates tend to be storage forms for IAA, from which IAA can be enzymatically released by the activity of specific IAA-amidohydrolases. One very good example for an IAA storage form is IAA-Leu, from which free IAA can be liberated by IAA-Leu amidohydrolases of the ILL family (Junghans et al. 2006). For other conjugates, different functions more related with plant development or abiotic/biotic stress tolerance have been postulated. For instance, IAA-Ala inhibits the induction of stem growth and the initiation of roots (Garay-Arroyo et al. 2014). Also, an increase in *GH3* gene expression has been reported during plant-pathogen interactions, in which elevated auxin levels were detected (Deeken et al. 2006; Ludwig-Müller et al. 2009; Siemens et al. 2006). An example of an up-regulated *GH3* gene is *GH3.5* (*WEIS1*) that possesses dual substrate specificity for both IAA and SA (Staswick et al. 2005; Zhang et al. 2007).

Moreover, an additional function has been detected for the IAA-Trp conjugate. IAA-Trp is considered a potent inhibitor of auxin signalling, capable of inhibiting several IAA-induced growth responses such as the stimulation of lateral root growth (Staswick 2009).

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Another mechanism influencing IAA homeostasis is its degradation, in particular through the oxidative metabolism of IAA. IAA catabolism is achieved through the enzymatic oxidation of its indole backbone (resulting in Ox-IAA or Ox-IAA conjugates), or via the oxidative decarboxylation of the side-chain (Normanly 2010). Some examples of these metabolites are 2-oxoindole-3-acetic acid (oxIAA) and ox-IAA-glucose (ox-IAA-Glc), considered to be the major degradation products of IAA in *Arabidopsis* (Kasahara 2015; Ljung 2013).

The main function of oxidative auxin conjugates is the control of the proper IAA concentration in the cell, since it has been observed that an increase of IAA levels after, e.g., IAA treatment (Östin et al. 1998), in IAA-overproducing lines (Novák et al. 2012; Stepanova et al. 2011) and after induction of IAA biosynthesis (Sairanen et al. 2012) translates into increases concentration of oxidized auxin conjugates. In addition, the occurrence of different oxidized conjugates depends very much on the cell type and of the plant species. For example, Sasaki et al. (1994) reported the accumulation of Ox-IAA-Asp in non-embryonic cells of carrot, but it was not present in embryonic cells.

1.8 Auxin biosynthesis pathways

It is suggested that different pathways for IAA production operate either in parallel to each other or in a redundant manner. The high degree of functional redundancy and multiplicity of pathways involved in hormonal formation underlines the outstanding importance of auxin for plant development (Cooke et al. 2002), especially in the generation of auxin gradients over plant tissues (Vanneste and Friml 2009).

In fact, it has been found that auxin biosynthesis is strongly influenced of the developmental stage as well as the given cell type. Consequently, the level of auxin biosynthesis is spacio-temporally controlled, providing adequate auxin amounts when the plant really needs it. The precise molecular mechanism(s) by which this process is controlled remains, however, in great parts unclear. Focusing on auxin biosynthesis, it is widely accepted that IAA is a derivative of the chorismate-pathway (Figure 1.4).

Figure 1.4 Schematic overview of L-Tryptophan biosynthesis.

Black arrows indicate the enzymatic reaction steps that conduct the conversion of chorismate into L-tryptophan (Trp). Abbreviations: ASA1 and ASB1: ANTHRANILATE SYNTHASE SUBUNITS α and β ; IGS: INDOLE-3-GLYCEROL PHOSPHATE SYNTHASE; TSA1: TRYPTOPHAN SYNTHASE α -SUBUNIT 1; TSB2: TRYPTOPHAN SYNTHASE β -SUBUNIT 2.

Chorismate is the precursor of the proteinogenic amino acid Trp, and is sequentially converted in the chloroplast via 1-(o-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate, indole-3-glycerol phosphate (IGP) and indole to Trp (Radwanski and Last 1995). As previously mentioned (c.f. Crosstalk of auxin) the two enzymes, ASA1 and ASB1 of *A. thaliana* catalyse the first reaction of the tryptophan biosynthetic pathway. In later reaction steps an IGP SYNTHASE (IGS) catalyses the conversion of 1-(O -carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate to IGP (Li et al. 1995). Ultimately, IGP is converted into Trp in a two step process, involving an heterotetrameric $\alpha_2\beta_2$ enzyme complex. This complex consists of two subunits: the first one is called TRP SYNTHASE $\alpha 1$ (TSA1) and catalyses the penultimate reaction of the pathway where IGP is converted to indole (IND). The second subunit is the TRP SYNTHASE $\beta 2$ (TSB2) and participates in the conversion of IND into Trp.

At present, Trp is assumed to be the major metabolic precursor of IAA. However, in older literature a direct conversion of the Trp-precursor IND to IAA has been suggested. The suggestion of this so-called Trp-independent pathway is based on metabolite quantification and feeding experiments in Trp biosynthesis mutants (Normanly, Cohent and Fink 1993; Wright et al. 1991). Normanly et al (1993) observed that feeding of labelled anthranilate ($[^{15}\text{N}]$ -

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anthranilate) to an Arabidopsis Trp biosynthesis mutant (*trp2-1*), resulted in a more pronounced ¹⁵N-incorporation in IAA than in Trp. In addition, it has been shown that endogenous amounts of IAA were remarkably higher in Trp biosynthesis mutants than in wild type plants (Kasahara 2015). For this reason, IGP has been proposed as a branch point intermediate in the Trp-independent pathway in Arabidopsis, also because IAA levels were significantly reduced in antisense IGS transgenic plants (Li et al. 1995; Ouyang, Shao, and Li 2000).

However, Müller and Weiler (2000) reported that the Trp-independent pathway was an artefact, as they observed high IGP and glucobrassicin (GB) levels in the Trp biosynthesis mutant *trp3-1*. They suggested that in previous publications IAA was most likely liberated from IGP and GB during IAA quantification, due to alkaline hydrolysis. Moreover, Yu et al. (2015) reported that a significant IAA level could be attributed to protein degradation-derived Trp.

Since then, it has not been conclusively demonstrated whether or not a Trp-independent pathway is operative in plants (Woodward and Bartel 2005). Nevertheless, a coordination of Trp-dependent and Trp-independent pathways could be happening in plants. This coordination theory can be based on the recently reported cytosolic INDOLE SYNTHASE (INS), a close paralog of plastidic TSA1 that functions in the Trp-independent pathway in Arabidopsis (Wang et al. 2015). This enzyme, in charge of converting IGP into IND, seems to have an important role in maintaining proper development during specific stages of plant development, as for instance, the establishment of the apical-basal patterning during early embryogenesis (Kasahara 2015). Nevertheless, Trp is present in cells in much greater amounts than indole and can be used for the rapid production of auxin at critical stages, likely being more important and necessary than indole (Rozov et al. 2013).

Regarding the Trp-dependent branch of IAA biosynthesis, there are different routes whose names are established according to their intermediates considered as hallmarks (Figure 1.5).

Figure 1.5 Proposed pathways of IAA biosynthesis in plants.

Solid arrows indicate the characterized enzymatic reaction steps in plants. Dashed arrows denote proposed steps for which the gene(s) and enzymatic function(s) have not been determined. The thin, grey and dashed arrow appearing in the left part of the figure denotes the Tryptophan-independent IAA biosynthesis pathway. All other arrows indicate steps of Trp-dependent IAA biosynthesis pathways. Red arrows show the reactions catalysed in the chloroplast. Blue arrows highlight the enzymatic reaction catalysed by AMI1 (principal enzyme of study in this thesis). A green circle surrounds the Brassicaceae species-reaction steps. Abbreviations: (TSB2) TRYPTOPHAN SYNTHASE β -SUBUNIT 2; (ISS1/VAS1) INDOLE SEVERE SENSITIVE 1/REVERSAL OF SAV3 PHENOTYPE; (TAA1/TAR2) TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1/TRYPTOPHAN AMINOTRANSFERASE RELATED 2; (YUC) YUCCA; (CYP79B2 CYP79B3) CYTOCHROME P450 79B2 and 79B3; (SUR2) SUPERROOT 2; (RTY1) ROOTY 1; (UGT74B1) UDP-GLUCOSYL TRANSFERASE 74B1; (CYP71A13) CYTOCHROME P450 71A13; (NIT) NITRILASE; (AMI1) AMIDASE1.

However, from all these pathways currently only one is completely disclosed in plants; the indole-3-pyruvic-acid (IPA)-pathway. The IPA-pathway is composed of two reaction steps: firstly, the conversion of Trp to IPA by the activity of a Trp-aminotransferase. Secondly, IPA is transformed into IAA by the virtue of flavin-monooxygenases.

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The Trp-aminotransferase that catalyzes the first reaction is TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1) and its activity is stimulated by the presence of pyridoxal 5'-phosphate (PLP) in the reaction mixture. In fact, this protein belongs to the superfamily of the α class of PLP-dependent enzymes (Stepanova et al. 2008). It was found in different screens for Arabidopsis mutants, i.e. in a screen for Arabidopsis mutants defective in shade avoidance response (SAS), a process in which plants adapt to changes in light quality by elongating stems and petioles (Stepanova et al. 2008; Tao et al. 2008). In addition, it was identified as a mutant resistant to the auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA) (Yamada et al. 2009). For this reason, the mutant received three different names according to the screening in which it was found: *shade avoidance mutant 3 (sav3)* (Tao et al. 2008); *weak ethylene insensitive 8 (wei8)* (Stepanova et al. 2008) or *transport inhibitor response 2 (tir2)* (Yamada et al. 2009). Later on, the corresponding gene was identified by positional cloning and renamed to *TAA1*. *TAA1* was reported to encode a protein homologous to aminotransferases, capable of catalysing the production of IPA from Trp. *In vivo* confirmation was provided by the observation that deficient mutants of the *TAA* family displayed considerably reduced IAA and IPA contents (Mashiguchi et al. 2011; Stepanova et al. 2011; Won et al. 2011). In contrast, induction of *TAA1* expression was shown to increase the endogenous amounts of IPA in Arabidopsis (Mashiguchi et al. 2011). Nevertheless, *TAA1* does not catalyse the rate-limiting step in auxin biosynthesis, because overexpression of *TAA1* under the control of the 35S promoter does not cause auxin overproduction phenotypes (Stepanova et al. 2008; Tao et al. 2008).

Along with *TAA1* there are closely related genes in the Arabidopsis genome. They have been referred to as TRYPTOPHAN AMINOTRANSFERASE RELATED 1 to 4 (*TAR1–TAR4*) (Stepanova et al. 2008). Studies on their function have suggested *TAR1* to be a paralog of *TAA1*, because both enzymes do not contain a N-terminal extension, consistent with their cytoplasmic localization (Stepanova et al. 2008; Tao et al. 2008). On the other hand, the *TAR2–TAR4* proteins contain such a N-terminal extension, suggesting that they most likely locate to the vacuole, similar to the onion or garlic alliinases and having a different function from that of *TAA1* (Stepanova et al. 2008). The second reaction step in the IPA-pathway is catalyzed by a bigger family of flavin containing monooxygenases, referred to as YUCCA enzymes (Mashiguchi et al. 2011; Stepanova et al. 2011; Won et al. 2011; Zhao et al. 2001). There are a total of 11 *YUCCA* (*YUC*) genes in Arabidopsis. Overexpression of each *YUCCA* gene leads to increased levels of IAA and results in the high-auxin phenotypes in Arabidopsis, suggesting that *YUCCA* proteins catalyse a rate-limiting step in IAA biosynthesis (Chen et al. 2014; Cheng, Dai, and Zhao 2006; Mashiguchi

et al. 2011; Novák et al. 2012; Zhao et al. 2001). However, each of them is expressed in a specific pattern and proposed to function redundantly to produce auxin in a spatio-temporally regulated manner during the different stages of plant development (Mcsteen 2010). For instance, *YUC3*, *YUC5*, *YUC7*, *YUC8*, and *YUC9* are expressed in root tissues including the proximal meristem and root caps in *Arabidopsis* seedlings (Chen et al. 2014; Cheng, Dai, and Zhao 2006). In contrast, *YUC1*, *YUC4*, *YUC10*, and *YUC11* are expressed in embryos (Cheng, Dai, and Zhao 2007). These specific expression patterns of the *YUCCA* genes are assumed to highlight the importance of the IPA pathway in different moments of the plant development.

Another completely disclosed Trp-dependent pathway for the biosynthesis of IAA is the IAM-pathway. However, this two-step route is assumed to be active in bacteria, but not in planta. In fact, it was the first auxin biosynthesis pathway characterized in plant pathogenic bacteria from different genera: *Agrobacterium*, *Azospirillum*, *Pseudomonas* and *Streptomyces*. Here, Trp is converted to IAM by a tryptophan-2-monooxygenase, encoded by the *aux1/iaaM/tms1* gene (Camilleri and Jouanin 1991; Gaudin et al 1993; Yamada et al. 1985). In a second step, IAM is transformed into IAA by an IAM-amidohydrolase, encoded by the gene *aux2/iaaH/tms2* (Camilleri and Jouanin 1991; Gaudin et al 1993; Mano et al. 2010; Nemoto et al. 2009a; Nemoto et al. 2009b; Yamada et al. 1985).

For many years, it was believed that the IAM-pathway does not exist in plants and that the observed conversion of IAM into IAA was just the result of bacterial contaminations, although several studies demonstrated the presence of IAM in plants [e.g.: in *Citrus unshui* (Takahashi et al. 1975), *Prunus jamasakura* (Saotome et al. 1993); *Curcubita maxima* (Rajagopal et al. 1994)]. All of those studies reported the presence of IAM in non-sterile plant extracts. This changed, when Pollmann et al. (2002) used sterile-grown *Arabidopsis* plants, providing unequivocal evidence for the occurrence of IAM in plants, refuting the arguments for a possible bacterial origin. By now, the occurrence of IAM in the plant kingdom has been confirmed for other plant species too, e.g. for tobacco, rice, maize and the seaweeds *Ulva fasciata* and *Dictyota humifusa* (Arai et al. 2004; Nemoto et al. 2009b; Stirk et al. 2009; Sugawara et al. 2009).

Although plants have been shown to contain IAM, corresponding enzymes involved in its formation and conversion to IAA remained undetected in plants. In particular the formation of IAM *in planta* remains enigmatic, as bioinformatics studies did not provide sufficient information to identify homologies between the correspondent gene from bacteria, *iaaM*, and any plant pendant (Mano and Nemoto 2012).

In 2003, however, Pollmann et al. were able to identify an IAM-specific amidohydrolase in

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Arabidopsis, referred to as AMIDASE 1 (AMI1), capable of catalysing the second reaction step in the IAM-pathway. AMI1 will be introduced in more detail in the following. Nonetheless, it cannot be excluded that AMI1 may also catalyse a common step of another Trp-dependent pathway: the indole-3-acetaldoxime (IAOx)-pathway.

To all we know today, the IAOx-pathway is restricted to the family of the *Brassicaceae*. This plant family is characterized by the production of a number of specific plant secondary metabolites related to plant defense, such as indole glucosinolates (IG) and the indole alkaloid camalexin (de Vos et al. 2008; Hansen and Halkier 2005; Mikkelsen et al. 2009). So, IAOx-pathway is not considered a common IAA biosynthesis route in plants.

Nevertheless, Irmisch et al. (2015) recently reported that the maize cytochrome P450 CYP79A61 could produce IAOx and another L-phenylalanine-derived compound (phenylacetaldoxime; PAOx) in heterologous systems. This finding suggests that the IAOx pathway might be more widely distributed in the plant kingdom than is currently thought. However, future work on this suggestion must be carried out to further confirm the finding.

In the IAOx-pathway, the first reaction is produced in the chloroplast by two cytochrome P450s enzymes, CYP79B2 and CYP79B3, converting Trp to IAOx (Hull, Vij, and Celenza 2000; Mikkelsen et al. 2000; Zhao et al. 2002). Once diffused to the cytoplasm, IAOx seems to be an important branching point from which a number of biosynthetic routes emerge, among others the biochemically complex formation of IGs.

In the production of IGs, a third cytochrome P450 monooxygenase, CYP83B1 (SUR2), catalyses the conversion of IAOx to its corresponding *N*-oxide, being the first step in IG biosynthesis. Besides its systematic name, CYP83B1, the enzyme possesses several other names, according to the screens in which it has been identified: RED1 (Hoecker et al. 2004), RTN1 (Bak et al. 2001) and ATR4 (Smolen and Bender 2002). The subsequent reaction in IG production is catalysed by SUR1 (Boerjan et al. 1995) also named, ROOTY (King et al. 1995), ALF1 (Celenza, Grisafi, and Fink 1995) or HOOKLESS1 (Lehman, Black, and Ecker 1996). Another identified enzyme that contributes to IG formation is UGT74B1 (Grubb et al. 2004)

IGs are considered potent plant defence compounds that, upon tissue damage, are able to release cyanogenic indole-3-acetonitrile (IAN) by the activity of myrosinases (Halkier and Gershenzon 2006). The same intermediate can be found in the camalexin-pathway where an enzyme, CYP71A13, has been described to convert IAOx directly into IAN (Nafisi et al. 2007). However, most important to IAA biosynthesis is the observation that IAN can be converted to

IAA by the virtue of nitrilases. In Arabidopsis, there are four nitrilases, NITRILASE 1-4 (NIT1-4), but only NIT1-3 seem to be involved in the IAA biosynthesis (Vorwerk et al. 2001). NIT4 has a different function, hydrolysing β -cyanoalanine to aspartic acid and ammonia (Janowitz et al. 2009; Piotrowski, Schönfelder, and Weiler 2001). Nevertheless, the action of NIT1-3 has to be revised because recent studies propose that they are more involved in IG metabolism, camalexin homeostasis and cyanide detoxification rather than in IAA biosynthesis (Nafisi et al. 2007; Su et al. 2011).

For some time, an alternative tryptamine-pathway was proposed to be operative in plants. Tryptamine was believed to directly derive from Trp by the activity of tryptophan decarboxylases (TDCs), before being further shunted into serotonin or indole alkaloids. Although it has been reported that overexpression of *TDC* did not cause higher IAA levels (Songstad et al. 1990), it was also proposed that YUCCA enzymes could catalyse the conversion of tryptamine to N-hydroxyl-tryptamine (another theoretical IAOx precursor) (Zhao et al. 2001). Subsequent studies, analysing the conversion of tryptamine into IAA, however, have drawn this hypothesis into question (Normanly 2010; Quittenden et al. 2009; Zhao 2010).

Alongside to the already mentioned intermediates, indole-3-acetaldehyde (IAAld) was also proposed as a precursor to IAA. IAAld oxidases that are likely responsible for the conversion of IAAld into IAA have been described from several plant species (Akaba et al. 1999; Bower, Brown, and Purves 1978; Sekimoto et al. 1998; Seo et al. 1998). In particular the enzyme ARABIDOPSIS ALDEHYDE OXIDASE 1 (AAO1) was suggested to catalyse this conversion, as the enzyme accumulated in consequence of rising amounts of IAAld in the *sur1* mutant (Seo et al. 1998). However, none of the four Arabidopsis IAAld oxidases (AAO1-4) displayed expression pattern that would suggest a participation in auxin biosynthesis (Seo et al. 2000). More importantly, the null mutant *aaol* does neither display auxin related phenotypes nor reduced IAA contents (Seo et al. 2004), which has drawn the participation of AAOs in auxin formation in question.

Finally, all these IAA biosynthesis pathways must be controlled to assure spatio-temporally controlled auxin biosynthesis. In this control, several transcription factors are assumed to modulate the expression of IAA biosynthesis-related genes. For example, LEAFY COTYLEDON2 (LEC2) is a B3 domain transcription factor that activates the expression of *YUC1*, *YUC2*, *YUC4* and *YUC10* genes in Arabidopsis (Barbara et al. 2013; Stone et al. 2008). Another transcription factor, STY1, member of the SHORT INTERNODES/STYLISH (SHI/STY) transcription factors family, activates the expression of *YUC4* resulting in increased IAA levels in Arabidopsis and

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may also positively regulate to *YUC8* (Eklund et al. 2010). There are also other transcription factors called INDETERMINATE DOMAIN (IDD) 14 and 16 whose targets are *TAA1* and *YUC5* genes. A further example is the so-called PHYTOCHROME-INTERACTING FACTOR (PIF) class of transcription factors. Specifically, PIF4 and PIF5 bind to the promoter of *YUC8*, and PIF7 binds to the promoters of *YUC8* and *YUC9* genes to increase IAA biosynthesis and promote shade avoidance responses including hypocotyl elongation (Kasahara 2015). In contrast, the SPOROCTELESS/NOZZLE (SPL/NZZ) transcription factor, a regulator of sporogenesis, has been shown to negatively regulate *YUC2* and *YUC6* genes (Li et al. 2008).

A combination of genetic, biochemical and metabolomic studies have led to the detection and association of a role for the gene *REVERSAL OF SAV3 PHENOTYPE (VAS1)*. *VAS1* is supposed to catalyse the conversion of IPA into Trp with an amino donor that is preferably L-methionine, establishing that *VAS1* modulates the concentration and lifetime of IPA (Zheng et al. 2013). L-methionine is an essential precursor of ethylene (ET), which settles a link between IAA and ET biosynthesis. It is known that *vas1* is a mutant allele of the *INDOLE SEVERE SENSITIVE 1 (ISS1)* gene and its role in Trp metabolism depends on the tissue and/or developmental stage of the plant (Pieck et al. 2015). This means that in meristematic tissues, where the expression of *TAA1* and *YUCCA* genes is highest, *ISS1/VAS1* modulates levels of IPA by reverting excess IPA into Trp (Zheng et al. 2013). However, in vegetative tissues, where IAA biosynthetic genes are not highly expressed, *ISS1/VAS1* functions in Trp degradation, by converting Trp into IPA (Pieck et al. 2015). Like this, the Trp-IPA regulation by *ISS1/VAS1* triggers the subsequent control of the auxin biosynthesis.

1.9 Amidase Family

The identification of a plant enzyme capable of catalysing the second step of the IAM-pathway has previously been mentioned. It is a homolog to the bacterial *iaaH* gene, identified and cloned from *A. thaliana*. The *in vitro*-analyses confirmed its enzymatic activity as a specific IAM-hydrolase. The enzyme was termed AMIDASE 1 (AMI1) (Pollmann, Neu and Weiler 2003). AMI1 belongs to an amidase signature family (AS), composed of 7 members in Arabidopsis. This family is widespread in nature, appearing not only in bacteria, but also in fungi, mammals and plants. Their members catalyse a huge range of reactions, as for instance the hydrolysis of a number of neuromodulatory fatty acids amides, responsible for a number of different functions, such as analgesia and induction of sleep (Neu et al. 2007).

The amidase family is characterized by a 50-130 amino acid spanning region, which gives the name to the family and is rich in glycine (Gly) and serine (Ser) residues. The catalytic centre is composed by three coordinated amino acid residues: Ser-*cis*Ser-Lys (Figure 1.6, from Neu et al. 2007). In contrast to this common reaction centre, some amidases from bacteria, e.g. from *Rhodococcus rhodochrous* strain J1 and *Sulfolobus solfataricus* seem to use another, alternative reaction centre, Cys-*cis*Ser-Lys (Cilia et al. 2005).

The proper spatial organization and composition of the catalytic triad is so important that the mutation of any of them, especially the serines, fully impairs enzymatic activity, blocking the conversion of IAM to IAA *in vitro*.

Figure 1.6 Amino acid-sequence comparison of AMI1 with five other members of the AS family (Neu et al. 2007).

The sequences are: Rr-J1, amidase from *Rhodococcus rhodochrous* J1 (GenBank D16207); PAM, peptide amidase from *Stenotrophomonas maltophilia* (GenBank CAC93616); MAE, malonamidase from *B. japonicum* (GenBank AF012735); RnFAAH, fatty acid amide hydrolase from *Rattus norvegicus* (GenBank NM_024132); AtFAAH, fatty acid amide hydrolase from *A. thaliana* (GenBank NM_125840); AMI1, amidase 1 from *A. thaliana* (GenBank NM_100769). Amino acid-positions are indicated. Conserved regions are boxed in grey. Proposed active-site residues are marked with asterisks. Secondary structure-prediction (PSIPRED [49]) of AMI1 is depicted above the aligned sequences. Arrows denote β -strands, barrels α -helices. The amidase consensus sequence motif (PS00571) as deposited in the PROSITE dictionary is underlined.

AMI1 from *Arabidopsis* is a cytosolic enzyme of 45 kDa that, as with other amidases, has a conic tri-dimensional structure composed by a central 11 β -sheet core covered with a layer of α -helices. Inside, several channels can be found. It is suggested that IAM enters by one of those channels and IAA and ammonium, the reaction products, exit on the opposite side of the channel. The hydrolysis is carried out in presence of water, which also enters the interior part

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of AMI1 through another pore of the molecule (Lehmann et al. 2010) (Figure 1.7, from Lehmann et al. 2010).

Unlike to other amidases, however, AMI1 does not tend to form dimers. Several essays including blue native gel electrophoresis (Pollmann, Neu and Weiler 2003), yeast two-hybrid interaction studies, and bimolecular fluorescence complementation experiments (BiFC) have discarded a dimerization of AMI1 (Neu et al. 2007). Moreover, AMI1 differs from the other amidases in its activity. Several AS family members are characterized by enzymatic bi-functionality showing the intrinsic amidase and additional esterase/peptidase activities. This is not the case for AMI1, which is seemingly unable to cleave ester bounds, as neither IAA-methyl ester nor IAA-glucosyl ester appeared to be suitable substrates for the enzyme.

Figure 1.7 The putative AtAMI1 reaction centre (Lehmann *et al.*, 2010).

Homology-based modelling was used to gain first insight into the putative structure and reaction mechanism of AMI1-like proteins. (A) The residues of the catalytic Lys³⁶-Ser¹¹³-Ser¹³⁷ triad are found in close vicinity to each other on the base of a cavity, which is connected with several channels that have been suggested to be involved in substrate and water supply as well as in product removal. (B) The conserved residues in the surrounding of the catalytic triad are highlighted in the model. Amino-acid positions are numbered according to the AtAMI1 full-length protein. Channels adjacent to the catalytic center are marked with red arrows. Calculated distances between the three amino-acid residues of the catalytic triad are given in Ångström.

Conclusively, it can be remarked that AMI1 from Arabidopsis and a close relative from tobacco, NtAMI1 (Nemoto et al. 2009b), share singular features within the AS family. AMI1 is a well-characterized enzyme whose function and main substrate have been studied *in vitro*.

Nonetheless, the question on its distribution in the plant kingdom and on how AMI1 unfolds its activity *in vivo* remains elusive. The precise participation of AMI1 in IAA biosynthesis is not totally clear, too, since it is not known whether the catalysed reaction forms part of an independent route or represents a complementary step of a bacteria-like IAM pathway in plants. Over time, several theories have been postulated: one of them proposed that nitrilases may be involved in the production of IAM as a by-product of their bi-functional activity. This would place AMI1 down-stream of the nitrilases (Pollmann et al. 2002). Another hypothesis suggests the possibility that AMI1 belongs to a so-called IAA synthase complex that catalyzes the conversion of Trp to IAA in a channelled reaction without the release of any detectable intermediate (Pollmann et al. 2009). Finally, a further hypothesis associates the AMI1 reaction with the IAOx-pathway (Sugawara et al. 2009) placing AMI1 down-stream of CYP79B2 and CYP79B3.

In order to address these remaining questions concerning AMI1 and its participation in IAA biosynthesis *in planta*, the presented study has been undertaken.

2 OBJECTIVES

OBJECTIVES

With respect to the powerful molecular biological and genetic tools *Arabidopsis thaliana* has been chosen as the plant system in this study. The availability of the complete genome sequence, the short generation times and the comparably easy genome organisation gave further reason to make this choice.

The major goal of my thesis is the disclosure of the physiological function of AMI1. Central to the presented work was the question whether AMI1 is involved in biosynthesis of auxin *in vivo* and how AMI1 is implemented into the complex network of auxin synthesis routes.

In order to comply with the set goal described above, the following objectives have been proposed:

- Distribution of AMI1-like enzymes in the plant kingdom.
- Comparison of *Arabidopsis* AMI1 with selected amidases from other plant species.
- Study of *AMI1* gene expression and *in vivo* function.
- Determination of the connection between AMI1 and other proteins related with the biosynthesis of IAA.
- In-depth analysis of the connection between the IAM- and IAOX-pathway.
- Query for novel molecular targets involved in IAA biosynthesis.

3 MATERIAL AND METHODS

3.1 Chemicals

Ampicillin	Duchefa, Haarlem, NL
Agarose	Conda, Madrid
Alkaline phosphatase	Conda, Madrid
Bacto-agar	Conda, Madrid
Bacto-trypton	Conda, Madrid
BASTA (Phosphinothricin)	Hoechst, Frankfurt, Germany
5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal)	Promega, Madison, USA
5-Bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc)	Duchefa, Haarlem, NL
Bromophenol blue	Sigma, München
CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate)	Thermo Scientific, Waltham, Massachusetts, USA
Coomassie Brilliant Blue G-250	Bio Rad, Hercules, California, USA.
Diethyl pyrocarbonate (DEPC)	Sigma, München
dNTPs (dATP, dCTP, dGTP, dTTP)	Takara Clontech, Saint-Germain-en-Laye France
Ethidium bromide	Sigma, München
Ethylenediaminetetraacetic acid (EDTA)	Sigma, München
Gene Ruler Mix (DNA)	Thermo Scientific, Waltham, Massachusetts, USA
Hygromycin	Duchefa, Haarlem, NL
Isopropyl- β -D-thiogalactopyranoside (IPTG)	Promega, Madison, USA, Madison, USA
Kanamycin	Duchefa, Haarlem, NL
Light Cycler 480 SYBR-Green I Master	Roche Diagnostics
2-(N-Morpholino) ethanesulfonic acid (MES)	Panreac Química, S.L.U., Barcelona.
Sodiumdodecylsulfate (SDS)	Applichem, Darmstadt
Octylphenol-polyethyleneglycolether (Triton X-100)	Sigma, München
Proteins for blotting	Powerplay, Isostar
Rifampicin	Duchefa, Haarlem, NL
RNasin RNase Inhibitor	Promega, Madison, USA
RQ1 buffer and enzyme	Promega, Madison, USA
Sephadex G50	GE Healthcare Europe, München
Silwet L-77	Lehle Seeds, Round Rock, USA
Spermidine	Sigma, München

TRIzol® reagent	Invitrogen, Paisley, UK
Tris-(hydroxymethyl)-aminomethane (Tris)	Duchefa, Haarlem, NL
Tween 20 Polyethylene glycol sorbitan monolaurate	Sigma, München
Yeast extract	Conda, Madrid

3.2 Enzymes

Gateway® LR Clonase™ II Enzyme Mix	Invitrogen, Paisley, UK
M-MLV Reverse Transcriptase	Promega, Madison, USA
PCR buffer; DNA polymerases	Roche Diagnostics
Platinum Taq DNA polymerase	Takara, Saint-Germain-en-Laye France
Restriction enzymes	Invitrogen, Paisley, UK Fermentas, St Leon-Rot, Germany
T4 DNA ligase	New England Biolabs, Frankfurt am Main, Germany Takara Clontech, Saint-Germain-en- Laye France

3.3 Antibodies

Antibodies	Antigen	
Mouse and rabbit (Fc) AP Conjugate	Heavy and light chain mouse/rabbit immunoglobulins	Promega, Madison, USA
α -AMI1 (rabbit-serum)	Native AMI1 protein heterologous expressed	Neu (2004)
α -c-myc-Mouse-IgG	c-myc epitope (EQKLISEEDLN)	Klon9.E10; Evan et al. 1985

3.4 Nucleic acids

3.4.1 Oligonucleotides

Oligonucleotides or primers used for conventional PCR were designed with the program Oligocalc: Oligonucleotide properties calculator (Kibbe WA, 2007); those used for qRT-PCR were elaborated with the program QuantPrime (Arvidsson et al. 2008). Primers to amplify genes from SALK lines were designed with T-DNA primer design program (Genome Express Browser Server (GEBD), 2005). In table 2.1, all oligonucleotides used in this work are listed. Primers were generally supplied by Sigma-Aldrich with the exception of those used for direct

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GATEWAY cloning, which were provided by Invitrogen (Paisley, UK).

Table 3-1 List of oligonucleotides used in this thesis.

Existing restriction sites are underlined. Abbreviations are set in accordance with IUPAC nomenclature for nucleic acids.

Primers for conventional PCR

Name	Sequence (5'-3')	Restriction sites
RTY-fwd	ATGAGCGAAGAACAACCACAC	-
RTY-rev	TTACATTTTCGAGATTATTATCAC	-

Primers to analyse zygosity of SALK lines

Name	Sequence (5'-3')	Restriction sites
pROK-LBa1	TGGTTCACGTAGTGGGCCATC	-
CYP71B22-1LP	AAATCACGGAACAAGATCTCG	-
CYP71B22-1RP	AAAAGCTTGCATGAGTTGGTG	-
CYP71B22-2LP	CTAGCCAGACATCCGAGAGTG	-
CYP71B22-2RP	TGGCCTGCTTACTAAATGTGG	-
CYP709B3LP	CATGAGTAGCGAAACAGGTC	-
CYP709B3RP	TTAATCACGGGTCCGTACAG	-
LP-AtAmi1-1	TGGCTTACAGTTCAATTTTACA	-
RP-AtAmi1-1	TCGATAGGGCCATGAGAGACG	-
LP-AtAmi1-2	CTGTGGGATTTTCGTTTCCG	-
RP-AtAmi1-2	CCAACACACCCTTCTCCTACAAAA	-
LP-AtAmi1-3	CTATCGAGTTCAATGAGATTGTTACAAAGG	-
RP-AtAmi1-3	TATAAAAATTATCTTTTCACAAGACCTC	-

Primers for Cloning

Name	Sequence (5'-3')	Restriction sites
OsAMI1-SacI-His2C-For	TAT <u>GAGCTCT</u> ATGGCGATGGCGGGTGGAG	SacI
OsAMI1-KpnI-His2C-Rev	TAT <u>GGTACCGT</u> GATTGGAGGACCAAGTTTTAG	KpnI
SbAMI1-SpeI-pUC-For	TATA <u>CTAGT</u> ATGGCGATGGGCGGCGATTAC	SpeI
SbAMI1-SmaI-pUC-Rev	TAT <u>CCCGGGG</u> GAGAGAGGAGTCTGGTGAGC	SmaI
MtAMI1-XhoI-His2B-For	TAT <u>CTCGAG</u> ATGGAAACAGCCTCAGACTATG	XhoI
MtAMI1-XbaI-His2B-Rev	TATT <u>CTAGAT</u> ATTTTTCAATGTTATCATAAATACTC	XbaI

<i>PtAMI1</i> - XhoI-His2B- For	TAT <u>CTCGAGAT</u> TGGAACGAGACCCGGATTATG	XhoI
<i>PtAMI1</i> - XbaI-His2B- Rev	TAT <u>CTAGAT</u> ATTTTTTCAGTGATCTCAACCTG	XbaI
AMI1-XhoI- pER8-For	TAT <u>CTCGAGAT</u> TGGAGGAGCAGAAGCTGATCTCAGAG	XhoI
AMI1-SpeI- pER8-Rev	TAT <u>ACTAGTT</u> CAAATAAATGCAGCAAGGGAATC	SpeI
FwdCYP71B 22-pDonr	<u>GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGTCGATCTCTC</u> TCTATTT*	-
RevCYP71B2 2-pDonr	<u>GGGGACCACTTTGTACAAGAAAGCTGGGTTTCACCACTGATGTG</u> GAGTTG*	-

Primers for qRT-PCR

Target Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>AtAMI1</i>	TGCATTCCGTCTCTCATGGCC	CGGCAATGGTCGATCTTCTCG
<i>UBQ10</i>	TTGGAGGATGGCAGAACTCTTGCT	AGTTTTCCCAGTCAACGTCTTAAC GAAA
<i>APT1</i>	TCGTGCTGTTCTTGCAACCG	GCGGAGGAGAAGAGGCGGAGT

* Specific recombination sites, attB, are underlined.

3.4.2 Vectors

Name	Use /Origin
pGEM®-T	Cloning / Promega, Madison, USA
pSP-EGFP	Cloning / Pollmann et al. 2006
pUC-GFPn	Cloning / Lehmann and Pollmann 2009
pBluescript SK(+)	Cloning / Agilent Technologies
pSP-AMI1-GFP	Cloning / Pollmann et al. 2006
pTrcHis2A	Prokaryotic expression / Invitrogen, Paisley, UK, Paisley, UK
pTrcHis2B	Prokaryotic expression / Invitrogen, Paisley, UK, Paisley, UK
pTrcHis2C	Prokaryotic expression / Invitrogen, Paisley, UK
pAMI1::GUS	Crossings / Neu 2008
pYUC9ox	Crossings / Hentrich et al. 2013
pER-AMI1ind	Analysis / Lehmann 2009
pGBKT7-AMI1	Cloning / Lehmann 2009
pQE-AMI1	Analysis / Neu et al. 2007

3.5 Biological material

3.5.1 Plant material and plant growth conditions

All experiments were carried out using *Arabidopsis thaliana* (L.) Heynh. ecotype Col-0 (from Nottingham Arabidopsis Stock Centre (NASC, stock N1092), *Oryza sativa* (L.) cv. Millin (obtained from Yanco Rice Research Institute, Yanco, NSW, Australia), *Sorghum bicolor* (L.) Moench var. Redland, *Medicago truncatula* (Gaert.) cv. Jemalong A17, or *Populus trichocarpa* (Hook.) (Botanical Garden, Ruhr-University Bochum, Germany).

The T-DNA insertion mutants for *AtAMI1* (At1g08980), in particular *ami1-2* and *ami1-3* (SALK_019823 and SALK_049701, respectively), and the corresponding T-DNA lines, *CYP71B22-1* (SALK_047773) *CYP71B22-2* (SALK_129670) and *CYP709B3* (SALK_011121) from the SALK collection were obtained from the NASC. In addition, Dr. Henrik Aronsson (University of Gothenburg, Sweden) kindly provided the line *ami1-1* (SALK_069970). A further ethylmethane sulfonate (EMS) null mutant for the gene At2g20610, *rt1*, was obtained from the NASC, stock number N8156. *AMI1* inducible lines (*AMI1ind*), constitutive *AMI1* overexpressor lines (*AMI1ox*), the overexpressor lines for *AMI1* with an internal mutation in its catalytic center (*AMI1^{mut}ox*) and the plants containing the construction *pAMI1::GUS* were obtained from a previous study (Neu 2008).

The *DR5::GUS* line, the *iaaMox* line, the *YUC-ox* line, the *cyp79b2 cyp79b3* double mutant, *rt1* as well as the *taa1* and *taa1 tar2* mutants have previously been described and were obtained from the corresponding laboratories (Barlier et al. 2000; Delarue et al. 1998; Klee et al. 1987; Romano et al. 1995; Stepanova et al. 2008; Ulmasov et al. 1997; Zhao et al. 2002; Zhao, Christensen, et al. 2001). The *NIT1ox* line was obtained in a previous study (Pollmann 1999). The overexpressor line *pTAA1::GFP:TAA100.2* and the *NIT2RNAi* knockdown line were provided by Dr. Anna Stepanova and Dr. Markus Piotrowski respectively.

After surface sterilization of the seeds was carried out using 70 % ethanol (v/v), sodium hypochlorite at 5 % (v/v) and sterilized water, *Arabidopsis* plants were grown under sterile conditions in solidified half-strength Murashige and Skoog (MS) medium supplemented with vitamins and 1 % sucrose in Petri dishes (Murashige and Skoog 1962). Following stratification (4 d, 4 °C), the plates were moved to a growth cabinet maintained at 16/8 h light/dark photoperiod and 22 °C. In rare cases, the stratification and growth was directly carried out in soil composed by 3:1 peat/vermiculite.

Leaf material from poplar plants was directly taken from mature trees grown in their natural environment, *S. bicolor* and *M. truncatula* seeds were sown onto a mixture of soil and sand

(2:1) and cultivated in a greenhouse at 22 to 24 °C during daytime and 18 to 20 °C over night (o/n), with a 16/8 h light/dark cycle. Rice was grown in MS medium containing 3 % sucrose (w/v) and 0.3 % gelrite (w/v) (Duchefa, Haarlem, NL). The photosynthetically active radiation was no less than $150 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Supplementary light, if required, came from sodium-vapor lamps.

For the exploration of Arabidopsis embryos, seeds were imbibed in water at 4 °C for one night. The next day, embryos were extracted from seeds by gently pushing their testa following the extraction process under a binocular (Leica MZ10F).

3.5.2 Plant treatments

With the objective to study the impact of various indolic compounds on the expression of *AMI1*, 100 mg of 14-days old Arabidopsis seedlings were treated with either indole-3-acetamide (IAM), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-pyruvic acid (IPA), indole-3-acetonitrile (IAN) or a mock control [0.5 % methanol (v/v) in liquid MS medium]. The different indolics were dissolved in MeOH and finally diluted in liquid MS medium to carry out the experiments. Concentrations varied with respect to the assay (20 μM for qRT-PCR experiments and 100 μM for RNA-Seq analysis). Treatment conditions were for qRT-PCR and RNA-Seq experiments of 3 h and 2 h, respectively, at RT in darkness, since some of the compounds are UV sensitive. After the incubation, the compound solution was discarded, seedlings thoroughly rinsed and dried on white paper, before they were frozen in liquid nitrogen and stored at -80 °C until their use. In some cases the effect of IAA and IAM, respectively, on the root system was analysed. For this purpose, plants were grown on vertical plates and root architecture was analysed after approximately 2 weeks. Compound concentrations varied from 0.01 to 200 μM . All assays were carried out in triplicate.

Regarding the analysis of *AMI1* expression by qRT-PCR in plants treated with different sugar concentrations, Arabidopsis thaliana seedlings (ecotype Columbia-0) were grown in liquid culture for 7 days on $\frac{1}{2}$ MS medium containing 0.5% glucose in constant light. After 7 days of growth, the medium was replaced with glucose-free medium for 24 h, and then seedlings were treated with 3% glucose, 3% mannitol, 3% glucose + 10 μM ABA or 3% mannitol + 10 μM ABA, and sampled at 6 h after treatment. Three independent sets of cultures were sampled for RNA isolation.

3.5.2.1 Induction of gene expression with β -estradiol

As previously mentioned in section 2.5.1, inducible *Arabidopsis* lines (AMI1ind) were used in this work. These lines contain the *AMI1* cDNA under the control of a β -estradiol inducible promoter (Zuo, Niu, and Chua 2000), conferring high expression of the transgene in the presence of the inductor. When these lines were employed, they had to be treated with β -estradiol in order to induce expression of the transgene. The steroid was diluted in MeOH and used according to the protocol of Zuo and colleagues (2000).

3.5.3 Bacteria strains

Cloning of all the vector constructs used in this thesis, and its subsequent purification, was carried out in *Escherichia coli*, strain DH5 α . Empty GATEWAY cloning vectors were maintained in the *E. coli* strain DB3.1. Recombinant protein expression was carried out in *E. coli* M15 [pREP4] or BL21-AI. Finally, *Agrobacterium* strain C58C1 was used to transform *Arabidopsis* plants (Table 2.2).

Table 3-2 Bacterial strains.

Bacteria strain	Genotype	Origin
<i>Escherichia coli</i>		
DB3.1	F ⁻ , <i>gyrA</i> 462, <i>endA</i> 1, <i>glnV</i> 44, Δ (<i>sr1-recA</i>), <i>mcrB</i> , <i>mrr</i> , <i>hsdS</i> 20(<i>r</i> _B ⁻ , <i>m</i> _B ⁻), <i>ara</i> 14, <i>galK</i> 2, <i>lacY</i> 1, <i>proA</i> 2, <i>rpsL</i> 20(<i>Smr</i>), <i>xyl</i> 5, Δ <i>leu</i> , <i>mtl</i> 1	Invitrogen, Paisley, UK Bernard and Couturier (1992)
DH5 α	F ⁻ , <i>endA</i> 1, <i>glnV</i> 44, <i>thi</i> -1, <i>recA</i> 1, <i>relA</i> 1, <i>gyrA</i> 96, <i>deoR</i> , <i>nupG</i> , <i>purB</i> 20, ϕ 80 <i>dlacZ</i> Δ M15, Δ (<i>lacZYA-argF</i>)U169, <i>hsdR</i> 17(<i>r</i> _K ⁻ <i>m</i> _K ⁺), λ ⁻	Thermo Scientific, Waltham, Massachusetts, USA
M15 [pREP4]	Nals, str ^s , rif ^s , <i>lac</i> , <i>ara</i> , <i>gal</i> , <i>mtl</i> , F ⁻ , <i>recA</i> ⁺ , <i>uvr</i> ⁺ , [pREP4, <i>lacIq</i> , Km ^r]	Qiagen, Hilden, Germany
BL21-AI	F ⁻ , <i>ompT</i> , <i>hsdS</i> _B (<i>r</i> _B ⁻ <i>m</i> _B ⁻), <i>gal</i> , <i>dcm</i> , <i>araB::T7RNAP-tetA</i>	Invitrogen, Paisley, UK
<i>Agrobacterium tumefaciens</i>		
C58C1	T-DNA, vir ⁺ , Rif ^r , gen ^r , [pMP90]	Koncz and Schell, 1986

To grow bacteria, the different strains were inoculated in lysogeny broth (LB) liquid medium (10 % tryptone (w/v), 5 % yeast extract (w/v), 10 % NaCl (w/v)) and incubated under agitation in a shaker at 180 rpm; or on LB solid medium (including 15 g/L agar). In both cases, the corresponding amount of antibiotics was added, in order to add selection pressure (Duchefa, Haarlem, NL; Table 2.3). Incubation was always at 37 °C during 16-20 h for *E. coli* and at 28 °C during 36 h for *A. tumefaciens*.

Table 3-3 Concentrations of used antibiotics in that thesis.

Antibiotic	<i>E. coli</i>	<i>A. tumefaciens</i>	Plants
Ampicillin	100 μ g/ml	—	
Kanamycin	50 μ g/ml	—	
Rifampicin	—	100 μ g/ml	
Hygromycin			15 μ g/ml

3.5.4 Preparation of competent cells

Chemically competent cells from *E. Coli* DH5 α and M15 were prepared according to the methods described by Inoue and colleagues (1990), with minor changes. Briefly, one individual colony was grown in LB liquid medium over a period of 16-20 h at 37 °C. Next, a dilution of 1:2500 from the previous saturated culture was inoculated in an Erlenmeyer-flask containing SOB medium (2 % tryptone (w/v), 0.5 % yeast extract (w/v), 10 mM NaCl (w/v), 2.5 mM KCl (w/v), 10 mM MgCl₂ (w/v), 10 mM MgSO₄ (w/v)) without selection pressure. The bacteria were kept at 25 °C, 130 rpm, over 16 h until they reached an OD_{600 nm} of 0.55-0.64. In that moment, bacterial growth was stopped on ice and the cells were harvested by centrifugation at 4 °C, 10 min, and 5000 rpm. After that, they were resuspended in 80 ml of TB buffer (10 mM PIPES, 15 mM CaCl₂, 250 mM KCl, pH 6.7) supplied with 55 mM MnCl₂ and incubated in an ice bath inside of a cold chamber (4 °C) for 10 min. Thereafter, they were centrifuged under the same conditions as before and the pellet was resuspended in 18.6 ml of TB, 55 mM MnCl₂ and 1.4 ml DMSO. After a brief manual agitation, cells were incubated on ice for another 10 min, before being divided in aliquots of 210 μ l and frozen in liquid nitrogen. Finally, they were stored at -80 °C until their use.

In order to obtain electrocompetent *E. coli* cells, a slightly adjusted protocol was used, employing different buffer systems. In brief, after growing the cells to an OD_{600 nm} of 0.55-0.64 and harvesting the bacteria by centrifugation (see above), they were resuspended in 100 ml of cold sterile milliQ water. Subsequently, they were again centrifuged under the same conditions. The last two steps were repeated twice. The final pellet was resuspended in 10 ml of 10 % glycerol (v/v) and it was again centrifuged in a new tube for 10 min at 5000 rpm to discard the supernatant. These last two steps were conducted twice. Next, the pellet was resuspended in 200 μ l of 10 % glycerol (v/v) by gentle pipetting. Finally, cells were divided in aliquots of 50-100 μ l volumes and frozen in liquid nitrogen. Finally, they were stored at -80 °C until they were used.

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Chemical competent *A. tumefaciens* C58C1 cells were obtained according to Kahl and Meksem, (2008). An individual colony was selected and grown in 10 ml of LB liquid medium in the presence of rifampicin at 28 °C o/n. The next day, the 10 ml were transferred to an Erlenmeyer flask containing 100 ml of LB containing rifampicin. The bacteria were grown until they reached an OD_{600 nm} of 0.8. Then, cell growth was stopped by transferring the bacteria to an ice bath, before centrifuging them for 5 min at 5000 rpm. The supernatant was discarded and the pellet was resuspended in 20 ml of 150 mM NaCl. Once more, cells were centrifuged in the same conditions, but now the pellet was resuspended in 2 ml of cold 20 mM CaCl₂. Ultimately, aliquots of 50 μ l were taken and frozen in liquid nitrogen. Aliquots were stored at -80 °C until been used.

3.5.5 Transformation of competent cells

Introduction of vector DNA into chemically competent *E. coli* cells was achieved according to the method of Inoue, Nojima, and Okayama (1990) with some changes. In brief, approx. 5-10 ng of DNA was added to an aliquot of competent cells (c.f. section 2.5.4). The mixture was incubated for 30 min on ice before being subjected to a 90 sec heat shock at 42 °C. Next, they were again incubated on ice over 2 min. After that, 700 μ l of LB liquid medium were added and the bacteria were incubated under constant agitation at 180 rpm, 37 °C, during 90 min. Thereafter, the bacteria were sedimented by centrifugation (4 min, 13000 rpm) and around 90 % of the supernatant was discarded. The remaining 10 % of the solution was used to redissolve the bacterial pellet. The dissolved bacteria were finally spread over petri dish containing LB solid medium supplemented with the corresponding antibiotic for the specific selection. Plates were incubated o/n at 37 °C to obtain individual colonies.

In case of transforming electrocompetent cells, the protocol according to the method of Ausubel et al. (2003) was used. Here, the way to introduce DNA into the cells is by electroporation and not by heat-shock. In brief, after the plasmid DNA was added to the cells, the mixture was incubated on ice for 30 min. Then, cells are electroporated by applying high voltage using an ECM® 630 Square Wave Pulse generator in combination with a wide array of BTX® specialty electrodes. The employed conditions were the following: 2500 V; 200 Ω and 25 μ F. Once electroporation had been carried out, 500 μ l of LB liquid medium were added and the mixture was incubated at 37 °C for 1 h under constant agitation. Then, the bacteria were centrifuged 4 min at 13000 rpm to discard the majority of the supernatant. The remaining liquid was used to dissolve the pellet and finally, it was spread over the correspondent petri dish with LB solid medium and specific antibiotics selecting for the introduced plasmid. Plates were incubated o/n at 37 °C.

To transform *A. tumefaciens* competent cells, an aliquot of cells was thawed on ice and 1 µg of vector DNA was added. The bacterial solution was shook over 5 min at 37 °C before being incubated on ice for 30 min. After that, 500 µl of LB liquid medium were added and the mixture was incubated at 28 °C, 180 rpm for 2-4 h. Then, the cells were precipitated (4 min, 13000 rpm) and approximately 90 % of supernatant was discarded. The remaining solution was used to dissolve the pellet. The bacteria were spread on petri dishes containing solidified LB medium supplemented with rifampicin and the specific antibiotic for the vector DNA introduced. In the end, bacteria were incubated for two days at 28 °C in order to facilitate colony growth.

3.6 Extraction of nucleic acids in Arabidopsis

3.6.1 Genomic DNA extraction

Arabidopsis seedlings subsequently used for total DNA extraction were grown two- to three-weeks on ½ MS medium plates under environmentally controlled conditions. Either around 100 mg of plant tissue or 20-25 mg of surface sterilized seeds was used for the extraction. Except of some minor modifications, the extraction procedure to obtain genomic DNA followed a previously published method (Edwards, Johnstone, and Thompson 1991).

Firstly, 400 µl of extraction buffer (200 mM Tris HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5 % SDS (w/v)) were added to the plant material. Next, the plant tissue was ground with a sterile pestle until a homogenous mix was obtained. All steps were carried out on ice. Subsequently, samples were centrifuged at 13000 rpm, 5 min at room temperature (RT). Thereafter, 300 µl of the supernatant were transferred into a clean reaction vessel and mixed with 300 µl of 2-propanol. The contained double-stranded nucleic acid chains were precipitating at -20 °C o/n. After one further centrifugation step of 20 min (13000 rpm) at RT, the supernatant was discarded. The pellet was washed with 300 µl of 70 % EtOH (v/v) followed by a centrifugation of 5 min at maximum velocity (13000 rpm). The supernatant was discarded and the remaining pellet taken to complete dryness. The remaining pellet was finally dissolved in 60 µl of distilled water.

3.6.2 RNA extraction

Arabidopsis seedlings of various developmental stages (according to the corresponding experiment) were frozen in liquid N₂ and ground with sterile pistils and mortars.

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RNA samples were extracted following the method described by Oñate-Sánchez and Vicente-Carbajosa (2008). To do so, samples were treated with 600 µl of phenol and 600 µl of extraction buffer (200 mM Tris HCl (pH 9); 0,4 M LiCl; 25 mM EDTA; 1 % SDS (w/v)). Then, samples were vortexed twice and centrifuged 2 min at 13000 rpm. The centrifugation separated the mixture in three phases: an organic phase containing proteins, an interphase, usually white, containing the DNA, and a colourless upper aqueous phase (containing RNA). That last one was transferred to a new clean tube and mixed twice with 600 µl phenol by vortexing. Again, samples were centrifuged 2 min at 13000 rpm, and the upper aqueous phase obtained in this step was transferred into a clean tube, avoiding transferring the DNA-containing interphase. Subsequently, 600 µl chloroform were added to the samples, shook twice and centrifuged 2 min at 13000 rpm. The volume of the resulting supernatant was estimated using a pipette before being transferred to another tube. The estimation of volume for each sample was used to calculate the exact quantity of 8 M LiCl to be added. It always corresponds to a 1/3 of the obtained volume. Once 8M LiCl was added, samples were vortexed twice, and stored on ice at -4 °C over 2 h or more.

After that time, samples were centrifuged 30 min at 4 °C and 13000 rpm. The supernatant was discarded and the pellet resuspended in DEPC treated water (300 µl), 30 µl of 3 M sodium acetate and 600 µl 100 % EtOH (v/v). Reaction mixtures were inverted manually four times and left o/n at -20 °C. The next day, after one centrifugation of 20 min, 13000 rpm at 4 °C, the supernatant was carefully discarded. Subsequently, the were washed with 150 µl of ice cold 80 % EtOH (v/v), before the pellet was dried at RT. Finally, the sediments were dissolved in 30 µl of DEPC treated water.

3.6.3 Complementary DNA (cDNA) preparation

To obtain cDNA, 2 µg of total RNA of the corresponding sample (green leaves or 10-days old seedlings from *A. thaliana*) were converted into cDNA. The reverse transcription reaction was carried out using the M-MLV reverse transcriptase (Promega, Madison, USA) and Oligo-dT₁₈ primers (Fermentas, Burlington, Canada) according to the manufacturer's instructions.

3.7 Quantification of nucleic acids

The concentration of nucleic acids was quantified by UV absorbance at 260 nm. Measurements were made using a Nanodrop® ND-1000 Spectrophotometer V3.1.2 (NanoDrop Technologies, Wilmington, USA). One absorbance unity at 260 nm is equivalent to 40 µg/µl of RNA and 50 µg/µl of DNA, respectively. Absorbance at 280 nm is indicating possible contaminations with

proteins or phenolic compounds. In order to ensure a preparation of sufficient quality, the absorbance ratio of 260/280 must be in the range of 1.8 to 2.0 (Sambrook and Russell 2001).

3.8 Quantitative and qualitative amplification of nucleic acids

3.8.1 Polymerase Chain Reaction (PCR)

To amplify DNA fragments by routine PCR, an enzymatic reaction was prepared with the following elements:

Components	Stock Concentration	Volume/Reaction
Reaction Buffer	10 X	5 μ l
dNTPs	10 mM	1 μ l
Forward Primer	10 μ M	1 μ l
Reverse Primer	10 μ M	1 μ l
DNA polymerase	5U/ μ l	0.5 μ l
Template DNA	50-100 ng/ μ l	1 μ l
DEPC ultrapure water	–	40.5 μ l
		Final Volume
	–	50 μ l

Standard amplification conditions were the following: 4 min of initial denaturation at 94 °C, followed by 35 cycles of 20 s at 94 °C for denaturation, 45 s at 54-62 °C allowing primer annealing (exact values are given with the experiments), 1 min/kb at 72 °C for extension, and a final extension at 72 °C for 7 min. Reactions were carried out in a *Primus 25 advanced* thermal cycler (peqLab, Erlangen, Germany). The sequence integrity of all DNA fragments designated for further cloning steps was verified by commercial sequencing (c.f. section 2.11.4).

3.8.2 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA and cDNA from 100 mg of starting material were prepared as described above. For qRT-PCR analysis, all experiments were carried out in triplicate using a LightCycler 480[®] II machine (Roche Diagnostics, Mannheim, Germany). The amplification of the cDNA was performed with gene specific primers for *AMI1* and for the housekeeping genes *UBQ10* and *APT1* (Czechowski et al. 2005; Gutierrez et al. 2008) used for normalizing expression levels (section 3.4.1, Table 3.1).

The cycling conditions employed were the following: pre-incubation of 10 s at 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C. Thereafter, melting curves were

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acquired by incubating the reaction mixtures for 5 s at 95 °C, 1 min at 65 °C, followed by heating up until 97 °C and a cooling step of 30 s at 40 °C.

SYBR Green I (Roche) was employed to monitor the kinetics of the PCR product in the qRT-PCR. Data analysis was conducted using the Light Cycler® 480 software version 1.5 SP4. The amount of the respective transcript was determined for at least two individual mutant lines; the normalized expression ratio of **AMI1** was calculated as the fold increase/decrease relative to the corresponding expression in control plant tissue ($2^{-\Delta \Delta C_t}$ method; Pfaffl 2004).

3.8.3 Gel electrophoresis and visualization of nucleic acids in agarose gels

DNA fragments were separated in agarose gels (0.8-1.5 % (w/v), according to fragment sizes to separate) in 1X TAE buffer (40mM Tris-Acetate; 1 mM EDTA; pH 8) and ethidium bromide dye at 0.02 % (v/v).

RNA samples were separated in 1 % agarose gels (w/v) under denaturing conditions, with MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA; pH 7), 0.02 % ethidium bromide (v/v) and 16 % formaldehyde (v/v).

The nucleic acids were visualized using UV light. Size estimation was made on the basis of commercial standards [GeneRuler Express DNA Ladder (#SM1551) and GeneRuler DNA Ladder Mix (#SM0331) of Thermo Scientific].

3.9 Transcriptomic analyses

3.9.1 Analysis of mRNA expression levels by microarray

Plants of the genotype Col-0, AMI1ind, *ami1-2*, and the *cyp79b2 cyp79b3* double mutant, were grown for 10 days under sterile conditions on MS medium. Approximately 100 mg have been weighed into micro reaction tubes. The material was frozen in liquid N₂ and stored at -80 °C until its further use. The RNA extraction was achieved as described in the section 3.6.2. Afterwards, the total RNA was subjected to further purification using the clean-up protocol of the RNasy Plant Mini Kit (Qiagen, Hilden, Germany), to obtain highly pure RNA.

Assessment of RNA purity and quantity were checked by gel electrophoresis on 1 % (w/v) agarose gels and absorbance measurement (Nanodrop® ND-1000 Spectrophotometer V3.1.2). In addition, the quality of the RNA was analyzed employing a Bioanalyzer Agilent 2100 [Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas (CSIC), Madrid] prior to its use in microarray analysis.

Five µg of each RNA extraction (final concentration of 0.5 µg/µl) were used to conduct the microarray analysis. Assays, including cDNA synthesis, biotin strands labeling, chip hybridization, scan and quantification of results and preliminary bioinformatics analyses, have been carried out by the Genomics Service of the CNB.

3.9.2 Analysis of mRNA expression by RNA-Sequencing (RNA-Seq)

Over the course of this work, a number of different RNA-Seq analyses were conducted. On the one hand, 22-days old plants from Col-0 and the lines *cyp71b22*, *cyp709b3*, as well as seeds from Col-0 and the cross ♂*ami1-2* ♀*rty1*, were used for transcriptomic analysis. On the other hand, 14-days old plantlets of the genotypes Col-0, *ami1-2* and AMI1ind were first treated over 2 h with liquid MS-medium + IAM at 100 µM before been prepared and used for the RNA-Seq analysis. In this latter case, 14-days old seedlings of Col-0 were also treated 3 h with liquid MS-medium + 0.5 % MeOH (v/v) to be used as the control.

Firstly, any residing DNA in the samples was removed. To do so, the samples were treated with RQ1 RNase-free DNase (Promega, Madison, USA) over a period of 30 min at 37 °C according to the manufacturer's instructions. Secondly, highly pure RNA was obtained using the clean-up protocol of the RNasy Plant Mini Kit (Qiagen, Hilden, Germany). Once the RNA was extracted, sufficiently high RNA quality for RNA-Seq analysis had to be tested. In a first step, the purity and quantity of total RNA was checked by gel electrophoresis analysis on 1 % (w/v) agarose gels and by absorbance measurements (Nanodrop® ND-1000 Spectrophotometer V3.1.2). Additionally, 5 µl of RNA were subjected to a Bioanalyzer Agilent 2100 (CNB; CSIC). Finally, 40 µg of each sample were sent to the Beijing Genomics Institute (BGI, www.genomics.cn), where RNA-Seq assays were conducted.

3.10 Vector cloning

3.10.1 Conventional cloning protocol

The DNA fragment to be cloned into a vector was usually obtained by routine PCR amplification (section 3.8.1) or restriction digest (section 3.10.4), and isolated in agarose gels (section 3.9). To cut the fragment from the gel agarose a sterile scalpel was used. Purification of fragments were carried out utilizing the NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) following the manufacturer's instructions.

In some cases and prior to all following steps, it was necessary to dephosphorylate the 5'-phosphate extremes with alkaline phosphatase (Invitrogen, Paisley, UK) to avoid religation of the plasmid DNA used in the reaction.

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The introduction of DNA into the plasmid produced closed circular DNA vectors, which facilitate the transformation of competent bacteria. DNA ligation was generally catalysed by a T4-DNA ligase (Promega, Madison, USA). Reaction conditions were the following:

Optimally, a molar ratio of 3:1 (insert:vector) is desirable. However, if it is not possible to obtain these ideal conditions, variations have also been shown to work successfully. For example, options reactions ranging from ration of 1:3 to 1:1 also lead to the desired vector, even though less efficiently. In any case, to calculate the insert:vector ratio, the following formula was employed

$$([\text{vector}] \times kb_{\text{insert}} / kb_{\text{vector}}) \times \text{molar ratio}$$

$$\text{Ex: } (50\text{ng}/\mu\text{l} \times 0,5\text{kb} / 3\text{kb}) \times \frac{3}{1} = 25\text{ng of DNA insert must be used in the ligation.}$$

A standard reaction mixture of 20 μl contained 2 μl 10X Buffer (T4 DNA, Promega, Madison, USA), 1 μl T4 DNA ligase (Promega, Madison, USA), vector and insert DNA (according to the molar ratio). The reaction mixtures were incubated at 16 °C and o/n. The product of that ligation reaction was used to transform competent bacteria (c.f. section 3.5.5). According to the introduced plasmid, the suitable selection pressure was applied by adding antibiotics to the solid LB-media plates. Then, various independent colonies were picked from the growth medium and used to inoculate liquid 4 ml LB-medium cultures. The cultures were incubated o/n at 37 °C to allow bacterial growth. Suitable selection pressure was maintained in the liquid cultures too, to prevent the growth of non-transformed clones.

3.10.2 GATEWAY Technology

The GATEWAY cloning system, invented and commercialized by Invitrogen (Paisley, UK), facilitates the efficient transfer of DNA-fragments between plasmids using a proprietary set of recombination sequences, the so-called att-sites, and two proprietary enzyme mixes, called BP Clonase and LR Clonase (Liang et al. 2013). The system is based on the site-specific recombination system utilized by bacteriophage lambda to shuttle sequences between plasmids bearing flanking compatible recombination attachment (*att*) sites (Hartley, Temple, and Brasch 2000).

Introduction of desired DNA fragments into suitable GATEWAY vectors has been carried out by two different ways. On the one hand, the desired fragments were amplified by conventional PCR adding *attB* recombination sites via extensions to the primers. The resulting fragments were integrated into the donor vector pDONR221 by performing a BP reaction. The obtained entry vector contained, in addition to the integrated DNA fragment, attL sites that are

essential for the subsequent production of the destination vectors. On the other hand, it is also possible to integrate target DNA fragments into so-called entry vectors, such as pENTR1, by conventional cloning employing suitable restriction enzyme combinations and ligation reactions. Once the entry vectors were constructed and the sequence integrity of the introduced DNA fragment was verified, the included DNA cassette flanked by the *attL* sites in the entry vectors was transferred into destination vectors by specific recombination reactions employing the *attR* sites in the destination vectors. This reaction was catalysed by a LR clonase enzyme mixture. It has to be highlighted that empty GATEWAY vectors, e.g. pDONR221, pENTR1 or pDEST17 contain a *ccdB* gene, whose expression leads to lethality in bacterial strains other than *E. coli* DB3.1. This is a measure to decrease the number of false-positive clones after conducting the recombination reactions.

In this work, the target DNA was introduced into suitable entry vectors by conventional cloning, if not stated otherwise. From there, the cassette was transferred into destination vectors using LR Clonase catalysed recombination reactions (Figure 3.1).

Figure 3.1 Scheme of recombination reactions employing *attL* and *attR* sites of an entry and destination vector, respectively.

The DNA fragment of an entry vector is exchanged by the lethal *ccdB* gene included in an empty destination vector. When the reaction is finished, the entry vector contains *attP* sites, while the destination vector has *attB* sites. The reaction is catalysed by the LR Clonase enzyme mixture (GATEWAY® Technology).

3.10.3 Extraction and purification of plasmid DNA

Extraction of vector DNA containing target inserts for subsequent restriction analysis or DNA sequencing is a crucial step in handling bacterial clones. A quick and easy way to extract and purify plasmid DNA on a small scale (1.5-3 ml culture volumes) is the mini-preparation technique, commonly called miniprep. However, other techniques that use bigger volumes than the miniprep were sometimes used too. These are: midipreps (15-25 ml culture volumen) and maxipreps (100-200 ml culture volumen). In all cases, the method is based on the alkaline lysis of bacteria (Birnboim and Doly 1979).

In order to obtain highly pure plasmid DNA, the Wizard®Plus SV Miniprep DNA Purification System (Promega, Madison, USA) was used. Five ml cultures inoculated with individual clones were grown o/n at 37 °C under constant agitation. Thereafter, 1.5 ml of the cultures were

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transferred to micro reaction tubes and the included bacteria harvested by centrifugation (2 min, at 13000 rpm). After discarding the supernatant, this step was repeated to harvest bacteria from a total volume of 3 ml. The remaining bacterial pellet was then treated according to manufacturer's instructions. In the end, the plasmid DNA was eluted in 50-100 μ L of sterile water. The DNA was stored at -20 °C until its use.

3.10.4 Verification of cloned DNA

Two procedures were used to corroborate if the DNA insert had been correctly introduced into the desired vector: restriction analysis and DNA sequencing.

DNA digestion by specific restriction enzymes (endonucleases) is a commonly used technique in molecular biology, providing a comparatively quick way to check the integrity of a construct by diagnostic digestion (Ausubel et al. 1995; Sambrook et al. 2001). DNA restriction was carried out using type II restriction endonucleases in the provided buffer systems according to the manufacturer's protocols.

In the framework of this project, commercial sequencing of double-stranded DNA was carried out by Stabvida (<http://www.stabvida.com/es/>) on ABI 3730 xl sequencer.

3.11 Biochemical methods

3.11.1 Protein purification

To express hexahistidine tagged amidases, *E. coli* M15 [pREP4] was transformed with the vector pQE-AMI1 (c.f. section 3.4.2). Appropriate selection pressure was applied by growing the bacteria in presence of both kanamycin and ampicillin as selection markers.

Pre-cultures of 30 ml LB liquid medium were grown from single colonies and used to freshly inoculated (1:10) 300 ml of LB liquid cultures. Bacteria were further grown as described by Neu et al. (2007). In brief, the bacteria were incubated at 37 °C under constant shaking. After 2 h, gene expression and thus, production of the recombinant proteins, were induced by adding 0.2 mM IPTG. However, for the expression of the other amidases analysed from plants other than *Arabidopsis*, induction conditions were adapted to the used vector systems. In general, 1 mM IPTG was added, when an OD_{600nm} of either 0.6, in case of the *MtAMI1* construct, or 1.3, for the *OsAMI1* and *PtAMI1* constructs, was reached. The cultures were transferred to either 4 °C (*MtAMI1*), 30 °C (*OsAMI1*, *SbAMI1*, *PtAMI1*) or 37 °C (*AtAMI1*) allowing protein expression to occur for at least 18 h. Thereafter, the bacteria were harvested by centrifugation (6600 rpm, 15 min, 4 °C), the supernatant was discarded and the pellet resuspended in 1/10 of the culture volume of lysis buffer (50 mM Na₂HPO₄ buffer pH 7.5, 300 mM NaCl, 15 mM imidazole). The samples were then snap frozen and kept at -80 °C until further use. Complete cell disruption

was subsequently achieved by a combination of incubation with lysozyme (45 min, 4 °C) and one burst of ultrasound (120 s) on ice. The soluble protein fraction obtained by centrifugation (15000 rpm, 15 min, 4 °C) was sterile filtered (0.20 µm) and used for Ni²⁺ affinity purification. The affinity-chromatography was carried out in accordance to the manufacturer's protocol (Qiagen, Hilden, Germany).

3.11.2 Analyses of proteins

3.11.2.1 SDS gel-electrophoresis

Protein samples were separated in SDS-polyacrylamide (PA) gels consisting of vertical 12.5 % separating gels and 6 % stacking gels (Laemmli 1970). Prior to loading onto the SDS-PA gels, all protein samples were denatured by a heat treatment (10 min, 90 °C) in the presence of SDS and 2-mercaptoethanol. The gels were run at 120 V for about 90 min at RT, stained with Coomassie solution (50 % MeOH (v/v), 0,05 % Coomassie brilliant blue R-250 (v/v), 10 % acetic acid (v/v) and 40 % H₂O), destained with 10 % MeOH (v/v) containing 5 % acetic acid (v/v) and subsequently photographed. For size estimation of the loaded proteins, one lane was loaded with a molecular weight standard (Spectra™ Multicolor Broad Range Protein Ladder, Thermofisher).

3.11.2.2 Western blotting and immunodetection of proteins

Denatured proteins were transferred from the SDS-PA gels onto nitrocellulose membranes (Whatman™ Protran™ BA85 nitrocellulose transfer membrane) using transfer buffer (20 mM Tris, 150 mM glycine, 0.1 % SDS (w/v), 20 % MeOH (v/v)) prepared according to Towbin, Staehelin, and Gordon (1979). The transfer was carried out for 2 h at 200 mA at 4 °C. For the visualization of proteins on the membrane after blotting, Ponceau S was used (0.5 % Ponceau S (w/v), 1 % glacial acetic acid (v/v)). For immunodetection, membranes were incubated for 1 h in TBST (50 mM Tris/HCl, pH 7.8; 150 mM NaCl; 1 mM MgCl₂; 0.05 % Tween-20 (v/v)) with 3-5 % protein extract "PowerPlay", then primary antibodies were added and the membranes further incubated for another 2 h. Afterwards the membranes were washed twice with TBST and twice with TBS (50 mM Tris/HCl, pH 7.8, 150 mM NaCl, 1 mM MgCl₂) for 10 min each. Secondary antibodies (HRPconjugate) were diluted 1:5000 or 1:7500, respectively, in TBS and the membranes were incubated for 2 h. After a subsequent washing step (twice with TBS), membranes were conditioned in AP buffer (100 mM Tris/HCl, pH 9.5; 100 mM NaCl; 5 mM MgCl₂) for another 20 min. Finally, specific bands were detected by colorimetric detection of alkaline phosphatase activity using the substrate BCIP (0.15 % (w/v)) and the oxidant NBT (0.31 % (w/v)). For the dilutions of the used specific antibodies refer to the previous section 3.3.

3.11.2.3 Assays for amidase activity

Two assays for the analysis of the enzymatic properties of amidases were conducted. On the one hand, the amidohydrolase activity was determined by analysing the ammonia produced during the reaction as described by Hiller and Van Slyke (1933). In this Bertholet reaction, ammonia is converted to chloroamine, which then reacts with phenol to yield the blue-coloured indophenol (Bolleter et al. 1961). Reactions were carried out in 0.3 ml 50 mM potassium buffer (pH 7.5) containing, if not stated otherwise, 5 µg purified protein and different substrates (10 mM). For background control, heat-inactivated enzyme (20 min, 100 °C), reactions with no substrate or no enzyme and empty-vector control samples, were used. After an incubation time of 4 to 6 h, aliquots of 100 µl were taken and the reaction was stopped by adding 100 µl each of 330 mM sodium phenolate, 20 mM sodium hypochlorite, and 0.01 % sodium pentacyanonitrosyl ferrate (III) (sodium nitroprusside) (w/v). Next, each sample was incubated for 2 min at 100 °C and diluted with 600 µl of water. The absorbance was read at 640 nm. Each experiment was calibrated using NH₄Cl solutions of known concentrations.

However, the amidohydrolase activity measurements for the characterization of the *k.o.* and inducible lines of *AM1*, were done in a different way to the previous described method: aliquots containing 200 µg proteins from two weeks old seedlings were incubated with 5 mM IAM in a total volume of 300 µl of 50 mM potassium phosphate buffer (pH 7.5) for 3 h at 30 °C. Finally, the amount of produced IAA was quantified by RP-HPLC (c.f. section 3.14.1).

On the other hand, the possibility of an amidase catalysed reverse reaction, forming IAM from IAA and ammonia, was also investigated. As before, 5 µg of purified protein were incubated in a total reaction volume of 0.3 ml 50 mM potassium buffer (pH 7.5) over a time period of 6 h at 30 °C. However, in this case, 10 mM IAA and 10 mM NH₄Cl were included as possible substrates. Again, for background control, heat-inactivated enzyme (20 min, 100 °C), and reactions with no IAA or no enzyme were used. The formation of IAM in the *in-vitro* reactions was monitored by UHPLC-MS (c.f. section 3.14.2).

3.12 Stable and transient plant transformation, plant crossing

Binary plant transformation vectors were introduced into the *A. tumefaciens* strain C58C1 [pMP90] (Koncz and Schell 1986) as it has explained before (c.f. 3.5.5). Suitable *A. thaliana* plants were transformed by floral dip according to Clough and Bent (1998). For each transformation, at least, three individual plants have been used. After the dipping, plants were placed in a sealed tray until the next day, ensuring a constantly high humidity. Transformed plants were kept in the greenhouse under controlled conditions and seeds were harvested

upon full maturation and stored at room temperature. Next, seeds were grown aseptically on medium as described, containing 10 µg/ml of the BASTA herbicide (Hoechst, Frankfurt, Germany). Homozygous lines were obtained after herbicide selection, following Mendelian distribution. The lines finally obtained in this thesis are listed in Table 3.4.

In order to generate the Arabidopsis lines ♂*ami1-2* / ♀*rty1* and ♂*ami1-2* / ♀*DR5::GUS*, mature siliques as well as open flowers and buds that already had a white tip, were carefully removed with fine scissors or forceps from the mother plants. Using two pairs of clean forceps, the meristems of too small flower buds were removed, until approx. 3 to 5 buds with a suitable size remained. Next, the flower buds were gently opened one by one and the still closed immature anthers were removed. After allowing the mother plant to grow for another 2 to 3 days, the emasculated inflorescence was then pollinated using an open mature flower of a father plant. Depending on the growth conditions, siliques containing the hybrid seeds were harvested after 15-25 days. For successful crossing it is necessary to protect the pistil against the cross-pollination with undesired pollen, thus, the pollinated mother plants were kept in small carrier bags. Followed by two to three rounds of self-pollination (depending of the line), the selection of the obtained crosses was done using two different ways: zygosis of T-DNA insertions within target genes was either analysed by conventional PCR using genomic DNA as template or, if possible, specific selection marker linked to the desired mutation were used to select the plants, following a Mendelian distribution. In case that the crossed lines contained reporter constructs, such as the *DR5::GUS*, additional β-glucuronidase assays (section 3.13) were carried out, to check for the functionality of the promoter-reporter construct.

Table 3-4 Lines of plants obtained by breeding or transformation with *Agrobacterium tumefaciens*.
Names of the lines and of the resistances that they contain are included in the table.

Method employed	Line obtained	Resistance to
Transformation by <i>A. tumefaciens</i>	<i>pAMI1::GUS/YUC9ox</i>	Kanamycin and BASTA
Transformation by <i>A. tumefaciens</i>	<i>pAMI1::GUS/rty1</i>	Kanamycin and BASTA
Breeding plant	♂ <i>ami1-2</i> / ♀ <i>rty1</i>	-
Breeding plant	♂ <i>ami1-2</i> / ♀ <i>DR5::GUS</i>	Kanamycin

For transient transformation assays, either epidermal cells of onion (*Allium cepa* L.) or Arabidopsis leaves were used. The plant tissue was bombarded using a biolistic helium gun device (DuPont PDS-1000/Bio-Rad, <http://www.biorad.com/>). Preparation and precipitation of plasmid DNA onto the microcarriers (gold particles) and particle bombardment were done essentially as described by (Díaz et al. 2002). The bombardment with each plasmid was

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performed three times using 1 µg of DNA. After bombardment, the plants were incubated for 24 h at 25 °C in the dark in Petri dishes containing MS agar medium. Fluorescent images were captured using an Axiophot (Zeiss) microscope.

3.13 Detection of β -glucuronidase activity in transgenic reporter lines

The determination of β -glucuronidase activity in plant tissues was conducted according to Jefferson *et al.*, 1987. Plant material (e.g. seeds, embryos, seedlings and leaves) was embedded in infiltration solution (100 mM Na₂HPO₄/NaH₂PO₄ pH 7.0, 20 mM X-Gluc, 500 mM EDTA pH 8.0, 20 % Triton X-100 (v/v)), and the samples were infiltrated by applying a vacuum of 540 mbar over 25 min. Next, they were incubated over night at 37 °C. Thereafter, the infiltration solution was discarded and the samples were subjected to consecutive washing steps with ethanol solutions of increasing concentrations (30, 70 and 100 %). In order to conserve the stained plant material for a short while, ethanol was changed to sterile water and tubes were kept at 4 °C. Images of the stained tissues were captured using a LEICA MZ10F (Leica Microsystems) system, equipped with a CCD colour Leica DFC 400C, of 5 megapixel camera.

3.14 Metabolomic analyses

All metabolomics analyses were carried out in triplicate, using a sample weight of approx. 100 mg of plant tissue per sample. The exact weight of each sample was measured prior to the extraction. The material was frozen in liquid nitrogen and stored at -80 °C until it was further use for extraction and subsequent mass spectrometric assessment.

3.14.1 RP-HPLC

Extraction procedure

Samples were extracted with 1 ml ethyl acetate, dried under vacuum and resuspended in the mobile phase (300 ml iso-hexane, 200 ml ethyl acetate with 2.5 % (v/v) aqueous formic acid and 10 % (v/v) methanol).

RP-HPLC settings

Separation of the compounds was carried out on a stationary silica phase (ZorbaxSil 5 µm, 250 mm x 4 mm i.d.; Knauer, Bad Homburg, Germany) using the mobile phase described above. The chromatographic separation was carried out isocratically with a flow rate of 1.5 ml/min, resulting in the following retention times: IAA = 2.7 min, IAM = 4.7 min). The amounts of IAA and IAM, respectively, were calculated by comparison of their peak areas with those of

reference compounds representing known concentrations of IAM or IAA. All values were corrected for non-enzymatic background activity.

3.14.2 UHPLC-MS

Extraction procedure

If not stated otherwise, all samples were extracted according to a previously described protocol (Corrales et al. 2014; Gullberg et al. 2004). In brief, to each sample 1 ml of a solvent system consisting of chloroform:MeOH:water (20:60:20, v/v/v) was added. The samples were incubated for 2 min at 70 °C, followed by an incubation of 1 h at RT under constant agitation. Later, samples were centrifuged (20 min, 13000 rpm, 4 °C) to sediment cell wall material and other cell debris. The supernatant was transferred to new micro reaction tubes and dried in a Speed-Vac concentrator, at 45 °C. At this stage, samples could be stored at -80 °C for a longer period of time. For the absolute quantification of IAM, corresponding samples were spiked with 50 pmol of [²H₅] indole-3-[¹⁵N] acetamide.

UHPLC-MS settings

Prior to their separation by ultra high performance liquid chromatography (UHPLC), the samples were resuspended in 100 µl MeOH + 0.1 % formic acid (v/v). Maximum solvation was achieved by rigorous vortexing and 10 min of ultra-sonication. The remaining insoluble particles were again sedimented (centrifugation 5 min at 13000 rpm) and the supernatant transferred into 400 µl HPLC autoinjector screw cap glass vials. UHPLC-MS analysis was performed on an Ultimate3000 RSLC system (Dionex, Sunnyvale, USA) coupled to a micrOTOF Q II hybrid mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source (ESI). Separation of the compounds was achieved on a Waters Acquity UHPLC BEH C₁₈ column (50 mm x 2.1 mm i.d., 1.7 µm) with a VanGuard pre-column (5 mm x 2.1 mm i.d., 1.7 µm) of the same material, and an 0.2 µm in-line filter set (Waters, Milford, USA), kept at 30 °C. The mobile phase A was composed of water containing 0.1 % formic acid (v/v); mobile phase B was acetonitrile containing 0.1 % formic acid (v/v). The flow rate was set to 0,4 ml/min and the following binary gradient profile was applied: from 0 to 2min, isocratic 98 % solvent A and 2 % solvent B; from 2 to 30 min linear gradient to 5 % A, 95 % B; at 30 min step gradient to 100 % B; isocratic for 1 min. Thereafter, the column was set to 98 % A, 2 % B and conditioned for 2 min before the next injection. The injection volume of the samples was 5 µl. Typical settings for the mass spectrometer were as follows: dry gas temperature, 200 °C; capillary voltage, 4500 V; nitrogen gas flow, 10 L/min. The instrument was operated in MRM mode. The following ion transitions were recorded: m/z 176 [M+H]⁺ to m/z 130 (endogenous IAA), m/z 175 [M+H]⁺ to m/z 130 (endogenous IAM), and m/z 181

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$[M+H]^+$ to m/z 135 ($[^2H_5, ^{15}N]$ -IAM; internal standard). Argon was used as the collision gas. The amount of the endogenous IAM was calculated from the signal ratio of the unlabelled over the stable isotope-containing mass fragment observed in the parallel measurements.

3.14.3 GC-MS

Extraction procedure

In case that absolute quantification of IAA by GC-MS was desired, corresponding stable isotope-labelled internal standards ($[^2H_2]$ -IAA at 50 pmol) was added to each sample prior to their extraction (c.f. section 3.5.2). The dried extract fractions were dissolved in 50 μ l MeOH, treated with 200 μ l diethyl ether. After that, they were vigorously vortex and sonicated (5 min), in order to achieve maximum solution of contained organic compounds. Remaining insoluble particles were separated by centrifugation (5 min, 12000 rpm). Afterwards, samples were transferred to aminopropyl cartridges (shorty NH_2 columns, 10 mg, Macherey & Nagel, Düren, Germany), washed twice with 200 μ l $CHCl_3$:2-propanol, 2:1 (v/v), and eluted two times with 200 μ l diethyl ether + 2 % acetic acid (v/v). Next, the combined eluates were taken to complete dryness. Methylation of the carboxyl function of IAA was done at RT over a time period of 20 min, dissolving the sediment in a mix of 26.4 μ l MeOH + 10 % acetone (v/v), 3.24 μ l diethyl ether and 0.36 μ l 3 M trimethylsilyldiazomethane (TMSDS). Finally, samples were transferred to glass vials and gas-tied sealed, before they were analysed by GC-MS in a randomized order.

GC-MS settings

Analysis was either performed on a Varian GC 3400 gas chromatograph coupled to a Finnigan MAT Magnum system or a Bruker Scion-455 gas chromatograph coupled to a Bruker Scion-TQ Triple Quadrupole mass spectrometer. Aliquots of 1 μ l of each sample were injected into the GC-MS systems. Compounds were separated on a 30 m x 0.25-mm i.d. fused silica capillary column with a chemical bond 0.25 μ m ZB35 stationary phase (Phenomenex). The injector temperature was 250 °C, and the column temperature was held at 50 °C for 1 min and then increased by 20 °C per minute to 250 °C and held for another 4 min at 250 °C. The transfer line temperature was set to 250 °C, and the ion source temperature was set to 200 °C. Ions were generated with 270 eV at a filament emission current of 80 mA. The dwell time was 100 ms, and the reactions m/z 189 to m/z 130 (endogenous IAA) and m/z 191 to m/z 132 ($[^2H_2]$ -IAA; internal standard) were recorded. Argon set at 2.0 mTorr was used as the collision gas. The amount of the endogenous IAA was calculated from the signal ratio of the unlabelled over the stable isotope-containing mass fragment observed in the parallel measurements.

3.15 Phylogenetic analysis of AMI1 in different plant species

The phylogenetic tree containing AMI1-like proteins from multiple plant species was performed comparing the translated nucleic acid sequence of *AMI1* from *A. thaliana* with orthologous protein sequences from plant amidases identified in different databases (mentioned in section 3.18). To carry out the phylogenetic grouping the Neighbour-Joining method was employed (Saitou et al. 1987). The percentage of replicate trees was calculated by a bootstrap test with 500 replicates (Felsenstein 1985). Evolutionary distances were computed using the Poisson correction method (Zuckerandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site.

3.16 In silico studies

The phylogenetic analysis was conducted using the MEGA5 software (Molecular Evolutionary Genetics Analysis, MEGA Software).

Alignments of nucleic and amino acid sequences were done with different programmes: Molecular Evolutionary Genetics Analysis, MEGA Software; ClustalW2 (EMBL-EBI) and PSIPRED. Chromatograms of sequencing reactions were studied using the ChromasPro 1.5 software.

In the case of handling microarray data, the genomic service of the CNB carried out initial bioinformatics analyses, applying algorithms for the statistical assessment of the data (*Linear Models for Microarray Data (LiMMA)*). To visualize the data, they were transferred with the FIESTA programme, developed in the CNB. Finally, the data were analysed with the help of Microsoft Excel software package. To analyse RNA-Seq data, the BGI company first quantifies gene expression by mapping reads to a reference genome. Next, they make a pairwise comparison to finally, cluster the genes according to their similar expression pattern. Then, we used the programme R in version 3.0.1 (R Core Team 2013) and Microsoft Excel software package to finish the analysis.

Gene Ontology enrichment analyses of differentially regulated genes (fold change ≥ 1.5 and p-value ≤ 0.05) in microarray and RNA-Seq analyses were performed using the Cytoscape 3.4.0 software with the BINGO application ((Maere, Heymans, and Kuiper 2005); agriGO (<http://bioinfo.cau.edu.cn/agriGO/>) (Du et al. 2010) and REVIGO (<http://revigo.irb.hr/>) (Supek et al. 2011) platforms.

Venn diagrams were performed using the Venny tool version 2.1.0 (<http://bioinfo.gp.cnb.csic.es/tools/venny>; Oliveros 2007-2015).

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Mass spectrometric data collected by UHPLC-MS or GC-MS were analysed and processed with the DataAnalysis 4.0 and MS Data Review 8.0 software (Bruker Daltonics, Bremen, Germany), respectively. Statistic analyses were either done using one-way ANOVA followed by Tukey's B post-hoc test to allow for comparisons among all means or by employing Student's *t*-test to allow for comparison between two means. Statistical analyses were conducted using PRISM version 5.03 (GraphPad Software, <http://www.graphpad.com/>).

Measurements of root system morphology were done with the WinRhizoTM image analysis software.

3.17 On-line molecular Tools

- Related with enzymatic digestion

- *Web map preferences* (https://pga.mgh.harvard.edu/web_apps/web_map/start).
- *Addgene's sequence analyser* (<https://www.addgene.org/analyze-sequence/>).

- Comparative platforms for the phylogenetic analysis

- Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>).
- Plant Genome Duplication Database (<http://chibba.agtec.uga.edu/duplication/>).

- Source of expression data

- GeneMANIA (<http://genemania.org>).
- Genevestigator V3 (<https://genevestigator.com/gv/>).
- Mockler lab, Donald Danforth Plant Science (<http://mocklerlab.org> web page).
- BAR, The Bio-Analytic Resource for Plant Biology (<http://bar.utoronto.ca/eplant/>).

4 RESULTS

RESULTS

4.1 Distribution of amidases in the plant kingdom

4.1.1 Phylogenetic analysis

One of the major objectives of this work was the functional characterization of the previously discovered *AMIDASE1* (*AMI1*) from *Arabidopsis thaliana* (Pollmann, Neu, and Weiler 2003). For this purpose, we firstly addressed the question whether *AMI1* occurrence is restricted to the *Brassicaceae*, as is the case for the cytochrome P450 enzymes CYP79B2 and CYP79B3 or the nitrilases NIT1-3 of *Arabidopsis* (Lehmann et al. 2010), or if *AtAMI1*-like proteins have a broader distribution in the plant kingdom. The analysis and comparison of the primary protein structure with the aim to identify homologies among proteins is a common tool that facilitates the estimation of the origin, evolution, and distribution of target proteins in nature. In a comprehensive query, we used the *Arabidopsis* IAM-hydrolase (*AtAMI1*, At1g08980) nucleic acid and the translated amino acid sequences, to search for IAM-hydrolase orthologs in different available databases (e.g., Phytozome, Plant Gene Duplication Database).

The *in silico* analyses provided indication for 60 *AtAMI1*-like proteins from 49 plant species covering the entire plant kingdom (Figure 4.1).

Figure 4.1 Cladogram of 60 AtAMI1-like proteins from plants, algae, mosses and bacteria.

A bootstrap consensus tree inferred from 500 replicates was generated to represent the evolutionary relationships of the proteins analysed. Species that belong to the same family are marked by a coloured name of the family. Species names are abbreviated as follows with gene/protein identifiers given in parenthesis: Ach: *Actinidia chinensis* (ACHN083681); Aco: *Aquilegia coerulea* (Aqua_05400135.1); Atr: *Amborella trichopoda* (AmTr_v1.0_scaffold00025.29); Aly: *Arabidopsis lyrata* (16043473); Ath: *Arabidopsis thaliana* (At1g08980); Atum: *Agrobacterium tumefaciens* (YP_001967410); Avit: *Agrobacterium vitis* (P25016); Bdi: *Brachypodium distachyon* (Bradi5g27490); Bra: *Brassica rapa* (Bra031652); Car: *Cicer arietinum* (XP_004495736); Cca: *Cajanus cajan* (C.cajan_06830); Ccl: *Citrus clementine* (AMI1:Ciclev10001213m.g, AMI2:Ciclev10001239m.g); Cgr: *Capsella grandiflora* (Cagra.4395s0082); Cla: *Citrullus lanatus* (Cla005242); Cpa: *Carica papaya* (Crubv10006860m); Cru: *Capsella rubella* (Carubv10009223m.g); Csa: *Cucumis sativus* (AMI1: Cucsa.182140.1; AMI2: Cucsa.182150.2); Csi: *Citrus sinensis* (orange1.1g044294m); Csu: *Coccomyxa subellipsoidia* (e_gw1.9.170.1); Egr: *Eucalyptus grandis* (AMI1: Eucgr.B01994.1, AMI2: Eucgr.B01996.1, AMI3: Eucgr.L03224.1); Esa: *Eutrema salsugineum* (Thhalv10007679m); Fve: *Fragaria vesca* (Mrna22572.1- v1.0-hybrid); Gma: *Glycine max* (AMI1: Glyma10g29640.1, AMI2: Glyma20g37660.1); Gra: *Gossypium raimondii* (AMI1: Gorai.007G295700.1); Lus: *Linum usitatissimum* (AMI1: Lus10029941, AMI2: Lus10004466); Mdo: *Malus x domestica* (AMI1: MDP0000302761, AMI2: MDP0000205696, AMI3: MDP0000205699, AMI4: MDP0000212087); Mes: *Manihot esculentum* (cassava4.1_010118m); Mgu: *Mimulus guttatus* (mgv1a006860m); Msp: *Micromonas sp.* (gw2.03.307.1); Mtr: *Medicago truncatula* (Medtr1g082750.1); Nta: *Nicotiana tabacum* (AB457638); Olu: *Ostreococcus lucimarinus* (C_Ch_11000198); Osa: *Oryza sativa* (AMI1: Os04g02780.1, AMI2: Os04g02754.1); Ppe: *Prunus persica* (Prupe.6G327700); Ptr: *Populus trichocarpa* (AMI1: Potri.013G024100, AMI2: Potri.013G024100.1); Pvu: *Phaseolus vulgaris* (Phvul.007G180900); Rco: *Ricinus communis* (30128.m008759); Spu: *Salix purpurea* (SapurV1A.0092s0290); Sbi: *Sorghum bicolor* (Sb02g039510.1); Sly: *Solanum lycopersicum* (Solyc10g086170.1.1); Stu: *Solanum tuberosum* (PGSC0003DMP400065555); Svi: *Setaria viridis* (Sevir.5G062800.1); Tae: *Triticum aestivum* (AK330138); Tha: *Thellungiella halophila* (Thhalv10007679m); Vca: *Volvox carteri* (Vocar.0009s0330.1); Vvi: *Vitis vinifera* (GSVIVT01000036001); Zma: *Zea mays* (GRMZM2G169087_T01).

With respect to the presented result, it can be concluded that AtAMI1-like proteins share a wider occurrence in the plant kingdom. Remarkably, all so far published plant genomes contain at least one AtAMI1-like sequence, which indicates a conserved and indispensable function of the enzyme in plant development.

4.1.2 Amino acid sequence comparison of AtAMI1 with other amidases

The partly high degree of sequence homology between the AtAMI1-like proteins compared in Figure 4.1 made it tempting to hypothesize that those enzymes may also share similar functions. Thus, it was decided to gain deeper insight into the functional properties of a selected number of candidates. Four candidate amidases both from monocot and dicot species, namely *Oryza sativa* OsAMI1 (Os04g02780), *Sorghum bicolor* SbAMI1 (Sb02g039510), *Medicago truncatula* MtAMI1 (Mt1g099370), and *Populus trichocarpa* PtAMI1 (Pt13g02200), were selected due to their high sequence identity of 57% (OsAMI1), 58% (SbAMI1), 66% (MtAMI1), and 68% (PtAMI1), respectively, relative to AtAMI1. As can be taken from Figure 4.2, a sequence alignment of the corresponding

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enzymes was done and in particular the region of the amidase signature motif shows a considerable sequence homology. Consequently, functional similarity of the enzymes seems highly likely.

Figure 4.2 Sequence alignment of five members of the amidase-signature family from plants.

The analysed sequences are *AtAMI1* (At1g08980), *MtAMI1* (Mt1g099370), *OsAMI1* (Os04g02780), *PtAMI1* (Pt13g02200), and *SbAMI1* (Sb02g039510). Amino acid positions are indicated. Sequence conservation among the polypeptides is color-coded in the alignment. Asterisks, appearing over the sequences aligned, mark the catalytic triad; the glycine- and serine-rich amidase-signature motif is underlined.

4.2 Functional analysis of the selected plant amidases

4.2.1 In vitro analysis of amidases activity

To assess whether the selected candidate amidases possess IAM-amidohydrolase activity *in vitro*, the respective cDNAs from *O. sativa*, *S. bicolor*, *M. truncatula* and *P. trichocarpa* were amplified by RT-PCR [primers in Table 3.1, (c.f. Material and Methods)] and introduced into a common cloning vector, pGEM-T (Promega), for sequence verification, before they transferred into suitable prokaryotic expression vectors. In case of the *S. bicolor* amidase, the cDNA fragment was inserted into the expression vector pTrcHis2A (Invitrogen) by using the *SpeI/SmaI* sites. The cDNA of the amidases from *Medicago* and poplar were integrated into the *XhoI/XbaI* site of the vector pTrcHis2B (Invitrogen), and the rice cDNA fragment was inserted into the *SacI/KpnI* site of pTrcHis2C (Invitrogen). In the four constructs a translational fusion of the amidases with a c-myc/hexahistidine double tag was generated. Then, protein expression was induced in *Escherichia coli* strain BL21-AI according to the protocol mentioned previously (c.f. Material and Methods, Section 3.11.1), to finally isolate the recombinant proteins from bacterial lysates by Ni^{2+} -nitrilotriacetate affinity chromatography. As displayed in Figure 4.3, due to general difficulties in expressing amidases in bacteria, the amount as well as the homogeneity of the purified protein fractions varied considerably. For this reason, the measured enzymatic activities have to be taken with some caution.

Figure 4.3 Expression and purification of recombinant amidases.

Hexahistidine tagged amidases were expressed in *E. coli* and purified as described in the experimental section. Aliquots of 12.5 μl of the corresponding elution fractions were used for SDS-polyacrylamide gel electrophoresis and subsequent coomassie-staining (A) and immunoblotting (B). For the immunoblot only the region between 75 and 37 kDa is shown.

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In order to compare the biochemical properties of the recombinant amidases to those of *At*AMI1 in terms of specific activity and substrate selectivity, equal amounts of total protein (5 μ g each) from desalted elution fractions were employed. In this series of experiments, substrate concentrations of 10 mM were used. The data for the substrate specificity are shown on a relative scale normalized to indole-3-acetamide (IAM) (100%) (Figure 4.4).

Figure 4.4 Analysis of the relative substrate conversion of recombinant amidases.

All assays were performed in a total reaction volume of 300 μ l at pH 7.5 and 30 $^{\circ}$ C (substrate concentration 10 mM, reaction time 4 to 6 h). All activities were normalized to the conversion of IAM (100%). Catalytic activities are corrected for background activities obtained for the non-enzymatic breakdown of substrates in heat-inactivated and empty- vector control samples. Maximum specific activities for IAM were 3070 pkat (mg protein) $^{-1}$ (*At*AMI1), 2378 pkat (mg protein) $^{-1}$ (*Sb*AMI1), 375 pkat (mg protein) $^{-1}$ (*Os*AMI1), 37 pkat (mg protein) $^{-1}$ (*Mt*AMI1), and 329 pkat (mg protein) $^{-1}$ (*Pt*AMI1). The data shown are means $\pm$ SE derived from $n \geq 3$ experiments. n.d.: not detected. Mean values within a graph are significantly

different ($p < 0.05$) where superscript letters differ.

Generally, the substrate preferences of the amidases from *O. sativa*, *S. bicolor*, *M. truncatula*, and *P. trichocarpa* were similar, but not identical, to those of AtAMI1. Under the used conditions, the best substrate found was phenyl-2-acetamide (PAM), a compound that is endogenous to *Phaseolus mungo* (Isogai et al. 1963; Isogai et al. 1967), while an occurrence in *A. thaliana* has not yet been described.

We further explored other enzymatic parameters of the enzymes, such as pH and temperature. The data are given together with the specific activities in Table 4.1.

Table 4-1 Comparison of the enzymatic parameters obtained for recombinant amidases from *S. bicolor*, *O. sativa*, *P. trichocarpa*, and *M. truncatula* with those of the *A. thaliana* AMI1.

(The data shown are means $\pm$ SE from at least three independent experiments).

Parameter	AtAMI1	SbAMI1	OsAMI1	PtAMI1	MtAMI1
Amino acid identity to AtAMI1 [%]	100	58	57	64	66
Intracellular localization	cytoplasm/ nucleoplasm	cytoplasm/ nucleoplasm	cytoplasm/ nucleoplasm	cytoplasm/ nucleoplasm	cytoplasm/ nucleoplasm
Specific activity (IAM) [pkat mg ⁻¹]	3070 $\pm$ 520	2378 $\pm$ 324	375 $\pm$ 52	329 $\pm$ 22	37 $\pm$ 3
Temperature optimum [°C]	37	45	27	37	35
pH optimum	7.5	6	7.5	7.5	7.5
Calculated molecular weight [kDa]	46	45	50	53	57

All enzymes shared similar enzymatic properties, with a pH between 6 and 7.5 and an optimum temperature around 37 °C. However, this last analysed parameter showed more profound differences between the analysed proteins than the pH value did. For instance, rice presented a lower optimum temperature than the others at 27 °C. This could possibly been explained by the differences in the habitat that these plants have. Whereas Medicago, Arabidopsis, poplar and sorghum grow on dry lands, rice grows in water covered paddy fields where temperature is usually lower than 35-40 °C, due to the constant vaporisation of water (Yokohari et al. 2001).

Along with the presented results, we also tried to determine the K_m values for IAM, but none of the enzymes followed a classical Michaelis-Menten kinetics, but rather followed a sigmoidal curve progression, suggesting an allosteric mode of action. Moreover, due to solubility constraints of the substrates and inhibitory effects of the organic solvent (either methanol or ethanol) substrate concentrations of more than 25 mM could not be assessed. At this concentration no saturation of the enzymatic reaction could be observed, thus not allowing the calculation of apparent K_m values (data not shown).

To continue with the analyses of the obtained homolog proteins, we studied their subcellular

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localization. To do this, chimeric GFP fusion proteins were used for the transient assays in leaf epidermal cells of *Arabidopsis thaliana*. In each experiment, 5 µg of plasmid DNA, facilitating the 35S promoter driven expression of the corresponding fusion construct, were transferred into the cells by particle bombardment (Figure 4.4) The used constructs were obtained by cloning the respective amidase fragments into the pSP-EGFP vector (c.f. Materials and Methods, section 3.4.2). The amidase fragment from *O. sativa* was obtained from the vector pTrcHis2C-OsAMI1, with a digestion with the restriction enzymes *SacI/KpnI* to be finally integrated into the same sites of pSP-EGFP. In case of *P. trichocarpa*, a *SacI/Sall* fragment was obtained from pTrcHis2B-PtAMI1 vector and also cloned into pSP-EGFP, resulting in the *PtAMI*:GFP fusion. Since all C-terminal GFP fusions of the Medicago amidase lacked *in vivo*-fluorescence, a N-terminal GFP-tag was fused to this amidase by using the vector pUC-GFPn (c.f. Materials and Methods, section 3.4.2). To obtain a suitable DNA fragment, the coding sequence was amplified by PCR, adding restriction sites for *BglII* and *Sall*, respectively (Table 3.1, c.f. Material and Methods). After subcloning of the resulting DNA fragment into pBluescript SK(+) and sequencing, the *MtAMI1 Spel/Sall* fragment was introduced into pUC-GFPn. In case of the *S. bicolor* amidase gene, the coding sequence was amplified using primers that added *SpeI/SmaI* sites to the corresponding DNA fragment. After cloning into pBluescript SK(+) and sequence analysis the *SpeI/SmaI* fragment was introduced into the *SpeI/Sall* site of pUC-GFPn.

Figure 4.5 Subcellular localization of amidase GFP fusion proteins in epidermis cells of *Arabidopsis thaliana*.

The images show typical results of confocal laser scanning microscopic studies (ZEISS LSM 510 Meta), using differential emission light filter sets. The upper row provides transmission images, followed by the GFP channel (GFP-fluorescence), showing the fluorescence of the GFP fluorophore between 500 to 530 nm. Next, the chlorophyll-autofluorescence is shown

in a separate channel (650 to 798 nm). In the bottom row, an overlay of the fluorescence channels is depicted. **(A)** Transformation with an empty GFP-vector (pSP-EGFP), cytoplasmic control. **(B)** Transformation with an AMI1:GFP construct from *A. thaliana* (Pollmann et al. 2006) (positive control). **(C)** Transformation of an epidermis cell with the *OsAMI*:GFP construct. **(D)** Transformation with the chimeric GFP:*MtAMI* construct. **(E)** Transformation with the *PtAMI*:GFP vector. **(F)** Transformation of the *SbAMI*1:GFP construct. Scale bars for each set of pictures are included in the figure.

Alike AMI1 of *A. thaliana*, all the enzymes appear located in the cytoplasm of the cells. Moreover, the microscopic assessment has provided evidence for a diffusion of all studied amidase-constructs into the nucleus and an accumulation in the nuclear pocket, thus, delivering results similar to those obtained for the Arabidopsis AMI1:GFP fusion construct.

To summarize, the examined enzymes presented typical amidase activity, converting preferentially PAM and IAM relative to other tested amides. In assays with empty vector controls and heat-denatured protein fractions, respectively, no considerable substrate conversion was detected. The highest background activity for the non-enzymatic conversion of the amide substrates to the corresponding free acids was detected for formamide and benzamide, with 0.14 % and 0.11 % of the enzymatic IAM conversion, respectively.

4.3 Reverse genetic approach to analyse the *in vivo*-function of AtAMI1

To investigate the involvement of *AMI1* in auxin biosynthesis *in vivo*, we took a reverse genetics approach. In this, we analysed *AMI1* loss- and gain-of-function mutants. First, we isolated three independent *ami1* loss-of-function alleles from the SALK collection. To do this, genomic DNA of each T-DNA insertion mutant was extracted (see protocols in Material and Methods, section 3.6.1) and used for PCR analysis. Three different primers were used (c.f. Material and Methods, Table 3.1), one of them corresponding to the left border of the T-DNA insertion (Lb = pROK-LBa1). The other two were the left and right genomic primers specifically designed for these SALK lines (LP-AtAMI1-X and RP-AtAMI1-X respectively). These latter two primers were designed with the use of the “T-DNA Primer Design” web-tool from the SIGNAL webpage (<http://signal.salk.edu/tdnaprimers.2.html>).

Subsequent sequencing of the PCR products showed that *ami1-1*, *ami1-2* and *ami1-3* carry either a single T-DNA insertion in intron 4 or tandem-insertions in exon 6 and 8, respectively (Figure 4.6A,B).

In addition, quantitative RT-PCR (qRT-PCR) experiments (primers in Table 3.1) revealed a supposedly functional knockout (*k.o.*) in the alleles *ami1-1* and *ami1-2*, while *ami1-3* showed remaining low expression levels, which point to the production of a most likely truncated *AMI1* version (Figure 4.6A and C). In agreement with this finding, *ami1-1* and *ami1-2* are characterized by a decreased IAM-hydrolase activity with reduced endogenous indole-3-acetic acid (IAA) levels and high IAM contents, while these values appear to be less pronounced in *ami1-3*. Comparing all three alleles, *ami1-2* shows the strongest effect with regard to its activity (Figure 4.6D). Also, *ami1-2* suffers the biggest

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decrease in gene expression (Figure 4.6C), and IAA contents (Figure 4.6F), while it contains the highest IAM levels (Figure 4.6E).

Figure 4.6 Characterization of *ami1* T-DNA insertion lines.

(A) Genomic *AMI1* region (At1g08980) showing the exon/intron structure and the T-DNA insertion sites in intron 4, exon 6, and exon 8 (top line). The resulting cDNA with fused exons, but without the 5' and 3' untranslated regions, is depicted below. Also given is the region coding for the amidase signature domain (AS) of the derived protein. The insertion site of the T-DNA in the alleles *ami1-1*, *ami1-2*, and *ami1-3* was analysed by sequencing. Genomic sequence and cDNA, but not the T-DNA, are drawn to scale. Lb, left border primer; ATG, start codon; TGA, stop codon. (B) PCR analysis of the mutant genotypes. Total genomic DNA was extracted from wild type (Col-0) and the T-DNA insertion lines. One T-DNA- and two gene-specific primers were used in three combinations for genotyping: The first reaction (1) contained two gene-specific primers, flanking the T-DNA insertion; the second (2) comprised the T-DNA left border primer (Lb) and the upstream gene specific primer; the third reaction (3) contained the T-DNA left border primer (Lb) and the downstream gene specific primer. (C) qRT-PCR analysis of *AMI1* gene expression in the T-DNA insertion lines (*ami1-1*; *ami1-2* and *ami1-3*) and in wild-type plants. Total RNA from 2-week-old plants was extracted and used for cDNA synthesis. Two gene-specific primers

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amplifying a 180 bp cDNA fragment were utilized to investigate *AMI1* transcript levels. Expression of the housekeeping gene *ADENINE PHOSPHORIBOSYL TRANSFERASE 1 (APT1)* was used to normalize gene expression levels among samples. **(D)** IAM hydrolase activity in *ami1* T-DNA insertion mutants. Crude extracts were incubated for 3 h at 30 °C with 5 mM IAM as substrate. The produced IAA was detected after separation by RP-HPLC. Given are means $\pm$ SD ($n = 5$). Similar results were obtained in two independent experiments. **(E)** Quantification of free IAM levels in the *ami1* T-DNA lines *ami1-1*, *ami1-2*, and *ami1-3* relative to the wild type. 2-week-old seedlings were used for the determination of IAA by LC-MS/MS analysis. **(F)** Quantification of free IAA levels by GC-MS/MS. Three independent biological replicates were assessed. Statistical analyses were determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Given is the standard deviation of the mean ($n = 9$).

Next, we examined expression of the auxin reporter *DR5::GUS* in *Arabidopsis* seedlings in order to investigate a putative impact of *AMI1* on auxin signalling and, thus, indirectly, on endogenous auxin contents. In the *ami1-2* background, the *DR5::GUS* expression was moderately reduced (Figure 4.7). In particular, leaf margins and root tips as well as lateral root primordia showed lower GUS activity than in the wild type (Figure 4.7). Taking together, these results demonstrate that *AMI1* contributes to cellular auxin homeostasis and provide compelling evidence for the postulated role of the gene in auxin biosynthesis.

Figure 4.7 Histochemical staining for GUS activity in *Arabidopsis*.

Depicted are *Arabidopsis* seedlings from the controls *DR5::GUS* and *Col-0* as well as the *ami1-2/DR5::GUS* line. In the *ami1-2* background, *DR5::GUS* expression appears in the tips of the leaves and roots and slightly in the leaf vasculature. However, the *DR5::GUS* control shows a considerably stronger expression affecting the complete longitude of the root, leaf margins and all veins of leaves. *Col-0* has been used as a negative control, not showing any staining.

By the virtue of the high sensitivity of roots towards altered auxin contents, the plant root system is an ideal read-out for the analysis of mutations that affect auxin homeostasis. Hence, we gathered comprehensive and statistically secured data on the root morphology of *ami1* mutants. To this end, we grew a large number of seedlings on vertical plates and scanned the roots of the plantlets while still attached to the plates. The root system morphology was examined using the WinRhizo™ image analysis software in terms of root length, root surface area, number of root branches, and the

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number of root tips, before shoot and root systems were dissected and their biomass was determined.

Figure 4.8 Analysis of the root phenotype of the three identified *ami1* alleles.

Seeds were sown on ½ MS-medium with different sucrose contents (0.1 or 1.5 % (w/v)). After scanning of the plants, characteristic root morphology traits were analysed using the WinRhizo™ software. Error bars represent the SE of at least three independent experiments carried out in triplicate. Different letters indicate significant differences between means ($P < 0.05$).

The two alleles *ami1-1* and *ami1-2* showed significant changes in total root length and root branch number when grown on low sucrose contents (0.1%, w/v), whereas *ami1-3* resembled the wild type. Interestingly, the root branching phenotype of *ami1-1* and *ami1-2* can be restored when mutants are grown on media containing elevated contents of sucrose (1.5%, w/v). To provide further support for this observation, we monitored *AMI1* promoter activity in plants that were grown on increasing amounts of sucrose in the media. As shown in Figure 4.9A, it was possible to confirm a considerable impact of sucrose contents on the activity of the *AMI1* promoter. Apparently, at high sucrose concentrations, *AMI1* gene expression appeared clearly repressed. Microarray analysis on the effect of sucrose and trehalose on gene expression levels in *Arabidopsis* provide further confirmation of our results, identifying *AMI1* among 40 genes that are down-regulated by both disaccharides (Aghdasi, Smeekens, and Schluepman 2008).

Figure 4.9 Response of *AMI1* expression to varying carbohydrate concentrations.

(A) Seeds of *pAMI1::GUS* were grown on media containing 0.1%, 1.5% or 6.0% of sucrose. Either 24 h, 44 h or 72 h after the beginning of seed germination, samples were collected and immediately stained for GUS activity. Photos were captured using a modular stereomicroscope; model MZ10F (Leica Microsystems). (B) *AMI1* expression evaluated by qRT-PCR. Plants were grown for 10 days on ½ MS media, then they were treated with different compounds (400 mM glucose; 400 mM glucose + 10 µM ABA; 400 mM mannitol and 400 mM mannitol + 10 µM ABA). The transcript levels of *AMI1* were normalized to the expression of *APT1* and *POLYUBIQUITIN 10 (UBQ10)*. Error bars represent the SE of at least three independent experiments carried out in triplicate. Statistical analyses were conducted by Student's *t*-test (****P* < 0.001).

It is known that some carbohydrates can produce osmotic stress by changing the balance of solutes maintained between the inside and outside of cells. In addition, the expression of many genes is co-regulated by sugar and abscisic acid (ABA) (Dekkers, Schuurmans, and Smeekens 2008). In order to exclude that the repression of *AMI1* expression by high carbohydrate levels is due to osmotic stress, rather than to sugar signalling, additional qRT-PCR studies have been carried out. As can be observed in Figure 4.9B, the addition of ABA, the principal mediator of osmotic stress responses, did not change the response of *AMI1* expression to glucose. In addition, neither the treatment with mannitol, a non-metabolizable osmoticum, nor with ABA and mannitol triggered a substantial repression of *AMI1* expression, which underlines that the regulation of *AMI1* expression is linked to sugar signalling.

To further analyse the impact of *AMI1* on plant development, we generated and analysed inducible *AMI1* gain-of-function mutants and selected three independent lines, *AMI1ind-1*, *AMI1ind-2*, and *AMI1ind-3*, for further investigation. These lines were generated cloning the *AMI1* gene in the vector (pER-8) containing the XVE cassette that allows β-estradiol induced gene expression (Lehmann 2009; Zuo, Niu, and Chua 2000). Plants containing the construct were selected in ½ MS medium with Hygromycin. The plants were used to analyse the *AMI1* overexpression (adding 10 µM of β-estradiol) in different assays (Figure 4.10).

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In Figure (4.10) shows the characterization of transgenic AMI1ind lines induced with β -estradiol. The degree of overexpression of *AMI1* on the transcriptional level ranges between a $\log_2$ ratio of 1.5 to 3.5 in the selected lines (Figure 4.10B). The result could also be confirmed on the post-translational level, with line AMI1ind-3 showing the strongest immuno signal for the introduced transgene (Figure 4.10A).

Figure 4.10 Characterization of AMI1ind lines.

(A) Expression levels of recombinant c-myc:AMI1 protein in inducible gain-of-function mutants in comparison to the wild type (WT). The analysed mutants were: AMI1ind-1; AMI1ind-2 and AMI1ind-3. 2-week-old seedlings grown on $\frac{1}{2}$ MS media supplemented with 10 μM β -estradiol under SD were harvested and total protein was isolated. The upper panel shows a Coomassie blue-stained gel, indicating even loading. The large subunit of the ribulose-biphosphate carboxylase/oxygenase (RbcL) was used as loading control. The lower panel displays a blot stained for the detection of recombinant c-myc:AMI1. (B) Analysis of *AMI1* gene expression in the three independent lines AMI1ind-1, AMI1ind-2, and AMI1ind-3 by qRT-PCR. (C) IAM hydrolase activity in extracts of wild-type plants and AMI1ind mutants. Samples containing 200 μg protein and 5 mM IAM as substrate were incubated for 3 h at 30 $^{\circ}\text{C}$. The enzymatically produced IAA was analysed photometrically at a wavelength

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of 280 nm after separation by RP-HPLC. Given are means $\pm$ SD ($n = 5$). Similar results were obtained in two independent experiments. **(D)** Comparison of endogenous IAM levels in *AMI1* overexpressing lines and in wild-type plants. Seedlings were grown for two weeks. Then, IAM levels were determined by LC-MS/MS. The results derive from three independent experiments. Given are means $\pm$ SE ($n = 5$). **(E)** Comparison of endogenous IAA levels in *AMI1* overexpressing lines against wild-type plants. The measurements were carried out as described before for IAM, but IAA was quantified by GC-MS/MS. Statistical assessment employed Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Complementing the observations made with the *k.o.* lines, *AMI1* activity, and IAA content in the gain-of-function mutants were found to be increased (Figure 4.10C, E), while the IAM levels were decreased (Figure 4.10D). It has to be noted that the degree of activity and IAA levels correlate with the expression levels found in the analysed lines. In the following, the phenotype of the *AMI1*ind lines was analysed (Figure 4.11). Remarkably, a phenotype resembling that of other auxin overproducers, e.g. *YUCCAox* or *gh3.5-1D* (M Hentrich, Sánchez-Parra, et al. 2013; Z. Zhang et al. 2007; Zhao, Christensen, et al. 2001), was observed. Some phenotypic characteristics were slightly smaller and blade shaped rosette leaves that showed epinastic growth. In addition, *AMI1*ind lines show premature flowering, which may relate *AMI1* with the regulation of flowering in *Arabidopsis* through the activation of genes encoding the floral integrators SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (*SOC1*), *LEAFY* (*LFY*), and *FLOWERING LOCUS T* (*FT*) in the inflorescence and floral meristems, and in leaves, respectively. Interestingly, a relationship between *AMI1* and *LFY* has already been assumed, due to altered expression level of *AMI1* in *LFY* gain- and loss-of-function mutants (Le et al. 2010; Lehmann et al. 2010; Mu et al. 2008).

Figure 4.11 Phenotype of inducible *AMI1*ind lines.

Induced overexpression of *AMI1* in the Col-0 background affects plant growth and development. Induced XVE::c-myc:*AMI1* mutants (*AMI1*ind) display distinct phenotypic alterations relative to the wild type.

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Assuming that AMI1 converts IAM to IAA not only *in vitro* but also *in vivo*, we next addressed the question to what extent IAM may alter the phenotype of plants overexpressing AMI1, as those are supposedly converting IAM more efficiently into IAA, which should result in a visible phenotype. Hence, we treated wild type and β -estradiol-induced AMI1ind-lines with different concentrations of either IAM (100-200 μ M) or IAA (0.1-10 μ M), to test for high-auxin phenotypes and possible effects on auxin signalling. For this reason, primary root length was determined. Figure 4.12A shows the phenotype of Col-0 and AMI1ind lines responding to different concentrations of IAM. Although wild-type Arabidopsis plants do also respond to high IAM concentration with enhanced formation of lateral roots and reduced primary root growth, the AMI1ind lines show a clear hypersensitivity towards IAM. Even without treatment, their primary roots appear slightly short. However, when IAM was added to the media, their primary root growth was markedly reduced. At very high IAM concentrations (200 μ M), AMI1ind lines basically stopped primary root growth in the favour of growing adventitious roots on the hypocotyls.

Figure 4.12 Root growth assay under treatment with IAM and IAA.

The growth behaviour of inducible XVE::c-myc:AMI1 mutant line (AMI1ind) and wild-type plants (Col-0) grown on supplemented $\frac{1}{2}$ MS media plates was analysed (A) Col-0 and AMI1ind lines were grown on plates without any supplemented IAM, or on plates containing 10 and 200 μ M of IAM, respectively. In the upper half, root tips are marked with arrows. In the lower part (200 μ M IAM), it was not possible to mark the tips, since AMI1ind lines did practically not grow any primary roots. (B) Root elongation of Arabidopsis wild-type (Col-0) and inducible XVE::c-myc:AMI1 mutant lines was quantified in two-week-old seedlings grown on medium containing indicated concentrations of either IAA or IAM. Results represent means $\pm$ SE of three independent experiments ($n \geq 20$).

Apart of the hypersensitivity of the AMI1ind lines towards IAM shown in the upper part of Figure 4.12B, the lower part of the Figure indicates that IAA signalling is seemingly not affected in the AMI1 overexpressing lines. Already at low IAA contents (< 0.1 μ M IAA), the AMI1ind lines behaved practically like the wild type. Thus, impairment of IAA perception through the ectopic overexpression

of *AMI1* can be excluded.

It has to be noted, that the constitutive overexpression of *AMI1*, driven by a Cauliflower Mosaic Virus 35S promoter, has previously been intended (Neu 2008). However, the degree of overexpression of the obtained BASTA resistant lines was always relatively low, not substantially surpassing the endogenous *AMI1* levels. Presumably, the constitutive overexpression of *AMI1* during embryo development leads to the formation of no viable embryos, which could explain why only mutants with relatively low constitutive expression levels of the transgene were isolated. In order to proof that lack of strong *AMI1* overexpressing lines is associated with the content of active *AMI1* during decisive stages of plant development, similar lines expressing a functional inactive *AMI1* variant, *AMI1_{mut}ox*, under the control of the 35S promoter were generated. *AMI1_{mut}ox* lines carry a mutation in serine 113 (S113A) of the Ser-*cis*Ser-Lys catalytic triade (Neu et al. 2007). Remarkably, for those mutants, it was no problem to identify numerous lines with considerable *AMI1* expression. These already available lines were as well subjected to root growth assays, in order to monitor their responses to rising amounts of IAM and IAA in the growth media, respectively. The mutants had only a very subtle if any deviant phenotype, when grown on IAA. Analysis of the root growth response towards IAM, however, revealed a weak hypersensitivity for the *AMI1ox* lines, consistent with the lower expression levels of the transgene. On the contrary, the *AMI1_{mut}ox* mutants showed hyposensitivity towards IAM at higher concentrations (Figure 4.13). Pointing towards a possible control of *AMI1* contents on the post-translational level.

Figure 4.13 Comparison of root growth response towards IAA and IAM of *AMI1ox* and *AMI1_{mut}ox*.

(A) Primary root length of wild-type *Arabidopsis* seedlings (WT) and 35S::*AMI1* overexpressor lines (*AMI1ox*) were measured at different concentrations of IAM and IAA. (B) Comparison of primary root growth of WT with 35S::*AMI1S113A*

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lines (*AMI1_{mutOx}*) grown on vertical plates containing rising amounts of either IAA or IAM. Results represent means $\pm$ SE of three independent experiments ($n \geq 20$).

4.4 Expression of *AMI1* during plant development

4.4.1 Regulation of *AMI1* expression by indolic compounds

The previously discussed results pointed clearly towards an enzymatic activity for *AMI1* converting IAM to IAA and, thus, an implication of the enzyme in auxin biosynthesis. To better the understanding of the regulation of *AMI1* gene expression, the response of *AMI1* towards several indolic compounds, i.e. IAA, IAM, indole-3-pyruvic acid (IPA), indole-3-acetonitrile (IAN), and indole-3-butyric acid (IBA), was analysed by qRT-PCR. Wild-type plants were treated for 3 h with solutions containing 20 μ M of the different compounds, before the RNA was extracted and qRT-PCR was performed.

Figure 4.14 qRT-PCR analysis of *AMI1* expression in response to selected indolic compounds.

2-week-old seedlings were harvested and used for RNA extraction and qRT-PCR analysis. The transcript levels of *AMI1* were normalized to the expression of *APT1* and *UBQ10* genes. Error bars represent the SE of at least three independent experiments carried out in triplicate.

As shown in Figure 4.14, both IAN and IBA have no substantial impact on *AMI1* expression. In contrast, *AMI1* expression appears to be induced by IAA and IPA, while the treatment with IAM represses its expression. It has to be remarked that the differential *AMI1* expression mediated by the application of IAA, IPA, and IAM was only minor, showing $\log_2$ ratios of $+1.25 \pm 0.16$, $+1.33 \pm 0.18$, and -0.87 ± 0.27 , respectively. Next, further qRT-PCR analyses with the aim to investigate the kinetics of the *AMI1* response towards IAA, IPA, and IAM were performed (Figure 4.15).

Figure 4.15 Monitoring of *AMI1* expression in response to selected indolic compounds by qRT-PCR analysis.

2-week-old plants were treated over 3 h at room temperature with 20 μ M IAA, IPA or IAM. Each hour 100 mg of material was harvested and used for RNA extraction and qRT-PCR analysis. The transcript levels of *AMI1* were normalized to the expression of *APT1* and *UBQ10* genes. Error bars represent the SE of at least three independent experiments carried out in triplicate.

The results demonstrated that the response to exogenously administered IAM was strongest after 2 h. In case of IAA and IPA, the experiments revealed that the response of *AMI1* towards IAA was quicker and stronger than the response to IPA. This suggests that activity of the instable intermediate IPA is possibly due to a previous enzymatic or autocatalytic conversion of IPA to IAA. Growing *pAMI1::GUS* lines on plates containing either IAA (1 μ M, 15 μ M, or 300 μ M) or IAM (1 μ M, 15 μ M, or 300 μ M) confirmed the observation of a relatively weak response. No striking differences in promoter activity could be observed (Figure 4.16). The obtained response profile was, however, somehow unexpected for a gene encoding for an enzyme involved in hormone biosynthesis, as the tight control of hormone contents is assumed to be paramount in a cellular environment, which contradicts positive feedback loops, but rather favour negative feedback loops. In any case, there are also examples in the literature that under certain conditions, such as osmotic stress, e.g. the accumulation of ABA can induce its own biosynthesis (Barrero et al. 2006).

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Figure 4.16 Response of *AMI1* expression to different indolic compounds concentrations.

Seeds of *pAMI1::GUS* were grown on media containing 1 μM, 15 μM or 300 μM of IAA (A) or 1 μM, 15 μM or 300 μM of IAM (B). Either 24 h or 44 h after the beginning of seed germination samples were collected and immediately stained for GUS activity. Photos were captured using a modular stereomicroscope; model MZ10F (Leica Microsystems).

Nevertheless, to conclusively exclude the possibility that AMI1 can also catalyse the reverse reaction, forming IAM from IAA and ammonia, even though this is highly unlikely from a biochemical and thermodynamic point of view, enzymatic assays using 5 μg of purified AMI1 (c.f. Material and Methods, section 3.11.1) and 10 mM of both IAA and NH₄Cl were carried out (c.f. Material and Methods, section 3.11.3). After 6 h of incubation, a 5 μl aliquot of each reaction mixture was directly injected to an UHPLC and the conversion of IAA to IAM determined using a coupled ESI-QTOF mass spectrometer. Positive controls, testing the formation of IAA from IAM, thereby proofing the general activity of the enzyme preparation were carried out in parallel. Under no circumstances a conversion of IAA to IAM was observed, while the principal activity of the protein could be proved (Figure 4.17). Hence, it can be concluded that AMI1 does not catalyse the reverse reaction, but only the formation of free acids from amide substrates.

Figure 4.17 Chromatograms and mass spectra obtained from the analysis of AMI1 enzymatic properties.

(A) Positive control sample, showing the enzymatic production of IAA from IAM (upper part). The lower part shows the mass spectra for IAM and IAA identified in the reaction mix, respectively. The retention times for IAM and IAA were 6.3 min and 8.6 min, respectively. (B) Representation of the chromatogram obtained for the reaction containing IAA and NH_4Cl as substrates. Only one peak at 8.6 min was detected. Both the retention time and the mass spectra shown in the lower part provide evidence that the peak contained IAA. All analyses were made in triplicate.

4.4.2 Analysis of *AMI1* expression in auxin biosynthesis-related mutants

The phenotype of some IAA biosynthetic knockout mutants cannot be rescued by the exogenous application of IAA. In contrast, the endogenous manipulation of cellular IAA levels by expressing the bacterial auxin biosynthesis gene *iaaM* (Klee et al. 1987; Romano et al. 1995) under the control of various *YUC* promoter is sufficient to complement the developmental defects in *yuc1yuc4* and *yuc1yuc2yuc6* double- and triple-mutants, respectively (Cheng, Dai, and Zhao 2006). To address whether altered endogenous auxin contents are capable of changing *AMI1* expression levels, we next examined *AMI1* transcript abundance in a number of auxin biosynthesis-related mutants including the *iaaM* overexpressing plant line *iaaMox*, overexpressor lines for *YUCCA 8* (*YUC8ox*), *YUCCA 9* (*YUC9ox*), *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*pTAA1::GFP:TAA1*), *NITRILASE 1* (*NIT1ox*), and in knockout mutants for genes *nitrilase1* (*nit1*), *rooty1* (*rty1*), *tryptophan aminotransferase of arabidopsis 1* (*taa1*), and the doubles of the cytochrome *p450 monooxygenases* *cyp79b2* and *cyp79b3* (*cyp79b cyp79b3*), as well as *tryptophan aminotransferase of arabidopsis 1* and *tryptophan aminotransferase-related 2* (*taa1 tar2*). Neither elevated nor reduced *NITRILASE1* contents had a profound effect on the expression of *AMI1* (Figure 4.18), confirming our previous

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results. Also in the mutants *taa1* and *taa1 tar2* (Stepanova et al. 2008), we only observed very moderate alterations in *AMI1* expression levels with $\log_2$ ratios of $+0.8 \pm 0.11$ and $+0.56 \pm 0.07$, respectively. In addition, we tested *AMI1* expression in four lines that showed enhanced *TAA1* expression (*pTAA1::GFP:TAA1*) (Stepanova et al. 2011; Stepanova et al. 2008). Notably, only in the strongest of those lines, *pTAA1::GFP:TAA1* 100.2, we were able to detect a considerable induction of *AMI1* gene expression with a $\log_2$ of $+1.5 \pm 0.09$. We observed, however, a much more pronounced effect when we analysed *AMI1* expression in plants known to overproduce IAA, i.e. *iaaMox*, *YUC8ox*, and *YUC9ox*. While we measured IAA levels in *iaaMox* to be on average 50% increased, it is already known that IAA levels in *YUC8ox* and *YUC9ox* are increased by approx. 100% and 85%, respectively (Hentrich et al. 2013). In the three lines, we detected *AMI1* inductions that reached up to a $\log_2$ ratio of $+5.04 \pm 0.21$ (*YUC8ox*).

Figure 4.18 Analysis of *AMI1* expression in auxin biosynthesis-related mutants by qRT-PCR.

2-week-old seedlings were harvested and used for RNA extraction and qRT-PCR analysis. The transcript levels of *AMI1* were normalized to the expression of *APT1*, and *UBQ10*. Error bars represent the SE of at least three independent experiments carried out in triplicate. Mutants analysed: *NIT1ox* (overexpressor of *NITRILASE 1*); *iaaMox* (overexpressor of *iaaM*); *YUC8ox* (overexpressor of *YUCCA 8*); *YUC9ox* (overexpressor of *YUCCA 9*) *pTAA1::GFP:TAA100.2* (overexpressor of *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1*); *nit1* (knockout of the *NITRILASE 1*); *taa1* (knockout *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1*); *taa1 tar2* (double knockout of *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* and *TRYPTOPHAN AMINOTRANSFERASE-RELATED 2*); *cyp79b2 cyp79b3* (knockout of *CYTOCHROME P450 MONOOXYGENASES* genes *CYP79B2* and *CYP79B3*); *rty1* (knockout of *ROOTY 1*).

Intriguingly, we also found an induction of *AMI1* expression in *cyp79b2 cyp79b3*, highlighting a possible functional relationship between *AMI1* and the two cytochrome P450 enzymes. This double mutant is characterized by drastically reduced IAM contents, while maintaining wild type-like IAA levels (Sugawara et al. 2009). Finally, we examined *AMI1* expression in *rty1*, a C-S lyase involved in glucosinolate biosynthesis allelic to *sur1* (King et al. 1995). In contrast to the other studied mutants, we found a repression of *AMI1* in this line. Although a mutation in the *rty1* gene (*At2g20610*) results in a more than 20-fold increase in IAA levels, the IAM contents appeared to be even more affected, showing an augmentation of about 34-fold (Sugawara et al. 2009). In summary, these results clearly confirm that *AMI1* expression responds to IAA and IAM levels, with IAA inducing the expression of *AMI1*, while IAM obviously represses its expression.

In order to substantiate this finding, we introgressed the *pAMI1::GUS* reporter into *YUC9ox* and *rty1* and monitored *AMI1* promoter activity over the course of seed germination. Confirming our previous results, we detected increased GUS activity in the *YUC9ox* background. Especially during the first 48 h after imbibition, we observed stronger GUS signals in the seedlings, whereas in the *rty1* genotype GUS activity appeared to be considerably reduced in the primary roots of young seedlings (Figures 4.19, 4.20, 4.21).

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Figure 4.19 *AMI1* promoter activity during seed germination.

Seeds of *pAMI1::GUS* were germinated on ½ MS medium. Starting at 24 h after the onset of germination, marked by the rupture of the testa, every 2 h (until 72 h post germination start) samples were collected and stained for GUS activity. Photos were taken with a modular stereomicroscope; model MZ10F (Leica Microsystems).

The GUS staining of the control line uncovered an increasing expression of *AMI1* with the on going of seedling development. Starting after about 48 h in the cotyledons, *AMI1* expression peaked 64 h after germination. The detected promoter activity matches considerably well with the previously determined IAM profile during seed germination and early seedling development (Pollmann et al. 2002). At later stages, especially in the primary root, the expression slowly decreased, while in root tips a clear GUS activity remained detectable.

Figure 4.20 *AMI1* promoter activity in *pAMI1::GUS/YUC9ox*.

Seeds of *pAMI1::GUS/YUC9ox* were grown on ½ MS medium. Once the germination process began, samples were taken every two hours between 24 h and 72 h post-germination. Samples were stained for GUS. Photos were taken employing a modular stereomicroscope; model MZ10F (Leica Microsystems).

Analysing the promoter activity of *AMI1* in *pAMI1::GUS/YUC9ox* (Figure 4.20), it becomes obvious that activation of the *AMI1* promoter is detectable earlier during seedling development. Moreover, GUS activity, especially in the primary roots, is more pronounced than in the control lines. As already mentioned, YUC9ox is characterized by substantially increased endogenous IAA level (Hentrich et al. 2013), which is likely the reason for the induced *AMI1* promoter activity. The obtained results confirm our qRT-PCR findings, pointing towards an induction of *AMI1* expression by IAA.

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Figure 4.21 *AMI1* promoter activity in *pAMI1::GUS/rty1*.

Seeds of *pAMI1::GUS/rty1* were grown on solidified ½ MS medium and between 24 h and 72 h every 2 h samples were collected and immediately stained for GUS activity. Photos were captured using a modular stereomicroscope; model MZ10F (Leica Microsystems).

In *pAMI1::GUS/rty1*, *AMI1* promoter activity very much resembles that of the control plants. If at all aberrant, the obtained GUS signal is slightly lower than in the control samples. Especially in roots at later stages (60 h-72 h) the *AMI1* promoter activity appears moderately suppressed. In fact, the data shown here do not fit too well with the qRT-PCR result, where IAM caused a considerable down-regulation of *AMI1*.

4.5 AMI1 and RTY1 do not act in the same pathway but possibly share a common precursor

Since the major portion of IAM in *Arabidopsis* has been shown to originate from IAOx and that a genetic blockade of glucosinolate biosynthesis translates into drastically elevated IAM levels, while not affecting endogenous indole-3-acetonitrile contents (CYP71A13/NIT-shunt), we next investigated whether *AMI1* and *RTY1* act in the same pathway or if they only share a common precursor. To do so, we crossed homozygous *ami1-2* with heterozygous *rty1* plants. After two rounds of selfing of the obtained hybrid lines, we selected for plants homozygous for the *ami1-2* mutation (c.f. figure 4.6) that still showed a pronounced *superroot* phenotype (Boerjan et al. 1995; King et al. 1995).

As presented in Figure 4.22, it was possible to identify three independent lines showing homozygosity for the *ami1-2* mutation.

Figure 4.22 Genotyping of the *ami1* mutation in *ami1-2/rty1* double mutants.

Leaves of 19 lines were used for PCR analysis and only three (the represented in the figure with the numbers 5, 15 and 17) were homozygous for the *ami1-2* mutation. Total genomic DNA was extracted from wild type (Col-0), *ami1-2* and the generated double mutant. To this end, one T-DNA and two gene specific primers were used in the following combinations: (A) T-DNA left border primer (Lb) and the upstream gene specific primer; (B) T-DNA left border primer (Lb) and the downstream gene specific primer; (C) two gene-specific primers, flanking the T-DNA insertion. Col-0 = negative control with DNA from wild-type plants; *ami1-2* = positive control with DNA from the *ami1-2* null mutant.

After confirmation of the homozygosity of the *ami1-2* mutation, the presence of the *rty1* mutation in those three lines was tested. As the mutant *rty1* originates from an EMS-mutagenesis experiment (King et al. 1995), cDNA sequencing was used to detect whether the lines carry a point mutation introduced in the *RTY1* gene. For this reason, cDNA from the three identified lines was prepared alongside to a wild-type control. Sequence integrity of the *RTY1* gene was investigated by PCR, comparing the three putative *ami1-2/rty1* lines with the Col-0 control. All amplified samples showed

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a signal with an estimated molecular weight of approx. 1300 bp, corresponding to the expected size of the *RTY1* cDNA. In order to detect point mutation(s) in the sequence of *RTY1*, being the causal link with the *rty1* mutation, the PCR products were commercially sequenced (StabVida).

Two out of the three lines homozygous for *ami1-2* presented a clear point mutation (transition) at bp 637 of the coding sequence, changing a cytosine to a thymine (Figure 4.23).

Figure 4.23 Sequence analysis of putative *ami1-2/rty1* mutants.

Alignment of wild-type *RTY1* cDNA with the *RTY1* cDNA sequences obtained from the three selected double mutants, homozygous for *ami1-2*. The figure shows a point mutation at position 637 of the sequence in two lines (#5 and #15). The detected mutation causes a change of a cytosine (C) into a thymine (T) (grey box). The sequencing trace files of the third assessed line (#17) showed two signals (C and T) at bp 637, thus, indicating heterozygosity for the *rty1* mutation (*rty1*/+). Nucleic acid positions are indicated.

At the protein level, the identified mutation most likely translates into a change of the primary amino acid structure too. Sequence translation revealed a change from Pro²¹³ to Ser (Figure 4.24).

```

RTY1 MSEEQPHANLAVPAFKTEKEPITQTRNGQSSVWRFGGSDKAAKASTVTLRGVIY
rty1 MSEEQPHANLAVPAFKTEKEPITQTRNGQSSVWRFGGSDKAAKASTVTLRGVIY
*****
RTY1 MLFDNCGKDVNKTILPLGHGDPVYPCFRTCIAEDAVVDVLRSGKGNSYGPGA
rty1 MLFDNCGKDVNKTILPLGHGDPVYPCFRTCIAEDAVVDVLRSGKGNSYGPGA
*****
RTY1 GILPARRAVADYMNRLPHKLTPEIDIFLTAGCNQIEIVFESLARPNANILLPR
rty1 GILPARRAVADYMNRLPHKLTPEIDIFLTAGCNQIEIVFESLARPNANILLPR
*****
RTY1 PGFPHYDARAAYSGLVVRKFDLLPEKEWEIDLEGIEAIADENTVAMVVIN:NNP
rty1 PGFPHYDARAAYSGLVVRKFDLLPEKEWEIDLEGIEAIADENTVAMVVIN:NNP
*****
RTY1 CGNVYSHDHLKKVAETARKLGIMVISDEVYDRTIFGDNPFVSMGKFASIVPVL
rty1 CGNVYSHDHLKKVAETARKLGIMVISDEVYDRTIFGDNPFVSMGKFASIVPVL
*****
RTY1 LAGISKGWVPGWKIGWIALNDPEGVFETTKVLQSIKQNLDPDTPATI IQAAL
rty1 LAGISKGWVPGWKIGWIALNDPEGVFETTKVLQSIKQNLDPDTPATI IQAAL
*****
RTY1 PAILEKADKNFFAKKNKILKHNVLDVCDRLKDIPCVVCPKKPESCTYLLTKLEL
rty1 PAILEKADKNFFAKKNKILKHNVLDVCDRLKDIPCVVCPKKPESCTYLLTKLEL
*****
RTY1 SLMDNIKDDIDFCVKLAREENLVFLPGDALGLKNWMRITIGVEAHMLEDALERL
rty1 SLMDNIKDDIDFCVKLAREENLVFLPGDALGLKNWMRITIGVEAHMLEDALERL
*****
RTY1 KGFCTRHA KKTETETESLQALKLSDNNLEM
rty1 KGFCTRHA KKTETETESLQALKLSDNNLEM
*****

```

Figure 4.24 Amino acid sequence alignment of RTY1 and *rty1*.

The analysed sequences are RTY1 (At2g20610) and the corresponding *rty1* mutant. Asterisks (*) indicate that both sequences have the same amino acid in the same position; points (.) mark amino acids that differ among the sequences. The grey box highlights the region containing the difference between both sequences: change of a proline (P) to a serine (S) at position 213 of the sequence.

After confirming that the *rty1* point mutation translates into an amino acid exchange (Pro²¹³Ser), the question whether this change also results in major changes of the secondary structure was addressed. As can be taken from the secondary structure prediction for RTY1 and *rty1* (Figure 4.25), it is likely that the mutation causes differences in the content of α -helices motifs (14 in RTY, 13 in *rty1*).

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Figure 4.25 Secondary structure prediction.

The figure shows the comparison of the predicted (PSIPRED server) secondary structures from RTY1 and *rty1* proteins. Pink barrels indicate predicted α -helical regions, while yellow arrows represent β -sheet motifs. The figure only shows the one section of the predicted secondary structure that contains the difference between RTY1 and *rty1*.

Analysis of the secondary structures of RTY1 and *rty1* disclosed that both sequences share the same number of β -sheets (11) in their structure. However, when focusing on the α -helices, it turned out that the two proteins seemingly differ in their number of α -helices. RTY1 contains 14 α -helical regions, whereas *rty1* only contains 13. This marked difference is located at the C-terminal end of the amino acid sequence, as shown in Figure 4.25.

4.5.1 Phenotypic analysis

Plants derived from the crossing of *ami1-2* and *rty1/+* were selected phenotypically choosing those, which displayed a clear root phenotype with a substantially increased number of root hairs, caused by the *rty1* mutation.

An example of the *rty1* phenotype is shown below.

Figure 4.26 Characteristic phenotype of *rty1* plants.

Mutant *ami1-2/rty1* seedling grown on soil, showcasing a root hair-phenotype.

After the selection of homozygous double mutants, we found that the offspring of these two lines were dramatically compromised in germination. The resulting seeds either did not germinate at all or aborted the germination process after a very short period of time (48-72 h), just after the radicle broke through the testa (Figure 4.27 (D-F)).

Figure 4.27 Seeds from *ami1-2/rty1* show a growth arrest.

(A) Seeds of *ami1-2/rty1* were grown on plates without IAA (a-c) or on corresponding plates containing 100 μ M IAA (d-f). (B) Comparison of seedlings of *ami1-2/rty1* and Col-0 grown on MS medium without and with IAA (100 μ M). Under both conditions, germination appeared to be aborted shortly after the radicle broke through the testa. However, it is possible to see that Col-0 plants arrive to germinate and later, to grow.

Applying exogenous IAA did not rescue the seedlings (Figure 4.27 (A-C)), suggesting that the double mutants possibly suffer from a genetic defect of the embryo that cannot easily be reverted by adding

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IAA during germination. To further investigate the role auxin and in particular AMI1 in germination, we first analysed the seed size of several auxin-related mutants in comparison to wild-type *Arabidopsis* seeds. As shown in Figure 4.28, the auxin-overproducing lines, i.e. AMI1ind and YUC9ox, are characterized by a 25 % increases in seed size, whereas the *ami1-2* and *cyp79b2 cyp79b3* mutants show significantly smaller seeds. However, *ami1-2/rty1* seeds do not significantly differ from those of wild-type or *rty1* seeds, suggesting the *rty1* mutation may be able to compensate for the lack of AMI1 during seed development.

Figure 4.28 Analysis of seed size for different auxin mutant lines.

The graphic shows the analysis of seed size in mm² from the lines Col-0; *ami1-2*; AMI1ind; YUC9ox; *cyp79b2 cyp79b3*; *rty1* and *ami1-2/rty1*. The biggest seeds belonged to the lines AMI1ind and YUC9ox, while the significantly smallest ones were those of *ami1-2* and the double mutant *cyp79b2 cyp79b3*. Values represent the mean of at least 20 seeds and error bars give the standard error of the mean. Statistic analyses were carried out using Student's *t*-test (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

However, to test whether the combination of the mutations in *ami1-2* and *rty1* has a pronounced effect on embryo development, we isolated embryos of *ami1-2*, *rty1* and *ami1-2/rty1* and compared their appearance and size to wild-type embryos (Figure 4.29). All examined embryos showed uncompromised polar growth with clearly developed hypocotyl, radicle, and cotyledons. The *ami1-2* mutation led to slightly smaller embryos, while those of the *rty1* mutant seemed to be bigger than the wild type. However, the *ami1-2/rty1* embryos were found to be substantially smaller than both the wild type and the parental lines, indicating that *rty1* does definitely not compensate for the functional loss of AMI1 in the *ami1-2* mutation. Moreover, our data indicate that, at least in *Arabidopsis*, the IAOx pathway plays an essential role in seed maturation and germination. Most obviously, other auxin biosynthesis pathways cannot compensate for the loss-of-function of AMI1 and RTY1. Furthermore, the additive phenotypic effect in *ami1-2/rty1* in comparison to the two

parental lines provides strong genetic evidence for a parallel action of the two enzymes. Most likely, IAOx represents a metabolic key branching point from which several pathways, e.g. the biosynthesis of glucosinolates, camalexin, and IAA (Glawischnig et al. 2004; Sønderby, Geu-flores, and Halkier 2010; Sugawara et al. 2009; Zhao et al. 2002) emerge.

Figure 4.29 Embryo phenotype of Col-0, *ami1-2*, *rty1-1* and *ami1-2/rty1-1*.

Seeds were imbibed over a period of 24 h in water. Thereafter, the embryos were prepared and analysed using a stereomicroscope (Leica Microsystems, MZ10F). (Scale bars = 1mm).

4.6 The expression of *AMI1* impacts several different functional groups in *A. thaliana*

With the aim of shedding light on the molecular function of AMI1, a series of microarray analyses were carried out. Firstly, the *ami1-2* transcriptome was compared to that of wild-type plants. Apart from a slightly smaller seed and embryo size, *ami1-2* plants are morphologically indistinguishable from wild type at the seedling stage, thus minimizing gene expression differences resulting from any morphological abnormalities 10 day-old seedlings were chosen for this study. The microarray analysis revealed 1075 differentially expressed genes, with 666 genes being up and 409 genes down-regulated in the mutant when compared to the wild type, according to the parameters used for the filtration (c.f. Material and Methods, section 3.16). The subsequently conducted Gene Ontology (GO) analysis (c.f. Annex, Figures S.1 and S.2) identified a significant proportion of the up regulated genes associated with stress responses, including responses to wounding and oxidative stress, as well as temperature and chemical stimuli. The latter GO category comprising genes that are related to responses to jasmonic and abscisic acid stimuli. In addition, there were other significantly overrepresented categories related to metabolic processes that included, among others, oxylipin and

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jasmonic acid metabolic processes (c.f. Annex, Table S.1). Remarkably, also among the 409 down-regulated genes there are several groups of genes belonging to the GO category of plant stress response-related genes, which highlights an involvement of *AMI1* in plant stress responses. In addition, our results provide a substantial body of evidence that *AMI1* is involved in plant hormone crosstalk, as a large number of jasmonic acid biosynthesis-related genes, i.e. *LOX3*, *AOC1*, *AOC3*, *AOS*, and *JAR1* appear to be significantly induced in *ami1-2*.

Moreover, we identified a group of genes related with the response to hormone stimulus among the down regulated genes. We observed a considerable repression of auxin signalling-related genes in the *ami1-2* background. This is in accordance with our previous result (c.f. Figure 4.6), showing a reduced GUS reporter activity in the *ami1-2/DR5::GUS* line, thus, pointing towards decreased auxin signalling in this line.

Enrichment analyses using a gene ontology approach often provide large and highly redundant results that are difficult to interpret. The REVIGO server, however, offers the possibility to summarize long and unintelligible lists of GO terms by finding representative subsets of terms through the utilization of a clustering algorithm that relies on semantic similarity measures (Supek et al. 2011). Hence, the multidimensional scaling and graph-based visualization tool of REVIGO was subsequently used to accurately render the non-redundant subdivisions and semantic relationships in the microarray data (Figure 4.30).

Figure 4.30 Scatterplot view of REVIGO for up- and down-regulated genes identified in the *ami1-2* microarray.

The scatterplot shows the cluster representatives (i.e. terms remaining after the redundancy reduction) in a two-dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities. The colour of the circles indicates the user-provided p-value (legend in upper part of the figure); size indicates the frequency of the GO term in the underlying GO database (circles of more general terms are larger).

Further improving the interpretation of the previously discussed GO results, an overrepresentation of genes involved in responses to various types of stresses was found among the up regulated genes. Intriguingly, the analysis also uncovered relationships with genes known to be involved in carbohydrate metabolism and regulation of responses to some received stimulus, such as the presence of bacteria or another pathogenic organism. This would explain the moderate *ami1-2* root phenotype that can be rescued by the application of carbohydrates and also suggest an intimate relationship of the AMI1 catalysed reaction with plant stress response. Moreover, the results imply that *AMI1* is involved in plant hormone crosstalk, particularly with ABA and SA.

To complement the obtained results for the *ami1-2* mutant, another microarray analysis was carried out using the overexpressor line AMI1ind. The results were again compared to Arabidopsis wild-type plants.

Here, the total number of differentially expressed genes was 1010, among which 388 were up regulated and 622 down regulated. Again, GO/Gene enrichment analyses have been performed, particularly focusing on functional connections between the groups of genes identified.

Studying the GO data obtained for the up-regulated genes in AMI1ind (c.f. Annex, Figure S.5), we detected an overrepresentation of genes involved in responses to chemical and abiotic stimulus, and here in particular in changes of temperature (c.f. Annex, Table S.2); GO categories that we already found enriched in the *ami1-2* mutant (c.f. Annex, Table S.1).

Analysing the distribution of down regulated genes in the AMIind in GO categories, we detected a clear enrichment of various functional groups. Many of the differentially expressed genes were allocated in functional groups related to plant defence processes, such as “response to chitin” or “innate immune defence”. In particular, we observed down regulation of a substantial number of genes that codify for TOLL/INTERLEUKIN RECEPTOR (TIR) domain-containing proteins that play an important role in plant defence (Nandety et al. 2013). Moreover, we observed the down regulation of several genes involved in cell death (c.f. Annex, Table S.2).

A representation of the corresponding REVIGO analyses for up- and down-regulated genes is shown in Figure 4.31.

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Figure 4.31 Scatterplot view of REVIGO for up- and down-regulated genes obtained from the analysis of differentially expressed genes in AMI1ind.

The representative clusters are shown in a two-dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities. Colours indicate the obtained p-value (legend in upper part of the figure); size reflects the frequency of the GO term in the underlying GOA database (bubbles of more general terms are larger).

As can be taken from Fig. 4.31, a significant up regulation of cellular and developmental processes, including carbohydrate metabolism, was found. This can be explained by the fact that AMI1ind is an inducible line that contains augmented IAA concentrations, which ultimately translates into alterations in the control of plant development (c.f. Figure 4.10E).

In addition, the GO analyses revealed the up regulation of several genes related to the response to heat, while the majority of abiotic/biotic stress-related genes appear down-regulated. This further underlines the relation of AMI1 with plant stress responses. Interestingly, carbohydrate metabolism is also affected in the *AMI1* overexpression line, which is possibly associated with the premature flowering phenotype. However, apparently the altered phenotype does not depend on the energy sensor kinases KIN10 and KIN11 (Sheen 2014), as they do not display differential expression in the microarray data.

As previously described, it was not absolutely clear where AMI1 acts in auxin biosynthesis (c.f. Figure 1.5). The work of Sugawara et al. (2009) provided evidence that 95% of the endogenous IAM in *Arabidopsis* originates from IAOx, which would position AMI1 downstream of CYP79B2 and CYP79B3 in the IAOx pathway, as the two cytochrome P450 monooxygenases are known to convert the IAA-precursor Trp into IAOx. Intriguingly, the *cyp79b2 cyp79b3* double knockout mutant and *ami1-2* display comparable phenotypes (Figure 4.28), which further underlines a possible functional

relationship between the gene products. However, experimental confirmation of the suggested relationship was still elusive.

In order to gain deeper insight into the relationship between the two P450 enzymes and *AMI1*, we decided to subjected *cyp79b2 cyp79b3* seedlings and wild-type plants to microarray analysis. The genome-wide transcriptional profiling disclosed a total of 429 and 544 up- and down-regulated genes, respectively. Among the up-regulated genes we detected *AMI1* (Fold-change = 1.83, p-value = 0.004). This result corroborates the previously obtained qRT-PCR results (Figure 4.18).

In order to analyse the obtained data in more detail, we compare them with the results obtained for the *AMI1* mutant lines. In first place, a GO enrichment analysis was carried out, employing the differentially expressed genes in the study (c.f. Annex Figures S.8-11). A filtered list of all significantly differentially expressed genes, as well as overrepresented groups of genes are included in the Table S.3.

The data in Table S.3 highlight that particularly defence-related genes become altered when *CYP79B2* and *CYP79B3* are mutated. As described before, in Brassica plants the IAOx pathway is involved in the formation of indole glucosinolates, which represent important defence compounds in this family. Thus, compensation of the loss of the IAOx pathway through the differential regulation of other defence mechanisms is not unexpected. For example, among the up-regulated genes, gene enrichment in the GO groups: defence response by callose deposition (GO Id.: 52542), defence response by cell wall thickening (GO Id.: 52482), innate immune response (GO Id.: 45087) and regulation of defence response (GO Id.: 31347) has been found, whereas processes related with the biosynthesis of glucosinolates (GO Id.: 19760), the response to wounding (GO Id.: 9611), and the biosynthesis of flavonoids (GO Id.: 9813), compounds involved in auxin transport, appear to be down regulated (c.f. Annex, Table S.3). *AMI1* expression is up-regulated in the *cyp79b2 cyp79b3* microarray, thereby confirming our previous qRT-PCR results and underlining a possible relationship between these genes. Moreover, this apparent connection may put further emphasis on the involvement of *AMI1* in defence-related processes.

To further clarify the results obtained, a subsequent REVIGO analysis was performed (Figure 4.32).

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cyp79b2 cyp79b3

Figure 4.32 Scatterplot view of for up- and down-regulated genes in the *cyp79b2 cyp79b3* genetic background.

The groups of genes are represented by clusters in a two-dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities. Colour indicates the user-provided p-value (legend in upper middle-hand corner) and size represents the obtained frequency of each GO term in the underlying GOA database (bubbles of more general terms are larger).

The obtained results confirmed the previous findings, underlining an overrepresentation of defence-related genes among the up regulated genes and an enrichment of genes related with glucosinolate and flavonoid biosynthesis among the down regulated genes. Differentially expressed genes involved in various processes related to carbohydrate metabolism also appeared to be overrepresented in the double knockout mutant. As shown in figure 4.32 and in the supplementary figures S.8 and S.9, particularly genes involved in the response to carbohydrates, especial to glucose, were detected to be up regulated. This finding is particularly intriguing; taking into account that *AMI1* expression is elevated in the explored double mutant and that its expression seems to be affected by sugar contents. Anyhow, without measuring sugar contents in *cyp79b2 cyp79b3*, a final conclusion on a putative regulatory circuit controlling *AMI1* expression via, e.g., glucose contents cannot be drawn. Neither does this observation allow placing *AMI1* downstream of *CYP79B2* and *CYP79B3* in a possible metabolic route. However, the obtained data corroborate the intimate and possibly complex relationship of *AMI1* and the two P450 enzymes, *CYP79B2* and *CYP79B3*.

Previously, studying the *ami1-2/rty1* double mutant, we observed that the germination process was always aborted (Figure 4.27). In addition, the analyses of embryos from plants of these mutant lines were tiny in comparison with those of the parental lines and/or wild-type plants (Figure 4.29). This led us to speculate that *AMI1* and *RTY1* are either important for successful seedling development or that an accumulation of IAM may inhibit proper development. In order to gain deeper insight into

the molecular basis of the abortion of the germination process and the extreme embryo phenotype of *ami1-2/rty1*, we decided to complete the data obtained up to this point, with a transcriptomic analysis. We performed a RNA-Sequencing (RNA-Seq) experiment, using extracted RNA from seeds of *ami1-2/rty1* plants (c.f. Materials and Methods, section 3.6.2).

As before, the resulting data was subjected to GO/Gene analysis for the identification of overrepresented functional groups and genes in these groups, respectively (c.f. Annex, Figures S.12 and S.13). In figure S.12 three groups of genes appear up regulated, although at low value of significance, as the yellow colour of their correspondent GO boxes shows. One of the three groups is composed by genes related to “localization”, which exert a negative regulation on transport-related genes. Also genes related to post-embryonic developmental processes appear negatively regulated, as the green arrow of the figure S.12 indicates. This result corroborates our previous observation of a repression of embryo development in this mutant. Finally, the third group of up regulated genes corresponds to the GO annotation of “response to stimulus”, and in particular to “response to chitin” (c.f. Annex, Figure S.12).

On the other hand, figure S.13 shows a large number of gene groups down regulated. In fact, it seems that the transcriptional machinery is severely affected, inhibiting any possibility of gene expression. Some examples for these down-regulated groups are “ribosome regulation” or “translation” (c.f. Annex Figure S.13; Table S.4). Of course, this blockade of the transcriptional elements has consequences on plenty of the other groups. For instance, genes pooled in the GO term “post-embryonic development” are affected. As expected, this group of genes is negatively affected, what could mean that even if those genes were up- or down-regulated, their transcription would be obstructed. In general, quite a big number of functional groups of genes, such as the group related to the “response to temperature”, appear to be significantly down-regulated in this mutant line (c.f. Annex, Figure S.13; Table S.4).

Doing a subsequent REVIGO analysis, we obtained the data presented in Figure 4.33 that corroborates the previously described results given in the GO figures and tables (c.f. Annex, Figures S.12, S.13 and Table S.4).

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Figure 4.33 Scatterplot view of REVIGO for up- and down-regulated genes generated on the basis of *ami1-2/rty1* RNA-Seq data.

The genes are grouped in clusters according to GO terms' semantic similarities. In the figure, it is showed the representatives clusters in a two-dimensional space. The legend, in the upper middle-hand corner, shows the different colours that represent the different obtained p-value in the RNA-Seq data. Clusters are bigger when they include more general terms.

Expectedly, we found the majority of enriched functional groups among the down-regulated genes. Re-assessing the GO analysis data, the REVIGO analysis provided only one enriched group among the up regulated genes: the group composed by “response to stimulus”-related genes.

With respect to the down-regulated genes, we again obtained groups related with cellular processes, cellular component biogenesis, gene expression, etc. as the most profoundly enriched groups among the down-regulated genes. Also, genes involved in the post-embryonic development and stress responses appeared significantly overrepresented.

4.7 Query for the enzyme working up-stream of AMI1

Apart from the functional characterization of AMI1, one important objective of this work has been the search for possible candidates that are capable of catalysing the reaction step up-stream of AMI1. This could be either the conversion of Trp to IAM or of IAOx to IAM. It is well established that several cytochrome P450 monooxygenases, e.g. CYP79B2, CYP79B3 and CYP83B1, are involved in the IAOx pathway, which is most likely a highly important pathway for the production of camalexin, indole glucosinolates and, to some extent, auxin in Arabidopsis. Alongside with flavin-containing monooxygenases, and due to their biochemical properties, members of this enzyme class appear to be the most probable candidates for the enzymatic production of IAM. For this reason, a detailed

analysis looking for corresponding genes showing differential regulation in the *ami1-2* and *cyp79b2/cyp79b3* microarray was carried out.

Table 4.2: List of differentially regulated genes in *ami1-2* and *cyp79b2 cyp79b3* microarrays. (Genes with significant changes in the expression are highlighted in red).

Gene	<i>ami1-2</i> vs WT microarray		<i>cyp79b2 cyp79b3</i> vs WT microarray	
	Fold-change	p-value	Fold-change	p-value
CYP94B1 (At5g63450)	+6.44	0.00009821	+1.13	0.83232513
CYP81D1 (At3g28740)	+4.43	0.00003987	-1.45	0.28996872
CYP94C1 (At2g27690)	+3.77	0.00016855	+3.56	0.07957492
CYP81F3 (At4g37400)	+2.26	0.03204994	+1.04	0.72077819
CYP82G1 (At3g25180)	+2.11	0.00019415	+1.09	0.74452625
CYP707A3 (At5g45340)	-2.67	0.00067379	+1.67	0.35839329
CYP71B22 (At3g26200)	-2.42	0.00014492	+1.73	0.04812773
CYP709B3 (At4g27710)	-1.15	0.11606600	-2.57	0.00007366

Overall, it was possible to identify 8 differentially regulated CYP genes (Table 4.2). Only 2 out of the 8 genes had unknown functions. These genes were *CYP709B3* and *CYP71B22*. Both were down-regulated in the *ami1-2* microarray with a fold-change of -1,15 and -2,42, respectively. However, it has to be remarked that the differential expression of *CYP709B3* in *ami1-2* is neither significant nor pronounced. Focussing on *CYP71B22*, the microarrays revealed a significant repression in the *ami1-2* background. On the contrary, in the *cyp79b2 cyp79b3* background, in which the IAOx-pathway is completely interrupted, the expression of *CYP71B22* is induced, rendering the latter gene a very interesting target for further studies. Nevertheless, based on these results and on the fact that the two genes appeared to be induced by auxin and through pathogen attack (Cartieaux et al. 2008; Goda et al. 2004; Pré et al. 2008), respectively, it was decided to further investigate on the two so far uncharacterized genes, trying to figure out whether they possibly play a role in auxin biosynthesis.

Corresponding T-DNA insertion lines from the SALK-collection were studied. For both genes T-DNA insertion lines could be identified in the collections [*CYP709B3*: SALK_011121; *CYP71B22*: SALK_047773 (named by us *CYP71B22-1*), SALK_129670 (called *CYP71B22-2*)]. It was possible to identify homozygous T-DNA insertion mutants for the lines *CYP709B3* and *CYP71B22-2* by PCR analysis (Figure 4.34). The SALK line *CYP71B22-1* did not present any T-DNA insertion, so we discarded it from further assays.

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Figure 4.34 Genotypization of *cyp71b22-1*, *cyp71b22-2* and *cyp709b3* T-DNA insertion lines.

Leaves of several plants from each mutant were used for individual PCR analysis. Representative examples for the obtained PCR results are shown. Total genomic DNA was extracted from wild type (Col-0), *cyp71b22-1*, *cyp71b22-2* and *cyp709b3*. For each plant sample three PCR reactions were performed. Herein, one T-DNA and two gene specific primers were used in three combinations for the analysis: The first reaction (A) contained two gene-specific primers, flanking the T-DNA insertion; the second reaction (B) contained the T-DNA left border primer (Lb) and the upstream gene specific primer; the third reaction (C) comprised the T-DNA left border primer (Lb) and the downstream gene specific primer. Col-0 = wild-type control.

Sequencing of the obtained PCR fragments revealed the loci of the T-DNA insertions. In case of *cyp709b3*, the T-DNA is located in the fourth exon of the gene, whereas the T-DNA in *cyp71b22* was integrated in the 3'UTR (untranslated region) (Figure 4.35).

Figure 4.35 Scheme of the position of the T-DNA insertion(s) in *cyp71b22* and *cyp709b3*.

In (A), the schematic structure of *CYP71B22* is showed. The gene is composed by only two exons joined by one intron. The T-DNA insertion was found to be located in the 3'UTR region. (B) The lower part of the figure shows the representation of the genomic organisation of the *CYP709B3* gene. Here, the T-DNA insertions are located in the fourth exon. Lb: Left border primer; Grey boxes: UTR motifs; White boxes: exons (numbers indicate the position that they have in the gene starting to count from the "ATG" initial).

Interestingly, the obtained mutants showed a retarded germination reminiscent of the *ami1-2/rty1* phenotype. In comparison with the wild type plants, the germination process was slower in these two lines, especially in *cyp71b22*. It was necessary to grow the seeds in plates under strictly controlled conditions to help them to establish viable seedlings. Once the seedlings started to develop the first leaves, it was possible to transfer them to soil. However, differences appeared again in silique production, being numerous for wild type but less productive in the *cyp* lines, suggesting that these genes could be important for optimal plant development and seed yield (Figure 4.36).

Figure 4.36 Comparison of adult wild type vs *cyp71b22* and *cyp709b3* plants.

The three images are showing the wild type (Col-0), *cyp71b22* and *cyp709b3* at a generative developmental stage. The number of ramifications and also of siliques are minor in the *cyp* lines relative to Col-0.

In a second approach, total RNA of the two mutants was extracted and subjected to next-generation sequencing. The RNA-Seq analysis confirmed one of the two genes, *CYP71B22*, as a highly promising candidate through its obvious transcriptional relationship with *AMI1*. In comparison to the *cyp709b3* mutant, in which *AMI1* expression did not appear to be affected at all (p-value = 2.41), *AMI1* expression levels were significantly down regulated in *cyp71b22* (p-value = 0.0024)

Apart from the differential expression of *AMI1* in *cyp71b22*, the RNA-Seq experiment disclosed additional highly interesting results. After filtering, 1295 differentially regulated genes were identified. Out of those genes, 838 appeared up regulated, while 457 were down regulated. In particular among the up regulated genes, the subsequently conducted GO and REVIGO analyses revealed an overrepresentation of genes involved in hormone levels, as for instance abscisic acid, or in response to several abiotic stresses, such as heat or high light intensities (Figure 4.37 and c.f.

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Annex, Figure S.14). The majority of the overrepresented genes are related with general responses to different abiotic and biotic stresses. For instance, we found a transcript, which codifies for a protein related with drought stress (*AT1G56280*) and also other genes, whose correspondent proteins are important to immunity of Arabidopsis to the downy mildew *Hyaloperonospora arabidopsidis* (*AT5G24530*). Furthermore, the results suggested a functional relationship of *CYP71B22* with carbohydrate metabolism and post-embryonic development, very much resembling the results observed for *AMI1*.

Focusing on the transcription of genes related with auxins, on the one hand, we detected up regulated several early response to auxin stimulus-genes. For instance: *IAA29* (p-value= $7,7 \cdot 10^{-6}$) or *IAA16* (p-value= $2,76 \cdot 10^{-23}$). On the other hand, in the Revigo analysis we observed that the genetic group of auxin biosynthesis appeared down regulated. Moreover, one of these genes was *AMI1* (p-value= 0,00249), which suggests the proposed relationship for these two genes.

Figure 4.37 Scatterplot view of REVIGO for up- and down-regulated genes based on *cyp71b22* RNA-Seq data.

This figure shows the most representative clusters obtained from the *cyp71b22* RNA-Seq data. These clusters are composed of genes grouped by GO terms similarities. Bigger clusters contain more general terms. The colour of each cluster indicates the user-provided p-value (legend in upper middle-hand corner).

In the case of *cyp709b3* plants, the RNA-Seq data only revealed an overrepresentation of groups related with the functions of CYP P450 proteins: lipid biosynthesis, cofactor metabolism, etc. (Figure 4.38 and c.f. Annex, Figure S.16), but none of the detected groups was involved in hormone stimulus, developmental processes or anything more connected with the previously groups observed in *cyp71b22* or *AMI1*.

Figure 4.38 Scatterplot view of REVIGO for up- and down-regulated genes calculated from the *cyp709b3* RNA-Seq data.

This two-dimensional space figure shows the most representative groups of genes that appear differentially regulated in the sample *cyp709b3*. The genes are grouped in clusters, according to GO terms similarities. Colours indicate the p-value of each cluster (legend in upper middle-hand corner); size represents the frequency of the GO term in the underlying GOA database (clusters of more general terms are larger).

While it was possible to identify the overrepresentation of a number of indole metabolism and auxin biosynthesis-related genes in *cyp71b22*, the differentially regulated genes in *cyp709b3* were mostly related with the biosynthesis of a wide range of auxin-unrelated compounds, such as fatty acids, pigments, other plant hormones and secondary metabolites.

From previous studies it was known that *AMI1* expression follows a circadian rhythm (communication S. Pollmann). Together with the regulatory influence that glucose and sucrose exert on the expression of *AMI1* (Figures 4.8 and 4.9), this may suggest a link of *AMI1* with the energy status of the plant. In any case, the circadian expression of both *CYP709B3* and *CYP71B22* was analysed employing data deposited in the Diurnal database hosted by the Mockler lab (Mockler et al. 2007). It turned out that both genes also share pronounced circadian expression profiles under short day conditions (8 h light, 16 h darkness) (Figure 4.39), but only the profile of *CYP71B22* matched with that of *AMI1*, although a moderate phase shift can be detected. The profile of *CYP709B3*, however, appeared more or less in anti-phase, which rules an enzymatic collaboration of the two gene products basically out. At the same time, this finding further underlines a possible functional connection of *AMI1* and *CYP71B22*.

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Figure 4.39 Diurnal expression profiles of the *AMI1*, *CYP709B3* and *CYP71B22* in short days.

Using the Diurnal database of the Mockler lab (<http://diurnal.mocklerlab.org>) the expression patterns for the studied target genes in plants grown under short day conditions (8 h light/ 16 h darkness) were retrieved.

Taking together, all of the presented results strongly suggest a functional relationship between *AMI1* and *CYP71B22*, making the latter an extremely promising candidate for an enzyme involved in the production of IAM. To verify the correctness of this educated guess, the *CYP71B22* cDNA was amplified and introduced into pDONR221. After sequence verification by commercial sequencing, the *CYP71B22* cDNA was transferred into the bacterial expression vectors pDEST15 and pDEST17

providing translational fusions of the cDNA with a GST- and His-tag, respectively on its 5'-extreme. Unfortunately, all trials of expressing the cDNA so far were unsuccessful. Apart of an only very low expression level, the recombinant protein was mainly found in the pellet fraction, suggesting a membrane localisation of the protein, which is a common feature of numerous cytochrome P450 enzymes. However, a thorough biochemical characterization of CYP71B22 is key and indispensable before drawing a final conclusion on the putative involvement of this enzyme in auxin biosynthesis.

4.8 Regulatory impact of the IAM:IAA ratio

With respect to the previously presented experiments, it appeared tempting to speculate that the main function of AMI1 is possibly not the production of IAA, but rather the control of cellular IAM levels, and thus the IAM:IAA ratio. This hypothesis is mainly based on the unexpected transcriptional regulation of *AMI1* gene expression (Figures 4.14 and 4.15) and the observation that auxin over-expressers produce bigger seeds, whereas plant lines over-accumulating IAM are characterised by smaller seeds (Figure 4.28) or, even more severe, by abortion of the germination process (Figure 4.27). From the genetic experiments presented in this work (Figure 4.18) and those previously published by Sugawara and colleagues (2009), it can be concluded that *AMI1* most likely participates in the IAOx-pathway in *A. thaliana*. Moreover, the data presented here point towards an intimate link between AMI1 and plant stress responses. For these reasons, it was decided to analyse the regulatory impact of IAM in closer detail. With the aim to scrutinize whether IAM can act as an independent signalling molecule, wild-type Arabidopsis, *ami1-2* and AMI1ind seedlings were treated for 2 h with 100 μ M IAM. Thereafter, total RNA was extracted and subjected to RNA-Seq experiments. It has to be kept in mind that the *ami1-2* mutant is substantially impaired in converting IAM to IAA, when compared to the wild type. On the contrary, AMI1ind plants show a considerably increased conversion of IAM to IAA. Thus, it can be concluded that in these lines auxin-related responses prevail.

Firstly, the comparison genes up- and down-regulated in the three genotypes, respectively, led to the identification of AMI1-independent regulatory effects. As can be taken from the Venn diagrams (Figure 4.40), only a very small number of genes, i.e. 3 down-regulated and 16 up-regulated genes, appeared differentially expressed upon IAM treatment, independent of AMI1. All of the remaining differentially regulated genes seem to be directly or indirectly attributable to AMI1-dependent responses to the treatment with IAM. Herein, the experiment using the *ami1-2* genetic background is of special interest, as the obtained results are not distorted by the conversion of IAM to IAA through either endogenous AMI1 (WT) or endogenous and recombinant AMI1 (AMI1ind).

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Figure 4.40 Venn diagram display of differentially expressed genes in wild-type, *ami1-2* and AMI1ind plants treated with IAM.

Data obtained from RNA-Seq analyses done for wild type, *ami1-2* and AMI1ind plants treated 2 h with IAM, were compared to each other to identify the overlapping genes after IAM-treatment among the down- (A) or up-regulated (B) genes.

As mentioned beforehand, we detect 3 down-regulated genes that are common for the three genotypes. These three genes are *At1g29500*, *At3g07195* and *At5g28630* (c.f. Annex, Table S.11). The first one is an early auxin response gene called *SMALL AUXIN UPREGULATED RNA 66* (*SAUR66*). The second one is a member of the resistance (R) protein family (RPM) that act as essential regulators of plant defence, playing central roles in case of pathogen infection. The third gene encodes for a glycine-rich protein of unknown function.

Regarding the group of 14 common genes found down regulated in wild type and *ami1-2*, the analysis revealed that is composed by a variety of transcripts. Some of them (*At5g52760* and *At4g18205*) are transcripts that code for transport proteins; others appear related with the biosynthesis of cell wall and its constituting components (*At1g0550*, *At5g10430*), respectively. In addition, there is a little group of genes composed by *At5g18080*, *At1g15580* and *At4g14560* that are part of the early auxin response. The first one is the *SMALL AUXIN UP RNA 24* (*SAUR24*) and the other two are the *Aux/IAA* genes *INDOLE-3-ACETIC ACID INDUCIBLE 5* and *1* (*IAA5* and *IAA1*), respectively. The latter group establishes a direct relation of AMI1 action with the response to auxin. On the other hand, the six common genes that are shared wild type and AMI1ind do not provide evidence for any kind of considerable relationship.

With respect to the up-regulated genes, the Venn diagram revealed 16 differentially up-regulated genes that do not depend on AMI1, as they appear in all three analysed genotypes. Some of these genes are related with defence processes. For instance, *At4g21440* and *At4g05100*, two MYB

transcription factors involved in wounding and osmotic stress response. Apart of that, the included transcripts do not share apparent functional relationships.

Focusing on the commonly up-regulated genes between wild-type Arabidopsis and *ami1-2*, we detect a number of interesting novel target genes for future studies. For example, a gene encoding for an ABC transporter (*At3g55130*), the gene *LATE EMBRYOGENESIS ABUNDANT 3* (*LEA3*; *At1g02820*) and a gene whose transcriptional response is blocked in JA biosynthetic and JA signalling mutants (*At1g61120*). These three genes may explain part of the impact of the IAM:IAA ratio on plant development, particularly when shifted towards IAM in the *ami1-2* null mutant. The T-DNA insertion line *ami1-2* is characterized by the accumulation of IAM, which may translate into an inhibition of IAA transport, impaired embryonic development (as detected in the *ami1-2/rty1* RNA-Seq assay) and finally by a possible connexion between AMI1 and JA metabolism. These genes are not only up regulated in *ami1-2*, but also in wild-type Arabidopsis, which is supposedly due to the addition of IAM that is not rapidly enough converted to IAA to maintain an unshifted IAM:IAA ratio.

Among the up-regulated genes common in WT and AMI1ind there is no specific aggrupation detectable that can directly be attributed to an effect caused by the administration of IAM, since both genotypes are still producing IAA. So, in these two lines the remaining IAM:IAA ratio does not facilitate the investigation of the pure IAM influence in the same way than in *ami1-2*. For this reason, we selected *ami1-2* as the most appropriate genotype to study the impact of IAM at the molecular level.

To further advance our understanding in this direction, we carried out detailed bioinformatics analyses for *ami1-2* and wt. The data used for this purpose were directly taken from the RNA-Seq experiments and from the analysis done with the GO and REVIGO platforms. The corresponding results are shown in figures 4.41 and 4.42.

First of all, we started with the line most affected by the addition of IAM, *ami1-2*. In this line, there were several down-regulated groups of genes related to defence processes, auxin, seed development, etc. (Figure 4.41 and c.f. Annex, Figures S.22 and Table S.8). The group of genes related to defence processes makes up the biggest functional group (13%). Here especially genes that are associated with the innate immune response peaked out. Some gene products of this group are disease resistance proteins from the TIR-NBS-LRR class; c.f. Annex, Table S.8. The supplementary table also provides information on several of these genes being related with cell death. Hypersensitive response (HR) is a process long known to be induced during plant defence responses. Under normal conditions, HR usually results in programmed cell death, what could explain the presence of these disease resistance proteins in the cell death group. On the other hand, we identified a group of auxin related genes (4.4%) that are principally involved in the early response to auxin stimuli, basically confirming the conclusions drawn on the basis of the Venn diagrams. In this

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group, we found several *INDOLE-3-ACETIC ACID* (IAA) genes and other auxin response proteins (c.f. Annex, Table S.8), principally confirming the observations made on the basis of the *ami1-2* microarray analysis (Table S.1).

Figure 4.41 also presents differentially regulated genes that are involved in seed development. And more important in this context, it was possible to associate those genes specifically with the arrest of seed development, which underlines the importance of maintaining proper internal IAM contents, in order to successfully drive the process. Moreover, it was possible to observe a big group of genes (8.03 %) involved in defence among the up-regulated ones. These defence-related genes are involved in the responses to JA stimulus and water depravation. Two responses that are usually induced in an enhanced triggered immune (ETI) defence process. Confirming a putative activity of AMI1 in the IAOx branch of auxin biosynthesis in Arabidopsis, we also identified a group of genes involved in glucosinolate biosynthesis in our studies, which are as well related to a basic and constitutive defence in plants.

Figure 4.41 Representation of the up- and down-regulated genes obtained from the *ami1-2*+IAM RNA-Seq dataset analyzed with the agriGO and REVIGO platforms.

Percentages for the down-regulated genetic groups were: 4.4 % for auxin-related genes; 1.3 % for genes involved in seed development; 13 % for plant defense-related genes; 3.7 % was constituted by transcription factors and 77.9 % of other genes. In case of the up-regulated genes the percentages were: 2.12 % auxin; 3.07 % for the group of seeds-related genes; 8.03 % for the involved in defense; 8.98 % for transcription factors; 0.7 % were considered unknown and the rest of genes constituted the 77.06 % of the total.

Notably, we identified a substantial number of genes related with seed and post-embryonic development in the RNA-Seq dataset, which further highlights the potentially important role of IAM and AMI1 in seed maturation and seedling establishment. Alongside, we found an important number of “flavonoid biosynthetic process”-related genes (c.f. Annex, Table S.8) to be differentially regulated.

For instance, a group of *TRANSPARENT TESTA* genes (*At5g13930*; *At3g55120* or *At5g07990*) was identified in our experimental approach.

Analysis of the RNA-Seq dataset for the WT samples treated with IAM disclosed the following gene groups as down regulated: response to auxin stimulus; seed and post-embryonic development (c.f. Annex, Figure S.19). Among the up-regulated groups, we again found those related with response to wounding, along with an enrichment of photosynthesis-related genes (c.f. Annex, Table S.7 and Figure S.18). The latter may further highlight the intimate relationship of *AMI1* with the energy status of the plant, which was previously detected analyzing the effect of glucose and sucrose on *AMI1* expression (c.f. Results, Figure 4.9) Moreover, as in the RNA-Seq data of *ami1-2*+IAM, the genes *CYP79B2* and *CYP79B3* also appear significantly up regulated.

A summary of the major results of this study is represented in the pie charts shown in figure 4.42. Intriguingly, we again found genes involved in post-embryonic development among the down-regulated genes. The genes of this group, however, appear to be mainly involved in the storage of energy reserves and protection of the seed, rather than with the continuity of developmental processes, which corroborates the negative regulatory effect of IAM on seed development previously detected in the GO analysis (c.f. Annex, Figure S.19).

Figure 4.42 Representation of the up- and down-regulated genes taken from the Wt+IAMRNA-Seq dataset after performing GO and REVIGO analyses.

The percentages of the down-regulated gene groups are: 6.5 % for auxin-related genes; 25 % for genes involved in seed development; 7.6 % for genes associated with plant defense; 1.08 % was constituted by transcription factors, 11.9 % of unknown genes and the rest of genes (others) were 47.8 %. In case of the up-regulated gene groups, the percentages the following: 2.24 % (seeds); 1.12 % for the genes involved in defense; 3.3 % for the transcription factors; 12.2 % were considered unknown and the rest of genes constituted the 79.7 % of the total.

Some of the up-regulated genes are again forming part of the photosynthesis-related processes. However, the groups of genes associated with auxin, plant defence and seeds practically do not appear substantially affected.

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Finally, we performed the same analysis for AMI1ind treated with IAM even though this line was considered as a control to contrast the fidelity of the obtained results for *ami1-2* and WT, due to its high endogenous IAA content. The results have been included in supplementary figures S.23 and S.24, as well as in Table S.9. As expected, it was possible to observe that administration of IAM substantially change the transcriptional profile of the plants. Obviously, we found a much stronger induction of auxin-related processes under these experimental conditions, which is likely due to the effective conversion of IAM into IAA in this line. All together these results provide further evidence for the involvement of AMI1 in the biosynthesis of IAA from IAM. However, the results on transcriptional changes obtained here have to be taken with some caution, because they actually represent a mixture of IAM- and IAA-dependent responses triggered by the unnaturally high conversion rate of IAM to IAA.

5 DISCUSSION

DISCUSSION

Hormones play a critical role in driving growth and development of multicellular organisms. Plants are no exception to this, since their organ development, reproduction, and responses to biotic and abiotic stresses are governed by the interplay of plant specific hormones, commonly referred to as plant or phytohormones. Among these signalling compounds, the class of auxins is particularly important for the successful development of plants. Especially indole-3-acetic acid (IAA), the most common auxin in the plant kingdom, is considered the best implicated in cell division, cell elongation and cell differentiation (Teale, Paponov, and Palme 2006; Woodward and Bartel 2005). IAA is controlling the growth and development of plants at virtually all possible steps, from embryo development to floral organ formation. Moreover, IAA appears to be involved response to environmental signals (Davies 2004; Thimann 197).

Despite the large amount of information that has been gathered on the function of auxins, the current knowledge of the processes that contribute to auxin biosynthesis and its spatio-temporal control is still far from complete. At present, we know that there are several biosynthetic pathways involved in IAA production. However, only one of these pathways is totally disclosed. This so-called IPA-pathway, due to its hallmark intermediate indole-3-pyruvic acid (IPA), is considered the main source of auxin *de novo*-biosynthesis in plants. It consists of a two step-conversion of L-tryptophan (Trp) to IAA. In the first reaction step two aminotransferases (TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1) and TRYPTOPHAN AMINOTRANSFERASE RELATED 2 (TAR2)) convert Tryptophan (Trp) into IPA (Stepanova et al. 2008). In the second one, several flavin-monooxygenases, also known as YUCCA enzymes are in charge of catalysing the transformation of IPA into IAA (Mashiguchi et al. 2011; Stepanova et al. 2011; Won et al. 2011).

Intriguingly, most recent studies have reported on the importance of the first reaction step in linking auxin biosynthesis likewise with Trp homeostasis and ethylene (ET) biosynthesis. These tight connections are facilitated by the enzymatic activity of another aminotransferase (REVERSAL OF SAV3 PHENOTYPE (VAS1)/INDOLE SEVERE SENSITIVE 1 (ISS1)), which has been identified in two independent mutant screens (Pieck et al. 2015; Zheng et al. 2013). VAS1/ISS1 catalyses the conversion of IPA into Trp, thereby coordinating both the cellular flux of Trp into the auxin pool and cellular L-methionine levels, the latter acting as the major precursor of ET biosynthesis.

Regarding the other IAA biosynthetic pathways, there are still many remaining gaps that need to be closed. One example for such a remaining open question is related to the enzyme AMIDASE1 (AMI1). Based on numerous *in vitro*-analyses, AMI1 is supposed to catalyse the conversion of indole-3-acetamide (IAM) into IAA (Pollmann, Neu, and Weiler 2003). However, among other things, this finding needs to be confirmed genetically. Moreover, it is still unknown if AMI1 is working in a

bacteria-like independent IAM pathway or whether AMI1 is incorporated in different type of pathway.

In case that AMI1 would work in a bacteria-like pathway, one would expect a two-step conversion of Trp into IAA. In the first reaction, Trp would be converted into IAM by the virtue of a tryptophan 2-monooxygenase, homologous to the described bacterial enzymes, e.g. *iaaM*, *aux1*, and *tms1*. As AMI1 is the plant homolog of the bacterial enzymes capable of converting IAM into IAA, i.e. *iaaH*, *aux2*, and *tms2*, it is supposed to act in the second reaction step. While AMI1 was initially identified through its substantial homology to its bacterial counterparts (Pollmann, Neu, and Weiler 2003), a similar approach looking for sequence homologies, particularly for conserved regions in *iaaM*, *aux1*, and *tms1*, did not provided any useful information for the identification of plant enzymes possibly catalysing the initial reaction. Anyway, according to the work of Sugawara et al. (2009) approximately 95 % of the endogenous IAM originates from indole-3-acetaldoxime (IAOx), which suggests that in *Arabidopsis* AMI1 acts somewhere downstream of the enzymes CYP79B2 and CPY79B3 in the IAOx pathway. To the best of our knowledge, this pathway appears to be specific for crucifers, although a first publication recently reported that a CYP79A61 enzyme might be capable of producing IAOx in maize (Irmisch et al. 2015). However, although overexpression of *CYP79A61* in *Nicotiana benthamiana* plants resulted in elevated phenylacetaldoxime concentrations, no accumulation of IAOx was detectable, which draws a general production of IAOx in maize into question. Hence, the IAOx pathway remains a unique feature of *Brassicaceae* plants, which can use this pathway along with the IPA pathway for the biosynthesis of auxin. In any way, with respect to genetic studies (Zhao et al. 2002) the major purpose of the IAOx pathway is most likely not the production of IAA, but rather the formation of defence compounds, as for instance indole-glucosinolate (IG) or camalexin (de Vos et al. 2008; Hansen and Halkier 2005; Mikkelsen et al. 2009; Radojčić et al. 2008).

Taken together, at the beginning of this work, neither the precise *in vivo*-function of AMI1 nor its integration in the complete network of enzymatic reactions that contribute to the production of IAA was known beyond doubt.

Thus, the principal objective of this thesis was a comprehensive *in vivo*-characterization of AMI1.

5.1 Importance of AMI1 in the Plant Kingdom

Due to the estimation that AMI1 might exclusively act in a *Brassicaceae*-specific metabolic route, we asked the question whether AMI1 homologs are restricted to this plant family or if they can also be found outside of the crucifers, which would suggest a more general role for the enzyme. Hence, we started to study the occurrence of AMI1-like proteins particularly in the plant kingdom using a bioinformatics approach.

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A phylogenetic analysis containing more than 50 sequences of AMI1 homologs displayed the widespread occurrence of this enzyme in plants (c.f. Results, Figure 4.1). The obtained cladogram showed that bacteria and green algae are the most separated groups of all the analysed species. Near to these two groups it is possible to find the monocotyledonous plants. This finding underlines the robustness of the analysis, since bacteria are evolutionarily closer related to the more ancient monocotyledonous plants than to dicotyledonous plants. In general, we observed that all plant genomes published so far contain at least one AMI1-like sequence. This high degree of conservation puts emphasis on a possibly important and ancient molecular function for AMI1 in plant development.

This new and substantially extended phylogenetic analysis corroborates the data published a number of years ago by Lehmann et al. (2010). They already provided the prospect that each plant genome could contain, at least, one *AMI1*-like sequence. However, their research was not based on a rich data basis, as genome-sequencing data were limited at that time. With the constantly increasing amount of publicly available genome datasets it was possible to underpin their initial hypothesis. Up to now, all of the analysed plant genomes contained one or more *AMI1* homolog sequences. On the basis of our phylogenetic assessment it seemed obvious that AMI1-like proteins are not restricted to the family of the *Brassicaceae*.

Nonetheless, this apparently widespread occurrence of AMI1 did not allow sound statements on the function of the found enzymes. To this reason, it has been decided to analyse the action of some AMI1-like enzymes from different plants, including *Oryza sativa* (Os), *Sorghum bicolor* (Sb), *Medicago truncatula* (Mt) and *Populus trichocarpa* (Pt). Apparently, the selected enzymes that were compared to AMI1 from *Arabidopsis thaliana* (At) comprised representatives for both mono- and dicotyledonous plants.

These five proteins were analysed using different assays, starting with a simple alignment of their amino acid sequences (c.f. Results, Figure 4.2), followed by the study of their affinity to a number of putative substrates (c.f. Results, Figure 4.4), and finishing up by the analysis of the enzymatic properties of each of them (c.f. Results, Table 4.1). The comparison of their primary amino acid composition revealed a high conservation of the amidase signature (AS) motif (Neu et al. 2007) in the enzymes (c.f. Results, Figure 4.2, the underlined zone). In particular the active site that is characterized by a catalytic triad composed of Ser-*cis*Ser-Lys active center, showed highest degree of conservation (c.f. Results, Figure 4.2). The enzymes showed preferences for the substrate very similar to those of AMI1 from *A. thaliana*, having more affinity for two out of the eight tested substrates, i.e. IAM and phenyl-2-acetamide (PAM) (c.f. Results, Figure 4.4).

With respect to the occurrence of these two substrates in plants, the IAM-hydrolysing activity IAA was firstly found in bacteria, but years later it was also detected in some plant species, like rice (Kawaguchi et al. 1991) and *Poncirus trifoliata* (Kawaguchi et al. 1993). After that, the IAM presence was reported in more plants as *Prunus jamasakura* (Saotome et al. 1993) and *Citrus unshiu* (Igoshi et al. 1971; Takahashi et al. 1975). However, the endogenous origin of IAM in these plants was questioned because during their studies, non-sterile tissue was used and the microbial contamination could have been the source of IAM. Finally, Pollmann et al. (2002) detected IAM in *A. thaliana* plants grown under strictly sterile conditions, unequivocally establishing IAM as a true endogenous metabolite of plants.

However, the occurrence of PAM *in planta* still remained unclear, although there were some indications that PAM can possibly derive from the turnover or breakdown of glucotropaeolin, a L-phenylalanine derived benzylglucosinolate (Wittstock and Halkier 2000). Nevertheless, the conversion of PAM into phenyl-2-acetic acid (PAA) by amidases has been shown to occur in a vast variety of different plant species including *Phaseolus mungo* (Okamoto, Isogai, and Koizumi 1967), *Solanum lycopersicum*, *N. tabacum*, and *Z. mays* (Wightman et al. 1982) as well as *Tropaeolum majus* (Jutta Ludwig-Müller and Cohen 2002). Moreover, it was known that the effects of PAA can phenocopy the impact of IAA on *A. thaliana* development. However, its efficiency is inferior, because relative to IAA a 50-fold higher PAA concentration is necessary to trigger the same physiological effect (Koepfli, Thimann, and Went 1938; Sánchez-Parra et al. 2014)(Cook and Ross 2016; Cook et al. 2016; Sánchez-Parra et al. 2014; Sugawara et al. 2015; Westfall et al. 2016).

From the evidences discussed above, it is clear that IAM is a true endogenous metabolite of plants. On the contrary, the occurrence of PAM is still in doubt. In any case, a rising number of recent publications seem to valorise the importance of PAA in plants (Cook and Ross 2016; Cook et al. 2016; Sánchez-Parra et al. 2014; Sugawara et al. 2015; Westfall et al. 2016).

Other characteristics common to the studied enzymes are their subcellular localization and their enzymatic parameters, such as the pH and temperature optimum (c.f. Results, figure 4.5; Table 4.1). All the tested enzymes displayed similar cellular localization pattern, in the cytosol. Moreover, all of them displayed highest conversion rates at a pH between 6 and 7.5, characteristic for cytosolic proteins, highlighting the comparable function of the polypeptides. Regarding the temperature optimum, OsAMI1 exhibited a slightly lower temperature optimum, at 27 °C, in comparison to the other polypeptides. This might be explained by differences in the plants' habitat. Whereas *A. thaliana*, *S. bicolor*, *P. trichocarpa* and *M. truncatula* grow on dry land, *O. sativa* as a halophyte has a high tolerance towards flooding. For the latter evaporation of water from the paddy fields, accounting for a slightly cooler microclimate (Yokohari et al. 2001), might be the reason for an

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evolutionary adjustment of the enzymatic parameters towards higher activities at lower temperatures.

When trying to determine the Michaelis-Menten constants (K_m) for IAM of the examined enzymes, it became apparent that none of the enzymes presented a classical Michaelis-Menten kinetics, but rather followed a sigmoidal curve progression, which resembled the results obtained for AtAMI1 (Pollmann, Neu, and Weiler 2003), and might indicate an allosteric mode of action.

In summary, it turned out that, all of the tested enzymes are indeed functionally related. They are capable of catalysing the conversion of IAM to IAA, even though with different specific activities. Nonetheless, our enzymatic studies provide clear evidence to support their recognition as plant IAM amidohydrolases.

Together with the observation that the *N. tabacum* homolog of AtAMI1 also encodes for a functional IAM-hydrolase (Nemoto, Hara, Suzuki, et al. 2009), the presented results highlight a widespread occurrence of IAM hydrolases throughout the plant kingdom. This is supportive of a conserved and presumably basic role of AMI1-like IAM-hydrolases in plant development and a tentative contribution to auxin biosynthesis in particular.

5.2 Functional characterization of Arabidopsis AMI1

Over the past years, a lot of insight into the enzymatic properties of AMI1 and AMI1-like enzymes has been gained. However, the exact determination of K_m values for their suggested substrate, IAM, was not possible. The estimated K_m value of Arabidopsis AMI1 for IAM ranged between 300 and 500 μM . With respect to the relatively low cellular concentrations of IAM (Sugawara et al. 2009), the enzyme's affinity to IAM appeared to be too low. In consequence, we decided to take a genetic approach to investigate the *in vivo*-function of AMI1.

Due to the outstanding genetic and bioinformatic resources available for *A. thaliana*, we decided to focus our work on the corresponding amidase from this model plant, AMI1. Firstly, we set out closely study a number of loss- and gain-of-function lines for *AMI1* that have been identified or generated previously. This kind of approach permits assessment of how the altered expression of a target gene impacts the phenotype and chemotype, respectively, of a plant. In this way, it is possible to compare these different scenarios with the wild-type habitus of the plant, and thereby learn on the involvement of the target gene in plant development.

At first, three independent T-DNA insertion mutants from the SALK collection, in the following referred to as *ami1-1*, *ami1-2* and *ami1-3*, were analysed. The random integration of T-DNA into the genome does not only create mutations in a gene, but it also marks the affected gene, thereby

allowing its identification by general molecular biological measures. T-DNA insertion mutants are very common molecular biological tools to study the loss of a gene, because in many cases the integration of a 4.500 to 5.000 bp T-DNA fragment into the genomic sequence of a gene results in a null mutation. However, the impact of the insertion locus should not be disregarded in this context (exon, intron or untranslated regions (UTRs)), as it can massively affect the mutation severity.

Regarding our three isolated mutant lines, the line *ami1-1* contained one T-DNA element in the fourth intron. However, the two other mutant lines, *ami1-2* and *ami1-3*, presented tail-to-tail orientated tandem T-DNA integrations each, in the sixth and eighth exon, respectively (c.f. Results, Figure 4.6A). The variation in the insertion loci was reflected in the results. Line *ami1-2* was the one with the strongest impact. Although the effect of the T-DNA insertion in *ami1-1* was nearly as pronounced as in *ami1-2*, it was slightly weaker, possible due to the integration in an intron. Sometimes insertions in introns have a weaker effect, because the gene disrupting T-DNA fragment can possibly be lost over the course of the mRNA splicing process.

In contrast to the T-DNA insertions in line *ami1-2* that is located in an exon more or less in the middle of the gene sequence, the T-DNA insertion locus in *ami1-3* was shown to be at the 3'-end of the gene, in the last exon. This opens up the possibility that the gene is transcribed at least in great parts, which may encode for an only little shorter enzyme that remains some of its enzymatic activity (c.f. Results, Figure 4.6). With respect to these considerations it was not unexpected to find that line *ami1-2* displayed the pronounced effect of the three isolated mutant alleles.

The overexpressor lines used in this thesis facilitated the tightly controlled expression of recombinant *AMI1* through the XVE cassette that allows β -estradiol induced gene expression (Zuo, Niu, and Chua 2000). In order to exclude positional effects being the reason for an observed phenotype, we analysed three independent inducible *AMI1* overexpression lines, *AMI1ind-1*, *AMI1ind-2* and *AMI1ind-3*. Despite minor variances in the expression level of the transgene and the therewith related strength of the phenotypic impact, all tested lines produced consistent results related to *AMI1* overexpression (c.f. Results, Figure 4.10).

Most importantly, and in agreement with our working hypothesis that *AMI1* is associated with IAA biosynthesis *in vivo*, it was possible to detect that the null mutants contained lower IAA, while the inducible lines were characterized by higher IAA contents relative to the wild-type. This observation was principally confirmed by either decreased IAM hydrolase activities in the knockout lines or higher enzymatic activities in the overexpressor lines. Consistently, matching the notion that IAM is the putative endogenous substrate for *AMI1* *in vivo*, we found an IAM accumulation in most *ami1* alleles, while the *AMI1ind* lines showed significantly lower IAM levels (c.f. Results, Figures 4.6 and 4.10). In summary, the in-depth analysis of the *AMI1* loss- and gain-of-function mutants provided circumstantial evidence for a contribution of *AMI1* to auxin biosynthesis *in vivo*. In order to confirm

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the observation that a loss of *AMI1* results in decreased auxin levels, we introgressed the *DR5::GUS* reporter into *ami1-2*. Although *DR5::GUS* actually reports on auxin signalling, it is often consulted as an indirect prove for endogenous auxin contents. GUS staining of the resulting *ami1-2/DR5::GUS* line revealed a moderate down-regulation of the GUS reporter relative to *DR5::GUS* control plants, thus confirming our mass spectrometric results (c.f. Results, Figure 4.7). However, since the *ami1* null mutants did not display a severe auxin-related phenotype, the importance of *AMI1* in auxin formation remained elusive.

On the first sight, the *ami1* mutant alleles showed no obvious phenotypic alteration. However, as mentioned above, we detected differences in the chemotype, with significantly reduced IAA levels in young seedlings. Apparently, those changes were not strong enough to substantially impact general plant development. By the virtue of the high sensitivity of the root towards altered auxin concentrations, changes of the root system represent an ideal read-out for the analysis of mutants that are likely to be affected in auxin homeostasis. For this reason, we decided to analyse the root system architecture of the *ami1* T-DNA insertion mutants in more detail. Eventually, we were able to detect a moderate root phenotype (c.f. Results, Figure 4.8). On the one hand, the *ami1-1* and *ami1-2* plants presented less root branches compared to wild-type *Arabidopsis*. On the other hand, the total root system length was decreased. Both effects are consistent with the reduced IAA concentrations in the two null mutants. Notably, the third T-DNA insertion mutant, *ami1-3*, very much resembled the wild-type phenotype. While growing the mutants on media containing rising amounts of sucrose, it became apparent that the addition of sucrose significantly repressed the *ami1* phenotype. Under those experimental conditions *ami1-1* and *ami1-2* resembled the wild type, suggesting a regulatory effect of sugar on *AMI1* expression. It was possible to confirm this finding by analysing *AMI1* promoter activity in response to various amounts of sucrose in the media, using a *pAMI1::GUS* reporter line (c.f. Results, Figure 4.9). In addition, we were also able to detect that glucose exerted a similar negative effect on *AMI1* gene expression. However, testing the response to either abscisic acid (ABA) or the sugar alcohol mannitol conclusively ruled out an osmotic effect. Further confirmation was achieved by using the Genevestigator platform (Hruz et al. 2008), basically corroborating our results regarding the transcriptional regulation of *AMI1* by sucrose and glucose, but not by mannitol (Figure 5.1).

Figure 5.1 Representation of *AMI1* expression levels for different treatments with sugars or sugar alcohol.

The labelling on the left side of the figure gives account of the different treatments. In the middle, red points represent *AMI1* expression according to a $\log_2$ -ratio value. The exact values of this ratio are given in the right part of the figure, where Fold-change and p-value are also showed. No filter was set for the values of Fold-Change and p-value, since we wanted to show that mannitol does not have an effect on *AMI1* expression.

In summary, both our experimental results and the *in silico*-analysis demonstrate that *AMI1* is transcriptionally repressed by sugars. Interestingly, *AMI1* seems to be among the only 40 genes that are likewise transcriptionally regulated by sucrose and trehalose (Aghdasi, Smeekens, and Schluepman 2008). Sugars and sugar contents, respectively, are reported to affect many developmental processes, including germination, early seedling growth, flowering and senescence (Gibson 2000, 2005; Smeekens 2000). A number of studies have highlighted the essential role of the trehalose precursor, trehalose-6-phosphate (T6P), as a pivotal signalling molecule in triggering metabolic changes to promote plant growth and development (Rolland, Baena-Gonzalez, and Sheen 2006; Wind, Smeekens, and Hanson 2010). Apart of that, T6P levels are closely related to those of sugars. For example, an increase in sucrose levels generally translates into a raise in T6P (Lunn et al. 2006; Martínez-Barajas et al. 2011; Schluepman et al. 2004; Wingler et al. 2012). However, alongside with this fairly novel insight into the regulatory role of T6P, trehalose is long known as a source of energy and protectant in a wide array of stresses. It was shown that trehalose is capable of stabilizing proteins and membranes under stress conditions (Crowe, Hoekstra, and Crowe 1992). Hence, *AMI1* is possibly functionally linked to either the plant energy status or plant stress responses or even both processes through the regulatory connection with the sugar status of the plant on the transcriptional level.

Analysing the phenotype of the inducible *AMI1ind* lines, it was possible to detect that they share typical auxin overproduction-related characteristics with a number of other IAA over-accumulating lines, such as *yucca*, *CYP79B2ox* or *rooty* (Zhao et al. 2002)(c.f. Results, Figure 4.11). On the one hand, this result again provided evidence for an involvement of *AMI1* in IAA biosynthesis. On the other hand, we detected a secondary phenotype that is not directly related with auxin effects. The

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examined *AMI1*ind lines displayed premature bolting and flowering, a process that is not easily explained by an increased IAA accumulation. Much more likely is a connection between *AMI1* and the master regulator genes involved in controlling floral development. Those master regulators may include the floral integrators SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), LEAFY (LFY), and FLOWERING LOCUS T (FT), respectively. In fact, a relationship between *AMI1* and *LFY* has already been assumed, due to altered expression levels of *AMI1* in *LFY* gain- and loss-of-function mutants (Le et al. 2010; Lehmann et al. 2010; Mu et al. 2008).

Our biochemical and mass spectrometric experiments provided clear evidences for an augmented auxin biosynthesis in *AMI1*ind. However, the observed IAA over-production triggered by the ectopic expression of *AMI1* was possible without manipulating the potential substrate pool of IAM, which suggests that the *AMI1* catalysed reaction is the rate limiting step in this route. Obviously, there is more substrate available than the endogenous enzyme can convert. Alternatively, it might also be considerable that another mechanism regulates *AMI1* activity in function of the available substrate content genetically. In any case, based on our results, we conclude that Arabidopsis seedlings contain an IAM pool sufficiently big enough to facilitate the manifestation of an auxin-related phenotype in *AMI1* over-expression lines in which a strong promoter that apparently does not underlie any *AMI1*-specific regulatory effects drives the expression of the recombinant gene. Otherwise it would not have been possible to detect increased IAA contents in these lines. The auxin levels measured in the *AMI1* mutants are taken together in Figure 5.2.

Figure 5.2 Schematic representation of the relationship of IAM and IAA pools in *AMI1* gain- and loss-of-function lines.

In the knockout lines, the substrate (IAM) accumulates as a consequence of the limited *AMI1* activity, while a genetically forced higher activity of the enzyme in the *AMI1*ind lines results in decrease of the IAM pool and significantly elevated IAA levels. IAM = indole-3-acetamide; IAA = indole-3-acetic acid.

Next, it has been tested whether a further increase of IAM contents through external administration may even enforce the auxin phenotype of the *AMI1*ind lines. The experiments revealed that, although the phenotype was already visible without supplementation of the medium, a further augmentation of the IAM pool led to a considerably stronger manifestation of the phenotype, disclosing a clear hypersensitivity of the *AMI1*ind lines towards IAM, while the responses to IAA turned out to be wild type-like (c.f. Results, Figure 4.12 A, B). At the same time, the results showed

that AMI1 is not working in saturation under normal growth conditions, since an enforcement of the phenotype was possible. From this we conclude that the plant possibly maintains a certain balance between IAA and IAM.

Another interesting evidence for the seemingly important control of the IAM pool size for plant development came from the attempt to constitutively express *AMI1* under the control of the Cauliflower Mosaic Virus 35S promoter. Although numerous trials have been conducted, it has never been possible to identify any 35S::*AMI1* line with a considerable over-expression of the recombinant gene. Because a similar experimental approach, over-expressing an enzymatically compromised version of AMI1, AMI1^{S113A} (Neu et al. 2007), directly led to the identification of several strong over-expressor lines and because we never observed any defect neither in seed numbers nor in silique sizes, we conclude that fertilization and embryogenesis are most likely not affected by low IAM contents. On the contrary, later developmental stages, i.e. seed maturation, are probably more sensitive to low IAM contents, which may result in no viable seeds. In this context, it has been reported that high IAA contents can confer parthenocarpic fruits. Parthenocarpy consists in fruit setting and growth in absence of fertilization, which usually results in seedless fruits (Mezzetti et al. 2004; Molesini et al. 2009; Pandolfini, Molesini, and Spena 2007; Rotino et al. 1997). In some of these studies, a *DefH9-iaaM* gene fusion, which is expressed specifically in placenta and ovules, was used to promote auxin synthesis during fruit development. However, in first instance, ectopic expression of *iaaM* does not lead to a direct over-production of IAA, but rather to an increased formation of IAM, which is the nominal reaction product of the *iaaM* tryptophan-2-monooxygenase. Taken together, the induced very strong accumulation of IAM, and subsequently IAA, can trigger parthenocarpy in fleshy fruits, something we did not observe neither in the *ami1* null mutants, nor in the AMI1ind lines, while a constitutive strong expression of AMI1 likely leads to no viable seeds, due to the reduction of the IAM pool size.

The post-translational modification of proteins represents another measure to control enzymatic activities. Over one hundred of such modifications are known to occur on various amino acid residues (Swarup 1998), including e.g. phosphorylations, acylations, and glycosylations. Many of those modifications are assumed to alter protein conformations and thereby change their enzymatic activity. *In silico*-predictions suggested the possibility that AMI1 could be post-translationally modified by phosphorylation. As such a putative modification could probably alter the molecular mode of action of AMI1 in auxin biosynthesis, raised the interesting question whether AMI1 can be indeed phosphorylated. Even more so because we often found AMI1 to occur in double bands in western blots, which cannot be due to possible cross-reactions with other AMI1-like proteins from *Arabidopsis*, as all of the possible candidates have significantly different molecular weights. However, all of our attempts, made in collaboration with the laboratory of Prof. Dr. Julia Kehr at the University

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Hamburg, Germany, investigating the putative phosphorylation of AMI1 failed (data not shown). Whilst we can most likely exclude a phosphorylation of AMI1, other types of post-translational modifications cannot be ruled out by our results.

5.3 Control of *AMI1* expression

After confirming the involvement of AMI1 in the conversion of IAM into IAA *in vivo*, we next addressed the question how and by which triggers *AMI1* gene expression can be controlled. Auxin biosynthesis is presumably tightly regulated since constitutive IAA accumulation has negative impacts on plant fitness and development (Hentrich et al. 2013 (b); Zhao et al. 2001). As described previously, auxin biosynthesis in plants seems to rely on a very complex network of biochemical reactions that include various pathways and a substantial number of mostly indolic metabolites. For this reason, and to better our understanding on the regulatory connections among the various metabolic pathways of IAA biosynthesis and their impact on *AMI1* expression, we firstly investigated the influence of a number of indolic compounds on *AMI1* gene expression by qRT-PCR. We detected that IAM and IAA intimately regulate *AMI1* expression (c.f. Results, Figure 4.14). While IAM repressed *AMI1* expression, administered IAA triggered the induction of *AMI1* expression. It is, however, noteworthy that the influence of IAM and IAA was only moderate, showing $\log_2$ ratios of -0.87 ± 0.27 and $+1.33 \pm 0.18$, respectively, relative to control samples. The administration of indole-3-pyruvic acid (IPA) also caused an induction of *AMI1* expression ($\log_2 +1.25 \pm 0.16$), similar to that of IAA. Taking the labile nature of IPA into account, and following up on the kinetics of the induction over time, it can be concluded that this effect is most likely due to the autocatalytic degradation of IPA to IAA by oxidation. In other words, we propose that the up-regulation of *AMI1* by IPA is a secondary effect actually triggered by IAA derived from the decay of IPA, rather than a primary IPA effect. The other tested compounds, i.e. indole-3-acetonitrile (IAN) and indole-3-butyric acid (IBA), are seemingly not involved in the transcriptional regulation of *AMI1*. Apparently, none of those compounds provoked a significant change of the expression level of *AMI1* (c.f. Results, Figure 4.14).

The exogenous application of chemical compounds to subsequently investigate corresponding plant responses represents a common tool in molecular plant biology, although the used concentrations are normally far higher than what a plant normally experiences. However, previous publications reported that the external application of IAA could not rescue a number of *yuc* double and triple knockout mutants, while side-specific endogenous induction of auxin biosynthesis was able to do so (Cheng, Dai, and Zhao 2006). Hence, we decided to confirm our previously described results by employing a number of auxin-related mutant lines with proven alteration of endogenous auxin and

auxin precursor levels, including *iaaMox*, *YUC8ox*, *YUC9ox*, *NIT1ox*, *nit1*, *rty1*, *cyp79b2/cyp79b3*, *pTAA1::GFP:TAA1*, *taa1*, and *taa1/tar2*. Neither elevated nor reduced NITRILASE 1 (NIT1) contents had a considerable effect on the expression of *AMI1*, confirming our previous result on the effect of IAN. Also in the mutants *taa1* and *taa1/tar2* (Stepanova et al. 2008), we only observed very subtle changes in *AMI1* expression with $\log_2$ ratio of $+0.8 \pm 0.11$ and $+0.56 \pm 0.07$, respectively. In addition, we tested *AMI1* expression in four lines that showed enhanced *TAA1* expression (*pTAA1::GFP:TAA1*) (Stepanova et al. 2011; Stepanova, Robertson-Hoyt, et al. 2008). Notably, only the strongest of those lines (#100.2) showed a substantial induction of *AMI1* gene expression at a $\log_2$ ratio of $+1.5 \pm 0.09$. A much more pronounced effect was, however, detectable when we analysed *AMI1* expression in plants known to overproduce IAA, i.e. *iaaMox*, *YUC8ox*, and *YUC9ox* (Hentrich et al. 2013 (a); Romano et al. 1995). In those three lines, the induction of *AMI1* expression reached up to a $\log_2$ ratio of $+5.04 \pm 0.21$ (*YUC8ox*). On the contrary, mutant plants that are characterized by much higher IAM than IAA contents, such as *rty1* (King et al. 1995) that is allelic to *sur1* (Michael Dalgaard Mikkelsen, Naur, and Halkier 2004; Sugawara et al. 2009), display a significant repression of *AMI1* gene expression. Interestingly, in the double mutant *cyp79b2/cyp79b3* the expression was clearly induced. The latter mutant is characterized by drastically reduced IAM contents, while maintaining wild type-like IAA levels (Sugawara et al. 2009; c.f. Results, Figure 4.18). While *rty1* supposedly contains IAM amounts that substantially exceed the endogenous IAA levels, which eventually results in a repression of *AMI1* gene expression, the nearly entire lack of IAM in *cyp79b2/cyp79b3* is seemingly likely responsible for the increased expression of *AMI1* in this line. Although there is no direct induction by elevated IAA levels, the lack of IAM obviously triggers the release of suppression, which ultimately results in a net increase of *AMI1* transcript levels. The obtained results nicely confirm the previously obtained findings on the IAA- and IAM-orchestrated regulation of *AMI1* gene expression.

In order to gain further *in vivo*-confirmation, we introgressed the *pAMI1::GUS* promoter-reporter construct into *YUC9ox* and *rty1*. The resultant *pAMI1::GUS/YUC9ox* and *pAMI1::GUS/rty1* plants were used as tools for the analysis of *AMI1* promoter activities in response to variations in endogenous IAA and IAM contents (c.f. Results, Figures 4.19-21). In particular at early developmental stages, it was possible to confirm the previously discussed results regarding the impact evoked by elevated IAA levels in *YUC9ox* on the expression level of *AMI1*. As expected, the *AMI1* promoter appeared to more activate in the *YUC9ox* genetic background relative to the *pAMI1::GUS* control line. In contrast, we were not able to detect a substantially weaker GUS signal in *pAMI1::GUS/rty1*. Probably this is because IAM may not directly suppress gene expression, but rather affects transcript stability, which can be assessed by qRT-PCR but not by promoter-reporter gene fusions that only give account of the transcriptional activity of the promoter. Apart of also testing the influence of IAM and IAA on *AMI1*

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promoter activity in *pAMI1::GUS*, again confirming the qRT-PCR results, a number of other factors, including gibberellin A₃ (GA₃), salicylic acid (SA), ET, and high light conditions, have been tested with respect to their influence on *AMI1* expression. If at all, we found a very weak effect for SA, augmenting the GUS signal at 15 µM SA in the medium. The other treatments appeared to have no striking regulatory effect on *AMI1* transcription (data not shown). Nonetheless, on the basis of all of the gathered data, we can conclude that IAM and IAA have a true regulatory influence on the expression of *AMI1*, repressing and inducing its expression, respectively.

Positive feedback loops, like the one described here, are not uncommon in the regulation of plant hormone biosynthesis. For example, it is known from the work of Sasaki et al. (2001) that jasmonic acid auto-activates its own biosynthesis. Another example for a positive feedback loop is the induction of several ABA biosynthesis genes by ABA (Barrero et al. 2006).

In nature, there are much more examples for this kind of positive feedback loops. Very recently, another positive feedback loop in relation with the maintenance of cellular auxin homeostasis has been reported. The presented publications reported on the identification of *DYOXIGENASE FOR AUXIN OXIDATION (DAO)* in various species, a gene that encodes for an enzyme that participates in IAA catabolism (Mellor et al. 2016; Porco et al. 2016; Zhang et al. 2016). DAO catalyses the tissue-specific oxidative inactivation of IAA, giving rise to oxIAA. The DAO reaction product, oxIAA, has been found to increase the formation of IPA, a key IAA precursor, which eventually promotes IAA biosynthesis. In turn, IAA is reported to induce the activity of DAO.

Although there are obviously plenty of examples for positive feedback loops, particularly in plant hormone biosynthesis, there is much less information on negative feedforward relationships in the field of phytohormone formation available. However, the phenomenon of feedforward inhibition was first reported by Ames and Hartman (1961) in the histidine biosynthetic pathway of *Salmonella typhimurium*. Later on, other groups confirmed this initial finding for several other organisms and biosynthetic pathways, and provided evidence for the functional effectiveness of feedforward inhibition. It is reported that in particular in branched pathways feedforward inhibition can provide a few advantages (Savageau and Jacknow 1979; Savageau 1979).

Finally, because we did not want to discard any possibility of regulation and biochemical activity *per se*, we also addressed the open question whether *AMI1* is capable of catalysing the reverse reaction as well, even though this appeared highly unlikely from a biochemical point of view (c.f. Results, Figure 4.17). Our experiments revealed that under the given conditions an *AMI1* catalysed reverse reaction, forming IAM from IAA and ammonia, has to be excluded. In conclusion, the *AMI1* catalysed reaction can definitively be included as unidirectional in the scheme of IAA biosynthesis.

5.4 Relationship of AMI1 and the IAOx pathway

Once we gained insight into that the *in vivo* function of AMI1, contributing to IAA biosynthesis, and into the regulation of *AMI1* gene expression, we next tackled the question in which IAA biosynthetic pathway AMI1 may work.

The widespread occurrence of AMI1-like enzymes in the plant kingdom contradicts an exclusive function in a plant family-specific pathway, such as the IAOx-pathway that is assumed to be restricted to the *Brassicaceae* (Pollmann, Müller, and Weiler 2006). However, according to the already mentioned work by Sugawara et al. (2009), the major portion of IAM (95%) in Arabidopsis has been shown to derive from IAOx. Interestingly, the genetic blockade of glucosinolate biosynthesis through the mutation of *sur1/rti1* leads to drastically elevated IAOx and IAM levels (Figure 5.3), but no alteration of the IAN pool.

Figure 5.3 The role of RTY1 in auxin homeostasis.

The figure shows the three Arabidopsis pathways that branch at the level of IAOx. One of those is likely involved in IAA biosynthesis, proceeding via the intermediate indole-3-acetonitrile (IAN) (left part of the figure). The IAM-dependent route in which AMI1 is acting is also participating in IAA production (middle of the figure). Finally, the third pathway is involved in the biosynthesis of indole glucosinolate (IG) (right part of the figure). All of the routes are composed by multiple reaction steps, which implicates the coordinated activity of a small number of enzymes in order to realize the multiple reactions

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indicated. A genetic blockade of the IG pathway (red cross) results in the accumulation of IAOx and IAM levels (as the red arrows indicates). Auxin homeostasis is considerably affected by such an alteration. Solid dark arrows indicate enzyme that have already been identified and characterized, dotted black arrows denote that neither a gene nor enzyme activity has yet been identified in Arabidopsis. CYTOCHROME P450 MONOOXYGENASE 79B2 and 79B3 (CYP79B2 CYP79B3), SUPERROOT 2 (SUR2), ROOTY 1 (RTY1), UDP-GLUCOSYL TRANSFERASE 74B1 (UGT74B1), CYTOCHROME P450 MONOOXYGENASE 71A13 (CYP71A13), NITRILASE 1-3 (NIT), AMIDASE1 (AMI1).

Thus, it can be assumed that the IAM- and the IG-shunt are closely related in Arabidopsis and, with IAOx, they are likely share the same precursor, although the enzyme catalysing the conversion of IAOx into IAM is yet to be disclosed. Why the IAN-dependent pathway via CYP71A13 and the nitrilases is not affected in the *sur1/rty1* background remains, however, elusive. *In vitro*-analyses of CYP71A13 heterologously expressed in either *Escherichia coli* or *N. benthamiana* highlight the involvement of CYP71A13 in the dehydration of IAOx, converting it into IAN. This observation is further supported by the ability of exogenously applied IAN to rescue the camalexin-deficient phenotype of *cyp71a13* mutants (Nafisi et al. 2007). However, obviously excess IAOx does not augment the metabolite flux through this pathway, suggesting a very tight metabolic control, with CYP71A13 being the protagonist in controlling the flux. On the other hand, it cannot be excluded that IAN is so rapidly converted into other intermediates that basically no IAN accumulation was detected in *sur1*. This could include the nitrilase-catalysed conversion of IAN into IAA and IAM, a reaction that has previously been proposed (Pollmann et al. 2002), or a further conversion of IAN into indole-3-carboxylic acid or dehydro-IAN (Müller et al. 2015), metabolites that have not been analysed in the work of Sugawara et al. (2009).

In any case, in order to further study the biochemical and metabolic relationship between AMI1 and RTY1, we decided to employ a genetic approach and cross the two available mutants, *ami1-2* and *rty1*. In case of enzymatic activities of the two enzymes in the same metabolic route, we expected a phenotype for *ami1-2/rty1* very similar to the single mutants. On the contrary, if the enzymes would act in parallel pathways, this would likely translate into more severe mutant phenotype, as the effect of the two individual mutations would add up.

After we confirmed homozygosity for the two mutation loci by RT-PCR-based genotyping and commercial sequencing (c.f. Results, Figure 4.23), we aimed at the molecular characterization of the *ami1-2/rty1* double mutant. However, herein we encountered serious problems, as the seeds obtained from homozygous mutant plants displayed a germination defect. The seeds aborted the germination process approx. three to four days after imbibition, more or less shortly after the radicle broke through the testa. At first, we assumed that this impairment might be related to reduced IAA biosynthesis rates in the mutant that, for what reason ever, cannot be compensated by

the other, not affected auxin biosynthetic pathways. For this purpose, we decided to supplement the plates with IAA in order to promote the germination process and rescue some of the seedlings (c.f. Results, Figure 4.27), in particular because it is known that IAA serves as an inductive signal for embryo and seed development (Mira et al. 2016; Raghavan 2004). Even more so, because the comparison of *ami1-2/rty1* seed size with those from other auxin-related lines, including the parental lines *ami1-2* and *rty1*, did not reveal significant differences in their proportions (c.f. Results, Figure 4.28). However, the application of IAA did not rescue the lethal phenotype of the *ami1-2/rty1* seedlings. So, it was concluded that the problem may lie in embryo itself that may not have the correct polar organization, which eventually prevents successful seed germination. In fact, the analysis of embryos from wild-type *Arabidopsis*, *ami1-2*, *rty1*, and *ami1-2/rty1* revealed a very intriguing result. While the *ami1-2* embryos appeared to be slightly smaller than wild-type embryos, and the *rty1* a little bit bigger, the size of the *ami1-2/rty1* embryos was drastically reduced (c.f. Results, Figure 4.29). Apart from the fact that AMI1 and RTY1 unequivocally act in parallel pathways, our results provide striking evidence that the IAOx-pathway in *Arabidopsis* is somehow involved in the regulation of seed maturation; a process that seemingly does not exclusively depend on general IAA contents or its spatio-temporally controlled biosynthesis. It is astonishing that the major auxin biosynthetic route via IPA cannot compensate for the loss of AMI1 and RTY1 action in a commonly as irrelevant considered side-branch for auxin and IG biosynthesis. At the same time, this may suggest that not IAA itself, but rather its precursor IAM is the driving force in this process. Moreover, it has to be concluded that IAOx represents a metabolic key branching point from which several pathways, e.g. the biosynthesis of glucosinolates, camalexin, and IAA emerge (Glawischnig et al. 2004; Sønderby, Geu-flores, and Halkier 2010; Sugawara et al. 2009; Zhao et al. 2002).

5.5 The expression of *AMI1* impacts several functional groups in *A. thaliana*

The phenotypic analysis of *AMI1* mutants facilitated the establishment of connections between *AMI1* expression levels and the sugar/energy status and flower development in *Arabidopsis*, respectively. However, in order to gain broader insight into possible interactions of *AMI1* with other genes, a co-expression analysis was performed. The correlated expression of genes is often providing evidence for possible functional relationships in given biological processes. As shown in Figure 5.4, the *in silico*-analysis revealed co-expression of *AMI1* with a number of very interesting genes under the selected parameters. Among those genes we found a sugar transporter (*STP14*, At1g77210) that is reported to be a galactose transporter with high expression levels in the seed endosperm and in cotyledons (Poschet, Hannich, and Büttner 2010). On the other hand, we found a L-galactose dehydrogenase (*LGALDH*, At4g33670) also to be co-regulated. L-GALDH contributes to the conversion of L-galactose

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into L-galactono-1,4-lactone, which is suggested to be the substrate for ascorbate synthesis in plants (Gatzek, Wheeler, and Smirnoff 2002). Further studies have associated ascorbate with an essential role in seedling development, as a corresponding *vtc2/vtc5* double null mutant that is basically devoid of ascorbate shows growth arrest immediately upon germination (Dowdle et al. 2007), a phenotype that closely resembles the *ami1-2/rty1* phenotype.

Figure 5.4 *AMI1* co-expression network in *A. thaliana* obtained using the GeneMANIA database.

Represented is the co-expression network showing the 25 genes with the best positive correlation to *AMI1*. The network has been calculated on the basis of 165 microarray datasets that have been performed and uploaded between 2009 and 2016.

Consistent with the roles of the already discussed genes that suggested that *AMI1* possibly contributes to embryo and seed development, an effect embryo arrest-related gene (Pagnussat et al. 2005), *MEE14* (At2g15890), was also disclosed to be co-expressed. *MEE14* encodes for CCG BINDING PROTEIN 1 (CBP1), which is proposed to connect transcription factors and the Pol II machinery to regulate pollen tube attraction (Li et al. 2015). Moreover, the analysis highlighted the co-expression of *AMI1* with two genes encoding for dormancy/auxin-associated proteins, i.e. *DYL1* (also referred to as *DRM1*, At1g28330) and *DRM2* (At2g33830). Excitingly, *DRM1* is transcriptionally repressed by both sucrose and glucose, very much like *AMI1*, which led to the conclusion that *DRM1*, among other sugar-modulated genes, contributes to *in vivo* sugar sensing in Arabidopsis (Gonzali et al. 2006). In

addition *DRM1* and *DRM2* have recently been associated with plant stress responses, particularly based on their varying responses after treatment with hormones that are generally related with stress: ABA, SA, and jasmonic acid (JA). However, *DRM1* and *DRM2* were also reported to respond to temperature stress (Rae et al. 2014). Finally, *AMI1* showed as well co-expression with the potassium transporter *POT12* (At1g60160) and *NLA*. The latter gene encodes for a RING-type ubiquitin E3 ligase, which is among the most interesting candidates, as is probably associates *AMI1* plant immune responses. It has been demonstrated that BAH1/NLA regulates the accumulation of SA and immune responses to *Pseudomonas syringae* DC3000 (Yaeno and Iba 2008).

In order to substantiate these intriguing results that hold out the prospect that *AMI1* is likely associated with embryo/seed development, *in vivo* sugar sensing, and plant stress responses, a transcriptomics approach was chosen and whole-genome DNA microarray analyses were performed, comparing the previously described *ami1* and *AMI1ind* mutants with wild-type *Arabidopsis* plants. We expected the microarray analyses to generally corroborate an involvement of *AMI1* in auxin biosynthesis, giving account on the differential expression of the classical auxin response-related genes, such as *Aux/IAA*, *ARF*, *SAUR*, and *GH3* genes. In addition, we had the hope that the arrays would shed light on some less obvious biological processes in which *AMI1* may also be involved. However, to our great surprise, the majority of differentially expressed genes identified in this study appeared to be related with responses to stress and plant defence. The number of genes directly related with auxin signalling was unexpectedly low. Among the groups of genes related with stress, we found an enrichment of genes that are known to respond to temperature stress, and here in particular to heat. In fact, this observation is in accordance with the preliminary results obtained by analysing the co-expression data.

It was possible to identify some heat shock proteins (HSPs) among the overexpressed genes in *ami1-2* that appeared to be merged in a single gene ontology (GO) grouping for oxidative stress (c.f. Annex, Table S.1). High temperature is commonly classified as abiotic stress. Abiotic stress situations usually generate ROS molecules, so it seems logical that we detected HSPs in this group. Furthermore, high temperatures usually cause water deficits that, as a consequence, result in the induction of ABA. Although the tested plants were not subjected to any type of temperature stress, we also found several genes related with responses to ABA (c.f. Annex, Table S.1), which may suggest that the observed response cascade is functionally linked on the transcriptional level.

Another important role of ABA in plant development is the control of seed dormancy (in cooperation with other hormones, e.g. SA, GA, ET or Brassinosteroids) until the environmental conditions comply with the requirements that most probably facilitate successful germination (Finkelstein, Gampala, and Rock 2002; Rodríguez-Gacio, Matilla-Vázquez, and Matilla 2009). Taking the severe *ami1-2/rty1*

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embryo phenotype into account, the overexpression of genes related with ABA in the *ami1-2* genetic background can likely be taken as a further confirmation for an involvement of AMI1 in embryo development and seed maturation in *Arabidopsis*, respectively.

Apart of the apparently tight connection between *AMI1* and ABA-dependent processes, the analysis of the *ami1-2* and *AMI1ind* microarray data revealed a possible crosstalk with JA and, thus, wound-triggered responses. Jasmonates are a type of oxylipins that are critical for the defence of plants against biotic stress cues, as for example, the attack of herbivore or pathogenic predators (Farmer and Ryan 1992; Hentrich et al. 2013 (a); Hu et al. 2013). In the *ami1-2* null mutant, we observed the significant up-regulation of basically all JA biosynthesis-related genes, including *LIPOXYGENASE 3* (*LOX3*), *ALLENE OXIDE SYNTHASE* (*AOS*), *ALLENE OXIDE CYCLASE 1* (*AOC1*) and *3* (*AOC3*), *12-OXOPHYTODIENOATE REDUCTASE 3* (*OPR3*), *ACYL-COA OXIDASE 1* (*ACX1*), and *JASMONATE RESISTANT 1* (*JAR1*) (c.f. Annex, Table S.1). In addition, a number of genes that encode for JASMONATE-ZIM-DOMAIN PROTEIN (JAZ)-transcriptional repressors, i.e. *JAZ3*, *JAZ9*, and *JAZ10*, also appeared to be induced in *ami1-2* (c.f. Annex, Table S.1), which suggests the operation of a negative feedback loop in JA signalling.

The JAZ transcriptional repressors are negative regulators that control the activity of downstream MYC transcription factors, which are ultimately required to realize appropriate jasmonate-mediated defence responses and JA-dependent developmental processes (Hu et al. 2013). The JA response is temporally divided in early and late responses (Mira et al. 2016). In the early response, the transcription factor JASMONATE INSENSITIVE 1 (*JIN1*/*MYC2*) is induced, which consequently results in the differential expression of other genes as for instance, those related to wounding response (e.g. *VSP1*). In the *ami1-2* microarray *VSP1* appears to be up regulated at a rate 1.27-fold, consistent with an involvement of AMI1 in suppressing JA synthesis under normal growth conditions. The late response to JA is controlled by *JAZ1* that represses, among other proteins involved in response to pathogens, *MYC2*. In contrast to this substantially induced response to wounding in the *ami1* null mutant, we find several other GO groups related with defence down regulated in *ami1-2*. These groups include genes particularly associated with the response to bacteria and chitin. Chitin is a polymer of *N*-acetyl-D-glucosamine found in fungal cell walls (Wan et al. 2008). Hence, it has to be concluded that the lack of AMI1 in the knockout background translates into the repression of responses to pathogenic bacteria and fungi (c.f. Annex, Table S.1).

Overall, the obtained results point towards the existence of numerous relationships between *AMI1* and different plant hormones, others than IAA. This suggests that AMI1 probably acts as a molecular hub that somehow contributes to the integration of multiple mostly stress related stimuli that are processes via a network of plant hormonal interactions (c.f. Annex, Table S.1). However, some of the

genes that can be found in this group are encoding AUXIN RESPONSIVE PROTEINS involved in auxin signalling, which further underpins the importance of *AMI1* for auxin homeostasis.

5.6 IAOx pathway is an important element for the development of brassica plants

The work presented here and the results reported by other laboratories provided evidence for an important role of the IAOx pathway in the biosynthesis of several secondary compounds in *Arabidopsis*, e.g. IAA, IGs, and camalexin. In particular IAOx has a pivotal position in this scenario, because it seems to be the branching point from which the different pathways emerge. The genetic blockade of the IAM-dependent pathway and the IG pathway, as prevalent in the *ami1-2/rty1* mutant, resulted in seeds with arrested germination. Interestingly, *cyp79b2/cyp79b3* double mutants do not display any considerable germination defects, thus a general importance of the end products of the IAOx-dependent pathways for seed germination can be excluded. However, there must be a molecular explanation for the severe seed germination phenotype of *ami1-2/rty1*. Since the IAN pathway is most likely not affected by mutations that cause accumulations and back-fluxes of metabolites (Sugawara et al. 2009), it can be concluded that the *ami1-2/rty1* phenotype is based on the accumulation of IAOx and/or IAM. Mass spectrometric analyses have shown that *cyp79b2/cyp79b3* double mutants contain wild type-like IAA contents, which suggests that the *ami1-2/rty1* phenotype is not based on low auxin contents. This fact is reaffirmed by the observation that application of IAA cannot rescue the *ami1-2/rty1* seedlings. To learn more on the general processes related with the IAOx pathway, we decided to assess differential gene expression in the *cyp79b2/cyp79b3* double mutant by microarray analysis. Herein, the relationship between the IAOx pathway and *AMI1* was of utmost importance.

As the two cytochrome P450 monooxygenases CYP79B2 and CYP79B3 are responsible for the production of approx. 95% of endogenous IAM in *Arabidopsis* (Sugawara et al. 2009), we speculated that *AMI1*-related reactions associated with the remaining 5% of cellular IAM become more obvious.

According to the previously discussed results (c.f. Results, Figures 4.14 and 4.15), IAM exerts an inhibitory effect on *AMI1* gene expression. Based on our qRT-PCR results, *AMI1* expression appeared to be induced in the *cyp79b2/cyp79b3* double mutant (c.f. Results, Figure 4.18), which is in accordance with the transcriptomic analysis. In the *cyp79b2/cyp79b3* microarray data, the expression of *AMI1* is also clearly induced (Fold-change = 1.83, p-value = 0.004). As already mentioned, the observed induction of *AMI1* in *cyp79b2/cyp79b3* can be attributed to the relaxed suppression of *AMI1* gene expression due to the significantly reduced IAM levels.

Nevertheless, in addition to only verify the qRT-PCR data, the analysis of the *cyp79b2/cyp79b3* microarrays revealed a wealth of interesting data. Expectedly, we found a number of genes related

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with glucosinolate metabolism down-regulated. Another large group of genes we found down regulated are associated with the formation of flavonoids. Flavonoids are metabolites that have several functions. Amongst others, they are assumed to contribute to the regulation of auxin transport (Kuhn et al. 2011; Lewis et al. 2011; Murphy, Peer, and Taiz 2000) and to responses to defence responses to pathogenic organisms (M. F. Cohen, Sakihama, and Yamasaki 2001; Dai et al. 1996; Jensen et al. 1998). Furthermore, they are considered as one of the most important pigments for the coloration of the flowers, fruits and seeds (Falcone, Rius, and Casati 2012; Y. Tanaka, Sasaki, and Ohmiya 2008). This last function again relates the IAOx-pathway with the development of reproductive organs in Arabidopsis.

In summary, we can record that if the IAOx route is blocked, the expression of genes that participate in the synthesis of important secondary metabolites, such as the indole glucosinolates and flavonoids, will be repressed. This is likely to have a negative effect on plant development and, possibly even more important, renders Brassica plants more susceptible to pathogen attacks.

Finally, it can be stressed that the main function of AMI1 is possibly not the plain production of IAA, but rather the control of IAM. Based on the obtained data, it seems that AMI1 enzymatic activity has implications for a number of biological processes, including responses to biotic and abiotic stresses, sugar signalling, and seed development.

5.7 Query for an enzyme working up-stream of AMI1

Previously we discussed the role of AMI1 in IAA biosynthesis and came to the conclusion that in *Brassicaceae* AMI1 is likely to operate in the IAOx pathway, since the predominant part of its substrate is produced via IAOx. However, assuming that AMI1 acts downstream of CYP79B2 and CYP79B3, there is still the question on how IAM is produced from IAOx. Alternatively assuming that AMI1 would contribute to a bacteria-like two-step pathway independent of IAOx, the enzyme upstream of AMI1 also remains to be identified.

After we gathered and analysed a large amount of transcriptomics data comparing the *ami1-2*, *AMI1ind*, and *cyp79b2/cyp79b3* mutant genotypes to the wild type, we asked the question if it would be possible to harness those data to identify candidate gene for molecular targets that are possibly involved in the production of IAM. With respect to this question, it appeared tempting to speculate that putative target genes are transcriptionally connected with the expression level of *AMI1* and/or the endogenous concentration of IAM and the relation between IAM and IAA, respectively. On the basis of a biochemical examination, we concluded that only two enzyme classes are likely to be involved in the formation of IAM. Those are, on the one hand, tryptophan-2-monooxygenases or, on

the other hand, cytochrome P450 monooxygenases. Thus, we screened the differentially expressed genes in the microarrays for candidates that fit into this classification. Mainly considering the data obtained in the *ami1-2* and *cyp79b2/cyp79b3* microarrays, it was possible to select eight possible candidates that could work in the uncharacterized enzymatic reaction. Comprehensive literature work facilitated a reduction of the candidate list to only two possible target genes, as all of the other have been functionally characterized, associating them with different processes. For the two remaining genes, *CYP71B22* and *CYP709B3*, however, there was no function association available.

First up, an *in silico*-study was carried out (Mockler et al. 2007), comparing the circadian expression pattern of *AMI1* with those of the two target genes (c.f. Results, Figure 4.60). It turned out that *CYP71B22* and *AMI1* share a very similar expression pattern, peaking after 12 and 15 h under short-day conditions, respectively, while the expression profile of *CYP709B3* appeared in anti-phase to that of *AMI1*. Although this slight phase shift between the expression pattern of *AMI1* and *CYP71B22* was detected, the results seemed to favour *CYP71B22* over *CYP709B3* as the best candidate for enzymatic cooperation with *AMI1*.

In order to substantiate this preliminary finding, we decided to use a reverse genetics approach. The use of T-DNA insertion lines facilitated the functional characterization of hundreds of genes over more than a decade now. In this thesis, we already employed such an approach successfully, isolating and characterizing the *ami1* mutant alleles. So, we next aimed for the identification of T-DNA insertion mutants for the two target genes. It was possible to find promising lines in the SALK collection (Alonso et al. 2003). After genotyping the lines and confirming the T-DNA insertion loci within the core sequence of the two genes, including 300 bp stretches of the 5'- and 3'-untranslated regions (c.f. Results, Figure 4.34 and 4.35), the mutant lines were subjected to transcriptomic analyses using a RNA-Seq approach. Transcriptomic analyses by RNA sequencing is currently considered as more precise and powerful than other existing technologies. It generates data with less background signal and is highly accurate in quantifying expression levels. Moreover, it requires less RNA quantity for the analyses (Z. Wang, Gerstein, and Snyder 2009).

The RNA-Seq data clearly confirmed *CYP71B22* as the best option. A gene enrichment analysis based on GO associations revealed an association of *CYP71B22* with processes related to phytohormones, such as for instance ABA, which is a well-described integrator of abiotic stress responses, in particular heat and drought (Suzuki et al. 2016; Verslues and Bray 2006). Intriguingly, all the plant hormone-related overrepressed groups were also identified as up-regulated in *ami1-2*. Moreover, among the down-regulated groups found in this RNA-Seq analysis we identified several genes related with post-embryonic development. Apart of a direct transcriptional link between *CYP71B22* and *AMI1* (*AMI1* appeared differentially expressed in *cyp71b22*, but not in *cyp709b3*), these observations clearly

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relate *CYP71B22* with *AMI1*. However, much more compelling was the finding that *CYP71B22* turned out to biological processes that are directly related with auxin biosynthesis and indole metabolism, which strongly emphasises the importance of *CYP71B22*.

On the one hand, the obtained results for the *cyp709b3* mutant disclosed that this gene mainly influences genetic groups related with, e.g., lipid biosynthesis and cofactor metabolism. In fact, there was no detectable overlap with the putative *AMI1* functions, but rather connections with generally monooxygenase-catalysed reactions, leading to the biosynthesis of a wide range of compounds including several fatty acids, pigments, plant hormones and various other secondary metabolites (Pan et al. 2009).

To sum up, we propose on the basis of the previously discussed results *CYP71B22* as a new possible player in IAA biosynthesis. More explicitly, we suggest that *CYP71B22* could probably catalyse the conversion of IAOx into IAM (Figure 5.5).

Figure 5.5 Proposed IAA-biosynthesis pathways in Arabidopsis

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The thin, grey and dashed arrow appearing on the left part of the figure denotes the Trp-independent IAA biosynthesis pathway. All the rest of the arrows refer to steps of Trp-dependent IAA biosynthesis pathways. Red arrows show the reactions catalysed in the chloroplast. Blue arrows highlight the enzymatic reaction catalysed by AMI1. The green circle surrounds the *Brassicaceae*-specific IAOx pathway. A dark orange dashed arrow indicates the step carried out by the proposed enzyme (CYP71B22). Solid dark arrows indicate those enzymes that have been suggested for the step; dotted black arrows denote that neither a gene nor enzyme activity has been identified in Arabidopsis yet. (TSB2) TRP SYNTHASE β -SUBUNIT 2; (ISS1/VAS1) INDOLE SEVERE SENSITIVE 1/REVERSAL OF SAV3 PHENOTYPE; (TAA1/TAR2) TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1/TRYPOTPHAN AMINOTRANSFERASE RELATED 2; (YUC) YUCCA; (CYP79B2 CYP79B3) CYTOCHROME P450 79B2 and 79B3; (SUR2) SUPERROOT 2; (SUR1) SUPERROOT 1; (UGT74B1) UDP-GLUCOSYL TRANSFERASE 74B1; (CYP71A13) CYTOCHROME P450 71A13; (NIT) NITRILASE; (AMI1) AMIDASE1; (CYP71B22) CYTOCHROME P450 71B22.

Nonetheless, apparently the functional properties of CYP71B22 need to be studied in closer detail, prior to drawing a final conclusion on its activity. This includes the genetic investigation of the relationship between *AMI1* and *CYP71B22*. The generation of an *ami1-2/cyp71b22* double mutant could be a very valuable tool for this purpose. On the other hand, classical biochemical studies and the application of a metabolomics approach will certainly help to achieve clarity on the activity of CYP71B22 and to what extent it contributes to auxin biosynthesis. Unfortunately, due to time constraints, it was not possible to follow up on this very promising results in the framework of the project.

5.8 The function of AMI1 in plants

With reference to the so far discussed results, we obtained compelling evidence for an intimate transcriptional regulation of *AMI1* by a positive feedback loop, provoked by IAA, and a feedforward inhibition triggered by IAM. Moreover, especially the phenotypisation and molecular biological analysis of the *ami1-2/rty1* double mutant raised the question whether the predominant function of AMI1 in Arabidopsis is related with the producing of IAA, or rather the control of endogenous IAM levels.

For this reason, and to test if IAM by its own right can function as a signalling molecule, we aimed at analysing the transcriptional influence of IAM in Arabidopsis. In this experiment we compared wild type, *ami1-2*, and AMI1ind seedlings treated with IAM to wild-type plants treated with a mock solution employing the RNA-Seq technology.

In this context it is necessary to recall that wild-type and AMI1ind plants are capable of converting the applied IAM into IAA. Especially in the AMI1ind line, which is characterized by increased IAM hydrolase activities and endogenous IAA contents, a rapid and effective conversion of IAM is expected (c.f. Results, Figure 4.10). However, for the *ami1-2* genetic background, the opposite phenomenon has to be anticipated. Most likely, the applied IAM will not be converted into IAA,

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resulting in a pronounced accumulation of IAM. Notably, such high IAM contents do not reflect any kind of natural situation, but may probably facilitate the identification of IAM-dependent processes.

To our great satisfaction, we found only very few differentially expressed genes shared between the different genotypes (c.f. Annex, Table S.10 and S.11). This underlines that most of the changes we observed depended likewise on AMI1 and IAM. When we compared the genes induced by exogenous IAM application in *ami1-2* with those previously identified in the microarrays, we found an overlap of approximately 5.5% and 1.3% for the up- and down-regulated genes, respectively. In general, it has to be remarked that the RNA-Seq analysis provided much bigger datasets. However, among the induced overlapping genes it was possible to identify those previously associated with plant hormones involved in abiotic and biotic stresses, including SA, JA and ABA. On the contrary, we found the genes apparently involved in post-embryonic development among the down-regulated ones, which is in accordance with the microarray data. Furthermore, we were capable of detecting evidence for a positive IAM-triggered effect on auxin response genes in the RNA-Seq dataset (c.f. Annex, Table S.8). Other up-regulated genes that we found in the RNA-Seq experiment utilizing IAM-treated *ami1-2* plants are those related with JA biosynthesis and response to JA.

The *ami1-2/rty1* mutant line is supposedly accumulating high IAM and IAOx levels. Unfortunately, we were not able to metabolically analyse this line, due to a lack of biomass. The suspected accumulation of endogenous IAM and/or IAOx is likely the cause for the germination arrest. The situation achieved in the *ami1-2* plants supplemented with IAM likely simulates the high IAM contents known to prevail in the *sur1 (rty1)* mutant. Such an assumption could explain why we observed the transcriptional repression of seed development-related genes in the corresponding RNA-Seq experiment. In addition, the genes of flavonoid and glucosinolate biosynthetic processes are up-regulated too. Therewith AMI1 may act as a counterpart to the CYP79B2/CYP79B3-dependent IAOx formation and its impact on gene expression levels. The fact that the accumulation of IAM mediates the induction of glucosinolate biosynthesis-related genes points towards a regulatory link between the IAM-dependent pathway and IG biosynthesis through the control of metabolite fluxes through the different IAOx shunts.

Overall, we realized that an excess of IAM changes the expression profile of a number of functional gene groups profoundly. So, it has to be concluded that the major role of AMI1 is the control of the endogenous IAM pool to regulate the expression of a subset of downstream genes.

In the wild-type samples treated with IAM, auxin response genes and seed-related genes were down-regulated, certainly due to the external addition of IAM that is not completely converted into IAA. The genes affected here in their expression are associated with seed development and particularly seed filling in Arabidopsis. For example, we detected several *LEA*, *SEED STORAGE PROTEIN* and *Oleosin* genes among the candidates (c.f. Annex Table S.7).

In summary, we were able to corroborate the relationship maintained between AMI1 and a number of genes involved in response to JA and abiotic stresses and, possibly even more interesting, between AMI1 and several target genes reported to be involved in post-embryonic and seed development. Notably, the AMI1 effect is mediated by the control of the endogenous IAM level that eventually contributes to the transcriptional control described. Moreover, the repression that IAM exerts over genes such as *CYP79B2*, *CYP79B3* and *CYP71B22* shows a classic negative feedback pattern (Figure 5.6). This fact supports the idea that the main function of AMI1 is to control IAM levels.

Figure 5.6 **Enzymatic regulation deduced from this work.**

The figure shows the hypothetical reaction cascade for Trp-dependent IAA biosynthesis via IAOx and IAM. Blue arrows denote the repressive effect that IAM possesses over *AMI1*, *CYP71B22*, *CYP79B2* and *CYP79B3*. Green arrows indicate the induction of gene expression. Abbreviations: (CYP79B2 CYP79B3) CYTOCHROME P450 79B2 and 79B3; (CYP71B22) CYTOCHROME P450 71B22; (AMI1) AMIDASE1; Trp (Tryptophane); IAOx (Indole-3-acetaldoxime); IAM (Indole-3-acetamide); IAA (Indole-3-acetic acid).

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6 CONCLUSIONS

CONCLUSIONS

1. The phylogenetic study demonstrated the widespread occurrence of AMI1-like proteins throughout the plant kingdom. In addition, the comparison of several plant amidases with *Arabidopsis* AMI1 provided evidence for a conserved role of AMI1-like IAM-hydrolases, suggesting that AMI1-like proteins have an essential function in plant development.
2. The characterization of AMI1-like proteins revealed IAM and PAM as the best substrates and IAM as putative endogenous substrate for AMI1.
3. From the study of loss- and gain-of-function lines of *AMI1*, we gained unequivocal insight into the contribution of AMI1 to auxin biosynthesis *in vivo*.
4. Moreover, we have observed that *AMI1* is transcriptionally repressed by sugars. This suggests a connection between *AMI1* and the energetic status of the plants. Also, this observation suggested a putative relationship between AMI1 and genes involved in floral development.
5. The analysis of the inducible *AMI1ind* overexpression lines led to the conclusion that the endogenous IAM pool is sufficiently big enough to produce auxin-related phenotypes after induction of *AMI1* gene expression. For this reason, we propose that plants seem to maintain a balance between IAM and IAA levels.
6. Regarding the regulation of *AMI1* gene expression, we have detected that is principally driven by IAM and IAA, where IAM has a repressive effect over *AMI1* and IAA induces it.
7. The measurement of the AMI1 catalysed reaction's natural direction has revealed that the obtention of IAM from IAA and ammonia has to be excluded. So, we conclude that the reaction driven by AMI1 must be included as unidirectional in the network of auxin biosynthesis routes.
8. During the functional characterization of AMI1, post-translational modifications of the protein, except of phosphorylations, could not be convincingly excluded.

9. The *ami1-2/rty1* double mutant provided evidence that the IAOx-pathway in Arabidopsis is likely to play a key role in seed maturation.
10. *In silico* co-expression studies suggested that *AMI1* could be involved in sugar sensing, plant stress responses and as a prominent determinant in post-embryonic development and seed maturation. These findings were confirmed by transcriptomics studies. Along with the confirmation, it was also possible to detect that *AMI1* is likely representing a molecular hub involved in plant hormone crosstalk.
11. The phenotype of the *ami1-2/rty1* double mutant is rather based on the accumulation of IAOx and IAM, than on low IAA contents. From this, together with a transcriptomics analysis performed with the *cyp79b2/cyp79b3* double mutant, it is deduced that the main function of *AMI1* could be to control the IAM pool of the plant.
12. Looking for a possible candidate to catalyse the conversion of either Trp or IAOx into IAM, we discovered two yet not functionally characterized genes: *CYP71B22* and *CYP709B3*. *In silico* analyses of their diurnal expression pattern along with RNA-Seq analyses pointed toward *CYP71B22* as the best candidate to act upstream of *AMI1* in the possibly initial enzymatic reaction step of the IAM-pathway.
13. The test whether IAM can function as a signalling molecule demonstrated that an excess of IAM has a profound influence on the expression profile of a large number of genes. This finding further underlines our hypothesis that the main role of *AMI1* is the control of the endogenous cellular IAM pool to regulate the expression of a subset of downstream genes.

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Up-regulated genes in *ami1-2*'s array

Figure S. 1 GO hieratical image containing all statistically significant terms for biological process of up-regulated *ami1-2*'s genes.

The node's colour is darker and more orange the more significant statistically. White nodes are not significantly overrepresented. The area of a node is proportional to the number of genes in the test set annotated to the corresponding GO category. In each circle is written the GO description.

Some overexpressed groups of genes from *ami1-2*'s array

Figure S. 2 GO hieratical image containing significant terms for biological process of up-regulated genes in *ami1-2*'s array.

The node's colour is darker and more orange the more significant statistically. White nodes are not significantly overrepresented. The area of a node is proportional to the number of genes in the test set annotated to the corresponding GO category. In each circle is written the GO description

Here, it is only represented the groups more significantly up regulated. Some of them are the groups related with “JA biosynthesis process”; response to stimulus and stress such as “response to temperature stimulus”.

Down-regulated genes in *ami1-2*'s array

Figure S. 3 GO hieratical image containing all statistically significant terms for biological process of down-regulated *ami1-2*'s genes.

The node's colour is darker and more orange the more significant statistically. White nodes are not significantly overrepresented. The area of a node is proportional to the number of genes in the test set annotated to the corresponding GO category. In each circle is written the GO description.

Down-regulated genes in *ami1-2*'s array

Figure S. 4 GO hieratical image containing significant terms for biological process of down-regulated genes in *ami1-2*'s array.

The node's colour is darker and more orange the more significant statistically. White nodes are not significantly overrepresented. The area of a node is proportional to the number of genes in the test set annotated to the corresponding GO category. In each circle is written the GO description. Here, it is only represented the groups more significantly down-regulated. Some infraexpressed groups are "defence response"; "response to bacterium" or "response to hormone stimulus".

Table S. 1 Representation of genes up- and down-regulated in *ami1-2*'s array.

The table shows the more overrepresented genes in the array for *ami1-2* plants with their correspondent Fold-Change and p-values.

Up-regulated (<i>ami1-2</i> 's array)			
JA Biosynthesis Process			
Gene		Fold-Change	p-value
LOX3 (Lipoxygenase 3)	AT1G17420	2,1	0,00304878
AOS (Allene oxide synthase)	AT5G42650	2,27	0,00052527
AOC1 (Allene Oxide Cyclase 1)	AT3G25760	2,34	0,00094297
AOC3 (Allene Oxide Cyclase 3)	AT3G25780	2,15	0,00667112
ACX1 (Acyl-CoA Oxidase 1)	AT4G16760	1,57	0,00402744
JAR1 (Jasmonate Resistant 1)	AT2G46370	4,19	0,00001934
OPR1 (12-oxophytodienoate reductase)	AT1G76690	1,56	0,00086725
OPR3 (OPDA-Reductase 3)	AT2G06050	1,83	0,00238468
Response to JA stimulus			
Gene		Fold-Change	p-value
JAZ2 (Jasmonate-Zim-Domain Protein 2)	AT1G74950	1,54	0,00626839
JAZ3 (Jasmonate-Zim-Domain Protein 3)	AT3G17860	2,18	0,00040825
JAZ9 (Jasmonate-Zim-Domain Protein 9)	AT1G70700	2,43	0,00033694
JAZ10 (Jasmonate-Zim-Domain Protein 10)	AT5G13220	2	0,01474088
ATPRB1 (Pathogenesis Related Protein)	AT2G14580	1,52	0,00433451
MYB32 (Myb Domain Protein 32)	AT4G34990	2,56	0,00407096
MYB34 (Myb Domain Protein 34)	AT5G60890	2,4	0,00044677
MYB47 (Myb Domain Protein 47)	AT1G18710	6,17	0,00047297
MYB74 (Myb Domain Protein 74)	AT4G05100	4,44	0,0002405
MYB108 (Myb Domain Protein 108)	AT3G06490	2,25	0,00017351
Putative Myb Family Transcription Factor	AT5G52660	1,79	0,00060104
ST2A (Sulfotransferase 2A)	AT5G07010	2,4	0,00027582
VTC5 (Vitamin C Defective 5)	AT5G55120	1,61	0,01203422
ESP (Epithiospecifier Protein)	AT1G54040	2,01	0,00253462
OEP16-1 (Outer plastid envelope protein 16-1)	AT2G28900	1,74	0,00104267
TPS04 (Terpene synthase 04)	AT1G61120	1,97	0,01967898
Response to Heat			
Gene		Fold-Change	p-value
HSP17.4 (Heat-Shock Protein Small (sHSP))	AT3G46230	2,46	0,00022303
HSP17.6C-CI (Heat Shock Protein Small (sHSP))	AT1G53540	3,35	0,00092778
HSFA2 (Heat Stress Transcription Factor (HsF))	AT2G26150	3,78	0,00051576
HSC70-3 (Heat Shock Cognate 70 kDa Protein 3)	AT3G09440	1,84	0,00004324
HSF4 (Heat Shock Protein 4)	AT4G36990	1,54	0,01187211
HSP17.4-CIII (Heat Shock Protein 17.4 kDa)	AT1G54050	3,11	0,00028404
HSP17.6A-CI (Heat Shock Protein)	AT1G59860	3,82	0,0003321
HSP17.6A (Heat Shock Protein 17.6A)	AT5G12030	5,3	0,00076544
HSP17.6II (Heat Shock Protein 17.6 kDa)	AT5G12020	3,05	0,00184065
HSP70T-2 (Heat Shock Protein 70T-2)	AT2G32120	1,73	0,00352971
HSP70 (Heat Shock Protein 70)	AT3G12580	2,58	0,00047962

Up-regulated (<i>ami1-2</i> 's array)			
Response to Heat			
Gene		Fold-Change	p-value
Hsp70B (Heat Shock Protein 70B)	AT1G16030	1,78	0,0035606
HSP90.1 (Heat Shock Protein 90.1)	AT5G52640	1,91	0,0037264
HSP101 (Heat Shock Protein 101)	AT1G74310	3,41	0,00013012
HSP23.5-M (Heat Shock Protein Small Mitochondrial)	AT5G51440	1,59	0,01944105
MBF1C (Multiprotein bridging factor 1C)	AT3G24500	3,04	0,00036458
COR47 (Cold-regulated 47)	AT1G20440	1,99	0,00009215
ABI1 (ABA insensitive 1)	AT4G26080	1,65	0,00029456
Protein_coding stress-inducible protein	AT4G12400	1,73	0,0064792
ERDJ3A (Oxidoreductase J Domain Protein)	AT3G08970	1,85	0,00051647
BAG6 (BCL-2-associated athanogene 6)	AT2G46240	3,28	0,0009235
NPQ1 (Non-photochemical quenching 1)	AT1G08550	1,54	0,00267514
GolS1 (Galactinol Synthase 1)	AT2G47180	1,73	0,00822632
Response to ABA Stimulus			
Gene		Fold-Change	p-value
ANNAT4 (Annexin Arabidopsis 4)	AT2G38750	1,6	0,00424248
LTI30 (Low Temperature-Induced 30)	AT3G50970	2,58	0,00081848
LTI78 (Low Temperature-Induced 78)	AT5G52310	4,61	0,00023951
KIN1 (Cold and ABA Inducible Protein)	AT5G15960	3,32	0,00111556
COR47 (Cold-Regulated 47)	AT1G20440	1,99	0,00009215
COR413-PM1 (Alpha Form of a Protein Similar to the Cold Acclimation)	AT2G15970	1,61	0,00255529
COR414-TM1 (Protein Similar to the Cold Acclimation)	AT1G29395	3,07	0,00050028
HVA22D (Homologous to a Eukaryote Specific ABA- and Stress-Inducible Gene)	AT4G24960	4,3	0,00012088
RD26 (Responsive to Desiccation 26)	AT4G27410	1,8	0,00070443
ERD10 (Early Responsive to Dehydration 10)	AT1G20450	1,8	0,0001729
ABI1 (ABA Insensitive 1)	AT4G26080	1,65	0,00029456
PP2C (Phosphatase 2C)	AT5G59220	1,98	0,01399705
PP2CA (Phosphatase 2CA)	AT3G11410	1,58	0,01873022
AFP1 (ABI Five Binding Protein)	AT1G69260	1,69	0,00065021
AFP2 (ABI Five Binding Protein 2)	AT1G13740	1,53	0,01005772
Myb Family Transcription Factor	AT5G52660	1,79	0,00060104
MYB32 (Myb Domain Protein 32)	AT4G34990	2,56	0,00407096
MYB34 (Myb Domain Protein 34)	AT5G60890	2,4	0,00044677
MYB74 (Myb Domain Protein 74)	AT4G05100	4,44	0,0002405
MYB102 (MYB-LIKE 102)	AT4G21440	2,67	0,00014979
MYB108 (Myb Domain Protein 108)	AT3G06490	2,25	0,00017351
MBF1C (Multiprotein Bridging Factor 1C)	AT3G24500	3,04	0,00036458
GBF3 (G-BOX Binding Factor 3)	AT2G46270	2,33	0,00114731
HB6 (Transcription activator)	AT2G22430	1,72	0,00061923
HVA22E (HVA22 homolog)	AT5G50720	1,63	0,00753574
PMZ (Putative Zinc Finger Protein (PMZ))	AT3G28210	2,81	0,00140191
TCP14 (Teosinte Branched 1,Cycloidea and PCF (TCP) 14)	AT3G47620	1,5	0,00178755
PRN (PIRIN) (Cupin-Domain Containing Protein)	AT3G59220	1,61	0,01203888
C3HC4-type RING Finger	AT1G63840	2,07	0,00006612
Receptor Protein Kinase-Related	AT3G22060	2,54	0,00005227

Up-regulated (<i>ami1-2</i> 's array)			
Response to Oxidative Stress			
Gene		Fold-Change	p-value
NADP-Dependent Oxidoreductase	AT5G17000	1,99	0,0000365
HSP17.4-CIII (Heat Shock Protein 17.4 kDa class III))	AT1G54050	3,11	0,00028404
HSP17.6A (Heat Shock Protein 17.6A)	AT5G12030	5,3	0,00076544
HSP17.6B-CI (Heat Shock Protein 17.6 kDa class I)	AT2G29500	1,99	0,01338295
HSP70 (Heat Shock Protein 70)	AT3G12580	2,58	0,00047962
HSP70T-2 (Heat-Shock Protein 70T-2)	AT2G32120	1,73	0,00352971
HSP101 (Heat Shock Protein 101)	AT1G74310	3,41	0,00013012
HSFA2 (Heat Stress Transcription Factor (Hsf) family)	AT2G26150	3,78	0,00051576
FER1 (Ferric Iron Binding)	AT5G01600	2,12	0,01216317
FER3 (Ferritin 3)	AT3G56090	2,89	0,00141986
FER4 (Ferritin 4)	AT2G40300	1,96	0,02072364
NADP-Dependent Oxidoreductase	AT5G16960	1,84	0,00090673
NADP-Dependent Oxidoreductase	AT5G16980	1,51	0,00441026
Response to Oxidative Stress	AT4G12000	3,41	0,00001819
TRX5 (Oxidoreductase, Acting on Sulfur Group of Donors)	AT1G45145	1,9	0,00092409
Stress-Inducible Protein	AT4G12400	1,73	0,0064792
HEMA2 (Glutamyl-tRNA Reductase)	AT1G09940	2,12	0,00027294
GSTU5 (Glutathione S-Transferase TAU 5)	AT2G29450	4,33	0,00017636
GolS1 (Galactinol Synthase 1)	AT2G47180	1,73	0,00822632
CRK11 (Cysteine-Rich RLK11)	AT4G23190	1,95	0,01085991
BAG6 (BCL-2-Associated Athanogene 6)	AT2G46240	3,28	0,0009235
TPS04 (Terpene Synthase 04)	AT1G61120	1,97	0,01967898
AER (2-Alkenal Reductase)	AT5G16970	1,67	0,00011994
FAD-Binding Domain-Containing Protein	AT4G20830	1,61	0,00488877
Unknown Protein	AT5G59080	1,92	0,00008844
Unknown Protein	AT5G19875	2,18	0,00298456
Response to Wounding			
Gene		Fold-Change	p-value
MES5 (Methyl Esterase 5)	AT5G10300	2,3	0,00663308
CYP79B3 (Cytochrome P450 79B3)	AT2G22330	1,54	0,00714396
LOX3 (Lipoxygenase 3)	AT1G17420	2,1	0,00304878
AOS (Allene Oxide Synthase)	AT5G42650	2,27	0,00052527
JAZ10 (Jasmonate-Zim-Domain Protein 10)	AT5G13220	2	0,01474088
ACX1 (Acyl-CoA Oxidase 1)	AT4G16760	1,57	0,00402744
JAR1 (Jasmonate Resistant 1)	AT2G46370	4,19	0,00001934
MYB102 (MYB-LIKE 102)	AT4G21440	2,67	0,00014979
OPR2 (12-Oxophytodienoic Acid Reductase)	AT1G76690	1,56	0,00086725
OPR3 (3-Oxophytodienoic Acid 3)	AT2G06050	1,83	0,00238468
Macrophage Migration Inhibitory Factor	AT3G51660	2,33	0,0002585
PP2-A13 (Phloem Protein 2-A13)	AT3G61060	1,59	0,00058637
Inositol 1,3,4-Trisphosphate 5/6-Kinase Protein	AT4G08170	3,43	0,00018818
CK1 (Choline Kinase 1)	AT1G71697	1,86	0,00023985
Phosphorylase Protein	AT4G24350	1,59	0,00118839
MATE (Efflux Family Protein)	AT1G66760	1,92	0,00178499
RAP2.6 (Related to AP2 6; ERF Ethylene Response Factor)	AT1G43160	7,29	0,0001167
TIFY10B	AT1G74950	1,54	0,00626839

Up-regulated (<i>ami1-2</i> 's array)			
Response to Wounding			
Gene		Fold-Change	p-value
HPL1 (Hydroperoxide Lyase 1)	AT4G15440	2,29	0,00080517
NUDT8 (Nudix Hydrolase Homolog 8)	AT5G47240	3,12	0,00000491
TOEP16-1 (Outer Plastid Envelope Protein 16-1)	AT2G28900	1,74	0,00104267
TPS04 (Terpene Synthase 04)	AT1G61120	1,97	0,01967898
Unknown Protein	AT5G53750	2,32	0,00014324
Unknown Protein	AT5G54170	2,13	0,00096171
Down-regulated (<i>ami1-2</i> 's array)			
Response to Bacterium			
Gene		Fold-Change	p-value
PTR3 (Peptide Transporter 3)	AT5G46050	-1,56	0,0399694
OSM34 (Osmotin 34)	AT4G11650	-3,79	0,00008138
MYB51 (MYB Domain Protein 51)	AT1G18570	-1,87	0,00018411
WRKY53 (Transcription Factor Member of WRKY)	AT4G23810	-1,86	0,02953252
WRKY60 (Transcription Factor Member of WRKY)	AT2G25000	-1,51	0,00310882
MPK3 (Mitogen-Activated Protein Kinase 3)	AT3G45640	-1,66	0,00059264
CYP81F2 (Cytochrome P450 81F2)	AT5G57220	-1,67	0,00124996
ELI3-2 (Elicitor-Activated Gene 3-2)	AT4G37990	-2,12	0,01230231
IRT1 (Iron-Regulated Transporter 1)	AT4G19690	-2,82	0,00035519
CBP60G (CAM-Binding Protein 60-like.G)	AT5G26920	-2	0,00457379
NRAMP4 (Manganese Ion Transmembrane Transporter)	AT5G67330	-1,65	0,00994483
CSLA09 (Mannan Synthase/ Transferase)	AT5G03760	-1,54	0,03398752
PBS3 (AVRPPHB Susceptible 3)	AT5G13320	-1,63	0,01368001
Response to Chitin			
Gene		Fold-Change	p-value
WRKY53 (Transcription Factor Member of WRKY)	AT4G23810	-1,86	0,02953252
Armadillo/Beta-Catenin Repeat Family Protein	AT1G23030	-2,28	0,00021171
ANAC036 (NAC Domain Containing Protein 36)	AT2G17040	-1,88	0,00002708
ERF13 (Ethylene-Responsive Element Binding Factor 13)	AT2G44840	-1,63	0,00402113
ERF (Ethylene Response Factor, Subfamily B-3)	AT5G51190	-3,04	0,00173101
RHA1B (Protein Binding / Zinc Ion)	AT4G11360	-1,68	0,00224523
MPK3 (Mitogen-Activated Protein Kinase 3)	AT3G45640	-1,66	0,00059264
ATL2 (Protein Binding / Zinc Ion RING-H2)	AT3G16720	-1,68	0,00041988
WRKY46 (Transcription Factor Member of WRKY)	AT2G46400	-2,04	0,00005791
PUB29 (Plant U-Box 29)	AT3G18710	-1,58	0,00643519
Response to Hormone Stimulus			
Gene		Fold-Change	p-value
PTR3 (Peptide Transporter 3)	AT5G46050	-1,56	0,0399694
MARD1 (Zinc-Finger Protein)	AT3G63210	-1,53	0,00054477
ACS6 (1-Aminocyclopropane-1-Carboxylic Acid (ACC) Synthase 6)	AT4G11280	-1,52	0,00533348
ERF (Ethylene Response Factor Subfamily B-6)	AT5G25190	-1,63	0,00253618
NAC1 (Transcription Factor)	AT1G56010	-1,74	0,00316626
ERF13 (Ethylene-Responsive Element Binding Factor 13)	AT2G44840	-1,63	0,00402113
MPK3 (Mitogen-Activated Protein Kinase 3)	AT3G45640	-1,66	0,00059264
PROPEP1 (Putative 92-aa Protein, precursor of AtPep1)	AT5G64900	-1,8	0,00071628

Down-regulated (<i>ami1-2</i> 's array)			
Response to Hormone Stimulus			
Gene		Fold-Change	p-value
ERF11 (ERF Domain Protein 11)	AT1G28370	-1,74	0,00065248
ETR2 (Ethylene Response 2)	AT3G23150	-1,86	0,00019062
PBS3 (AVRPPHB Susceptible 3)	AT5G13320	-1,63	0,01368001
ICK1 (Cyclin-Dependent Protein Kinase Inhibitor)	AT2G23430	-1,61	0,00900987
RALFL23 (Ralf-Like 23)	AT3G16570	-1,51	0,00296391
DFL1 (Dwarf in Light 1)	AT5G54510	-1,54	0,00533771
AMY1 (Alpha-Amylase-Like)	AT4G25000	-4,87	0,00117781
ARR16 (Arabidopsis Response Regulator 16)	AT2G40670	-1,61	0,03653917
Ethylene-Responsive Element-Binding Protein	AT1G72360	-1,51	0,00659226
GA3OX1 (Glibberellin 3-Oxidase 1)	AT1G15550	-1,53	0,02147986
MYB51 (MYB Domain Protein 51)	AT1G18570	-1,87	0,00018411
SAUR_E (Small Auxin Up RNA; auxin-responsive protein)	AT4G34750	-1,69	0,00102644
Auxin-Responsive Protein	AT2G37030	-1,61	0,01690404
Auxin-Responsive Protein	AT2G21220	-1,72	0,01062801
Auxin-Responsive Protein	AT1G56150	-3,65	0,00007446
Auxin-Responsive Protein	AT4G34760	-1,72	0,00722531
Auxin-Responsive Protein	AT4G38860	-2,8	0,00309755
Auxin-Responsive Protein	AT4G38840	-1,98	0,00776349
PR4 (Pathogenesis-Related 4)	AT3G04720	-2,41	0,00010449
Defense Response			
Gene		Fold-Change	p-value
PTR3 (Peptide Transporter 3)	AT5G46050	-1,56	0,0399694
AtRLP26 (Receptor Like Protein 26)	AT2G33050	-1,74	0,0035977
PROPEP1 (Putative 92-aa protein, precursor of AtPep1)	AT5G64900	-1,8	0,00071628
PDF1.4 (Pathogenesis-Related Protein)	AT1G19610	-1,73	0,02714662
Serine-Type Endopeptidase Inhibitor	AT5G43580	-1,99	0,0316567
CBP60G (Cam-Binding Protein 60-Like.G)	AT5G26920	-2	0,00457379
Disease Resistance Protein (TIR-NBS class)	AT1G72920	-1,96	0,00363902
WRKY53 (Transcription Factor Member of WRKY)	AT4G23810	-1,86	0,02953252
OSM34 (Osmotin 34)	AT4G11650	-3,79	0,00008138
PROPEP3 (Elicitor Peptide 3 Precursor)	AT5G64905	-2,85	0,00117175
MLP-Related Major Latex Protein	AT1G23120	-1,69	0,01000287
BAP1 (Bon Association Protein 1)	AT3G61190	-1,51	0,00463496
PBS3 (AVRPPHB Susceptible 3)	AT5G13320	-1,63	0,01368001
ATTI1 (Serine-Type Endopeptidase Inhibitor)	AT2G43510	-3,45	0,00506056
Disease Resistance Protein (TIR-NBS class)	AT4G09420	-2,15	0,00067045
PP2-A8 (Phloem Protein 2-A8)	AT5G45070	-2,07	0,00694668
ELI3-2 (Elicitor-Activated Gene 3-2)	AT4G37990	-2,12	0,01230231
NRAMP4 (Inorganic Anion Transmembrane Transporter)	AT5G67330	-1,65	0,00994483
PROPEP2 (Elicitor Peptide 2 Precursor)	AT5G64890	-1,64	0,00453175
ERF (Ethylene Response Factor Subfamily B-3)	AT5G61600	-1,84	0,00337785
ATL2 (Protein Binding / Zinc Ion RING-H2)	AT3G16720	-1,68	0,00041988
MYB51 (MYB Domain Protein 51)	AT1G18570	-1,87	0,00018411
PR4 (Pathogenesis-Related 4)	AT3G04720	-2,41	0,00010449
WRKY60 (Transcription Factor Member of WRKY)	AT2G25000	-1,51	0,00310882
CYP81F2 (Cytochrome P450 81F2)	AT5G57220	-1,67	0,00124996

Up-regulated genes in AMI1ind's array

Figure S. 5 GO hierarchical image containing all statistically significant terms for biological process of up-regulated AMI1ind's genes.

Groups of overrepresented genes are grouped in nodes with different colours. When the color of the node is darker and more orange, the genetic group will be more significant statistically. White nodes are not significantly overrepresented. In addition, the area of a node is proportional to the number of genes in the test set annotated to the corresponding GO category. In each node is written the GO description.

Down-regulated genes in AMI1ind's array

Figure S. 6 GO hieratical image containing all statistically significant terms for biological process of down-regulated AMI1ind's genes.

Groups of overrepresented genes are grouped in nodes with different colours. The node's colour darker and more orange, the more significant statistically. White nodes are not significantly overrepresented. In addition, the area of a node is proportional to the number of genes in the test set annotated to the corresponding GO category. In each node is written the GO description.

Down-regulated genes in AMI1ind's array

Figure S. 7 GO hieratical image containing significant terms for biological process of down-regulated genes in AMI1ind's array.

The node's colour is darker and more orange the more significant statistically. White nodes are not significantly overrepresented. The area of a node is proportional to the number of genes in the test set annotated to the corresponding GO category. In each circle is written the GO description. Here, it is only represented the groups more significantly down-regulated. Some infraexpressed groups are "response to chitin" or "innate immune response".

Table S. 2 Representation of genes up- and down-regulated in AMI1ind's array.

The table shows the more overrepresented genes in the array for AMI1ind plants with their correspondent Fold-Change and p-values.

Up-regulated AMI1ind's array			
Response to Heat			
Gene		Fold-Change	p-value
HsP17.4-CIII (Heat Shock Protein 17.4 kDa class III)	AT1G54050	2,2	0,0113339
HsC70-3 (Heat Shock Cognate 70 kDa Protein 3)	AT3G09440	2,07	0,00896387
HsP70b (Heat Shock Protein 70B)	AT1G16030	1,89	0,00612822
HsP90.1 (Heat Shock Protein 90.1)	AT5G52640	1,92	0,01950751
HsP101 (Heat Shock Protein 101)	AT1G74310	3,07	0,00463863
HsP23.5-M (Heat Shock Protein Small Mitochondrial 23.5 kDa)	AT5G51440	1,56	0,03342945
HsC70-2M (Heat Shock Protein Mitochondrial 70-2)	AT5G09590	1,58	0,02192495
ABA3 (ABA Deficient 3)	AT1G16540	1,68	0,00254775
Stress-Inducible Protein	AT4G12400	1,67	0,02607503
DREB subfamily A-2 (Transcription Factor of ERF/AP2 family)	AT2G40350	1,95	0,04543541
BAG6 (BCL-2-Associated Athanogene 6)	AT2G46240	2,83	0,00046256
SKP2B (Ubiquitin-Protein Ligase AtSKP2)	AT1G77000	1,72	0,01500974
MBF1C (Multiprotein Bridging Factor 1C)	AT3G24500	2,55	0,00965789
Down-regulated AMI1ind's array			
Innate Immune Defense			
Gene		Fold-Change	p-value
Disease resistance protein (TIR-NBS-LRR class)	AT5G58120	-3,04	0,04976928
Disease resistance protein (TIR-NBS class)	AT3G04210	-2,03	0,01673859
Disease resistance protein (TIR-NBS class)	AT1G66090	-2,67	0,03032003
Disease resistance protein (TIR-NBS class)	AT1G72940	-1,91	0,00833913
Disease resistance protein (TIR-NBS class)	AT1G72900	-1,84	0,02060321
Disease resistance protein (TIR-NBS-LRR class)	AT5G41740	-2,85	0,02537127
Disease resistance protein (TIR-NBS-LRR class)	AT5G22690	-2,44	0,00043368
Disease resistance protein (TIR-NBS class)	AT1G72950	-1,84	0,00198347
WRKY53 (Transcription Factor Member of WRKY)	AT4G23810	-3,2	0,0122355
WRKY70 (Transcription Repressor Factor Member of WRKY)	AT3G56400	-1,75	0,0263869
BON1 (BONZAI 1) (calcium-dependent phospholipid binding)	AT5G61900	-1,66	0,04249462
PROPEP1 (Encodes a putative 92-aa protein)	AT5G64900	-2,48	0,00489587
CBP60G (Calmoduline-binding protein 60-Like.G)	AT5G26920	-2,7	0,00670693
TCH2 (TOUCH 2) (Calcium ion binding)	AT5G37770	-3,44	0,00718876
OSM34 (Osmotin-like protein 34)	AT4G11650	-3,47	0,01163013
NPR3 (NPR1-like protein 3)	AT5G45110	-1,97	0,02437329
MKS1 (MAP kinase substrate 1)	AT3G18690	-1,94	0,00027156
ACD2 (Accelerated cell death 2)	AT4G37000	-1,58	0,00432524
SYP122 (Syntaxin of plants 122)	AT3G52400	-2,24	0,02151143
PUB23 (Plant U-Box 23)	AT2G35930	-5,69	0,00969236
PROPEP2 (Elicitor peptide 2 precursor)	AT5G64890	-1,63	0,04557638
Transmembrane receptor	AT2G32140	-3,4	0,01059801
PR4 (Pathogenesis-Related 4)	AT3G04720	-2,02	0,01536482

Down-regulated AMI1ind's array			
Response to Chitin			
Gene		Fold-Change	p-value
MYB77 (Transcription factor)	AT3G50060	-2,14	0,00016418
MYB73 (MYB Domain Protein 73)	AT4G37260	-2,15	0,00072861
ANAC036 (NAC Domain Containing Protein 36)	AT2G17040	-1,99	0,02466186
ANAC062 (NAC Domain Containing Protein 62)	AT3G49530	-3,16	0,02093236
WRKY22 (Transcription Factor Member of WRKY)	AT4G01250	-1,94	0,04527615
WRKY33 (Transcription Factor Member of WRKY)	AT2G38470	-4,66	0,02640409
WRKY46 (Transcription Factor Member of WRKY)	AT2G46400	-4,73	0,01363039
WRKY53 (Transcription Factor Member of WRKY)	AT4G23810	-3,2	0,0122355
WRKY70 (Transcription Repressor Factor Member of WRKY)	AT3G56400	-1,75	0,0263869
C3HC4-type RING finger (Zinc Finger)	AT5G59550	-2,74	0,00937861
C3HC4-type RING finger (Zinc Finger)	AT3G46620	-2,32	0,00013225
C3HC4-type RING Finger (Zinc Finger)	AT1G20823	-3,19	0,01126121
C3HC4-type RING Finger (Zinc Finger)	AT5G27420	-2,98	0,00402403
C3HC4-type RING Finger (Zinc Finger)	AT5G66070	-1,85	0,0415417
AN1-like (Zinc Finger)	AT3G52800	-2,48	0,02292832
AZF2 (Arabidopsis Zinc-Finger Protein 2)	AT3G19580	-3,62	0,04930111
ATL2 (Zinc Ion Binding RING-H2 Protein)	AT3G16720	-2,09	0,00117893
STZ (Salt Tolerance Zinc Finger)	AT1G27730	-2,89	0,01562584
SZF1 (Salt-Inducible Zinc Finger 1)	AT3G55980	-4,9	0,01306269
Protein Binding / Zinc Ion	AT4G15975	-1,79	0,04605321
PUB23 (Plant U-Box 23)	AT2G35930	-5,69	0,00969236
PUB29 (Plant U-Box 29)	AT3G18710	-2,13	0,0022204
U-box domain-containing protein	AT5G64660	-2,36	0,04134017
BT5 (BTB and TAZ Domain Protein 5)	AT4G37610	-2,06	0,00444077
ERF5 (Ethylene-Responsive Element Binding Factor 5)	AT5G47230	-1,79	0,00932438
ERF6 (Ethylene-Responsive Element Binding Factor 6)	AT4G17490	-2,85	0,00243582
ERF13 (Ethylene-Responsive Element Binding Factor 13)	AT2G44840	-4,14	0,0261502
EDA39 (Embryo Sac Development Arrest 39)	AT4G33050	-1,98	0,02961167
Scarecrow Transcription Factor	AT3G46600	-2,06	0,0232515
CZF1 (Transcription Factor)	AT2G40140	-2,27	0,03684562
HSFA4A (Heat Stress Transcription Factor (Hsf) family)	AT4G18880	-1,9	0,042781
AP2 (Domain-Containing Transcription Factor)	AT5G51190	-2,62	0,01788096
MPK3 (Mitogen-Activated Protein Kinase 3)	AT3G45640	-3,77	0,00028638
Armadillo/Beta-Catenin Repeat Family Protein	AT1G23030	-1,9	0,01179512
Regulation of Programmed Cell Death			
Gene		Fold-Change	p-value
SYP122 (Syntaxin of Plants 122)	AT3G52400	-2,24	0,02151143
RBOHD (Respiratory Burst Oxidase Homologue D)	AT5G47910	-2,71	0,00155992
FAH1 (Fatty Acid Hydroxylase 1)	AT2G34770	-1,54	0,03135677
ACD2 (Accelerated Cell Death 2)	AT4G37000	-1,58	0,00432524
ACS6 (1-Aminocyclopropane-1-Carboxylic Acid (ACC) Synthase 6)	AT4G11280	-4,78	0,01937469

Up-regulated genes in *cyp79b2 cyp79b3*'s array

Figure S. 8 GO hieratical image containing all statistically significant terms for biological process of up-regulated *cyp79b2 cyp79b3*'s genes.

Groups of overrepresented genes are grouped in nodes with different colours. When the colour of the node is darker and more orange, the genetic group will be more significant statistically. White nodes are not significantly overrepresented. In addition, the area of a node is proportional to the number of genes in the test set annotated to the corresponding GO category. In each node is written the GO description. The groups of genes more significantly up regulated are part of defense and response to glucose stimulus groups.

Down-regulated genes in *cyp79b2 cyp79b3*'s array

Figure S. 10 GO hieratical image containing all statistically significant terms for biological process of down-regulated *cyp79b2 cyp79b3*'s genes.

Groups of overrepresented genes are grouped in nodes with different colours. When the colour of the node is darker and more orange, the genetic group will be more significant statistically. White nodes are not significantly overrepresented. In addition, the area of a node is proportional to the number of genes in the test set annotated to the corresponding GO category. In each node is written the GO description.

Table S. 3 Representation of genes up- and down-regulated in *cyp79b2 cyp79b3*'s array.

These following tables show the more overrepresented genes in the array for *cyp79b2/b3* plants with their correspondent Fold-Change and p-values.

Up-regulated <i>cyp79b2 cyp79b3</i> 's array			
Response to Defense			
Gene		Fold-Change	p-value
CYP83B1 (Cytochrome P450 83B1)	AT4G31500	2,12	0,00183954
PTR3 (Peptide Transporter 3)	AT5G46050	3,13	0,00009242
Disease Resistance Family Protein	AT2G34930	7	0,00000035
Disease Resistance Protein (TIR class)	AT1G61100	2,02	0,0017957
FLS2 (Flagellin-Sensitive 2)	AT5G46330	3,21	0,00007137
PP2-A5 (Phloem Protein 2 A5)	AT1G65390	6,75	0,00000053
TIR-NBS-LRR class (Disease Resistance Protein)	AT3G44400	2,16	0,00224305
PMR6 (Powdery Mildew Resistant 6)	AT3G54920	2,76	0,00011556
PEN3 (Penetration 3)	AT1G59870	2,23	0,0021836
MLO8 (Mildew Resistance Locus O 8)	AT2G17480	2,03	0,00445647
RLP12 (Receptor Like Protein 12)	AT1G71400	2,17	0,00146374
RLP33 (Receptor Like Protein 33)	AT3G05660	2,01	0,00281491
RLP22 (Receptor Like Protein 22)	AT2G32660	2,83	0,00017536
Peroxidase	AT5G64120	2,98	0,00018518
Putative peroxidase	AT5G39580	8,75	0
NSL1 (Necrotic Spotted Lesions 1)	AT1G28380	2,23	0,00245734
TIR-NBS-LRR class (Disease Resistance Protein)	AT5G22690	3,59	0,00004682
MKS1 (MAP Kinase Substrate 1)	AT3G18690	2,36	0,00110495
SYP121 (Syntaxin of Plants 121)	AT3G11820	2,54	0,0006555
CCR2 (Cold Circadian Rhythm 2)	AT2G21660	3,18	0,00005559
BIK1 (Botrytis-Induced Kinase 1)	AT2G39660	2,4	0,00076142
TIP (TCV-Interacting Protein)	AT5G24590	2,24	0,00111197
Protease Inhibitor/Seed Storage/Lipid Transfer Protein (LTP)	AT4G12470	3,66	0,00002911
Response to Glucose			
Gene		Fold-Change	p-value
DYL1 (Dormancy-Associated Protein-Like 1)	AT1G28330	5,34	0,00000053
TAG1 (Triacylglycerol Biosynthesis Defect 1)	AT2G19450	3,43	0,00002683
XLG2 (Extra-Large GTP-Binding Protein 2)	AT4G34390	2,48	0,00101868
Stress-Responsive Protein	AT4G37220	3,09	0,00005033
OEP16-1 (Outer Plastid Envelope Protein 16-1)	AT2G28900	2,46	0,00038597
ASN1 (Glutamine-Dependent Asparagine Synthase 1)	AT3G47340	5,47	0,00000053

Down-regulated <i>cyp79b2 cyp79b3</i> 's array			
Response to flavonoid biosynthetic process			
Gene		Fold-Change	p-value
Chalcone-Flavanone Isomerase Family Protein	AT5G05270	-2,75	0,00011854
UDP-glucuronosyl/UDP-glucosyl transferase family protein	AT1G06000	-3,21	0,00003384
TT5 (Transparent Testa 5)	AT3G55120	-2,36	0,00038843
MYBL2 (MYB-like 2)	AT1G71030	-2,39	0,00056554
F3H (Flavanone 3-Hydroxylase)	AT3G51240	-2,93	0,00007137
TT4 (Transparent Testa 4)	AT5G13930	-2,7	0,00013205
FLS (Flavonol Synthase)	AT5G08640	-3,83	0,00000772
PAP1 (Production of Anthocyanin Pigment1)	AT1G56650	-2,81	0,00010557
Response to Glucosinolate Biosynthetic Process			
Gene		Fold-Change	p-value
CYP79B3 (Cytochrome P450 79B3)	AT2G22330	-7,91	0
CYP79F1 (Cytochrome P450 79F1)	AT1G16410	-5,03	0,0000007
CYP83A1 (Cytochrome P450 83A1)	AT4G13770	-2,7	0,00012854
AOP2 (Alkenyl Hydroxalkyl Producing 2)	AT4G03060	-5,87	0,00000035
FMO GS-OX1 (Flavin-Monooxygenase Glucosinolate S-Oxygenase 1)	AT1G65860	-2,24	0,00059483
FMO GS-OX2 (Flavin-Monooxygenase Glucosinolate S-Oxygenase 2)	AT1G62540	-2,83	0,00009733
FMO GS-OX3 (Flavin-Monooxygenase Glucosinolate S-Oxygenase 3)	AT1G62560	-4,01	0,00000614
SOT18 (Desulfo-Glucosinolate Sulfotransferase 18)	AT1G74090	-2,06	0,00112231
MYB28 (MYB Domain Protein 28)	AT5G61420	-2,99	0,00008803
MYB29 (MYB Domain Protein 29)	AT5G07690	-4,9	0,0000007
Bile acid:sodium symporter family protein	AT4G12030	-2,85	0,00008628
BCAT4 (Branched-Chain Aminotransferase 4)	AT3G19710	-2,25	0,0005438
Response to Jasmonic Acid Stimulus			
Gene		Fold-Change	p-value
MYB Transcription Factor	AT3G09600	-2,65	0,0001836
MYB Transcription Factor	AT1G49010	-2,31	0,00058045
MYB3 (MYB Domain Protein 3)	AT1G22640	-2,34	0,00054415
MYB28 (MYB Domain Protein 28)	AT5G61420	-2,99	0,00008803
MYB29 (MYB Domain Protein 29)	AT5G07690	-4,9	0,0000007
MYB47 (MYB domain protein 47)	AT1G18710	-2,01	0,00181745
MYB111 (MYB Domain Protein 111)	AT3G46130	-2,23	0,00073038
MYBL2 (Arabidopsis MYB-like 2)	AT1G71030	-2,39	0,00056554
WRKY70 (Transcription Factor)	AT3G56400	-2,27	0,00100623

Down-regulated <i>cyp79b2 cyp79b3</i> 's array			
Response to Jasmonic Acid Stimulus			
Gene		Fold-Change	p-value
LOX2 (Lipoxygenase 2)	AT3G45140	-2,46	0,00025848
TAT3 (Tyrosine Aminotransferase 3)	AT2G24850	-3,25	0,00004594
PAP1 (Production of Anthocyanin Pigment 1)	AT1G56650	-2,81	0,00010557
FAD-binding domain-containing protein	AT2G34810	-2,15	0,00090013
TRP1 (Telomeric Repeat Binding Protein 1)	AT5G59430	-2,13	0,00085085
VSP1 (Vegetative Storage Protein 1)	AT5G24780	-2,65	0,00016747
CCA1 (Circadian Clock Associated 1)	AT2G46830	-7,41	0
SQE3 (Squalene Epoxidase 3)	AT4G37760	-2,12	0,00093941
TT4 (Transparent Testa 4)	AT5G13930	-2,7	0,00013205
NTP2 (Nitrate Transporter)	AT2G26690	-2	0,00158965
DNA-binding family protein	AT1G70000	-2,44	0,00030794
Bile Acid:Sodium Symporter Family Protein	AT4G12030	-2,85	0,00008628
VTC2 (Vitamin C Defective 2)	AT4G26850	-5,68	0,00000053
LHY (Late Elongated Hypocotyl)	AT1G01060	-3,58	0,00001385

Up-regulated genes in *ami1-2/rty1* RNA-Seq

Figure S. 12 GO hieratical image containing all statistically significant terms for biological process of up-regulated *ami1-2/rty1* genes

These nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean "is a"; green arrows indicate "negative regulate"; a gross dashed arrow means "two significant nodes" and a thin dashed black arrow denotes "one significant nodes". There are not groups to be really significantly overrepresented.

Down-regulated genes in *ami1-2/rty1* RNA-Seq

Figure S. 13 GO hieratical image containing all statistically significant terms for biological process of down-regulated *ami1-2/rty1* genes

The nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The colour of nodes is darker and redder when more significant statistically is. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean “is a”; green arrows indicate “negative regulate”; a gross dashed arrow means “two significant nodes” and a thin dashed black arrow denotes “one significant nodes”. The principal nodes of each biological process are significantly down-regulated. The most affected by the two mutations (*ami1-2* and *rty1*) are the genes related with “translation”, “ribosome regulation”, “post-embryonic development” and “response to temperature”.

Table S. 4 Representation of genes down-regulated in *ami1-2/rty1*'s RNA-Seq.

These following tables show the more overrepresented genes in the RNA-Seq for *ami1-2/rty1* plants. These genes present a FDR ≤ 0.001 and a Fold Change > 2 .

Down-regulated <i>ami1-2/rty1</i>'s RNA-Seq	
Ribosome Regulation	
Gene	
BBC1 (Breast Basic Conserved 1)	AT3G49010
NUC-L1 (Nucleic Acid Binding)	AT1G48920
Acidic Ribosomal Protein P0-related	AT1G25260
Ribosomal Protein L7Ae/L30e/S12e/Gadd45	AT4G12600
P40 (Structural Constituent of Ribosome Acidic Protein)	AT1G72370
40S Ribosomal Protein S24 (RPS24A)	AT3G04920
60S Ribosomal Protein L5 (RPL5B)	AT5G39740
60S Ribosomal Protein L12 (RPL12B)	AT3G53430
60S Ribosomal Protein L28 (RPL28A)	AT2G19730
60S Ribosomal Protein L31 (RPL31C)	AT5G56710
60S Ribosomal Protein L32 (RPL32A)	AT4G18100
60S Ribosomal Protein L32 (RPL32AB)	AT3G55280
60S Ribosomal Protein L34 (RPL34C)	AT3G28900
60S Ribosomal Protein L34 (RPL34)	AT1G69620
60S Ribosomal Protein L37 (RPL37A)	AT1G15250
60S Ribosomal Protein L37a (RPL37aC)	AT3G60245
60S Ribosomal Protein L38 (RPL38B)	AT3G59540
60S Ribosomal Protein L37 (RPL37B)	AT1G52300
Post-Embryonic Development	
Gene	
Emb1138 (Embryo Defective 1138)	AT5G26742
Emb1211 (Embryo Defective 1211)	AT5G22640
Emb2726 (Embryo Defective 2726)	AT4G29060
Emb3010 (Embryo Defective 3010)	AT5G10360
CCR2 (Cold, Circadian Rhythm and RNA Binding 2)	AT2G21660
CRF3 (Cytokinin Response Factor 3)	AT5G53290
CYP707A2 ((+)-Absciscic Acid 8'-Hydroxylase Activity)	AT2G29090
DET1 (De-Etiolated 1)	AT4G10180
DG1 (Delayed Greening 1)	AT5G67570
GR-RBP2 (Glycine-Rich RNA-Binding Protein 2)	AT4G13850
MEE58 (Material Effect Embryo Arrest 58)	AT4G13940
NRP1 (NAP1-Related Protein 1)	AT1G74560
PKP-BETA1 (Plastidic Pyruvate Kinase Beta Subunit 1)	AT5G52920
PRR5 (Pseudo-Response Regulator 5)	AT5G24470
UBQ1 (Ubiquitin Extension Protein 1)	AT3G52590

Down-regulated <i>ami1-2/rty1</i> 's RNA-Seq	
Translation	
Gene	
Emb3010 (Embryo Defective 3010)	AT5G10360
Emb2726 (Embryo Defective 2726)	AT4G29060
ElongationFactor P (EF-P)	AT3G08740
Elongation Factor 1B Alpha-Subunit 2 (eEF1Balpha2)	AT5G19510
PGY1 (PIGGYBACK1; Ribosomal Protein L10aP)	AT2G27530
KRS-1 (LYSYL-tRNA Synthetase 1)	AT3G11710
BBC1 (Breast Basic Conserved 1)	AT3G49010
tRNA-Phe	ATCG00320
P40 (Structural Constituent of Ribosome Acidic Protein)	AT1G72370
Ribosomal Protein P0-related	AT1G25260
Ribosomal Protein L3 Plastid (RPL3P)	AT3G17465
Ribosomal Protein 5B (RPS5B)	AT2G37270
Ribosomal Protein S15 (RPS15)	AT1G04270
Ribosomal Protein L16 (RPL16B)	AT4G18730
Ribosomal Protein L23AB (RPL23AB)	AT3G55280
Ribosomal Protein L29	AT1G07830
Ribosomal Protein L34 (RPL34)	AT1G69620
30S Ribosomal Protein	AT5G24490
40S Ribosomal Protein S2 (RPS2C)	AT2G41840
40S Ribosomal Protein S3A (RPS3aA)	AT3G04840
40S Ribosomal Protein S3A (RPS3aB)	AT4G34670
40S Ribosomal Protein S4 (RPS4B)	AT5G07090
40S Ribosomal Protein S4 (RPS4D)	AT5G58420
40S Ribosomal Protein S7 (RPS7B)	AT3G02560
40S Ribosomal Protein S7 (RPS7C)	AT5G16130
40S Ribosomal Protein S9 (RPS9C)	AT5G39850
40S Ribosomal Protein S12 (RPS12A)	AT1G15930
40S Ribosomal Protein S12 (RPS12C)	AT2G32060
40S Ribosomal Protein S13 (RPS13A)	AT3G60770
40S Ribosomal Protein S14 (RPS14B)	AT3G11510
40S Ribosomal Protein S15 (RPS15D)	AT5G09510
40S Ribosomal Protein S18 (RPS18B)	AT1G34030
40S Ribosomal Protein S24 (RPS24A)	AT3G04920
40S Ribosomal Protein S25 (RPS25B)	AT2G21580
60S Ribosomal Protein L4/L1 (RPL4D)	AT5G02870
60S Ribosomal Protein L5 (RPL5B)	AT5G39740
60S Ribosomal Protein L7A (RPL7aA)	AT2G47610
60S Ribosomal Protein L7A (RPL7aB)	AT3G62870
60S Ribosomal Protein L7 (RPL7C)	AT2G44120
60S Ribosomal Protein L10A (RPL10aC)	AT5G22440
60S Ribosomal Protein L11 (RPL11D)	AT5G45775
60S Ribosomal Protein L12 (RPL12B)	AT3G53430
60S Ribosomal Protein L13A (RPL13aB)	AT3G24830

Down-regulated <i>ami1-2/rty1</i> 's RNA-Seq	
Translation	
Gene	
60S Ribosomal Protein L37a (RPL37aC)	AT3G60245
60S Ribosomal Protein L13A (RPL13aD)	AT5G48760
60S Ribosomal Protein L15 (RPL15A)	AT4G16720
60S Ribosomal Protein L15 (RPL15B)	AT4G17390
60S Ribosomal Protein L17 (RPL17A)	AT1G27400
60S Ribosomal Protein L18 (RPL18C)	AT5G27850
60S Ribosomal Protein L22-2 (RPL22B)	AT3G05560
60S Ribosomal Protein L26 (RPL26A)	AT3G49910
60S Ribosomal Protein L27 (RPL27C)	AT4G15000
60S Ribosomal Protein L28 (RPL28A)	AT2G19730
60S Ribosomal Protein L31 (RPL31C)	AT5G56710
60S Ribosomal Protein L32 (RPL32A)	AT4G18100
60S Ribosomal Protein L34 (RPL34C)	AT3G28900
60S Ribosomal Protein L37 (RPL37A)	AT1G15250
60S Ribosomal Protein L37 (RPL37B)	AT1G52300
60S Ribosomal Protein L38 (RPL38B)	AT3G59540
60S Ribosomal Protein L39 (RPL39A)	AT2G25210
60S Acidic Ribosomal Protein P0 (RPP0B)	AT3G09200
Response to Temperature	
Gene	
HSFA2 (Heat Stress Transcription Factor (Hsf)	AT2G26150
HSP15.7-CI (Related Small Heat Shock Protein-like 15.7 kDa class I)	AT5G37670
HSP17.4-CIII (Heat Shock Protein 17.4 kDa class III)	AT1G54050
HSP17.6A-CI (Heat Shock Protein 17.6 kDa class I)	AT1G59860
HSP17.6B-CI (Small Heat Shock Protein 17.6 kDa class I)	AT2G29500
HSP17.6C-CI (Small Heat Shock Protein 17.6 kDa class I)	AT1G53540
HSP17.6II (Heat Shock Protein 17.6 kDa class II)	AT5G12020
HSP17.8-CI (Heat Shock Protein 17.8 kDa class I)	AT1G07400
HSP18.2 (Heat Shock Protein 18.2)	AT5G59720
HSP21 (Heat Shock Protein 21)	AT4G27670
HSP22.0 (Small Heat Shock Protein)	AT4G10250
HSP23.6-M (Small Heat Shock Protein Mitochondrial 23.6)	AT4G25200
HSA32 (Heat-Stress-Associated 32)	AT4G21320
HSP60 (Heat Shock Protein 60)	AT3G23990
MBF1C (Multiprotein Bridging Factor 1C)	AT3G24500
MTO3 (Methionine Over-Accumulator 3)	AT3G17390
TCH4 (Touch 4; Hydrolase)	AT5G57560
Monodehydroascorbate Reductase	AT1G63940
Ribosomal Protein L23AB (RPL23AB)	AT3G55280
60S ribosomal protein L26 (RPL26A)	AT3G49910
60S Ribosomal Protein P0 (RPP0B)	AT3G09200
CPN60B (Chaperonin 60 Beta)	AT1G55490
SAG21 (Senescence-Associated Gene 21)	AT4G02380

Down-regulated <i>ami1-2/rty1</i> 's RNA-Seq	
Response to Temperature	
Gene	
2-Cys Peroxiredoxin, Chloroplast (BAS1)	AT3G11630
GR-RBP2 (Glycine-Rich RNA-Binding Protein 2)	AT4G13850
PMH1 (Putative Mitochondrial RNA Helicase 1)	AT3G22310
KCS10 (3-Ketoacyl-CoA Synthase 10)	AT2G26250
CCR2 (Cold, Circadian Rhythm and RNA Binding 2)	AT2G21660
VTC2 (Vitamin C Defective 2)	AT4G26850

Up-regulated genes in *cyp71b22*'s RNA-Seq

Figure S. 14 GO hieratical image containing all statistically significant terms for biological process of up-regulated *cyp71b22*'s RNA-Seq.

These nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean "is a"; green arrows indicate "negative regulate"; red arrows denote "positive regulate"; a gross dashed arrow means "two significant nodes" and a thin dashed black arrow denotes "one significant nodes". The majority of up-regulated groups are part of response to stimulus, such as response to hormone, to fungus, etc.

Down-regulated genes in *cyp71b22*'s RNA-Seq

Figure S. 15 GO hieratical image containing all statistically significant terms for biological process of down-regulated *cyp71b22*'s RNA-Seq.

These nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean "is a"; a gross dashed arrow means "two significant nodes" and a thin dashed black arrow denotes "one significant nodes". Response to stimulus and polycarbohydrate processes are the most significantly down-regulated groups of genes in *cyp71b22*.

Table S. 5 Representation of genes up- and down-regulated in *cyp71b22*'s RNA-Seq.

These following tables show the more overrepresented genes in the RNA-Seq for *cyp71b22* plants. These genes present a FDR $\leq$ 0.001 and a Fold Change > 2 .

Up-regulated <i>cyp71b22</i> 's RNA-Seq	
Response to Auxin Stimulus	
Gene	
MYB (Myb Domain Protein)	AT3G09600
MYB (Myb Domain Protein)	AT1G49010
MYB3 (MYB Domain Protein 3)	AT1G22640
MYB4 (Myb Domain Protein 4)	AT4G38620
MYB15 (MYB Domain Protein 15)	AT3G23250
Auxin-Responsive Protein	AT2G21210
Auxin-Responsive Protein	AT4G38840
Auxin-Responsive Protein	AT1G29430
Auxin-Responsive Protein	AT5G18020
Auxin-Responsive Protein	AT1G20470
Auxin-Responsive Protein	AT2G46690
ARF10 (Auxin Response Factor 10)	AT2G28350
IAA1 (Indole-3-Acetic Acid Inducible 1)	AT4G14560
IAA13 (Indole-3-Acetic Acid Inducible 13)	AT2G33310
IAA16 (Indole-3-Acetic Acid Inducible 16)	AT3G04730
AUX2-11 (Auxin Inducible 2-11)	AT5G43700
HAT2 (Transcription Factor)	AT5G47370
Transcription Factor	AT1G70000
RVE2 (Reveille 2)	AT5G37260
LHY (Late Elongated Hypocotyl)	AT1G01060
DFL1 (Dwarf in Light 1)	AT5G54510
BT1 (BTB and TAZ Domain Protein 1)	AT5G63160
BT5 (BTB and TAZ Domain Protein 5)	AT4G37610
ACS4 (1-Aminocyclopropane-1-Carboxylate Synthase 4)	AT2G22810
PBS3 (AVRPPHB SUSCEPTIBLE 3)	AT5G13320
LCL1 (LHY/CCA1-like 1)	AT5G02840
TRB3 (Telomere Repeat Binding Factor 3)	AT3G49850
CCA1 (Circadian Clock Associated 1)	AT2G46830
Response to ABA Stimulus	
Gene	
PTR3 (Peptide Transporter 3)	AT5G46050
HY5 (Elongated Hypocotyl 5)	AT5G11260
MYB (Myb Domain Protein)	AT3G09600
MYB3 (Myb Domain Protein 3)	AT1G22640
MYB4 (Myb Domain Protein 4)	AT4G38620
MYB7 (Myb Domain Protein 7)	AT2G16720
ARF10 (Auxin Response Factor 10)	AT2G28350
AKT2/3 (Arabidopsis Potassium Transport 2/3)	AT4G22200

Up-regulated <i>cyp71b22</i> 's RNA-Seq	
Response to ABA Stimulus	
Gene	
HB-12 (Homeobox 12)	AT3G61890
Transcription Factor	AT1G70000
TRFL9 (TRF-LIKE 9)	AT3G12560
CIPK1 (CBL-Interacting Protein Kinase 1)	AT3G17510
Protein Kinase	AT3G22060
Serine/Threonine Kinase	AT4G23260
DR4 (Peptidase Inhibitor)	AT1G73330
MBF1C (Multiprotein Bridging Factor 1C)	AT3G24500
GBF3 (G-BOX Binding Factor 3)	AT2G46270
ATDI19 (Related with Drought Stress)	AT1G56280
LCL1 (LHY/CCA1-like 1)	AT5G02840
CCA1 (Circadian Clock Associated 1)	AT2G46830
PP2C (Phosphatase 2C)	AT5G59220
RVE2 (Reveille 2)	AT5G37260
RGL2 (RGA-Like 2)	AT3G03450
LHY (Late Elongate Hypocotyl)	AT1G01060
RHA2A (RING-H2 Finger Protein)	AT1G15100
Phenylpropanoid Biosynthetic Process	
Gene	
ARF10 (Auxin Response Factor 10)	AT2G28350
Disease Resistance-Responsive Protein	AT1G65870
MYB3 (Myb Domain Protein 3)	AT1G22640
2OG-Fe(II) (Oxidoreductase)	AT4G25300
O-Methyltransferase 2	AT1G77520
C4H (Cinnamate-4-Hydroxylase)	AT2G30490
CYP711A1 (Cytochrome P450 711A1)	AT2G26170
DMR6 (Downy Mildew Resistant 6)	AT5G24530
UGT78D1 (UDP-Glucosyl Transferase 78D1)	AT1G30530
CAD (Cinnamyl-Alcohol Dehydrogenase)	AT1G09490
CAD4 (Cinnamyl-Alcohol Dehydrogenase 4)	AT3G19450
PAL1 (Phe Ammonia Lyase 1)	AT2G37040
SRG1 (Senescence-Related Gene 1)	AT1G17020
Defense Response to Fungus	
Gene	
Peroxidase	AT5G39580
Peroxidase	AT5G64120
WRKY60 (Transcription Factor Member of WRKY)	AT2G25000
Protease Inhibitor	AT2G38870
MIPS2 (MYO-Inositol-1-Phosphate Synthase 2)	AT2G22240
MLO6 (Mildew Resistance Locus O 6)	AT1G61560
pEARLI 1(Lipid Transfer Protein)	AT4G12480
LURP1 (Late Upregulated in Response to Hyaloperonospora Parasitic)	AT2G14560

Up-regulated <i>cyp71b22</i> 's RNA-Seq	
Defense Response to Fungus	
Gene	
MLO12 (Mildew Resistanc Locus O 12)	AT2G39200
PP2C (Phosphatase 2C)	AT2G30020
Protease Inhibitor	AT4G12470
WRKY70 (Transcription Factor Member of WRKY)	AT3G56400
Response to Heat	
Gene	
MBF1C (Multiprotein Bridging Factor 1C)	AT3G24500
HSP17.6A (Heat Shock Protein 17.6A)	AT5G12030
HSP17.6II (Heat Shock Protein class II 17.6 kDa)	AT5G12020
HSP17.8CI (Heat Shock Protein class I 17.8 kDa)	AT1G07400
HSP26.5-P (Heat Shock Protein class P-related 26.5 kDa)	AT4G21870
HSP70 (Heat Shock Protein 70)	AT3G12580
HSC70-2 (Heat Shock Cognate 70 kDa protein 2)	AT5G02490
HSP90.1 (Heat Shock Protein 90.1)	AT5G52640
HSP101 (Heat Shock Protein 101)	AT1G74310
HSP23.5-M (Heat Shock Protein Small Mitochondrial 23.5 kDa)	AT5G51440
HSF4 (Heat Shock Factor 4)	AT4G36990
CYP71B2 (Cytochrome P450 71B2)	AT1G13080
MIPS2 (Myo-Inositol-1-Phosphate Synthase 2)	AT2G22240
GolS1 (Galactinol Synthase 1)	AT2G47180
BAP1 (Bon Association Protein 1)	AT3G61190
Stress-Inducible Protein	AT4G12400
Unknown Protein	AT2G19310
Flavonoid Biosynthetic Process	
Gene	
ARF10 (Auxin Response Factor 10)	AT2G28350
HY5 (Elongated Hypocotyl 5)	AT5G11260
2OG-Fe(II) (Oxidoreductase)	AT4G25300
CYP711A1 (Cytochrome P450 711A1)	AT2G26170
DMR6 (Downy Mildew Resistant 6)	AT5G24530
UGT78D1 (UDP-Glucosyl Transferase 78D1)	AT1G30530
SRG1 (Senescence-Related Gene 1)	AT1G17020
Response to High Light Intensity	
Gene	
HSP17.6A (Heat Shock Protein 17.6A)	AT5G12030
HSP70 (Heat Shock Protein 70)	AT3G12580
HSP101 (Heat Shock Protein 101)	AT1G74310
Stress-Inducible Protein	AT4G12400
GolS1 (Galactinol Synthase 1)	AT2G47180
KAT2 (Potassium Channel in Arabidopsis thaliana 2)	AT4G18290
MIPS2 (Myo-Inositol-1-Phosphate Synthase 2)	AT2G22240
Unknown Protein	AT2G19310

Down-regulated <i>cyp71b22</i> 's RNA-Seq	
Carbohydrate Metabolic Process	
Gene	
UDP-Glucose:Sterol Glucosyltransferase	AT1G43620
Glycosyl Hydrolase 17 Protein	AT1G64760
Glycosyl Hydrolase Family 17	AT1G32860
Glucosamine/Galactosamine-6-Phosphate	AT1G13700
Glycerophosphoryl Diester Phosphodiesterase Protein	AT5G08030
GATL7 (Galacturonosyltransferase-Like 7)	AT3G62660
GoIS3 (Galactinol Synthase 3)	AT1G09350
SEX1 (Starch Excess 1)	AT1G10760
SEX4 (Starch-Excess 4)	AT3G52180
SBE2.1 (Starch Branching Enzyme 2.1)	AT2G36390
SUS1 (Sucrose Synthase 1)	AT5G20830
Catalytic/Trehalose-Phosphatase	AT2G22190
TPS10 (Trehalose phosphate synthase)	AT1G60140
UDP-3-O-[3-Hydroxymyristoyl] N-Acetylglucosamine Deacetylase	AT1G24880
ISA3 (Isoamylase 3)	AT4G09020
MUR4 (Murus 4)	AT1G30620
CSLG3 (Cellulose Synthase/Transferase)	AT4G23990
FMO GS-OX4 (Flavin-Monooxygenase Glucosinolate S-Oxygenase 4)	AT1G62570
DPE1 (Disproportionating Enzyme)	AT5G64860
DPE2 (Disproportionating Enzyme 2)	AT2G40840
AMY3 (Alpha-Amylase-Like 3)	AT1G69830
BAM5 (Beta-Amylase 5)	AT4G15210
LDA (Limit Dextrinase)	AT5G04360
TRANS11 (Protein Transmembrane Transporter/Catalytic)	AT4G18270
Phosphoglucomutase Putative	AT1G70820
MEE31 (Maternal Effect Embryo Arrest 31)	AT3G02570
Response to Starch Catabolic	
Gene	
DPE1 (Disproportionating Enzyme)	AT5G64860
DPE2 (Disproportionating Enzyme 2)	AT2G40840
SEX1 (Starch Excess 1)	AT1G10760
LDA (Limit Dextrinase)	AT5G04360
AMY3 (Alpha-Amylase-Like 3)	AT1G69830
ISA3 (Isoamylase 3)	AT4G09020
BAM5 (Beta-Amylase 5)	AT4G15210
Response to Oxylipin	
Gene	
LOX2 (Lipoxygenase 2)	AT3G45140
ST2A (Sulfotransferase 2A)	AT5G07010
PLA2A (Phospholipase A 2A)	AT2G26560
AOC1 (Allene Oxide Cyclase 1)	AT3G25760
AOC2 (Allene Oxide Cyclase 2)	AT3G25770

Down-regulated <i>cyp71b22</i> 's RNA-Seq	
Response to Hormone Stimulus	
Gene	
AHB2 (Arabidopsis Haemoglobin 2)	AT3G10520
ERF2 (Ethylene Responsive Element Binding Factor 2)	AT5G47220
Ethylene-Responsive Element-Binding Protein	AT5G25190
COR47 (Cold-Regulate 47)	AT1G20440
COR413-PM1 (Related with cold acclimation)	AT2G15970
COR414-TM1 (Related with Cold Acclimation Protein)	AT1G29395
KIN1 (Cold and ABA Inducible Protein)	AT5G15960
KIN2 (Cold Responsive 6.6)	AT5G15970
RCI2A (Rare-Cold-Inducible 2A)	AT3G05880
LTI30 (Low Temperature-Induced 30)	AT3G50970
LTI78 (Low-Temperature-Induced 78)	AT5G52310
ABF1 (Abscisic Acid Responsive Element-Binding Factor 1)	AT1G49720
DFL2 (Dwarf in Light 2)	AT4G03400
TCH4 (Touch 4) (Hydrolase/ xyloglucan:xyloglucosyl transferase)	AT5G57560
CORI3 (Coronatine Induced 1)	AT4G23600
PLC1 (Phospholipase C 1)	AT5G58670
HVA22E (HVA22 homolog in Arabidopsis)	AT5G50720
MYBR1 (Myb Domain Protein R1)	AT5G67300
MYBL2 (Myb Domain Protein-Like 2)	AT1G71030
SNRK2.2 (SNF1-Related Protein Kinase 2.2)	AT3G50500
CIPK20 (CBL-Interacting Protein Kinase 20)	AT5G45820
ELF3 (Early Flowering 3)	AT2G25930
LTP3 (Lipid Transfer Protein 3)	AT5G59320
GA2 (GA Requiring 2)	AT1G79460
Auxin-Responsive Protein	AT1G56150
HHP2 (Heptathelical Transmembrane Protein2)	AT4G30850
CAT1 (Catalase 1)	AT1G20630
BT4 (BTB and TAZ Domain Protein 4)	AT5G67480
AILP1 (Unknown Protein)	AT5G19140
Response to ABA Stimulus	
Gene	
CORI3 (Coronatine Induced 1)	AT4G23600
COR47 (Cold-Regulated 47)	AT1G20440
COR414-TM1 (Related with Cold Acclimation Protein)	AT1G29395
COR413-PM1 (Related with Cold Acclimation Protein)	AT2G15970
RCI2A (Rare-Cold-Inducible 2A)	AT3G05880
KIN1 (Cold and ABA Inducible Protein)	AT5G15960
KIN2 (Cold Responsive 6.6)	AT5G15970
LTI30 (Low Temperature-Induced 30)	AT3G50970
LTI78 (Low-Temperature-Induced 78)	AT5G52310
ABF1 (Abscisic Acid Responsive Element-Binding Factor 1)	AT1G49720
CIPK20 (CBL-Interacting Protein Kinase 20)	AT5G45820

Down-regulated <i>cyp71b22</i> 's RNA-Seq	
Response to ABA Stimulus	
Gene	
PLC1 (Phospholipase C 1)	AT5G58670
MYBR1 (Myb Domain Protein R1)	AT5G67300
MYBL2 (Myb Domain Protein-Like 2)	AT1G71030
CAT1 (Catalase 1)	AT1G20630
LTP3 (Lipid Transfer Protein 3)	AT5G59320
SNRK2.2 (SNF1-Related Protein Kinase 2.2)	AT3G50500
HVA22E (HVA22 Homolog in Arabidopsis)	AT5G50720
ELF3 (Early Flowering 3)	AT2G25930
Response to Cold Acclimation	
Gene	
COR15A (Cold-Regulated 15A)	AT2G42540
COR47 (Cold-Regulated 47)	AT1G20440
COR413-PM1 (Related with Cold Acclimation Protein)	AT2G15970
COR414-TM1 (Related with Cold Acclimation Protein)	AT1G29395
KIN1 (Cold and ABA Inducible Protein)	AT5G15960
LT130 (Low Temperature-Induced 30)	AT3G50970
SEX1 (Starch Excess 1)	AT1G10760
CBF2 (C-Repeat/Dre Binding Factor 2)	AT4G25470

Up-regulated genes in *cyp709b3*'s RNA-Seq

Figure S. 16 GO hieratical image containing all statistically significant terms for biological process of up-regulated *cyp709b3*'s RNA-Seq.

These nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean "is a"; green arrows indicate "negative regulate"; a gross dashed arrow means "two significant nodes" and a thin dashed black arrow denotes "one significant nodes". The majority of up-regulated groups are part of response to stimulus and lipid localization.

Down-regulated genes in *cyp709b3*'s RNA-Seq

Figure S. 17 GO hieratical image containing all statistically significant terms for biological process of down-regulated *cyp709b3*'s RNA-Seq.

These nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean “is a”; green arrows indicate “negative regulate”; a gross dashed arrow means “two significant nodes” and a thin dashed black arrow denotes “one significant nodes”. The majority of down-regulated groups are part of response to temperature stimulus and different metabolic processes.

Table S. 6 Representation of genes up- and down-regulated in *cyp709b3*'s RNA-Seq.

These following tables show the more overrepresented genes in the RNA-Seq for *cyp709b3* plants. These genes present a FDR $\leq$ 0.001 and a Fold Change > 2 .

Up-regulated <i>cyp709b3</i> 's RNA-Seq	
Lipid Metabolic Process	
Gene	
TPS-CIN (Terpene Synthase-Like Sequence-1,8-Cineole)	AT3G25830
Fatty Acid Desaturase	AT1G06350
Cyclopropane Fatty Acid Synthase	AT3G23510
GDSL-Motif Lipase/Hydrolase	AT3G14220
GDSL-Motif Lipase/Hydrolase	AT5G45670
GDSL-Motif Lipase/Hydrolase	AT3G53100
GDSL-Motif Lipase/Hydrolase	AT4G30140
GDSL-Motif Lipase/Hydrolase	AT5G45960
GDSL-Motif Lipase/Hydrolase	AT5G33370
II Extracellular Lipase	AT1G20120
Lipase Class 3	AT1G51440
Lipase Class 3	AT2G30550
DOX1 (Lipoxygenase)	AT3G01420
TLL1 (Triacylglycerol Lipase-Like 1)	AT1G45201
LUP1 (Lupeol Synthase 1)	AT1G78970
HPL1 (Hydroperoxide Lyase 1)	AT4G15440
MIPS2 (Myo-Inositol-1-Phosphate Synthase 2)	AT2G22240
Enoyl-CoA Hydratase/Isomerase	AT3G24360
Dehydrodolichyl Diphosphate Synthase	AT5G58770
CYP711A1 (Cytochrome P450 711 A1)	AT2G26170
CYP96A15 (Cytochrome P450 96 A1)	AT1G57750
SQP1 (Squalene Monooxygenase)	AT5G24150
ROT3 (Rotundifolia; Member of the Cytochrome P450 90C)	AT4G36380
AOC3 (Allene Oxide Cyclase 3)	AT3G25780
AOC4 (Allene Oxide Cyclase 4)	AT1G13280
BR6OX2 (Brassinosteroid-6-Oxidase 2)	AT3G30180
RD20 (Responsive to Dessication 20)	AT2G33380
Response to Reactive Oxygen Species	
Gene	
HSP70 (Heat Shock Protein 70)	AT3G12580
Stress-Inducible Protein	AT4G12400
MIPS2 (Myo-Inositol-1-Phosphate Synthase 2)	AT2G22240
PAP17 (Acid Phosphatase)	AT3G17790
GalS1 (Galactinol Synthase 1)	AT2G47180
EGY3 (Ethylene-Dependent Gravitropism-Deficient and Yellow-Green-Like 3)	AT1G17870
Unknown Protein	AT2G19310

Up-regulated <i>cyp709b3</i> 's RNA-Seq	
Response to Other Organism	
Gene	
TGA1 (Transcription Factor)	AT5G65210
POT (Proton-Dependent Oligopeptide Transport)	AT1G72140
RD20 (Responsive to Dessication 20)	AT2G33380
CAX3 (Cation Exchanger 3)	AT3G51860
NSP1 (Nitrile Specifier Protein 1)	AT3G16400
DMR6 (Downy Mildew Resistant 6)	AT5G24530
LURP1 (Late Upregulated in Response to Hyaloperonospora Parasitica)	AT2G14560
Latex Protein-Related	AT4G23670
ACD6 (Accelerated Cell Death 6)	AT4G14400
PYK10 (Beta-Glucosidase/Copper Ion Binding /Fucosidase/Hydrolase)	AT3G09260
VSP2 (Vegetative Storage Protein 2)	AT5G24770
MIPS2 (Myo-Inositol-1-Phosphate Synthase 2)	AT2G22240
BGL2 (Beta-1,3-Glucanase 2)	AT3G57260
AIG1 (AVRRPT2-Induced Gene 1)	AT1G33960
YSL3 (Yellow Stripe Like 3)	AT5G53550
NUDT6 (Nudix Hydrolase Homolog 6)	AT2G04450
DOX1 (Lipoxygenase Encodes an Alpha-Dioxygenase)	AT3G01420
PBS3 (AVRPPHB Susceptible 3)	AT5G13320
HSP70 (Heat Shock Protein 70)	AT3G12580
HSP90.1 (Heat Shock Protein 90.1)	AT5G52640
AOC3 (Allene Oxide Cyclase 3)	AT3G25780
AUX1 (Auxin Resistant 1)	AT2G38120
ABC Transporter	AT2G39350
Protease Inhibitor	AT2G38870
WRKY60 (Transcription Factor)	AT2G25000
Peroxidase	AT4G36430
RDR1 (RNA-Dependent RNA Polymerase 1)	AT1G14790
CSLA09 (Mannan Synthase/Transferase)	AT5G03760
Response to Fungus	
Gene	
PBS3 (AVRPPHB Susceptible 3)	AT5G13320
Protease Inhibitor	AT2G38870
WRKY60 (Transcription Factor)	AT2G25000
MIPS2 (Myo-Inositol-1-Phosphate Synthase 2)	AT2G22240
RD20 (Responsive to Dessication 20)	AT2G33380
LURP1 (Late Upregulated in Response to Hyaloperonospora Parasitica)	AT2G14560
AOC3 (Allene Oxide Cyclase 3)	AT3G25780
DMR6 (Downy Mildew Resistant 6)	AT5G24530
PYK10 (Beta-Glucosidase/ Copper Ion Binding / Fucosidase/ Hydrolase)	AT3G09260

Up-regulated <i>cyp709b3</i> 's RNA-Seq	
Response to ABA Stimulus	
Gene	
PP2C (Phosphatase 2C)	AT5G59220
AKT2/3 (Arabidopsis Potassium Transport 2/3)	AT4G22200
CVP2 (Cotyledon Vascular Pattern 2)	AT1G05470
LTP3 (Lipid Transfer Protein 3)	AT5G59320
LTP4 (Lipid Transfer Protein 4)	AT5G59310
MBF1C (Multiprotein Bridging Factor 1C)	AT3G24500
CDPK2 (Calcium-Dependent Potein Kinase 2)	AT1G35670
Benzodiazepine Receptor	AT2G47770
RAB18 (Responsive to ABA 18)	AT5G66400
RD20 (Responsive to Dessication 20)	AT2G33380
CIPK1 (CBL-Interacting Protein Kinase 1)	AT3G17510
DR4 (Peptidase Inhibitor)	AT1G73330
MYB102 (Myb-Like 102)	AT4G21440
Myb Transcription Factor	AT3G09600
LTI65 (Low-Temperature-Induced 65)	AT5G52300
GBF3 (G-Box Binding Factor 3)	AT2G46270
Down-regulated <i>cyp709b3</i> 's RNA-Seq	
Cold Acclimation	
Gene	
KIN1 (Cold and ABA Inducible Protein)	AT5G15960
KIN2 (Cold Responsive 6.6)	AT5G15970
COR414-TM1 (Related with Cold Acclimation)	AT1G29395
COR15A (Cold-Regulated 15A)	AT2G42540
COR15B (Cold Regulated 15B)	AT2G42530
CCR2 (Cold, Circadian Rhythm and RNA Binding 2)	AT2G21660
LTI30 (Low Temperature-Induced 30)	AT3G50970
LTI78 (Low-Temperature-Induced 78)	AT5G52310
Low Temperature and Salt Responsive Protein	AT4G30660
Hydrophobic Protein	AT4G30650
STZ (Salt Tolerance Zinc Finger)	AT1G27730
ELF3 (Early Flowering 3)	AT2G25930
ELIP1 (Early Light-Inducable Protein)	AT3G22840
GR-RBP2 (Glycine-Rich RNA-Binding Protein 2)	AT4G13850
GR-RBP8 (RNA Binding)	AT4G39260
CBF2 (C-Repeat/DRE Binding Factor 2)	AT4G25470
OEP16-1 (Outer Plastid Envelope Protein 16-1)	AT2G28900
SEX1 (Starch Excess 1)	AT1G10760
GI (Gigantea)	AT1G22770

Down-regulated <i>cyp709b3</i> 's RNA-Seq	
Pigment Metabolic Process	
Gene	
ELIP2 (Early Light-Inducible Protein 2)	AT4G14690
LZF1 (Light-Regulated Zinc Finger Protein 1)	AT1G78600
PORA (Protochlorophyllide Oxidoreductase A)	AT5G54190
PORB (Protochlorophyllide Oxidoreductase B)	AT4G27440
CLH2 (Chlorophyllase)	AT5G43860
MAT (O-Malonyltransferase)	AT3G29590
Starch Metabolic Process	
Gene	
QQS (Qua-Quine Starch)	AT3G30720
SEX1 (Starch Excess 1)	AT1G10760
SEX4 (Starch-Excess 4)	AT3G52180
ISA3 (Isoamylase 3)	AT4G09020
DPE1 (Disproportionating Enzyme)	AT5G64860
Chlorophyll Metabolic Process	
Gene	
ELIP2 (Early Light-Inducible Protein 2)	AT4G14690
LZF1 (Light-Regulated Zinc Finger Protein 1)	AT1G78600
PORA (Protochlorophyllide Oxidoreductase A)	AT5G54190
PORB (Protochlorophyllide Oxidoreductase B)	AT4G27440
CLH2 (Chlorophyllase)	AT5G43860
Lipid Localization	
Gene	
LTP (Lipid Transfer Protein)	AT5G48490
LTP (Lipid Transfer Protein)	AT4G12490
LTP (Lipid Transfer Protein)	AT2G10940
LTP (Lipid Transfer Protein)	AT4G12500
ELP (Extensin-Like Protein)	AT1G12090
Structural Constituent of Cell Wall	AT3G22142
CWLP (Cell Wall-Plasma Membrane Linker Protein)	AT3G22120

Up-regulated genes in Wt+IAM RNA-Seq

Figure S. 18 GO hieratical image containing all statistically significant terms for biological process of up-regulated Wt+IAM genes.

These nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean "is a"; gross dashed arrow means "two significant nodes" and a thin dashed black arrow denotes "one significant nodes". The groups of genes appearing to be more significantly up-regulated, correspond with genes from "response to wounding" and "photosynthesis".

Figure S. 19 GO hieratical image containing all statistically significant terms for biological process of down-regulated Wt+IAM genes.

These nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean "is a"; green arrows indicate "negative regulate"; a gross dashed arrow means "two significant nodes" and a thin dashed black arrow denotes "one significant nodes". The groups of genes appearing to be more significantly down regulated, correspond with genes from "lipid localization", "seed development" and "response to auxin stimulus".

Table S. 7 Representation of genes up- and down-regulated in Wt+IAM's RNA-Seq.

These following tables show the more overrepresented genes in the RNA-Seq for Wt+IAM plants. These genes present a FDR $\leq$ 0.001 and a Fold Change > 2 .

Up-regulated Wt+IAM RNA-Seq	
Photosynthesis	
Gene	
ND1 NDHA (NADH dehydrogenase)	ATCG01100.1
Subunit IV of the Cytochrome b6/f Complex	ATCG00730.1
PSII D2 protein PSBD	ATCG00270.1
CP43 (Subunit of the Photosystem II Reaction Center)	ATCG00280.1
AtFER3 (Ferritin 3)	AT3G56090.1
Chlorophyll Binding Protein D1	ATCG00020.1
Response to Wounding	
Gene	
TPS04 (Terpene Synthase 04)	AT1G61120.1
Wound-Responsive Protein	AT4G10265.1
Inositol 1,3,4-Trisphosphate 5/6-Kinase	AT4G08170.2
HPL1 (Hydroperoxide Lyase 1)	AT4G15440.1
VSP2 (Vegetative Storage Protein 2)	AT5G24770.1
AtNUDT8 (Nudix Hydrolase Homolog 8)	AT5G47240.1
AtMYB102 (MYB-LIKE 102)	AT4G21440.1

Down-regulated Wt+IAM RNA-Seq	
Lipid Localization	
Gene	
NWMU1-2S (Albumin 1 2S Seed Storage Protein 1)	AT4G27140.1
NWMU2-2S (Albumin 2 2S Seed Storage Protein 2)	AT4G27150.1
NWMU2-2S (Albumin 4 2S Seed Storage Protein 4)	AT4G27170.1
OLEO1 (Oleosin 1)	AT4G25140.1
OLEO2 (Oleosin 2)	AT5G40420.1
Oleosin Glycine-Rich Protein	AT3G01570.1
At2S3 (Lipid Binding /Nutrient Reservoir)	AT4G27160.1
LTP4 (Lipid Transfer Protein 4)	AT5G59310.1
Protease Inhibitor/Seed Storage/Lipid Transfer Protein (LTP)	AT5G54740.1
Seed Development	
Gene	
LEA protein (putative late embryogenesis abundant protein)	AT3G15670.1
LEA protein (putative late embryogenesis abundant protein)	AT2G42560.1
LEA protein (putative late embryogenesis abundant protein)	AT4G21020.1
LEA protein (putative late embryogenesis abundant protein)	AT3G17520.1
OLEO1 (Oleosin 1)	AT4G25140.1
OLEO2 (Oleosin 2)	AT5G40420.1
AtS1 (Seed Gene 1)	AT4G26740.1
PER1 (Antioxidant/ Thioredoxin Peroxidase)	AT1G48130.1

Down-regulated Wt+IAM RNA-Seq	
Response to Auxin Stimulus	
Gene	
Auxin-Responsive Protein	AT1G29500.1
Auxin-Responsive Protein	AT1G29450.1
Auxin-Responsive Protein	AT5G18080.1
IAA1 (Indole-3-Acetic Acid Inducible)	AT4G14560.1
IAA5 (Indole-3-Acetic Acid Inducible 5)	AT1G15580.1
GH3.3 (Indole-3-Acetic Acid Amido Synthetase)	AT2G23170.1

Up-regulated genes in *ami1-2*+IAM RNA-Seq

Figure S. 20 GO hieratical image containing all statistically significant terms for biological process of up-regulated *ami1-2*+IAM genes.

These nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean "is a"; red arrows denote "positive regulate"; green arrows indicate "negative regulate"; a gross dashed arrow means "two significant nodes" and a thin dashed black arrow denotes "one significant nodes". Five additional blue arrows point groups of genes related to "JA biosynthesis process"(1); "response to JA stimulus"(2); "response to H₂O deprivation"(3); "Flavonoid byosinthetic process"(4) and "Glucosinolate biosynthetic process"(5).

Up-regulated genes in *ami1-2*+IAM RNA-Seq

Figure S. 21 GO hieratical image containing significant terms for biological process of up-regulated genes in *ami1-2*+IAM's RNA-Seq.

The nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean "is a"; a gross dashed arrow means "two significant nodes" and a thin dashed arrow denotes "one significant nodes". Here, it is only represented the groups more significantly up-regulated. They correspond to five different groups, pointed with blue arrows. Number 1 is pointing the group of genes related with JA biosynthesis process; the number 2 points a group of genes related with response to JA stimulus; the number 3 belongs to the group of genes to water deprivation; number 4 denotes the group of flavonoid biosynthetic process and number 5 the genes related with glucosinolate biosynthetic process.

Down-regulated genes in *ami1-2*+IAM RNA-Seq

Figure S. 22 GO hieratical image containing all statistically significant terms for biological process of down-regulated *ami1-2*+IAM genes.

These nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean "is a"; red arrows denote "positive regulate"; green arrows indicate "negative regulate"; a gross dashed arrow means "two significant nodes" and a thin dashed black arrow denotes "one significant nodes". The groups of genes appearing to be more significantly down regulated, correspond with genes from "response to auxin stimulus", "innate immune response" and "apoptosis".

Table S. 8 Representation of genes up- and down-regulated in *ami1-2*+IAM's RNA-Seq.

These tables show the more overrepresented genes in the RNA-Seq for *ami1-2*+IAM plants. These genes present a FDR ≤ 0.001 and a Fold Change > 2 .

Up-regulated <i>ami1-2</i> +IAM's RNA-Seq	
JA Biosynthesis Process	
Gene	
LOX2 (Lipoxygenase 2)	AT3G45140.1
AOC1 (Allene Oxide Cyclase 1)	AT3G25760.1
AOC2 (Allene Oxide Cyclase 2)	AT3G25770.1
ACX1 (Acyl-CoA Oxidase 1)	AT4G16760.1
DAD1 (Defective Anther Dehiscence 1)	AT2G44810.1
Response to JA Stimulus	
Gene	
MYB21 (Arabidopsis thaliana MYB Domain protein 21)	AT3G27810.1
MYB29 (Arabidopsis thaliana MYB Domain Protein 29)	AT5G07690.1
MYB34 (MYB Domain Protein 34)	AT5G60890.1
MYB47 (MYB Domain Protein 47)	AT1G18710.1
MYB74 (MYB Domain Protein 74)	AT4G05100.1
MYB94 (MYB Domain Protein 94)	AT3G47600.1
MYB108 (MYB Domain Protein 108)	AT3G06490.1
MYB Family Transcription Factor	AT3G09600.1
LOX2 (Lipoxygenase 2)	AT3G45140.1
CCA1 (Circadian Clock Associated 1)	AT2G46830.1
SQE3 (Squalene Epoxidase 3)	AT4G37760.1
VTC2 (Vitamin C Defective 2)	AT4G26850.1
RVE2 (Reveille 2)	AT5G37260.1
TPS04 (Terpene Synthase 04)	AT1G61120.1
LHY (Late Elongated Hypocotyl)	AT1G01060.2
VTC5 (Vitamin C Defective 5)	AT5G55120.1
PAP1 (Production of Anthocyanin Pigment 1)	AT1G56650.1
AtPRB1 (Pathogenesis Related Protein)	AT2G14580.1
LDOX (Leucoanthocyanidin Dioxygenase)	AT4G22880.1
RNS1 (Ribonuclease 1)	AT2G02990.1
FAD-Binding Domain-Containing Protein	AT2G34810.1
TT4 (Transparent Testa 4)	AT5G13930.1
COR13 (Coronatine Induced 1)	AT4G23600.1

Up-regulated <i>ami1-2</i> +IAM's RNA-Seq	
Response to H ₂ O Deprivation	
Gene	
ANAC019 (Arabidopsis NAC Domain Containing Protein 19)	AT1G52890.1
LTI30 (Low Temperature-Induced 30)	AT3G50970.1
LTI65 (Low-Temperature-Induced 65)	AT5G52300.1
KIN1 (Cold and ABA Inducible Protein)	AT5G15960.1
KIN2 (Cold Responsive 6.6)	AT5G15970.1
ABI2 (ABA Insensitive 2)	AT5G57050.1
RAB18 (Responsive to ABA 18)	AT5G66400.1
ABA-Responsive Protein-Related	AT3G02480.1
RD20 (Responsive to Dessication 20)	AT2G33380.1
RD28 (Responsive to Desiccation 28)	AT2G37180.1
PIP2A (Plasma Membrane Intrinsic Protein 2A)	AT3G53420.1
AtHVA22D (Specific ABA- and Stress-Inducible gene)	AT4G24960.1
AOC1 (Allene Oxide Cyclase 1)	AT3G25760.1
LOX2 (Lipoxygenase 2)	AT3G45140.1
AtHB-7 (Arabidopsis thaliana Homeobox 7)	AT2G46680.1
PIP2B (Plasma Membrane Intrinsic Protein 2)	AT2G37170.1
PIP1;4 (Plasma Membrane Intrinsic Protein 1;4)	AT4G00430.1
PP2C (Putative Protein Phosphatase 2C)	AT5G59220.1
AtMRP4 (Arabidopsis thaliana Multidrug Resistance-Associated Protein 4)	AT2G47800.1
BHLH100 (Basic Helix-Loop-Helix Protein 100)	AT2G41240.1
AtHB-12 (Arabidopsis thaliana Homeobox 12)	AT3G61890.1
ALDH7B4 (Aldehyde Dehydrogenase 7B4)	AT1G54100.1
RD22 (Nutrient Reservoir Responsive to Dehydration 22)	AT5G25610.1

Up-regulated <i>ami1-2</i> +IAM's RNA-Seq	
Flavonoid Biosynthesis Process	
Gene	
UGT73C6 (UDP-Glucosyl Transferase 73C6)	AT2G36790.1
At5MAT (O-malonyltransferase/ transferase)	AT3G29590.1
Chalcone-Flavanone Isomerase Family Protein	AT5G05270.1
TT4 (Transparent Testa 4)	AT5G13930.1
TT5 (Transparent Testa 5)	AT3G55120.1
TT7 (Transparent Testa 7)	AT5G07990.1
F3H (Flavanone 3-Hydroxylase)	AT3G51240.1
PAP1 (Production of Anthocyanin Pigment 1)	AT1G56650.1
LDOX (Leucoanthocyanidin Dioxygenase)	AT4G22880.1
RNS1 (Ribonuclease 1)	AT2G02990.1
DFR (Dihydroflavonol 4-Reductase)	AT5G42800.1
Glucosinolate Biosynthesis Process	
Gene	
FMO GS-OX1 (Flavin-Monooxygenase Glucosinolate S-Oxygenase 1)	AT1G65860.1
FMO GS-OX2 (Flavin-Monooxygenase Glucosinolate S-Oxygenase 2)	AT1G62540.1
FMO GS-OX3 (Flavin-Monooxygenase Glucosinolate S-Oxygenase 3)	AT1G62560.1
FMO GS-OX4 (Flavin-Monooxygenase Glucosinolate S-Oxygenase 4)	AT1G62570.1
CYP79B3 (Cytochrome P450 79B3)	AT2G22330.1
CYP79F1 (Cytochrome P450 79F1)	AT1G16410.1
CYP79F2 (Cytochrome P450 79F2)	AT1G16400.1
MYB29 (MYB Domain Protein 29)	AT5G07690.1
MYB34 (MYB Domain Protein 34)	AT5G60890.1
BCAT4 (Branched-Chain Aminotransferase 4)	AT3G19710.1
SOT17 (Sulfotransferase 17)	AT1G18590.1
AKN2 (APS-Kinase 2)	AT4G39940.1

Down-regulated <i>ami1-2</i> +IAM's RNA-Seq	
Innate Immune Response	
Gene	
Disease Resistance Protein (TIR-NBS-LRR class)	AT5G22690.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT5G46510.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT1G56540.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT4G19520.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT1G66090.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT5G41750.2
Disease Resistance Protein (TIR-NBS-LRR class)	AT4G16960.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT4G19530.1
Transmembrane Receptor	AT2G32140.1
Transmembrane receptor	AT2G20142.1
Transmembrane Receptor ATP Binding	AT4G16940.1
PMR6 (Powdery Mildew Resistant 6)	AT3G54920.1
PR1 (Pathogenesis-Related Gene 1)	AT2G14610.1
BGL2 (Beta-1,3-Glucanase 2)	AT3G57260.1
CCR2 (Cold Circadian Rhythm, and RNA Binding 2)	AT2G21660.1
ACD6 (Accelerated Cell Death 6)	AT4G14400.1
Response to Auxin Stimulus	
Gene	
Auxin-Responsive Protein	AT1G29430.1
Auxin-Responsive Protein	AT5G18080.1
Auxin-Responsive Protein	AT2G18010.1
Auxin-Responsive Protein	AT3G03850.1
Auxin-Responsive Protein	AT5G18030.1
Auxin-Responsive Protein	AT1G29500.1
Auxin-Responsive Protein	AT4G34770.1
IAA1 (Indole-3-Acetic Acid Inducible 1)	AT4G14560.1
IAA5 (Indole-3-Acetic Acid Inducible 5)	AT1G15580.1
IAA19 (Indole-3-Acetic Acid Inducible19)	AT3G15540.1
IAA29 (Indole-3-Acetic Acid Inducible 29)	AT4G32280.1
SAUR68 (Small Auxin Upregulated 68)	AT1G29510.1
HAT2 (Transcription Factor)	AT5G47370.1
TTL3 (Tetratricopeptide-Repeat Thioredoxin-Like 3)	AT2G42580.1
EXGT-A1 (Endoxyloglucan Transferase)	AT2G06850.1
ACS4 (1-Aminocyclopropane-1-Carboxylate Synthase 4)	AT2G22810.1

Down-regulated <i>ami1-2</i> +IAM's RNA-Seq	
Death	
Gene	
Disease Resistance Protein (TIR-NBS-LRR class)	AT5G22690.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT5G46510.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT1G56540.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT4G19520.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT5G66900.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT1G66090.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT4G16960.1
Disease Resistance Protein (TIR-NBS-LRR class)	AT5G41750.2
Disease Resistance Protein (CC-NBS-LRR class)	AT1G15890.1
Transmembrane Receptor ATP Binding	AT4G16940.1
ACD6 (Accelerated Cell Death 6)	AT4G14400.1

Up-regulated genes in AMI1ind+IAM RNA-Seq

Figure S. 23 GO hieratical image containing all statistically significant terms for biological process of up-regulated AMI1ind+IAM genes.

These nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean "is a"; red arrows denote "positive regulate"; green arrows indicate "negative regulate"; a gross dashed arrow means "two significant nodes" and a thin dashed black arrow denotes "one significant nodes". The groups of genes that are more significantly up-regulated correspond to genes related with temperature stresses.

Down-regulated genes in AMI1ind+IAM RNA-Seq

Figure S. 24 GO hieratical image containing all statistically significant terms for biological process of down-regulated AMI1ind+IAM genes.

These nodes in the image are classified into ten levels, which are associated with corresponding specific colours. The node's colour is darker and redder the more significant statistically. Inside the box of the significant terms, the information includes: GO term, adjusted p-value, GO description, item number mapping the GO in the query list and background, and total number of query list and background. But when those term whose adjusted p-value is higher than the cut off set by the user, only GO information will be given in the box. Black arrows mean “is a”; red arrows denote “positive regulate”; green arrows indicate “negative regulate”; a gross dashed arrow means “two significant nodes” and a thin dashed black arrow denotes “one significant nodes”. The groups of genes that are more significantly down-regulated correspond to genes from flavonoid biosynthetic process.

ANNEX

Table S. 9 Representation of genes up- and down-regulated in AMI1ind+IAM's RNA-Seq.

These two tables show the more overrepresented genes in the RNA-Seq for AMI1ind+IAM plants. These genes present a FDR ≤ 0.001 and a Fold Change > 2 .

Up-regulated AMI1ind+IAM's RNA-Seq	
Heat Acclimation	
Gene	
AtFP6 (Farnelysated Protein 6)	AT4G38580.1
AP2 (Domain-Containing Transcription Factor)	AT2G40340.1
HsP18.2 (Heat Shock Protein 18.2)	AT5G59720.1
COR47 (Cold-Regulated 47)	AT1G20440.1
SKP2B (Ubiquitin-Protein Ligase AtSKP2)	AT1G77000.1
DREB2B (DRE/CRT-Binding Protein 2B)	AT3G11020.1
Cold Acclimation	
Gene	
COR15A (Cold-Regulated 15A)	AT2G42540.2
COR413-PM1 (Similar to the cold acclimation protein WCOR413 in wheat)	AT2G15970.1
COR414-TM1 (Similar to the cold acclimation protein WCOR413 in wheat)	AT1G29395.1
COR47 (Cold-Regulated 47)	AT1G20440.1
LT130 (Low Temperature-Induced 30)	AT3G50970.1
KIN1 (Cold and ABA Inducible Protein)	AT5G15960.1
DREB1A (Dehydration Response Element B1A)	AT4G25480.1
ERD10 (Early Responsive to Dehydration 10)	AT1G20450.1
RAB18 (Responsive to ABA 18)	AT5G66400.1
CBF1 (C-REPEAT/DRE Binding Factor 1)	AT4G25490.1

Down-regulated AMI1ind+IAM's RNA-Seq	
Flavonoid biosynthesis Process	
Gene	
Chalcone-Flavanone Isomerase	AT5G05270.1
F3H (Flavanone 3-Hydroxylase)	AT3G51240.1
TT4 (Transparent Testa 4)	AT5G13930.1
TT7 (Transparent Testa 7)	AT5G07990.1
UGT78D1 (UDP-Glucosyl Transferase 78D1)	AT1G30530.1
At5MAT (O-Malonyltransferase)	AT3G29590.1
LDOX (Leucoanthocyanidin Dioxygenase)	AT4G22880.1
FLS5 (Flavonol Synthase 5)	AT5G63600.2

Table S. 10 Groups of up-regulated genes obtained by Venn diagram from the RNA-Seq data of Wild type + IAM, *ami1-2* + IAM, AMI1ind + IAM.

In parenthesis is set the number of common genes between the different lines of plants.

Common Up-regulated Genes in RNA-Seqs with IAM treatment			
Wt, <i>ami1-2</i> , AMI1ind (16)	Wt, <i>ami1-2</i> (20)	Wt, AMI1ind (11)	<i>ami1-2</i> , AMI1ind (81)
AT2G42540.2	AT3G27250.1	AT5G44210.1	AT3G01345.1 AT4G35190.1
AT5G10410.1	AT1G14250.1	AT2G42530.1	AT1G53480.1 AT3G04000.1
AT1G09350.1	AT2G15020.1	AT1G70640.1	AT5G54585.1 AT2G39030.1
AT4G21440.1	AT1G60590.1	AT1G73510.1	AT1G51780.1 AT1G62710.1
AT4G05100.1	AT3G28220.1	AT2G17830.1	AT1G52690.1 AT5G64230.1
AT5G59220.1	AT2G36590.1	AT3G20395.1	AT5G24080.1 AT5G57050.1
AT3G25770.1	AT5G63450.1	AT3G56090.1	AT1G04220.1 AT3G29575.1
AT3G55500.1	AT1G52000.1	AT1G29395.1	AT2G28190.1 AT1G17940.1
AT5G45950.1	AT3G55130.1	AT4G15550.1	AT1G69480.1 AT2G32510.1
AT1G53470.1	AT5G24420.1	AT5G52310.1	AT1G05680.1 AT1G10370.1
AT4G33930.1	AT1G18710.1	AT4G33905.1	AT4G37990.1 AT4G26080.1
AT1G56600.1	AT1G02820.1		AT2G37770.2 AT5G67620.1
AT1G05100.1	AT5G52400.1		AT1G73390.3 AT4G22530.1
AT2G39980.1	AT5G54300.1		AT5G50360.1 AT5G39050.1
AT1G21790.1	AT1G61120.1		AT2G33380.1 AT2G47780.1
AT2G36750.1	AT5G40390.1		AT1G48100.1 AT3G28007.1
	AT3G21670.1		AT2G36770.1 AT5G24570.1
	AT1G73480.1		AT2G46370.4 AT1G69260.1
	AT1G68570.1		AT3G48240.1 AT3G24420.1
	AT1G01030.1		AT5G37300.1 AT4G01870.1
			AT3G17130.1 AT1G02205.3
			AT2G46680.1 AT5G19440.1
			AT2G45570.1 AT2G43520.1
			AT1G07430.1 AT1G54100.1
			AT3G46670.1 AT3G04010.1
			AT5G06760.1 AT3G50970.1
			AT4G33467.1 AT1G10585.1
			AT2G36780.1 AT4G34230.1
			AT3G14810.1 AT1G09530.1
			AT2G34810.1 AT5G66400.1
			AT3G02010.1 AT1G10060.2
			AT1G04570.1 AT4G14060.1
			AT5G47330.1 AT3G19270.1
			AT1G52890.1 AT4G35160.1
			AT3G56260.2 AT5G15970.1
			AT3G15670.1 AT2G43820.1
			AT4G22620.1 AT2G16630.1
			AT2G22200.1 AT4G24960.1
			AT4G23600.1 AT2G02990.1
			AT4G10960.1 AT5G15960.1
			AT1G53490.1

ANNEX

Table S. 11 Groups of down-regulated genes obtained by Venn diagram from the RNA-Seq data of Wild type + IAM, *ami1-2* + IAM, AMI1ind + IAM.

In parenthesis is set the number of common genes between the different lines of plants.

Common Down-regulated Genes in RNA-Seqs with IAM treatment			
Wt, <i>ami1-2</i> , AMI1ind (3)	Wt, <i>ami1-2</i> (14)	Wt, AMI1ind(6)	<i>ami1-2</i> ,AMI1ind (22)
AT1G29500.1	AT1G15580.1	AT4G36700.1	AT3G44350.2
AT3G07195.1	AT5G18080.1	AT5G39580.1	AT3G57260.1
AT5G28630.1	AT4G14560.1	AT2G36690.1	AT3G03660.2
	AT1G10550.1	AT4G08040.1	AT2G14610.1
	AT5G52760.1	AT2G44370.1	AT2G22810.1
	AT5G03120.1	AT1G23730.1	AT3G23800.1
	AT1G74440.1		AT1G15890.1
	AT1G15125.1		AT1G68870.1
	AT5G14920.1		AT5G06930.1
	AT5G65390.1		AT1G35710.1
	AT5G10430.1		AT5G10760.1
	AT4G18205.1		AT4G38560.2
	AT1G26945.1		AT4G00970.1
	AT4G23220.1		AT1G30135.1
			AT5G47370.1
			AT2G31110.2
			AT4G25020.1
			AT3G54790.1
			AT1G10340.1
			AT1G12080.2
			AT2G07680.1
			AT4G23180.1

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