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**IN-DEPTH ANALYSIS OF JASMONATE-MEDIATED
INDOLE-3-ACETIC ACID (IAA) BIOSYNTHESIS AND ITS
IMPLICATION IN PLANT DEVELOPMENT**

TESIS DOCTORAL

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“Jede wirkliche Geschichte ist eine
unendliche Geschichte”

Michael Ende “*Die unendliche Geschichte*”

“...Nobody said it was easy
It's such a shame for us to part
Nobody said it was easy
No one ever said it would be this hard
Oh take me back to the start

I was just guessing
At numbers and figures
Pulling the puzzles apart

Questions of science
Science and progress
Do not speak as loud as my heart...”

Coldplay “*The Scientist*”

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Summary

Indole-3-acetic acid (IAA), the main naturally occurring auxin in plants, is an essential plant hormone that has been associated with the regulation of many aspects of plant development and growth. However, despite auxin biology is one of the classical fields of plant research, its biosynthesis, spatio-temporal regulation, molecular action and the crosstalk with other hormones remain uncertain. Actually, although multiple pathways are proposed for auxin biosynthesis, many previous genetic and biochemical studies revealed that the indole-3-pyruvic acid (IPA) pathway is the main source of IAA in plants. This pathway involves the action of two classes of enzymes, tryptophan-pyruvate aminotransferases (TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1)/TRYPTOPHAN AMINOTRANSFERASE RELATED (TAR)) and flavin monooxygenases (YUCCA). Among these families, the presence of the *YUCCA* genes in multiple plant genomes point out an essential role in biosynthesis and plant development. Current evidences indicate that exogenous application of methyl jasmonate (MeJA) or endogenous production of jasmonic acid (JA), a hormone with a prevalent role in plant defense to necrotrophic pathogens and insect herbivores, is sufficient to trigger IAA biosynthesis through the up-regulation of *YUCCA8* and *YUCCA9*.

The work described in this thesis aims at the elucidation of the molecular auxin action by in-depth analysis of 35S::*YUCCA8* (YUC8ox) and 35S::*YUCCA9* (YUC9ox) *Arabidopsis* transgenic lines, which overproduce IAA. To this end we carried out histochemical analyses and quantitative lignification assays. These analyses allow us to finally determine that the overproduction of auxin cause an abnormal secondary growth, mainly characterized by a stimulated xylem and interfascicular fibers differentiation, as well as increased lignification, which is enriched in H-lignin deposition. Moreover, whole-genome transcriptomic analysis comparing YUC8ox and YUC9ox to wild-type Col-0 plants, revealed a cluster of up-regulated genes capable of modifying cell wall properties. Among those a group of *XYLOGLUCAN ENDOTRANSGLYCOSYLASE/HYDROLASE (XTH)* genes peaked out. Furthermore, when we analyzed the chemotype of the overexpression lines, we discovered significantly increased levels of ethylene (ET), a gaseous plant hormone with a prominent role in cell division. In conclusion, the results presented here highlight the importance of the hormonal cascade JA-IAA-ET in plant development. We propose a mechanistic model in which the activation of IAA production mediated by JA-induced *YUCCA8* and *YUCCA9* enzymes, stimulate or inactivate the production of ET to regulate vascular cell division or epidermal cell expansion in a

tissue-specific manner. In addition, considering the observed down-regulation of different chitin and water deficit responses genes in the studied YUC8/9ox lines, we determine that the JA-IAA crosstalk is an on-off “switch” mechanism to fine-tune the balance between growth and defense processes.

The physiological aspects underlying the IAA-ET induced secondary growth and cell expansion have been also unveiled in this thesis. Our results show that YUCCA8 and YUCCA9 mediate a positive defense response against the herbivore *Tetranychus urticae*, as well as, an improved drought tolerance for YUC9ox, thereby underlining a role of JA-IAA-ET in plant response.

Finally, this thesis strives to shed light on the molecular relationship that share these two seemingly antagonistic hormones, jasmonic acid and auxin. Herein, we analyzed different mutants defective in known jasmonate-induced transcriptional factors. Our transient expression analysis in *Nicotiana benthamiana* and *Arabidopsis* protoplasts clearly demonstrate that MYC2, as well as its closest homologs MYC3 and MYC4, function as a transcriptional regulators of YUCCA8 and YUCCA9 through its direct binding to different G-boxes located at the promoter of the mentioned YUCCA genes. This study provides for the first time a molecular proof that links jasmonic acid signaling with auxin biosynthesis.

Resumen

El ácido-3-indol-acético (AIA) es la auxina principal presente en las plantas. Esta hormona vegetal regula diversos procesos fisiológicos, siendo esencial para el correcto crecimiento y desarrollo de las plantas. Sin embargo, a pesar de que el estudio de esta auxina es uno de los campos de estudio más antiguos en biología vegetal, algunos aspectos de su biosíntesis, regulación espacio-temporal, mecanismos moleculares de acción, así como la interacción con otras hormonas, está aún por determinarse. En la actualidad, aunque se han descrito diversas rutas para la biosíntesis del AIA, el desarrollo de numerosos estudios bioquímicos y genéticos ha permitido determinar que la ruta del indol-3-piruvato (IPA) es la principal fuente de producción de AIA en plantas. La biosíntesis de AIA por la ruta metabólica del IPA comprende la acción consecutiva de dos clases de enzimas, las triptófano-piruvato aminotransferasas (TRIPTOFANO AMINOTRANSFERASA DE ARABIDOPSIS1 (TAA1)/TRIPTOFANO AMINOTRANSFERAS RELACIONADAS (TAR)) y las monooxigenasas dependientes de flavina (YUCCA). Entre estas familias, los genes *YUCCA* muestran una amplia distribución, siendo posible encontrarlos en diferentes genomas de plantas. Este hecho remarca el importante papel que dichos genes desempeñan, no sólo en lo relativo a la biosíntesis de la AIA, sino también en el desarrollo vegetal. Por otro lado, estudios recientes indican que la aplicación exógena de metil jasmonato (MeJA) o la producción endógena de ácido jasmónico (JA), una fitohormona implicada en la regulación de la respuesta de defensa frente a patógenos necrótrofos e insectos herbívoros, es suficiente para estimular la producción de AIA a través de la activación transcripcional de los genes *YUCCA8* y *YUCCA9*.

El trabajo descrito en esta tesis tiene como objetivo elucidar los mecanismos moleculares de acción de la auxina mediante la caracterización detallada de las líneas transgénicas de *Arabidopsis 35S::YUCCA8* (YUC8ox) y *35S::YUCCA9* (YUC9ox), las cuales son sobreproductoras de AIA. Para lograr el objetivo planteado, se han realizado análisis histoquímicos, así como ensayos de cuantificación de la lignina. En concreto, se ha demostrado que un aumento exagerado de la producción de auxina da lugar a un crecimiento secundario anormal, en el cual se ha observado tanto una diferenciación del xilema y fibras interfasciculares, así como una lignificación, ambas altamente estimuladas. Igualmente, la caracterización de la composición de la lignina ha permitido determinar la alta deposición de unidades de lignina tipo H. Además, el desarrollo de microarrays comparando plantas Col-0 (silvestre) con las líneas transgénicas YUC8ox y YUC9ox, ha permitido revelar un

incremento en la expresión en un grupo de genes implicados en la regulación de procesos asociados con la modificación de la pared celular de las plantas. Entre los genes encontrados, los conocidos como XILOGLUCANO ENDOTRANSGLICOSIDASA/HIDROLASA (XTH) fueron particularmente relevantes. Así mismo, se ha corroborado que las plantas YUC8ox y YUC9ox presentan un incremento en los niveles de etileno (ET), una hormona gaseosa implicada en la regulación de la división celular. En conclusión, los datos presentados en este trabajo demuestran la importancia de la cascada hormonal JA-AIA-ET en el desarrollo de las plantas y desvelan un modelo de acción en el cual la activación de las enzimas YUCCA8 y YUCCA9 por JA estimula la producción de AIA, lo cual induce o inhibe la biosíntesis de etileno en determinados tejidos para regular la división celular en el cambium o la expansión celular en la epidermis. Además de esto, considerando la inactivación de la expresión en genes de respuesta a quitina y sequía, el presente estudio permite determinar que la interacción establecida entre JA y AIA actúa como mecanismo interruptor “encendido-apagado” en el balance entre crecimiento y defensa.

Por otro lado, el trabajo de investigación aquí presentado ha estudiado los aspectos fisiológicos subyacentes al crecimiento secundario estimulado por la acción conjunta de IAA-ET. Se ha observado que tanto YUCCA8 como YUCCA9 confieren resistencia frente al ácaro herbívoro *Tetranychus urticae*, igualmente se ha visto que las plantas YUC9ox presentan mayor tolerancia a estrés por sequía, de esta manera estos resultados demuestran el papel esencial que desempeña la triple interacción JA-AIA-ET en la respuesta de las plantas a estrés.

Finalmente, el mecanismo molecular por el cual interaccionan estas dos hormonas aparentemente antagonistas, ácido jasmónico y auxina, ha sido también dilucidado durante la realización de este estudio. Con este fin, se han analizado diferentes mutantes defectivos en varios factores de transcripción dependientes de jasmonato. Mediante ensayos de expresión transitoria en *Nicotiana benthamiana* y en protoplastos de *Arabidopsis*, se han confirmado que el factor de transcripción MYC2, así como sus dos homólogos más cercanos MYC3 y MYC4, actúan como reguladores transcripcionales de YUCCA8 y YUCCA9 a través de su unión directa a diferentes cajas G, localizadas en el promotor de los genes YUCCA mencionados. La presente tesis doctoral aporta por primera vez una prueba molecular que une la señalización del ácido jasmónico con la biosíntesis de auxinas.

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LIST OF ABBREVIATIONS

In addition to the standard units of the "International System of Units" (SI) and their prefixes for multiples or fractions and, also, to common abbreviations for chemical elements and compounds set forth in the rules of the "International Union for Pure and Applied Chemistry" (IUPAC), we highlight the following list of abbreviations used in this work:

2,4-D	2,4-dichlorophenoxyacetic acid
4-MU	4-methylumbelliferone
4-MUG	4-methylumbelliferyl- β -D-glucuronide
5-MT	5-methyltryptophan, phytotoxic Trp analog
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i> (L.) HEYNH.
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i> C58C1
ABA	Absciscic acid
ABCB	ABCB/MDR/PGP (ABC-B/MULTI DRUG RESISTANCE/P-glycoproteins)
ABP1	AUXIN BINDING PROTEIN
AIR	alcohol-insoluble residues
Amp, Amp ^r	Ampicillin, Ampicillin resistance
<i>APT1</i>	Adenine Phosphoribosyl Transferase 1 (At1g27450)
attL/R	DNA attachment sites for homologous recombination
AUX1/LAX	AUXIN1/LAX
AuxRE	auxin response element
<i>axr1</i>	<i>auxin resistant 1</i> loss-of-function mutant
β -MeEtO	β -Mercaptoethanol
bp	Base pairs
BSTFA	N,O-Bis (trimethylsilyl) trifluoroacetamide
CaMV (35S)	35S promoter from the cauliflower mosaic virus
cDNA	DNA complementary to the mRNA
COI1	CORONATINE-INSENSITIVE 1
Col-0	Ecotype Columbia-0 (<i>A. thaliana</i>)
Da	Dalton (1 Da = $1,66 \cdot 10^{-27}$ kg)
DAB	3,3-Diaminobenzidine reagent
DMSO	Di-methyl-sulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxynucleoside triphosphate
DR5	Auxin synthetic promoter
DTT	1,4-Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i> DH5 α /DB3.1
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERF	ETHYLENE RESPONSIVE FACTOR

ET	Ethylene
FMO	Flavin monooxygenase
FW	Fresh weight
GC-MS/MS	Gas chromatography-Mass spectrometry
G-Lignin	Coniferyl alcohol
GUS	β -glucuronidase
h	Hour, time
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEYNH.	HEYNHOLD, GUSTAV
H-Lignin	<i>p</i> -coumaryl alcohol
IAA	Indole-3-acetic acid
IAAld	Indole-3-acetaldehyde
IAM	Indole-3-acetamide
IAN	Indole-3-acetonitrile
IAOx	Indole-3-acetaldoxime
IBA	Indole-3-butyric acid
IF	Interfascicular fibers
IPTG	Isopropyl- β -D-thiogalactopyranoside
IPyA	Indole-3-pyruvic acid
JA	Jasmonic acid
JA-Ile	(+)-7- <i>iso</i> -Jasmonoyl-L-Isoleucine
JAs	Jasmonates
Kan, Kan ^r	Kanamycin, Kanamycin resistance
KOCH	KOCH, CARL LUDWIG
L.	LINNÉ, CARL VON
LB	Luria & Bertani, bacterial nutrient medium
m/z	Mass-to-charge ratio
MALDI-TOF/MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MeJA	Methyl jasmonate
MeOH	Methanol
MES	2-(N-morpholino)ethanesulfonic acid
min	Minute, time
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger-RNA, here also Poly(A) ⁺ -mRNA
MS	Murashige & Skoog medium
<i>N. benthamiana</i>	<i>Nicotiana benthamiana</i> L.
NAA	1-naphthaleneacetic acid
NPA	1-naphthylphthalamic acid
OD ₆₀₀	Optical density at a wavelength of 600 nm
OLIMP	OLigo Mass Profiling
OPDA	cis (+)-12-oxophytodienoic acid
ox	Overexpression
<i>p</i>	Value of the statistical significance test
<i>P. syringae</i>	<i>Pseudomonas syringae</i>
PAA	Phenylacetic acid
PAT	Polar auxin transport

PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIN	PIN-FORMED auxin transporter
<i>Pst</i> DC3000	<i>Pseudomonas syringae pv tomato</i> DC 3000
qRT-PCR	quantitative real-time polymerase chain reaction
Rif	Rifampicin
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature
RWC	Relative water content
SA	Salicylic acid
SDS	Sodium dodecyl sulphate
SE	Standard error of the mean
S-Lignin	Sinapyl alcohol
<i>T. urticae</i>	<i>Tetranychus urticae</i> KOCH
TAAR1/TAR	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 / TRYPTOPH AMINOTRANSFERASE RELATED
TAE	Tris-acetate EDTA
TAM	Tryptamine
TBS	Tris buffered saline
T-DNA	Transfer-DNA
T-DNA LB, RB	Insertion sequences of the T-DNA (left/right border)
TMCS	trimethylchlorosilane
Tris	Tris-(hydroxymethyl)-aminoethane
Triton X	Octylphenol polyethylene glycol ether
Tween	Polyoxyethylene sorbitan monolaurate
<i>UBI10</i>	Ubiquitin 10 (At4g05320)
<i>uidA</i>	Gene sequence of β -glucuronidase (GUS)
UTR	Untranslated region
UV	Ultraviolet
v/v	volume/volume
w/v	weight/volume
WT	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
X-GlucA	Cyclohexylammonium salt of 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid
XTH	XYLOGLUCAN ENDOTRANSGLYCOSYLASE/HYDROLASE
Xy	Xylem
YUC8	Member of the YUC family in <i>A. thaliana</i> (CAA22980; 48,1 kDa; <i>AtYUC8</i> ; At4g28720)
YUC9	Member of the YUC family in <i>A. thaliana</i> (AAC16744; 47,4 kDa; <i>AtYUC9</i> ; At1g04180)

CHAPTER 1:

INTRODUCTION

1.1 Auxin: An overview of its action and regulatory mechanisms

The heterogeneity of the environment exerts a potential selective force on every living organism. This is particularly remarkable in plants, where the sessile lifestyle determines the need of driving continuously rapid responses to external stimuli. Such adaptive plasticity, conferred by the permanent activity of meristematic tissues, involves both physiological processes and morphological adaptations (Depuydt & Hardtke 2011). Thus, during the course of evolution, plants have evolved a whole battery of bioactive signaling molecules in order to regulate their growth and development (Hoffmann et al. 2011; Pozo et al. 2015).

The term ‘hormone’ refers to chemical molecules that at very low concentration exert their function either locally, at or near the site of biosynthesis, or in distant organs or tissues (Santner et al. 2009). The existence of these substances was firstly observed in animals and it was the mammalian physiologist Starling (1905) who coined the terminology from the Greek “ὁρμᾶν” (*horman*), or “to stimulate” in English (Weyers & Paterson 2001). In botany, Francis and Charles Darwin firstly introduced the idea of a transmissible influence migrating between one part of the plant to another in response to light and gravity, in their book *The Power of Movements in Plants* (1880). They empirically demonstrated that after exposing the tip of an etiolated coleoptile to a phototropic stimulus, the lower part experimented differential growth and active bending movement. Posteriorly, Julius von Sachs (1887) described the correlation phenomenon, in which determinate “messengers” move across the plant body to control growth and development. However, H. Fitting (1909, 1910) was the first to use the word hormone to explain the induction of post-pollination responses in orchids (*Phanerosis amabilis*) after application of aqueous pollen extracts. Later on, in 1926, Frits Went isolated the transmissible messenger detailed by Darwin and Darwin (1880), from decapitated oat (*Avena sativa*) coleoptiles, by allowing the substance to diffuse into agar blocks, which were demonstrated to retain a growth promoting activity when placed on top of the decapitated coleoptile. After the determination of the chemical structure of indole-3-acetic acid (IAA) in 1935, Frits Went named this molecule «auxin», which derived from the Greek word “αὐξάνω” (*auxano*) meaning “to grow or to expand”, giving rise to the discovery of the first phytohormone (Thimann & Went 1934; Woodward & Bartel 2005; Teale et al. 2006; Kutschera & Niklas 2009; Abel & Theologis 2010; Enders & Strader 2015).

Actually, the molecule IAA is recognized as the principal auxin in plants, being ubiquitously distributed within this kingdom (Teale et al. 2006). Moreover, it has

been shown that IAA is also synthesized in bacteria (Spaepen & Vanderleyden 2011), fungi (Fu et al. 2015) and algae (Buggeln & Craigie, 1971). Nonetheless, the term auxins comprise not only IAA, but also a group of important structural related molecules that cause profound changes in plant growth and development (Bonner and Bandurski 1952) (Figure 1). For example, indole-3-butyric acid (IBA) is an auxin closely related to IAA, which has been demonstrated to be important in root initiation (Zimmerman & Wilcoxon 1935; Epstein & Ludwig-Müller, 1993). Another indolic auxin, the halogenated 4-chloroindole-3-acetic acid (4-Cl-IAA), has been mainly isolated in diverse members of the family *Leguminosae* (*Fabaceae*) and pine seeds, and its function has been associated with pea pod (*Pisum sativum*) development (Normanly et al. 1995; Normanly et al. 2010). Phenylacetic acid (PAA) is also a naturally occurring auxin species that can be found widespread in plants (Sánchez-Parra et al. 2014). Likewise, 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA), are two synthetic auxin derivatives with important economic value as herbicide or fruit growth enhancer, respectively (Bonner & Bandurski 1952; Ortolá et al. 1991; Teale et al. 2006).

Figure 1: Chemical structure of the naturally occurring and synthetic active auxins in plants (Teale et al. 2006; Korasick et al. 2013).

Endogenous IAA is associated with the regulation of numerous embryonic and post-embryonic processes, ranging from cell division, cell elongation, cell differentiation, tropisms, apical dominance, emergence of new organ primordia, initiation of lateral and adventitious root growth, senescence, leaf abscission, to flowering (Woodward

& Bartel 2005; Hentrich et al. 2013a;). This wide diversity of functions reflects the extraordinary importance of IAA as versatile modulator of the plant life cycle. However, despite auxin biology is one of the oldest fields of plant physiology, its homeostasis and metabolism remain in parts uncertain. In this respect, the emergence of *Arabidopsis thaliana* as model organism of choice in plant biology (Meinke et al. 1998; Koornneef & Meinke 2010), has contributed to provide new insights into the molecular and biochemical basis of IAA metabolism (biosynthesis, conjugation and degradation), transport and signaling.

In plants, IAA is mostly biosynthesized in the shoot apex and basipetally transported in a cell-to-cell manner towards the root (Domagalska & Leyser 2011). Apart of that, other organs, such as cotyledons, expanding leaves and primary and lateral root tips, are also reported to have the capacity to produce IAA *de novo* (Ljung et al. 2001; Ljung et al. 2005). The active basipetal transport of auxin is known as polar auxin transport (PAT), and results in a spatio-temporal auxin distribution required for the generation and maintenance of local auxin maxima and gradients (Petrášek & Friml 2009; Domagalska & Leyser 2011). Interestingly, the design of the synthetic promoter DR5, composed of multiple repeats of the TGTCTC auxin response element (AuxRE), facilitated the effective monitoring of auxin signaling, and thereby analysis of the role of PAT in plant development (Tanaka et al. 2006). For example, analysis of the DR5 activity has demonstrated that PAT is indispensable for establishing the embryo apical-basal axis, leaf vein patterning, primordia formation, phyllotaxis and root meristem patterning in *Arabidopsis* (Friml et al. 2003; Bainbridge et al. 2008).

Much of the cell-to-cell auxin movement, is based on the chemical characteristics of IAA. IAA is a weak organic acid composed of an indole ring structure coupled to a side chain harboring a terminal carboxyl group. Therefore, the chemiosmotic model explains that, as a consequence of the proton gradient generated by the proton pumps localized in the plasma membrane, in the acidic environment of the apoplast ($\text{pH} \approx 5.5$) the carboxyl group of IAA is protonated, making the molecule less polar ($\text{IAA}^- + \text{H}^+ \leftrightarrow \text{IAA-H}$). In this form it can passively diffuse across the membrane (Figure 2). However, in the cytosol, where the pH is less acidic ($\text{pH} \approx 7$), the IAA molecule exists in its deprotonated, negatively charged form (IAA^-), which prevents its free diffusion through the plasma membrane. As a result, the hydrophilic form remain trapped inside the cell and needs the aid of specific transporters or efflux carriers to escape (Tanaka et al. 2006; Ljung 2013). On the other hand, the canalization theory of Sachs (1969) postulated that cells experiencing diffusion of auxin in a certain direction will increase their capacity to transport the hormone,

leading to the distribution of auxin in channels (Petrášek & Friml 2009; van Berkel et al. 2013). Experiments with radioactive IAA in the *Arabidopsis* defective mutant *pin1*, demonstrated a reduced PAT in the inflorescence axis (Okada et al. 1991). In addition, the loss-of-function mutant *pin2* (also called, *ethylene insensitive root1*, *agravitropic1* or *wavy6*) fails in root gravitropism (Luschnig et al. 1998; Křeček et al. 2009). The observed results gave rise to the identification of the auxin transporters PIN-FORMED (PIN). This proteins belong to a family of auxin efflux carriers, which display asymmetric localization and distribution dynamics within the cell membrane and facilitate efficient and directional auxin export out of the cell (Tanaka et al. 2006; Křeček et al. 2009). In other words, in response to auxin, PIN transporters change their subcellular localization to direct the auxin flow and create auxin gradients to drive accumulation of auxin maxima in specific types of cells. So far, in addition to PIN1 and PIN2, another six PIN homologues have been described in *Arabidopsis*. Among them, PIN3, PIN4 and PIN7 are also localized at the plasma membrane, and are respectively involve in tropism, root meristem patterning and apical-basal polarity establishment in early embryogenesis (Tanaka et al. 2006). While PIN5, PIN6 and PIN8, together with some recently identified PIN-LIKES auxin efflux proteins, have been associated to the endoplasmic reticulum (ER) (Mravec et al. 2009; Dal Bosco et al. 2012; Simon et al. 2016). This indicates a possible auxin flow from the cytoplasm to the lumen of the ER, however, the physiological role of the intracellular IAA transport remains uncertain (Figure 2).

Other proteins that play a role in auxin efflux are the ABC-B/MULTI DRUG RESISTANCE/ P-glycoproteins (ABCB/MDR/PGP) (Tanaka et al. 2006) (Figure 2). These ATP-binding cassette (ABC) transporters are widely distributed in bacteria, yeast, plants and animals (Aryal et al. 2016). In *Arabidopsis*, the ABC subfamily B (ABCB) include 21 members grouped into three clusters (Geisler & Murphy 2006). Among the subfamily, ABCB1, ABCB4, ABCB14, ABCB15, ABCB19, and ABCB21 have been characterized to be involve in the directional transport of IAA and IAA derivatives (Kaneda et al. 2011; Cho & Cho 2013). However, unlike PIN proteins, these plasma membrane-associated proteins are mainly symmetrically distributed over the plasma membrane and do not display particular dynamics. This suggests that ABCB proteins function as basal auxin transporters and could be implicated in the regulation of the cellular auxin homeostasis (Cho & Cho 2013). Importantly, it has been observed that ABC4 and ABC21, which are expressed in the root epidermis and the abaxial side of cotyledons, respectively, can function either as efflux or influx carriers, depending on the cytoplasmic IAA concentration (Kamimoto et al. 2012).

On the other hand, the description of the T-DNA insertion knockout mutant *AUXIN1* (*AUX1*) in *Arabidopsis*, which is characterized by reduced sensitivity to the synthetic auxin 2,4-D and agravitropic response (Bennett et al. 1996), led to the discovery of a class of auxin influx carriers (Figure 2). This novel finding challenged the idea that auxin transport from the apoplast into the cytosol is only controlled by passive diffusion. At present, four conserved transporter genes have been identified in *Arabidopsis*, including *AUX1* and the *AUX1*-like genes *LAX1*, *LAX2* and *LAX3*, that share auxin import activity. Furthermore, it has been demonstrated that, alike the ABCB transporters, this family of proteins does not display a polar distribution. A recent study carried out by Fábregas et al. (2015) using the quadruple loss-of-function mutant *aux1lax1lax2lax3*, documented that the impaired vasculature observed was attenuated under long day conditions. The authors suggest that the active influx and passive diffusion mechanisms can be affected by the photoperiod. Therefore, the short day conditions would be relevant for protein-mediated IAA import, whereas the passive inward movement might function under long day conditions.

Apparently, auxin distribution is dynamically modified in response to both developmental and environmental cues. For example, the analysis of the *Arabidopsis aux1lax1lax2* mutant showed alteration in the divergence angle of leaf primordia at the shoot apical meristem. This observation provides evidence for the cooperation between PIN1 and the AUX1/LAX family to coordinate phyllotactic pattern maintenance (Bainbridge et al. 2008). Gray et al. (1998) reported that the auxin-induced hypocotyl expansion is increased at higher temperatures (Gray et al. 1998). Furthermore, it has been demonstrated that cell elongation in response to blue light is caused by the lateral reorganization of PIN1 and PIN3 (Keuskamp et al. 2010). In pea epicotyls, the radioactivity from applied ³H-labelled IAA is asymmetrically distributed in epidermal peels in response to light and gravity stimuli, promoting correctional bending (Haga & Iino 2006). Thus, it is crucial that cells sense and interpret changes in auxin allocation, in order to conduct an efficient regulation of the plant developmental programs. In this line, pioneer studies in the field of auxin response led to the identification of the *auxin resistant 1* (*axr1*) gene mutation in a reverse genetic analysis of several allelic *Arabidopsis* mutants. The AXR1 gene encodes a protein related to the N-terminus of the ubiquitin-activation enzyme (E1). The resulting loss-of-function mutant plant, *axr1*, does not only exhibit a pleiotropic phenotype, but also displays resistance to exogenously applied 2,4-D and severe defects in auxin response (Estelle & Somerville 1987).

Figure 2: A model of auxin transport across the plasma membrane (modified from Petrášek & Friml, 2009).

It can be recorded that the transport of auxin into the cell proceeds from the apoplast to the cytosol through passive diffusion and active influx, being the latter mediated by the AUXIN1/AUXIN-LIKE (AUX/LAXs) carriers. Efflux of auxin from the cell to the apoplast is mediated by the PIN-FORMED proteins (PINs) and the ABC-B/MULTI DRUG RESISTANCE/P-glycoproteins (ABCB/MDR/PGP), denoted in the scheme as ABCBs, and from the cytosol into the endoplasmic reticulum (ER) by the PIN. The direction of the polar auxin transport is also showed.

Currently, it is known that the primary response to an auxin signal is initiated in a rapid and specific manner, at very low physiological concentration of 10^{-8} to 10^{-6} M (Abel & Theologis 1996). Early auxin-mediated responses are characterized by alterations in gene expression. Among the regulated early genes three gene families are experimental shown to be significantly induced within just a few minutes, i.e. the SMALL AUXIN-UP RNA (SAUR), the Aux/INDOLEACETIC-3-ACID (Aux/IAA), and

the GRETCHENHAGEN-3 (GH3) genes (Teale et al. 2006). Indeed, the mRNA expression of the SAUR group, whose function is commonly associated with calcium responses and cell elongation, is induced by auxins within 2 to 5 min, even in presence of the protein synthesis inhibitor cycloheximide (CHX) (Abel & Theologis 1996; Li et al. 2015), suggesting that early primary auxin responses are independent of *de novo* protein synthesis.

On the other hand, auxin acts by means of triggering transcriptional cell reprogramming, which is mainly determined by the modulation of the two gene families *Aux/IAA* and *AUXIN RESPONSIVE FACTORS (ARFs)*, which consist of 29 and 23 members in *Arabidopsis*, respectively (Paponov et al. 2008). The aforementioned *Aux/IAA* genes, encode a family of transcriptional repressors that inhibit auxin signaling by recruiting the co-repressor TOPLESS (TPL) and directly binding to the ARF transcription factors (Szemenyei et al. 2008) (Figure 3). The ARF transcription factors, in turn, are able to repress or activate expression of auxin-responsive genes, through their binding to the auxin-responsive element motif (ARE: TGTCTC) in the promoters of downstream target genes. Interestingly, all *Aux/IAA* proteins share a conserved motif, also called degron, which directly engages with the TRANSPORT INHIBITOR RESISTANT 1 (TIR1)/AUXIN SIGNALING F-BOX 1 to 5 (AFB1-5) family of auxin receptor. TIR1/AFB1-5 are F-box components that, together with the CULLIN1 (CUL1), the ARABIDOPSIS S-PHASE KINASE-ASSOCIATED PROTEIN1 (ASK1) and the RING-BOX1 (RBX) proteins, forms the nuclear protein complex SCF^{TIR1/AFB}-Type E3 ubiquitin ligase (Ruegger et al. 1998; Teale et al. 2006; Shabek & Zheng 2014; Santner et al. 2009). Although, many questions regarding the auxin signaling are still unsolved, the discovery of the auxin receptors presented a tremendous advance in our understanding of the sophisticated auxin signal transduction cascade. It is known that at low IAA levels the TIR1/AFBs-Aux/IAA-ARF complex blocks IAA signaling, while increased concentrations of IAA promotes the interaction of IAA with TIR1/AFB1-5. The binding of IAA to the receptors triggers Aux/IAA ubiquitination at a specific lysine residue, followed by the subsequent degradation of the repressors via the 26S proteasome, thereby relieving the repression of ARFs and initiating the expression of IAA-responsive target genes (Santner & Estelle 2009; Hoffmann et al. 2011) (Figure 3).

As aforementioned TIR1 and others AFB F-box proteins are known to function as auxin receptors. Further *in vitro* evidence using extracts of maize coleoptiles (*Zea mays* L.), indicated that IAA and NAA were also perceived by another receptor located at the plasma membrane (Hertel et al. 1972). One decade later, the proposed receptor was purified and identified as AUXIN BINDING PROTEIN 1 (ABP1) (Löbner &

Klämbt 1985). Later, ABP1 was found to be expressed in the lumen of the ER and in the plasma membrane (Napier et al. 2002). The occurrence of ABP1 has been reported both for monocotyledonous and dicotyledonous plants, and it has been associated with early auxin responses (Robert et al. 2010). In fact, ABP1 apparently modulates cell elongation, which is known to be one of the fastest auxin responses with a lag period of 10-25 min (Abel & Theologis 2010). Besides this, David et al. (2007) reported a prominent role of ABP1 in the regulation of cell division events. In their work, the authors demonstrated that immunoblocking of ABP1 in tobacco BY2 cell cultures inhibited the G1/S1 progression during the cell cycle. In *Arabidopsis*, overexpression of ABP1 was shown to increase PIN internalization, which was further characterized to proceed via clathrin-mediated endocytosis (Robert et al. 2010).

Figure 3: SCF^{TIR1}-mediates auxin signaling in *Arabidopsis* (Santner et al. 2009; Hoffmann et al. 2011).

a) TRANSCRIPTION INHIBITOR RESPONSE (TIR1) and the AUXIN SIGNALING F-BOX (AFB), are F-box proteins that can function as auxin receptors. Focusing on TIR1 it is known that it forms part of the SCF protein complex, which comprises the ARABIDOPSIS S-PHASE KINASE PROTEIN 1 (ASK1), CULLIN1 (CUL1), and RING-BOX1 (RBX1). In presence of biological levels of IAA, the bioactive hormone is bound to the bottom of a cavity located at the surface of the TIR1 receptor. **b)** Through the binding of IAA, the affinity of TIR1 for the Aux/INDOLEACETIC-3-ACID (Aux/IAA) transcriptional repressors increases. Subsequently, the SCF E3 ligase mediates the ubiquitination of the bound Aux/IAA protein and their degradation via the 26S proteasome. **c)** The ubiquitin-mediated proteolysis of Aux/IAA results in the liberation of the co-repressor TOPLESS and the AUXIN RESPONSIVE FACTOR (ARFs), thus, permitting ARFs to promote transcription of the auxin responsive genes.

Along with the transport and perception of auxin, the cellular homeostasis of the active hormone plays an essential role. The physiologically active free IAA can be conjugated to sugars, *myo*-inositol, amino acids or proteins. The conjugation occurs via ester- or amide-linkages, mediated by the action of UDP-glucose transferases and amino acid conjugate synthetases, respectively (Ljung et al. 2002; Normanly et al. 2010; Korasick et al. 2013). Among the latter group the already mentioned GH3 family of acyl acid-amido synthetases can be found (Westfall et al. 2013). The GH3 family of enzymes is present in several plant genomes, being composed of 19 members in *Arabidopsis*. *GH3* genes appear to be in part regulated by the auxin signaling pathway, putting forward the hypothesis that the group II of GH3 enzymes serves to counteract cellular auxin maxima by inactivating free IAA via conjugation (Staswick et al. 2005; Woodward & Bartel 2005; Ludwig-Müller 2011). Analysis using an auxin overproducer mutant, *sur2*, reported increased activity of the IAA conjugation system (Barlier et al. 2000), further validating the concept of a plausible function of the conjugation enzymes in decreasing the auxin signal to maintain auxin homeostasis.

Auxin conjugates are generally assumed to be inactive hormone derivatives that either mark the first step in hormone degradation or represent storage or mobile long distance transport forms of which the active hormone can eventually be liberated (Normanly et al. 2010). Storage of IAA conjugates has been observed in seeds, representing an important hormone source during early seedling development (Ljung et al. 2002). Nonetheless, the precise function of IAA-conjugates during plant development is still a matter of active investigation. As already mentioned, some of the IAA-conjugates, such as IAA-Ala, IAA-Leu, IAA-Phe, are regarded to be storage compounds that can release free IAA if indicated. Likewise, other amino acid conjugates, e.g. with Asp and Glu are thought to represent the first intermediates in IAA catabolism. In addition, IAA-Trp has been observed to act as an anti-auxin inhibiting plant growth. Intriguingly, the IAA-Trp effect seems not to interfere with the general auxin signaling process (Ludwig-Müller 2011; Westfall et al. 2013). Furthermore, high-molecular weight IAA-polypeptide conjugates have been described to be present in diverse species, like for example beans (*Phaseolus vulgaris*), *Arabidopsis* or strawberries (*Fragaria* sp), suggesting a possible function of the hormone in post-translationally influencing protein function or stability (Ludwig-Müller 2011; Korasick et al. 2013). Finally, besides IAA it is known that other auxin compounds, including 4-Cl-IAA and IBA can form conjugates, although both are usually less abundant than IAA (Staswick et al. 2005).

1.2 *De novo* auxin biosynthesis in plants

As mentioned above, auxin affects virtually every aspect of the plant life cycle (Hoffmann et al. 2011). However, despite the tremendous importance of auxin and the massive research efforts of plant physiologists to unravel the intricacies of auxin biology, it is still not fully understood how plants synthesize their major growth hormone, IAA. The scenario is further complicated by the lack of an entirely auxin-deficient mutant, indicating that such mutants are lethal, or most likely, the existence of multiple pathways and functional redundancy of enzymes within the pathways.

Since the discovery of IAA in the 1930s, many genetic and biochemical studies have clearly demonstrated that L-tryptophan (L-Trp) is the most important precursor of IAA (Zhao 2010a; Mashiguchi et al. 2011; Won et al. 2011; Ljung 2013; Gao & Zhao 2014). Alternatively, there is mounting evidence that indicates that IAA can be produced in a Trp-independent pathway that may branch from indole-3-glycerol phosphate (IGP) and/or indole intermediates (Figure 4-a). The latter pathway has been proposed on the basis of the following observations: (i) stable-isotope labeling experiments showed that several tryptophan synthetase-deficient mutants such as the *orange pericarp* of maize, and the *trp2* or *trp3* of *Arabidopsis*, accumulate higher levels of IAA (Normanly et al. 1995); (ii) silencing of INDOLE-3-GLYCEROL PHOSPHATE SYNTHASE (IGS) causes a diminution of the IAA and Trp levels (Ouyang et al. 2000). A recent study published by Wang et al. (2015) provide new data not only for the participation of a cytosol-localized indole synthetase (INS) in the Trp-independent IAA biosynthesis by catalyzing the conversion of IGP to indole, but also for the implication of the Trp-independent pathway during embryogenesis. Nevertheless, still very little is known about the enzymatic mechanisms, metabolic intermediates and spatiotemporal regulation implicated in the production of IAA through the Trp-independent pathway.

With respect to the Trp-dependent pathways, it is currently assumed that three IAA biosynthetic routes act either in parallel or in a developmentally regulated fashion (Figure 4). Each of these putative pathways is termed according to the main metabolic intermediate generated from Trp, namely: indole-3-acetaldoxime (IAOx)-pathway, indole-3-acetamide (IAM)-pathway, and the indole-3-pyruvic acid (IPyA)-pathway. At this point, it is important to mention that a fourth route, designed as the tryptamine (TAM)-pathway [Trp → TAM → N-hydroxyl-TAM → IAOX → IAAld → IAA] was also believed to participate in the conversion of Trp to IAA (Woodward & Bartel 2005; Pollmann et al. 2006a; Stepanova et al. 2011). However, the operability

of this pathway has recently been drawn into questioned and accordingly is not represented in Figure 4 (discussed in detail later).

Turning to the currently proposed Trp-dependent IAA biosynthetic pathways (Figure 4b and c), it has been reported that the IAOx route is seemingly restricted to the *Brassicaceae*, which contains *Arabidopsis* and other cruciferous plants (Figure 4c) (Woodward & Bartel 2005; Zhao 2010a; Mano & Nemoto 2012). In *A. thaliana*, it is known that the cytochromes P450 monooxygenases CYP79B2 and CYP79B3 catalyze the conversion of L-Trp to IAOx (Hull et al. 2000; Mikkelsen et al. 2000). Subsequently, a third P450 polypeptide, CYP83B1 or SUPEROOT2 (SUR2), and a C-S lyase known as SUPEROOT1 (SUR1), can use IAOx as putative intermediate to produce defense-related compounds, i.e. indole glucosinolates (IGs) and camalexin (Bak et al. 2001; Zhao et al. 2002; Mikkelsen et al. 2004; Nafisi et al. 2007). In addition, presumably under normal growth conditions, a considerable portion of the produced IAOx can be converted to IAA via two intermediates, indole-3-acetonitrile (IAN) and IAM (Pollmann et al. 2006a; Sugawara et al. 2009). Phenotypical and biochemical approaches have revealed that inactivation of both *SUR1* and *SUR2*, favoured the accumulation of IAOx and IAA resulting in the manifestation of auxin-overproduction phenotypes, such as hypocotyl elongation, epinastic cotyledons and a massive induction of adventitious root growth (Boerjan et al. 1995; Delarue et al. 1998; Barlier et al. 2000; Sugawara et al. 2009; Maharjan et al. 2014), suggesting an increased flux towards IAA biosynthesis mediated by a changed metabolite flux in the IAOx-dependent pathway branch.

The IPyA pathway seemingly constitutes the major route for Trp-dependent *de novo* production of IAA (Figure 4b). Herein, a family of aminotransferases including, e.g., TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) catalyze the conversion of L-Trp to IPyA. In a subsequent reaction, the YUCCA enzymes contribute to the conversion of IPyA to IAA (Zhao 2010a; Zhao 2010b; Mashiguchi et al. 2011; Stepanova et al. 2011; Won et al. 2011; Zhao 2014). This IAA biosynthetic route will be discussed in closer detail later in this chapter. A second IPyA-pathway variant has been suggested on the basis of earlier observation that some plant-growth promoting rizhobacteria, such as *Enterobacter cloacae*, *Azospirillum brasilense*, *Pseudomonas putida* and *Pantoeae agglomerans*, are capable of converting IPyA into indole-3-acetaldehyde (IAAld) by the virtue of indole pyruvate decarboxylases (IPDs) (Spaepen & Vanderleyden 2011) (Figure 4b). However, no conclusive evidences for enzymes catalyzing the rate-limiting step for the conversion of IAAld into IAA were found in these microbes (Pollmann et al. 2006a; Zhao 2010a). In plants, IAAld has been identified as a natural constituent of pea,

being mostly present in roots (Quittenden et al. 2009). In addition, Barlier et al. (2000) proved the capability of *A. thaliana* to convert [³H]-L-Trp to labeled IAAld. The authors also demonstrated *in vivo* that, even though at a minor amount, IAAld was further oxidized to give rise to IAA. In view of these result, it is assumed that a bacteria-like IPyA pathway can also operate in plants, and hence, IPyA can be converted into IAAld and then into IAA by the consecutive action of two types of enzyme families: the IPDs and the indole aldehyde oxidases (AOs) (Mano & Nemoto 2012). However, while the analysis of enzyme extracts demonstrated a strong preference of the *ARABIDOPSIS* ALDEHYDE OXIDASE1 (AAO1) for the substrate IAAld (Seo et al. 1998), the *in planta* participation of the IPDs during the IAA biosynthesis is still questioned. In fact, Stepanova et al. (2011) documented that generation of both defective and overexpression plants of all four *Arabidopsis* IPDs had no detectable impact on IAA production, since neither of the mutants manifested auxin-related phenotypic alterations.

Finally, an additional route proceeding via IAM seems to be operative in plants (Figure 4b). The latter biosynthetic pathway was firstly identified to occur in several bacteria of the genera *Agrobacterium*, *Pseudomonas* and *Streptomyces* (Glickmann et al. 1998; Pollmann et al. 2002). In all of these phytopathogenic strains it is accepted that auxin enhances the virulence and promotes the formation of tumors and galls, with the intention of sequester plant cells for nutrient productions (Zhao 2010a; Spaepen & Vanderleyden 2011). Nowadays, it is estimated that over 80 % of the bacteria isolated from the rhizosphere are capable of synthesizing IAA (Spaepen & Vanderleyden 2011). It is well established that the bacterial IAM-pathway proceeds from L-Trp in a two-step reaction catalyzed by the action of two enzymes to IAA. The first enzyme is a tryptophan-2 monooxygenase called *iaaM*, which convert L-Trp to IAM. Whereas the second, is an indole-3-acetamide hydrolase (*iaaH*), involved in the hydrolysis of IAM into IAA (Kemper et al. 1985; Woodward & Bartel 2005; Pollmann et al. 2006a; Mano & Nemoto 2012). So far, this *iaaM/iaaH* pathway is the first IAA biosynthetic route that has been completely deciphered. Advances in analytical chemistry, resulting in highly sensitive mass spectrometric technologies, facilitated the confirmation of the occurrence of IAM as an endogenous compound of many plants, including, among others, *A. thaliana*, rice (*Oryza sativa*), maize (*Zea mays*) and tobacco (*Nicotiana tabacum*) (Pollmann et al. 2002; Sugawara et al. 2009). Shortly after, the molecular cloning of the bacterial *iaaH*-related *ARABIDOPSIS* AMIDASE 1 (*AtAMI1*) further highlighted the operability of a similar IAM-pathway in higher plants (Pollmann et al. 2003; Pollmann et al. 2006b). Likewise, the *in silico* and molecular comparative analysis carried out by

Sánchez-Parra et al. (2014) demonstrated the broad distribution of the plant AMI1-like enzymes throughout the plant kingdom, as well as the probable common function of these polypeptides in several monocot and dicot species.

Figure 4: Potential routes of IAA biosynthesis in *Arabidopsis* (Woodward & Bartel 2005; Tzin & Galili 2010; Sánchez-Parra et al. 2014).

a) L-Tryptophan (Trp) synthesis pathway in the chloroplast. **b)** and **c)** show the Trp-dependent pathways of IAA production. **b)** Summary of the L-Trp derived IAA pathways postulated in plants. **c)** Glucosinolates and *de novo* IAA production in the *Brassicaceae* family. Dashed lines indicate assumed reaction steps for which the corresponding enzymes have yet to be identified. The enzymes abbreviations are: AAO, ARABIDOPSIS ALDEHYDE OXYDASE; AMI, AMIDASE; ASA, ANTHRANILATE SYNTHASE SUBUNIT ALPHA; ASB, ANTHRANILATE SYNTHASE SUBUNIT BETA. CYP71A13, CYTOCHROME P450 MONOOXYGENASE 71A13; CYP79B2/B3, CYTOCHROME P450 MONOOXYGENASE 79B2/B3; CYP83B1 (SUR2), CYTOCHROME P450 MONOOXYGENASE 83B1 (SUPERROT 2); IGS, INDOLE-3-GLYCEROLPHOSPHATE SYNTHASE, NIT, NITRILASE; PAI, PHOSPHORIBOSYLANTHRANILATE ISOMERASE; PAT, PHOSPHORIBOSYLANTHRANILATE TRANSFERASE1; SUR1, SUPERROOT 1 (S-ALKYL-THIOHYDROXIMATE LYASE); TAA1, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1; TAR, TRYPTOPHAN AMINOTRANSFERASE RELATED 2; TSA, TRYPTOPHAN SYNTHASE ALPHA; TSB1-2, TRYPTOPHAN SYNTHASE BETA1-2.

1.3 The two-step TAA1/TAR-YUC pathway as the main auxin biosynthetic route in plants.

As already mentioned, indole-3-pyruvic acid (IPyA) has been unambiguously identified as an intermediate of IAA biosynthesis in bacteria and plants (Woodward & Bartel 2005). In *A. thaliana*, the current proposed model for the IPyA pathway involves the conversion of Trp into IPyA and its subsequent conversion into IAA, which is mediated by the activity of two classes of enzymes: tryptophan-pyruvate aminotransferases, namely TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1)/TRYPTOPHAN AMINOTRANSFERASE RELATED 2 (TAR2) — and flavin monooxygenases —YUCCA— (Stepanova et al. 2008; Stepanova et al. 2011; Mano & Nemoto 2012b; Zhao 2012). Nevertheless, the elucidation of this route for the production of auxin has been one of the most puzzling pieces of work in modern plant biology.

Not long ago, TAA1/TAR and YUCs were believed to act in two separated pathways (Zhao et al. 2001; Tao et al. 2008; Stepanova et al. 2008). Independent *in vitro* analyses suggested that TAM was utilized as substrate by the YUC enzymes, which were assumed to catalyze the hydroxylation of the amino group of TAM to produce *N*-hydroxytryptamine (Zhao et al. 2001; Expósito-Rodríguez et al. 2007; LeClere et al. 2010). Subsequently, *N*-hydroxytryptamine was tentatively proposed to be converted into IAOx and IAOx, in turn, into IAAld (Woodward & Bartel 2005). These assumptions led to the postulation of an additional TAM-dependent pathway to be operative *in planta*. However, several biochemical and genetic studies challenged the implication of the YUCs enzymes, as well as the related intermediates, in the TAM-pathway. Almost in parallel to the *in vitro* observations, (Kang et al. 2007) reported that serotonin (5-hydroxytryptamine) is accumulated in rice (*Oryza sativa*) seedlings supplemented with either Trp or TAM. In consequence, the authors proposed an alternative TAM-pathway [Trp → TAM → serotonin], mediated by the enzymes TRYPTOPHAN DESCARBOXYLASE (TDC) and TRYPTAMINE 5-HYDROLASE (T5H). Prior to this study, a revised characterization of the mass spectrometric properties of *N*-hydroxytryptamine not only argued the inconsistency of the conclusions given by Zhao et al. (2001), Expósito-Rodríguez et al. (2007) and LeClere et al. (2010), but also confirmed the possibility of TAM-derived serotonin formation (Tivendale et al. 2010). In addition, using labeled $^{13}\text{C}_3$ -IAOx, Sugawara et al. (2009) proved that the quadruple *Arabidopsis* mutant *yuc1yuc2yuc4yuc6* does not exhibit alteration in the IAOx levels, underlining that IAOx most likely only derives from the CYP79B2/B3 shunt. However, final empirical proof arguing against a participation of YUC enzymes in the TAM-pathway was provided by Mashiguchi et

al. (2011). Using liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS/MS), the authors confirmed the incapability of the *yuc1yuc2yuc4yuc6* loss-of-function line to accumulate TAM *in vivo*.

Most recent genetic studies discovered several *Arabidopsis* mutants, namely: *shade avoidance 3 (sav3)*, *weak ethylene insensitive 8 (wei8)*, and *transport inhibitor response 2 (tir2)*, in which a gene encoding a tryptophan aminotransferase appeared to be impaired (Stepanova et al. 2008; Tao et al. 2008; Yamada et al. 2009). The identified protein was named as TAA1 and the four closely TAA1-related proteins encoded in the *A. thaliana* genome were, thus, denominated as TRYPTOPHAN AMINOTRANSFERASE RELATED 1 to 4 (TAR1-4) (Stepanova et al. 2008). The identification of the TAA1/TAR family of proteins is considered a breakthrough discovery in the field of auxin biosynthesis. Three independent groups presented multiple biochemical lines of evidences that TAA1 is specifically involved in the *in planta* formation of IPyA from L-Trp in the course of IAA biosynthesis. For example, it has been demonstrated that both *sav3* and *tir2* mutants cannot metabolize the toxic 5-methyl Trp (5-MT), a Trp analog that inhibits Trp synthesis, and hence, generating hypersensitive mutants (Tao et al. 2008; Yamada et al. 2009). Likewise, Yamada et al. (2009) demonstrated that exogenous application of IPyA and IAA in a concentration of 10 μ M and 5 nM, respectively, rescued the short hypocotyl phenotype observed in the *tir2* mutant. Furthermore, using recombinant TAA1 expressed in bacteria, it was possible to identify IPyA as the enzymatic reaction product using HPLC-MS techniques (Stepanova et al. 2008; Tao et al. 2008).

On the other hand, the described phenotypic similarities between several *taal1/tar* and *yuc* loss-of-function mutants, for instance the observed vascular defects in the *taal1tar2* double and the *yuc1yuc2yuc4yuc6* quadruple mutants, as well as the overlapping expression pattern of both gene families, suggested that the TAA1/TAR and YUC family members possibly operate in the same IAA biosynthetic route (Zhao et al. 2001; Strader & Bartel 2008; Stepanova et al. 2008; Yamada et al. 2009; Stepanova et al. 2011; Won et al. 2011). In support of this idea, Phillips et al. (2011) demonstrated that the maize mutant *vanishing tassel2 (vt2)/sparse inflorescence (spi1)*, co-orthologues of *TAA1* and *YUC*, respectively, has a slightly more severe phenotype than the single defective mutant *vt2*. This result, in addition to the observation that the double mutant does not exhibit a significant reduction in auxin levels in comparison to *vt2* (Phillips et al. 2011), also prompted the authors to hypothesize that VT2 and SPI1 enzymes could be acting in the same pathway. This concept of a possible tandem action of TAA1/TAR and YUC families was further confirmed by pharmacological, biochemical and genetic studies. Stepanova et al.

(2011) reported that blocking the *in vivo* TAA1/TAR activity with kynurenine resulted in the suppression of the high-auxin phenotype of 35S::YUC1. Analysis of the IPyA levels in the double *wei8-1tar2-1* and quadruple *yuc1yuc2yuc4yuc6* deficient mutants demonstrated that, while the *taal1/tar2* plants accumulate only trace levels of detectable IPyA, the *yuc* mutants accumulate IPyA (Mashiguchi et al. 2011; Won et al. 2011). Finally, genetic approaches showed that overexpression of YUC1 (35S::YUC1) do not totally alleviate the developmental defect of *wei8tar2-2* or the shade avoidance phenotype of *taal1sav3-1* (Won et al. 2011). All the presented results suggested the activity of the YUCs downstream of TAA1/TAR and, thus, the establishment of the prevailing two-step IPyA pathway in auxin biosynthesis. In fact, today, the IPyA pathway is commonly recognized as the main source of IAA in plants (Stepanova et al. 2011; Korasick et al. 2013) (Figure 5).

Based on their sequence homology, YUC proteins belong to clade II of the flavin-containing monooxygenases (FMO) family of enzymes (Schlaich 2007). Alike other FMOs, the YUC group of proteins contains: (i) a FAD-binding motif (GxGxxxG) located proximal to the N-terminus, (ii) an identical but less conserved NADP-binding site (GxGxxxG) placed at the centre of the protein, (iii) a characteristic plant FMO-identifying motif (FxGxxxHxxxY/F)-binding site, and (iv) a FMO-binding motif (LATGY) relatively conserved among FMOs of yeasts, animals and plants (Schlaich 2007; Cha et al. 2015). The FMO family members commonly utilize NADPH and FAD as cofactors and molecular oxygen as a co-substrate to transfer a single oxygen atom into the organic substrate (Schlaich 2007; Dai et al. 2013). Considering the FMO structure and requirements, a recent study revealed the biochemical mechanism of YUC-mediated IAA biosynthesis. Purifying recombinant YUC6 and its FAD cofactor from *Escherichia coli*, Dai and co-workers (2013) reported that the *in vitro* decarboxylation of IPyA by YUC6, producing IAA, occurs in a sequential manner. Firstly, the enzyme bound-FAD cofactor molecule is reduced to FADH₂ by using electrons provided by NADPH. Following the reduction, the FADH₂-enzyme complex binds oxygen at the 4 α -position of the flavin molecule, resulting in the C4 α -hydroperoxy flavin adenine dinucleotide or FAD-OOH. Finally, the two oxygen atoms react with the substrate IPyA to catalyze its decarboxylation to IAA accompanied by the simultaneous release of CO₂ and H₂O (Figure 5).

Figure 5: The main auxin biosynthesis pathway in plants (Zhao 2012; Dai et al. 2013).

The first step in the IPyA pathway is catalysed by the TAA1/TAR family, which transfer the amino group from Trp to an alpha keto acid to generate IPyA and another amino acid. Subsequently, the YUCCA family of flavin-containing monooxygenases catalyse the oxygen and NADPH-dependent reaction finally producing IAA.

1.4 The role of YUCCA enzymes in plant development

The first characterization of a *YUCCA* (*YUC*) flavin-containing monooxygenase (*FMO*) gene was reported in a T-DNA activation-tagging screen for long hypocotyl mutants (Zhao et al. 2001). Through the insertion of four copies of the cauliflower mosaic virus (CaMV) 35S transcriptional enhancer flanking the *YUC* gene, two independent *Arabidopsis* mutants were isolated, termed “*yucca*”. It was shown that both mutants displayed an increased expression level of the *YUC1* gene. The dominant plant mutants exhibited the expected trait of longer hypocotyl, but also showed additional phenotypes resembling those of plants with elevated auxin levels, as for instance, *sur1* (Boerjan et al., 1995;) and the *iaaM* overexpression line (Romano et al. 1995). Similar to the *yucca* plants (*Yucca* sp. L.), mature leaves of the *YUC1* activation-tagging mutant were blade shaped, curled downwards with semi-erect growth habits. Hence, according to this similarity, the mutant was named YUCCA. In the same study, Zhao et al. (2001) confirmed that, in the *yucca* mutants, the endogenous free auxin levels were enhanced by at least 50 % in comparison to the wild-type. Likewise, overexpression of *YUC1* promoted an increased resistance to 5-MT, which suggests that the YUC enzyme can metabolize 5-MT, thereby decreasing its toxic effect (Zhao et al. 2001). On the basis of these observations, the authors concluded that *YUC1* plays a prominent role in Trp-dependent auxin biosynthesis.

Currently, it is known that the *Arabidopsis* genome contains 11 YUCCA genes — termed *YUC1* to *YUC11* — (Figure 6), whose products are involved in the rate-limiting step of the IPyA pathway of auxin biosynthesis (Mashiguchi et al. 2011; Stepanova et al. 2011; Won et al. 2011; Yunde Zhao 2010a).

Figure 6: Phylogenetic tree of the YUCCA family proteins from *Arabidopsis* (according to Cheng et al. (2006)).

The bootstrap consensus tree was inferred using the Neighbour-Joining methods. Bootstrap values based on 100 replicates and the TAIR accession number for each gene are shown.

Importantly, it has been demonstrated that the phenotype of YUC1 plants was phenocopied by other *Arabidopsis* YUC gain-of-function lines (Cheng et al. 2006; Lee et al. 2012; Hentrich et al. 2013b; Chen, Q. et al. 2014). However, while the overexpression of each of the YUC genes causes obvious developmental phenotypes, the same does not occur when single YUC genes are inactivated (Cheng et al. 2006; Zhao 2014), suggesting that the members of this FMO family have overlapping functions. By contrast, inactivation of different YUC gene combinations leads to severe developmental defects and even plant lethality (Cheng et al. 2006; Cheng et al. 2007). This finding not only confirms the redundant functionality of the YUCs, but also highlights the essential role of the YUC-mediated auxin biosynthesis for plant growth and development. In this line, analysis of the *yuc1yuc2yuc6* triple and *yuc1yuc2yuc4yuc6* quadruple loss-of-function mutants have revealed strong floral and vascular defects, mostly characterized by scarce flower generation, diminished flower size, aberrant organization of the flower organs, and altered vascular tissue formation in flowers and leaves (Cheng et al. 2006). Deactivation of YUC10 and YUC9 in *yuc1yuc4* defective plants led to dramatic embryo defects, which resulted in seedlings lacking hypocotyl and root, and hence, consisting basically in cotyledons with only little vasculature (Cheng et al. 2007). In addition, simultaneous inactivation of five YUC genes (YUC3, YUC5, YUC7, YUC8 and YUC9) generated seedlings that failed to achieve normal formation of root meristems, primary root

growth, and gravitropic root response (Chen, Q. et al. 2014). It is therefore clear, that *Arabidopsis* use the different sets of *YUC* genes to synthesize auxin for shoot and root development. Remarkably, Cheng et al. (2007) proposed that both auxin biosynthesis and transport act synergistically to co-regulate plant development. This affirmation arose from the observation that disruption of *pin1* or *aux1* in different combination of *yuc* loss-of-function mutants partially blocked leaf formation, a phenotype that is not observed when the *yuc*, *aux1* or *pin1* mutants were analysed independently.

On the other hand, the introgression of the *iaaM* gene, under the control of the *YUC6* promoter, into different *yuc* knockout mutants demonstrated that the *pYUC6::iaaM* construct can rescue the developmental disorders observed in the *yuc1yuc2yuc6* triple mutant, but not the floral defects of the *yuc1yuc4* double mutant (Cheng et al. 2006). From this it can be deduced that tissue-specific auxin biosynthesis, mediated by *YUC* enzymes, is indispensable for normal auxin functions. Furthermore, RNA *in situ* hybridization and analysis of *YUC* promoter β -glucuronidase (GUS) reporter lines have demonstrated that *YUC* genes are not ubiquitously expressed, but rather restricted to particular cell types and developmental stages (Cheng et al. 2006; Cheng et al. 2007; Hentrich et al. 2013a). In other words, the expression pattern of the *YUC* genes are regulated spatially and temporally to control auxin levels during development.

The importance of the *YUC* family of genes is underlined by the fact that *YUC*-homologous genes have been identified in many plant genomes including, tobacco (Rozov et al. 2012), rice (Yamamoto et al. 2007), maize (Gallavotti et al. 2008), pea (Tivendale et al. 2010), petunia (Tobena-Santamaria et al. 2002) tomato (Expósito-Rodríguez et al. 2007) and poplar (Ye et al. 2009). Functional characterization of the several *YUC* homologs have revealed that these genes also conduct auxin biosynthesis and play critical roles in plant development (Gallavotti et al. 2008; Tobena-Santamaria et al. 2002). Nonetheless, the future research in this area is likely to focus on clarifying the biochemical role of *YUC*s and *YUC*-likes enzymes in auxin biosynthesis and plant growth and development.

1.5 General mechanism of interaction between auxin and jasmonates

In their natural environment, plants are under continuous biotic and abiotic stresses that represent considerable threats for the survival of the plant and its offspring. Therefore, being sessile organisms, the rapid perception and integration of external signals with endogenous developmental programs is of key importance for the adaptational capacity and, thus, fitness of a plant. In this context, the perception of

environmental stimuli alters the concentration of several signaling molecules, including plant hormones, intracellular Ca^{2+} levels, and secondary compounds such as, e.g., inositol phosphate, sugars or reactive oxygen species (Pozo et al. 2015; Verma et al. 2016). Among these, the phytohormones are considered the most important plant substances for regulating physiological and molecular responses.

Phytohormones are small chemical messengers derived from secondary metabolism, which take centre stage in the integration and transduction of environmental demands into plastic responses to shaping plant architecture (Santner & Estelle 2009; Kazan 2013). The major hormones produced by plants include the “classical” five, i.e. auxins, gibberellins (GA), cytokinins (CK), abscisic acid (ABA), ethylene (ET), and the relatively new compounds salicylic acid (SA), jasmonates, brassinosteroids (BR) and strigolactones (Kende & Zeevaart 1997; Bari & Jones 2009; Verma et al. 2016). Regarding their function, auxins, BR, CK, GA and strigolactones are known to play decisive roles in the orchestration of growth and development (Pozo et al. 2015). In contrast, ABA, SA, jasmonates and ET are considered to play crucial roles in mediating plant defense responses against pathogens and abiotic stresses, such as drought, light, salinity or high temperatures (Bari & Jones 2009; De Bruyne et al. 2014). Diverse studies have indicated that investing in defense usually comes at the expense of plant growth (McGuire & Agrawal 2005; Walters & Heil 2007; Kempel et al. 2011; Leone et al. 2014). A phenomenon known as the “growth-defense tradeoff” (Huot et al. 2014). For example, it have been reported that the induction of stem elongation in response to light competition increase the susceptibility of *Arabidopsis* and *Chenopodium album*, against different pathogens such as the bacterium *Pseudomonas syringae* or the herbivore *Spodoptera exigua* (Kurashige & Agrawal 2005; Griebel & Zeier 2008).

In this framework, it is known that jasmonic acid (JA) and its metabolites, collectively known as jasmonates (JAs), are plant growth inhibitors, mainly involved in counteract biotic stress, i.e. against herbivore and pathogen attack (Antico et al. 2012; Studham & MacIntosh 2012). Earlier observations reported the inhibition of primary root growth in *Arabidopsis* seedlings grown on media supplemented with 0.1 μM methyl jasmonate (MeJA) (Staswick et al. 1992). Further *in vivo* analyses demonstrated that induction of endogenous JAs in *Arabidopsis* is sufficient to impair primary root growth and leaf expansion (Zhang & Turner 2008); and that in leaves, JAs exert their growth inhibitory effect through the suppression of mitosis, by arresting the cell cycle in G1 prior to the S transition (Noir et al. 2013). Contrary to the growth inhibitory effect triggered by the mentioned lipid-derived oxylipins, indole-3-acetic acid (IAA) is the most important plant growth hormone (Woodward

& Bartel 2005; Abel & Theologis 2010; Zhao 2010b; Mano & Nemoto 2012). However, despite these two natural hormone regulators i.e., JA and IAA, have seemingly nothing in common, a wave of studies have demonstrated that, far from acting in separate modules, both phytohormones interact either antagonistically or synergistically to regulate plant fitness (Hentrich et al. 2013a; Dombrecht et al. 2007; Sun et al. 2009).

The first indications of the existence of JA-IAA crosstalk arouse from the observation that both hormones share a conserved signal transduction mechanism that makes use of the 26S-proteasome system (Hoffmann et al. 2011; Pérez & Goossens 2013; Shabek & Zheng 2014) (Figure 7). In very simplified terms, oxylipins, such JA and its precursor 12-oxo-phytodienoic acid (OPDA), are synthesized from chloroplastic linolenic acid via the octadecanoid pathway (Dar et al. 2015). It is well-characterized that after a wound or pathogen attack the production of the bioactive JA, jasmonoyl-L-isoleucine (JA-Ile), is stimulated (Huot et al. 2014). Subsequently, the synthesized JA-Ile is perceived by the F-box protein CORONATINE INSENSITIVE 1 (COI1) (Chini et al. 2007). Analogously to auxin perception (See Section 1.1), JA-ILE enables the SCF^{COI1} complex to bind and ubiquitinate specific repressor proteins, here the so-called JASMONATE ZYM DOMAIN (JAZ) family of transcription repressors, which are in turn labeled for degradation by the 26S proteasome, thereby relieving the repression of MYC transcription factors and triggering the expression or different subsets of JA-responsive genes (Kazan & Manners 2008; Chini et al. 2009; Fernández-Calvo et al. 2011). Intriguingly, auxin and JA perception are sharing parts of their machinery. During a mutant screen it became evident that a newly identified MeJA insensitive mutants was allelic to the *axr1* defective mutant, which is impaired in auxin signaling, suggesting that AXR1 contributes to both the perception of IAA and JA (Tiryaki & Staswick 2002). Likewise, it has been demonstrated that a point mutation of a SCF subunit of *Arabidopsis* resulted not only in reduced auxin response, but also in a diminished expression of several specifically JA-induced genes, indicating a reduction in JA sensitivity (Ren et al. 2005). Another possible convergence point is the co-repressor TOPLESS, which has been demonstrated to be recruited by both Aux/IAA and JAZ through the ETHYLENE RESPONSIVE FACTORS (ERF)-associated amphiphilic repression (EAR) motif to exert their repressive function (Pauwels et al. 2010; Hoffmann et al. 2011). In addition, it has been observed that JA and IAA cooperate spatiotemporally to regulate flower development and fertility. The *Arabidopsis* double *arf6arf8* null mutant, which displayed decreased sensitivity to IAA and failed to form normal flowers, exhibited both JA deficiency and reduced transcription of different octadecanoid biosynthetic

genes in their flowers (Nagpal et al. 2005). A further example for oxylipin-IAA interaction at the signal transduction level came from the investigation of adventitious root initiation. (Gutierrez et al. 2012) found that through the regulation of several ARFs, IAA modulates JA-homeostasis in an antagonistic manner to stimulate adventitious rooting.

Apart of the JA-IAA interactions described above, plant literature also contains other examples of intimate interplay between those two phytohormones. In rice coleoptiles the IAA-gradient generated during gravitropism is accompanied by a reciprocally oriented JA-gradient to co-regulate asymmetrical growth (Gutjahr et al. 2005). Furthermore, it has been documented that auxin positively affects the accumulation of transcript levels of the JA-biosynthetic genes *LOX2* and *AOS* (Tiryaki & Staswick 2002). Conversely, jasmonates have been shown to influence auxin biosynthesis. In a forward genetic study for seedlings that did not show lateral root (LR) formation when grown in presence of MeJA, Sun et al. (2009) found a mutant with decreased auxin levels. Genetic analysis of this *Arabidopsis* mutant termed *jasmonate-induced defective lateral root1* (*jdl1*) revealed a defect in the Trp-biosynthetic gene *ANTHRANILTE SYNTHASE1 α1* (*ASA1-1*) to be the reason for the phenotype (Sun et al. 2009). As described earlier, Trp is considered to be the main precursor for the synthesis of IAA and glucosinolates (Woodward & Bartel, 2005). In their work, Sun et al. (2009) also found that exogenous MeJA application reduced protein levels of the IAA efflux transporters PIN1 and PIN2, as well as gene expression of the auxin influx *AUX* in the *jdl1/asa1* mutant roots. Later on, Sun et al. (2011) clarified that JA not only suppresses the expression of *PIN2*, but also inhibits *PIN2* endocytosis to modulate subcellular distribution and abundance. Overall, the finding suggested that JAs control LR formation through the modulation of IAA biosynthesis and transport. In summary, in view of the arduous work of researchers it is clear that a tight molecular interconnection between auxin and JA exists. Moreover, it can be concluded that auxin-oxylipin crosstalk is essential for the fine-tuning of plant development.

Figure 7: JA signaling model in *Arabidopsis* (Hoffmann et al. 2011; Yan et al. 2013).

a) At high levels, the bioactive jasmonate JA-Ile is perceived by the F-box receptor CORONATINE INSENSITIVE1 (COI1). **a)** and **b)** The SCF^{COI1}-E3 ligase multiprotein complex, including the COI1 and the ARABIDOPSIS S-PHASE KINASE PROTEIN 1 (ASK1), CULLIN 1 (CUL1), and RING-BOX 1 (RBX1), mediates the ubiquitination and degradation of the JASMONATE ZIM DOMAIN (JAZ) transcriptional repressors through the 26S proteasome, thereby releasing the repressor complex-NINJA-TOPLESS and the MYC family of transcriptional factors. **c)** This culminates with the activation of the JA-responsive genes.

1.6 Role of YUC8 and YUC9 in JA-IAA crosstalk

Since the discovery of the YUCCA family of flavin-like monooxygenases in 2001 by Zhao and co-workers, many projects focus on the understanding of both the molecular details by which YUCCA gene expression is regulated, and their

physiological role in plant development. This includes the discovery of a possible role as hubs for the interconnection with other hormonal pathways. In this context, two independent transcriptomic analyses revealed that among the group of up-regulated genes two *YUCCA* genes, *YUC8* and *YUC9*, were differentially induced by OPDA and MeJA (Mueller et al. 2008; Pauwels et al. 2008). Meanwhile, it has been found that wound-induced formation of MeJA in *Arabidopsis* leaves is sufficient to trigger *YUC9* expression (Hentrich et al. 2013a).

Similar to the *YUC1* gain-of-function mutant, the group of Pollmann and coworkers (Hentrich et al. 2013a) has recently demonstrated that *Arabidopsis* *35S::YUC8* and *35S::YUC9* overexpression lines (*YUC8ox* and *YUC9ox*), which constitutively express both enzymes, are phenotypically characterized by elongated hypocotyls, shorter primary root, epinastic cotyledons, narrow elongated leaf blades and petioles and increased apical dominance (Figure 8a-c, e-g).

Figure 8: Phenotype of the *YUC8* and *YUC9* overproducer mutants and the double *yuc8yuc9* loss-of function mutant (Hentrich et al. 2013a).

a-d) One week-old and **e-h)** six week-old plants of the *Arabidopsis* wild type (Col-0) as well as the *YUC8ox*, *YUC9ox* and the *yuc8yuc9* line. Scale bar = 1 mm in the upper images and 5 mm in the lower images.

Further analyses on the chemotype of *YUC8ox* and *YUC9ox* lines, showed a significant increase of free IAA levels compared to wild-type (Hentrich et al. 2013a). In addition to the initial phenotypic analysis some of the overexpression lines

displays an abnormal secondary growth of the stem (Figure 9a). Indeed, in some cases the secondary growth was so strong that the stem was no longer able to follow the growth and cracked open from the bottom to the top (Hentrich, et al. 2013b) (Figure 9b-c).

Figure 9: Analysis of the stem phenotype of 10 to 12 weeks-old *Arabidopsis* plants (Hentrich et al. 2013b).

a) Stem cross sections of the *YUC8/9* overexpression lines compared to Col-0. **b)** and **c)** detail of the stem in *YUC8ox*. Noted the busted stem in the lateral.

Likewise, Hentrich et al. (2013a) also used both *35S::YUC8* and *35S::YUC9* overexpression vector to carry out a transient expression experiment in *Nicotiana benthamiana* (Domin) leaves (Figure 10). The authors observed that leaves infiltrated with *Agrobacterium tumefaciens* ((Smith & Townsend) Conn.) harboring the overexpression constructs exhibited strong curling compared with leaves agroinfiltrated with an empty vector (Figure 10a-c). Analysis of the epidermal cells revealed that the overexpression of *YUC8* and *YUC9* in leaves led to a remarkable increase in the pavement cell size (Figure 10d-f).

Figure 10: Transient expression in *Nicotiana benthamiana* leaves (Hentrich et al. 2013a).

Leaves of *N.benthamiana* 18 h post-infiltration with *A.tumefaciens* harbouring either an empty vector or a construct expressing *YUC8* or *YUC9* under the control of the 35S promoter. **a-c)** Leaves phenotype and **d-f)** leaf epidermis detail 18 hours post-infiltration. Pavement cells are colored. Scale bar = 50 μ m.

Importantly, inferred from the *YUC8* and *YUC9* expression pattern Hentrich et al. (2013a) demonstrated that these two auxin biosynthetic genes are spatially and temporally regulated. Thus, *YUC8* transcript levels peak in young seedlings, i.e. 3 and 7 days after germination, and decline thereafter. In adult plant (6-8 week-old), *YUC8* expression is higher in roots, young sinks leaves, mature source leaves and flower buds, being just found in the stems as a trace. On the other side, *YUC9* expression, is remarkably higher 7 days after germination and predominantly restricted to roots in adult plants, with minor detection in sink leaves. In addition, analysis of the *pYUC8::GUS* and *pYUC9::GUS* reporter lines confirmed the consistent expression of both genes in root tips, but also a partial overlapping expression in lateral roots, suggesting a redundant role in lateral root formation. The *YUC8* and *YUC9* promoter activity also provided evidence for the spatio-temporal regulation of auxin biosynthesis mediated by *YUC8* and its essential role in reproductive development, whereas *YUC9* is more related to early embryo development.

Coming back to the role of *YUC8* and *YUC9* in IAA-JA crosstalk, in their work Hentrich et al. (2013a) reported that the exogenous application of several oxylipins, i.e. MeJA, OPDA and coronatine, a bacterial toxin that mimics the effect of the

bioactive JA-Ile (Zheng et al. 2012), have differential impact on *YUC8* and *YUC9* gene expression (Figure 11a-b). The qRT-PCR results indicated a transient down-regulation of *YUC8* at short-term oxylipin treatment, with a subsequent recovery throughout the treatment period. In contrast, *YUC9* transcripts levels increased progressively along the treatment time. However, using the T-DNA insertion mutants *yuc8* and *yuc9*, independently, it was possible to reveal an increase in transcript abundance of both genes also after short-term MeJA treatment, meaning that both *YUC* genes complement functionally and compensate for a loss function of function of the other (Figure 11c).

Figure 11: Oxylipin impact on the transcriptional response of *YUC8* and *YUC9* (Hentrich et al. 2013a).

Quantitative RT-PCR analysis for *YUC8* and *YUC9* genes after different durations of treatment with oxylipins: OPDA, MeJA and Coronatine: **a)** *YUC8* and **b)** *YUC9* and **c)** transcript levels in the *yuc8* and *yuc9* knockout mutant backgrounds after short-term (2h) and long-term (7d) MeJA treatment. In this experiment the relative expression levels of the genes were normalized to levels in the control samples, with bars showing the standard error (SE). Significant difference between *YUC8* or *YUC9* and Col-0 by *t*-test at $P < 0.01$ are indicated **. Different letters indicate significant differences between means ($P < 0.05$).

Consistent with the idea of complementary functions shared by the different YUC family members proposed by (Cheng et al. 2006), the phenotypical analysis of the aforementioned *yuc8* and *yuc9* knockout mutants, does not shown any obvious developmental defect. Indeed, the lack of a distinguishable defect was maintained even in the absence of both *YUC8* and *YUC9* (Figure 8 d and h). Nevertheless, GC-MS analysis demonstrated a significant reduction of the auxin levels produced in response to MeJA (20 μ M) application in roots of both single-knockout mutants,

and in hypocotyls of the *yuc8* mutant. These observation suggest that the differential effect of MeJA over *YUC8* and *YUC9* occurs in an organ dependent manner. Moreover, coupling the two mutations in the *yuc8yuc9* mutant, results in a strongly impaired IAA production following MeJA treatment in cotyledons, hypocotyls and roots. Clearly indicating a role for both enzymes, YUC8 and YUC9, in MeJA-induced IAA production (Hentrich et al. 2013a).

Further proof for the JA-mediated *YUC8* and *YUC9* transcriptional response came from the examination of the *YUC8* and *YUC9* gene expression in the *coi1* knockout mutant background after various oxylipins treatment (Figure 12). As already mentioned, it has been shown that COI1 is a critical component of the jasmonate perception machinery (Chini et al. 2009). It has been observed that inactivation of COI1 causes a nearly complete loss of transcriptional responses of both *YUC8* and *YUC9* after MeJA stimulation. In view of this data, Hentrich et al. (2013a) tentatively speculated that oxylipins regulate *YUC8* and *YUC9* transcription via a COI1-dependent pathway.

Figure 12: *YUC8* and *YUC9* expression in the *coi1* mutant background (Hentrich et al. 2013b).

Transcript abundance was evaluated after the treatment with various oxylipins: OPDA, MeJA and coronatine. The data reflect the relative expression levels relativized to the reference genes in the mock treated plants. Means are given with their SE. Significantly different means are reflected by distinct letters ($P < 0.05$).

Taken together, the presented results provide strong evidences supporting that *YUC8* and *YUC9* both contribute to the auxin biosynthesis in *Arabidopsis*. In addition, the data on the regulation of *YUC8* and *YUC9* demonstrate a parallel role of these two YUC proteins in maintaining auxin homeostasis and MeJA-induced IAA production. Finally, the analyses highlight the intimate relationship

established between JA-signaling and IAA biosynthesis, not only by regulating the production of the Trp-precursor (Sun et al. 2009), but also by direct modulation of the IAA synthesis-related genes. Nonetheless, further studies in this field will help to clarify the molecular basis concerning the JA-mediated *YUC8* and *YUC9* transcriptional regulation.

1.7 Secondary growth development in the *Arabidopsis thaliana* stem

In flowering plants, the body formation is initiated from a totipotent zygotic cell (Machida et al. 2013). Proliferation of the cell, followed by differentiation, leads to the formation of a mature embryo, constituted by two distinctive growth foci called meristems, one or two cotyledons, a radicle, and a pattern of tissue that comprises epidermis, ground tissue and vascular tissue (Miyashima et al. 2013). After germination, the embryonic primary root apical meristem (RAM) and shoot apical meristem (SAM) take over to the post-embryonic formation organs, and they start generating the main root and aerial organs, i.e. leaves, flowers and stem, respectively (Berleth & Jürgens 1993; Machida et al. 2013). The SAM and RAM meristems are major sources of permanent growth. However, lateral or secondary meristems are equally important to model plant architecture and determine developmental plasticity upon environmental stimuli (Perianez-Rodriguez et al. 2014).

Vascular tissue comprises two functionally distinct domains: (i) xylem, involved in the transport of water, nutrients and minerals from the root system to the shoot, as well as providing mechanical support to the plant, and (ii) phloem, which mediates the shoot to root transport of autotrophic energy source, photoassimilates, small regulatory RNAs and signaling molecules, such as plant hormones and peptides (Buhtz et al. 2008; De Rybel et al. 2015). As just mentioned, the vascular system is formed in its initials during embryogenesis. In *Arabidopsis*, it is well-defined that vascular initiation occurs in the early globular embryo, through the generation of ground tissue and vascular stem cell initials, also called procambium. After subsequent periclinal divisions (parallel to the axis/surface) of the procambium during the late globular stage, heart stage and torpedo stage, the mature embryo displays a radial vascular pattern with the vascular tissue in the center (Miyashima et al. 2013). These embryonic-derived provascular tissues will generate the vascular system in the primary root and hypocotyl, whereas the shoot apical tissue is derived from the SAM (Nieminen et al. 2015). On the other hand, in the embryonic cotyledons only a procambial tissue is extended in an apical-basal axis and will generate the leaf veins (De Rybel et al. 2015). Final growth and

maintenance of the vascular tissue pattern take places post-embryonically, through cell divisions in zones of the plant with mitotic activity, i.e. the primary and lateral meristems (procambium). Thus, the vascular anatomy of *Arabidopsis* differs depending on the plant organ (Figure 13). In shoots, the vasculature is located in separate collateral vascular bundles with the primary xylem towards the pith parenchyma cells (Figure 13a) (Figure 14a). In young roots, the vascular tissue is similar to the mature embryo and displays a bisymmetric vascular patten consisting in a central xylem axis flanked by procambium and two poles of primary phloem (Figure 13f) (Nieminen et al. 2015; De Rybel et al. 2015).

Figure 13: Schematic representation of the vascular development in *Arabidopsis* (modified from Nieminen et al. (2015) and De Rybel et al. (2015)).

a-b) stem and **d-f)** root. SAM and RAM are indicated. **a)** and **f)** represent young developmental stages, **b)** and **e)** intermediate developmental stages, **c)** and **d)** mature developmental stages.

In addition to the development, growth and maintenance of the primary vascular tissues, *Arabidopsis* and some other plant species undergo secondary growth to enlarge the girth of the plant organs (Barra-Jiménez and Ragni, 2017). During this event, a secondary lateral meristem called vascular cambium, originates from the procambium, starts to divide periclinally to give rise to the secondary xylem (inwards) and the secondary phloem (outwards) (Schuetz et al. 2013; Nieminen et al. 2015; De Rybel et al. 2015). The vascular cambium functions as a stem cell niche, forming a continuous ring in an organ-specific manner. Regarding the *Arabidopsis* inflorescence stem (Figure 13b-c) (Figure 14a-b), the vascular cambium develops in two different anatomical regions, within the vascular bundles, where it is termed fascicular cambium, and between them, called interfascicular cambium; thus, the interfascicular cambium progresses laterally until the two fascicular cambium connect and become indistinguishable (Sehr et al. 2010; Sanchez et al. 2012). Nonetheless, histological and transcriptomic analysis have revealed that the secondary growth of the *Arabidopsis* inflorescence stem is limited to the base (Little et al. 2002; Ko & Han 2004; Sehr et al. 2010). In roots (Figure 13e-f), actively dividing cambial cell located just after the rosette cells produces the secondary xylem and the secondary phloem, resulting in a radially symmetric vascular pattern (Nieminen et al. 2015).

Figure 14: Comparison of the stem cross-section during different developmental stages in *Arabidopsis* (according to Sanchez et al. (2012)).

a) young stem exhibiting primary growth, **b)** mature stem with secondary growth.

Secondary or lateral growth implicates that cells with xylem and phloem identities differentiate mainly into tracheary and sieve elements, respectively, in order to create functional conductive tissues (De Rybel et al. 2015). On the xylem side of the cambium, the cells go through several stages of differentiation that involve cell division, expansion, maturation, lignification, secondary cell wall thickening, and programmed cell death, in which all cellular process are terminated (Fukuda 1997a; Oh et al. 2003; Ko & Han 2004; Ko et al. 2004). The resulting mature xylem includes three main cell types: (i) xylem parenchyma, involved in storage functions (ii) fibers, implicated in mechanical support for the plant, and (iii) the aforementioned tracheary (vessel) elements, which facilitate water and solute transport (Myburg et al. 2013; Schuetz et al. 2013). Besides this functions, plant secondary cell walls play important roles in preventing pathogen invasion, not only by working as a physical barrier against initial pathogen colonization, but also by supplying antimicrobial compounds, which are liberated during cell wall degradation (Miedes et al. 2014). Both fibers and tracheary element possess thick secondary walls, composed of a complex mixture of cellulose (40-80 %), hemicellulose (10-40 %), lignin (5-25 %) and cell wall proteins (Handakumbura & Hazen 2012; Kumar et al. 2016). Cellulose microfibrils crosslink with hemicellulose to form the framework of the secondary cell wall. Hemicellulose, such as xylans, are needed for the normal assembly and structural strength of the secondary cell wall. While lignin, which is hydrophobic and inert, impregnates the cellulose and hemicellulose network to reinforce strength and provide hydrophobicity to the cell wall (Zhong & Ye 2015). Cell wall composition is influenced by diverse developmental and environmental stimuli. Thus, for example, transcriptomic analyses on cotton (*Gossypium hirsutum*) have revealed an increased lignification in response to the infection of the wilt fungus *Verticillium dahlia* (Xu et al. 2011a). In gymnosperms the compression wood, which forms on the lower side of a leaning stem, produce higher contents of the *p*-coumaryl alcohol (Boyd 1973; Ralph et al. 2004). Static leans drive the formation of tension wood in the upper stem of dicotyledonous angiosperms, which is characterized by an additional inner, cellulose-rich, gelatinous secondary cell wall (Love et al. 2009; Zhong & Ye 2015). Furthermore, alteration on lignin contents have been observe under low temperatures, water deficit, and some other abiotic stresses (Moura et al. 2010).

CHAPTER 2:

OBJECTIVES

Objectives

Despite auxin is one of the oldest fields of study, its biosynthesis, regulation and action remains in part uncertain. Over the last several years mounting evidence indicates that plant hormones, particularly the naturally occurring auxin, indole-3-acetic acid (IAA), play an important regulatory role in the control of vascular cambium initiation and secondary growth transition, i.e. differentiation of cells into mature phloem and xylem elements, and stimulation of associated events as secondary cell wall deposition and thickening. Actually, the construction of transgenic plants with altered levels of hormones has provide a new approach for investigating the hormonal regulation of plant growth and development. On the other hand, physiological and genetic studies have shown that the seemingly antagonistic hormones jasmonic acid (JA) and IAA, are linked by two flavin monooxygenases (*YUCCA*) genes, *YUCCA8* and *YUCCA9*, both recognized as key players in IAA biosynthesis. Nevertheless the molecular details by which *YUCCA8* and *YUCCA9* genes are regulated, and its physiological role are still unknown. Therefore, the objectives of the work described in this thesis are:

Objective 1: The elucidation of the molecular auxin action in secondary growth by in-depth analysis of *YUCCA8* and *YUCCA9* overexpressor lines.

Objective 2: To analyze the physiological role of YUC8 and YUC9 in biotic and abiotic stresses.

Objective 3: To disclose the jasmonate-induced transcription factor(s) involve(s) in the regulation of *YUC8* and *YUC9* expression.

CHAPTER 3:

MATERIALS & METHODS

3.1 List of equipment, chemicals and materials.

3.1.1 Instruments

Autoclave	
Laboratory Steam Sterilizer Med 20	Selecta, Abrera, Barcelona (Spain)
Balances	
Analytical balance Si-234	Denver Instruments, Bohemia, NY (USA)
Digital balance MXX-2001	Denver Instruments, Bohemia, NY (USA)
Centrifuges	
Centrifuge 5414 R	Eppendorf AG, Hamburg (Germany)
High-speed centrifuge Avanti® J-26XP and rotors: JLA-16.250 and JA-25.50	Beckman Coulter Life Sciences, Indianapolis (USA)
Centrifuge Sigma 1-15PK and rotor 12024-H	Sigma Laborzentrifugen GmbH, Osterode am Harz (Germany)
Centrifuge 5804 R	Eppendorf AG, Hamburg (Germany)
Centrifuge Sigma 4-15c Qiagen and rotor 2 x 96	Qiagen, Hilden (Germany)
Digital Camera	
Canon EOS 600D	Canon Inc. Tokyo (Japan)
Electrophoresis system	
Electrophoresis PowerPac™ Power Supply	Bio-Rad Laboratories, Hercules, CA (USA)
Horizontal electrophoresis chambers:	
-Mini ReadySub-Cell GT Cell	Bio-Rad Laboratories, Hercules, CA (USA)
-Wide Mini-Sub Cell GT Cell	
Electroporation equipment	
Electroporation cuvette	Cell Projects Ltd, Harrietsham, Kent (UK)
ECM® 630 Electroporation System	Genetronics Inc, San Diego, CA (USA)
GC-MS/MS system	
SCION TQ GC-MS/MS equipped with:	
-451 gas chromatograph	Bruker Daltonik GmbH, Bremen (Germany)
-CLC CombiPal autosampler	
-Triple quadrupole mass spectrometer	
GC capillary columns:	
-ZB-35 (30 m x 0.25 mm, 0.25 µm film)	Phenomenex, Aschaffenburg (Germany)
-DB 5-MS (30 m x 0.25 mm, 0.25 µm film)	Bruker Daltonik GmbH, Bremen (Germany)
Gel documentation	
Molecular Imager® Gel DOC™ XR System	Bio-Rad Laboratories, California (USA)
B&W Sony UP-D898 Digital Video Printer	Sony, Tokyo (Japan)
Incubator shakers	
Certomat BS1	B.Braun Biotech Inc.Allentown, PA (USA)
Hotplate and Stirrer	
1203 hotplate&stirrer	Jenway, Stone (UK)
Laminar flow cabinet	
Airstream horizontal laminar flow clean benches AHC-AD1	Esco, (Singapore)
Airstream horizontal laminar flow clean benches AHC-AD1 H-100	Teslar® Life Sciences Solutions, Madrid (Spain)
Mass spectrometer	
UltrafleXtrem™ Smartbeam II Laser MALDI-TOF	Bruker Daltonik GmbH, Bremen (Germany)
MALDI target plate	Kratos Analytical Ltd. Manchester (UK)

Microscope equipment	
Stereomicroscope Leica MZ10F	Leica Microsystems, Wetzlar (Germany)
Leica DFC 400C camera	Leica Microsystems, Wetzlar (Germany)
PCR thermocycler	
PeqSTAR 96 Universal Gradient	PEQLAB GmbH, Erlangen (Germany)
LightCycler® 480 System	Hoffmann-La Roche, Basel (Switzerland)
pH meter	
pH-meter basic 20+	Crison, L' Hospitalet de Llobregat, Barcelona (Spain)
Sample Concentrator	
Sample Concentrator and Dri-Block® DB-3D	Techne, Stone (UK)
Scanner	
HP Scanjet 5590 Digital Flatbed Scanner	HP, Palo Alto, CA (USA)
Spectrophotometer and Fluorimeter	
NanoDrop 1000 Spetophotometer	Thermo Fisher Scientific, Waltham, MA (USA)
OD600 DiluPhotometer	IMPLEN, Westlake Village, CA (USA)
Ultrospec 3300 <i>pro</i> UV/Visible Spectrophotometer	Amersham Biosciences, Little Chalfont (UK)
TECAN GENios Pro 96/384 Multifunction Multimode Microplate Reader	MTX Lab Systems, Bradenton, FL (USA)
Thermomixer and thermoblock	
Thermomixer R	Eppendorf AG, Hamburg (Germany)
Tissue Lysser	
Retsch MM300 TissueLyser	Qiagen, Hilden (Germany)
Vacuum system	
Speed-vac concentrator Plus 5305	Eppendorf AG, Hamburg (Germany)
Membrane vacuum pump stand PC 620 NT	Vacuubrand, Wertheim (Germany)
Extraction vacuum manifold 20-port	Waters Corporation, Milford, MA, (USA)
Vibratome	
Vibratome 1000 plus	Technical Products International Inc, St.Louis, MO (USA)
Ultrasonic bath	
Banasonic 5510E-DTH	Branson Ultrasonics Corporation, Danbury,CT, (USA)

All other equipment used corresponds to usual laboratory standards.

3.1.2 Chemicals

Acetonitrile, LiChrosolv®	Merk KGaA, Darmstadt (Germany)
Acetosyringone	Sigma-Aldrich, St. Louis, MO (USA)
Acetyl bromide	Sigma-Aldrich, St. Louis, MO (USA)
Agarose D1 Medium EEO	Conda, Torrejón de Ardoz, Madrid (Spain)
Antibiotics	Duchefa Biochemie, Haarlem (Netherlands)
Bacto™ agar	Conda, Torrejón de Ardoz, Madrid (Spain)
Boron trifluoride diethyl etherate	Sigma-Aldrich, St. Louis, MO (USA)
Bromophenol blue	Sigma-Aldrich, St. Louis, MO (USA)
BSTFA + TMCS, 99:1 (Sylon BFT)	Sigma-Aldrich-Supelco, St. Louis, MO (USA) Supelco
Chloral hydrate	Sigma-Aldrich, St. Louis, MO (USA)
Chloroform	VWR International, Radnor, PE (USA)
Congo Red reagent	Sigma-Aldrich, St. Louis, MO (USA)
3,3-Diaminobenzidine reagent (DAB) 99%	Sigma-Aldrich, St. Louis, MO (USA)
2,5-Dihydroxybenzoic acid (DHB) matrix	Sigma-Aldrich, St. Louis, MO (USA)
Diethyl ether	Sigma-Aldrich, St. Louis, MO (USA)
1,4-Dioxane	Sigma-Aldrich, St. Louis, MO (USA)
DMSO	Sigma-Aldrich, St. Louis, MO (USA)
dNTPs (dATP, dCTP, dGTP, dTTP)	Invitrogen™, Carlsbad, CA (USA)
DTT	VWR International, Radnor, PE (USA)
EDTA disodium dihydrate	Duchefa Biochemie, Haarlem (Netherlands)
Ethanol	Sigma-Aldrich, St. Louis, MO (USA)
Ethidium bromide	Sigma-Aldrich, St. Louis, MO (USA)
Fluorescent brightener 28	Sigma-Aldrich, St. Louis, MO (USA)
Formaldehyde	VWR International, Radnor, PE (USA)
Formamide	J.T. Baker Chemical Company, Phillipsburg, NJ (USA)
Formic Acid	Sigma-Aldrich, St. Louis, MO (USA)
[¹³ C ₆]-D-glucose	Cambridge Isotope Laboratories, Tewksbury, MA (USA)
Glycerol	J.T. Baker Chemical Company, Phillipsburg, NJ (USA)
Hormone stable isotopes	Isotopes Inc. (Pointe-Claire, Canada); OlChemIm Ltd (Olomouc, Czech Republic)
Hydroxylamine hydrochloride	Sigma-Aldrich, St. Louis, MO (USA)
IPTG	Duchefa Biochemie, Haarlem (Netherlands)
β-MeEtO	Sigma-Aldrich, St. Louis, MO (USA)
MES monohydrate	Duchefa Biochemie, Haarlem (Netherlands)
4-MUG	Duchefa Biochemie, Haarlem (Netherlands)
4-MUN	Sigma-Aldrich, St. Louis, MO (USA)
Methoxyamine hydrochloride	Sigma-Aldrich, St. Louis, MO (USA)
Methyl jasmonate, Kosher	Sigma-Aldrich, St. Louis, MO (USA)
MOPS	Sigma-Aldrich, St. Louis, MO (USA)
Monolignols	Sigma-Aldrich, St. Louis, MO (USA)
Murashige and Skoog medium	Duchefa Biochemie, Haarlem (Netherlands)
N-lauroylsarcosinate (Sarkosyl)	Sigma-Aldrich, St. Louis, MO (USA)

Paraformaldehyde	Sigma-Aldrich, St.Louis, MO (USA)
Phenol-Roti®	Carl Roth, Karlsruhe (Germany)
Phloroglucinol dye	Sigma-Aldrich, St.Louis, MO (USA)
Phyto-agar	Duchefa Biochemie, Haarlem (Netherlands)
Polyethylene glycol-4000 (PEG)	Sigma-Aldrich-Fluka, St.Louis, MO (USA)
Polyethylene glycol-8000 (PEG)	Invitrogen™, Carlsbad, CA (USA)
Potassium permanganate	Sigma-Aldrich, St.Louis, MO (USA)
Protease Peptone # 2	Difco, Hamburg (Germany)
Pyridine	Sigma-Aldrich, St.Louis, MO (USA)
SDS	Appllichem/BIOMOL
Toluidine Blue O	Sigma-Aldrich, St.Louis, MO (USA)
Tris/HCl	Sigma-Aldrich, St.Louis, MO (USA)
Trimethylsilyl diazomethane solution (in 2M Diethyl ether)	Sigma-Aldrich, St.Louis, MO (USA)
Triton™ X-100	Sigma-Aldrich, St.Louis, MO (USA)
Trypan blue reagent	Sigma-Aldrich, St.Louis, MO (USA)
Tryptone	Duchefa Biochemie, Haarlem (Netherlands)
Tween® 20, SigmaUltra	Sigma-Aldrich, St.Louis, MO (USA)
Vitamin mix	Duchefa Biochemie, Haarlem (Netherlands)
X-Gal	Promega, Madison, WI (USA)
X-GlucA	Duchefa Biochemie, Haarlem (Netherlands)
Xylene cyanol	Sigma-Aldrich, St.Louis, MO (USA)
Yeast extract	Duchefa Biochemie, Haarlem (Netherlands)

All other chemicals were ordered in the required purity from the following suppliers: Duchefa (Haarlem, Netherlands), Fisher Scientific (Waltham, MA, USA), Merck (Darmstadt, Germany), Sigma-Aldrich (St.Louis, MO, USA) and VWR (Radnor, PE, USA).

3.1.3 Materials

Chromabond® NH ₂ shorty columns (10 mg)	Macherey-Nagel GmbH & Co.KG, Düren (Germany)
Crimp top conical microvial	Chromacol Uni-VL Supelco #27312
Crimp seals with PTFE/rubber septa (8 mm)	Chromacol Uni-VL Supelco #27312
LightCycler® 480 multiwell plate 96, white	Hoffmann-La Roche, Basel (Switzerland)
Minisart® syringe filters	Sartorius, Götting (Germany)
Nylon mesh with 60 µm pore size (NY6000010)	Merck Millipore, Billerica, MA (USA)
PD-10 desalting column	GE Healthcare Europe, Madrid (Spain)
Razor blades, chrome platinum	BIC, Clinchy (France)
Stainless steel balls (5.5 mm)	Retsch Technology GmbH, Haan (Germany)
Syringe (2.5 ml)	Becton Dickinson Plastipak™, Agustin de Guadalix, Madrid (Spain)
Well clear flat bottom microtiter plate (96)	Falcon®, Tewksbury, MA (USA)

3.2 Enzymes and kits

Cellobiase (<i>Aspergillus niger</i>)	Sigma-Aldrich, St.Louis, MO (USA)
Cellulase (<i>Trichoderma reesei</i> ATCC 2692)	Sigma-Aldrich, St.Louis, MO (USA)
Cellulase R-10 (<i>Trichoderma Viride</i>)	Duchefa Biochemie, Haarlem (Netherlands)
endo- β -1,4-glucanase (EG) (<i>Aspergillus niger</i>)	Megazyme Inc. Chicago, IL (USA)
Gateway® LR Clonase™ Enzyme mix	Invitrogen™, Carlsbad, CA (USA)
GenRuler™ 1kb DNA Ladder	Thermo Fisher Scientific, Waltham, MA (USA)
LightCycler® 480 SYBR Green I Master	Hoffmann-La Roche, Basel (Switzerland)
MALDI mass standard kit	Bruker Daltonik GmbH, Bremen (Germany)
Mazeroryme R-10 (<i>Rhizopus</i> sp.)	Duchefa Biochemie, Haarlem (Netherlands)
M-MLV reverse transcriptase	Promega, Madison, WI (USA)
NucleoSpin® Extract II kit	Macherey-Nagel GmbH & Co.KG, Düren (Germany)
Oligotex mRNA Mini Kit	Qiagen N.V., Hilden (Germany)
pGEM® -T vector	Promega, Madison, WI (USA)
Plasmid DNA purification maxi kit	Qiagen N.V., Hilden (Germany)
Platinum® Taq DNA Polymerase High Fidelity	Thermo Fisher Scientific, Waltham, MA (USA)
Proteinase K	Invitrogen™, Carlsbad, CA (USA)
Quick Start™ Bradford protein Assay Kit	Bio-Rad Laboratories, California (USA)
Restriction endonucleases	Takara Bio Inc. K Kusatsu, Shiga (Japan); New England Biolabs Inc. Ipswich, MA (USA); Fermentas GmbH, St.Leon-Rot (Germany)
RNAasin	Takara Bio Inc. K Kusatsu, Shiga (Japan)
RNeasy MinElute Cleanup Kit	Qiagen N.V., Hilden (Germany)
Taq polymerase	Takara Bio Inc. K Kusatsu, Shiga (Japan)
T4 DNA ligase kit	Invitrogen™, Carlsbad, CA (USA)
Wizard® Plus minipreps DNA purification system	Promega, Madison, WI (USA)

3.3 Nucleic acids

3.3.1 Primer

Standard PCR primers spanning 19-26 bp were manually designed to have a melting temperature (T_m) of 50-60 °C, a GC content of approximately 50 %, minimal probabilities for primer-dimer formation and self-ligation. Oligonucleotide properties were analysed using the webserver OligoCalc (www.basic.northwestern.edu/biotools/oligocalc.html) (Kibbe 2007). For amplification of full length open reading frames (ORFs), the primers included the start-ATGs and stop-codons.

Primers used for qRT-PCR analyses were designed using the QuantPrime tool (www.quantprimer.de) (Arvidsson et al. 2008) as follows: length of 20-24 bp, T_m of 61-67 °C, a GC content of 45-55 %, and an amplicon size of 170-190 bp. All oligonucleotides used were synthesized by the company Sigma-Aldrich and are listed in Table 1.

Table 1: List of primers for cloning, expression analysis and sequencing. Forward (For) or reverse (Rev) orientation is indicated in the primer name. Binding sites for restriction endonucleases are underlined in the sequence.

Primers for cloning	
Primer Name	Primer sequence (5'-3') and restriction sites
Promoter YUC8-(919) For	TAT <u>GGATCC</u> AAAAGTGCAGCGTCTACCAAAA
Promoter YUC8-(919) Rev	TATTCTAGATTAGGTACGGAAAATGTGATT
Promoter YUC8-(3) For	TAT <u>GGATCC</u> TCCGTACCTAAAAATTGGATT
Promoter YUC8-(3) Rev	TATTCTAGATGCTTGACGACGAAGTAATAAT
Promoter YUC8-(Ø) For	TAT <u>GGATCC</u> TCGTCGTCAAGCATTATCACTGTT
Promoter YUC8-(Ø) Rev	TATCCATGGTCTAGATGGAAGTTGTATTGGAAATGGTTT
Promoter YUC9-(919) For	TATAAGCTTAACAAAATTAGGACCCGCTCT
Promoter YUC9-(919) Rev	TATTCTAGAGATTGAATTATATGGTAAACTCAA
Promoter YUC9-(3) For	TATAAGCTTACCACGAAGAAAATAACATCTC
Promoter YUC9-(3) Rev	TATCCATGGTCTAGAGTTAAGAGTTATAACGAGACTG
Promoter YUC9-(Ø) For	TATAAGCTTCAAATTATTCACATTAATAAAATAATC
Promoter YUC9-(Ø) Rev	TATCCATGGTCTAGATTTTCTTGAGTGAGTTTTTGAATG
ORF MYC2 For	TATGGTACCATGACTGATTACCGGCTACAACCAACGA
ORF MYC2 Rev	TATGCGGCCGCTTAACCGATTTTGGAAATCAAACCTTGCTCTGA
ORF MYC3 For	TATGGATCCATGAACGGCACAACATCATCA
ORF MYC3 Rev	TATGATATCTCAATAGTTTTCTCCGACTTTCGT
ORF MYC4 For	TATGGATCCATGTCTCCGACGAATGTTCAAGTAACCGA
ORF MYC4 Rev	TATGATATCTCATGGACATTCTCCAACTTTCTCCGTT
pENTRY-SP1 For	TATCTGATAGTGACCTGTTTCGTTGCA
pENTRY-SP1 Rev	TATGGAGATCCGTGACGCAGTAGC

Table 1 continued ↓

Primers for expression analyses	
Primer Name	Primer sequence (5'-3')
APT1-qPCR For	TCGTGCTGTTCTTGCAACCG
APT1-qPCR Rev	GCGGAGGAGAAGAGGCGGAGT
UBI10-qPCR For	TTGGAGGATGGCAGAACTCTTGCT
UBI10-qPCR Rev	AGTTTTCCCAGTCAACGTCTTAACGAAA
XTH15-qPCR For	GCACCGTCACTGCTTACTACTTG
XTH15-qPCR Rev	AGTGTGGAAGTTCTTGTTGGG
XTH16-qPCR For	AATGGCCGGAACAGCATTCTCC
XTH16-qPCR Rev	AAGCTGCATGTCGATTCTTCCG
XTH23-qPCR For	AGTTCTTGGGAAACCTAAGTGGTG
XTH23-qPCR Rev	GGAGTTCCATCCACGGAGAATATG
XTH24-qPCR For	ACGGCCAGCTTCTTACTCTCTC
XTH24-qPCR Rev	TCTCATCCCAAGTGGATCCTTCG
XTH25-qPCR For	GGAACCGTCACTGCCTACTACTTG
XTH25-qPCR Rev	GTGGAAATCAGCGGTTGGATCG
XTH30-qPCR For	CTTCCGGCGGCAAATACAAAGC
XTH30-qPCR Rev	GGCGCGTTGATGGGAATTAATGG
XTH31-qPCR For	ATCCACTGGGAGTGGGTTCAAG
XTH31-qPCR Rev	ATAAGGCTTCCCTGGCGTTGTC
XTH33-qPCR For	TGGTAGACAACATTCCGTTAGGC
XTH33-qPCR Rev	ACTCCACGTCAGCAACGGAAAC
YUC8-qPCR For	CGTCTCAAGCTTCACCTTCC
YUC8-qPCR Rev	AGCCACTGGTCTCATCGAAC
YUC9-qPCR For	TTCTCGCCACCGGTTATCGTAG
YUC9-qPCR Rev	AGCGATGTTAACGGCGTCTACTG
Primer for sequencing	
Primer Name	Primer sequence (5'-3')
T7 For	TAATACGACTCACTATAGG
SP6 Rev	ATTTAGGTGACACTATAG
M13 For	TGTAAAACGACGGCCAGT
M13 Rev	CAGGAAACAGCTATGACC
pBT-10-Seq Rev	TATTTGGGGTTTCTACAGGACGGACCAT
pENTRY-SP1 Rev	TATGGAGATCCGTGACGCAGTAGC
35S-Seq For	AGTGGAAAAGGAAGGTGGCT

3.3.2 Vectors

During the present work different vectors were used to generate constructs for promoter-reporter gene analyses. All vectors used are listed in the Table 2.

Table 2: Vectors employed in this work.

Vector	Resistance	Description	Reference
pGEM®-T	Ampicillin	<i>LacZ</i> gene	Promega (Madison, WI, USA)
pBT-10-GUS	Ampicillin	TATA- <i>uidA</i> -NosA3'	Sprenger-Haussels & Weisshaar (2000)
pSP-Entry1	Ampicillin	Gateway	S. Pollmann (not published)
pMDC163	Kanamycin	Gateway- <i>uidA</i> -NosA3'	Curtis & Grossniklaus (2003)
pEARLEY201	Kanamycin	35S-Gateway	Earley et al. (2006)
p35S-HA-GW	Ampicillin	35S-Gateway-3xHA-tag	Ehlert et al. (2006); Weltmeier et al. (2006)

3.4 Biological material

3.4.1 Bacteria

All bacteria strains from *Escherichia coli* and *Agrobacterium tumefaciens* used for both plasmid and construct propagation, as well as the *Pseudomonas syringae* strain used for infection are listed in Table 3.

Table 3: Bacteria strains and their genotype.

Bacteria Strain	Genotype	Reference
<i>E. coli</i> DH5 α	F ⁻ <i>endA1 glnV44 thi1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ80dlacZΔM15Δ (<i>lacZYA-argF</i>) U169, hsdR17(<i>r_K⁻m_K⁺</i>), λ^-.</i>	Woodcock et al. (1989); Grant et al. (1990)
<i>E. coli</i> DB3.1	F ⁻ <i>gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20 (<i>r_B⁻, m_B⁻) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1.</i></i>	Bernard & Couturier (1992)
<i>A. tumefaciens</i> C58C1 (pMP90)	Chromosomal-Rif ^r ; Helper plasmid pMP90 (pTiBo542DT-DNA) Gm ^r .	Koncz & Schell (1986)
<i>P. syringae</i> pv. <i>tomato</i> (DC 3000)	Spontaneous Rif resistant. Derivative of DC52 wild-type.	Cuppels (1986)

3.4.2 Bacteria growth and glycerol stock preparation

Culturing and manipulation of bacteria was performed under strictly sterile conditions in the laminar flow cabinet. All media were autoclaved (20 min at 121 °C) prior to their use.

Cultivation of *E. coli* and *A. tumefaciens* was performed in Luria-Bertani (LB) liquid medium [1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl, pH 7.0

adjusted with 1 N NaOH] (modified from Bertani, 1951). Furthermore, *E. coli* and *A. tumefaciens* were also grown on LB solid medium, for which the LB medium was supplemented with 1.5 % (w/v) Bacto™ Agar (DIFCO).

Pseudomonas syringae pv *tomato* DC 3000 (*Pst*DC3000) was grown using liquid King's B Medium [1 % (w/v) protease peptone # 2(DIFCO), 1.5 % (v/v) glycerol, 0.15 % (w/v) K₂HPO₄, pH 7.5 adjusted with 1 M HCl, after autoclaving was added 0.5 % (v/v) 1 M MgSO₄ filter sterilized (0.45 µm)] (Cold Spring Harb Protoc 2009). When *Pst*DC3000 was grown on plates the medium used was solid LB.

Depending on the resistance conferred by the bacterial strain the culture media were supplemented with the appropriated selective antibiotic/s (25 µg ml⁻¹ streptomycin for *E. coli* DB3.1, 50 µg ml⁻¹ rifampicin (in DMSO) for *A. tumefaciens* and 75 µg ml⁻¹ rifampicin (in DMSO) for *Pst*DC3000). Furthermore, when transformed bacteria were cultured the used medium was supplemented with the selective antibiotic encoded by the plasmid (100 µg ml⁻¹ ampicillin, 50 µg ml⁻¹ kanamycin) (See Section 3.8). All antibiotics were sterilized by filtration before usage (0.45 µm) and diluted according the given final concentrations. To ensure the quality of media reagents used and to exclude the presence of contaminations, a negative control of the solid and liquid culture containing the appropriate antibiotic but no bacteria inoculum was included in each experiment.

For the long-term preservation of transformed or non-transformed *E. coli*, *A. tumefaciens* or *Pst*DC3000 clones glycerol stocks were prepared. For this purpose, 4 ml of LB medium containing antibiotics were inoculated with the desired bacterial culture and incubated overnight at 37 °C (200 rpm) for *E. coli* and 28 °C (180 rpm) for *A. tumefaciens* and *Pst*DC300. Afterwards, 500 µl of the culture were mixed with 500 µl of sterile storage medium [65 % glycerol, 25 mM Tris-HCl, pH 8.0 adjusted with 1 M HCl, 100 mM MgSO₄ (sterilized by filtration (0.45 µm) and added after autoclavation)]. Glycerol stocks were snap frozen in liquid N₂ and stored at -80 °C.

3.4.3 Plant material and growth conditions

The present work was mainly conducted using the plant species *Arabidopsis thaliana* (L.) Heynh. The analyses were carried out using the wild-type Columbia ecotype (Col-0), the constitutive overexpression mutant lines *35S::YUCCA8* (YUC8ox) and *35S::YUCCA9* (YUC9ox) (Hentrich et al. 2013a), the T-DNA insertion lines *yuc8* and *yuc9*, the double T-DNA mutant line *yuc8yuc9* (Hentrich et al. 2013a), and the single, double and triple T-DNA insertion lines *myc2*, *myc3*, *myc4*, *myc2myc3*, *myc2myc4*, *myc3myc4* and *myc2myc3myc4* (Fernandez-Calvo et al. 2011). All mentioned transgenic lines are generated in the Col-0 genetic background.

For sterile plant growth, seeds were surface-sterilized as follows: 70 % ethanol 2 min (2 min centrifugation at 12000 rpm), 5 % sodium hypochlorite 5 min (2 min centrifugation at 12000 rpm), and then rinsed three times with autoclaved H₂O. Afterwards, sterilized seeds were plated on sterile solidified half-strength Murashige and Skoog (MS) medium (Murashige & Skoog 1962) (Murashige & Skoog 1962), supplemented with 1 x MS vitamins mix (Duchefa), 1 % (w/v) sucrose, and 0.6 % (w/v) phyto-agar. Petri dishes containing seeds on MS medium were sealed with millipore tape and placed for 2 days at 4 °C. After 48 h stratification at 4 °C in darkness to synchronize germination, plants were grown under 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ cool-white fluorescent light at 22 °C with a 16:8 h (light/dark) photoperiod in controlled plant germination chambers.

When mature plants were needed, 10 day-old seedlings were transferred to plastic pots containing a 3:1 mixture of peat and vermiculite. After transplantation seedlings were covered with a clear plastic wrap for approximately 1 week, in order to maintain high humidity and prevent seedlings from transplantation shock. Plants were grown for 3 to 4 weeks or until inflorescence stems was fully developed in controlled environment chambers (22 °C, 16 h light/8 h dark and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity).

For *P. syringae* (PstDC3000) infection assays 15 day-old seedlings grown on MS medium in controlled plant germination chambers (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, at 22 °C) with a 16:8 h (light/dark) photoperiod were used.

For *Arabidopsis* mesophyll protoplast isolation, sterilized seedlings were directly germinated in pots containing soil (3 peat /1 vermiculite) for 4 to 5 weeks. Plants were grown under the same light intensity and temperature conditions described above, but with 8 h day and 16 h dark photoperiod.

In addition, part of this work made use of the plant species *Nicotiana benthamiana* (L.). Seedling were provided and germinated by the greenhouse service of the Centre for Plant Biotechnology and Genomics (CBGP) greenhouse service. 14 day-old seedlings were transplanted to independent 13 cm diameter pots containing peat-based soil. Plants were grown under controlled greenhouse conditions (25 °C and 40-65 % relative humidity) and a long-day photoperiod (16 h light/ 8 h dark) for 2 to 3 weeks.

3.4.4 Methyl jasmonate treatment

Induction of *YUCC8* and *YUCC9* prior to the analysis of gene expression by qRT-PCR or the quantification of free IAA levels (See Sections 3.7.11 and 3.9.6) was performed according to Hentrich et al. (2013a). Thus, ten days-old *Arabidopsis* seedlings (See

Section 3.4.3) were transferred to either an induction solution of the following composition: 50 μ M MeJA (Sigma-Aldrich), solved in liquid MS media containing 0.5 % MeOH (v/v), or a control solution (liquid MS media, 0.5 % MeOH (v/v)), and incubated at room temperature (RT) for the indicated period of time.

3.4.5 Plant cell wall isolation

Inflorescence stem of 16 cm and 24 cm height, were frozen in liquid nitrogen (N_2) and ground with 5.5 mm stainless steel balls (Retsch) on a Retsch MM300 TissueLyser (Qiagen) (25 Hz, 2.30 min at 4 °C). For lignin assays cell wall material was isolated by removing soluble components with consecutive washes with: dH_2O (750 rpm shaking, 30 min at 98 °C), 70 % ethanol (750 rpm shaking, 30 min at 76 °C), 1:1 v/v chloroform:methanol (750 rpm shaking, 30 min at 59 °C), and 100 % acetone (750 rpm shaking, 30 min at 54 °C). For glucose and extracellular polysaccharides evaluations cell wall isolation was carried out by washing at RT with 70 % ethanol, 1:1 v/v chloroform:methanol, and 100 % acetone. After the isolation processes, the alcohol-insoluble residues (AIR) were dried under vacuum (45 min at 30 °C).

3.4.6 Stem tissue fixation and sectioning

The first 10 cm, immediately above the rosette, of independent inflorescence stems were cut off in 1 cm pieces in separated microcentrifuge tubes. Fixation was carried out by embedding stem pieces in 10 ml of 4 % paraformaldehyde in 10 mM phosphate buffered saline (PBS) [137 mM, NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , pH 7.4 adjusted with HCl 37%]. To prepare this solution, 100 ml of PBS were heated at 60 °C, while stirring, under a fumehood. Subsequently, 4 g of paraformaldehyde (Sigma-Aldrich) were added and dissolved by raising the pH with NaOH (1 N) until solution clears. Afterwards, paraformaldehyde solution was filtered and the pH was adjusted to 6.9 with HCl 37 %. Embedded samples were then vacuum infiltrated (1.5 min, 400 mmHg) to assist paraformaldehyde penetration and incubated overnight at 4 °C. Finally, samples were washed two times with 10 mM PBS to completely remove paraformaldehyde and stored at 4 °C in 10 mM PBS (pH 7.2) until sectioning (Nagar et al. 1995).

Afterwards, fixed tissues were half-embedded vertically into 7 % agarose (Conda), in sterile H_2O water, before sectioning. Each agarose block contained four to five, inflorescence stem pieces from independent biological replicates. Then, inflorescence stems 1 cm tall pieces were sliced in transversal 100 μ m thick cross-sections using a Vibratome 1000 plus (Technical Products International) according the following parameters: 50 Hz frequency, program 5 speed, program 4 amplitude.

Collected sections were transferred to a new microcentrifuge tube containing 10 mM PBS (pH 7.2) and stored at 4 °C until used for histochemical analysis.

3.5 Biotic and abiotic stress assays

3.5.1 Plant-Arthropod interactions

The *Tetranychus urticae* (Kock) spider mite colony used in this experiments was kindly provided by Isabel Díaz (CBGP, UPM). The mite colony was raised on bean plants (*Phaseolus vulgaris*, cultivar “California Red Kidney”, Stokes, Thorold, Ontario, Canada) in growth chambers at 24 °C, 60 % relative humidity and with a 16 h light / 8 h dark photoperiod, for more than 100 generations.

For plant damage assays 10 adult female mites, isolated from infested bean plants, were carefully placed on leaves of 3 to 4 week-old *A. thaliana* plants (See Section 3.4.3). A total of 10 plants for each genotype were used for spider mite infestation. Beside this, 4 plants from each genotype were maintained as treatment control. The mites were allowed to feed for 4 days in growth chambers (24 °C, 60 % relative humidity with a 16/8 h (light/dark regime)). After 3 days, whole rosettes of the infested and control plants were scanned (HP Scanjet 5590 Digital Flatbed Scanner) at a resolution of 1200 dpi. Leaf injury was calculated as mm² of affected tissue using Adobe Photoshop CS (Adobe Systems, San Jose, CA) software according to (Cazaux et al. 2014). Based on this protocol, scanned leaves were overlaid with a grid of 0.25 mm x 0.25 mm and the damaged area was covered with red dots of known pixel size (52 pixels) within grid units for which there was damage covering more than half of the grid units. Finally, total area damage was calculated according the formula:

$$\text{Area damage [mm}^2\text{]} = \text{number of dots} \times 0.25 / 0.25 \quad [1]$$

Here, the number of dots was calculated from the total number of pixels (derived from the histogram tool) divided by the number of pixels per dot (52 pixel/dot). Plant damage was assessed in five infested independent samples from each genotype.

3.5.2 *Pseudomonas syringae* infection assay

This experiment was realized using the hemibiotrophic bacteria *Pseudomonas syringae* pv *tomato* DC 3000 (*Pst*DC3000) virulent strain DB4H2, which was kindly provided by Dr. Emilia Solanilla (CBGP, UPM). For plant infection analysis, 15 day-old seedlings, were inoculated with *Pst*DC3000 using the flooding method described by Ishiga et al. (2011). In brief, a single colony of *Pst*DC3000 from a selection plate

was inoculated in 5 ml of liquid KB medium (See Section 3.4.2), containing 75 $\mu\text{g ml}^{-1}$ rifampicin (Duchefa). The pre-culture was incubated at 28 °C overnight with continuous shaking (200 rpm). Then, 100 μl of the pre-culture were used to inoculate 100 ml of liquid KB medium, which was incubated again at 28 °C over 16-24 h under continuous shaking (180 rpm). After the incubation, log-phase cultures OD_{600} (0.6-1) were transferred to a 50 ml centrifuge tube and centrifuged at 5000 rpm for 10 min using a JLA-16.250 rotor (Avanti J-26XP, Beckam Coulter). Supernatants were discarded and the obtained pellets were resuspended in 10 ml filter sterilized (0.45 μm) 10 mM MgCl_2 to enhance bacterial motility and adhesion. Afterwards, bacteria suspension was diluted to an OD_{600} of 0.01 (5×10^6 , colony-forming units ml^{-1}) with sterile deionized H_2O , supplemented with 0.02 % of the surfactant Tween®20 (Sigma-Aldrich). A total of 40 ml of this bacterial suspension was then transferred into petri plates containing the seedlings. The inoculated plates were incubated for 3 min at RT, before the bacteria solution was removed. To provide an adequate experimental control, independent petri plates containing seedlings of each genotype were treated with sterile H_2O along the same time of 3 min. After the infection, all seedlings were incubated under controlled conditions (22 °C, 16 h light/8 h dark and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity) for 3 days.

Seedlings immune response was evaluated by measuring pathogen growth levels in the leaf tissue. This procedure was performed based on Liu et al. (2015). According with this protocol, the leaves 3 and 4 from two different seedling were harvested 3 days after infection and surface sterilized in 1 ml of 70 % ethanol for 1 min. Subsequently, they were placed in microcentrifuge tubes and homogenized in 500 μl sterile 10 mM MgCl_2 . After homogenizing, 20 μl from the bacterial suspension were used to prepare a series of dilutions (1:10, 1:100, 1:1000; 1:10000; 1:100000; 1:1000000). These dilutions were then plated on LB medium (See Section 3.4.2) containing 75 $\mu\text{g ml}^{-1}$ rifampicin (Duchefa). Colony numbers were counted after incubating the plates at 28 °C for 2 days and the number of colony forming units was calculated using the following equation:

$$\text{colony forming units [cfu/leaf]} = \frac{T \times 10^R}{20} \times \frac{500}{2} \quad [2]$$

Where T is the number of colonies within each technical replicate and R the dilution number. The experiment was repeated twice, independently, obtaining similar results. Six plants per genotype were used in each infection assay. Similarly, six plants from each genotype were used as control. All infections were performed during morning hours. Pictures of disease symptoms after 3 days post bacteria inoculation were taken with a digital Canon EOS 600D.

3.5.3 Water deprivation analysis

3.5.3.1 Drought stress tolerance assay

For drought stress treatment, fifteen 10 day-old seedlings of *Arabidopsis* were transplanted to (8 cm x 8 cm x 8 cm) plastic pots containing a 3:1 mixture of peat and vermiculite and transferred to plant growing chambers (See Section 3.4.3). Before transplanting, each pot was filled with the same amount of soil mixture and equally watered to ensure the same amount of water at the beginning of the experiment. To avoid transplantation shock, the seedlings were covered with a transparent plastic wrap for approximately 1 week immediately after transplantation. When the cover was completely removed, each pot was watered daily with 40 ml of water for 1 week. Then, irrigation was stopped for 15 days before being resumed for another 10 days applying the same conditions as before. To provide an experimental control, a group of five plants of each line were irrigated without interruption. The survival rate of each genotype was calculated by counting the numbers of green, healthy plants. Similar results were obtained in two independent experiments.

3.5.3.2 Evaluation of relative water content

Relative water content (RWC) was determined according to a modified protocol published by González & González-Vilar (2003), using 3 week-old soil grown *Arabidopsis* plants (See Section 3.4.3). For each genotype fifteen whole rosettes were detached and immediately weighed to obtain the fresh weight (FW). Then, the harvested rosettes were hydrated by immersing them in 10 ml of water for 4 h at 4 °C in the dark. After hydration, the excess of water was removed from the plant surface and samples were again weighed to obtain the turgid weight. Subsequently, rosettes were dried at 60 °C in an oven and weighed again at different time points (2, 4, 6, 8 and 24 h). The RWC was calculated as follows (Barrs & Weatherley 1962):

$$\text{Relative water content} = [(FW - DW)/(TW - DW)] \times 100 \quad [3]$$

Where FW is the initial fresh weight, TW is turgid fresh weight and DW is dry weight.

3.6 Computational assays

3.6.1 *In silico* analysis of *YUCCA* promoter sequences

The complete nucleotide sequences of both *YUCCA8* and *YUCCA9* from *A. thaliana* genes were used in queries utilizing the basic local alignment search tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul et al. 1990), in order to identify the orthologous genes in *A. lyrata* L. subsp. *lyrata* O’Kane & Al-Shelbaz. To analyse the degree of promoter conservation, the full-length 5'-non-coding sequences of the *A. thaliana* *YUCCA8* and *YUCCA9* genes, as well as those of the *A. lyrata* homolog genes, were used to construct pairwise sequence alignments using the EMBOSS Matcher software (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) (Rice et al. 2000) provided by the European bioinformatics institute (EMBL-EBI).

MYC2 binding motifs in the promoter sequences were predicted by running target promoter sequences against known *cis*-regulatory elements in the AtPan collection (<http://atpan.itps.ncku.edu.tw/>) (Chen et al. 2012) and PlantCate (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot et al. 2002) database. All promoter sequences used in this work were retrieved from the NCBI gene database (<https://www.ncbi.nlm.nih.gov/gene/>) using the corresponding gene accession numbers: At4g32450 (*YUC1*), At4g13260 (*YUC2*), At1g04610 (*YUC3*), At5g11320 (*YUC4*), At5g43890 (*YUC5*), At5g25620 (*YUC6*), At2g33230 (*YUC7*), At4g28720 (*YUC8*), At1g04180 (*YUC9*), At1g48910 (*YUC10*), At1g21430 (*YUC11*).

3.6.2 *In silico* expression analysis

YUC8 (At4g28720) and *YUC9* (At1g04180) e-northern analyses were conducted at the e-norther/Expression Browser tool of the Botany Array Resource (BAR) (http://bar.utoronto.ca/affydb/cgi-bin/affy_db_exprss_browser_in.cgi) (Toufighi et al. 2005). The gene expression levels in root and shoot tissues were analyzed in the *Arabidopsis* WT (Col-0) under control and drought at treatment durations of 0.25, 0.5, 1, 3, 6, 12 and 24 h.

3.7 Molecular biological methods

3.7.1 Purification of plasmid DNA from *Escherichia coli*

The isolation of plasmid DNA from *E. coli* was carried out using the commercial Wizard® Plus Minipreps DNA Purification System (Promega), which is based on the alkaline lysis protocol (Bimboim & Doly 1979; Green & Sambrook 2012). Here, the principle is the ability of silica resins to bind alkaline extracted plasmid DNA under high salt conditions. According to the manufacturer’s recommendations, a single colonies of transformed *E. coli* cells was transferred into a 4 ml LB culture

containing appropriate selective antibiotics, and incubated overnight at 37 °C/200 rpm. After the incubation, 1.5 ml of the culture were transferred to a microcentrifuge tube and the contained bacteria were harvested by centrifugation at speed of 8000 rpm at room temperature for 5 min. This step was repeated twice and the final bacterial pellet was re-suspended in 250 µl of the supplied cell re-suspension solution. Bacteria were then lysed by adding 250 µl of the cell lysis solution and repeated inversions. When the suspension turned clear, 10 µl of alkaline protease solution were added to the reaction and incubated 4 min, to inactivate endonuclease and other proteins released during the lysis process. Bacterial proteins and chromosomal DNA were then precipitated by the addition of 350 µl of neutralization solution. Subsequently, cell debris and precipitated cell components were removed by centrifugation at 11000 rpm at room temperature for 10 min. Afterwards, the supernatants were transferred to the provided spin columns containing the silica resin and subjected to centrifugation at 13000 rpm for 60 s. The salt concentration was decreased by washing the columns with 750 µl of column wash solution, diluted in 95 % ethanol, followed by a centrifugation at 13000 rpm for 2 min. This step was repeated using 250 µl of column wash solution. Finally, the plasmid DNA was eluted from the column with 50 µl deionized autoclaved H₂O. The resulting DNA concentration and purity was determined by spectrophotometry at a wavelength of 260 nm (See Section 3.7.5). Purified samples were stored at -20 °C until they were used or they were directly used for cloning, restriction digest or sequencing purposes.

3.7.2 Preparative purification of plasmid DNA from *E. coli*

In case that a high DNA concentration was needed, the desired plasmids were purified according the manufacturer's instructions using the QIAGEN® Plasmid Maxi Kit. Briefly, a single colony of *E. coli* from an antibiotic selective plate was inoculated in a 5 ml LB starter liquid culture maintaining the appropriate selective pressure. One hundred microliter of the overnight grown pre-culture (37 °C/200 rpm) were used to inoculate 250 ml LB liquid medium in a 1 l conical flask, also containing the selective antibiotic(s). After an overnight incubation under the previously described conditions, the culture was centrifuged for 10 min at 4 °C/4000 rpm in a JLA-16.500 rotor (Avanti J-26XP, Beckam Coulter). The resulting bacterial pellet was re-suspended in 10 ml of pre-chilled buffer P1 containing 100 µg ml⁻¹ RNase, and cells were lysed by adding 10 ml of buffer P2 through mixing of the solution by inversion. Neutralization was then achieved by adding 10 ml of buffer P3. The precipitated cellular components were separated by centrifugation for 30 min at 4000 rpm and 4 °C. After that, the supernatant was transferred into a provided

column previously equilibrated with 10 ml of buffer QBT, and plasmid DNA was retained by allowing the supernatant enter in the resin by gravity-flow. Possible contaminants were removed by two consecutive washing steps with 30 ml buffer QC, containing 95 % ethanol. By adding 15 ml of buffer QF to the resin the plasmid DNA was eluted. The DNA was then precipitated by the addition of 10.5 ml isopropanol followed by centrifugation at 13000 rpm and 4 °C for 30 min in a JA 25.50 rotor (Avanti J-26XP, Beckam Coulter). The pellet was washed with 70 % ethanol, re-centrifuged at 13000 rpm for 5 min at room temperature, allowed to air dry and finally re-suspended in 300 µl of autoclaved deionized H₂O. Subsequent to the extraction, the DNA content and quality were quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific) (See Section 3.7.5). Purified samples were either directly used or stored at -20 °C until further use.

3.7.3 Polymerase chain reaction (PCR)

The selective amplification of DNA fragments was performed by standard PCR in a total volume of 50 µl containing: 2.5 U of Platinum® Taq DNA Polymerase High Fidelity (Thermo Scientific), which is composed of a mixture including a DNA proofreading activity enzyme (exonuclease activity 3' → 5') and a Taq (*Thermus aquaticus*) polymerase, 5 µl of the supplied buffer, 50 mM of MgSO₄, 0.2 µM of each of the two specific primers, 20 mM of dNTP, 10-50 ng of the DNA template, and autoclaved deionized H₂O. The primer sequences used are listed in Table 1 (See Section 3.3.1). PCRs were carried out in a peqSTAR (PEQLAB) thermocycler. The conditions for amplification were as follows: an initial single DNA denaturation step at 94 °C for 2 min and 30 reaction cycles including a denaturation step at 94 °C for 20 s, a primer annealing step for 45 s, and an elongation step at 72 °C for 1 min per kbp of expected product. Then, a final extension step at 72 °C for 5 min was added. For subsequent cloning of the PCR product into pGEM-T vectors (See Section 3.8.1), the final extension step at 72 °C was prolonged to 30 min in order to generate 3'-A-tailed PCR fragments. The annealing temperature of oligonucleotides was adjusted to the T_m of the used primers (annealing temperature = T_m - 5 °C). To ensure quality of the PCR reagents used, a negative control of the PCR mix containing no template was used for each amplification. PCR products were directly analysed by agarose gel electrophoresis (See Section 3.7.4) or stored at -20 °C until further use.

3.7.4 Denaturing agarose gel electrophoresis of DNA

In first instance, amplified PCR products (See Section 3.7.3) were analysed by denaturing agarose gel electrophoresis. Electrophoresis gels were prepared by

melting 0.8 - 1.2 % (w/v) agarose in 1 x TAE buffer [40 mM Tris/HCl pH 7.5, 20 mM sodium acetate, 1 mM EDTA, 50 $\mu\text{g } \mu\text{l}^{-1}$ ethidium bromide]. The amount of agarose was chosen with respect to the size of the DNA fragment to be separated. After the agarose solidified again, gels were submerged into running buffer (1 x TAE) and mounted in a Bio-Rad horizontal electrophoresis cell. Prior to gel loading, samples were diluted 1:3 with DNA loading dye buffer [50 % (v/v) glycerol, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol], and loaded into the wells. The molecular weight marker GenRuler™ 1kb DNA Ladder (Thermo Scientific) was added to one lane, in order to estimate the approximate size and quantity of the nucleic acid fragments. Electrophoresis was carried out run for approximately 45 min with constant voltage (80-100 V) using a Bio-Rad PowerPac mini power supply. After running the gels, DNA fragments were visualized using a short wavelength UV transilluminator (Gel DOC XR+System (BioRad)), photographed using a CCD camera and printed using a Sony UP-895MDW paper printer for documentation.

3.7.5 Purification of PCR products

For cloning purposes, the PCR products (See Section 3.7.3) were purified using the NucleoSpin® Extract II kit according the manufacture's instructions (Macherey & Nagel). Briefly, 50 μl of the amplified DNA was dissolved in 100 μl of the high-salt buffer NT. Afterwards, the sample was applied to a column containing a silica membrane and centrifuged for 1 min at 8900 rpm. Contaminants and non-bound compounds were removed by a subsequent washing step with 600 μl of the buffer NT3, containing ethanol. Samples were then centrifuged for 1 min at 8900 rpm, to allow the buffer entering into the column, and finally centrifuged for 2 min at 8900 rpm to completely dry the membrane. Fragments were eluted from the column using the alkaline buffer NE [5 mM Tris/HCl, pH 8.5]. After purification, the concentration and purity of the obtained DNA was assessed using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Absorbance was measured at wavelengths 230, 260 and 280 nm. Nucleic acid concentration was calculated according to the absorbance at 260 nm. The 260/230 nm and 260/280 nm absorbance ratios were used to estimate DNA quality. Samples with absorbance ratio values below 1.8 were not used for further experiments, due to insufficient purity. In addition, samples was analysed by agarose gel electrophoresis, before the DNA fragments were directly used or stored at -20 °C.

3.7.6 Enzymatic digestion of DNA

Restriction digests were performed by incubating 1 μg plasmid DNA with 5 to 10 U of the corresponding restriction endonuclease (Fermentas GmbH, New England

Biolabs or Takara-Clontech) and the specific buffer, in a final 25-50 µl reaction volume, at the appropriate temperature, usually 37 °C, for at least 1 h. After incubation, the reaction was inactivated by incubating samples at 65-80 °C for 20 min. For double enzyme digestions with non-compatible buffers, sequential digest were performed. Digested DNA fragments were directly analysed by agarose gel electrophoresis (See Section 3.7.4), and the desired bands were then excised from the gel using a scalpel and purified essentially as described in Section 3.7.5. However, prior to the DNA cleanup, the gel pieces containing the restriction fragments were submerged in 100 µl of NT buffer and heated at 50 °C until the agarose was completely melted (5 to 10 min).

3.7.7 Isolation of total RNA

Arabidopsis RNA was isolated from ten day-old seedlings (See Section 3.4.3). Aliquots of 100 mg were ground with mortar and pestle in liquid N₂ and stored at -80 °C until samples processing. Total RNA extraction was performed using the phenol:chloroform method, coupled to lithium chloride precipitation, modified from (Box et al. 2011). According to this procedure, the homogenized tissue was resuspended in 600 µl of extraction buffer [0.2 M Tris/HCl pH 9, 0.4 M LiCl, 25 mM EDTA, 1 % SDS]. After resuspension, 600 µl of phenol, equilibrated with 10 mM Tris/HCl, pH 8, (Carl Roth) were added. The aqueous phase containing the RNA was separated from the organic phase, which contains denatured proteins and DNA, by centrifuging 2 min at 13000 rpm. In order to improve RNA recovery, one additional step of phenol extraction was performed. Posteriorly, 600 µl of chloroform were added to the collected aqueous phase and the mixture was newly subjected to centrifugation for 2 min at 13000 rpm. Samples were then incubated 2 h at 4 °C with 1/3 volumes of 8 M LiCl and subsequently centrifuged for 30 min at 4 °C. After this, samples were resuspended in 300 µl sterile deionized H₂O, 3 µl of 3 M sodium acetate (pH 5.2), and 600 µl of 100 % pre-chilled ethanol, and were incubated overnight to allow RNA precipitation. Afterwards, the precipitated total RNA was rinsed with 80 % ethanol, air-dried and resuspended in 20 µl RNase-free water and stored at -80 °C until its use.

3.7.8 Spectrophotometry and denaturing agarose gel electrophoresis of RNA

In first instance, the quantity and quality of the extracted total RNA (See section 3.7.7) was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Absorbance was measured at the wavelengths 230, 260 and 280 nm. Nucleic acid concentration was calculated using 1 µl of the RNA sample, according to the absorbance at 260 nm. The 260/230 nm and 260/280 nm absorbance ratios were

used to estimate RNA quality. Samples with absorbance ratio values below 1.8 were not used, since they were considered to contain substantial amounts of contaminants that absorb at 280 nm, e.g. proteins, or at 230 nm, e.g. carbohydrates, EDTA, guanidine isothiocyanate, and phenol.

The quality of the isolated RNA (See section 3.7.7) was further evaluated by observing the integrity of 28S and 18S ribosomal RNA bands in a denaturing gel electrophoresis. For this analysis, 2 µg of the isolated RNA, from each sample, were diluted in 3 volumes of RNA denaturing buffer [25 mM MOPS/NaOH pH 7.0, 6.25 M NaAc, 1.25 EDTA, 62.5 % (v/v) deionized formamide, 1.14 M formaldehyde, 50 µg µl⁻¹ ethidium bromide]. This mixture was incubated for 10 min at 70 °C and then briefly cooled on ice to ensure RNA secondary structures denaturation. Afterwards, samples were loaded onto 1.2 % (w/v) agarose gel [20 mM MOPS/NaOH pH 8.0, 1.25 mM EDTA, 6.25 mM NaAc, 6 % (v/v) formaldehyde] with 2 µl loading dye orange G (Leica), submerged into the correspondent running buffer [20 mM MOPS/NaOH pH 7.0, 3.7 % (v/v) formaldehyde]. The gel was run for approximately 30 min with constant voltage (70 V) prior to visual inspection under UV light using a Gel DOC XR+System (BioRad).

3.7.9 mRNA purification from total RNA

Due to the low abundance of mRNA molecules (1-5 %) in the total cellular RNA pool, and the existence of rare mRNA species. Prior to transcriptomic analysis of *YUCCA9* by quantitative real-time polymerase chain reaction (qRT-PCR), the polyA⁺ mRNA was additionally purified from the isolated total RNA (See Sections 3.7.7) using the Oligotex mRNA Mini Kit (QIAGEN). This purification procedure is based on the capacity of the polyadenylated mRNA tails to hybridize under high salt concentration with dT₃₀ oligomers, which are coupled to solid polystyrene-latex particles (Oligotex). By passing the polyA mRNA containing solution over the column the mRNA can be purified by association with the immobilized polydT oligomers. The extraction was performed following the manufacturer's instruction. For this, 80 µl of the extracted total RNA, from each independent sample, were adjusted to 250 µl with RNase free H₂O and immediately mixed with 250 µl of OBB buffer and 15 µl of Oligotex. The suspension was then incubated 3 min at 70 °C for 10 min at 25 °C and finally centrifuged over 2 min at 13000 rpm. The resulting pellet was resuspended in OW2 buffer, transferred to the supplied column and centrifuged twice (1 min, 13000 rpm). Finally, 60 µl of pre-warmed (70 °C) OEB buffer were added to the column and the mRNA was eluted by centrifugation (1 min,

13000 rpm). Samples containing polyA⁺ mRNA were stored at -80 °C until they were used.

3.7.10 First strand cDNA synthesis

Isolated total RNA and mRNA (See Sections 3.7.7 and 3.7.9) samples were reverse-transcribed into complementary DNA (cDNA) using the RNA-dependent DNA polymerase from Moloney Murine Leukemia Virus (M-MLV) with intrinsic reverse transcriptase activity. The M-MLV reverse transcriptase is characterized by a weak RNAase H activity and is, hence, adequate to avoid degradation of long RNA templates during cDNA synthesis. To this end, 2 µg of the total RNA or the polyA⁺ mRNA were incubated for 15 min at 48 °C with 1 µl (1 µg) of oligo dT₁₈-primer (Promega) and RNase-free water (to complete a final reaction volume of 17.25 µl). This first step allows the formation of polyA⁺ RNA-oligo dT hybrids. After incubation, 7.75 µl of a mix containing, 5 units of M-MLV reverse transcriptase (Promega), 5 µl of the M-MLV reaction buffer [50 mM Tris/HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT], 1 mM of dNTPs, 0.5 units of RNA inhibitor (RNasin) (Takara) and autoclaved deionized water, were added to the mixture. Immediately, samples were incubated for 1 h at 42 °C and diluted 1:1 with sterile RNase-free water. Subsequently, generated RNA-cDNA hybrid were incubated for 5 min at 75-90 °C and stored at -20 °C, until they were used to amplify by PCR (See Sections 3.7.3, 3.7.4 and 3.7.5) or in (q)RT-PCRs (See Section 3.7.11).

3.7.11 qRT-PCR analysis

Quantitative real time RT-PCR was carried out utilizing the ready to use PCR mix LightCycler® 480 SYBR Green I Master (Roche). This master mix is composed of the FastTaq polymerase, the corresponding reaction buffer, dNTPs (with dUTP instead of dTTP), MgCl₂, and the SYBR Green dye. The qRT-PCR method is based on the ability of the SYBR Green fluorescent reporter to intercalate into double-stranded DNA. Accordingly, it is possible to monitor the amount of DNA amplified by a polymerase after each PCR cycle since the fluorescence signal evoked by the dye increases in direct proportion to the contained DNA. Following the instruction manual, the PCR reaction mixtures contained 3.5 µl of the cDNA synthesized templates (See Sections 3.7.10), the LightCycler® 480 SYBR Green I master mix and 1 µM transcript-specific primer pair, in a final volume of 25 µl. In Table 1, the utilized oligonucleotide sequences are listed (See Section 3.3.1). The PCR was initialized by activating the hot-start polymerase at 95 °C for 5 min. Transcript amplification was performed over 45 cycles. Each cycle included a denaturation step at 95 °C for 10 s, an annealing step at 60 °C for 20 s, and a final extension step at 72 °C for 30 s. In

order to analyse the melting dynamic of the amplified products, a dissociation step was added at the end of the last extension phase [5 s at 95 °C, 1 min at 65 °C, continuous 97 °C and 30 secs at 40 °C]. qRT-PCRs were performed using a LightCycler® 480 (Roche) thermocycler. For data accuracy, three independent biological repeats were loaded in triplicates into a LightCycler® 480 Multiwell Plate 96 (Roche). To exclude the presence of primer dimers and contaminations, control samples containing no template were included in each assay. *APT1* and *UBIQ10* were selected as the reference genes for data normalization (Czechowski et al. 2005). The threshold cycle number (C_T), defined as the PCR at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold, was used to calculate the relative gene expression levels according the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001; Schmittgen & Livak 2008):

$$\Delta C_T = C_T (\text{genen of interest}) - C_T (\text{normalization gene}) \quad [4]$$

$$\Delta\Delta C_T = \Delta C_T (\text{sample of interest or treated}) - \Delta C_T (\text{control sample or untreated}) \quad [5]$$

$$\text{fold change: } 2^{-\Delta\Delta C_T} \quad [6]$$

3.7.12 Microarray

3.7.12.1 Total RNA cleanup

For microarray analysis, the isolated total RNA (See Section 3.7.7) of 10 day-old seedlings was subjected to an additional cleanup and concentration using the RNeasy MinElute Cleanup Kit (Qiagen). This commercial kit is based on the capability of RNA to selectively bind to a silica-membrane in presence of guanidine-thiocyanate and ethanol. According to the manufacturer's instruction, 80 µl of RNase-free water were added to 20 µl of total RNA, to complete a volume of 100 µl. Then, 350 µl of RLT buffer and 250 µl of 100 % ethanol were added. Samples were transferred to the supplied RNeasy Mini spin columns and centrifuged for 15 s at 10000 rpm. After centrifugation, 500 µl of RPW buffer were added to the RNeasy Mini spin column and centrifuge again (15 s, 10000 rpm). Next, 500 µl of 80 % ethanol were added and columns were centrifuged for 2 min at 10000 rpm. To completely remove the ethanol an additional 5 min centrifugation step at 10000 rpm was performed. Finally, the RNA was eluted using 30 µl RNase-free water and samples were stored at -80 °C until subjecting it to microarray analysis.

3.7.12.2 Microarray generation

Preparation of cDNA, biotin-labelled cDNA and hybridization to the Affimetrix GeneChip® Arabidopsis ATH1 Genome Array was executed at the genomics service unit at the National Centre of Biotechnology (Madrid, Spain) [<http://www.cnb>.

csic.es/~genomica/]. Prior to the microarray analysis, the RNA integrity was assessed, to exactly measure the RNA concentration and to exclude possible genomic contaminations. Analyses were run on an Agilent 2100 Bioanalyzer (Agilent Technologies). Samples displaying RNA integrity numbers (RIN) above a value of 7 were considered adequate for further processing.

For the microarray analyses, Affymetrix ATH1 Gene Chips®, which contain probes of approximately 24000 different *Arabidopsis thaliana* genes, were employed. For each genotype three independent biological replicates were carried out. Microarray results were visualized using the FIESTA Viewer tool [<http://bioinfogp.cnb.csic.es/tools/FIESTA/>] (Oliveros 2007). Gene expression profiles were analysed setting the following arbitrary parameters: fold-change ≤ -2 or ≥ 2 and LiMMA p-value $< 0,05$. Subsequently, functional clustering of selected genes was achieved using the bioinformatics resource DAVID version 6.7 [<https://david.ncifcrf.gov/>] (Huang et al. 2009a; Huang et al. 2009b) by setting the classification stringency to medium. Comparative analysis and Venn diagrams to illustrate overlap of genes between genotypes was generated using the online tool VENNY 2.1, [<http://bioinfogp.cnb.csic.es/tools/venny/>] (Oliveros 2007). To inspect the function of the common YUC8ox and YUC9ox down-regulated genes, gene ontology (GOs) annotation analysis was conducted with the web-based tool and database agriGO [<http://bioinfo.cau.edu.cn/agriGO>] (Du et al. 2010). Singular enrichment analysis (SEA) was used for GO enrichment analysis. Default values including the Fisher statistical method and the Yekutieli (false discovery rate (FDR) under dependency) multi-test adjustment method were utilized for the advanced options. Significant GO terms (p-values < 0.05) were retrieved.

3.7.13 Protoplast isolation and transfection

Prior to protoplast isolation, all employed solutions were freshly prepared and autoclaved at 121 °C for 20 min. Only CaCl₂ and MgCl₂ were sterilized by filtration using a 0.45 µm filter. Polyethylene glycol (PEG) was not sterilized.

Mesophyll protoplasts were prepared from rosette leaves of 3 to 4 weeks-old vegetative *A. thaliana* (Col-0) plants (See Section 3.4.3) essentially according to the method of (Yoo et al. 2007; Alonso et al. 2009). This procedure is based on the direct introduction of DNA into protoplasts from *Arabidopsis* leaves released by enzymatic cell wall digestion. To comply with this goal, fresh enzyme solution [20 mM MES (pH 5.7 adjusted with 1 N NaOH), 20 mM KCl, 400 mM mannitol, 10 mM CaCl₂, 1.5 % (w/v) cellulase R-10 (Duchefa), 0.4 % (w/v) macerozyme R-10 (Duchefa)] was prepared as described in the following. Firstly, sterile MES, KCl, and mannitol were

mixed, heated for 3-5 min to 70 °C, and cooled down to RT (25 °C). Enzymes were then added and the solution was heated to 55 °C to inactivate DNases and proteases and to enhance enzyme solubility. Finally, the mixture was again cooled down to RT, the sterile CaCl₂ was added and the ready-to-use solution was transferred to a sterile Petri dish. After the preparation of the enzyme solution well-expanded *Arabidopsis* leaves, usually leaf numbers 5 to 7, were detached from the plant and the middle part was cut in 0.5-1 mm leaf strips using a razor blade. Leaf strips were immediately submerged into the enzyme solution (20 leaves for each 10 ml of enzyme solution), and digested at 25 °C in darkness for 10 h. After digestion, the enzyme/protoplasts solution was diluted with 10 ml W5 solution [2 mM MES (pH 5.7 adjusted with 1 N NaOH), 5 mM KCl, 154 mM NaCl, 125 mM CaCl₂], shook to allow protoplasts liberation from the leaves, and filtered through a nylon mesh with 60 µm pore size (NY6000010, Merck), to remove undigested leaf tissue. Protoplasts were then pelleted by centrifugation at 100 rpm in a JLA-16.250 rotor (Avanti J-26XP, Beckam Coulter), at room temperature for 2 min and re-suspended in 10 ml new W5 solution. This step was repeated once more, and subsequently protoplasts were incubated on ice for 5 h. During the recovery time the efficiency of the procedure was evaluated by checking the protoplasts number and morphology under the microscope using a 25 µl aliquot of the preparation.

Transfection of plasmid DNA was performed employing the PEG-calcium fusion protocol (Mathur & Koncz 1998; Yoo et al. 2007). Here, protoplasts were harvested by another gentle centrifugation step (see above). The supernatant was removed and the sedimented protoplasts re-suspended in 7 ml of MMg solution [4 mM MES (pH 5.7 adjusted with 1 N NaOH), 15 mM MgCl₂, 400 mM mannitol]. For transactivation, 9 µg of each reporter plasmid and 14 µg of the effector plasmid were used (See Sections 3.7.2). For normalization, 3 µg of the 35S::NAN plasmid (Kirby & Kavanagh 2002) were used (See Section 3.7.1). Plasmid mixtures were added in a total volume of 20 µl per 200 µl of protoplast suspension. Subsequently, 220 µl of freshly prepared PEG solution [40 % (v/v) PEG 4000 (Fluka), 100 mM CaCl₂, 200 mM mannitol] were added. After 25 min of incubation, the protoplast suspension was diluted with 800 µl of W5 solution, to relax the protoplasts and enable DNA diffusion into the cell. Next, the solution was centrifuged at 100 rpm for 1 min at RT. After centrifugation, the supernatant was discarded and the protoplasts were re-suspended in 250 µl WI solution [2 mM MES (pH 5.7 adjusted with 1 N NaOH), 20 mM KCl, 500 mM mannitol]. The protoplast solution was then incubated for 12-16 h at room temperature. Following the incubation, protoplasts were harvested by centrifugation (2 min, 100 rpm), the supernatant was removed,

and the sedimented protoplasts were snap frozen in liquid N₂ and stored at -80 °C until they were used for GUS and NAN reporter activities analysis.

3.7.14 Agroinfiltration

Transient expression analyses were performed using 5 to 6 week-old wildtype *N. benthamiana* plants essentially as described by Ma et al. (2012). This procedure is based on the ability of *A. tumefaciens* to transfer part of its DNA (transferred DNA or T-DNA), which is flanked by the right- and left-border sequences, into the host plant cell, where it becomes integrated into the genome of infected cells (Gelvin 2003). Briefly, the *Agrobacterium* strain carrying the desired construct was cultivated in 3 ml of LB supplemented with appropriate antibiotic(s) (See Sections 3.4.2). To enhance expression yields, an *A. tumefaciens* strain carrying the P19 suppressor of gene silencing from tomato bushy stunt virus (TBSV) was included in the experiment and cultivated in 6 ml of LB with the adequate selective pressure (See Section 3.4.2). After incubation for 24 h at 28 °C in and incubation shaker (180 rpm), the *Agrobacteria* were centrifuged for 10 min at 4000 rpm in a 5804R centrifuge (Eppendorf) and re-suspended in 2 ml or 3 ml of sterile agroinfiltration media (MA) [1 M MES/KOH pH 5.6, sterilized by filtration, 1 M MgCl₂] depending on the volume of the pre-culture. Subsequently, the OD was measured at 600 nm in a spectrophotometer (Amersham Biosciences) and necessary volumes of *Agrobacteria* containing the desired constructs and the P19 strain were calculated according the following equation:

$$V_{construct/P19} = n \times V_{final} \times 0.3 / OD_{600}(construct/P19) \quad [7]$$

Where $V_{construct/P19}$ is the volume needed for agroinfiltration, n is the number of infiltrated plants, OD_{600} is the optical density obtained and V_{final} is the volume of infiltration suspension used (3 ml). The final volume was adjusted to 2 ml with MA and then 1 ml of 450 µM acetosyringone (Sigma-Aldrich) was added to activate the *Agrobacteria* virulence region. For co-infiltration of three bacterial strains the cultures were mixed in a 1:1:1 ratio. Afterwards, the infiltration solution was incubated for 4 h at RT in darkness to allow bacteria to recover. Following the incubation, the leaves 3, 4 and 5 of each tobacco plant were agroinfiltrated with 3 ml of the bacterial solution using a 2.5 ml syringe. To enhance stomata opening and facilitate the entrance of the pathogen solution into the leaves, plants were watered 3 hour prior to bacteria inoculation. Plants were maintained under greenhouse condition (See Section 3.4.3) for at least 3 days. To ensure that the reporter activity was not false-positive, an *Agrobacterium* strain harboring no additional cons-

tract was included in each experiment as negative control. Results represent data obtained from three independent *N. benthamiana* plants per reporter construct.

3.8 Cloning procedures

3.8.1 Incorporation of PCR products in pGEM-T vectors

Amplified 3'-A-tailed PCR products (See Sections 3.7.3) were purified using the NucleoSpin® Extract II kit (See Section 3.7.5) and ligated into the pGEM®-T vector (Promega) (See Section 3.3.2). This high copy number-plasmid, carrying the *lacZ* gene and the T7 and SP6 RNA polymerase promoters flanking a multiple cloning site (MSC), was employed as an intermediate cloning vector.

The ligation method, mediated by the T4 DNA ligase enzyme, is based on the complementarity between the 3'-desoxyadenosine, produced by the Taq polymerase during the PCR product amplification, and the desoxythymidine residues contained at the 3'-terminal cloning site of the T-vector. The ligation was carried out according to the manufacturer's instructions, in a final reaction volume of 10 µl containing 1 x rapid ligation buffer (supplied), 1 U of T4 DNA ligase (Promega), 50 ng µl⁻¹ vector and autoclaved deionized H₂O. The PCR insert was added in a molar ratio of 3:1 of insert to vector. The appropriate insert concentration was calculated according to the equation:

$$\frac{ng\ of\ vector \times kb\ size\ of\ insert}{kb\ of\ vector} \times \frac{3}{1} = ng\ of\ vector \quad [8]$$

Ligation was carried out by incubating the reaction mixture at 4 °C overnight.

3.8.2 Ligation of restriction digest fragments

Ligation of restriction fragments into the pBT-10, pMDC-163 vectors and pSP-Entry1 (See Section 3.3.2) was carried out using the T4 DNA ligase kit (Invitrogen). The ligation process is based on the insert:vector annealing by restoration of the covalent phosphodiester bond, between the 3'-hydroxyl (OH) and the 5'-phosphate ends of endonuclease digested nucleic acid molecules, by the T4 ligase (Nelson & Cox 2011). To accomplish this goal, both vector and DNA inserts, were digested with the appropriate restriction enzymes and gel-purified (See Section 3.7.6). After purification of the DNA inserts, a 20 µl ligation reaction was prepared according to the provided instructions (Invitrogen): 1 U T4 DNA ligase (Invitrogen), 4 µl of 5 x Ligase Reaction Buffer [250 mM Tris/HCl pH 7.6, 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25 % (w/v) polyethylene glycol-8000 (PEG)] and autoclaved deionized water. Ligation was generally performed using a molar ratio of 3:1 of insert over

vector DNA. Insert amount was adjusted to 50 ng of vector (See Sections 3.8.1). Ligation was incubated at 16 °C for at least 16 h.

3.8.3 Generation of plasmid constructs using Gateway technology

The Gateway™ cloning technology is based on the bacteriophage λ mediated compatible *att*-site recombination reactions, which was utilized in the preparation of overexpression constructs for transient expression analyses. For this purpose, LR reactions, that serves to mobilize DNA fragment from the entry clones to the destination vectors through the recombination of *attL* $\times$ *attR* specific sites by the LR Clonase™, were performed. Thus, the target DNA fragments, previously cloned into the vector pSP-Entry1 (See Section 3.8.2) that contains the attL1-GW-attL2 cassette, were transferred into the destination vectors p35S-HA-GW and pEarlyGate-210, harbouring the attR1-GW-attR2 cassette by LR reactions with the Gateway® LR Clonase™ Enzyme mix according to manufacturer's instructions (Invitrogen). Briefly, to execute homologous recombination 100 ng of the entry clone were mixed with 100 ng of the desired destination vector and 2 μ l of the LR enzyme supplied buffer. The solution was adjusted to a total volume of 10 μ l with TE buffer pH 8.0 [10 mM Tris/HCl pH 8.0, 1 mM EDTA]. Subsequently, 2 μ l of LR clonase enzyme mix were added and the reaction mixture was incubated at 25 °C for 60 min. After the incubation, 2 μ l of Proteinase K solution (Invitrogen) was added and the samples were transferred to 37 °C for 10 min to eliminate LR clonase activity.

In case that the entry clone and destiny vector exhibited the same antibiotic resistance, a PCR amplification-based (PAB) method was performed prior to the LR recombination reaction. This technique increases the recombination efficiency by avoiding the entry clone antibiotic cassette during the LR reaction (Kumar et al. 2013). PCR reactions were performed in a volume of 50 μ l as detailed in Section 3.7.3. Specific primers that flank the attL sites of the entry vector were used for amplification [Table 1 (See Section 3.3.1)]. Cycling conditions for PCRs were as follows: an initial DNA denaturation step at 94 °C for 4 min, 35 cycles of a denaturation step at 94 °C for 20 s, a primer annealing step at 48 °C for 45 s, and an elongation step at 72 °C for 3.5 min, followed by a final extension step at 72 °C for 7 min. Subsequently, PCR products were purified (See Section 3.7.5) and employed for LR reactions as described above.

3.8.4 Generation of chemical competent *E. coli* cells

Chemically competent *E. coli* strain DH5 α cells (See Section 3.4.1) were prepared essentially as described by Inoue et al. (1990). Briefly, *E. coli* DH5 α from glycerol stocks were plated on sterile LB plates and incubated overnight in an incubator at

37 °C. Then, single colonies were grown overnight in 4 ml of sterile LB liquid culture, in an incubator shaker overnight at 200 rpm and 37 °C. Thereafter, 100 µl of the pre-culture were used to inoculate 250 ml of sterile SOB media [2 % (w/v) tryptone, 0.25 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, adjusted to pH 7 with 1 N NaOH, after autoclaving 10 mM MgCl₂ and 10 mM MgSO₄ sterilized by filtration (0.45 µm) were added (Hanahan 1983)] contained in 1 l Erlenmeyer flasks. The culture was incubated under constant agitation (110 rpm) at 25 °C until the cells reached mid-log phase (OD₆₀₀ 0.55-0.64). Subsequently, the bacterial culture was cooled on ice for 10 min and the bacteria cells were harvested by centrifugation for 10 min at 4000 rpm and 4 °C using a JLA-16.250 rotor (Avanti J-26XP, Beckman Coulter). Afterwards, the supernatant was discarded and the bacterial pellet was re-suspended in 80 ml of pre-chilled sterile transformation buffer (TB) [15 mM CaCl₂, 250 mM KCl, 10 mM PIPES, adjusted to pH 6.7 with 1 M KOH, after autoclaving 55 mM MnCl₂ (sterilized using a 0.45 µm filter) were added]. The re-suspended bacteria were incubated on ice for 10 min, centrifuged for 10 min at 4000 rpm and 4 °C, and re-suspended in 18.6 ml TB containing 7 % DMSO, before being again incubated on ice for 10 min. Finally, cells were divided into 210 µl aliquots and snap frozen in liquid nitrogen. Competent cells were stored at -80 °C until their further use.

3.8.5 Transformation of chemical competent *E. coli*

Chemically competent *E. coli* cells (See Section 3.8.4) were transformed according the heat-shock transformation method described by Inoue et al. (1990). This transformation procedure is based on the use of a calcium rich environment to counteract the electrostatic repulsion between the highly hydrophilic plasmid DNA and the cell membrane of bacterial. A subsequent increase in temperature is used to permeabilize the cell membrane by creating pores, which facilitates the movement of the DNA molecule into the bacteria. For each transformation, one aliquot of competent *E. coli* cells was thawed on ice. Then, approximately 10-20 µl of the desired ligation reaction (See Sections 3.8.1 and 3.8.2) were mixed with the bacteria suspension and incubated on ice for 30 min. Next, the cells were heat-treated for 90 s at 42 °C, returned to ice for 2 min and then incubated in 700 µl of LB at 37 °C, while shaking (200 rpm) for 1 h to enable cell recovery. Afterwards, the sample was centrifuged for 4 min at 4000 rpm and 600 µl of the supernatants were removed. After re-suspension in the remaining supernatant, 100 µl of cells were plated onto LB agar plates containing appropriate contents of antibiotics to facilitate the selection of transformed cells (See Section 3.3.2 and 3.4.2). In addition, pGEM-T vector allows positive clones color screening by the insertional inactivation

of the α -peptide coding region of the enzyme β -galactosidase, LacZ gen. Blue/white screening was performed by supplementing plated with 4 μ l of 1 M isopropyl-1-thio- β -D-galactopyranoside (IPTG) (Sigma), filter sterilized (0.20 μ M), and 40 μ l of 20 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (Promega), dissolved in DMSO, prior to plating out cells.

3.8.6 Generation of electro-competent *E. coli*

The *E. coli* strain DB3.1 (See Section 3.4.1), tolerant growth inhibition by the *lethal D* (*letD*) gene product of the F plasmid, was chosen for propagating empty gateway plasmids containing the *ccdB* operon (Miki et al. 1992). DB3.1 electro-competent cells were prepared as described by Dower et al. (1988). According to this protocol, *E. coli* DB3.1 cells from a glycerol stock were plated onto LB agar (25 μ g ml⁻¹ streptomycin) petri dishes and incubated overnight at 37 °C with vigorous shaking at 200 rpm (See Section 3.4.2). Independent colonies from the fresh agar plates were then used to inoculate a 4 ml LB (25 μ g ml⁻¹ streptomycin) liquid broth pre-culture that was grown overnight at 37 °C and an agitation of 200 rpm. One hundred microliter of this culture were subsequently used to inoculate 250 ml of sterile liquid LB, in a 1 l Erlenmeyer flask, which was incubated at 28 °C and 200 rpm shaking. Cultures were grown to an OD₆₀₀ = 0.5-1. After growth, cultures were chilled on ice for 10 min and centrifuged for 15 min at 4 °C at 3000 rpm in a JLA-16.250 rotor (Avanti J-26XP, Beckam Coulter). To decrease the ionic strength of the cell suspension for electroporation at high voltages, cell pellets were washed twice in 100 ml of cold 1 mM HEPES (pH 7.0), sterilized by filtration (0.45 μ m), and centrifuged as mentioned above. After centrifugation, the supernatant was removed and cells were re-suspended in 10 ml of pre-chilled 10 % glycerol and again centrifuged for 15 min at 4 °C and 4000 rpm in a JLA-16.250 rotor (Avanti J-26XP, Beckam Coulter). The resulting cell pellets were re-suspended in 1 ml cold 10 % glycerol. This concentrated suspension was divided into 50 μ l aliquots, frozen in liquid N₂ and stored at -80 °C until used for transformation.

3.8.7 Transformation of electro-competent *E. coli*

Electroporation of electro-competent *E. coli* cells (See Section 3.8.6) was carried out as previously detailed (Dower et al. 1988; Green & Sambrook 2012), using a ECM® 630 Electroporation System (Genetronics). This process is based on the application of controlled direct current electrical pulses to the bacteria cells, in order to generate a transmembrane potential. The introduction of the desired DNA molecules is then facilitated by the permeation or “pore formation” of the cell membrane. To achieve this, approximately 10-25 ng of the plasmid DNA to be transformed, in a volume of

1 μl , were mixed with 50 μl aliquot of competent cells thawed on ice. The electroporation apparatus was set to deliver an electrical pulse of 25 μF capacitance, 2500 V voltage, and 200 Ω resistance. The electro-competent cells/DNA mixture (See Section 3.8.3) was transferred into a pre-chilled electroporation cuvette with 0.2 cm path length (Cell Projects) and the correspondent electrical pulse was delivered to the cells. The electrode configuration provided a time constant of 4 to 5 milliseconds (Time constant = Resistance x Capacitance), and a 12.5 kV cm^{-1} field strength (Field strength = Voltage / Cuvette path length). This information was registered in the electroporation machine. Following pulse delivery, cells were immediately diluted with 500 μl LB liquid media, transferred to a sterile 1.5 ml microcentrifuge tube and incubated for 1 h at 37 $^{\circ}\text{C}$ under constant shaking (200 rpm). Cells were then centrifuged for 4 min at RT and 4000 rpm and 400 μl of the supernatant were removed. After re-suspension in the remaining supernatant, 100 μl of cells were plated onto LB agar plates containing appropriate antibiotics for plasmid DNA containing bacteria selection (See Sections 3.3.2 and 3.4.2).

3.8.8 Generation of chemical-competent *Agrobacterium tumefaciens*

The preparation of chemically competent *A. tumefaciens* cells was performed as described by Berberich et al. (2008). *A. tumefaciens* strain C58C1 cells (See Section 3.4.1) from a glycerol stock were plated onto LB agar plates containing 50 $\mu\text{g ml}^{-1}$ rifampicin (Rif) and incubated overnight at 28 $^{\circ}\text{C}$. A single colony was selected and used to inoculate a 10 ml liquid LB culture containing 50 $\mu\text{g ml}^{-1}$ Rif and incubated overnight in an incubator shaker 28 $^{\circ}\text{C}$ /180 rpm (See Section 3.4.2). Afterwards, the culture volume was adjusted to 100 ml using sterile LB in 250 ml conical flask and cells were grown at 28 $^{\circ}\text{C}$ /200 rpm to an OD_{600} of 0.8 (approximately 4 h). The culture was placed on ice for 10 min and then centrifuged at 10 min at 4 $^{\circ}\text{C}$ /4000 rpm in a JLA-16.250 rotor (Beckman Coulter). Thereafter, the supernatant was removed and the pellet was re-suspended in 20 ml of 150 mM cold NaCl, sterilized by filtration (0.45 μm filter), and centrifuged for 5 min at 4 $^{\circ}\text{C}$ /4000 rpm. After the centrifugation, the supernatant was discarded and the pellet was re-suspended in 2 ml pre-chilled 20 mM CaCl_2 . Finally, 50 μl aliquots were prepared, frozen in liquid nitrogen and stored at -80 $^{\circ}\text{C}$.

3.8.9 Transformation of chemically competent *A. tumefaciens* cells

Heat shock-mediated transformation of chemically competent C58C1 cells (See Section 3.8.8) has previously been described (Kahl & Meksem, 2008). Accordingly, 1 μg of the purified plasmid DNA (See Section 3.7.1) in a maximum volume of 10 μl ,

were added to 50 µl of a bacteria suspension and mixed carefully. The mixture was immediately incubated for 5 min at 37 °C while shaking at 200 rpm. After the initial incubation, cells were transferred to ice and kept there for 30 min. Subsequently, 500 µl of sterile LB medium were added and bacteria cell were incubated over 2-4 h at 28 °C in dark to enable cell recovery. Cells were centrifuged for 10 min at 3200 rpm, and re-suspended in 100 µl of supernatant. The cell suspension was then plated onto solid LB plates containing the appropriated antibiotics for the selection of transformants (See Sections 3.3.2 and 3.4.2).

3.8.10 Screening for recombinants by colony PCR

Identification of cells that contain the desired insert at the right orientation was performed by colony PCR. To achieve this, independent colonies of transformed *E. coli* or *A. tumefaciens* cells were picked and transferred to sterile deionized water. Cells were boiled at 100 °C for 10 min to evoke cell lysis, and then centrifuged at 9700 rpm for 5 min. Following the centrifugation, 5 µl of the super-natant were transfer to a PCR reaction mixture containing 1.5 U Taq polymerase (TaKaRa), 2.5 µl of supplied 10 x PCR buffer (including Mg²⁺), 400 µM of dNTPs, 0.5 µM of each specific primer and autoclaved deionized water ad 25 µl. Primers used for colony PCRs are listened in Table 1 (See Section 3.3.1). PCRs were carried out in a peqSTAR (PEQLAB) thermocycler and PCR conditions were as follows: an initial single DNA denaturation step at 95 °C for 7 min, and 35 x [denaturation step at 94 °C for 60 s, a primer annealing step at 50-55 °C for 45 s, and an elongation step at 72 °C for 1 min per kbp of expected product]. To ensure quality of the PCR reagents used, for each colony PCR reaction a negative control of the PCR mix containing no template was included. PCR products were analysed by agarose gel electrophoresis (See Section 3.7.4).

3.8.11 DNA sequencing

After all cloning procedures, but in particular when DNA fragments were obtained by error-prone PCR, plasmids were isolated (See Section 3.7.1) and 1.5-2 µg were sent out for commercial sequencing (STAB VIDA, Portugal). The primers used for sequencing are listened in Table 1 (Section 3.3.1). Samples were sequenced according to the Sanger method, based on the chain terminator principle (Sanger et al. 1977), using an ABI 3730 XL DNA analyser equipment (Applied Biosystems), which enable readings of up to 1000 bps. All received sequences were analysed by sequence comparison with the original cloned DNA region retrieved from the NCBI database (<http://www.ncbi.nlm.nih.gov/>) or from “The Arabidopsis Information Resource” (TAIR) (<https://www.arabidopsis.org/>). Multiple sequence alignments were created

in CLUSTAL OMEGA (Thompson et al. 1994; Larkin et al. 2007; Sievers et al. 2011) (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), employing sequences in FASTA mode.

3.9 Biochemical methods

3.9.1 Glucose quantification

Saccharification of isolated cell walls was based on the method of Van Acker et al. (2013). According with this protocol, 10 mg of the extracted lignocellulosic biomass or AIR (See Section 3.4.5) were incubated overnight with 70 % ethanol at 55 °C, washed with 100 % acetone and dried under vacuum (45 min at 30 °C). Then, samples were dissolved in 1 ml sodium acetate/acetic acid buffer solution (combined 0.2 M sodium acetate, 0.2 M acetic acid, pH 4.8) and incubated 30 min at 55 °C. Enzymatic glucose liberation was carried out by adding a mixture of cellulose, from *Trichoderma reesei* ATCC 2692, and cellobiase, from *Aspergillus niger*, (Sigma-Aldrich) in a 5:3 ratio. Both enzymes were previously desalted via a PD10 column (GE Healthcare Europe), according to the manufacture's guidelines. The released glucose was extracted by using ethyl acetate (Baltes et al. 1998). Each sample was spiked with [¹³C₆]-D-glucose (Cambridge Isotope Laboratories) at a final concentration of 15 ng µl⁻¹. Vacuum dried extracts (60 min, 30 °C) were successively derivatized by adding 30 µl of 15 mg ml⁻¹ methoxyamine hydrochloride (Meox) diluted in dry pyridine (16 h at room temperature) and BSTFA+1% TMCS (1 h at room temperature).

The concentration of glucose was then measured by gas chromatography mass spectrometry (GC-MS/MS) as described in the following: 1 µl of the derivatized sample was injected splitless using an CLC CombiPal autosampler into a BRUKER Daltonics 451 gas chromatograph equipped with a 30 m x 0.25 mm i.d. fused silica capillary column with a chemically bonded 0.25 µm DB 5-MS stationary phase (BRUKER Daltonics). The injector temperature was set to 270 °C. After 1 min, the split was opened (1:100). During the first 1.10 min a pressure pulse (30 psi) supported sample application onto the column. The gas flow rate through the column was adjusted to 1 ml min⁻¹, the column temperature was held at 70 °C for 2 min, then increased by 20 °C min⁻¹ to 325 °C, and held there for 5 min. The column effluent was introduced into the ion source of a Scion-TQ triple quadrupole mass spectrometer (BRUKER Daltonics). The transfer line and the ion source temperatures were maintained at 250 °C. Ions were generated by a 70 eV electron beam at an ionization current of 80 µA, and 30 spectra s⁻¹ were recorded in the mass range of 50 to 600 m/z. Under the given conditions the retention time was 12.67 min for the 1Z- and 12.76 min for the 1E-isomer. The m/z 319 fragment of

the endogenous glucose MEOX 5TMS was selected and subjected to further sub-fragmentation by colliding it with argon gas regulated to a constant pressure of 1.5 mTorr in the collision cell. From the resulting fragments only the transitions of m/z 319 to m/z 73, m/z 319 to m/z 129, and m/z 319 to m/z 157, were considered. The transition that gave the strongest signal (m/z 319 to m/z 73) was considered as the quantifier ion used for the subsequent calculation of the amount of glucose in the sample. The two other transitions served as qualifier ions. The corresponding transitions for the $[^{13}\text{C}_6]$ -D-glucose MEOX 5TMS m/z 324 fragment (m/z 323 to m/z 73, m/z 323 to m/z 133, and m/z 323 to m/z 162) were recorded simultaneously. Each sample was run in three biological and three technical replicates. Glucose contents were calculated by comparison to the spiked internal standard $[^{13}\text{C}_6]$ -D-glucose.

3.9.2 Lignin content determination

Total lignin contents were assessed by the acetyl bromide method adapted from the protocol described by Hatfield et al. (1999) and Foster et al. (2010). Portions of 1 mg of the extracted cell wall material (See Section 3.4.5) were dissolved in 25 % acetyl bromide in glacial acetic acid and incubated 3 h at 50 °C. After the incubation, 2 M sodium hydroxide and 0.5 M hydroxylamine hydrochloride were added. Absorbance was measured at 280 nm (λ) using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). For each plant line three independent samples were evaluated and read in triplicates. The percentage of acetyl bromide soluble lignin (% ABSL) was calculated according the *Bouguer-Lambert-Beer* law:

$$A = \epsilon \times l \times c \quad [9]$$

With A as the absorbance (280 nm), $\epsilon = 15.69 \text{ g}^{-1} \text{ cm}^{-1}$ as the standard coefficient, and $l = 0.1 \text{ cm}$ as the NanoDrop path length. Final lignin concentration (c) was expressed as $\mu\text{g lignin mg}^{-1} \text{ cell wall}$.

3.9.3 Lignin monolignol analysis

The lignin composition was determined by the thioacidolysis method according to Foster et al. (2010). In brief, 100 μl of a solution containing 2.5 % boron trifluoride diethyl etherate (BF_3)-10 % ethanethiol (EtSH) in dioxane was added to 2 mg aliquots of AIR material (See Section 3.4.5) and incubated 4 h at 100 °C. Following the incubation, the reaction was stopped by cooling samples on ice for 5 min and adjusting the pH to 3-4 using 0.4 M sodium bicarbonate. Then, samples were extracted by ethyl acetate (0.5 ml) and the organic phases were dried using a sample concentrator FSC496D (Techne). Afterwards, samples were derivatized for 1 to 2

hours at RT with 30 μ l of BSTFA (N,O-Bis (trimethylsilyl) trifluoroacetamide) in presence of 1% TMCS (trimethylchlorosilane), which acts as a catalyst of the silylation reaction. Identification and quantification of lignin-derived monomers were performed by gas chromatography-mass spectrometry (GC-MS/MS) on a SCION triple quadrupole (TQ) detector (Bruker) using three biological replicates run in triplicates. Retention times of 12.98 min, 14.42 min, and 15.82 min could be determined for *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S), respectively. Peaks were identified by characteristic ion transitions for each monomer: m/z 234 to m/z 191 for S-, m/z 294 to m/z 73 for G-, and m/z 189 to m/z 115 for H-monolignol units. Lignin monomer quantification was performed using external calibration for each independent lignin monomer with commercial *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S) standards (Sigma-Aldrich). Finally, the relative composition of lignin monomers was quantified by setting the total monolignol peak area to 100 % and express the corresponding contents for each monolignol relative to this value. The quantification provided absolute values given in μ mol monolignol monomer mg^{-1} cell wall material.

3.9.4 OLigo Mass Profiling (OLIMP) of extracellular polysaccharides

Analysis of inflorescence stems cell wall glycostructures was adapted from the method described by Günl et al. (2010). Isolated AIR (See Section 3.4.5) were solved in 100 μ l of 50 mM ammonium formate (pH 4.5 adjusted with formic acid). After full dissolution, 0.4 units of *endo*- β -1,4-glucanase (EG) from *Aspergillus niger* (Megazyme) were added to the cell wall solution and the reaction was incubated overnight at 37 °C, while shaking. Xyloglucan pattern were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) in collaboration with Dr. Julia Kehr (University of Hamburg, Hamburg, Germany). To this end, 0.5 μ l of DHB matrix (10 mg ml^{-1} 2,5-dihydroxybenzoic acid in steriled H_2O) (Sigma-Aldrich) was spotted onto the MALDI target plate (Kratos Analytical) followed by the addition of 1 μ l of the digested xyloglucan solution and 0.5 μ l of the matrix solution. Then, samples were dried at atmospheric pressure (RT) and spectra were recorded on a Bruker UltrafleXtrem Smartbeam II Laser MALDI-TOF mass spectrometry instrument in positive reflectron mode with an acceleration voltage of 20 kV. The obtained spectra represent oligosaccharide ions m/z values and their relative peak intensity, expressed as the percentage of the most intense peak. To optimize instrument parameters and calibrate the mass scale prior to sample analysis a mass standard kit (Bruker), which contains a mixture of known peptides, was employed. All analyses were carried out in triplicates using three

biological replicates of each genotype. Mass data were analyzed using the mMass software package (Strohalm et al. 2008).

3.9.5 Analysis of endogenous ethylene production

Arabidopsis ethylene production was measured by gas chromatography in collaboration with Dr. Ronald Pierik (University of Utrecht, Utrecht, Netherlands) according to Millenaar et al. (2005). Following this protocol, approximately 350 mg freshly weight plants (See Section 3.4.3), were transferred to a 2.5 ml syringe for 20 min leaving 1 ml air space. The ethylene produced by the plant material during the 20 min incubation diffused into this headspace. Subsequently, the headspace was transferred into a second syringe and injected into a gas chromatograph (Synspec GC955-100 equipped with a photoionization detector (PID) and a 160 cm Haye Sep R column filled with Haye Sep 80/100 mesh, Synspec, Groningen, the Netherlands) for analysis. This procedure took less than 25 min from harvesting the plant to injecting into the GC, which is fast enough to prevent wounding-induced ethylene production.

3.9.6 Multiplex plant hormone analysis

The quantitive analysis of acidic phytohormones, namely indole-3-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) was carried with minor modifications out according to Carrasco Loba & Pollmann (2017). In brief, approximately 100 mg of 10 day-old *Arabidopsis thaliana* seedlings (See Section 3.4.3) were harvested in microcentrifuge tubes containing 1 ml of methanol, 50 pmol [$^2\text{H}_2$]-IAA, 25 pmol [$^2\text{H}_4$]-SA, 50 pmol [$^2\text{H}_6$]-ABA and 30 pmol [$^2\text{H}_5$]-JA as internal standards. The stable isotope-labelled compounds were purchased at C/D/N Isotopes Inc. (Pointe-Claire) or OlChemIm Ltd (Olomouc, Czech Republic). To each tube three 5.5 mm stainless steel balls (Retsch) were added. The samples were then heated at 60 °C, while shaking, for 3 min. After heating, seedling tissues were homogenized in two rounds of 1 min each, at 30 Hz, using a Retsch MM300 TissueLyser (Qiagen). After removing the steel balls, samples were centrifuged at 11000 rpm for 1 min and supernatants were carefully transferred into new microcentrifuge tubes and dried at 60 °C under vacuum. Subsequently, the obtained residues were solved in 50 µl methanol and 200 µl diethyl ether were added for samples pre-cleaning. Samples were sonified for 5 min in an ultrasonic bath (Bansonic 5510E-DTH, Branson Ultrasonics Corporation) and centrifuged at 11000 rpm for 5 min. The solution was loaded onto an aminopropyl solid-phase extraction column (Chromabond NH_2 shorty 10 mg, Macherey-Nagel GmbH) previously conditioned with two washing steps with 200 µl diethyl ether, and

passed through the column by gravity flow to improve the association of the acidic compounds to the NH₂-matrix. After sample passing, the column was washed twice with 200 µl of freshly prepared chloroform:2-propanol (2:1, v/v). The bound material was finally eluted with two times 200 µl of diethyl ether containing 2 % acetic acid, before being taken to complete dryness.

Plant hormone concentrations were analysed by gas chromatography-mass spectrometry (GC-MS/MS). To this end, samples were firstly derivatized to their methyl ester forms, which are characterized by an increased volatility, due to the lower melting point. Therefore, samples were dissolved in 25 µl methanol, transferred to a 600 µl crimp top conical microvial (Chromacol Uni-VL Supelco #27312) and dried in a gentle stream of N₂. Subsequently, samples were re-dissolved in 20 µl of the freshly prepared derivatization solution [88 % acetone:methanol (9:1, v/v), 11.8 % diethyl ether, 1.2 % Trimethylsilyl diazomethane, 2 M in diethyl ether]. Immediately, the vials were closed with 8 mm crimp seals with PTFE/rubber septa and samples were incubated at RT for 30 min. After incubation, 1 µl of the derivatized sample was injected splitless using a CLC CombiPal autosampler into a BRUKER Daltonics 451 gas chromatograph equipped with a stationary phase ZB-35 (30 m x 0.25 mm, 0.25 µm film) fused silica capillary column (Phenomenex). Helium at a flow rate of 1 ml min⁻¹ was used as the mobile phase for separation. The injector temperature was set to 250 °C and the column was held at 50 °C for 1.2 min, then temperature was increased by 30 °C min⁻¹ to 120 °C, and finally to 325 °C by 10 °C min⁻¹ and held there for 4 min more. The column effluent was introduced into the ion source of a Scion-TQ triple quadrupole mass spectrometer (BRUKER Daltonics). The transfer line and the ion source temperatures were maintained at 250 °C and 200 °C, respectively. Ions were generated by a 70 eV electron beam at an ionization current of 80 µA, and 30 spectra s⁻¹ were recorded in the mass range of 50 to 600 m/z. Under the given conditions the retention time for the endogenous methylated plant hormones was 13.6 min for the MeIAA, 16.2 min for the MeABA, 11.6 min for the MeJA and 6.7 min for the MeSA. For quantification, the selected precursor ions and corresponding diagnostic product ions given in Table 2 were used.

Table 4: Diagnostic fragment ions.

Compound	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
MeSA	6.7	152	120	10
[³ H ₄]-MeSA	6.7	156	124	10
MeJA	11.6	151	108	10
[³ H ₃]-MeJA	11.6	154	111	10
MeIAA	13.6	189	130	10
[³ H ₂]-MeIAA	13.6	191	132	10
MeABA	16.2	162	133	15
[³ H ₆]-MeABA	16.2	168	139	15

Subfragmentation of the precursor ions was achieved by collision with argon gas regulated to a constant pressure of 2 mTorr. Phytohormone contents were calculated by comparison of the peak area for the endogenous compound to the peak area of the respective spiked internal standard.

3.9.7 Fluorometric detection of β -glucuronidase (GUS) activity in *Arabidopsis* protoplast

The analysis of GUS activity levels was basically performed as previously described Jefferson et al. (1987) and Kirby & Kavanagh (2002). The β -glucuronidase, encoded by the *uidA* locus of *E. coli*, is a hydrolase that catalyses the cleavage of a wide variety of β -glucuronides. The fluorometric assay is based on the hydrolysis of the artificial substrate 4-methylumbelliferyl- β -D-glucuronide (4-MUG), liberating the fluorescent molecule 4-methylumbelliferone (4-MU) (Gallagher 1992):

The fluorescence intensity generated through 4-MU production over a given period of time can be used to quantify the activity of the GUS enzyme. For this analysis, frozen protoplasts (See Section 3.7.13) were re-suspended in 150 μ l of GUS extraction solution [50 mM sodium phosphate buffer Na₂HPO₄/NaH₂PO₄ pH 7.5, 10 mM EDTA, 0.1 % (v/v) Triton X-100, 0.1 % (w/v) sodium lauroylsarcosinate (Sigma-Aldrich), 0.05 % (v/v) β -MeEtO]. An aliquot of 100 μ l of the suspension was transferred to a 96-well clear flat bottom microtiter plate (Falcon) and mixed with 100 μ l GUS extraction solution containing 4 mM 4-MUG. Protoplasts were then incubated at 37 °C in the dark for 10 min (*T*₀). After the incubation, 100 μ l of the

protoplast/4-MUG solution were transferred to a new 96-well plate and the reaction was stopped by the addition of 100 µl of GUS stop solution [200 mM Na₂CO₃]. The remaining protoplast/substrate solution was further incubated at 37 °C in darkness for 1 h (T₆₀). Subsequently, fluorescence was measured at 360 nm excitation and 460 nm emission (56 gain, 10 flashes, 50 % mirror) using a TECAN Genios Pro fluorescence spectrometer (MTX Lab Systems). The GUS activity was calculated using the following equation:

$$GUS - Activity [pmol/min]: \frac{\Delta F/10}{t} \quad [10]$$

Where ΔF is the difference in fluorescence intensity T₆₀-T₀, 10 are the fluorescence units corresponding to 1 pmol of hydrolysed 4-MUG. GUS activity was quantified in triplicates to ensure accuracy.

3.9.8 Quantitative GUS analysis and protein assay in agroinfiltrated *Nicotiana benthamiana* leaves

For the analyses of GUS activity two leaf discs (See Section 3.7.14) were placed in a microreaction tube, frozen in liquid nitrogen (N₂), and ground with 5.5 mm stainless steel balls in a Retsch MM300 TissueLyser (Qiagen), and re-suspended in 150 µl of GUS extraction solution (See Section 3.9.7). Then, samples were centrifuged for 5 min at 11000 rpm and 10 µl of the supernatant were utilised for total protein content determination (Bradford 1976) using bovine-γ-globulin as the protein standard (Bio-Rad). GUS expression levels were quantified by fluorometric analysis and repeated in triplicates as described in Section 3.9.7. Normalization of the GUS-activity was performed according the following equation:

$$GUS \text{ Activity } [units] = \frac{GUS - Activity}{\text{mg of protein}} \quad [11]$$

3.9.9 Fluorometric detection of neuroaminidase (NAN) activity

NAN activity was determined according to Kirby & Kavanagh (2002). The NAN gene is a codon-optimized derivative of the *nanH* gene, which encodes a cytoplasmic sialidase of *Clostridium perfringens*. The fluorometric method utilized here is based on the hydrolysis of the artificial substrate 4-MUN by NAN to produce the fluorescent molecule 4-methylumbelliferone (4-MU), with the reaction:

Quantification of the fluorescent reaction product over time is used to determine the activity of the NAN enzyme. To achieve this, 10 µl of the previously re-suspended protoplasts in GUS extraction solution (See Sections 3.7.13 and 3.9.7) were transferred to a 96-well clear round bottom microtiter plate (Falcon) and mixed with 10 µl NAN extraction solution [50 mM $\text{N}_2\text{HPO}_4/\text{NH}_2\text{PO}_4$ pH 7.0, 10 mM EDTA, 0.1 % (v/v) Triton X-100, 0.1 % (w/v) sodium lauroylsarcosinate] containing freshly added 0.05 % (v/v) β -MeEtOH and 1 mM 4-MUN. The protoplasts were then incubated at 37 °C in the dark for 10 min (T_0). After the incubation, 3.3 µl of the protoplast suspension was transferred to a 96-well clear flat bottom microtiter plate (Falcon) containing 200 µl of NAN stop solution [330 mM Na_2CO_3]. The remaining protoplast/4-MUN solution was incubated at 37 °C in darkness for 1 h (T_{60}). Afterwards, the fluorescence was measured as described before. NAN activity was calculated as (Gallagher 1992):

$$\text{NAN} - \text{Activity} [\text{pmol/min}] = \frac{\Delta F/10}{t} \quad [12]$$

Where ΔF is the difference in fluorescence $T_{60}-T_0$, 10 are the fluorescence units corresponding to 1 pmol of hydrolysed 4-MUN. To ensure data accuracy NAN activity were measured in triplicates. Normalization of the GUS-activity was performed by calculating the ratio of GUS and NAN activities, represented as relative GUS/NAN units, according the equation:

$$\frac{\text{GUS}}{\text{NAN}} - \text{Activity} [\text{units}] = \frac{\text{GUS} - \text{Activity}}{\text{NAN} - \text{Activity}} \quad [13]$$

3.10 Histochemical Analysis

3.10.1 Secondary elements visualization

Fully developed inflorescence stems (~24 cm tall) were collected and directly processed for secondary cell wall element visualization by histochemical studies (See Sections 3.4.3 and 3.4.6).

3.10.1.1 Toluidine Blue staining

Stem tissue structure was studied after Toluidine blue staining. For this, stem sections were incubated 5 min with 1 ml of 0.05 % (w/v) Toluidine Blue O (Sigma-Aldrich) in autoclaved H_2O . After that, the Toluidine dye was rinsed out by serial washes with dH_2O (Pradhan Mitra & Loqué 2014) .

3.10.1.2 Phloroglucinol-HCl staining

Phloroglucinol staining for lignin detection was conducted by incubating 100 μm stem cross-sections over 5 min in 1 ml of a phloroglucinol solution in strong hydrochloric acid, which is also known as the Wiesner reagent. For this reagent, 3 % (w/v) phloroglucinol (Sigma-Aldrich) in absolute ethanol was diluted 2:1 (v/v) in 37 N HCl (Hossain et al. 2012; Pradhan Mitra & Loqué 2014).

3.10.1.3 Mäule staining

For the Mäule reaction, used in lignin structure analysis, cross-sections were treated with successive incubations as follows: 1 ml of 0.5 % potassium permanganate solution (KMnO_4) in dH_2O for 2 min; washed with dH_2O to completely remove the potassium permanganate solution excess; 1 ml of 3 % HCl until the deep brown colour cleared from the sections (3-5 min); and 1 ml of 14.8 M ammonium hydroxide (NH_4OH) for 1 min. Because Wiesner and Mäule solutions cause sample deterioration when samples dry out, photographs were taken within 5-10 min after the reaction (Pradhan Mitra & Loqué 2014).

3.10.1.4 Ultraviolet light (UV)

Additionally, autofluorescence of phenolic compounds of secondary plant cell walls were visualized under excitation at 360/400 nm ultraviolet (UV) light using unstained sections (Pradhan Mitra & Loqué 2014).

3.10.1.5 Calcofluor White staining

Analysis of cell wall cellulose, carboxylated polysaccharides and callose was performed by Calcofluor White M2R staining. To this end, 100 μm thick stem cross-sections were incubated for 5 min with 1 ml of 0.02 % (v/v) fluorescent brightener 28 (Sigma-Aldrich) in distilled water. After incubation, samples were rinsed 3 to 4 times with dH_2O to remove excess brightening agent (Pradhan Mitra & Loqué 2014).

3.10.1.6 Congo Red staining

To additionally visualize cellulose, 1 ml of 0.5 % (w/v) Congo Red reagent (Sigma-Aldrich) in dH_2O , was added to the cross-sections. Samples were incubated for a total of 13 min, softly shaking each 5 min. Then, samples were washed with dH_2O to remove excess dye (Pradhan Mitra & Loqué 2014).

3.10.2 Trypan blue staining

To visualize spider mite-induced cell death (See Section 3.5.1), five plants from each genotype were harvested 4 days after spider mite treatment. The collected leaves from independent samples were placed in 15 ml plastic tubes and stained with

5 ml of lactophenol-trypan blue solution (TB) [10 ml lactic acid (Sigma), 10 ml phenol, 10 ml glycerol, 10 ml trypan blue (Sigma), and 10 ml of deionized H₂O] (Koch & Slusarenko 1990). Before being used, this TB solution was diluted 1:2 with 100 % ethanol. Subsequently, the solutions containing leaves were boiled for 1 min and destained over 30 min at room temperature in 2 ml of chloral hydrate solution [5 g of chloral hydrate dissolved in 2 ml of deionized H₂O]. After destaining, the chloral hydrate solution was renewed and left overnight to allow total discoloration. Finally, the chloral hydrate solution was removed and 2 ml of 50 % glycerol were added. Leaves were then placed on a microscope slide, covered with a coverslip and inspected under bright-field illumination. Two experiment control plants of each genotype were utilized in parallel for cell death analysis.

3.10.3 DAB staining

H₂O₂ accumulation after spider mite attack was visualized using the DAB staining method (Thordal-Christensen et al. 1997). For this purpose, 5 plants of each genotype were harvested 4 days after spider mite treatment (See Section 3.5.1). Thereafter, 2 leaves per plant were placed in a 15 ml plastic tube and immediately covered with 5 ml of DAB solution [0.1 % (w/v) of 3,3-diaminobenzidine-HCl (pH 3.8)]. Leaves submerged in the solution were vacuum infiltrated for 5 min and incubated overnight. Afterwards, leaf tissues were cleared by three subsequent washes with 5 ml of 1:1 ethanol:chloroform; each washing step took 2 h. Finally, leaves were mounted onto a microscope slide using 50 % glycerol and covered with a coverslip. For each genotype two control plants, without any spider mite treatment, were additionally stained alongside with the treated leaves.

3.10.4 Histochemical GUS assay

Histochemical analyses of GUS activity were performed as described by Jefferson *et al.* (1987). For this, four leaves disc from each agroinfiltrated leaf (See Section 3.7.14) were placed in a microreaction tube and immersed 1 ml of GUS staining solution [100 mM sodium phosphate buffer pH 7.0, 0.5 M EDTA pH 8.0, 20 % (v/v) Triton X-100, 20 mM X-Gluc (Duchefa)]. Afterwards, the leaf discs were vacuum infiltrated for 10 min at 540 mbar followed by an overnight incubation at 37 °C in darkness. Thereafter, leaf discs were cleared by discarding the GUS solution and incubating them in 10 % (v/v) ethanol, 30 % (v/v) ethanol, 70 % (v/v) ethanol and 100 % (v/v) ethanol. Each incubation step was carried out for 1 h.

3.10.5 Light microscopy

After the various staining procedures (See Sections 3.10.1, 3.10.2, 3.10.3 and 3.10.4), samples were placed on a microscope slide and covered with a cover glass. Then,

100 µm sections and complete plant leaves were examined using a light stereomicroscope Leica MZ10F at a magnification of x8 x40 x60 x80 (Leica Microsystems). Images were captured using a Leica DFC 400C camera (Leica Microsystems). Samples stained either with Toluidine Blue, phloroglucinol, Mäule, trypan blue, DAB and GUS were observed under bright-field illumination. Calcofluor and Congo Red stained sections were examined by fluorescence microscopy using an excitation of 360/400 nm (UV) and 460/500 nm (blue-violet), respectively.

3.11 Statistical Analyses

All generated data are represented as the arithmetic mean, calculated for each independent sample ($X_1 \dots X_n$) as:

$$\bar{X} = \frac{1}{n} \sum_{i=1}^n X_i \quad [14]$$

And their correspondent standard error of the mean (SE), whose calculation directly depends on the standard deviation (SD):

$$SD = \sqrt{\frac{\sum_{i=1}^n (X_i - \bar{X})^2}{n-1}} \quad SE = \frac{SD}{\sqrt{n}} \quad [15]$$

Data were statistically analysed using STATGRAPHICS® Centurion XVI (Statpoint Technologies, INC.) Two-tailed Student's *t*-test assuming equal variances was used to study for significant differences among means. For multiple comparisons one-way analysis of variance (ANOVA) was carried out. In both statistical tests the result was represented as *p*-value and a significance value of 0.05 (95 % confidence level) or 0.01 (99 % confidence level) was used for alpha. Prior to the statistical analysis with the pertinent test, all data were also tested for normality using the Kolmogorov-Smirnov test.

CHAPTER 4:

RESULTS

4.1 In-depth analysis of the *YUC8* and *YUC9* overexpression lines to elucidate the molecular basis of observed auxin actions

4.1.1 Stimulation of ethylene biosynthesis in *YUC8ox* and *YUC9ox* lines

Previous work has demonstrated that the constitutive overexpression of the indole-3-acetic acid (IAA) biosynthetic genes *YUC8* and *YUC9*, likely results in increased ethylene production, as these lines are less sensitive to the ethylene inhibitor 2-aminoisobutyric acid (AIB) than wild-type plants (Col-0) (See Section 1.6). In agreement with this result, quantitative transcript analyses revealed the up-regulation of a small number of ethylene biosynthesis-related genes, like e.g. *ACS4* (At2g22810) and *ACS11* (At4g37770), as well as some ethylene signalling-related genes, such as *EIL1* (At2g27050) and *ETR2* (At3g23150) in the stable *35S::YUC8* and *35S::YUC9* overexpression lines (*YUC8ox* and *YUC9ox*, respectively) (Hentrich et al. 2013b).

Therefore, to provide conclusive evidence for the induction of ethylene biosynthesis through the overexpression of either *YUC8* or *YUC9*, we explored ethylene (ET) formation by gas chromatography (GC) and compared ET production in both overexpressors lines with that in wild-type (WT) control plants.

To perform this experiment, whole rosettes from the *Arabidopsis thaliana* WT, as well as the homozygous *YUC8ox* and *YUC9ox* lines, were harvested. All plant material was grown on soil under standard climatic conditions (See Section 3.4.3). The overexpressor lines, *YUC8ox* and *YUC9ox*, were kindly provided by the Dr. Mathias Hentrich (Ruhr-University, Bochum, Germany). After harvesting, samples were placed into a syringe to allow ethylene accumulation. Then, the air phase contained in the syringe was analyzed as described in Section 3.9.5. The ethylene content analysis was carried out in collaboration with Dr. Ronald Pierik (University of Utrecht, Utrecht, Netherlands).

Confirming our previous data, the results of the GC analysis demonstrated that ET production was indeed significantly increased in *YUC8ox* and *YUC9ox* (Figure 15), showing ET contents 4.46-fold and 7.45-fold, respectively, higher than in WT *Arabidopsis*. The obtained data verify a possible crosstalk between jasmonate (JA), indole-3-acetic acid (IAA) and ET. Consequently, with respect to these and previous results (Dombrecht et al. 2007; Sun et al. 2009; Hentrich et al. 2013a; Hentrich et al. 2013b), it can be speculated that JA produced in response to wounding, triggers the local overproduction of IAA through *YUC8* and/or *YUC9* action, which in turn promotes the overproduction of ET, resulting in increased secondary/radial growth.

Figure 15: Ethylene production

The levels of ethylene produced by 350 mg of whole rosettes from *Arabidopsis* WT (Col-0) seedling and both overexpressor lines, YUC8ox and YUC9ox, were determined by GC analysis after 20 min of ethylene accumulation in a 2.5 ml syringe. The obtained results are expressed as μmol of ethylene produced in 1 hour per g of fresh weight (FW). In the experiment three biological samples were utilized. Means are given with their SE. Asterisks indicated significant difference between each transgenic line and WT by Student's *t*-test at $**P < 0.01$.

4.1.2 Microscopic analysis of YUC8ox and YUC9ox stem morphology.

Apart from the higher tolerance towards AIB, strong overexpressor lines of both *YUC8* and *YUC9* are also characterized by aberrant secondary growth of the stem and a higher degree of lignification (See Section 1.6). To gain deeper insight into the mechanistic and conceptual implications that the elevated auxin contents in YUC8ox and YUC9ox may exert on secondary growth, we investigated the morphological consequences on stem and cell wall architecture and structure, respectively, by histochemical analyses.

Accordingly, the basal parts of the mature inflorescence stems from 8 week-old plants grown on soil under long day conditions were fixed using 4 % paraformaldehyde and sectioned transversally using a vibratome (Technical Products International). Afterwards, 100 μm cross-sections were histochemically stained either with toluidine blue O, phloroglucinol/HCl, Mäule, calcofluor white, or congo red and analyzed by light microscopy (See Sections 3.4.6 and 3.10.1).

Representative histochemical images for three independent biological replicates per genotype are represented in Figure 16. We observed that, along the main inflorescence stem secondary growth WT plants was detectable at the stem base. We therefore concentrated the analysis in the 1 to 5 cm immediately above the rosette. The Toluidine blue staining, which stains acidic tissue components (Sridharan & Shankar 2012), revealed that the cortex cells in the stems of YUC8ox and YUC9ox plants exhibited a bigger size, while the area of the xylem (Xy) and interfascicular

fibers (IF) showed to be unusually expanded Figure 16a-c. Treatment with phloroglucinol/HCl, which reacts with the cinnamaldehyde end groups of lignin in secondary xylem elements (Lewis & Yamamoto 1990), resulted in a more intense fuchsia-red color in the YUC8ox and YUC9ox sections, pointing towards a higher level of lignification in the transgenic lines (Figure 16d-f). The elevated lignin contents in YUC8ox and YUC9ox were further confirmed by UV microscopy, which disclosed increased auto-fluorescence of phenolic groups of the lignin biopolymer in YUC8ox and YUC9ox (Figure 16g-i). Next, we carried out a Mäule staining to study alterations in syringyl lignin (S-lignin) contents in the investigated genotypes. As depicted in Figure 16j-l, the Mäule staining showed a more reddish coloration in wild type indicating an enrichment of syringyl lignin units (S-lignin) in Col-0. In contrast, the yellow-brown coloration visible in YUC8ox and YUC9ox lines can be attributed to a decrease or lack of S-lignin (Pradhan Mitra & Loqué 2014). Using the calcofluor white stain, employed to visualize cellulose, callose and other non- or weakly substituted β -glucans (Fagard et al. 2000), it was possible to stain the epidermis, cortex and pith in all studied genotypes. The brilliance, however, is absent in the xylem and interfascicular fibers (Figure 16m-o). This is due to the fact that the diffusion efficiency of calcofluor white significantly decrease in highly lignified tissues (Pradhan Mitra & Loqué 2014), thereby providing a negative stain for lignified tissues. In contrast, the congo red reagent directly stains β -(1 $\rightarrow$ 4)-glucans, and in particular cellulose (Verbelen & Kerstens 2000), thereby facilitating an estimation of cellulose contents in the studied tissues. As can be taken from Figure 16p-r, the cross-sections of YUC8ox and YUC9ox show a higher cellulose deposition in the fascicular and interfascicular cambium in comparison to WT.

In summary, the obtained results suggest a possible expansion of the Xy and IF area. Furthermore, the presented histochemical experiments are indicative not only for elevated cellulose and lignin contents in YUC8ox and YUC9ox lines, but also for a possible alteration of the lignin composition highlighted by a putative shift in monolignol ratios.

Figure 16: Histochemical analysis of cell wall.

100 μm thickness cross-sections from the inflorescence stem (1-5 cm immediately above the rosette) of WT (Col-0), YUC8ox and YUC9ox plants were stained either with **a-c**) Toluidine blue, **d-f**) phloroglucinol, **g-i**) UV light, **j-l**) Maüle, **m-o**) calcofluor, or **p-r**) congo red staining. The position of the xylem (Xy) and interfascicular fibers (IF) are indicated. Scale bar = 100 μm .

4.1.3 Overexpression of *YUC8* and *YUC9* affects secondary cell wall development in *Arabidopsis* stems

The histochemical analysis revealed an unusual expanded Xy and IF secondary growth in the YUC8ox and YUC9ox (See Section 4.1.2). Thus, to further analyze this feature, we assessed the cell number in both Xy and IF regions, as well as the IF width, in the transgenic lines YUC8ox and YUC9ox and compared them with the values for *Arabidopsis* WT (Col-0).

To this end, captured images from Toluidine blue stained cross-section were submitted to Xy and IF quantification analysis using the image processing program ImageJ (Schneider et al. 2012; Schindelin et al. 2015).

Figure 17a shows a significantly increased cell numbers for both transgenic lines in comparison to the WT. Similarly, IF stack height was 2-fold and 1-fold increase in YUC8ox and YUC9ox, respectively, when compared to the WT (Figure 17b). Importantly, from this observation it can be concluded that *YUCCA8*- and *YUCCA9*-mediated IAA overproduction, presumably in conjunction with the induced formation of ET, causes drastic augmentation of cell expansion growth and cell division.

Figure 17: Secondary growth quantitative analysis.

Arabidopsis WT, YUC8ox and YUC9ox inflorescence stem cross-sections (100 μm), were stained with Toluidine blue and used for the quantitative characterization of xylem (Xy) and interfascicular fibers (IF) using the program ImageJ (Schneider et al. 2012; Schindelin et al. 2015). **a)** Number of cells in the Xy and IF areas. **b)** Comparison of the IF stack height, expressed in μm. All values are means ($\pm$ SE) of three replicates. Significant difference between YUC8ox or YUC9ox and WT by *t*-test at $P < 0.01$ are indicated **.

4.1.4 Assessment of total lignin contents and lignin composition in *YUC8* and *YUC9* overexpressing plants

Based on the analysis of gene expression levels, it has been concluded that the formation of extracellular polysaccharides during early stages of inflorescence stem developmental surpasses the production of lignin, whereas lignin biosynthesis is seemingly dominant over polysaccharide production at later developmental stages (Vanholme et al. 2012). Taking this observation into account, we analyzed the lignin content in wild-type *Arabidopsis* and the overexpressor lines *YUC8ox* and *YUC9ox* at two developmental stages. Analyses were performed with inflorescence stems that reached either 16 cm or 24 cm of height, representing early and later developmental stages, respectively, applying a spectrophotometric method.

To perform this experiment, mature inflorescence stems (16 cm or 24 cm height) of plants grown on soil under long day conditions were harvested. Then, cell walls were isolated and subsequently we quantified total lignin contents in the extracted lignocellulosic material using the acetyl bromide method (See Sections 3.4.5 and 3.9.2).

The results showed that the two overexpressor lines possessed slightly elevated total lignin contents relative to the WT, but the observed increase was not statistically significant (Figure 18). However, when looking at the more developed plants (24 cm stem height), we found the expected significant increase in total lignin contents in *YUC8ox* and *YUC9ox*. Surprisingly, the lignin content in *YUC9ox* plants did even significantly surpass the lignin levels in *YUC8ox* plants.

Figure 18: Comparative analysis of the lignin content

1 mg extracted cell walls from mature inflorescence stems (16 and 24 cm height) of the *Arabidopsis* WT plants, and the *YUC8ox* and *YUC9ox* transgenic lines, were analyzed for lignin content by the acetyl bromide methods. Lignin quantities are expressed in µg of lignin per mg of cell wall. Data are Means ± SE (n = 3 biological replicates). Different letters indicate significant differences between means ($P < 0.05$, ANOVA).

In order to gain deeper insight into the lignin composition of the genotypes under study and, thus, confirm the preliminary results obtained by the Mäule staining, a second experiment was conducted analyzing monolignol ratios by gas chromatography-mass spectrometry (GC-MS/MS).

Here, isolated cell wall fractions from the inflorescence stems of 24 cm tall stems were subjected to thioacidolysis. This degradative method proceeds via the cleavage of β -O-4 ether bonds in the lignin biopolymer and facilitates the determination of modulations in lignin composition by calculating monolignol ratios (Holtman et al. 2003). After complete decomposition of the cell wall preparations, we determined p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin unit levels by GC-MS/MS and calculated the H:G:S monolignol ratios for mature stems (See Sections 3.4.5 and 3.9.3).

As can be taken from Figure 19a, our experiments revealed that significantly reduced G and S contents characterize the lignin polymer in YUC8ox and YUC9ox plants, with H lignin levels appearing to be significantly increased. Both the number of substitutes on the ring structure of the various monolignols and their chemical properties decisively determine the molecule's readiness to form linkages in the radical polymerization process. A shift of the H:G:S ratio towards a less substituted monolignol, in particular H-lignin, such as that observed in YUC8ox and YUC9ox cell walls, most likely changes the polymerisation degree of the lignin polymer by reducing the possible points of linkages (Figure 19b).

In conclusion, the presented data on secondary cell wall composition indicated that the transgenic lines YUC8ox and YUC9ox exhibit a higher lignin content when the inflorescence stem reach the later developmental stages. Furthermore, the observed increased lignin content was associated with increased levels of the β -O-4 linked H-lignin, which in consequence supposes a decreased degree of lignin polymerization in the YUC8ox and YUC9ox lines.

Figure 19: Quantitative analysis of monolignol ratios thioacidolysis yield

a) Two mg of isolated cell wall material from WT, YUC8ox and YUC9ox mature inflorescence stems were used for GC-MS/MS quantification of H, G and S lignin monomers after complete thioacidolic acid-mediated degradation. Monolignol concentrations were calculated with respect calibration curves established for each lignin monomer. Monolignol concentrations were measured as μmol lignin monomer mg^{-1} cell wall. Relative abundance of the lignin monomers was normalized by setting the sum of all monolignol peak areas to 100%. Means are given with their SE ($n = 3$ biological replicates). Different letters indicate significant differences between means ($P < 0.05$, ANOVA). **b)** Chemical structure of the aromatic lignin units: H-lignin, G-lignin and S-lignin. Coupling sites possibilities are colored in grey (Hatfield & Vermerris 2001).

4.1.5 Analysis of enzymatic cellulose to glucose conversion

Staining of stem cross-sections with calcofluor white and subsequent microscopic assessment indicated a substantial increase in cellulose levels for YUC8ox and YUC9ox lines. However, apart from more cellulose, the two investigated lines contain as well significantly higher levels of total lignin. Considering the possibility of a potentially lower degree of polymerisation of the lignin polymer implicated by the shift of the H:G:S lignin ratio towards the less substituted H-lignin unit, we next addressed the question whether enzymatic breakdown of cellulose from cell walls of YUC8ox and YUC9ox plants is perhaps more efficient.

To evaluate the consequences of the observed structure modification of the cell wall, we quantified the enzymatic cellulose to glucose conversion, also referred as saccharification (See Sections 3.4.3 and 3.4.5). As described in Section 4.1.4 for

quantification of total lignin contents, we tested cell walls from inflorescence stems representing two different developmental stages (16 cm and 24 cm).

To carry out this study, prior to enzymatic hydrolysis, extracted lignocellulosic material was washed with ethanol and pretreated with an acetic acid buffer. After this, the enzymatic hydrolysis of cellulose was carried using a mixture of cellulase from *Trichoderma reesei* and cellobiase from *Aspergillus niger* in a 5:3 ratio (pH4.8). Hereafter, quantification of liberated D-glucose was carried out by GC-MS/MS using [$^{13}\text{C}_6$]-D-glucose as (Cambridge Isotope Laboratories) internal standard (See Section 3.9.1).

The enzymatic hydrolysis revealed only little differences in saccharification when focussing on less lignified younger plants (16 cm stems) (Figure 20). In the more developed plants, however, saccharification appears to be considerably less effective in YUC8ox and YUC9ox relative to wild type. It seems as if the moderate shift in monolignol ratios in the overexpressor lines is not sufficient to effectively change the polymerisation properties of the lignin polymer. As a consequence, the higher amount of total lignin in YUC8ox and YUC9ox considerably hampers the enzymatic liberation of D-glucose.

Figure 20: Quantification of glucose yield.

Ten mg of the extracted lignocellulosic biomass from *Arabidopsis* WT and the overexpressor lines, YUC8ox and YUC9ox, derived from inflorescence stems with a height of 16 and 24 cm, respectively, were analyzed for glucose yield after treatment with cellulase and cellobiase. Glucose content was calculated as a ratio of sample glucose to the internal standard $^{13}\text{C}_6$ -D-glucose. Results are expressed as μmol of glucose per mg of cell wall. Given are means $\pm$ SE ($n = 3$ biological replicates). Different letters indicate significant differences between means ($P < 0.05$, ANOVA).

4.1.6 Transcriptome characterization of YUC8ox and YUC9ox lines.

To better understand the molecular correlations that are triggered by the elevated IAA contents in YUC8ox and YUC9ox, we performed genome-wide cDNA microarray analyses. These microarray are based on the principle of complementary base-pairing of nucleic acids, which allows parallel probing of complex mixtures of gene transcripts.

To do so, seedling were cultivated on MS agar medium, supplemented with 1 % sucrose, under sterile conditions (22 °C, 16 h light/8 h dark and 100 $\mu\text{mol}/\text{m}^2 \text{ s}^{-1}$ light intensity). Total RNA of 10 day-old seedlings of *A. thaliana* WT, YUC8ox, and YUC9ox plants was extracted. The conducted microarrays employed the 24 K Affymetrix ATH1 Gene Chip® and were performed in collaboration with the genomic unit leaded by Dr. José Manuel Franco (CNB-CSIC, Madrid, Spain) (See Sections 3.7.7 and 3.7.12).

We compared the total numbers of the up- and down- regulated genes from YUC8ox or YUC9ox seedlings with the respective Col-0 control plants. Changes in the gene expression was considered as significant when the fold change was $\geq$ than 1.99-fold or $\leq$ than -1.99-fold, and the LIMMA p-value was lower than 0.05. Classification of genes based on functional similarity was carried out using the DAVID 6.7 program. These tool generates a gene-to-gene similarity matrix using more than 75000 terms from 14 functional annotation sources, which allows the identification of highly related genes (Huang et al. 2009a; Huang et al. 2009b).

The microarray results indicated that a total of 590 genes in YUC8ox and 486 genes in YUC9ox showed differential expression. Identification of functionally related gene groups based on gene ontology and biological process classification highlighted a group of cell wall related genes, which represent 6 % and 20% of the up-regulated genes in YUC8ox and YUC9ox, respectively (Figure 21). Among those cell wall-related genes, a group of members of the *xyloglucan endo-transglycosylase/hydrolase* (XTH) gene family, assumed to be involved in hemicellulose modifications, appeared to be very prominent, which attracted our attention.

Figure 21: Transcriptome changes of YUC8ox and YUC9ox in comparison to Col-0.

Pie charts showing the functional groups of significantly up-regulated genes in the two studied YUC overexpression lines. Genes were separated into functional categories according to gene ontology and biological process classification using DAVID (Huang et al 2009a; Huang et al 2009b). The criterion for differential expression was ≥ 1.99 -fold changes with a LiMMA p-value < 0.05 .

To further corroborate the outcome of the genome-wide transcript analysis, we performed a quantitative real time (qRT)-PCR with cDNA obtained from 10-day old Col-0, YUC8ox and YUC9ox seedling. All procedures with respect to plant growth conditions, total RNA extraction, cDNA synthesis, and qRT-PCR execution are described in Sections 3.7.7, 3.7.10 and 3.7.11.

The experiments confirmed for four out of the six genes the transcriptional up-regulation observed in the YUC8/9ox microarrays. The detected up-regulation of *XTH30* transcription is apparently a false positive result in YUC9ox, whereas the induction of *XTH33* expression seems to be restricted to the YUC8ox lines (Figure 22).

Figure 22: Relative expression of selected *XTH* genes in the wild-type *Arabidopsis* and both YUC8ox and YUC9ox lines.

Induction of gene expression of six previously identified *XTH* genes has been validated by qRT-PCR analysis. cDNA was prepared from either the YUC8ox or YUC9ox background and transcript levels were normalized to the reference gene *APT1* and then compared to the WT. Three biological replicates were run in triplicates and averaged. Means are given with their standard error (SE). A two fold-change between the WT seedlings and the respective overexpressor line was considered as differentially regulated (* fold-change ≥ 2).

To follow up on this finding, we decided to address the question whether the *XTH* genes are involved in the modulation of hemicellulose profiles. To this end, extracellular sugars were analyzed using the OLigo Mass Profiling (OLIMP) method. This technique offers a rapid and likewise sensitive way to measure plant cell wall glycostructures, being based on the enzymatic release of oligosaccharides from wall material facilitated by specific glycosylhydrolases. Subsequently, the analysis of solubilized oligosaccharide mixtures is conducted using matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Günl et al. 2011)

Cell walls from 24 cm tall inflorescence stems of YUC8ox, YUC9ox, and WT, were incubated with ethanol in order to remove starch and convert the hemicellulose fraction into monomeric sugars. After this, the remaining polysaccharides were enzymatically digested with a commercial endo- β -1,4-glucanase (EG) to release xyloglucans (XyG). Samples were then analyzed by MALDI-TOF MS in collaboration with Dr. Julia Kehr (University of Hamburg, Hamburg, Germany) (See Section 3.9.4).

The identification of xyloglucans after enzymatic digestion was performed according to Lerouxel et al. (2002) using a BRUKER MALDI-TOF/TOF.

The obtained results indicated that, in terms of xyloglucan polysaccharides (XyGs), the YUC8ox and YUC9ox derived cell wall preparations share spectra very similar to that of the wild type (Figure 23). Nevertheless, the more detailed inspection of the signal intensities determined that several XyG oligosaccharides, including XXXG, XXLG/XLXG, XXFG, XXFG+10Ac XLLG, XLLG+10Ac displayed incremented levels in the transgenic lines, YUC8ox and YUC9ox, in comparison to WT. Interestingly, YUC9ox exhibited higher abundance of these fragments in its cell walls. Alongside with the altered lignin composition, these results suggest that the decreased lignin polymerization degree observed in Section 4.1.4, probably facilitates a more effective breakdown of the cell wall-bound hemicellulose.

Figure 23: MALDI-TOF mass spectrometric analysis of xyloglucan oligosaccharides.

One milligram of cell wall material from *Arabidopsis* WT (Col-0) and the overexpressor lines, YUC8ox and YUC9ox, derived from 24 cm tall inflorescence stems, was utilized for OLIMP analysis. MALDI-TOF MS spectra of xyloglucan fragments were obtained after treatment with *endo*-1,4- β -D-glucanase (EG). Xyloglucans subunits were named according to the nomenclature introduced by Fry et al. (1993.) Herein, each letter corresponds to the last substitute sugar present in the xyloglucan side-chain: D-xylose (X), D-glucose, L-fucose (F), D-galactose (L). *O*-acetylated fragments are also indicated. Mass spectra represent data from three biological replicates run in triplicates. WT (top), YUC8ox (middle) and YUC9ox (bottom) panel. Data were analyzed using the mMass software package (Strohalm et al. 2008).

On the other hand, focusing on the YUC8ox and YUC9ox down-regulated genes, we found 347 genes and 147 genes to be repressed in YUC8ox and YUC9ox, respectively (See Supplementary Figure X). When searching for commonly down-regulated genes in the two genotypes, we were able to identify 64 commonly repressed genes (Figure 24a). These 64 genes were further functionally classified according to gene ontology (GOs) terms using the agriGO platform (Du et al. 2010), and 71 significantly enriched GO terms were identified. Intriguingly, among those commonly repressed genes, a group of chitin response-related genes turned out to be enriched in both examined genotypes (Figure 24b) (Figure 25). Chitin is a polymer of *N*-acetyl-D-glucosamine (GlcNAC) found in yeast and fungal cell walls, the exoskeleton of insects, and nematode egg shells. It has been reported that chitin is an important component of fungal pathogenicity and in controlling plant parasitic nematodes (Mian et al. 1982; Spiegel et al. 1986; Wan et al. 2008a; Wan et al. 2008b) . Thus, it can be speculated that the YUC8/9-mediated local production of IAA may contribute to regulating the chitin signal cascade either to modulate it after the perception of an attack by biotic predators or to prime the defence response.

In addition, GO terms associated with abiotic stress response, specially related to water deprivation, were found to be significantly enriched (Figure 25). This suggested that both *YUC8* and *YUC9* genes might be implicated in drought stress response.

Figure 24: Comparative analysis of negatively regulated genes between WT and either YUC8ox and YUC9ox.

a) Venn diagram showing common genes whose expression is down-regulated in YUC8ox and YUC9ox lines. Comparison between the *Arabidopsis* WT and the overexpressor lines, YUC8ox and YUC9, was performed using VENNY 2.1 (Oliveros 2007). **b)** List of the common YUC8ox and YUC9ox chitin response-related genes.

Figure 25: Gene Ontology (GO) analysis of YUC8ox and YUC9ox down-regulated genes.

Hierarchical tree graph of overrepresented GO terms in biological processes generated by singular enrichment analysis (SEA) using AgriGo (Du et al. 2010). Boxes represent GO terms with the corresponding definition labelled with the GO number and statistical information. Numbers indicate the genes in the input list associated with the number of total genes that GO term group. The box colors indicate levels of statistical significance with yellow = 0.05; orange = e^{-05} and red e^{-09} .

4.2 Molecular characterization YUC8 and YUC9 in relation to their role in biotic and abiotic stress responses in *Arabidopsis*.

4.2.1 Analysis of the YUC8- and YUC9-mediated defence against *Tetranychus urticae*.

As explained in Section 4.1.6, our microarray analysis identified 15 chitin related genes whose expression was downregulated in the *YUC8* and *YUC9* overexpression lines. It has been established that this glucosamine-based biopolymer, not only present in the fungi cell wall but also in the insect and mite exoskeleton, functions as an elicitor of the expression of diverse defence response-related genes (Eckardt 2008). To test whether the observed gene repression leads to a higher susceptibility to chitin-bearing pests, we performed a plant damage assay using the spider mite *Tetranychus urticae*.

In these experiment were tested wild-type *Arabidopsis*, the *yucca* single-, and double-knockout mutants (*yuc8ko*, *yuc9ko* and *yuc8/yuc9ko*), as well as the overexpression lines (*YUC8ox* and *YUC9ox*) (See Section 3.4.3). Ten adult female spider mites were placed on 10 plants of each genotype and allowed to feed for 4 days. All *A. thaliana* plants (3 to 4 weeks-old) were grown on soil under long day conditions. The *Tetranychus urticae* London strain used in this work was kindly provided by Dr. Isabel Díaz (CBGP-INIA, UPM, Madrid, Spain) and was adapted to feed on bean plants (*Phaseolus vulgaris*). To determine the possible defense phenotype of the different genotypes, we quantified the foliar damage after 4 days of spider mite feeding (See Section 3.5.1). The plant damage assay was performed in collaboration with Dr. Estrella Santamaria Fernandez (CBGP-INIA, UPM, Madrid, Spain) (See Section 3.5.1).

The result revealed, by eye, that both overexpression and knockout mutants manifested less signs of damage when compared with WT (Figure 26a). However, after damage quantification (Figure 26b), the *yuc* single mutants did not show significant differences to the wild type, although the suffered damage tended to be slightly reduced. Interestingly, *yuc8yuc9ko* double mutant displayed indeed significantly reduced damaged areas (70 mm² damage). Apparently, adult mites preferred to feed on wild type and knockout plants, which exhibited on average approximately 40 % more plant damage than *YUC8ox* and *YUC9ox* (Figure 26b). Remarkably, as represented in Figure 26b, *YUC8ox* and *YUC9ox* showed the least amount of area with damage (37 mm² and 15 mm² damage, respectively) (See Supplementary Material Figure S1).

Figure 26: Plant damage assay after spider mite (*Tetranychus urticae*) herbivory.

a) Pictures of representative leaves from Arabidopsis WT (Col-0), *yuc8ko*, *yuc9ko*, *yuc8/yuc9ko*, YUC8ox and YUC9ox after 4 days of *T. urticae* feeding. Leaf (top) and leaf detail (bottom). Diagnostic features of plant damage are indicated with arrows. **b)** Quantification of the total plant damage area (expressed in mm²) in WT and YUC8/9 mutants. Data are means $\pm$ SE. Five biological replicates per genotype were assessed. Statistical analysis was performed using Student's t-test comparing between the WT plants and each mutant genotype. Asterisks indicate significant difference at * $P < 0.05$ and ** $P < 0.01$. Scale bar = 1 mm.

Additionally, a second group of plants was used to evaluate the spider mite herbivory response, in terms of cell death, by the trypan blue exclusion test (See Section 3.10.2). This assay is based on the principle that intact membranes from viable cells exclude the specific dye trypan blue giving a clear cytoplasm. However, dead cells are permeable to the dye, which consequently results in a blue stained cytoplasm. Consistent with the leaf damage assay, this analysis revealed a visibly higher cell death in WT in comparison with YUC8ox, YUC9ox, *yuc8ko*, *yuc9ko* and

yuc8yuc9ko (Figure 27a). Interestingly, YUC8ox showed higher number of dead cells than YUC9ox, which presented almost no detectable blue coloured cells.

On the other hand, it has been documented that reactive oxygen species (ROS), such as free radicals and hydrogen peroxide (H_2O_2), accumulate during the wound response and that they are required for triggering defence gene induction (De Puyseleir et al., 2011). Hence, to further understand the response to spider mite feeding, we stained a third group of plants using 3,3'-diaminobenzidine (DAB) in order to document a putative H_2O_2 accumulation (See Section 3.10.3). This method is based on the peroxidase-catalysed of DAB- H_2O_2 , which forms an insoluble brownish precipitate that can be visualised after removal of chlorophyll. We found that spider mite herbivory triggered a strong accumulation of H_2O_2 in WT and moderate accumulation in *yuc9ko*, *yuc9ko*, and *yuc8yuc9ko*. Consistent with the cell death experiment, YUC8ox showed a higher H_2O_2 accumulation than YUC9ox, where the brown precipitate was almost imperceptible (Figure 27b).

Figure 27: Histochemical analysis after spider mite herbivory.

Staining of *Arabidopsis* WT, *yuc8ko*, *yuc9ko*, *yuc8/yuc9ko*, YUC8ox and YUC9ox leaves after 4 days of *T. urticae* feeding. **a)** Trypan blue, **b)** DAB. Arrows indicate cell death and H_2O_2 accumulation, respectively. Entire leaves (top row) and leaf details (bottom row). Scale bar = 1 mm.

4.2.2 *Pseudomonas* infection

To further decipher the role of YUC8 and YUC9 in biotic stress responses in *Arabidopsis*, we analyzed the response of the *YUC8/9* knockout and overexpression mutants to infection by the hemibiotrophic pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*PstDC3000*) using the flood-inoculation method (Ishiga et al. 2011).

To carry out this analysis, 15 day-old seedlings were submerged in a *PstDC3000* bacterial suspension. The bacteria strain was kindly provided by Dr. Emilia Solanilla (CBGP-INIA, UPM, Madrid, Spain). Three days after inoculation, we evaluated the bacterial growth using 2 leaves of each genotype (See Section 3.5.2).

The results determined that the single and double loss-of-function mutants, *yuc8ko*, *yuc9ko* and *yuc8yuc9ko*, showed the same symptoms and bacterial growth than the WT (Figure 28a and b). Interestingly, YUC8ox seedling showed enhanced resistance against *PstDC3000* (5 % decrease in bacteria growth). In contrast, the bacterial growth in leaves of YUC9ox was significantly promoted when compared to WT (Figure 28b) (See Supplementary Material Figure S2).

Figure 28: Resistance assay against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*PstDC3000*) using the seedling flood-inoculation method.

P. syringae infection was performed on *Arabidopsis* WT (Col-0), *yuc8ko*, *yuc9ko*, *yuc8yuc9ko*, YUC8ox and YUC9ox. Petri dishes containing 15 day-old seedlings of each genotypes, grown on solid MS medium, were covered with *PstDC3000* suspension at a dilution of 5×10^6 colony-forming units (CFU) $\cdot$ ml⁻¹ for 3 min. **a)** Representative picture of the disease phenotypes three days after bacteria inoculation. **b)** *PstDC3000* growth 3 days post-inoculation. Bacterial population are expressed as log (cfu/leaf). Two leaves per plant and four biological replicates per genotype were used. Asterisks indicated significant difference between WT and each mutant genotype by Student's t-test at *P < 0.05 and ** P < 0.01.

4.2.3 Drought stress experiments and water content

In addition to the observed implications of YUC8 and YUC9 in biotic stress responses, our expression analyses suggested a possible role for YUC8 and YUC9 in plant response to water deprivation (See Section 4.1.6). A connection between YUCCA enzymes and an increased drought tolerance has already been reported for potato plants the overexpress *Arabidopsis YUC6* (Kim et al. 2013). In order to inspect the function of YUC8 and YUC9 in this context, we performed an *in silico* expression analysis of both *YUC8* and *YUC9* genes under drought stress conditions using the Botany Array Resource (BAR) Expression Browser program (See Section 3.6.2). This online tool allows the creation of an electronic Northern in order to analyse expression profiles of particular genes by scanning all the experimental conditions contained in the database (Toufighi et al. 2005). Interestingly, we observed that the transcriptional response to drought of *YUC8* and *YUC9* is different in shoot and root tissues. Whereas in shoots *YUC8* and *YUC9* seem to be induced by long-term treatments, i.e. 24 h treatment, their response in root tissues was detected earlier (Figure 29). Importantly, in root tissue *YUC8* and *YUC9* exhibited an alternating expression pattern, suggesting a compensatory effect due to a possible redundant function of YUC8 and YUC9.

Figure 29: Expression profile of *YUC8* and *YUC9* under drought stress conditions.

Differential expression of the auxin biosynthetic genes, *YUC8* and *YUC9*, in drought stress experiments performed on root and shoot tissues. The *in silico*-analysis was conducted using the e-Northern tool (http://bar.utoronto.ca/affydb/cgi-bin/affy_db_exprss_browser_in.cgi). Data refer to different experiments using 10 day-old seedling of wild-type *Arabidopsis*. The duration of the applied drought stress treatment and the expression levels are indicated. Significant differences in the expression are indicated with asterisks (* fold-change ≥ 2).

On the basis of the above discussed results, we performed a drought stress assay. For this, well-watered 3 week-old plants of each genotype, *Arabidopsis* WT (Col-0), *yuc8ko yuc9ko* and *yuc8yuc9ko*, were subjected to a prolonged drought period of 15 days. Eventually, the survival rate was calculated 10 days after re-watering (See Sections 3.4.3 and 3.5.3.1).

Similar to the WT plants, both single loss-of-function mutant lines exhibited a survival rate of 0 %, while the survival rate of *yuc8yuc9ko* lines (15 %) after drought stress stimulus was visibly higher than of the control (0 %) (Figure 30a). Intriguingly, only *YUC9* overexpression conferred an increased drought tolerance and exhibited a stay-green phenotype. All of the *YUC9ox* plants survived the imposed drought stress conditions, whereas the *YUC8ox* plants showed to be as drought sensitive as the wild type, displaying a survival rate of 0 %. Although the increased drought tolerance of the *yuc8yuc9ko* double null mutant is somewhat unexpected, and may possibly be explained by compensatory effects of other *YUCCA* family members, our observations clearly suggest that at least *YUC9* is seemingly likely involved conferring drought stress tolerance (See Supplementary Material Figure S3).

To further investigate the role of *YUC8* and *YUC9* in drought stress we also evaluated the relative water content (RWC), which is considered an important indicator of the plant water status. The RWC reflects the balance between water supply to the leaf tissue and the transpiration rate (Teulat et al. 1997). Thus, to determine the RWC whole rosettes of the *A. thaliana* WT and the genotypes *yuc8ko*, *yuc9ko*, *yuc8yuc9ko*, *YUC8ox* and *YUC9ox* were sampled after 3 weeks of growth on soil under long day conditions. After the harvest, the plant rosettes were immediately weighed. Subsequently, samples were submerged in water during four hours, weighed again, and finally were dried at 60 °C over 2, 4, 6, 8 and 24 h. After each drying period samples were weighed. RWC was calculated according to Barrs & Weatherley (1962) (See Section 3.5.3.2).

In the conducted assay, *yuc8ko* displayed the highest initial RWC. However, *yuc8ko* displayed also the fastest decrease in RWC, suggesting a high water consumption of this line through transpiration (Figure 30b). In contrast, the genotype *yuc9ko* exhibited the lowest initial RWC, which could indicate a reduced water absorption capability. Remarkably, the RWC values of the *yuc8yuc9ko* plants resembled very much those of the WT, which on the one hand confirms the results obtained for the single mutants, as it appears highly likely that the line with the highest and with the lowest RWC value show compensating effects. On the other hand, the induction of other *YUC* genes in response to the lack of *YUC8* and *YUC9* in the double knockout line cannot be excluded. Most importantly, we observed that the RWC of *YUC8ox* is

dramatically decreased. This result point to a high transpiration rate of this overexpression mutant, and could explain the extremely low survival rate obtained in the drought stress experiments. Moreover, consistent with the obtained resistance against drought, YUC9ox was able to maintain the water content longer than the rest of lines.

Figure 30: Role of YUC8 and YUC9 in drought stress

Analyses were performed using 3 week-old *Arabidopsis* WT, *yuc8ko*, *yuc9ko* *yuc8/yuc9ko*, YUC8ox and YUC9ox plants **a)** Drought stress experiment. Well-watered plants of each genotype were grown under standard long-day conditions (16 h light/8 h dark) but without irrigation for 15 days. Photographs were taken after a re-watering period of 10 days. Survival rate is indicated in parenthesis (n = 15). **b)** Relative water content. Whole rosettes of each genotype were detached and weighed (fresh weight). Thereafter, plants were weighed after 4 h submerged in water (turgid weight). Subsequently, samples were dried at 60°C and weighed at the indicated time points. Water content was calculated as the percentage of the ratio fresh weight/turgid weight at time zero.

In summary, the expression analysis revealed a different expression pattern of *YUC8* and *YUC9* between shoot and root with respect to the time of treatment. Furthermore, the observed expression pattern invite to speculate about a potential compensatory effect of both genes. However, this hypothesis will need further investigation. On the other hand, we found that rather *YUC9* than *YUC8* has a possible role in plant response to water deficit by conferring tolerance to drought.

4.2.4 ABA, MeJA and SA levels are altered in *YUC8* and *YUC9* knockout and overexpression mutants.

Among the known plant hormones, abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are known to play important roles in mediating responses to multiple biotic and abiotic stresses (Forcat et al. 2008; Verma et al. 2016). Here, we demonstrated the implication of *YUC8* and *YUC9* in the response to spider mite attack and drought stress responses (See Sections 4.2.1 and 4.2.3). Furthermore, we also provided evidence for the *YUC8*- and *YUC9*-overexpression mediated promotion of ET biosynthesis (See Section 4.1.1). Thus, we decided to address the question whether the overexpression of *YUC8* and *YUC9* also influences the production of the remaining defence response hormones, which include ABA, JA and SA. In order to confirm the observed chemotype in the auxin-overproducing *YUC8ox* and *YUC9ox* we also evaluated the levels of the growth hormone IAA.

To carry out this experiment, *Arabidopsis* (Col-0), *yuc8ko*, *yuc9ko*, *yuc8yuc9ko*, *YUC8ox* and *YUC9ox* seedlings were grown for 10 days on soil under sterile conditions (See Section 3.4.3). Then, approximately 100 mg of plant material were sampled, extracted with methanol, and analyzed by GC-MS (See Section 3.9.6). Endogenous hormone contents are expressed as a ratio of the phytohormone to the applied corresponding stable isotope-labelled internal standard.

As previously reported (Hentrich et al. 2013a), the *yuc8* and *yuc9* knockout lines showed no differences in the IAA levels in comparison to WT (Figure 31a), while the *yuc8/yuc9* genotype exhibited a moderately decreased level of IAA (approx. 0.2-fold decreased). On the contrary, the *YUC8ox* and *YUC9ox* lines displayed significantly higher IAA contents than WT plants (0.8-fold and 0.4-fold increased IAA levels, respectively).

Intriguingly, when comparing the levels of ABA it turned out that the content of this drought stress related signalling molecule is significantly decreased in the *yuc8ko* (0.5-fold decrease) and *yuc9ko* lines (0.6-fold decrease) relative to WT (Figure 31b). In contrast, *YUC9ox* plants showed a slight increase in the ABA levels (0.3-fold increment). However, this increase was not significant, neither were the differences found for *YUC8ox* and the *yuc8yuc9ko* double mutant. It has been reported that ABA triggers stomatal closure in response to drought (Leckie et al. 1998). Thus, along with the data from the RWC (See Section 4.2.3), it can be speculated that impaired ABA production in *yuc8ko* and *yuc9ko* may inhibit stomatal closure promoting water loss. In contrast, the increased fitness of *YUC9ox* could be the consequence

of a better water stress responsiveness through a tighter regulation of the stomatal aperture.

A closer inspection of the levels of JA in genotypes *yuc8ko*, *yuc9ko*, *yuc8yuc9ko* and YUC8ox did not reveal significant changes compared to WT (Figure 31c). In YUC9ox, however, a significant increase of JA levels of 0.76-fold was detected, which puts further emphasis on the cross-talk between IAA and JA mediated by YUC9.

On the other hand, when analysing the SA data, no significant alteration was perceived for the *yuc8ko*, *yuc9ko*, *yuc8yuc9ko* and YUC9ox lines (Figure 31d). Nevertheless, here YUC8ox displayed a moderate augmentation of the SA levels (0.5-fold) in comparison to the WT. SA has widely been associated with response of plants to biotrophic and hemibiotrophic pathogens by triggering systemic acquired resistance (SAR) (Conrath 2006; Chen et al. 2009; Vlot et al. 2009). Taking this involvement of SA into account, the observed elevated SA content in YUC8ox can explain the observed improved resistance to *Pst*DC3000 of this mutant plant.

Figure 31: Phytohormone levels in the *Arabidopsis* WT and several YUCCA8/9 mutants.

Quantification of plant hormone levels in 10 day-old seedlings of the following genotypes: *Arabidopsis* WT, *yuc8ko*, *yuc9ko*, *yuc8yuc9ko*, YUC8ox and YUC9ox by gas chromatography-mass spectrometry. **a)** Free indole-3-acetic acid (IAA) levels. **b)** Absciscic acid (ABA). **c)** Jasmonic acid (JA). **d)** Salicylic acid (SA). The IAA, ABA, JA and SA contents were calculated as the ratio of the endogenous hormone to the respective internal standard. Data are normalized to the control plants and expressed as μmol of IAA per g of FW. The given values represent means $\pm$ SE. For each genotype three independent samples were analyzed. Statistical analysis was performed using Student's *t*-test individually comparing the mean of WT with those of the different mutants. Asterisks

4.3 Disclosing the jasmonate-induced TF(s) that regulate *YUC8* and *YUC9* expressions

4.3.1 The jasmonate signalling components MYC2, MYC3 and MYC4 are required for auxin production after methyl jasmonate treatment.

As already mentioned, our previous work (Hentrich et al. 2013a) demonstrated that the transcriptional response of the *Arabidopsis thaliana* indole-3-acetic acid (IAA) biosynthetic genes *YUC8* and *YUC9* towards MeJA is almost inhibited in the *coi1* mutant (See Section 1.6). Mainly based on this experiment, it is expected that the transcriptional regulation of the two IAA biosynthetic genes proceeds via the CORONATINE INSENSITIVE 1 (COI1) pathway. COI1 collaborates with the transcriptional repressors of the JASMONATE ZIM DOMAIN (JAZ) family and the MYC transcription factor family in the integration and procession of JA responses, forming a core COI-JAZ-MYC signalling module (Chini et al. 2009).

In order to substantiate our proposed working hypothesis, we first measured the MeJA-triggered production of IAA in *Arabidopsis thaliana* wild-type plants, the *myc* single mutants *myc2*, *myc3* and *myc4*, the double mutants *myc2/myc3*, *myc2/myc4* and *myc3/myc4*, and the triple mutant *myc2/myc3/myc4* by GC-MS analysis.

To perform this experiment, 10 day-old seedlings of *Arabidopsis* WT and the *myc* mutants, grown on solid MS medium under standard sterile conditions (22 °C, 16 h light/8 h dark and 100 $\mu\text{mol}/\text{m}^2 \text{ s}^{-1}$ light intensity) were harvested. Subsequently, samples were incubated for 4 h with either 50 μM of MeJA or a control solution (0.5 % methanol, v/v). After the treatment, approximately 100 mg of plant material was harvested for each sample and subjected to solid-phase extraction of organic acid and analysis by GC-MS (See Sections 3.4.3 and 3.9.6).

The result indicated that in WT seedlings the MeJA treatment stimulated IAA production by more than 4-fold when compared to mock-treated WT seedlings (Figure 32). In contrast, the IAA production in response to the MeJA treatment was clearly impaired in all *myc* single mutants, as well as in the *myc2/myc3* and *myc2/myc4* double and *myc2/myc3/myc4* triple mutants. Indeed, the strongest effect was visible in the triple mutant, which experimented a 0.5-fold reduction of IAA levels in comparison to the respective mock-treated seedlings. This additive effect observed in the triple mutant indicates that MYC2, MYC3 and MYC4 transcription factors are presumably involved in mediating IAA accumulation in response to oxylipin treatment. However, it is remarkable though that compared to the *myc3/myc4* mock treated seedlings, the *myc3/myc4* mutant exhibited

approximately 3.5-fold increased IAA levels after the MeJA treatment. Suggesting an essential role of MYC2 in the regulation of *YUC8* and *YUC9* expression.

In summary, this results confirm the hypothesis of a possible JA-IAA crosstalk and provide further evidence for a direct implication of the jasmonate signalling components MYC2, MYC3 and MYC4 as transcriptional regulators of *YUC8* and *YUC9*.

Figure 32: Levels of free endogenous indole-3-acetic acid after MeJA treatment.

IAA levels were evaluated by gas chromatography-mass spectrometry analysis using 10 day-old *Arabidopsis* WT, *myc2*, *myc3*, *myc4*, *myc2/myc3*, *myc2/myc4*, *myc3/myc4* and *myc2/myc3/myc4* seedlings. IAA accumulation was calculated as a ratio of endogenous IAA to the stable isotope-labelled d_2 -IAA. The final relative IAA content after the MeJA treatment is expressed in reference to the respective control treated plants as $\mu\text{mol IAA} \cdot \text{fresh weight}^{-1}(\text{g})$. For each genotype three independent samples were analyzed in triplicates. Data are means $\pm$ SE. Statistical analysis was performed using Student's t-test comparing between the mock treated seedlings and each respective MeJA treated WT or *myc* loss-of-function mutant. Significant differences between means are indicated with asterisks (* $P < 0.05$, ** $P < 0.01$).

4.3.2 *YUC8* and *YUC9* expression is suppressed in different *myc* loss-of-function mutant backgrounds

In addition to the IAA content assay, it appeared interesting to us interesting to check whether the expression levels of *YUC8* and *YUC9* would be affected after MeJA treatment in the single, double and triple *myc* mutants. To this end, we performed a series of qRT-PCR analyses to quantify transcript levels in the respective mutant backgrounds (Figure 33).

We employed *A. thaliana* WT seedlings as well as the *myc* knockout mutants. The plants were grown on solidified MS medium for 10 days under sterile standard conditions (See Section 3.4.3). As described in Section 1.6 (Figure 11), the expression of *YUC8* is slightly induced after 3 h of MeJA treatment in WT seedling, whereas the transcriptional responses of *YUC9* was shown to occur substantially earlier when similarly treated with MeJA. Taking this information into account, samples used for the quantification of *YUC8* expression were transferred to either 50 μ M MeJA solution or a control solution (0.5 % methanol, v/v) and incubated along 4 h. Nevertheless, samples used for *YUC9* expression analysis were also transferred to oxylipin-containing or control solution, but incubated only over 2 h. After the chemical treatment, samples were used for RNA extraction and cDNA synthesis (See Sections 3.4.4, 3.7.7, 3.7.9 and 3.7.10). Thereafter, the relative expression of *YUC8* and *YUC9* was analysed by qRT-PCR (See Section 3.7.11).

In agreement with the results on the IAA contents, we observed that the abundance of *YUC8* transcripts increased 1.3-fold in WT seedlings, after the MeJA treatment relative to mock-treated WT plants (Figure 33a). In contrast, the analysis of the *myc* single mutants, *myc2* and *myc4*, the *myc* double mutants *myc2myc4* and *myc3myc4*, and the *myc* triple mutant *myc2myc3myc4*, revealed a lack of transcriptional response of *YUC8* after treatment. Surprisingly, in the *myc3* mutant *YUC8* transcription was slightly induced after 4 h of MeJA treatment (0.67-fold induction) in comparison to the respective mock control. In addition, it was found that in the *myc2myc3* background the transcription of *YUC8* was rather induced than suppressed after 4 h of MeJA treatment (1.4-fold induction). Taken together, these data suggest that the transcriptional control of *YUC8* is mainly exerted by the action of MYC2 and MYC4 transcription factor.

Similar to *YUC8*, but to a stronger degree, *YUC9* transcript levels increased in WT seedlings after 2 h of MeJA treatment (8.7-fold induction) (Figure 33b). This highlights that the expression of both auxin biosynthetic genes, *YUC8* and *YUC9*, is

activated in response to MeJA. Importantly, we found impairment of the transcriptional response to MeJA in *myc2*, *myc3*, *myc2myc3*, *myc2myc4*, *myc3myc4* and *myc2myc3myc4* seedlings. Remarkably, in the *myc4* background *YUC9* expression was highly induced by MeJA (7.8-fold induction), suggesting a possible role of MYC2 and MYC3 as major regulators of the *YUC9* gene transcription in response to oxylipins. It is noteworthy that we did not observe full suppression of *YUC8* and *YUC9* transcription in the *myc2myc3myc4* triple mutant (Figure 33a-b). Hence, it has to be concluded that other transcription factors other than the MYCs also contribute to the transcriptional regulation of *YUC8* and *YUC9*.

Figure 33: Relative expression of *YUC8* and *YUC9* genes after methyl jasmonate treatment in wild-type seedlings and *myc* mutants.

a) Quantitative reverse transcriptase PCR for *YUC8* after 4 h of MeJA treatment. **b)** qRT-PCR of *YUC9* after 2 h of MeJA treatment. The *YUC8* and *YUC9* transcript levels were relativized to the reference genes *APT1* and *UBI10* and then normalized to the mock-treated (0.5 % MeOH) plants. Three biological replicates were run in triplicates and averaged. Means are given with their standard error (SE). A two fold-change between the mock treated seedlings and the respective *myc* knockout mutant was considered as differentially regulated (* fold-change ≥ 2).

4.3.3 *In silico* analysis of *YUCCA* promoter sequences indicates a conserved MYC2, MY3 and MYC4 binding sites in the promoter of *YUC8* and *YUC9*.

Our first analyses supported the idea that the bHLH transcription factors MYC2, MYC3 and MYC4 decisively contribute to the regulation of both *YUC8* and *YUC9* expression. Thus, to further confirm this hypothesis we firstly aimed at the identification of the conserved core promoter region for *YUC8* and *YUC9* by computational analysis (See Section 3.6.1).

For this analysis, we conducted a NCBI nucleotide BLAST search using the *A. thaliana YUC8* and *YUC9* coding sequences (CDS) to identified the homolog genes in the closest relative *Arabidopsis lyrata* L. subsp. *lyrata* O’Kane & Al-Shelbaz (Beck et al. 2007; Beilstein et al. 2008). Then, the full-length promoter sequences from the *A. thaliana YUC8* and *YUC9* genes, as well as the respective *A. lyrata* genes encoding for *YUC8* and *YUC9*-homologs, XM_002869432 and XM_002869432, respectively, were retrieved from the NCBI database and aligned using the online EMBOSS Matcher tool for pairwise sequence alignment. The sequence alignment was performed using the corresponding pair of homolog genes, *i.e.* *A. thaliana YUC8*-*A. lyrata YUC8* and *A. thaliana YUC9*-*A. lyrata YUC9*. Following the determination of similarities in the promoter sequences, the predicted conserved regions obtained for the *A. thaliana YUC8* and *YUC9* promoter were used to identify and isolate the equivalent promoter sequences for the rest of the *A. thaliana YUCCA* family members: *YUCCA1* (pYUC1); *YUCCA2* (pYUC2); *YUCCA3* (pYUC3); *YUCCA4* (pYUC4), *YUCCA5* (pYUC5), *YUCCA6* (pYUC6), *YUCCA7* (pYUC7), *YUCCA10* (pYUC10) and *YUCCA11* (pYUC11). Finally, all of the promoter sequences were subjected to an *in silico*-analysis using the bioinformatics tools AtPan (Chen et al. 2012) and PLantCARE (Lescot et al. 2002) to search for MYC2 *cis*-acting elements, which included the G-box, known as the preferred DNA binding site for MYC2, MYC3 and MYC4, and sixteen described G-box variants (Fernandez-Calvo et al. 2011). To ensure the incorporation of all the possible G-box variants the promoter sequences were also manually inspected.

As shown in Figure 34, for each *A. thaliana YUCCA* gene a conserved region of approximately 3000 bp upstream of the transcription start was isolated and used for the prediction of MYC2 binding motifs. Intriguingly, we found that the jasmonate responsive elements, G-box and G-box variants, are highly enriched in all *YUCCA* promoter sequences. However, we detected that only pYUC8 and pYUC9 contain a specific “tandem” DNA binding motif configuration. This “tandem” includes two canonical G-boxes (5'-CACGTG-3') and one G-box variant 9 (5'-CACGTC-3') at the nucleotide positions -535, -555 and -571 in case of pYUC8, and -1240,-1247 and -

1272 in pYUC9. In addition, we found that this particular configuration is followed by the G-box variant 3 (5'-CATGTG -3') in the nucleotides positions -140 and -207 of pYUC8 and pYUC9, respectively. Remarkably, the G-box variant 3 was relatively close of a 5'-TATAAA-3' sequence, positions -153 (pYUC8) and -267 (pYUC9). This sequence was identified as the consensus TATA-box, which is commonly recognized as a transcription enhancer (Kiran et al. 2006).

In conclusion, the prediction of putative *cis*-elements prompted us to hypothesize that this particular G-box configuration may be crucial for the transcriptional regulation of both *YUC8* and *YUC9*.

Figure 34: Schematic representation of the distribution of *cis*-regulatory elements (G-box and G-box variants) in the promoter of *Arabidopsis YUCCA* genes.

The conserved promoter region (from -3000 to -1) upstream to the transcriptional start point (ATG) of the eleven *YUCCA* family members is shown. All putative G-boxes are colour-coded (square) and assigned with a specific sequence. Numbers indicate the corresponding G-box sequence, with number 1 being the canonical G-box. Different colours indicate different experimental MYC2 binding affinities (Fernandez-Calvo et al. 2011).

4.3.4 Exploring the transactivation of the *YUC8* and *YUC9* promoter GUS reporter construct by MYC2, MYC3 and MYC4.

As described above, we discovered that the 5' non-coding sequences of the promoter of both *YUC8* and *YUC9* exhibited a “tandem” sequence composed by two canonical G-boxes and the G-box variants referred to as variant number 9. In addition, both promoters also contained an additional G-box variant designed as number 3 (See Section 4.3.3). With the goal to verify whether these putative DNA binding sites are indeed involved in the transcriptional regulation of *YUC8* and *YUC9*, we decided to perform a transient transactivation effector-reporter experiment in *Nicotiana benthamiana* leaves.

To prepare the effector plasmid, the independent whole *MYC2*, *MYC3* or *MYC4* open reading frames (ORFs) were isolated by PCR amplification from WT *A. thaliana* plants (Col-0) (See Section 3.7.3). The sequences were fused to the *Cauliflower mosaic virus* (CaMV) 35S promoter to generate three constructs, *35S::MYC2*, *35S::MYC3*, and *35S::MYC4*, that facilitate constitutive and strong expression of *MYC2*, *MYC3* and *MYC4* (Figure 35a). As reporter, a series of truncated promoter fragments from *YUC8* or *YUC9*, containing the tandem DNA motifs or the final *cis*-acting element (#3), as well as a promoter segment containing none of these regulatory sequences (Ø), were amplified by PCR from the pSP-ENTR2-*YUC8/9*Prom3kb vector construction (Hentrich *et al.*, 2013). After isolation, all promoter fragments were fused to the β -glucuronidase (GUS) reporter gene. This resulted in the generation of three promoter constructs for p*YUC8*, i.e. *191::GUS*, *3::GUS* and *Ø::GUS*, and three promoter constructs for p*YUC9*, i.e. *191::GUS*, *3::GUS* and *Ø::GUS* (Figure 35a). In this series of experiments, it was necessary that the constructs contained corresponding right border (RB) and left border (LB) sites in both the effector and reporter plasmid, in order to facilitate the transfer of the incorporated fragments into *N. benthamiana* host cells and test their interaction under *in planta* conditions (Gelvin 2003) (See Section 3.8.3) (See Supplementary Material Figures S4 and S5 for full cloning strategy details).

Subsequently, we investigated GUS expression levels after *Agrobacterium tumefaciens*-mediated plant transformation. To do so, 3-4 week-old *N. benthamiana* plants, cultured on soil under greenhouse conditions, were co-infiltrated with mixed cultures of *A. tumefaciens* clones harbouring the corresponding reporter and effector plasmids (See Section 3.7.14). As negative control we also infiltrated an empty vector control along with the *35::MYC2*, *35::MYC3* and *35::MYC4* plasmids constructions, respectively, in independent *N. benthamiana* leaves. After infiltration, plants were incubated over 3 days under standard greenhouse

conditions. Discs from the transformed *N. benthamiana* leaves were then analyzed by histochemical GUS staining (See Section 3.10.4).

The observed blue coloration revealed that the GUS expression, driven by the *191::GUS* and *3::GUS* constructs of both *YUC8* and *YUC9* promoters, was strongly activated in presence of either of the transcription factors MYC2, MYC3 or MYC4 relative to the fusion construct $\emptyset::GUS$ or the empty vector control pMDC-163 (Figure 35b). At the same time, the detected weak blue staining perceived for the $\emptyset::GUS$ construct and the negative control indicated a very weak background activity.

Figure 35: Transactivation assays in tobacco plants.

a) Scheme of the constructs utilized in the transient reporter-effector assay. Full-length *MYC2*, *MYC3* and *MYC4* coding sequences were cloned into the 35S containing vector pEARLY 201. The indicated attB1 and attB2 sites represent specific recombination sites that facilitate the integration of DNA fragments by homologous recombination using the Gateway cloning system (Invitrogen). The truncated promoted fragments were inserted into the *KpnI*/*XbaI* sites in the pMDC163 vector, upstream of the *uidA* reporter gene encoding for a bacterial β -glucuronidase, which is followed by a *Nos* terminator cassette. Numbers refer to the G-box sequences contained in the different promoter fragments. The presence of the TATA-box is also indicated. The predicted MYC binding sites contained in each fragment are indicated. Ø is utilized to refer a promoter fragment lacking the investigated cis-regulatory sequences (#919 and #3).

b) Histochemical GUS staining of *N. benthamiana* leaf discs agroinfiltrated with the 191::GUS, 3::GUS and Ø::GUS constructs of pYUC8 and pYUC9 in presence of the constitutively expressed MYC2, MYC3 and MYC4 transcription factors. Empty vector pMDC163 was employed as the negative control. GUS activity assays were directly performed in leaf discs treated with X-Gluc as substrate.

In a second experiment, similarly agroinfiltrated leaf discs were also utilized to quantify the transactivation of GUS reporter gene expression employing a fluorometric technique (See Section 3.9.8). Notable, in agreement with the histochemical results the quantitative enzymatic assay showed that the *191::GUS* and *3::GUS* promoter-reporter constructs for both *YUC8/9* promoters displayed significant GUS activation when compared to the $\emptyset::GUS$ construct and the negative control in presence of MYC2 (Figure 36a). GUS activity of *191::GUS* and *3::GUS* increased 2-fold and 0.8-fold in pYUC8, and 2.3-fold and 1.3-fold in pYUC9, respectively.

However, when the *N. benthamiana* leaves were co-infiltrated with different pYUC8 constructs and *35::MYC3*, the enzymatic analysis indicated that only the *191::GUS* construct was significantly activated, with almost 1.5-fold increase of the GUS activity compared to the negative control (Figure 36b). It is noteworthy that the absence of GUS activity in *3::GUS* could point out that MYC3 does not effectively bind to the 5'-CATGTG-3' element #3. Nevertheless, the analysis of the *YUC9* promoter draw this possibility into question since the fluorometric assay revealed a strong activation of the GUS activity in *191::GUS* and *3::GUS* relative to the empty vector control (2.5-fold and 11.5-fold increased GUS activity, respectively).

On the other hand, we observed that the transcription factor MYC4 significantly activated the *3::GUS* construct in the pYUC8, whereas in the pYUC9, both *191::GUS* and *3::GUS* showed 4.5-fold and 4-fold increased GUS activity levels, respectively, when compared to the negative control (Figure 36c). In contrast to MYC2 and MYC3, the presence of MYC4 only resulted in a moderate GUS activity for the $\emptyset::GUS$ construct of pYUC8. This promoter fragment contains the G-box variant #11 (5'-CAAATG-3') in its sequence, suggesting that this *cis*-acting element could be important in the transcriptional regulation of *YUC8* exerted by MYC4.

In summary, the histochemical and fluorometric assays demonstrate that the studied *cis*-regulatory elements most likely play an essential role in the regulation of *YUC8* and *YUC9* gene expression *in vivo*. In addition, the results highlight MYC2 as the powerful positive regulator of both auxin biosynthetic genes, most probably through the interaction with the promoter G-box elements included in the “tandem” 1-9-1 (5'-CACGTG - CACGTC - CACGTG-3'), and/or the final *cis*-regulatory G-box #3 (5'-CATGTG-3'). The results also suggest that MYC3 and MYC4 could act as possible co-operator in the transcriptional regulation of *YUC8* and *YUC9*.

Figure 36: Fluorometric quantification of GUS activity in *N. benthamiana* leaves agroinfiltrated with different *YUC8* and *YUC9* promoter fragments.

a) Relative GUS activity of the *191::GUS*, *3::GUS* and *Ø::GUS* reporter constructs co-transformed with the effector construct *35::MYC2*, **b)** *35::MYC3* and **c)** *35::MYC4*. Empty vector pMDC163 was employed as the negative control. GUS activity was normalized to the negative control and expressed as pmol 4-methylumbelliferone (MU)/mg protein/min. Protein amount was determined using the Bradford method. Data are presented as the means $\pm$ SE. Three different leaves per plant were agroinfiltrated. The quantification of GUS activity for each promoter construct was measured in triplicates. Statistical analysis was performed using Student's t-test comparing between the negative control and each promoter construct. Significant differences between means are indicated with asterisks (* $P < 0.05$, ** $P < 0.01$).

4.3.5 MYC2 activates GUS expression through the interaction with the *YUC8* and *YUC9* in promoter in *Arabidopsis* mesophyll protoplast.

To independently validate whether the *YUC8* and *YUC9* promoters are targets of MYC2 by an alternative *in planta* method, we performed a transient effector-reporter assay in mesophyll protoplast of *A. thaliana*.

In this experiment, constructs analogous to those used for transient expression in *N. benthamiana* leaves were employed. They only differ in the plasmid backbone used for cloning. Thus, for the effector plasmid we generated constructs containing the *Arabidopsis* MYC2 complete coding sequence was fused to the CaMV 35S promoter, giving rise to a *35S::MYC2* construct (Figure 37a). For the reporter

constructs, the truncated pYUC8 and pYUC9 fragments, harbouring either the “tandem” 191-DNA motifs, the final G-box #3, or none of these regulatory sequences, were fused to the *GUS* reporter gene, resulting in the corresponding 191::*GUS*, 3::*GUS* and $\emptyset$::*GUS* constructs (Figure 37a) (See Sections 3.3.2 and 3.8). A detailed cloning strategy is described in the Supplementary Material Figures S4 and S5.

To investigate the transcriptional activity, leaf mesophyll protoplasts were isolated from 4 to 5 week-old seedlings of *A. thaliana* (Col-0) grown on soil under short day conditions (8 h light/ 16 h dark). The isolated protoplast were subsequently co-transfected with different combinations of the generated effector and reporter plasmids. In addition, an empty pBT-10 vector was co-transfected with the 35::*MYC2* effector constructs as negative control. For each plasmid co-transfection combination a positive control consisting of a reporter plasmid harbouring the NAN gene under the control of a 35S promoter (35::*NAN*) was also included. This synthetic sialidase reporter gene has been tested as a GUS-compatible reporter gene (Kirby & Kavanagh, 2002). All plasmids were co-transfected in a ratio of 15:3:1. After 16 h of transient expression, protoplasts were used to carry out a fluorometric assay in order to quantitatively evaluate the GUS enzyme activity (See Sections 3.9.8, 3.9.9 and 3.7.13).

The relative values of GUS activity revealed that, in comparison to the negative control, the transcription factor MYC2 significantly activates the GUS reporter gene of the 919::*GUS* and 3::*GUS* constructs in both pYUC8 and pYUC9 Figure 37b. Remarkably, the detected enzymatic GUS activity for the construct pYUC9- $\emptyset$::*GUS*, showed an increment of approximately 1.5-fold in comparison to the empty vector control. This could be explained by the presence of the G-box variants 5 (5'-CACGCG-3') and 10 (5'-TACGTG-3') in the promoter sequence used for these particular constructs. However, this conclusion is mainly based on the observation that no significant differences were found when compared to the negative control, which might be due to the high variability registered for the $\emptyset$::*GUS* samples.

Importantly, the presented results further confirm the *in vivo* functionality of the *cis*-acting elements analyzed. Furthermore, they support the idea that the MYC2 transcription factor can directly bind to this G-box elements to promote *YUC8* and *YUC9* gene expression.

Figure 37: Relative GUS activity of *YUC8* and *YUC9* promoter fragments co-transfected with MYC2 in *Arabidopsis* leaf protoplasts.

a) Scheme of the constructs used in the transient expression analysis. The full-length coding sequence of MYC2 was cloned into the vector p35S-HA-GW, harbouring the CaMV 35S promoter. The attB1 and attB2 sites represent specific recombination sites used in the Gateway system (Invitrogen). The truncated promoter fragments were inserted into the *KpnI/HindIII* and *XbaI* sites in the plasmid pBT-10, upstream of the *uidA* reporter gene encoding GUS and the *Nos* terminator cassette. Number indicate the G-box sequences carried by the different promoter fragment. The TATA-box is also indicated. **b)** Transcriptional activity assay in *A. thaliana* mesophyll protoplasts co-transfected with the 191::GUS, 3::GUS and Ø::GUS reporter constructs and the effector constructs 35::MYC2. Ø::GUS refers to a promoter region lacking the cis-regulatory elements under study (#191 and/or #3). The pBT-10 empty vector was used as the negative control. GUS activation levels, fluorometrically quantified, were relativized to the NAN reporter gene activity and then normalized to the empty pBT-10 vector. Final GUS activity was expressed as pmol 4-methylumbelliferone (MU)/min. Means are given with their standard error (SE). Three aliquots per protoplast suspension were analyzed. Similar results were obtained in two independent experiments. Statistical analyses were performed using Student's t-test comparing between the negative control and each promoter construct. Asterisks indicated

CHAPTER 5:

DISCUSSION

5.1 Towards deciphering the role of auxin in biotic stress responses

Since the Industrial Revolution in the nineteenth century, the excessive exploitation of nature has generated a threatening resource depletion (Jordan 2002; Saini et al. 2015). Deforestation by anthropogenic or natural effects, such as fires, diseases, pollution or climatic changes has slowed down during the last 25 years by 0.08 %. However, the total forested land in 2015 was estimated to be approx. 30.6 %, with only 7 % of it planted for industrial activities (FAO, 2015). Thus, a better comprehension of the secondary growth will allow wood production optimization by genetic and metabolomic engineering.

Previous studies associated auxin biosynthesis with plant stress responses towards wounding through the induction of two *YUCCA* genes involved in auxin biosynthesis (Hentrich et al. 2013a). An initial inspection of constitutive *YUC8* and *YUC9* overexpression lines revealed an aberrant secondary growth in these lines (See Section 1.6). Plant secondary growth is defined as the radial expansion of the stem that occurs after an elongating period. In other words, secondary growth determines the stem diameter and wood formation in gymnosperm and dicot trees, through the massive production of secondary xylem and phloem by periclinal cell division and differentiation of a lateral meristem, the vascular cambium (Fukuda 1997b; Barra-Jiménez & Ragni 2017). Historically, progress in understanding the metabolomics, molecular and genetic basis controlling secondary growth has been restricted to trees. However, despite recent advances in large-scale EST sequencing and the possibility of producing transgenic forestry species (Allona et al. 1998; Kajita et al. 2004), the slow growth and long generation times of trees makes the advances in this field laborious and limited.

The recent finding that the herbaceous model species *Arabidopsis thaliana* undergoes secondary growth when kept from flowering (i.e. decapitated plants) and under short day conditions (Little et al. 2002; Ko & Han 2004), has contributed significantly to disclose the basis of secondary tissues formation (See Section 1.7). *Arabidopsis* secondary growth has been observed to occur in root, hypocotyl and stem (Nieminen et al. 2015). Moreover, a comparative analysis between *Arabidopsis* and poplar hypocotyls has demonstrated that the produced secondary xylem of both plant species exhibit striking similarities in terms of tissue organization, albeit *Arabidopsis* lacks the radial vascular rays (Chaffey et al. 2002). Nevertheless, further studies might provide new insight into the regulation of secondary growth.

The transition from primary to secondary growth involves cell wall thickening and lignification of secondary xylem cells, which finally results in the formation of a

secondary cell wall highly enriched in lignocellulosic material (Plomion et al. 2001). The secondary cell walls are the major source of plant biomass and constitute one of the most promising feedstocks utilized as natural and renewable resource for biofuel production and papermaking industry (Kádár et al. 2004; Hatfield & Fukushima 2005; Haghighi Mood et al. 2013). Furthermore, recent studies have highlighted a number of plant derived phenolic compounds, e.g. lignin, flavonoids, tannins, etc., as potential anti-cancer pharmaceuticals (Anantharaju et al. 2016). Nonetheless, despite its economic and developmental importance, the molecular and biochemical mechanisms involved in secondary growth formation remain poorly understood.

5.1.1 Auxin overproduction, mediated by YUC8 and YUC9, induce secondary stem growth by increasing xylogenesis and interfascicular cambium division

At the beginning of the presented work, it was known that YUCCA, a key family of indole-3-acetic acid (IAA) biosynthetic enzymes, is involved in several aspects of the ontogeny of the vascular system and secondary growth formation. For example, recent genetic studies, have demonstrated a diminished vascular formation in leaves of the *yuc1yuc4yuc6* and *yuc1yuc2yuc4yuc6* loss-of-function mutants (Cheng et al. 2006). In petunia plants the overexpression of *FLOZZY* (35S::FLZ), the ortholog of YUCCA from *Arabidopsis*, led to an aberrantly hard and woody stem (Tobeña-Santamaria et al. 2002). In a similar experiment, Hentrich et al. (2013b) described that radial growth in inflorescence stems of the gain-of-function lines YUC8ox and YUC9ox is increased (See Section 1.6, Figure 8). Nonetheless, it wasn't until cross sections of YUC8/9ox mutant stems were made and inspected, which revealed particularly interesting phenotypic alterations.

First of all, the study presented here was able to confirm that *Arabidopsis thaliana* inflorescence stems (> 20 cm height) undergo secondary growth even under long day conditions (Figure 16). Interestingly, our histochemical analyses of *Arabidopsis* wild-type and YUC8/9 gain-of-function lines, demonstrated a normal progression in secondary vasculature development and tissue pattern establishment. Staining with Toluidine Blue O, a polychromatic stain, showed that the secondary xylem (Xy) is visible as radial cell fibers in the inner part of the vascular bundles (See Section 4.1.2, Figure 16a-c). Also, an activation of the interfascicular cambium, which connects the adjacent vasculature bundles to form a closed circle through interfascicular fiber (IF) formation was detected. However, the Toluidine blue staining clearly showed a change in the amount of secondary vasculature present in the YUC8ox and YUC9ox stems, further confirming that the overproduction of indole-3-acetic acid, driven by the constitutive expression of YUC8 and YUC9

enzymes promotes an aberrant secondary growth of the plant shoot. Secondary xylem, which typically stains blue, was present in much higher amount in the YUC8/9ox mutants than in the WT stems of equal diameters (Figure 16a-c). Our subsequent quantitative phenotypic analyses, i.e. cell number Xy and IF area and IF stack height in stem cross sections, have demonstrated that the observed abnormal secondary growth results from an increased xylogenesis and the massive augmentation of interfascicular cambium division (Figure 17). The Toluidine staining further revealed that the observed alteration can apparently be associated to the increased number of smaller cells, rather than to an aberrant cell expansion in the stems. Thus, this study provides evidences to support the hypothesis that auxin is an important regulator of cambial and interfascicular cambial activity by stimulating mitosis. Furthermore, our results strongly underline the remarkably important role of IAA in Xy formation and IF differentiation during the transition of primary to secondary growth.

Current evidences coming from genetic and biochemical studies in *Populus trichocarpa* and *Arabidopsis thaliana* highlight the central role of plant hormones in the regulation of secondary plant growth (Groover & Robischon 2006) and various phytohormones have been described as possible mediators of secondary growth (Brackmann & Greb 2014). Indole-3-acetic acid (IAA), the main auxin in plants, has been reported to be essential for the developmental control of the vascular cambium activity and consequently of secondary xylem (wood) formation (Little et al. 2002). The main contribution to this observation is that decapitation of shoot tips, which constitute the main source of IAA, results in the inhibition of cambial activity in several conifers and hardwood species. Development of the vascular cambium can be restored by the exogenous application of auxin onto the inflorescence stem (Savidge 1988; Sundberg et al. 2000). Similarly, Klee et al. (1987) showed that the overexpression of the bacterial auxin biosynthesis gene tryptophan-2-monooxygenase (*iaaM*) in *Petunia hybrid* significantly increased the number of secondary xylem and phloem cells in the stem. Moreover, in a *Zinnia elegans* cell culture system, trans-differentiation of mesophyll cells into tracheary elements (TE), which constitute the xylem conductive cells in vascular plants, is seemingly dependent of auxin and cytokinins (Fukuda 1997b).

On the other hand, a clear evidence of the auxin involvement in xylem formation was observed analysing the vascular phenotype of the *auxin insensitive 1* (*axr1*) mutant, which is impaired in auxin signaling (Bishopp et al. 2011). In their work, the authors revealed a complete lack of protoxylema in *Arabidopsis* mutated roots. Similarly, in *Arabidopsis* stems, the *axr1* mutant displays a low activity of the

interfascicular cambium, which results in reduced secondary growth (Agusti et al. 2011). Moreover, a correct auxin transport has also been highlighted to be a prerequisite for a correct cambial activity and secondary xylem development. Auxin transport occurs from cell to cell in a basipetal manner, from the shoot apex to the base. The different spatial hormone distribution, generated by a directional transport or polar auxin transport (PAT), creates local auxin maxima, which have been shown to be essential for xylem development in this organ (Sachs 1969). In agreement, local application of the auxin transport inhibitor 1-naphthylphthalamic acid (NPA) to *Arabidopsis* stems was demonstrated to enhance xylem proliferation in the zones adjacent to the treatment areas, due to an increased accumulation of auxin (Gälweiler et al. 1998; Little et al. 2002). Moreover, current mathematical and experimental evidences have highlighted the auxin influx carrier AUX1, LAX1, LAX2 and LAX3 to have a relevant role in xylem differentiation under short day conditions (Fàbregas et al. 2015). It is well known that the direction of the auxin flux depends of the asymmetric localization of the PIN-FORMED (PIN) auxin efflux carrier proteins in the plasma membrane of the transporting cell (van Berkel et al. 2013). A recent study showed that PIN1, together with LAX3, are strongly expressed in cambial tissue, meristems and xylem of aspen hybrid (*P. tremula* x *P. tremuloides*) (Schrader et al. 2003). In this work, the authors also suggested a complex interaction of AUX/LAX and PIN transport processes that is regulated by developmental and environmental signals. In *Arabidopsis*, the expression of *PIN1* and *PIN3* has been determined in secondary thickened stems (Gälweiler et al. 1998; Agusti et al. 2011). In addition, mutant screens carried out in *Arabidopsis* led to the identification of the *MONOPTEROS* (*MP*) gene, also referred to as auxin response factor (ARF5). This gene encodes a transcription factor involved in the establishment of vasculature and plant body pattern during embryonic and post-embryonic development (Hardtke & Berleth 1998; Konishi et al. 2015). The loss-of-function mutant *mp* has misaligned tracheary elements and exhibits reduced expression of *PIN1* (Aida et al. 2002). This finding supports the “canalization of auxin flow hypothesis” in the auxin-mediated formation of vascular tissue. Additionally, MP has been proposed to directly regulate the expression of *AtH8*, which encode a HD-ZIP transcription factor that promotes procambial cell proliferation (Schuetz et al. 2013)

5.1.2 Auxin-induced ethylene biosynthesis promotes cell division and secondary xylem differentiation in stem cambial meristems

In addition to auxin, the volatile plant hormone ethylene has also emerged as a positive regulator of cell division and cambial activity. Ortega-Martínez et al. (2007) reported that the defective *ethylene overproduced* (*eto1*) mutant, which produces

excessive amounts of ethylene, displayed aberrant cell division in the organising centre of *Arabidopsis* roots. The observed cell division in the quiescent centre (QC) was abolished when *eto1* plants were grown on media supplemented with the chemical inhibitor of ethylene biosynthesis, 1-aminoethoxyvinyl glycine (AVG). Furthermore, application of the ethylene precursor aminocyclopropane-1-carboxylic acid (ACC), indicated that stimulation of cambial cell division and secondary xylem growth in *Populus* is ethylene-dependent (Love et al. 2009).

In this work, we also analysed ethylene production by gas-chromatography and found that the content of this hormone was clearly increased in both IAA overproducer lines, YUC8ox and YUC9ox (Figure 15). This result is consistent with the previously reported up-regulation of several ethylene biosynthesis and signaling-related genes in YUC8ox and YUC9ox lines (Hentrich et al. 2013b). Consequently, these results confirm that IAA overproduction stimulates a concomitant overproduction of ethylene. Congruent with our results, earlier studies indicated that IAA is capable of stimulating ethylene production through the activation of specific *ACC-synthase* genes (*ACS*), which encode enzymes involved in a rate-limiting step of ethylene biosynthesis (Jones & Kende 1979; Yu et al. 1979; Yang & Hoffman 1984; Abel et al. 1995). Moreover, biochemical studies highlighted the implication of both auxin and ethylene in the regulation of radial stem growth in conifers and hardwood trees (Savidge 1988). These observations prompted us to suggest that auxin promotes ethylene biosynthesis to synergistically regulate cambial cell divisions and secondary xylem and interfascicular fiber formation.

The existence of an intimate crosstalk between ethylene and auxin to regulate several biological processes, such as root elongation, differential growth of the hypocotyl, and root hair initiation and elongation is well stabilised (Stepanova et al. 2005; Stepanova et al. 2007; Růžicka et al. 2007; Muday et al. 2012). However, less is known about the implication of this crosstalk in relation with the control of secondary growth. In the shoot apical meristem (SAM) the transcription factor family WUSCHEL-RELATED HOMEODOMAIN (WOX), is considered to play critical roles in the regulation of stem cell formation and maintenance (Dolzblasz et al. 2016). Genetic studies have demonstrated that the establishment of auxin gradients mediated by the polar distribution of PIN1, are essential for the regulation of WOX expression during somatic embryogenesis (Palovaara & Hakman 2009; Su et al. 2009). Among the WOX family, WOX4 has been observed to promote proliferation of stem cells in the procambium/cambium in *Arabidopsis* by acting as a downstream target of the ligand-receptor pairs CLV3/ESR1-LIKE 41 (CLE41) and the related CLE42, and leucine-rich repeat receptor-like kinase PHLOEM INTERCALATED WITH XYLEM (PXY). In turn,

the PXY receptor coordinates the orientation of the cell division plane in the procambium (Hirakawa et al. 2010; Etchells et al. 2013; Suer et al. 2011). Analysis of *pxy* and *wox4* loss-of-function mutants, which exhibit normal and reduced cambium division, respectively, revealed a group of APETALA2/Ethylene-Responsive Factors (AP2/ERF) to be up-regulated in both mutants (Etchells et al. 2012). In the same work, Etchells and coworkers (2012) demonstrated that two of the aforementioned AP2/ERF transcription factors, ERF109 and ERF018, are required for cell division during primary and secondary growth. By contrast, when analysing the inflorescence stem of *ethylene insensitive 2* (*ein2*), the only single mutant in which ethylene signaling is totally abolished, a normal vascular phenotype was detectable. On the contrary, the *pxy/ein2* mutant showed a dramatic reduction in vascular cell division, indicating the essential role of PXY and EIN2 in the maintenance of vascular cell number.

On the other hand, with reference to a plausible ethylene-mediated auxin response, it has also been demonstrated that ethylene signaling induces auxin biosynthesis in *Arabidopsis* root tips. This induction is mediated by modulation of two anthranilate synthase genes WEAK ETHYLENE INSENSITIVE 2/ANTHRANILATE SYNTHASE α 1/TRANSPORT INHIBITOR RESPONSE 1 (WEI2/ASA1/TIR1) and WEAK ETHYLENE INSENSITIVE 2/ANTHRANILATE SYNTHASE β 1 (WEI7/ASB1), which encode enzymes participating in a rate-limiting step of tryptophan biosynthesis (Stepanova et al. 2005; Růžicka et al. 2007). In addition, Růžicka et al. (2007) demonstrated the upregulation of several auxin efflux and influx proteins, such as PIN1, PIN2, PIN4 and AUX1 in response to ethylene. These authors suggest that expression of PIN1, PIN2 and AUX1 in the vasculature might favour the transport of auxin to the basal part of the inflorescence stem and root. Summarizing all these evidences and based on our obtained results, is tempting to speculate on the existence of a strictly spatio-temporally regulated positive feedback loop between auxin and ethylene biosynthesis. Furthermore, the auxin-mediated CLV41/42-PXY-WOX pathway might be acting in parallel with ethylene signaling to regulate cell division and the differentiation of secondary xylem during *Arabidopsis* vascular development. Nevertheless, crosstalk between auxin and ethylene appears to be very complex. In fact, this was already suggested by Stepanova et al. (2007) whose transcriptomic and genetic work on *Arabidopsis* roots demonstrated the existence of auxin-mediated, auxin-dependent and auxin-independent ethylene responses.

Artificial weights applied to the apical part of immature inflorescence stems were shown to trigger auxin accumulation and transport and subsequently promote the development of secondary xylem (Ko et al. 2004; Mazur et al. 2016). Whole-

transcriptomic profiling of the weight-induced stems, revealed the up regulation of several auxin carriers and auxin responsive genes, thereby providing evidences supporting the hypothesis that auxin is a crucial determinant in secondary xylem dynamics in *Arabidopsis* stems (Ko & Han 2004). Similar secondary weight induction results were obtained in *Arabidopsis* Col-0 plants with, at least, 15 cm tall stems grown under short-day conditions (Sehr et al. 2010). In their work, Sehr et al. (2010) described a differential expression of several jasmonate signaling genes, e.g. COI1 and MYC2, as well as ethylene-related genes in the treated plants. Moreover, the authors demonstrated that the exogenous application of jasmonic acid (JA) has a positive impact in interfascicular cambium-derived tissue extension, xylem and fiber cell wall thickening and phloem formation. This finding could translate into the hypothesis that mechanical intra-tissue forces, exerted by the aberrant secondary growth, could stimulate both JA and ET production in a branch independent of the IAA secondary growth regulatory pathway. In fact, growth responses to mechanical stimuli, i.e. wind or touch, a process referred to as thigmomorphogenesis, have been well documented in pine species (*Pinus pinaster*, *Pinus radiata*, and *Pinus sylvestris*), castorbean (*Ricinus comunis*) or cucumber (*Cucumis sativus*) (Jacobs 1954; Jaffe 1973; Valinger 1992; Sellier & Fourcaud 2009), highlighting a phytohormone-based regulation of these mechanical responses. Love and coworker (2009) recently demonstrated the implication of ethylene during tension wood (TW) formation in response to leaning, using transgenic ethylene-overproducing and -insensitive hybrid aspen (*Populus tremula* x *Populus tremuloides*).

Nonetheless, considering the following observations: (i) the recent demonstration of the importance of an effective auxin transport in TW formation (Yu et al. 2017); (ii) the implication of auxin in the local regeneration of mechanically wounded stem regions through *de novo* vessel formation, as well as the induction of circular vessel development, which are characterized by secondary cell walls (Mazur et al. 2016), (iii) the jasmonate inducibility of the auxin biosynthetic genes *YUC8* and *YUC9* (Hentrich et al. 2013a) (Figure 11), it appears to be possible that mechanical stress as wounding, caused for instance by a pathogen attack, initiates a signaling cascade including the crosstalk between jasmonate, auxin, and ethylene, to promote secondary growth in the stem and thus providing additional plant support and protection.

5.1.3 Auxin overproduction significantly increases H-lignin content in secondary cell walls

One of the main characteristics of secondary xylem, tracheary elements, and interfascicular fiber cells is the deposition of a secondary cell wall surrounding the

primary cell wall. The secondary cell wall consists of three types of polymers, i.e. lignin, cellulose and hemicellulose (Taylor-Teeple et al. 2014). Among these components lignin, a complex aromatic biopolymer, plays a central role preserving the integrity of the plant cell wall, providing mechanical support and strength, enabling the transport of water and nutrient from roots to the whole plant body, and acting as a physical barrier against invading pathogens, and other environmental stresses (Bonawitz et al. 2014; Liu 2012).

In this work, we provide empirical evidence that auxin induces the transition from primary growth to secondary growth in *Arabidopsis*. We examined lignin contents in both YUC8ox and YUC9ox gain-of-function mutants, as a major feature of secondary wall formation. Through staining with phloroglucinol-HCl, a polyphenolic specific stain, we further corroborated the toluidine blue data. There was a massive formation of mature secondary xylem and interfascicular fiber tissues in the YUC8ox and YUC9ox lines, made visible by the strong fuchsia-red color of the lignified cell walls (Figure 16d-f). The presence of phenolic material was also confirmed by the UV absorption in the section of highest lignin accumulation under UV-light (Figure 16g-i). In addition, we analysed the lignin content by the acetyl bromide method and found a significant augmentation in the total lignin content of YUC8ox (40 % increase) and YUC9ox (66,6 % increase) mature inflorescence stems compared to WT plants of the same developmental stage (Figure 18). This result is in agreement with the observed Xy and IF lignification observed in the histochemical analyses. Moreover the observed increased lignin deposition in 24 cm tall inflorescence stem is consistent with the increased secondary xylem formation obtained in plants with, at least, 15 cm tall stems grown under short-day conditions (Ko et al. 2004; Sehr et al. 2010). Indicating that secondary xylem formation strongly depends on plant height.

In terms of lignin composition, the phenylpropanoid lignin polymer, is mainly derived from three related hydroxycinnamyl alcohols *p*-coumaryl, coniferyl, and sinapyl alcohols (called monolignols). After their synthesis in the cytosol from L-phenylalanine and L-tyrosine, monolignols are translocated to the apoplast/cell wall, where they are oxidized into the H, G, and S lignin units. Finally, the lignin units or monomers are integrated into the cell wall matrix and attached to one another via radical polymerization, in a process known as lignification (Ralph et al. 2004; van Parijs et al. 2010; Liu 2012; Vanholme et al. 2012). The maintenance of the S:G:H ratio appears to be indispensable for normal growth. Indeed, disruption of shared phenylpropanoid pathway enzymes acting upstream of both G and S lignin results in dramatic developmental abnormalities. For instance, Li et al. (2010)

demonstrated that silencing of the *Arabidopsis* gene *HYDROXYCINNAMOYL-CoA:SHIKIMATE/QUINATE HYDROXYCINNAMOYL TRANSFERASE (HCT)* causes a dwarf phenotype. A stunted growth phenotype was also recorded for the *Arabidopsis* T-DNA mutant *reduced epidermal fluorescence 8 (ref8)*, defective in *p*-coumarosylshikimate 3'-hydroxylase (C3'H) (Franke et al. 2002a; Franke et al. 2002b).

Surprisingly, the Mäule staining and thioacidolysis followed by GC-MS/MS analysis of the monolignol composition, expressed as the H:G:S ratio, revealed that the overproduction of auxin leads to H-enriched lignin. (Figures 16 j-l and 19a). Whereas the G lignin was significantly reduced and S lignin were only present in trace amounts. This result suggests that, in spite of the aforementioned evidences, S and G are not completely necessary. Consequently, sufficient amounts of H lignin are apparently enough to facilitate normal plant growth and development. Consistent with this hypothesis Bonawitz et al. (2014) proved that the triple *Arabidopsis* mutant *med5a/5b/ref8-1*, which is defective in C3'H and the transcriptional regulator mediator complex subunits MED5a and MED5b (Kornberg 2007), not only rescues the dwarfish phenotype of *ref8-1*, but also exhibited high levels of H lignin and scarce G and S lignin. However, it remains unclear whether MED5 subunits control monolignols synthesis, transport and polymerization, or if they are rather implicated in cell wall integrity.

Quite some time ago, Aloni et al. (1990) reported that auxin stimulation induced lignin formation with a decreased content of S units, in the secondary xylem fraction of *Coelus blumei*, while in the phloem fraction S units appeared enrichment. Furthermore, Pasrija & Thakur (2012) reported a strong reduction of the *AtMED5* transcript levels after IAA treatment (40 % decreased levels). Therefore, one possibility is that auxin might act as negative regulator of *MED5a* and *MED5b* to control the quantitative relationship between G and S lignin, in response to developmental or environmental cues. The idea of an auxin-mediator regulatory pathway is supported by a recent study carried out by Ito et al. (2016). In this work, the authors reported a physical interaction between several auxin-signaling components and MAB2, a mediator complex involve in lateral root initiation. Further elucidation of the molecular mechanism underlying MED5 function, as well as the possible implication of auxin, will help to shed light onto lignin synthesis and deposition processes and may reveal additional targets for bioengineering.

Another possibility for the differential lignin monomer composition arise from the observation that ethylene has also been associated with the regulation of lignin biosynthesis and thus could alter lignin composition. Thus, for example,

biochemical and transcriptomic analyses in mung bean (*Vigna radiata*) have revealed that exogenous and endogenous ethylene strongly increases the activity of *PHENYLALANINE AMIDE LYASE* (*PAL*) and *PERODIXASE* (*POD*) genes (Huang et al. 2013), two families of proteins involved in the biosynthesis and polymerization of lignin monomers, respectively (Vanholme et al. 2010). A similar *POD* activation has also been found in auxin overproducer tobacco *35S-iaaM/iaaH* mutants, which exhibited increased contents of ethylene (Sitbon et al. 1999). In *Arabidopsis*, the characterization of a mutant in the chitinase-like protein *AtACTL1*, *elp1*, revealed that the observed ectopic deposition of lignin was accompanied with increased production of ethylene (Zhong et al. 2002). Alternatively, wounding and jasmonate have also been associated with lignin biosynthesis through the transcriptional regulation of a series of wound-induced genes, as for instance the *CAFFEIC ACID-O-METHYL TRANSFERASE* (*COMT*) (Vélez-Bermúdez et al. 2015), which encode a protein implicated in the synthesis of G-lignin (Vanholme et al. 2010). In young *Eucalyptus gunnii*, it has been shown that lignin relatively poor in S-lignin, which differs from the normal “developmental” lignin, is deposited in xylem zones 24 h after mechanical wounding (Hawkins & Boudet, 1995).

On the other hand, in contrast to the complex G- and S-lignin biosynthetic routes, with around 9 different steps, respectively. H-lignin production can be reached after 5 biosynthetic steps (Liu 2012; Vanholme et al. 2012), which would be less costly to the plant with regards to energy required for synthesis of the lignin. Therefore, based on our results together with the mentioned effect of plant hormones on lignin biosynthesis, it can be proposed that auxin, jasmonate and ethylene could together act as mediators stimulating H-lignin deposition as a rapid response to the elevated rate of xylem and interfascicular fiber division to reinforce wall rigidity and allow plants to continue with their growth program. This explanation could be extended as response to mechanical forces or pathogen attack, where the reinforcement of cell walls with lignin must be carefully orchestrated to rapidly produce an effective protective barrier. This is supported by the observation of a the rapid deposition of H-lignin unit in potato tuber tissue and cucurbit leaves after as a non-host resistance response (Hammerschmidt 1984; Hammerschmidt et al. 1985).

5.1.4 Lignocellulosic recalcitrance is affected by lignin content rather than lignin composition in the YUC8ox and YUC9ox plants

Cellulose, the predominant component of secondary cell walls, constitutes the primary carbohydrate source for lignocellulose-based bioethanol production. This biopolymer is formed of a linear chain of D-glucose linked by β -(1,4)-glucosidic bonds (Haghighi Mood et al. 2013). The production of polysaccharides requires the

activation of glucose by the addition with UDP. This polymerization process gives rise to a crystalline cellulose species, which can extend to more than 10000 glucose molecules (Schuetz et al. 2013). In secondary cell walls, lignin is tightly bound to cellulose and hemicellulose, thereby hindering the accessibility and activity of hydrolytic enzymes during the bioconversion process used in bioethanol and bio-based chemical production (Mood et al., 20013). In this context, extensive studies were centred in tailoring monolignol biosynthesis to control lignin content and/or composition. Recently, Huntley et al. (2003) demonstrated that hybrid aspen with increased S-lignin units, but with no altered lignin content, presented improved pulp yields and an increase availability of cell wall polysaccharides. This is probably due to S units having less free binding position and consequently form fewer covalent bonds (van Parijs et al. 2010; Xu et al. 2011). Following up on this assumption, it can be expected that an increase in H-units strongly reduce lignin condensation, complexity, and thereby, recalcitrance of the cell wall. In fact, Bonawitz et al. (2014) demonstrated that the substantial increase in H lignin, accompanied by a reduction in the S:G ratio in the *med5a/med5b/ref8-1* Arabidopsis triple mutant gives rise to a high saccharification efficiency.

To test whether the diminished content of the “developmental” lignin monomers (S:G) could be compensated by an altered content of cellulose, we used the YUC8ox and YUC9ox transgenic lines in a detailed study employing histochemical and metabolomics approaches. Unexpectedly, the calcofluor-treated xylem tissues showed no binding of this fluorochrome, which is normally used for rapid detection of cellulose. As already mentioned in Section 4.1.2, this is caused by a decreased diffusion efficiency of the calcofluor white stain in highly lignified tissues (Pradhan Mitra & Loqué 2014) (Figure 16m-o). This finding, however, rather confirmed the strong xylem lignification observed in both YUC8ox and YUC9ox mutants. In contrast to this result, the Congo red stain performed in this study revealed a substantially increased content of cellulose (Figure 16 p-r). Furthermore, the analysis indicated that the increased cellulose contents are most pronounced in the vascular cambium and interfascicular cambium (tissues with a high rate of cell division). In plants, cellulose biosynthesis takes place at the plasma membrane by a large rosette-shaped protein complex, the cellulose synthase complex (CSC) composed of cellulose synthase (CESA) proteins (Kumar et al. 2016). Immunolocalization experiments performed in poplar stem cross-sections demonstrated the presence of several CESAs in the vascular cambium (Song et al. 2010). The observations reported here further demonstrate the active formation of secondary walls in *Arabidopsis* stems. Nonetheless, relatively little is known about

the function of auxin in cellulose biosynthesis. The observed increased cellulose amount could be a result of the massive formation of xylem *per se*. Alternatively, our results prompted us to suggest that, as we hypothesised before, the increased cellulose content could be caused as a response of the H-lignin enrichment, possibly to implement cell wall reinforcement.

In order to investigate if the lignin composition observed in the YUC8ox and YUC9ox lines could improve saccharification efficiency through enzyme hydrolysis, we measured cellulose yield by GC-MS/MS. Interestingly, our saccharification analysis of YUC8ox and YUC9ox plants show that H lignin-enriched cell walls do not reduce biomass recalcitrance (Figure 20), presumably due to the elevated lignin content of this plants. However, a positive relationship between reduced lignin content and increase availability of cell wall polysaccharides has been shown in studies on biomass derived from alfalfa (*Medicago truncatula*) (Chen & Dixon 2007) and switchgrass (*Panicum virgatum*) (Xu et al. 2011b). Our recent observations support the idea of H lignin being in fact sufficient to reinforce strength of the cell wall. It will be interesting to determine the molecular and biochemical basis of the regulation of pathogen-induced lignin to resolve the mechanism of auxin-induced elevate glucose content and H lignin deposition.

5.1.5 Auxin-mediated differential regulation of cell wall remodelling genes

In the framework of this work, we provide novel insight supporting the notion that auxin plays an integral role in secondary growth in *Arabidopsis* inflorescence stems. In addition, we used YUC8ox and YUC9ox seedlings to explore the effect of auxin in early-stages of plant growth. It has to be remarked that in *Arabidopsis* secondary growth transition occurs in mature stems and roots (Baum et al. 2002; Ko et al. 2004; Ko & Han 2004; Sehr et al. 2010; Vanholme et al. 2012). Hence, there is good reason to expect that young seedlings only exhibit primary vascular differentiation. In line with this expectation, our whole-genome expression analyses show no secondary growth-related genes differentially expressed. Intriguingly, we observed that only 19 % and 20 % for YUC8ox and YUC9ox, respectively, of the differentially expressed genes were commonly regulated in both genotypes, which appears relatively low. This can possibly be explained as a consequence of uneven ectopic expression levels of the two *YUC* genes, although both constructs are driven by the same 35S CaMV promoter or by different biological half-life times for the two investigated monooxygenases.

Nevertheless, our microarray analyses and subsequent functional categorization clearly revealed that the expression of some cell wall-related genes is highly induced

in the auxin overproducer seedlings (Figure 21). Moreover, using qRT-PCR we further confirm the reproducibility of the observed expression pattern for four *XYLOGLUCAN ENDOTRANSGLYCOSYLASES/HYDROLASES* (*XTH15*, *XTH23*, *XTH24*, and *XTH25*) (Figure 22). The expression analyses also allow us to determine an increased expression of *XTH33* in the YUC8ox line. The *XTHs* (formerly named *XET*) genes encode a family of enzymes highly important for xyloglucan polymer remodelling during cell expansion (Rose et al. 2002).

The positive effect of auxin in cell expansion has been well documented (Rayle & Cleland 1992; Paque et al. 2014a; Ko & Han 2004; Perrot-Rechenmann 2010; Chapman et al. 2012). In a previous study, Hentrich and coworkers (2013a) demonstrated that both auxin overproducer lines, YUC8ox and YUC9ox, exhibited elongated hypocotyls and petioles (Figure 7). Hypocotyl elongation has also been registered in other auxin overproducer mutants as for instance *superroot1-1* (*sur1-1*) (Boerjan et al. 1995; King et al. 1995), the *iaaM* overexpression line (Romano et al. 1995) and the 35S::*YUC7* mutant (Lee et al. 2012). Hypocotyl growth is the result of a highly controlled series of cell expansion events, whereas division in the external layer is not supposed to significantly contribute to the growth process (Gendreau et al. 1997). On the other hand, YUC8ox and YUC9ox transfection of *Nicotiana benthamiana* leaves with *Agrobacterium tumefaciens* harbouring either a 35S::*YUC8* or 35S::*YUC9* binary vector, displayed significantly expanded pavement cells (Hentrich et al. 2013a) (Figure 9). In line with this idea, analysis of transgenic *Arabidopsis* plants expressing the β -glucuronidase-encoding GUS reporter under the control of the potential regulatory regions upstream of the *XTH15*, *XTH23*, *XTH25* and *XTH33*, reported that these *XTHs* are expressed in young hypocotyls and cotyledons, while *XTH24* is seemingly restricted to the cotyledon at early developmental stages (Becnel et al. 2006). Thus, it is tempting to speculate that the auxin-mediated increase in *XTH* expression occurs in response to the stimulated cell expansion. It is also interesting to mention that *XTH* enzymes also play an important role in vasculature morphogenesis. Thus for example, *XTH15::GUS* was clearly found to be expressed in the vasculature of the inflorescence stem (Becnel et al. 2006). Bourquin et al. (2002) demonstrated the activity of *XTHs* during secondary growth by assessing the incorporation of labelled sulforhodamine xyloglucan oligosaccharides into poplar stems sections. The authors observed a more intense labelling in xylem fibers during their secondary wall thickening. In line with this study, the transcriptomic analysis carried out by Ko & Han (2004) disclosed that three of the 27 *XTH* genes on the gene chip (*XTH24*, *XTH28* and *XTH32*) were preferentially expressed in secondary stems of *A. thaliana*. This suggests that the

XTH gene family is expressed in various different cell types and have diverse functions including hypocotyl and leave expansion and xylem formation.

Cell expansion is an irreversible increase in cell size and plays a crucial role in plant growth and adaptive processes (Perrot-Rechenmann 2010). Expansion growth requires cell wall loosening, which involves modification and remodelling of cell wall components, resulting in the relaxation of wall tension (Cosgrove 2005). In plant cell walls, xyloglucan, which constitute the major hemicellulose, binds non-covalently via hydrogen-bonds to cellulose microfibrils forming an intricate polysaccharide network (Rose et al. 2002; Ko & Han 2004). Cell wall loosening can be regarded as a breakage of these crosslinks between cellulose and hemicellulose, a process mediated by expansins, and cleavage of the hemicellulose polymer by the XTHs (Cosgrove 1998; Becnel et al. 2006). It has been suggested that auxin-mediated cell wall loosening and expansion is triggered by membrane hyperpolarization through the modulation of ion fluxes across the plasma membrane, also known as the “acid growth theory” (Rayle & Cleland 1992). According to this theory, auxin is perceived by the auxin receptor AUXIN BINDING PROTEIN1 (ABP1), which in turn activates a proton ATPase at the plasma membrane, inducing extrusion of H^+ to the apoplast and, therefore, acidification of the extracellular space and activation of cell wall proteins, such as expansins and XTHs. The provoked acidification also activates K^+ inward directed channels, which allow the uptake of water to increase the cell turgor (Cosgrove 2005; Perrot-Rechenmann 2010). Surprisingly, our whole-genome microarray analysis did not show differential expression of the *ABP1* gene in the auxin overproducer lines, YUC8ox and YUC9ox. ABP1-mediated cell expansion has been observed to vary in a cell- and tissue-depend manner (Paque et al. 2014). Thus, it is plausible that minor differences in the expression of *ABP1* could be masked due to the use of whole seedlings instead of isolated hypocotyls. Nonetheless, the activated *XTH* expression found in YUC8ox and YUC9ox provides empirical evidence supporting the auxin-induced acid growth theory. On the other hand, the inhibitory effect of ethylene in leaf cell expansion (Onkokesung et al. 2010), invites to think that the synergistic effect of IAA on ET may be tissue specific, probably limited to plant shoot and roots.

In summary, we demonstrate that auxin induces the differential expression of several *XTH* genes already during early developmental stages, which led us to examine the possible consequences in mature stems by oligosaccharide mass profiling (OLIMP). Most notably, no major changes in the hemicellulose composition were observed (Figure 23).

5.1.6 Growth-defense tradeoff

The microarray data generated for YUC8ox and YUC9ox seedlings revealed that auxin overproduction affects the expression of genes involved in biotic and abiotic stress adaptation. In particular genes related to chitin response were among the most negatively affected in both transgenic lines (Figure 24). The polymer of *N*-acetyl- β -D-glucosamine, commonly termed chitin, is a structural component of fungal cell walls and insect and spider mite exoskeletons, as well as nematode egg shells (Punja & Zhang 1993; Merzendorfer and Zimoch, 2003; Son et al., 2012; Van Leeuwen et al., 2012). However, it has never been reported in plant cell walls. Chitin and its fragments, chitooligosaccharides and chitooligomers, are widely recognized elicitors of plant defense responses via the microbe-associated molecular pattern (PAMPs)-trigger immunity system (Miya et al. 2007; Wan et al. 2008a; Iizasa et al. 2010; Son et al. 2012), being also an important activator of JA-signaling (Turner et al. 2002).

Among the GO categories of commonly down-regulated genes in YUC8ox and YUC9ox we found an overrepresentation of those genes related to processes associated to drought, cold and wounding stress responses Figure 25. Taken together, these results indicate that a proportion of the commonly down-regulated genes are related to stress-signaling pathway genes, possibly to counteract a hyper-response of the defense machinery after activation of the auxin-induced secondary growth. Consistent with this conclusion, it has been demonstrated that plant defense activation entails negative impacts on photosynthetic genes and reallocation of nutrients (Huot et al. 2014; Smakowska et al. 2016). From a biological point of view, it make sense that plants undergoing active growth require significant amount of energy and, therefore, inactivate the costly defense-associated transcriptional machinery to arbitrate growth-defense conflicts. Based on our results, we conclude that jasmonate-induced auxin biosynthesis through the modulation of *YUC8* and *YUC9* activity, is an on-off “switch” mechanism to fine-tune the balance between growth and defense processes.

5.1.7 The jasmonate-auxin-ethylene crosstalk model

As explained in Section 1.6 both *YUC8* and *YUC9* auxin biosynthetic genes are clearly induced by the defense related hormone JA. Moreover, Hentrich et al. (2013a) described that expression of the *YUC9* gene was dramatically increased after mechanical wounding, which translates in the local production of auxin. To better understand the physiological function of this IAA peak, part of this work has focused on the characterization of the overexpression lines *35S::YUC8* and

35S::*YUC9* (*YUC8ox* and *YUC9ox*), which constitutively express both enzymes. Therefore, despite the generated auxin production is exaggerated and static, these auxin-overproducing mutants are useful for understanding the implication of the IAA peak in plant growth-defense regulation.

The implication of the JA-IAA-ET module in root growth responses has been recently described (Hentrich et al. 2013a; Hentrich et al. 2013b). The results presented here highlight the importance of this hormonal cascade in plant stem secondary growth regulation. Furthermore, the obtained results facilitated the formulation of a novel model explaining how the crosstalk between the three plant hormones contributes to the strictly spatio-temporal regulation of mechanical stress or wounding responses.

According with our model (Figure 38), the mechanical disruption of tissue integrity in plant shoots will trigger a local JA accumulation, which triggers JA-related defense responses. In turn, the activation of the JA-signaling pathway will induce the expression of both *YUC8* and *YUC9* genes, and therewith drive local IAA production and the subsequent accumulation of ET. The synergistic interaction between these two hormones, i.e. IAA-ET, will contribute to initiate cambial cell divisions, secondary xylem and interfascicular fiber formation and H-lignin deposition. On the other hand, the stimulation of IAA and ET production will have an inhibitory effect on the plant defense machinery. Most likely, this enables the plant the redirect resources and energy from defense processes to growth.

Through photosynthesis, plants convert light energy into chemical energy in the form of carbohydrates. Because the major part of photosynthesis occurs in leaves, it is physiologically reasonable that after a pathogen attack plants optimize leaf cell size to improve energy capturing. It has been demonstrated that *YUC8ox* and *YUC9ox* transgenic lines exhibited epinastic cotyledons and narrow elongated leaf blades. Thus, our model proposes that, while IAA-ET crosstalk stimulates the mitotic activity of secondary meristems in stems, in leaf epidermal cells IAA could promote expansion by inhibiting ET in a tissue-specific manner. This conclusion is supported by the pavement cell expansion observed in *Nicotiana benthamiana* leaves after transfection with the 35S::*YUC8* or 35S::*YUC9* binary vectors (Hentrich et al. 2013a) (Figure 10).

Figure 38: Model illustrating the physiological mode of action of the crosstalk between jasmonate, auxin, and ethylene in the framework of stress response in aerial parts.

5.2 IAA regulates biotic and abiotic responses in *Arabidopsis thaliana* through the action of YUC8 and YUC9

The human population increases in a rate of approximately 250000 people every day. According to recent estimations, the current world population of 7.3 billion people will reach 8.5 billion by 2030 (UN DESA, 2015). Hence, one of the major challenges of this century is to cope with the increased demand of food and other plant products, which must increase by at least 40 % (Bazzaz 2001). Against this backdrop, the generation of genetic modified crops has emerged as a promising biotechnological solution to improve nutritional content or simply increase crop yield. The development of “Golden Rice”, as a solution to vitamin A deficiency in developing countries is an important example for the potential of this new technology (Ye et al. 2000). Nonetheless, biotechnology is not free of risk, and it has been critically commented that it could have negative impact on allergenicity and the environment (Key et al. 2008; Mittler & Blumwald 2010). A better understanding of the mechanism driving plant development and plant adaptations towards biotic and abiotic stresses will contribute to diminish the possible risk and help the optimization of genetically modified plants in the future.

Because plants are intrinsically sessile organism, the rapid perception and integration of environmental signals is crucial for their successful development.

Auxin is a well-known mediator that, at low concentrations, regulates many aspects related to plant growth and developmental (Woodward & Bartel 2005; Hoffmann et al. 2011). The lack of a mutant plant completely lacking auxin is a clear indication that auxin plays crucial developmental and adaptive roles. However, the implication of auxin as a regulator of biotic stress responses is not fully deciphered.

In recent years, significant progress has been made towards understanding how auxin is synthesized. The recognition of the participation of the *YUCCA* proteins in the IPA pathway of auxin biosynthesis in *Arabidopsis*, has greatly contributed to the advances made in this field (Cheng et al. 2006; Stepanova et al. 2011; Won et al. 2011). The presence of the *YUCCA* genes in multiple plant genomes further highlights their essential and conserved role in plant development (Tobeña-Santamaria et al. 2002; Yamamoto et al. 2007; Ye et al. 2009; Rozov et al. 2012). Nevertheless the different physiological implications of the numerous *YUCCA* isogenes are still under scrutiny.

5.2.1 Auxin depletion in the *yuc8/yuc9ko* mutant confers enhanced resistance to *Tetranychus urticae* through fine-tuning of growth/defense responses

The finding of an implication of JA-IAA-ET crosstalk in plant defense responses motivated us to examine more closely the hormone crosstalk in herbivore challenged plants. For this end, we used *Tetranychus urticae* Koch (Acari: Tetranychidae), the two-spotted spider mite, which is a polyphagous herbivore that has been documented to feed on over thousand different plant species, which include more than a hundred crop plants (Sarmiento et al. 2011; Zhurov et al. 2014; Bensoussan et al. 2016).

To study the particular and possible mutualistic role of YUC8 and YUC9 in *T. urticae* infestation we compared *yuc8ko*, *yuc9ko* and *yuc8/yuc9ko* knockout mutants. In addition, we have analysed the transgenic lines YUC8ox and YUC9ox, to evaluate the costs and benefits of auxin overproduction in response to herbivore attack. The plant damage and trypan blue staining results performed in the framework of the project uncovered that exposure of the single *yuc8* and *yuc9* knockout *Arabidopsis* mutants to these predatory chelicerate results in a slightly but not significant increased resistance to the mite, when compared to the WT infected plants. Likewise, our data demonstrate that the absence of both YUC8 and YUC9 proteins, confers a significant increased resistance to *T. urticae* (Figure 26 and Figure 27a). The reduced plant damage after infestation was also validated by the scarce accumulation of reactive oxygen species (ROS) (Figure 27b), whose generation upon biotic stresses (Kotchoni & Gachomo 2006; Maffei et al. 2007; de Puyssseleyr et al. 2011; Kerchev et

al. 2012; War et al. 2012). In view of the obtained results, we hypothesised that a wound response independent of the JA-induced auxin biosynthesis might be responsible for this reduced feeding damage. Consistent with our data, Zhurov et al. (2014) demonstrated that *Arabidopsis* mutants disrupted in JA biosynthesis and JA signaling were severely damaged after *T. urticae* feeding. Similarly, in tomato, it has been observed that both JA and salicylic acid (SA) collaborate to promote tolerance against *T. urticae* (Ament et al., 2004). Onkokesung et al. (2010) demonstrated that JA and ET activate *Nicotiana attenuata* defense through the inhibition of IAA-mediated process, i.e. cell expansion and plant cell wall relaxation, in response to the herbivore *Manduca sexta* oral secretion elicitation. On the other hand, a recently published work in *N. attenuata* showed the accumulation of auxin at the site of damage by *M. sexta*. This auxin accumulation was accompanied by a rapid activation of two YUCCA-like proteins (Machado et al., 2016). However, this study did not provide evidence for an altered tolerance against herbivory. These apparent contradictions underline the importance of additional research to elucidate the role of auxin in pathogen/herbivore attack. Here, we suggest that the reduced auxin levels exhibited by the *yuc8/yuc9ko* mutant plants (Figure 31a), facilitate the activation of defense-response pathways and thereby, redirecting the plant resources from further growth into the production of defense-related metabolites. In line with this, flux experiments using labelled carbon (C) and nitrogen (N) in *N. attenuata*, demonstrated that activation of the defense machinery reconfigure C and N allocation to different organs or to defense compounds synthesis, e.g. nicotine and phenol amides (Ullmann-Zeunert et al. 2013; Schwachtje et al. 2006).

In addition, exposure of the auxin overproducer lines, YUC8/9ox, to spider mites further disclosed reduced plant-damage, cell death and H₂O₂ accumulation, in comparison to treated WT plants (Figure 26, Figure 27a-b). Importantly, we confirmed increased auxin levels in both overexpression mutants (Figure 31a). Surprisingly, we also demonstrated elevated MeJA level contents in YUC9ox lines (Figure 31c). Based on the ability of MeJA to inhibit root growth (Hentrich et al. 2013a), we determined that roots are the main source of the observed increased MeJA production, which together with IAA mediated YUC9ox primary root growth inhibition, severely more accused than in the YUC8ox mutant. Feeding of *T. urticae* on plant leaves proceed without damaging the epidermal cell layer, by the insertion of their stylet between the pavement cells or through the open stoma to reach the internal leaf mesophyll tissue (Bensoussan et al. 2016). We speculate that epidermal cell expansion is the cause of this enhanced tolerance, rather than IAA-mediated

immune activation. We propose that expansion of pavement cells in leaves will reduce stylet penetration paths, and hence limit spider mite feeding. Furthermore, we suggest that lignification promoted by the IAA-ET interaction contributes to the observed higher tolerance towards biotic foes by increasing leaf rigidity and possibly reducing leaf nutritional values. In particular the latter aspect is based on the observation that the spider mites actively leave the YUC8/9ox leaves, which seemingly likely indicates that the spider mites detest feeding on those leaves. Induction of lignin deposition has previously been observed in response to herbivore attack (War et al. 2012). Moreover, mutant experiments have confirmed that down-regulation of lignin biosynthesis genes promotes susceptibility against different pathogens in several plant species (Miedes et al. 2014).

In summary, based on the increased resistance reported for the *yuc8/yuc9ko* double mutant and the YUC8/9ox lines we conclude that, despite elicitation of the JA-mediated response is a primary event, the emerging tissue-specific effects of IAA and ET play an important role in conferring resistance to herbivory by creating a second protective barrier. It would be interesting to study plants expressing GUS under the control of either *YUC8* or *YUC9* promoter, as well as JA and IAA accumulation after *T. urticae* infestation in the *yuc8ko*, *yuc9ko*, *yuc8/yuc9ko* mutants, to disentangle the mechanism of how plants coordinate and fine-tune JA and IAA biosynthesis and integration over the course of spider-mite herbivory.

5.2.2 *Pst*DC3000-induced defense response requires reduced IAA levels

Pseudomonas syringae is a gram negative, rod shaped bacterium with polar flagella, capable of promoting disease in a wide variety of plant hosts including *Arabidopsis* (Katagiri et al. 2002). In nature, *P. syringae* survives as an epiphyte on the leaf surface and subsequently enters the leaf tissue through wounds or natural openings such as stomata (Katagiri et al. 2002; Hurley et al. 2014). Actually, based on the host-specificity, more than 50 *P. syringae* pathovars have been identified. Among them, the *P. syringae* pv. *tomato* DC3000 (*Pst*DC3000) is one of the most widely used in plant-pathogen interaction studies (Xin & He 2013). It has been documented that *Pst*DC3000 activates *Arabidopsis* PAMP-triggered immunity (PTI) through its perception by leucine-rich repeat receptor kinases (LRR-RKs), e.g. FLAGELLIN SENSING 2 (FLS2) (Ishiga et al. 2011; Liu et al. 2015).

The *Pst*DC3000 infection data obtained here clearly show that the resistance of the loss-of-function mutants does not differ from that of wild-type plants (Figure 28a-b). In agreement with this result, the SA content neither showed significant changes (Figure 31d). SA is a phenolic hormone and affects plant defense against biotrophic

and hemi-biotrophic pathogens and the establishment of the systemic acquired resistance (Huot et al. 2014). Previously, it has been reported that SA exerts a negative effect on IAA signaling (Naseem et al. 2015). Wang et al. (2007) demonstrated that the SA over-accumulation mutant *cpr6* exhibited lower IAA contents. The authors also indicated that SA promotes the repression of the auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1). Moreover, *P. syringae* infection experiments on the *auxin resistant2/IAA7* (*axr2*) mutant, which is impaired in auxin signaling, showed enhanced resistance (Kazan & Manners 2008). All these evidences together suggest that a partial suppression of auxin content or signaling is not sufficient to increase resistance to *PstDC3000*. Consistent with the aforementioned evidences, the confirmed elevated content of IAA in *YUC9ox* translates into an increased susceptibility to *PstDC3000* (Figure 28a-b and Figure 31a). Surprisingly, unlike *YUC9ox*, *YUC8ox* showed an opposite effect, conferring enhanced resistance towards *PstDC3000*. Nevertheless, this transgenic line also showed elevated SA levels (Figure 31d). Considering the lower JA accumulation in *YUC8ox* (Figure 31c), one possible explanation maybe a mutual antagonistic effect between SA and JA. The antagonistic relationship between SA and JA has been previously described (Spoel et al. 2007). In agreement with this, Leon-Reyes et al. (2010) reported that SA suppresses the transcription of two JA-responsive marker genes *PLANT DEFENSINE 1.2* (*PDF1.2*) and *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*).

This result prompted us to speculate that that *YUC8* and *YUC9* might have different roles in the regulation of the biological trade-off between resistance to biotrophs and necrotrophs.

5.2.3 *YUC9* has a prominent role in conferring drought tolerance.

Abiotic stresses, and in particular drought, are the primary cause of plant loss worldwide (Key et al. 2008). Drought is defined as a period of water scarcity that limits plant productivity in a natural or agricultural system (Verslues et al. 2006). Current evidences have indicated that *YUC8* and *YUC9* play a prominent role in the regulation of abiotic stress responses in *Arabidopsis* (Stavang et al. 2009). In addition to this, Sun et al. (2012) demonstrated the induction of *YUC8* under high temperatures. They suggested that the transcriptional factor PHYTOCHROME-INTERACTING FACTOR4 (PIF4), is a direct regulator of *YUC8* expression. Moreover, our *in silico*-expression results revealed that the expression of *YUC8* and *YUC9* in 10 day-old seedlings is basically restricted to roots after drought stress (Figure 29). Only *YUC9* seems to up-regulated in plant shoots during long-term water deprivation.

Importantly, our drought experiments demonstrated that YUC8 and YUC9 are implicated in water deficit responses, since their absence promote plant death under drought condition (Figure 30a). In agreement with this observation, *yuc8ko* and *yuc9ko* lines exhibited a significant reduction in their abscisic acid (ABA) contents (Figure 31b). After the perception of water deprivation, the plant hormone ABA induces stomatal closure (Leckie et al. 1998; Campalans et al. 1999; Tuteja 2007; Fang & Xiong 2015). Both *yuc8ko* and *yuc9ko* lines exhibit a rapid decrease in water content when compared to the wild type, probably through the inhibition of ABA-mediated stomatal closure (Figure 30b). Our observations implicate that YUC8 and YUC9 could mediate drought stress responses by regulating ABA homeostasis. In support of our hypothesis, YUC1 and YUC6 have been reported to be involved in the regulation of *Arabidopsis* ABA-inhibited seed germination (Liu et al. 2013).

We also found that the *yuc8/yuc9ko* double mutant possesses an increased tolerance towards drought, no altered ABA levels and a WT-like water loss pattern (Figure 30a-b and Figure 31b). On the basis of these findings, it can be predicted that simultaneous inactivation of both YUC8 and YUC9 is likely to induce a compensatory ABA-independent pathway in response to drought. Consistently, Cha et al. (2015) described a novel thiol-reductase activity in YUC6, which, under water deficit, could function as an activator of the ROS pathway. These authors also demonstrated that *Arabidopsis* plants overexpressing YUC6 or YUC7 displayed enhanced tolerance to water deficit. In potato, the overexpression of *AtYUC6* also resulted in an increase in drought tolerance (Kim et al. 2013). Altogether, this suggests that IAA is seemingly a potent modulator of water deficit responses and that YUCCA genes play a decisive role in the modulation of this tolerance.

Our results unequivocally evidence an enhanced drought tolerance in the YUC9ox line and reduced water loss (Figure 30a-b). However, we do not detect changes in the levels of ABA in this line (Figure 31b). Thus, although the induction of ABA responses by IAA has been described (Hansen & Grossmann 2000), we make the increased lignin deposition and the differential expression of XTHs mainly responsible for the altered drought stress response in YUC9ox. Several lignin biosynthesis genes have been observed to be up-regulated after drought stress in different plant species (Le Gall et al. 2015). Beside this, overexpression of *CaXTH3*, a hot pepper (*Capsicum annum*) xyloglucan endotransglucosylase/hydrolase homologous to *Arabidopsis* XTHs, in both hot pepper and tomato (*Solanum lycopersicum*), has been shown to enhance tolerance to drought and salinity stress (Cho et al. 2006; Choi et al. 2011). In contrast to YUC9ox, YUC8ox plants were more susceptible to drought stress and exhibited increased water loss, supporting the

hypothesis of a possibly different function of these enzymes. Consistent with our finding, Lee et al. (2012) reported that while YUC9 expression is activated upon drought, YUC8 transcript accumulation does not appear to be affected by the stress treatment. Alternatively, an explanation for the differential behaviour of the YUC8/9 lines may be related with the expansion of their xylem vessel area. In poplar, Junghans et al. (2006) described that exposure of the species *Populus euphratica* and *Populus x canescens* to salt-stress conditions clearly resulted in a reduction of the stem diameter, accompanied by increased xylem cell wall strength, measured as the relation between cell wall thickness and maximum span of cell wall. A reduction in the metaxylem has also been reported in salt treated leaves of sorghum (*Sorghum bicolor*) (Baum et al. 2000). For this reason, and taking into account that YUC8ox displayed increased IF stack height (Figure 17b), it is presumable that the reaction of secondary cambia, balanced with the lignification rate, follows an optimum curve in the modulation of transpiration during water deficit stress.

With respect to the here presented results we therefore highlight YUC9 but not YUC8, as a potential tool to engineer plants with higher drought tolerance. Our data support the notion that overexpression of *YUC9* induces drought resistance by preventing desiccation through the massive deposition of lignin in the shoot and leaves, where the majority of the water loss occurs (Baum et al. 2000; Junghans et al. 2006). Moreover, despite the ABA levels of the YUC9ox line were not significantly changed compared to wild-type, the decreased ABA levels reported in the *yuc9ko* suggest a possible IAA-ABA interplay during water deficit conditions. The possibility of a role for hormonal crosstalk in drought tolerance conferred by *YUC9* overexpression needs, however, further examination.

5.3 Unravelling the molecular basis of jasmonate-mediated auxin biosynthesis

As already outlined before, plants are sessile organisms that have been forced to develop a wide variety of strategies to rapidly adapt to an ever-changing environment. As a consequence, plants developed a tremendously high degree of plasticity, which involves the permanent activity of meristematic tissues, *de novo*-organogenesis and regeneration, and direct growth responses to external stimuli (Domagalska & Leyser 2011; Hoffmann et al. 2011). The integration of environmental signals and the aforementioned developmental plasticity implicates, among others, the action of small bioactive molecules collectively termed plant hormones or phytohormones. This group of mobile chemical signals are produced at very low concentration and enable to specifically regulate a plethora of plant processes, i.e.

growth, development, reproduction and immune response (Werner et al. 2001; Tanaka et al. 2006; Avanci et al. 2010; Pieterse et al. 2009; Zhao 2010; Brewer et al. 2013; Denancé et al. 2013; McAtee et al. 2013). This capability of phytohormones to act as versatile modulators of the life cycle of plants makes them potent candidates for genetic and metabolomic engineering by plant biotechnologists.

It is assumed that plant hormones can exert their regulatory action in an independent manner. Nevertheless, recent research puts emphasis on the fact that the degree of cooperation and crosstalk between phytohormones is of key importance in driving plant responses to their ever-changing environment (Kazan & Manners 2008; Hoffmann et al. 2011; Gallego-Bartolome et al. 2012). In this context, jasmonates and auxin make no exception and several lines of evidence indicate that the connectivity of the pathways might be particularly relevant in the control of the growth-defense trade-off. For instance, the inactivation of *GH3.9*, a gene involved in the conjugation of free auxin, has been shown in the jasmonate resistant mutant *jar1* (Khan & Stone 2007). The biochemical analysis of the *arf6arf8* mutant has demonstrated a strong reduction of JA levels in flowers, suggesting a possible link between auxin signaling and JA biosynthesis (Nagpal et al. 2005). However, the intricacies of the jasmonate and auxin crosstalk regulation are still largely unsolved.

5.3.1 The promoters of *YUC8* and *YUC9* are direct targets of MYC2

Interconnectivity of JA signaling and IAA biosynthesis have previously been reported. (Dombrecht et al. 2007) reported increased auxin contents in a transgenic *Arabidopsis* line overexpressing MYC2. Furthermore, it has been demonstrated that *ANTHRANILATE SYNTHASE α1* (*ASA1*) gene expression can be induced through the application of 20 μM MeJA (Sun et al. 2009). As mentioned in Section 1.6, Hentrich et al. (2013a) described the positive impact of oxylipin treatments on auxin production through the up-regulation of *YUC8* and *YUC9*, both recognized as key players in auxin biosynthesis. In addition, the authors further described a lack of MeJA-mediated induction of *YUC8* and *YUC9* transcription after various oxylipins treatments in the *coronatine insensitive 1* (*coi1*) mutant (Figure 12), suggesting that both auxin biosynthetic genes could be downstream targets of the JA signaling pathway (See Section 1.5).

The JA signaling pathway facilitates the perception of environmental stimuli and transduction of the signal. To this end, JA activates several types of transcription factors including WRKYs, MYBs, ERF1, ORAs, and MYCs among others (Santner & Estelle 2007; Fernández-Calvo et al. 2011). The MYC family of basic helix-loop-helix (bHLH) transcription factors is conserved in all dicotyledonous plants and plays an

important role in almost every aspect of JA-mediated responses (Schweizer et al. 2013; Cai et al. 2014).

Using single, double and triple loss-of-function mutants of *MYC2*, *MYC3* and *MYC4* we have characterized the molecular mechanism involved in the regulation of *YUC8* and *YUC9*. Our GC-MS experiments highlighted that IAA production after MeJA induction, is considerably dependent on the key member of the MYC family, *MYC2* (Figure 32). Furthermore, we observed that *MYC3* and *MYC4* additionally contribute to auxin homeostasis, thereby confirming that these two transcription factors are phylogenetically closely related to *MYC2*. Apart of that, our transcriptomic analysis revealed that *MYC2* is central in the regulation of *YUC8* and *YUC9* responses towards JA stimuli (Figure 33). We furthermore confirmed that *MYC3* and *MYC4* might also have a role in the transcriptional regulation of *YUC8* and *YUC9*, respectively. However, due to the fact that we detected an induction of *YUC8* and *YUC9* expression in some of the loss-of-function backgrounds after MeJA treatment, the possibility that alternative regulatory mechanism(s) also contribute to the transcriptional control of *YUC8* and *YUC9* in the investigated process cannot fully be excluded. For example, *PIF4*, which triggers hypocotyl elongation under high-temperatures, has recently been described as transcriptional regulator of *YUC8* through its binding to the G-motif (Sun et al. 2012). The jasmonate inducible ETHYLENE RESPONSE FACTOR109 (*ERF109*) has been reported to modulate auxin biosynthesis through the regulation of *ASA1* and *YUCCA2* transcription through directly binding to the core 5'-GCCGCC-3' motif in the promoter of these genes (Cai et al. 2014). However, the aforementioned *cis*-element, called GCC-box, is not contained in the promoter region of neither *YUC8* nor *YUC9*.

MYC2, *MYC3* as well as *MYC4* have been demonstrated to be induced by jasmonate and are known to bind with similar, although not identical affinities to the core 5'-CACGTG-3' motif, called G-box, and its variants (Chini et al. 2009; Fernández-Calvo et al. 2011). We analysed the presence of these JA-responsive element in the promoter sequence of the eleven *YUCCA* members in close detail (Figure 34). Our results identified an overrepresentation of G-box and G-box variants. Moreover, we detected a specific G-box motif configuration composed by the “tandem” 1-9-1 (5'-CACGTG - CACGTC - CACGTG-3') and a subsequently following *cis*-regulatory G-box 3 (5'-CATGTG-3') in the non-coding region of *YUC8* and *YUC9*. The identification of several MYC binding elements in the *YUCCA* family promoters, highlight the complex network of interactions between JA signaling and auxin biosynthesis. Furthermore, the results described here attribute a potentially central

role in mediating *YUC8* and *YUC9* expression to the transcription factors MYC2, MYC3 and MYC4.

To gain deeper insight into the molecular mechanism involved in the interplay of MYC2/MYC3/MYC4 and *YUC8* and *YUC9*, we performed an effector-reporter assay in *N. benthamiana*. In this assay, we demonstrated *in vivo* that *YUC8* and *YUC9* promoters become activated in presence of all three MYC transcription factor, when the 1-9-1 “G-boxes tandem” or the 3 G-box variant was included in the non-coding region (Figure 35b and 36). From this we conclude that *YUC8* and *YUC9* are specific targets of MYC2, MYC3 and MYC4. Mesophyll leaf protoplasts have been proposed as a versatile and powerful system for functional genomics in the model plant *A. thaliana* (Abel & Theologis 1996; Yoo et al. 2007). To practically exploit this tool, we carried out an effector-reporter assay in *Arabidopsis* leaf protoplasts. To this end, we used MYC2, under the transcriptional control of the CaMV 35S promoter as the effector. Just like in *N. benthamiana*, the results obtained here clearly corroborated that MYC2 functions as a direct regulator of *YUC8* and *YUC9*. In brief, we were able to find that MYC2 can drive the expression of *YUC8* and *YUC9* through the direct binding to the 1-9-1 “G-boxes tandem” or the 3 G-box variant (Figure 37b). Herewith, we further confirmed the operability of these *cis*-regulatory elements *in vivo*.

At least to our knowledge, the results presented here demonstrate, for the first time that the transcription factor MYC2 is a molecular integrator directly linking jasmonate signaling with auxin biosynthesis to fine-tune plant defense and plant architecture remodeling. In our genetic efforts to identify new components involved in the regulation of JA-IAA crosstalk, we determined that both MYC3 and MYC4 are also involved in the expression modulation of *YUC8* and *YUC9*. It will be of particular interest to shed light onto the precise role on each transcription factor, as well as the interaction of these two hormones at the signaling and transport level in future studies.

5.4 Future prospects

Traditionally, lignin has been considered as a residual material or a low value product used in pulping processes. However, due to the current natural resource limitation for industrial use, lignin has emerged as a valuable, renewable feedstock for new chemical products. At present, lignin has been proposed to be appropriate for the manufacturing of phenolic resins, adhesives, polyolefins (polypropylene, polystyrene, etc.), and epoxy resins, which are especially relevant in electronic systems (Stewart, 2008). Indeed, Kosbar et al. (2001) demonstrated in a research for IBM the possibility of incorporating lignin to printed wiring boards. Alongside with chemical studies, additional efforts have been made to improve the environmental “friendliness” of lignin extraction methods for pulp and paper industry, which actually involve caustics and sulfonates (Santos Ramos et al., 2009). On the other hand mounting evidences indicate that lignin deposition is stimulated by developmental and environmental cues to provide additional support and protection (Xu et al. 2011a; Miedes et al. 2014; Le Gall et al. 2015). Within this context, a better understanding of the lignin biosynthesis, assembly, and polymerization regulatory mechanisms will have significant impact on plant biotechnology.

Our data support that *YUC8* and *YUC9* overexpression triggers an increased lignin deposition, most probably through the stimulation of ethylene production. In addition, we demonstrated that modulation of both *YUC8* and *YUC9* expression enhances the resistance against herbivory and, in case of *YUC9*, also increase drought tolerance. This results suggests that *YUC* overexpression could be an effective means for bioengineering by plant biotechnologists. Nonetheless, the underlying molecular bases of the identified IAA-ET crosstalk and the increased lignification need to be further examined.

CHAPTER 6:

CONCLUSIONS

Conclusions

The analyses of the implications of auxin overproduction mediated by YUC8 and YUC9 on secondary growth at tissue and biochemical level has allowed us to draw the following conclusions:

1. Auxin promotes stem diameter increment by stimulating the mitotic activity of the vascular cambium and interfascicular cambium.
2. In plant shoots, auxin favoured the transition from primary growth to secondary growth by promoting secondary xylem and interfascicular xylem differentiation and secondary cell wall formation.
3. Auxin stimulates lignification and cellulose deposition into secondary cell walls.
4. Auxin induces ethylene biosynthesis to synergistically drive cell division, secondary growth transition and lignin deposition.
5. Lignification of both overexpression mutants, YUC8ox and YUC9ox, is enriched in H-lignin units.
6. The auxin-mediated increase of cellulose contents is mainly restricted to the cambium and interfascicular cambium.
7. Auxin controls cell wall remodelling through the modulation of the expression of *XYLOGLUCAN ENDOTRANSGLYCOSYLASES/HYDROLASES (XTHs)*, which are essential for the auxin induce cell expansion and possibly favour xylem formation.
8. Our transcriptomic analysis of the *35S::YUC8* and *35::YUC9* transgenic lines indicates that the elevation of auxin causes a dramatic down-regulation of diverse stress-response gene groups to shut-down defence responses and promote plant growth.

In addition, the analysis of the role of both YUC8 and YUC9 in plant tolerance to biotic and abiotic stresses has allowed us to formulate the following conclusions:

9. The constitutive overexpression of *YUC8* and *YUC9* enhances *Tetranychus urticae* feeding tolerance by making spider mite stylet penetration more difficult.
10. The transgenic line overexpressing *YUC8* showed higher resistance against the pathogen *Pseudomonas syringae* pv. *tomato* DC3000, likely by increasing the levels of salicylic acid.

11. Single or double suppression of *YUC8* and *YUC9* genes is not sufficient to abolish salicylic acid- and jasmonate-induced defense.
12. The drought tolerance experiments demonstrated that *YUC8* and *YUC9* play an important role in the modulation of ABA levels during the water-deficit resistance. Moreover, *Arabidopsis* plants overexpressing *YUC9* exhibit water-deficit resistance most probably by avoiding plant desiccation due to the massive lignification.

Finally, biochemical and genetic studies on JA-IAA crosstalk, as well as the analysis of *MYC2*, *MYC3* and *MYC4* as possible interactors of these two hormonal pathways have enabled us to conclude that:

13. Indole-3-acetic acid production and *YUC8* and *YUC9* expression after MeJA induction treatment are dependent on *MYC2*.
14. The closest homologs to *MYC2*, *MYC3* and *MYC4*, may also contribute to auxin homeostasis through the transcriptional regulation of *YUC8* and *YUC9*.
15. *MYC2*, *MYC3* and *MYC4* bind to the *cis*-DNA “tandem” motif 1-9-1 (5'-CACGTG - CACGTC - CACGTG-3') and a subsequently following *cis*-regulatory G-box 3 (5'-CATGTG-3') to act as transcriptional activators of *YUC8* and *YUC9*.

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SUPPLEMENTARY MATERIAL

Figure S1: *Tetranychus urticae* non-infested control plants.

Pictures of representative leaves (top) and histochemical analysis with trypan blue (middle) and DAB (bottom) from *Arabidopsis* WT (Col-0), *yuc8ko*, *yuc9ko*, *yuc8/yuc9ko*, YUC8ox and YUC9ox non-infested with the spider mite.

Figure S2: *PstDC3000* non-infected control plants.

Figure S3: Drought stress tolerance experiment.

a) Picture showing the appearance of the different genotypes (3 weeks-old) prior to the drought stress treatment. **b)** Control plants of *Arabidopsis* WT, *yuc8ko*, *yuc9ko*, *yuc8/yuc9ko*, YUC8ox and YUC9ox continuously irrigated.

Figure S4: Overview of the generation of 35S:MYCx effector constructs for transient expression analysis *in planta*.

The full-length *MYC* open reading frames were amplified from *A. thaliana* (Col-0) cDNAs by PCR using the primers listed in Table 1 (See Section 3.3.1). The nucleotide sequence of *MYC2*, *MYC3* and *MYC4* were available in the TAIR database under the accession number At1g32640, At5g4760, and At4g17880 respectively. Amplified PCR products were ligated into the pGEM-T vector. Afterwards, the *MYC* sequences included into the pGEM-T vector were excised by restriction enzyme digestion with *KpnI/NotI*, in case of *MYC2*, and *BamHI/EcoRI*, in case of *MYC3* and *MYC4* and introduced into the *KpnI/BamHI-NotI/EcoRI* -linearized pSP-Entry1 vector, which contains the attL1-GW-attL2 cassette. For *Arabidopsis* protoplast transfection the target fragments in the entry clones were transferred by LR recombination into the destination vector p35S-HA-GW, including the CaMV 35S promoter and the attR1-GW-attR2 cassette. In case that the generated constructs were utilized for *N. benthamiana* agroinfiltration the entry clones were transferred by LR recombination into the destination vector p35S-201, that, besides the 35S promoter and the Gateway cassette, also contains the corresponding right border (RB) and left border (LB) sequences.

Figure S5: Preparation of *pYUCx::GUS* reporter constructs for transient expression assays in *N. benthamiana* and *A. thaliana*.

Truncated promoter fragments containing the DNA motif tandem 1-9-1 (5'-CACGTG - CACGTC - CACGTG-3'), the final *cis*-regulatory G-box #3 (5'-CATGTG-3') or none of these regulatory (Ø) elements, were amplified by PCR using suitable oligonucleotides (See Section 3.3.1) from pSP-ENTRY2-YUC8/9Prom3kb-GUS and inserted into the high-copy number vector pGEM-T vector. Then, the promoter fragments were excised using the endonucleases recognition sites incorporated during the PCR process, i.e. *HindIII*, *KpnI* and *XbaI*, and ligated into the linearized pBT-10 or pMDC-GUS plasmids, upstream of the *uidA* reporter gene encoding GUS. The generated constructs pBT10-GUS-pYUCx were used for *Arabidopsis* protoplast transfection, whereas the pMDC1163-GUS-pYUCx, which contains the LB and RB border, were utilized for agrobacterium mediated transient expression in *N. benthamiana*.