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Dissertação de Mestrado
Mestrado em Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Trabalho realizado sob orientação da
Doutor Martin Alejandro Darino
e da
Prof. Doutora Teresa Lino-Neto

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DECLARAÇÃO

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Study of IAS8, an *Ustilago maydis* effector able to induce auxin signalling in plants

Abstract

One of the most often used models to study effectors and biotroph-plant interactions is the smut fungus *Ustilago maydis.* This biotrophic fungus is responsible for the corn smut disease which causes up to 1 billion US dollars' worth of damages in the US alone. Besides maize, *U. maydis* can infect several other important crops such as wheat, barley and sugar cane. To be able to accomplish plant infection, this fungus relies on a set of proteins referred to as effectors. Effectors are thought to be used to supress host defences and/or alter the host's metabolism.

During a heterologous screen in *Nicotiana benthamiana,* the effector IAS8 was identified. This effector was proven to induce auxin signalling in a DR5 assay in *N. benthamiana*. Furthermore, a Co-IP, using IAS8 as bait, was performed in *N. benthamiana* and sent for mass spec analysis. This work aims to study this effector and its interaction with candidate proteins, topless related protein 3 (NbTPR3) and Ubiquitin-Specific Protease 6 (NbUBP6), as well as to perform a functional analysis by performing a DR5 assay in *Arabidopsis thaliana* inducible lines and to evaluate the phenotypes caused by the effector in *Marchantia polymorpha*. Our results show that the effector, IAS8, induces auxin signalling in three-fold in *A. thaliana* in a DR5 assay. *M. polymorpha* mutants, constitutively expressing IAS8, seem to have no gemma cups and are impaired in growth. Furthermore, our results show that IAS8 interacts with NbTPR3, NbUBP6 and a *Z. mays* ortholog of NbUBP, ZmUBP, as well as two orthologs in *A. thaliana*, AtUBP12 and AtUBP13. Moreover, we show that IAS8 interacts with the MATHUCTH domains of NbUBP6 and ZmUBP.

The effector IAS8 alone induces auxin signalling in *A. thaliana* by three-fold and drastically impairs the morphology of *M. polymorpha*. Its interaction with NbTPR3 and NbUBP6 hints at that it might be able to manipulate other hormones and their respective signalling. Moreover, our results allude that the pathway through which IAS8 induces auxin signalling is highly conserved. The effector IAS8, here partially characterized, seems to be one of the reasons why *U. maydis* is a master manipulator of hormones and their signalling.

Estudo do IAS8, um efector de *Ustilago maydis* capaz de induzir sinalização da auxina em plantas

Resumo

Ustilago maydis é um fungo biotrófico capaz de infetar colheitas economicamente importantes, como o milho, cevada, trigo ou cana-de-açúcar. Este fungo chega a causar por ano, só nos Estados Unidos da América, danos anuais de quase um mil milhão de dólares. *U. maydis* é um modelo proeminente no estudo de interações biotróficas entre plantas e fungos. Para completar o seu ciclo de vida, *U. maydis* tem de estabelecer uma relação biotrófica com a planta. Para estabelecer esta relação, o fungo emprega efectores (proteínas, hormonas ou outras moléculas) para suprimir as defesas ou para alterar o metabolismo da planta.

Os efectores putativos de *U. maydis* foram avaliados pela sua capacidade de indução da via de sinalização de auxina num ensaio de indução de DR5 em *Nicotiana benthamiana*. Neste ensaio foram identificados vários efectores, entre eles o IAS8. O IAS8 foi usado como presa num ensaio de co-imunoprecipitação em *N. benthamiana*. As amostras resultantes foram analisadas por espectrometria de massa. Esta análise revelou possíveis interações com topless related protein 3 (NbTPR3), repressor da via de sinalização da auxina, e Ubiquitin-Specific Protease 6 (NbUBP6), uma enzima capaz de clivar moléculas de ubiquitina. Este trabalho pretende validar estas possíveis interações, recorrendo a ensaios de co-imunoprecipitação, validar indução da via de sinalização da auxina em *Arabidopsis thaliana* e avaliar o fenótipo causado pelo IAS8 em *Marchantia polymorpha.* Os nossos resultados indicam que o efector IAS8 induz via de sinalização da auxina num ensaio de indução de DR5. Para além disso, os mutantes de *M. polymorpha* que expressam constitutivamente o efector, IAS8, parecem ter um defeito no crescimento e também parecem não ter as estruturas para reprodução assexuada, as taças de gemas. Validou-se também que o efector, IAS8, interage com NbTPR3 e NbUBP6 e ainda com os seus ortólogos ZmUBP, AtUBP12 e AtUBP13. Em conclusão, o efector induz via de sinalização da auxina 3 vezes mais em *A. thaliana* e afeta drasticamente o fenótipo em *M. polymorpha.* Além disto, a interação do IAS8 com NbTPR3 e NbUBP6 indica que IAS8 poderá interferir com outras vias de sinalização hormonais.

Table of contents

Abbreviations and Acronyms

ABA Abscisic Acid

ABC ATP-Binding Cassette

AFB Auxin Signalling F-BOX

Ala Alanine

ARFs Auxin-Response Factors

ARM Agrobacterium Resuspension Medium

At *Arabidopsis thaliana*

ATP Adenosine Triphosphate

Aux/IAA auxin/IAA protein

AUX/LAX AUXIN1/LIKE-AUX1

AuxRE Auxin Responsive Elements

BRs Brassinosteroids

cDNA complementary DNA

CKs Cytokinins

Co-IP Co-Immunoprecipitation

CPS Copalyl Diphosphate Synthase

C-terminal Carboxy-terminal

DNA Deoxyribonucleic Acid

dNTP Deoxynucleoside Triphosphate

DTT Dithiothreitol

DUB Deubiquitinating Enzymes

E1 Ub-activating enzyme

E2 Ub conjugating enzyme

E3 Ub ligase enzyme

ER Endoplasmic Reticulum

NADPH Nicotinamide Adenine Dinucleotide Phosphate Hydrogen

ETI Effector Triggered Immunity

eYFP enhanced Yellow Fluorescent Protein

GA Gibberellins

GFP Green Fluorescent Protein

gly Glycine

HA Tag, Human influenza

HR Hypersensitive Response

IAA Indole-3-Acetic-Acid

IAAld Indole-3-Acetaldehyde

IAM Indoleacetamide

IAN Indole-3-Acetonitrile

IAOx Indole-3-Acetaldoxime

IAS Inducers of The Auxin Signalling

IPyA Indole-3-Pyruvic Acid

JA Jasmonate

KAO Kaurenoic Acid Oxidase

KO Kaurene Oxidase

KS Kaurene Synthase

LDS Lithium Dodecyl Sulphate

Leu Leucine

lys Lysine

Mp *Marchantia polymorpha*

MS Mass Spectrometry

myc myelocytomatosis oncogene epitope tag

Myr Myristoylation signal

Nb *Nicotiana benthamiana*

NES Nuclear Export Signal

NLS Nuclear Localization Signal

N-terminal Amino-terminal

OD Optical Density

PAGE Polyacrylamide-gelelectrophoresis

PAMP Pathogen-Associated-Molecular Pattern

PCR Polymerase Chain Reaction

PIN PIN-FORMED

PRR Pattern Recognition Receptor

PTI PAMP-Triggered Immunity

PTRE1 Proteasome Regulator1

RNA Ribonucleic Acid

ROS Reactive Oxygen Species

RT Room Temperature

RT-PCR Reverse Transcriptase PCR

SA Salicylic Acid

SAR System Acquired Response

SDS Sodium Dodecyl Sulphate

TAA Tryptophan Aminotransferase of Arabidopsis

TIR1 Transport Inhibitor Resistant 1

TPL Topless

TPR3 Topless Related Protein 3

Trp Tryptophan

Ub Ubiquitin

UBP Ubiquitin-Specific Protease

YFP Yellow Fluorescent Protein

Y2H Yeast-2-Hybrid

Y3H Yeast-3-Hybrid

Zm *Zea mays*

List of figures

List of tables

1. Introduction

1.1. *Auxins*

Plants have several biosynthetic pathways dedicated to producing small signalling compounds called hormones. Hormones or phytohormones, can either act near their site of production or travel and act on some other parts of the plant (Fahad et al., 2015). Phytohormones are divided based on their chemical composition into several classes: auxins, gibberellins (GA), abscisic acid (ABA), cytokinins (CK), salicylic acid (SA), ethylene (ET), jasmonates (JAs), brassinosteroids (BR), strigolactones and peptide hormones. All of which play an important role in several processes like development, growth, and responses to both biotic and abiotic stresses (Table 1).

Table 1. Some known functions of phytohormones.

Hormone	Function
Abscisic Acid	✓ Seed germination and development (Bari & Jones, 2009). ✓ Regulate the plant's water status (Fahad et al., 2015). ✓ Stress adaptation (Nemhauser et al., 2006; Pacifici et al., 2015).
Auxins	✓ Apical dominance (Nemhauser et al., 2006; Park et al., 2011). ✓ Regulation of photo- and gravitropism (Fahad et al., 2015). ✓ Important role in plant defence (Kazan & Manners, 2009).
Brassinosteroids	✓ Vascular differentiation (Fahad et al., 2015). ✓ Nucleic acids and protein biosynthesis (Fahad et al., 2015). ✓ Reproductive growth, production of flowers and fruit (Fahad et al., 2015).
Cytokinins	✓ Vascular differentiation and nutrient mobilization (Fahad et al., 2015). ✓ Anthocyanin production (Bari & Jones, 2009; Fahad et al., 2015). ✓ Chloroplast biogenesis (Bari & Jones, 2009; Fahad et al., 2015).
Ethylene	✓ Cell expansion along transverse axes (Achard et al., 2003). ✓ Shortening and thickening of the hypocotyls (Achard et al., 2003). ✓ Plant defence responses against insects, microbial pathogens and necrotrophic fungi (Bari & Jones, 2009; Yang et al., 2013).
Gibberellins	✓ Regulate expansion along longitudinal axes (Nemhauser et al., 2006). ✓ Seed germination (Davière & Achard, 2013; Jones & Dangl, 2006). ✓ Stem elongation and leaf expansion (Olszewski et al., 2002).

Jasmonates	✓ Seed germination (Kunkel & Brooks, 2002; Lacombe & Achard, 2016). ✓ Fruit ripening (Bari & Jones, 2009; Kunkel & Brooks, 2002). ✓ Plant defence responses against insects and necrotrophic fungi (Kunkel & Brooks, 2002; Lacombe & Achard, 2016).
Peptide Hormones	✓ Meristem organization (Matsubayashi & Sakagami, 2006). ✓ Callus growth (Bari & Jones, 2009; Matsubayashi & Sakagami, 2006). ✓ Meristem organization (Matsubayashi & Sakagami, 2006).
Salicylic Acid	✓ Plant defence against hemi- and biotrophic pathogens (Bari & Jones, 2009). ✓ Ion uptake and transport (Fahad et al., 2015). ✓ Thermo-tolerance (Fahad et al., 2015).
Strigolactones	✓ Regulate various developmental responses (Lacombe & Achard, 2016). ✓ Positive regulators of primary root elongation (Pacifici et al., 2015). ✓ Negative regulators of adventitious root formation (Pacifici et al., 2015).

Auxins were discovered by Kögl and Haagen-Smit in 1931 and are involved in several plant processes such as regulation of expression along longitudinal axes, differentiation of vessels, cell division in the cambium, apical dominance, root initiation, regulation of photo- and gravitropism (Kurepin et al., 2014; Nemhauser et al., 2006; Pacifici et al., 2015; Park et al., 2011). Their effects depend mostly their concentration in the cell, which depends mostly on their metabolism, transport and conjugation (Enders & Strader, 2015).

1.1.1. *Auxin biosynthesis, transport and storage*

Indole-3-acetic acid (IAA) is the most common bioactive form of auxin in *Arabidopsis thaliana* (Ljung, 2013). The biosynthesis of the IAA occurs through two routes: the tryptophan (Trp)-dependent and the Trp-independent one (Figure 1). Most of the enzymes involve in the Trp-independent pathways remain unknown. However, several Trp-dependent auxin biosynthesis pathways have been well described, such as, the indole-3-acetaldoxime (IAOx) pathway, the indole-3-acetamide (IAM) pathway, the indole-3-pyruvate (IPyA) pathway, the tryptamine (TAM) pathway, the indole-3-acetonitrile (IAN) pathway and indole-3-acetaldehyde (IAAld) pathway (Gao et al., 2015; Enders, & Strader, 2013; Mano & Nemoto, 2012).

The IPyA pathway is the main contributor of free IAA in plants (Zhao, 2012). The conversion of Trp to IAA is a two-step process. The Tryptophan Aminotransferase of Arabidopsis (TAA) is

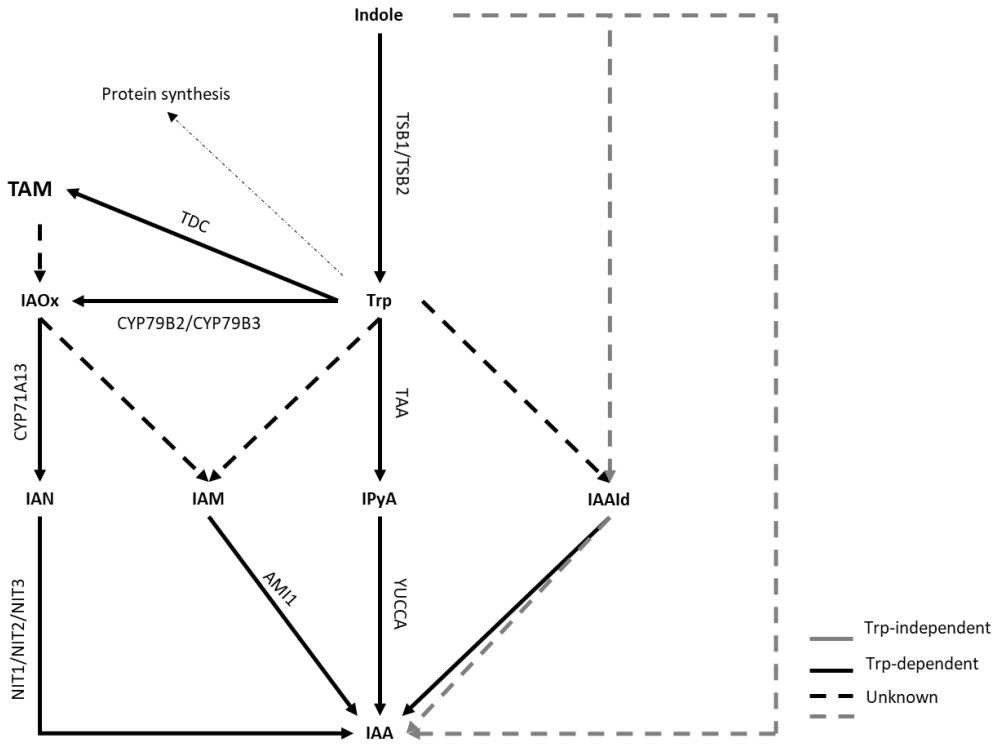

Figure 1. IAA biosynthetic pathways, both tryptophan in- and dependent routes. Indole-3-acetic acid (IAA), Indole-3-acetaldehyde (IAAld), Indoleacetamide (IAM), Indole-3-acetonitrile (IAN), Indole-3-acetaldoxime (IAOx), Indole-3-pyruvic acid (IPyA), Tryptamine (TAM), Tryptophan (Trp). Adapted from: Enders & Strader, 2015; Korasick et al., 2013; Mano & Nemoto, 2012

responsible for the conversion of Trp to IPyA and then a flavin monooxygenase-like enzyme called YUCCA converts the IPyA to IAA.

Being a weak acid, IAA, is only partially ionized in the cytoplasm (Zazímalová et al., 2010). At a low pH, IAA converts to its non-ionized form, IAA-H, which is less polar and can diffuse through cell membranes. The IAA, being a polar molecule, is trapped inside the cell and can only leave with the help of efflux carriers (Ljung, 2013). Some families of IAA transporters have been identified, the PIN-FORMED (PIN), the ATP-binding cassette (ABC) family and the AUXIN1/LIKE-AUX1 (AUX/LAX) family (Figure 2). The PIN family is involved in the polar transport of IAA and the differential localization of these proteins determines the direction of transport. According to the length of a hydrophilic loop in the middle of their poly-peptide chain, PIN family are divided into 'long' and 'short' PINs. Long PINS, such as PIN 1, 2, 4 and 7 were shown to be involved in direct transport of auxin. Short PINs have been hypothesized to be used to alter cytoplasmic levels of free IAA. This type of PINS has been found in plasmatic membrane as well as in the membrane of

endoplasmic reticulum (ER). Their location is not static and, through transcytosis-like mechanisms, they can be quickly re-localized (Zazímalová et al., 2010). In contrast, the ABC family position in the plasma membrane is stable. These proteins are ATP-dependent transporters and usually function when auxin must move against gradient. However, they require activation/folding assisted by chaperone proteins (Zazímalová et al., 2010).

Diverging from the previously mentioned families, the AUX/LAX are symporters of auxin/H+ which are responsible for the influx of auxin from the apoplast to the cytoplasm. Their specificities and location contribute for the differences to auxin response (Enders & Strader, 2015). Besides this cell-to-cell transport, auxins can also be transported through the vascular tissues from the source tissues to the rest of the plant.

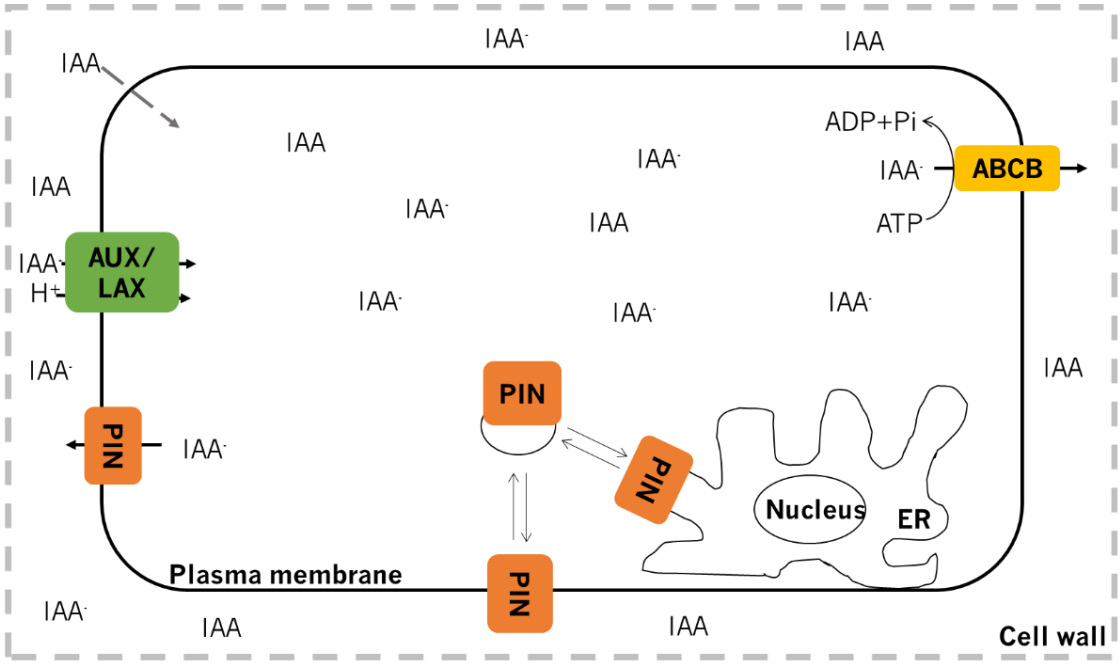

Figure 2. Schematic representation of the several types of transport of both IAA and IAA⁻. Three families of auxin transporters have been identified: PIN, the ABC family and the AUX/LAX. PINs are responsible for polar transport. A specific type of PINs, short PINs, have been found in the ER and were hypothesised to be involved in regulating the levels of free auxin in the cell. These PINs can be re-localized through transcytosis-like mechanisms. AUX/LAX are symporters of auxin/H+ which are responsible for the influx of auxin from the apoplast to the cytoplasm. The ABC family are ATP dependent transporters that are usually involved in the transport against gradient. Adenosine triphosphate (ATP), Adenosine diphosphate (ADP), AUXIN1/LIKE-AUX1 (AUX/LAX), ATP-binding cassette B (ABCB), Endoplasmic reticulum (ER), Indole-3-acetic acid (IAA), PIN-FORMED (PIN). Adapted from: Enders & Strader, 2015; Zazímalová et al., 2010.

In combination with metabolism and transport, the interconversion of the IAA conjugates is responsible for the differential gradient of auxin in plants (Korasick et al., 2013). These conjugates, which can be ester-linked simple and complex carbohydrate conjugates, amide-linked

amino acid conjugates, and amide-linked peptide and protein conjugates, are a form of storage (Ljung, 2013) and have long been thought of as an inactive form of auxin. However, IAA–Ala and IAA–Leu have been shown to inhibit root elongation in *A. thaliana* and IAA–Trp functions as an inhibitor of auxin-induced growth (Korasick et al., 2013).

1.1.2. *Auxin signalling*

Auxin can modulate several developmental and growth processes by regulating gene expression. Genes regulated by auxin possess auxin responsive elements (AuxRE) in their promoters. These elements are recognized by the auxin-response factors (ARFs), which bind to the AuxRE specifically. Furthermore, the bind of ARFs to AuxRE has been shown to be independent of auxin levels (Figure 3) (Ulmasov et al., 1999). In low levels of auxin, the auxin/IAA (Aux/IAA)

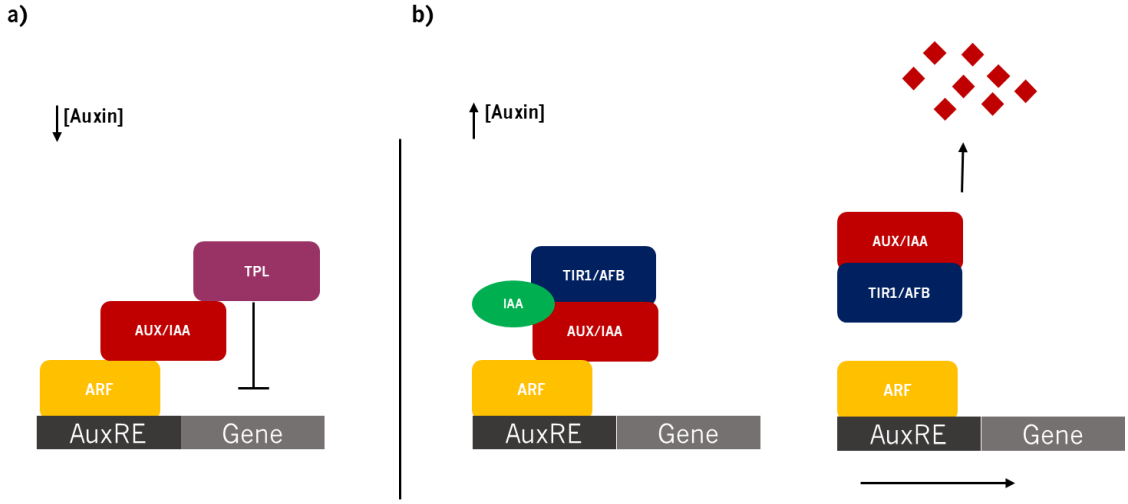

Figure 3. Auxin Signalling. a) When the concentration of auxin, the auxin/IAA (Aux/IAA) proteins function as repressors by recruiting Topless (TPL), which in turn recruits protein remodelers, inhibiting gene expression. When the concentration of auxin rises, IAA stabilizes the binding of the Transport Inhibitor Resistant 1 (TIR1) / Auxin Signalling F-BOX (AFB) receptors to the repressor auxin/IAA (Aux/IAA) proteins causing Aux/IAA ubiquitination and degradation via the proteasome. Adapted from: Pacifi et al., 2015.

proteins function as repressors by recruiting Topless (TPL), which in turn recruits cromatin remodelers (Choi et al., 2018). Thus, transcription does not occur. In high concentration of auxin, IAA stabilizes the binding of the Transport Inhibitor Resistant 1 (TIR1) / Auxin Signalling F-BOX (AFB) receptors to the repressor Aux/IAA causing Aux/IAA ubiquitination and degradation via the proteasome (Pacifici et al., 2015). When auxin levels become low, the rate at which AUX/IAA proteins are degraded is lower, thus their concentration in the cell increases over time eventually becoming enough to repress auxin signalling by forming the complex with ARFs and TPL.

1.1.3. *Auxin and plant defence*

Phytohormones play an important role in the plant's immunity system. The immune system relies heavily on the coordination of various defence pathways and on the recognition of the pathogen-associated molecular patterns (PAMPs) (Kamoun & Zipfel, 2016; Yang et al., 2013). PAMPS are highly conserved molecules among pathogens, like chitin, glucans and flagellins. These molecules are recognized by receptors in the plasma membrane, usually referred to as pattern recognition receptors (PRRs), thus setting off the PAMP-triggered immunity response (PTI) (Kamoun & Zipfel, 2016; Kazan & Lyons, 2014; Kazan & Manners, 2009; Lanver et al., 2017; Yang et al., 2013). The PTI is a fast response that involves a rise of cytosolic Ca^{2+} levels and production of reactive oxygen species (ROS) by the PRR-triggered Nicotinamide adenine dinucleotide phosphate oxidase and Respiratory Burst Oxidase Homologue Protein D. Besides their role in plant's defence, ROS also act as a messenger promoting stomatal closure which restricts the entry of microorganisms (Couto & Zipfel, 2016). To overcome PTI, the secretion of effectors arose as a strategy to suppress elicitation of the plant's immunity responses and to manipulate the host's physiology to support its own growth and development (Lanver et al., 2017). Effectors can be hormones, proteins or their functional equivalents like some microorganisms have been found to produce (Bari & Jones, 2009; Fahad et al., 2015; Kurepin et al., 2014). Effectors can be recognized by resistance proteins (R genes), consequently activating effector triggered immunity (ETI). ETI may involve not only a hypersensitive response (HR), which involves programmed cell death at the infection site, but also the triggering of a system acquired response (SAR), through hormones such as SA, JA and ET (Couto & Zipfel, 2016; Kamoun & Zipfel, 2016; Kazan & Lyons, 2014; Kazan & Manners, 2009; Lanver et al., 2017).

SA, JA and ET are commonly known as defence hormones. The cocktail of SA, JA and ET allows for a network of cross-talk that fine tunes the immunity response (Jones & Dangl, 2006). For instance, SA is usually involved in the plant's response to hemi- and biotrophic pathogens which contrasts with the function of the JA/ET pathways, that are generally involved in responses to both necrotrophic pathogens and insect herbivores (Bari & Jones, 2009). The interaction between SA and JA mediated defences is often antagonistic. This has been proven by the exogenous application of SA which down-regulated genes involved in the biosynthesis of JA, thus compromising the JA signalling (Koornneef & Pieterse, 2008).

Auxins' role in plant defence depends largely on its cross talk with other hormones. They have been described as having a synergetic relationship with JA signalling (Kazan & Manners, 2009). However, auxin has also been described to make hosts susceptible to invasion and have been described as major antagonists of SA signalling (Yan & Dong, 2014). Furthermore, IAA was shown to induce cell wall loosening in rice (Fu et al., 2011).

Either for its role in plant defence or for its role in plant growth and development, many organisms have been shown to alter auxin levels in plants or auxin signalling directly. For instance, *P. syringae* avoids the accumulation of auxin in the cell level by inserting the HopM1 effector that recruits the proteasome to degrade MIN7, thus preventing it from enabling auxin's influx by PIN5 (HopM INTERACTOR 7) (Hirano et al., 2010). In another study, *P. syringae*'s effector AvrRpt2 was shown to induce IAA biosynthesis (Chen et al., 2007). In contrast, the effector AvrBs3 of *Xanthomonas campestris* was shown to upregulate auxin-induce expansin-like gene (Maroiset al., 2002). Through mutations in the components of auxin signalling or by interfering with transport, noted that the resistance of *A. thaliana* to *Plectosphaerella cucumerina* and *Botrytis cinerea* had been compromised (Llorente et al., 2008). Since the SA- or JA-mediated defence pathways had not been altered, not even upon infection by *P. cucumerina,* and that the infection by this necrotrophic fungus usually results in the downregulation of auxin responsive genes, it was postulated that the auxin signalling is important for resistance to this necrotrophic fungus.

1.2. *Ubiquitination*

Ubiquitination is characterized by the addition of ubiquitin molecule by its glycine (gly) residue to a lysine (lys) residue of the target protein (Heideker & Wertz, 2015; Jeong et al., 2017). This post-translation modification can influence activity, abundance, trafficking, or localization of proteins (Stone, 2014).

The ubiquitination process (Figure 4) starts with the activation of a ubiquitin (Ub) molecule by the E1 (the Ub-activating enzyme), via ATP hydrolysis. Thus, the enzyme forms a thioester bond between its cysteine side chain group and the C-Terminus of the Ub. The Ub is then shuttled to the cysteine residue of the E2 (Ub conjugating enzyme) in an ATP-dependent manner. Even though, E2 is known to be able to transfer the Ub directly to the target protein, most commonly, an E3 (Ub ligase enzyme) mediates the transfer of the ubiquitin between the E2 and the target protein. The

Ub is covalently attached by an isopeptide bond between its the C-terminal gly and a lys of the target protein. Since Ub has seven lys residues, polyubiquitination is possible thus, protein ubiquitination can vary structure wise (Burger & Seth, 2004; Gyrd-Hansen, 2017; Heideker & Wertz, 2015; Hicke, 2001; Stone, 2014).

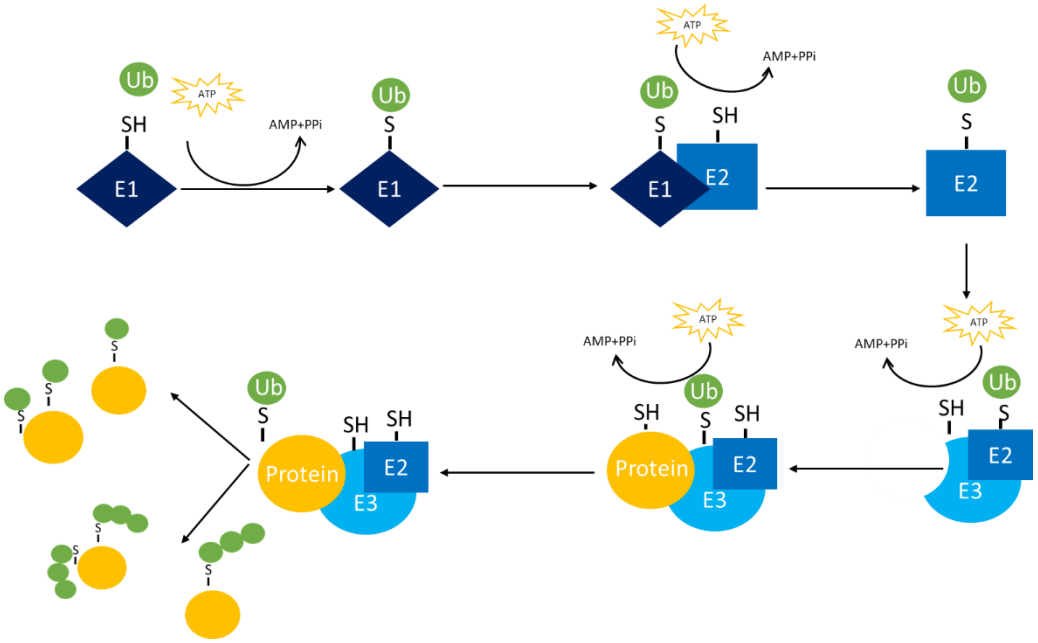

Figure 4. Protein ubiquitination. The ubiquitin is activated by the E1 (the Ub activating enzyme), via ATP hydrolysis. A thioester bond between E1's cysteine side chain group and the C-Terminus of the Ub is formed. The Ub is then shuttled to the cysteine residue of the E2 (Ub conjugating enzyme) in an ATP-dependent manner. Even though, E2 is known to be able to transfer the Ub directly to the target protein, most commonly, an E3 (Ub ligase enzyme) mediates the transfer of the ubiquitin between the E2 and the target protein. The Ub forms an isopeptide bond between its the C-terminal gly and lys of the target protein. Ubiquitin (Ub), Adenosine Triphosphate (ATP), Adenosine Monophosphate (AMP). Adapted from: Stone, 2014.

These different structures originated by protein ubiquitination may each be recognized by specific Ub receptors. Therefore, mono- and polyubiquitination is thought to have different purposes. For instance, monoubiquitylation was shown to lead to the export of proteins from the nucleus to the cytoplasm (Carter et al., 2007). In contrast, the polyubiquitination of the Lys^{48} residue targets the substrate for degradation by the 26S proteasome (Chau et al., 1989).

Allowing for quick responses and physiological flexibility, ubiquitination is a reversible process. The deubiquitylation reaction is catalysed by deubiquitinating enzymes (DUB), which hydrolyses the peptide bond between ubiquitin molecules, **Erro! A origem da referência não foi encontrada.**. Thus, rescuing the target proteins from their ubiquitinated purpose and allowing them to resume their biological function. Besides reversing ubiquitination, DUBs have been found to process ubiquitin chains into single ubiquitin molecules as it's being translated (**Erro! A origem**

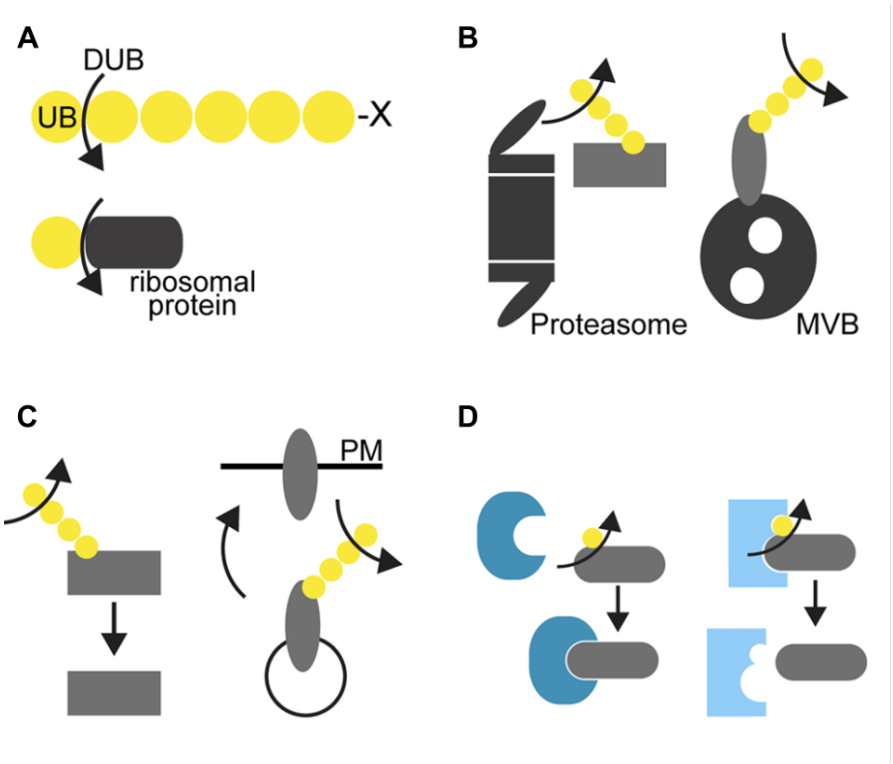

Figure 5. Cellular function of DUBs. (A) Ubiquitin is translated as tandem ubiquitin repeats. In the cell, ubiquitin can exist as a mono- or polymer, or fused to a peptide or to ribosomal proteins in plants. DUBs process the peptide bond between ubiquitin and its fusion protein to produce ubiquitin monomers that can be then conjugated to its substrate proteins. **(B)** DUBs can remove ubiquitin chains from its target proteins and recycle ubiquitin molecules prior to degradation by the 26S proteasome (left) or before the sequestration into the intraluminal vesicles of the multivesicular body (right). Deubiquitylation can start at the distal end as shown here or at the proximal end or in the interior of polyubiquitin chains. **(C)** Removal of the ubiquitin chains by DUBs can inhibit their recognition by the degradation machinery and thus rescues them from degradation regardless whether the protein is a cytosolic proteasomal substrate (left) or a membrane cargo (right). **(D)** Ubiquitylation can serve as an interaction signal for the modified protein. By removing the ubiquitin molecule, DUBs could change the protein-protein interactions, either by enabling (left) or by disabling the binding of the unmodified protein to its interacting protein. From: Isono & Nagel, 2014.

da referência não foi encontrada.a). They have also been found to hydrolise free ubiquitin chains into molecules (**Erro! A origem da referência não foi encontrada.**b). Another important function is to cleave off ubiquitin molecules from target proteins prior to their degradation either by the 26S proteasome or by vacuolar proteases (**Erro! A origem da referência não foi encontrada.**c and d) (Isono & Nagel, 2014).

In *A. thaliana* ~50 candidate DUBs genes have been found. These genes were classified into 5 families: ubiquitin-specific proteases (UBPs), ubiquitin C-terminal hydrolases, ovarian tumour proteases, Machado–Joseph domain proteases, and JAB1/MPN/MOV34 domain proteases (An et al., 2018). Through a bioinformatics approach, 27 UBP sequences were uncovered in *A. thaliana* (Isono & Nagel, 2014). UBPs are cysteine proteases. These proteins have in their catalytic domain a cysteine box. Along with this catalytic domain, UBPs usually have other domains that seem to be

involved in protein-protein interactions. Identifying UBPs' targets has been the main way their functions have been elucidated. So far, UBPs have been shown to play an essential role in regulating many developmental processes (Table 2).

Table 2. Deubiquitinating enzymes known functions.

Name	Function
UBP1 and UBP2	Offer resistance to canavanine (Yan et al., 2000).
UBP 3 and 4	Implicated in gametogenesis and pollen development (Doelling et al., 2007).
UBP12 and 13	Regulate root meristem development by interacting with RGF1 receptor (RGFR1) and its close homolog RGFR2 (An et al., 2018). Positively regulate JA responses by deubiquitinating MYC2 (Jeong et al., 2017). UBP12 can supress Cf-9-mediated hypersensitive response (Ewan et al., 2011).
UBP14	Plays a key role in embryogenesis (Doelling et al., 2001).
UBP24	Negative regulator of ABA signalling (Zhao et al., 2016).
UBP26	Regulation of DNA and H3 methylation by H2B deubiquitination by UBP26 (Sridhar et al., 2007).

1.3. *Ustilago maydis*

One of the most often used models to study effectors and biotroph-plant interactions is the smut fungus *Ustilago maydis.* This biotrophic fungus is responsible for the corn smut disease (Figure 6b) which causes up to 1 billion US dollars' worth of damages in the US alone (Chavan & Smith, 2014). Besides maize, *U. maydis* can infect several other important crops such as wheat, barley and sugar cane (Lanver et al., 2017). Unlike most smut fungi, *U. maydis* is unable to spread through the plant systematically thus remaining locally confined in the areal parts of the plant. *U. maydis* infection is characterized by pronounced chlorosis, less chlorophyll, reduced rates of CO_2 assimilation in infected leaf tissue, the accumulation of anthocyanins and by the formation of tumours (Doehlemann et al., 2008; Lanver et al., 2017). Its infection process relies heavily on reprogramming of both plant signalling and metabolism and in altering the pace and pattern of plant's cell division.

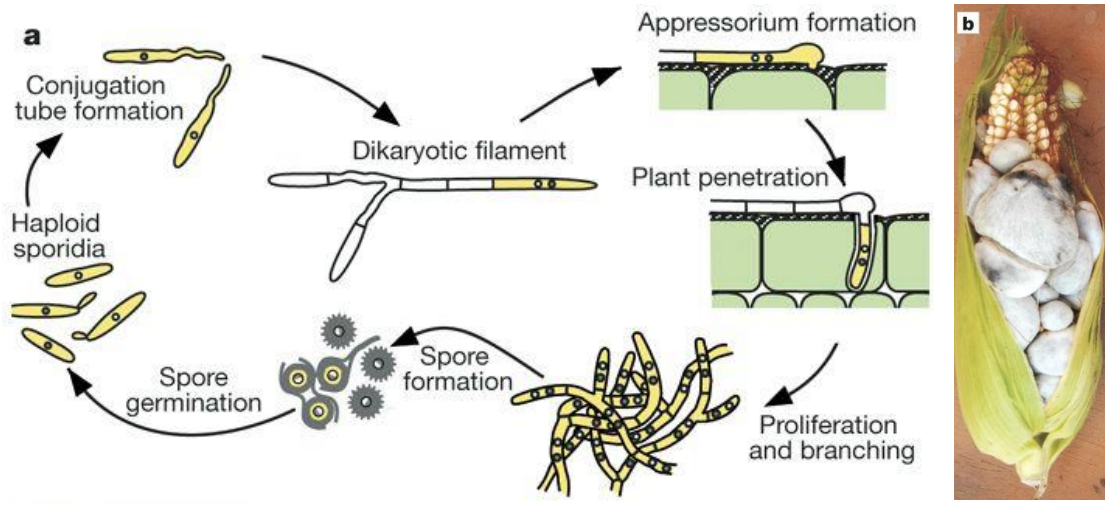

Figure 6. Life Cycle of *U. maydis*. a) Following mitotic divisions, haploid cells start budding off. After detection of a compatible mate, the budding programme ceases and cells develop conjugation tubes that are directed towards each other. After cell fusion, a filamentous cell cycle-arrested dikaryon is produced. These retraction septa enable filament elongation and the formation of an infective structure (appressorium) in extended infectious hyphae. Hyphal tip cells develop appressoria in specific locations on the leaf surface and then penetrate plant cells. With the onset of plant tumour formation, fungal hyphae are mainly detected intercellularly. Subsequently, the two nuclei of the dikaryon fuse, followed by the substantial proliferation of diploid cells that form huge aggregates in apoplastic cavities. Hyphae then fragment and undergo spore development. Diploid spores are released when tumours break open. Meiosis takes place in germinating spores. The four resulting haploid nuclei migrate into a promycelium, in which they become delineated by septa. b) Tumour formation on maize. Adapted from: Kämper et al., 2006

Being a biotrophic fungus, *U. maydis* needs living tissue to complete its life cycle, which can be divided into two stages: the saprophytic phase and the pathogenic phase (Kämper et al., 2006). The saprophytic stage begins when diploid spores, called teliospores, germinate and meiosis occurs (Figure 6a). The 4 haploid nuclei produce during meiosis migrate forming a structure called promycelium. Soon after, septation occurs followed by mitosis after which the haploid cells bud off from the promycelium, growing and then entering the vegetative stage of the lifecycle. In this stage, the haploid yeast-like saprophytic cells grow by polar budding (Lanver et al., 2017). On the maize leaf surface, two haploid cells mate forming the pathogenic form, a filamentous cell cycle-arrested dikaryon. Once the mechanochemical signals, like hydrophobicity and hydroxyfatty acids, are perceived, the infection structures, appressoria, are formed. There by releasing lytic enzymes degrade the cell wall and penetrate the epidermal layer of the plant. At first, the dikaryotic hyphae establishes itself in the apoplast, where molecules such as effectors and

nutrients are exchanged. And not before long, the hyphae cross the mesophyll and get established around the vascular bundle (Tanaka et al., 2014). At later stages of the infection, the fungus promotes host's cells grow and mitotic division leading to the formation of tumours. After which, the hyphae start to fragmentize and differentiate into teliospores, which are released to the air when the plant tissue surrounding the tumour breaks. Once teliospores germinate, they re-start the lifecycle (Brefort et al., 2014; Doehlemann et al., 2008; Lanver et al., 2017; Tanaka et al., 2014).

Several authors have linked tumour formation, cell enlargement and proliferation to the involvement of phytohormones. One hormone linked to tumour formation was IAA. Since it was discovered by Turian and Hamilton (1960) that the tumours showed high levels of auxins and that this fungus produces IAA, through Trp-derived intermediates, it was implied that the two might be correlated. However, upon testing the tumour inducing ability of a quadruple-mutant *U. maydis* for the four enzymes involved in IAA biosynthesis, it was shown that, even though auxin levels were much lower, tumour formation still occurred (Reineke et al., 2008). A microarrays analysis of maize infected by *U. maydis* showed that 3 auxin synthesis genes and 19 auxin-responsive genes were induced and that, in later stages of infection, genes involved in the SA pathway were down-regulated (Doehlemann et al., 2008).

U. maydis and its respective host plant are one of the few eukaryote models' systems that allow the study roles of pathogen effectors at a functional, genome-wide level (Lanver et al., 2017). This biotrophic pathogen has a highly compact, completely sequenced genome of 20.5 Mb (Djamei et al., 2011). Through a bioinformatic approach, the data of *U. maydis* secretome was analysed. Effectors were defined as proteins with a secretion signal, which was identified by SignalP 4.0, and the absence of transmembrane domains, which were identified by TMHMM 2.0c (Presti et al., 2015). It has been found that out that 467 are potentially secreted (Lanver et al., 2017). Of those, 386 genes are exclusively expressed during its biotrophic stage (Djamei et al., 2011). However, so far only a few effectors have been characterized.

One of the effectors identified is the chorismite mutase 1 (Cmu1) which is an enzyme involved in redirecting the chorismite, from the plastid to the cytosol, to the phenylpropanoid pathway and thus lowering its availability for SA biosynthesis (Djamei et al., 2011). Tin2 induces the anthocyanins biosynthesis, by masking the motif of ZmTTK1 thus protecting it from degradation. Then, the ZmTTK1 activates the genes involved in anthocyanins biosynthesis. Rewiring the metabolites into this pathway is aimed at preventing lignification of the vascular

bundle, which limits the hyphae's access to nutrients (Tanaka et al., 2014). Protein essential for penetration 1 (Pep1) was found to be involved in penetration and in inhibiting the plant's immunity responses by interfering with the peroxidase POX12, which is one of the major sources of H_2O_2 in the apoplast (Doehlemann et al., 2009). Seedling efficient effector1's (See1) deletion affects tumour formation in maize leaves but not in tassel floral tissues which means that this is the first identified organ-specific effector. While in interaction with Suppressor of G2 allele of skp1 (SGT1), See1 modulates of immune responses and reactivation of DNA synthesis in leaf cells (Redkar et al., 2015). Effectors' functions have been hard to prove so far, mainly due to other effectors with overlapping functions.

1.4. *The role of* A. thaliana *in auxin signalling assays*

A. thaliana is the go-to model of plant-based-biology. It's a simple angiosperm, whose life cycle can be completed 6-8 weeks. It starts with seed germination, formation of the rosette plant and then comes the flowering. Self-pollination often occurs as the bud opens. However, it can be crossed by applying pollen to the stigma surface. Several hundred siliques, or ovaries where the seeds mature, are formed before on-set senescence (Meinke et al., 1998).

This small plant is amenable to most known tissue culture and transformation techniques. Another factor that makes it an attractive model system, is its small genome, which has been completed sequence and has several bioinformatic curated data bases available (Feldmann & Goff, 2014)[1].

Several studies have used *A. thaliana* to evaluate auxin signalling. To do such evaluations, auxin inducible promoters have been identified, such as IAA2 and GH3 and synthetic promoters have been developed like DR5 (Wells et al., 2013). The DR5 is an inducible promoter that was established in 1997 (Ulmasov et al., 1997) and has since been used by several authors. For instance, while studying lateral roots development, the polar transport of auxin was inhibited with NPA and the GUS activity in transgenic DR5::GUS lines was evaluated and showed auxin signalling in plants treated with NPA was confined to the root apex (Casimiro et al., 2001). Another study evaluated the effects of Proteasome Regulator1 (PTRE1) on auxin signalling by assessing the induction of DR5::GFP in PTRE1 mutants (Yang et al., 2016).

1.5. *The role of* Marchantia polymorpha *in auxin signalling assays*

Marchantia polymorpha L. is a liverwort that has slowly become an important experimental model. The gametophytic generation of *M. polymorpha* dominates its life cycle. The haploid spore germinates and forms the protonema. Resulting from cell divisions of an apical cell of the protonema, the thallus emerges. Being a dioecious species, it has female (archegonium) and male organs (antheridium) on different thallus. On the dorsal side of the mature thallus, in the gemmae cups, the multicellular gemmae are produced, which allow for the liverwort to reproduce asexually. Inside the archegonium, an antherozoid and an egg meet, fertilization occurs, and a zygote is produced. The zygote develops into a sporophyte as the archegonium grows into a calyptra, a structure that confers protection to the young sporophyte. The sporophyte later suffers meiosis, which occurs while still in the sporangium, and the spores are then released in a downward fashion, since the sporophyte hangs upside down beneath the sporangium (Figure 7) (Alam & Pandey, 2016; Shimamura, 2015).

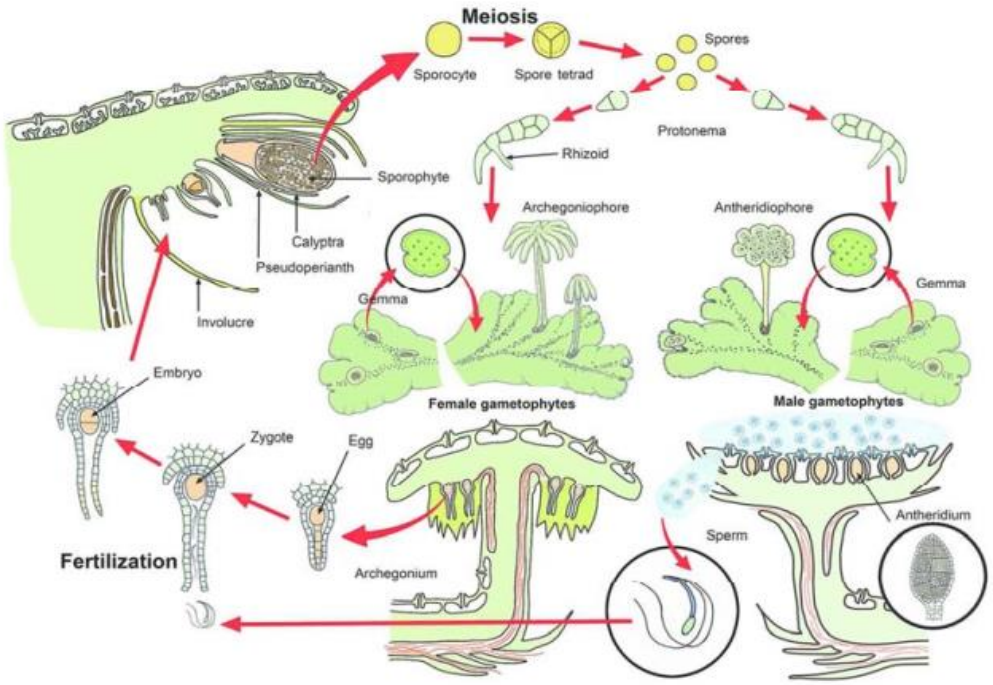

Figure 7. The life cycle of *Marchantia polymorpha* **L.** The male gametophyte forms antheridiophores, that hold the antheridia with the antherozoids (sperm). The female gametophyte forms archegoniophores, which hold the archegonia, each of which contains a single egg cell. During sexual reproduction, the antherozoids are released and swim towards the archegonia. Fertilization occurs and the sporohyte is formed and develops in the archegoniophore. Spores are formed in the sporophyte through meiosis and are finally released. Both, male and female gametophytes are capable of asexual reproduction through the formation of gemmae in the gemma cups. Source: Shimamura, 2015

This liverwort is in a key position of evolutionary events and thus has a pivotal role in improving our understanding of the genetic basis of evolutionary and developmental biology of land plants (Alam & Pandey, 2016). Belonging the Marchantia family, *M. polymorpha,* contrasts from other land plants by lacking vascular system and lignified cell walls, and by having a less redundant genetic networks (Ishizaki et al., 2016; Shimamura, 2015). Additionally, it has a short life cycle, a small completely sequenced genome (~280 Mb) and several transformation techniques developed specifically for it. Moreover, the haploid generation allows the production of genetically homogeneous lines, which can be propagated asexually. All of this characteristics make it an exemplary model organism (Alam & Pandey, 2016; Ishizaki et al., 2016).

Evidence found in charophytes and bryophytes of auxin-mediated responses and of regulation of auxin levels, suggested that auxin signalling might have arisen from that of the early land plants. Because, common to other plants, auxin regulates this liverwort's growth as it does in other land plants. By using auxin responsive promoters in *M. polymorpha*, it was proven that the auxin-mediated transcription had already been established when liverworts separated from other land plants (Ishizaki et al., 2012).

Studying auxin related responses in *M. polymorpha,* has been done by using biosensors, such as DR5 or GH3, as well as by evaluating auxin related phenotypes. One example is the strong growth defect phenotype that was found by applying exogenously several types of auxin on wild type *M. polymorpha* gemmalings, which was characterized by an arrest in the growth of the plants after the first bifurcation (Ishizaki et al., 2012). Another example is the dormancy of gemmae which was shown to be positively regulated by auxin synthesized by the IPyA pathway in the apex of the thallus (Eklund et al., 2015).

1.6. *The role of* Nicotiana benthamiana *in protein production*

Nicotiana benthamiana is an angiosperm with a short life cycle that belongs to the Solanaceae family. Its genome (3 Gb), which has been sequenced, consists of 19 chromosomes. Additionally, *N. benthamiana* is host for several virus, fungi and bacteria. In fact, this plant is amenable to agrobacterium-mediated transformation. All of this makes it an important research model for protein studies in plant biology (Bombarely et al., 2012; Goodin et al., 2008; Ma et al., 2012).

N. benthamiana has large easily infiltratable leaves that allow for transient expression mediated by *Agrobacterium tumefaciens* strains. This allows for a rapid small- scale protein production. Furthermore, this system has been employed to assess gene function, host–pathogen interaction, protein localization and protein–protein interaction (Ma et al., 2012). To evaluate protein-protein interactions, several authors have used this system to perform Co-immunoprecipitations (Co-IP) assays and this technic have been used to confirmed interaction between effectors proteins and their target proteins. For instance, it was confirmed by Co-IP in *N. benthamiana* the interaction between Tin2 and the maize protein kinase ZmTTK1 (Tanaka et al., 2014). Another study employing the same method proved the interaction between See1 and the suppressor of G2 allele of skp1, STG1 (Redkar, 2014).

1.7. *Scientific problem and objectives*

To identify *U. maydis'* effector proteins able to induce the auxin signalling, a heterologous screen was performed in *N. benthamiana* by other members of the Djamei group. Three hundred putative effectors were cloned into a plant destination vector where the Cauliflower mosaic virus promoter 35S (35S) drove the expression of each of the effector. A second plant vector was designed, where the DR5 promoter was fused to the enhanced yellow fluorescent protein (eYFP). Each effector, by agrobacterium-mediated transformation, was co-infiltrated with the reporter construct, DR5::eYFP, into *N. benthamiana* leaves. As a negative control, the reporter DR5::eYFP was co-infiltrated with a construct where the 35s promoter drove the expression of the mCherry gene. Those effectors, which can induce the auxin signalling, will lead to the activation of the DR5 promoter and therefore, eYFP fluorescence emission. Three days post infection, leaves discs were cut it and the fluorescence emission was evaluated using a microplate reader. During the screen several effectors were identified as inducers of the auxin signalling (IAS), among them IAS8. To verify the results of the screen, this experiment was repeated 3 more times (Figure 8). The experiment shows that IAS8 induces the auxin signalling reporter, DR5, in *N. benthamiana.*

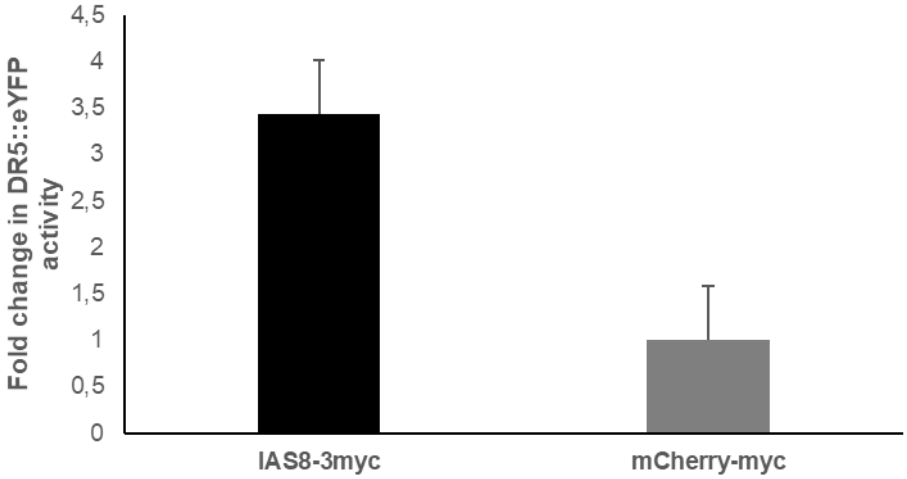

Figure 8. IAS8 induces auxin signalling in *N. benthamiana*. *N. benthamiana* was grown in controlled short-day conditions (8 h light/16 h dark) at 22 °C. The plants were co-infiltratrated with both DR5::eYFP and IAS-3myc, and the negative control, mCherry-myc. The fold change was then calculated by dividing the samples by the negative control, mCherry. The values are mean ± SD, n=3. Source: Darino, unpublished.

Next, a mis-localization study was performed to study in which subcellular compartments IAS8 exerts its function. The effector was fused to different signal peptides to drive the effector to the nucleus (Nuclear localization signal, NLS), cytoplasm (Nuclear export signal, NES) and plasma membrane (Myristoylation signal, Myr). Each of the constructs were co-infiltrated with the DR5::eYFP reporter. We observed that when IAS8 was driven to the cytoplasm or the membrane, it was unable to induce DR5. However, when driven to the nucleus, the IAS8 remained able to induce DR5. Thus, indicating that this effector has a nuclear localization (Figure 9).

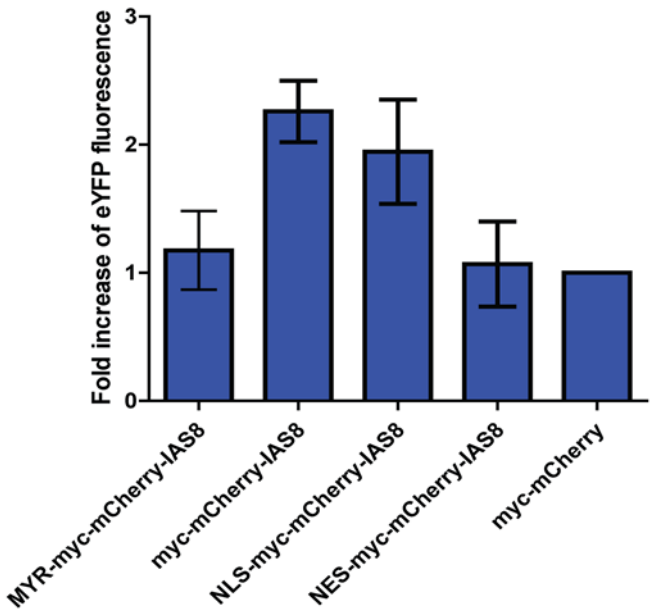

Figure 9. IAS8 induces auxin signalling in a DR5 induction assay and is only functionally active when present in the nucleus. *N. benthamiana* was grown in controlled short-day conditions (8 h light/16 h dark) at 22 °C. The plants were co-infiltratrated with both DR5::eYFP and IAS-mCherry-3myc and with different constructs to drive IAS8 to the nucleus (Nuclear localization signal, NLS), cytoplasm (Nuclear export signal, NES) and plasma membrane. (Myristoylation signal). The mCherry was used as a negative control. Source: Darino, unpublished.

Our main objective in this assay is to evaluate the induction of auxin signalling by a DR5 induction assay, in *A. thaliana*, and, by evaluation the phenotype caused by the effector, in *M. polymorpha*. To try an elucidate a possible mechanism, a Co-IP was performed in *N. benthamiana* followed by Mass spectrometry (MS). Among the several hits, a ubiquitin-specific protease, designated as NbUBP6, and Topless related protein 3 (NbTPR3), a known repressor of the auxin signalling, were found (Data no shown). The secondary objectives are to verify these interacting partners found in the MS approach. Furthermore, we aim to validate that IAS8 is also able to interact with *Zea mays* UBPs, a maize UBP (ZmUBP) with homology with NbUBP6 was cloned and Co-IP experiments were performed in *N. benthamiana*.

2. Materials and Methods

2.1. *Materials*

2.1.1. *Chemicals*

All the chemicals used in this study were mainly obtained from Roche, Sigma-Aldrich, Qiagen, Centic Biotec and Analytic Jena. Restrictions enzymes, DNA ligases and polymerases, and the size standard for agarose gel electrophoresis ladders, both 100 bp and 1 kb, were obtained from NEB. The antibodies used in this study are described below (Table 3).

Table 3. List of antibodies used in this study.

Type	Antibody name	Derived animal	Monoclonal or Polyclonal	Dilution used for Western blot	Company
Primary antibody	anti-Myc	mouse	monoclonal	1:2500	Sigma Aldrich
	anti-HA	mouse	monoclonal	1:5000	Molecular Biology Services
	anti-mCherry	rabbit	polyclonal	1:1000	abcam
	anti-H3	rabbit	polyclonal	1:1000	Sigma Aldrich
Secondary antibody	anti-mouse (IgG)	sheep	polyclonal	1:20000	GE Healthcare
	anti-rabbit (IgG)	donkey	polyclonal	1:30000	GE Healthcare

2.1.2. *Buffers and Other Solutions*

Buffers and solutions used in this study were prepared according to the protocols from Sambrook et al. (1989) and Ausubel et al. (2003). Solutions separately prepared are described in the respective section. For sterilization the solutions were autoclaved 5 minutes at 125 °C or a filter with pore size of 0,2 µm was used in case of heat sensitivity of the solutions.

2.1.3. *Commercial Kits and Additional Material*

The commercial kits used in this study are listed below (Table 4).

Table 4. List of commercial kits.

Name	Company	Purpose
InnuPrep Doublepure	Analytik Jena	DNA elution from agarose gel
Quiagen buffers and silica gel filter columns	Centic biotec	plasmid DNA extraction from *E. coli*
µMACSTM anti-myc Isolation Kit	Miltenyi Biotec	Co-immunoprecipitation
RapidOut DNA removal Kit	Thermo Scientific	To remove DNA from RNA samples
PeqGold Plant RNA Kit	VWR	To isolate RNA
SuperSignal West Pico PLUS Chemiluminescent Substrate	Thermo Scientific	Immunodetection
RevertAid H Minus First Strand cDNA Synthesis Kit	Thermo Fisher	cDNA synthesis

2.1.4. *Bioinformatic Software*

The software *CLC* Main *Workbench* (Qiagen) was used to analyse Sanger sequencing data, for primer design and the generation of vector maps. The image lab (BioRad) software was used to treat the immunodetection pictures. The Prism 7 (GraphPad Software) was used for the statistical analysis of the results.

2.1.5. *Gene information*

The genes used in this study are described in Table 5.

Table 5. Gene information.

Species	Name	Description	Application
Zea mays	ZmUBP	Ubiquitin carboxyl-terminal hydrolase	Co-IP
Nicotiana benthamiana	NbTPR3	Topless-related protein 3	Co-IP
Nicotiana benthamiana	NbUBP6	Ubiquitin carboxyl-terminal hydrolase	Co-IP
Nicotiana benthamiana	NbUBP26	Ubiquitin carboxyl-terminal hydrolase	Co-IP
Ustilago maydis	IAS8	Putative effector	Co-IP, DR5 assays.

2.1.6. *Oligonucleotides*

Several oligonucleotides were design during this study to sequence, clone DNA fragments and to perform RT-PCR. The oligonucleotides and their applications are listed below (Table 6).

Table 6. List of oligonucleotides used in this study.

Name	Sequence	Application
F1_IAS8	CGCCTTCGAAACTGGTGGAG	To sequence IAS8 and for RT-PCR
F2_IAS8	TCGAGACAGAGCGGACACTG	to sequence IAS8 and for RT-PCR
GG35SPro_seqF	TCAAAGCAAGTGGATTGATG	Green Gate 35S promoter forward sequencing primer
IAS8Rev	CTGGCGCGTGCTCTGTTCCG	for RT-PCR
IAS8Rev	CAAGAATGTATCTGGCCTCG	for RT-PCR
MpEFI_fwd	TCACTCTGGGTGTGAAGCAG	for RT-PCR
MpEFI_rev	GCCTCGAGTAAAGCTTCGTG	for RT-PCR
NbMATHUBP6.1_R	ATATggtctcActgaATGGGACCAGTAATCAATGACT	To clone and sequence NbUBP6 Domains
NbUBP26_seq1	ATTTATCCAAACTCTAGAGC	to sequence NbUBP26
NbUBP26_seq2	CAAACAATCAATTTCAGCTG	to sequence NbUBP26
NbUBP26_seq3	TCTTCCGTCGGAGCCACTGC	to sequence NbUBP26
NbUBP26_seq4	GTCTAAGGACGTACATCAGC	to sequence NbUBP26
NbUBP26_seq5	GGAAGAAAATCACATCTGCG	to sequence NbUBP26
NbUCTH6.1_F	atatGGTCTCaGGCTgtATGTCCCATGACTCTAAGAAGGAG	To clone and sequence NbUBP6 Domains
NbUCTH6.1_R	ATATggtctcActgaGCATATAATCTTATCCTTGTC	To clone and sequence NbUBP6 Domains
NbUSP76.1_F	atatGGTCTCaGGCTgtATGGTTGCAAAAGAATTAGGT	To clone and sequence NbUBP6 Domains
pJET-fwd	TGGAGCAGGTTCCATTCATTG	Sequencing
pJET-rev	GTTCCTGATGAGGTGGTTAGCATAG	Sequencing
Ubi_Term_SeqR	GAAAGAGATAACAGGAACGG	Greengate sequencing primer

ZmMATH domainv2_R	ATATggtctcActgaACCTGTTTCTTTTTTCGAGTC	to clone MATH domain from *Z. mays*
ZmUBP_seq1	CTTAAGAATCAAGGTGCTAC	to sequence *Z. mays* UBP
ZmUBP_seq2 (v2)	TGAATATGATTACATGCGAG	to sequence *Z. mays* UBP
ZmUBP_seq3	GACATCGCTGAGCATTTGCG	to sequence *Z. mays* UBP
ZmUBP_seq4	TGTGAAGGCTCTGGGAAAGC	to sequence *Z. mays* UBP
ZmUBP23640_seq5	ACATCTACTGGATATGCTTG	to sequence *Z. mays* UBP

2.1.7. *Bacterial strains, media and culture conditions*

2.1.7.1. Culture conditions and media of *Escherichia coli*

For cloning purposes, the *Escherichia coli* used are listed in Table 7. The cells were grown at 37 °C, 28 °C or at room temperature (RT) depending on the inserted protein's toxicity in dYT or LB liquid medium or in LB agar plates (Table 8). The selection of the transformed cells was done using either the antibiotics Ampicillin or Spectinomycin added to the medium in a concentration of 100 mg/L. Liquid cultures were left shaking overnight at 180 rpm.

2.1.7.1. Culture conditions and media of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens (Table 7) was grown at 28 °C, in LB liquid medium or in LB agar plates (Table 8) with the following antibiotics: Rifampicin, Gentamycin, Spectinomycin, each in 50, 10 and 100 mg/L concentration respectively for selection purposes. To the liquid medium, 10 mM MES-NaOH (pH 5,6) and 0,02 mM Acetosyringone were added. Glycerol stocks were generated with equal amounts of overnight-grown-liquid culture and 50% Glycerol (w/v) were mixed and stored at -80 °C.

Table 7. Bacterial strains used in this study.

Species	Strain	Genotype	Application	Reference
Escherichia coli	Mach1	F- Φ80lacZΔM15 ΔlacX74 hsdR(rK–, mK+) ΔrecA1398 endA1 tonA		Thermo Fisher Scientific
	TOP10	pHuLUC3/TOP10 (PTA 10989)	Cloning	Invitrogen
Agrobacterium tumefaciens	GV3101/pMP90 pSOUP	C58C1: pGv3101 RifR; pTiC58 ΔTDNA GentR; pSoup TetR	Plant Infection	Koncz & Schell, 1986; Hellens et al., 2000

Table 8. List of mediums use for bacteria cultivation in this study.

Medium	Components
dYT liquid medium	1,6% Tryptone (w/v) 0,5% Yeast extract (w/v) 0,5% NaCl (w/v)
LB liquid medium	1% (w/v) Tryptone 0,5% (w/v) Yeast extract 1% (w/v) NaCl
LB agar	1% (w/v) Tryptone 0,5% (w/v) Yeast extract 1% (w/v) NaCl 1.5% Bacto-agar
SOC medium	2% Bacto-Tryptone (w/v) 0,5% Bacto-Yeast extract (w/v) 10 M NaCl 2,5 mM KCl 10 M $MgCl_2$ 20 M Glucose

2.2. *Microbiological methods*

2.2.1. *Generation of chemo-competent* E. coli *cells*

To be able to transform *E. coli*, chemo-competent cells were generated. Between 15-20 mL of dYT media was inoculated with the respective *E. coli* strain and allowed to grow over night at 37 °C with shaking (180 rpm). Of this preculture, 2 mL were used to inoculate 100 mL dYT media, containing 10 mM $MgCl_2$ and 10 mM $MgSO_4$, which were incubated again at 37 °C with shaking (180 rpm) until the OD_{600} of 0,4-0,6 was reached. The culture was then incubated at 4 °C for 30 minutes. While working at 4 °C, the cell culture was centrifuged for 8 minutes at 3000 rpm. The supernatant was removed, the cells were resuspended in 33 mL of RF1 solution (Table 9) and allowed to rest for 30 minutes. Afterwards, the cells were centrifuged at 3000 rpm for 8 minutes, resuspended in RF2 solution and incubated again for 30 minutes. Then, the mixture was divided in 50 µL aliquots, which were shocked frozen in liquid nitrogen and stored at -80 °C.

Table 9. Composition of the solutions used to generate chemo-competent *Escherichia coli*.

Solution	Composition
RF I set pH of the RF I-solution to 5,8 with 0,2 M acetic acid, filter-sterilize and keep at 4 °C.	100 mM RbCl 12 g/L 50 mM $MnCl_2$ x 4 H_2O 9,9 g/L 30 mM Potassium acetate 30 mL/L of 1 M stock (pH 7,5) 10 mM $CaCl_2$ x 2 H_2O 1,5 g/L 15% (w/v) Glycerol 100% 150 g/L 1 M Potassium acetate [M=98,13 g/mol] 98,1 g/L, set pH to 7,5 with acetic acid (~1 drop !)
RF II set pH of the RF II-solution to 6,8 with NaOH, filter-sterilize and keep at 4 °C.	10 mM MOPS 20 mL/L of a 0,5 M stock, pH 6,8 10 mM $RbCl_2$ 1,2 g/L 75 mM $CaCl_2$ x 2 H_2O 11,0 g/L 15 % (w/v) Glycerol 100% 150 g/L 0,5 M MOPS [M=209,3 g/mol] 104,6 g/L

2.2.2. *Heat-shock transformation of chemo-competent* E. coli

The transformation of *E. coli* was achieved by the following protocol. 50 µL of chemo-competent *E. coli* cells were left to melt on ice, afterwards, 150 ng of DNA or 2 µL of ligation mixture was added. The mixture was incubated for 10 min on ice and then heat-shocked for one minute at 42 °C. Afterwards, the cells rested on ice for 2 minutes after which 200 µL of SOC was

added. the recovery took place at 37 °C for one hour, shaking at 650 rpm. If the construct's inferred resistance was ampicillin, this step wasn't performed. The cells were centrifuged at maximum speed, approximately half of the supernatant was discarded. Afterwards, the cells were resuspended, plated on LB plates with the corresponding antibiotic and incubated overnight at 37 °C.

2.2.3. *Generation of electro-competent* A. tumefaciens *cells*

To generate electro-competent cells, the *A. tumefaciens* cells were grown in LB liquid medium with Rifampicin, Gentamycin and Spectinomycin, overnight, at 28 °C, while shaking at 180 rpm. Then, they were centrifuged at 3000 rpm for 10 min at 4 °C and afterwards washed with sterile water and one time with 10% glycerol. At which point, they were resuspended in 10% glycerol, aliquoted, frozen in liquid nitrogen and stored at -80 °C.

2.2.4. *Transformation of electro-competent* A. tumefaciens

Transforming *A. tumefaciens* was done by the following protocol. The 100 µL aliquots of *A. tumefaciens*' cells were thawed on ice. Afterwards, 200 ng of DNA was mixed in. The mixture was then transferred into a cold 1,5 mm cuvette in which the electroporation occurred at 2,4 V. Upon this, 1 mL of SOC was added and the culture is transferred into a new tube. Then, it was incubated for 3 hours at 28 °C with shaking (180 rpm). After which, the suspension was centrifuged for 10 minutes at 3000 *g*. Approximately 900 µL of the supernatant were discarded, the cells were resuspended in the remainder, plated in LB medium, with the appropriate antibiotics, and incubated for 2 days.

2.3. *Molecular biology methods*

2.3.1. *Mini preparation of plasmid DNA from* E. coli

DNA was extracted from *E. coli* performing the next protocol. The *E. coli* cells were grown over night, at 37 °C, in 2 mL of liquid mL dYT medium containing the respective antibiotics. Afterwards, the cultures were centrifuged at 13000 rpm for 1 minute and resuspended in 250 µL of P1-buffer (Table 10) containing RNaseA. Followed

Table 10. Composition of the buffers used in plasmid isolation.

Buffer	Composition
P1-buffer	50 mM Tris-HCl, pH 8,0 10 mM EDTA 100 µg/mL RNaseA
PB-buffer	5 M Guanidine hydrochloride 30% Isopropanol
PE-buffer	10 mM Tris-HCl, pH 7,5 80% Ethanol

by the addition of 250 µL of P2-buffer (Table 10) (Qiagen) and then everything was gently mixed by inverting the tubes several times. Next, incubated for 3 minutes and then 350 µL of N3-buffer (Qiagen) were added. After which, the tubes were again inverted several times and centrifuged at 13000 rpm for at least 10 minutes. The supernatant was transferred to the columns with silica gel filters (CenticBiotec), centrifuged at 13000 rpm for 1 minute. Thus, binding the DNA onto the columns, which was then washed with 500 µL of PB buffer (Table 10) under high salt conditions. Then, centrifuged again at 13000 rpm for 1 minute. To remove the salt, the column was washed with 750 µL of PE buffer (Table 10), centrifuged again at 13000 rpm for 1 minute. To remove the ethanol, the columns were centrifuged at 13000 rpm for 2 minutes. Finally, to elute the DNA 30-50 µL of water was added and let to rest at room temperature for 2 minutes before centrifuging them at 13000 rpm for 1 minute.

2.3.2. *RNA extraction from plant material*

The *M. polymorpha* tissue was harvested, frozen in liquid nitrogen and ground to a fine powder using mortal and pistil. The Kit PeqGold Plant RNA was used to extract RNA from about

100 mg of tissue. To clean the sample from DNA contaminations, the RapidOut DNA removal Kit (Thermo Scientific) was used.

2.3.3. *Golden Gate cloning*

All the cloning in this study was accomplished by employing the golden gate technique. Golden gate is a highly efficient cloning technique that allows for the assembly of several DNA fragments. This is possible by using type II enzymes, such as BsaI or SapI, that cleave outside of their recognition sites leaving a 5' or 3' overhang. By designing specific overhangs, to make the fragments compatible, it is possible to form side-directed ligations. The products, after cleavage and ligation, lack the enzyme's recognition sites and are therefore protected from being re-digested. So, after several cycles, the product accumulates.

A Golden Gate reaction contained the following components:

Modules (approximately 100 ng)	0,5 µL
ATP	0,5 µL
T4 ligase buffer	1 µL
MQH_2O	4,25 µL
BsaI	0,5 µL
T4 ligase	0,5 µL

The detailed cleavage and ligation programme is listed in Table 11.

Table 11. Thermocycler conditions for Golden gate reaction.

Step	Temperature	Time	Comment
1	37 °C	10 min	initial cleavage
2	37 °C	5 min 10 cycles	cleavage
3	16 °C	10 min 10 cycles	ligation
4	37 °C	10 min	cleavage of unligated products
5	16 °C	10 min	ligation
6	50 °C	5 min	heat inactivation

The Golden gate modules used to build the constructs (Table 13) used in this study are described in Table 12.

Table 12. List of golden gate modules used in this study.

Name	Description	Module
pGG103	Plant destination Vector	A-G
pGG454	Low copy plant destination vector pGG103 (pMB1+rop gene)	A-G
35S	Constitutive promotor of Cauliflower mosaic virus	A-B
EF2	Constitutive Promoter of *M. polymorpha*	A-B
Dummy	N-terminal dummy	B-C
mCherry	mCherry	C-D
3xmyc tag	Linker-triple myc tag	D-E
3xHA tag	Linker-triple ha tag	D-E
mCherry-myc Tag	mCherry-Linker (SG)-Myc-Stop	D-E
Ubq10T	Ubiquitin terminator	E-F
HygR	Plant resistance to hygromycin	F-G
BastaR	Plant resistance to Basta	F-G

Table 13. List of constructs and their applications in this study.

Construct	Application
pGG103-35s-omega-IAS8-3xMyc-Ubq10-HygR	Bait construct for Co-IP
pGG103-DR5Pro:GFP -HYGR	DR5 induction assays
pGG103-35S-omega-AtUBP12-3HA-UbqT-BastaR pGG103-35S-omega-AtUBP13-3HA-UbqT-BastaR pGG103-35S-omega-NbMATH (6.1)-3HA-UbqT-BastaR pGG103-35S-omega-NbMATH-UCTH (6.1)-3HA-UbqT-BastaR pGG103-35S-omega-NbUBP6-3HA-UbqT-BastaR pGG103-35S-omega-NbUCTH (6.1)-3HA-UbqT-BastaR pGG103-35S-omega-NbUCTH-USP7 (6.1)-3HA-UbqT-BastaR pGG103-35S-omega-mcherry-3myc-UbqT-BastaR	Prey construct for Co-IP in *N. benthamiana*

pGG103-35s-omega-NbTPR3-3HA-Ubq10-HygR	
pGG103-EF2 -Omega-mCherry_CD-3myc-UbqTer-2x35S-HygR-35ST pGG103-EF2-Omega-IAS8-3Myc-UbqTer-2x35S-HygR-35ST	To express in *M. polymorpha*
pGG-35s:XVE-dummy-Mcherry-Myc-Ubq10-BastaR pGG-35s:XVE-dummy-IAS8-Mcherry-Myc-Ubq10-BastaR	To Induce expression in *A. thaliana*
pGG454-35S-omega-ZmUBP-3HA-UbqT-BastaR pGG454-35S-omega-ZmMATH-3HA-UbqT-BastaR pGG454-35S-omega-ZmMATH-UCTH-3HA-UbqT-BastaR pGG454-35S-omega-ZmUCTH-3HA-UbqT-BastaR pGG454-35S-omega-ZmUCTH-USP7-3HA-UbqT-BastaR	Prey construct for Co-IP in *N. benthamiana*

2.3.4. *DNA ligation*

With the purpose of DNA amplification, the DNA fragment was inserted in the expression vector pJET. For each ligation reaction the following volumes were pipetted:

ATP	0,5 µL
Destination vector	0,5 µL
Insert	2 µL
MQH_2O	5,5 µL
T4 ligase	0,5 µL
T4 ligase buffer	1 µL

The mix was then incubated for one hour at room temperature. Chemo-competent *E. coli* cells were then transformed with it according to the protocol described in 2.2.1 and the plasmid was isolated as described in 2.3.1.

2.3.5. *PCR*

2.3.5.1. *Direct PCR*

To discern which of *E. coli's* colonies had the correct plasmid a direct PCR was performed. Single colonies were picked and inoculated in 2 mL of dYT or LB medium with the respective antibiotic. The cultures were incubated for at least 15 min, at 37 °C and shaking at 180 rpm. To

amplify the section of the desired DNA, the following reaction was prepared, and the tubes were transferred into a thermocycler set to the conditions described in Table 14.

OneTaq© Quick-Load© 2x Master Mix (NEB)	5 µL
H_2o	5 µL
Forward primer (100 µm)	0,1 µL
Reverse primer (100 µm)	0,1 µL
Liquid culture	1 µL

Table 14. Thermocycler conditions for direct PCR reaction.

Step	Time	Temperature
1. Initial denaturation	1 min	94 °C
2. Denaturation	30 sec	94 °C
3. Primer annealing	20 sec	primer specific
4. Elongation	1 min/kb	68 °C
Go to step two (40x)		
5. Final elongation	5 min	68 °C
6. Cooling	∞	12 °C

2.3.5.2. *Q5 PCR*

For further cloning, DNA fragments were amplified employing a polymerase chain reaction using the Q5 polymerase and running the PCR program described in Table 15.

5x Q5 reaction buffer	5 µL
Mono Q H_2O	18 µL
dNTP (10 mM)	0,5 µL
Forward primer (100 µm)	0,125 µL
Reverse primer (100 µm)	0,125 µL
Q5 polymerase	0,25 µL
Template DNA	1 µL

Table 15. Thermocycler conditions for Q5 PCR reaction.

Step	Time	Temperature
1. Initial denaturation	30 sec	98 °C
2. Denaturation	10 sec	98 °C
3. Primer annealing	20 sec	primer specific
4. Elongation	20 sec/kb	72 °C
Go to step two 40x		
5. Final elongation	5 min	72 °C
6. Cooling	∞	12 °C

2.3.5.3. *RT-PCR*

Reverse transcriptase PCR (RT-PCR) was performed to analyse the expression of the constructs in *M. polymorpha*. RNA was isolated as described in section RNA extraction from plant material (2.3.2.). To synthesize cDNA, from the previously isolated RNA, the the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher). Samples were used for PCR or stored at -80 °C. The cDNA was amplified by a Q5 PCR reaction, as the one described in 2.3.5.2, and the samples were run in an agarose gel electrophoresis (2.3.6).

2.3.6. *Agarose gel electrophoresis*

Agarose matrixes were used to separate DNA fragments. The DNA-grade agarose was weighed according to the desired final concentration, 0,8-2%, and dissolved in TAE buffer (Table 16) by boiling. The Peq-green dye was added in a final concentration of 4 µL/100 mL. The gel was left to solidify for about 15 minutes. After loaded with 1XDNA loading dye (Table 16), it ran for 20-30 min at a voltage of 125 V. The stained DNA was excited by UV-light and photographed. If DNA purification was the purpose, the region of the gel with the proper size was cut, under UV-light, and eluted (2.3.7).

Table 16. Composition of solutions used in agarose gel electrophoresis.

Solution	Reagent
TAE buffer	40 mM Tris 1 mM EDTA, pH 8,0 20 mM acetic acid
6xDNA loading dye (Dissolved in TE buffer)	50% (v/v) Saccharose 0,1% (v/v) Bromophenol blue

2.3.7. *DNA elution from agarose gel*

After agarose gel electrophoresis, the candidate fragment with the appropriate size was excised and eluted according to the protocol for "DNA extraction from agarose gel slices", using the innuPREP DOUBLEpure kit (Analytik Jena).

2.3.8. *Sequencing*

The samples were delivered to the Vienna Biocentre's Molecular Biology Services. The samples contained 150 ng of DNA and 0,5 µL of a specific primer and water to fulfil the volume of 7,5 µL.

2.4. *Biochemical methods*

2.4.1. *Protein crude extraction from plant tissue*

To obtain a crude extract from plant tissue, frozen (-80 °C) tissue was ground with metal beads for 1 min and 30 s at 25 Hz using the Mixer Mill (Retsch MM 400). To 50 mg of tissue, 250 µL of 1xLDS buffer was added. The mixture was then heated at 75 °C for 10 min and then centrifuged for 3 min at 1300 rpm. Protein quantification of the samples was performed by an Amido Black. In an SDS polyacrylamide gel electrophoresis, 20 µg of protein was loaded.

2.4.2. *Amido Black*

Amido Black was used to measure protein concentration of the extract. To 190 µL of MQ H_2O, 10 µL of crude extract was added. Followed by the addition of 800 µL Amido Black Staining solution (Table 17). The mixture was vortexed and then centrifuged at 1300 rpm for 10 min. The supernatant was decanted, and the pellet was washed with 1 mL Amido Black Washing solution (Table 17). The samples were centrifuged again for 10 min at 1300 rpm, the supernatant was removed, and the pellet was dried in a SpeedVac for 10 min. to dissolve the pellet, 250 µL of NaOH (0,2 M) were added. The OD_{620} was measured. Protein concentration was calculated using the calibration curve. For the calibration curve the same procedure was performed with triplicates of 5, 10, 20, 30 µg BSA in 200 µL MQ H_2O.

Table 17. Composition of the solutions while performing an Amido Black for a final volume of 250 mL.

Solution	Reagent	Amount
Staining solution	10% (v/v) Acetic acid	25 mL
	90% (v/v) Methanol	225 mL
	0,05% (m/v) Amido Black	0,125 g
Washing solution	10% (v/v) Acetic acid	25 mL
	90% (v/v) Ethanol	225 mL

2.4.3. *SDS polyacrylamide gel electrophoresis*

The proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to LaemmLi (1970). Firstly, the proteins were mixed with 1xLDS loading dye (Table 18) and incubated for 10 min at 95 °C. Thus, the proteins are coated with negatively charged

Table 18. Composition of LDS Buffers.

Buffer	Composition
4xLDS	40% Glycerol 1 M Tris-HCL-pH 8,5 2 mM EDTA 8% LDS (Lithium Dodecyl sulphate) MQ H_2O 0,02% Bromphenol Blue
1xLDS buffer + DTT (denaturing buffer)	1xLDS 100 mM DTT

SDS molecules and therefore the negative charge will correlate with each protein's molecular mass. This will allow for their separation, in an electric field, by size. Following its denaturation, the proteins were loaded on a vertical SDS polyacrylamide gel composed by the stacking and resolving gel (Table 19). The stacking gel concentrates the proteins before entering the resolving gel. Thus, all proteins start from the same point and, when in the resolving gel, are separated by size because the smaller the proteins will progress faster in the gel. The acrylamide percentage, which is directly

Table 19. Composition of the gels used for SDS polyacrylamide gel electrophoresis. Amount for 1 SDS gel (10,5x11,5x1,5 cm).

	Resolving gel (10%)	**Stacking gel (4%)**
Reagents	**Volume in µL**	**Volume in µL**
1,25 M Bis-Tris	1825	14125
MQ H_20	1430	715
30% acrylamide/bisacrylamide	1660	3325
10% SDS	50	25
TEMED	2,5	2,5
10% APS	25	12,5

correlated with the density of the meshed molecular network, can be adjusted for a better

resolution. When analysing a set of smaller proteins, higher acrylamide percentages are preferred and vice versa. 1X Tris-Mes was used as a running buffer. The separation of proteins was achieved with a constant current of 60 V until the proteins reached the resolving gel, after which, the current was increased to 80 V. The PageRuler Prestained Protein ladder (ThermoFisher Scientific) was used as a reference for the protein's molecular weight.

2.4.4. *Immunological protein detection - Western blot analysis*

After separation by SDS-PAGE, a Trans-Blot Turbo Transfer System (Bio-Rad) is used. In a cassette, the filter paper soaked in Transfer-blot Buffer was placed. The gels were positioned onto the Trans-Blot Turbo mini nitrocellulose membrane and are sandwiched between filter paper, which were previously soaked in Transfer-Blot Buffer. The transfer was accomplished by applying 1,3 A, 25 V for 10 min. The membranes were then blocked for 1 h at RT by adding 5% skimmed milk powder dissolved in blocking buffer (Table 20). The membranes were washed using washing buffer (Table 20) and then immunoblotted for 1 h at RT, using the antibody as described in Table 3. After incubation with an antibody, the membranes were washed 4x with washing buffer. For development in ChemiDocTouch Imaging System, the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) kit was used.

Table 20. Composition of the buffers used in immunodection.

Buffer	Reagent
Blocking pH 7,2	100 mM Tris 200 mM NaCl 0,05% Tween
Washing pH 7,2	200 mM NaCl 50 mM Tris 0,05% Tween

2.4.5. *Co-immunoprecipitation*

To validate protein-protein interactions, *Nicotiana benthamiana* leaves were transformed according to the protocol in section 2.5.2.2. After 2 days, the tissue was collected, and shock frozen in liquid nitrogen. The samples were ground with metal beads for 1 min and 30 s at 25 Hz

using the Mixer Mill (Retsch MM 400). To 300 mg of tissue, 2 mL of cold extraction buffer (Table 21) was added, and the samples thawed on ice. Afterwards, the samples were sonicated for 5 min (15 s on/15 s off) (BioRuptur). To clean up the samples, they were centrifuged 3 times for 10 min at 20.000 *g* at 4 °C. After each the supernatant was transferred into a new tube. Of the extract, 100 µL were taken for the input which were mixed with 4xLDS Buffer+ DTT warmed at 95 °C. To the rest of the extract, 30 µL of anti-Myc magnetic beads (µMACS MicroBeads, Miltenyi Biotech) were added, the mixture was then incubated for 1 h at 4 °C.

The magnetic columns (µ Column, Miltenyi Biotech) were set on the magnetic stand and separator (µMACS Separator + MACS MultiStand Miltenyi Biotech). The columns were equilibrated with 2x 200 µL of IP buffer (Table 21). After equilibration, the samples were loaded on the respective column, which were then washed 4 times with 300 µL of IP buffer. The proteins were specifically eluted by incubating for 10 min with the myc-peptide solution (Table 21). To the elution, 4xLDS buffer + DTT, warmed to 95 °C, was added. Then, the samples were incubated at 75 °C for 10 min, centrifuged for 3 min at 13000 rpm and frozen with liquid nitrogen for long term storage.

Table 21. Composition of the solutions used in the co-immunoprecipitation assays.

Solution	Reagent
IP Buffer	50 mM HEPES-KOH pH 7,5 140 mM NaCl 1 mM EDTA 0,1% Triton X-100 10% Glycerol 1 mM PMSF (stock 100 mM) 1X (1 tablet per 50 mL) EDTA-Free Protease Inhibitor cocktail (Roche, Cat. No. 11873580001)
Extraction buffer	2% Polyvinylpyrrolidone (PVPP) IP buffer
Myc-peptide solution	500 µg of myc-peptide / mL of IP Buffer

2.5. *Plant methods and growth conditions*

During this study three different plant species were used: *Arabidopsis thaliana*, *Nicotiana benthamiana* and *Marchantia polymorpha*.

2.5.1. *A. thaliana*

2.5.1.1. *Growth conditions*

For seed production and to perform DR5 assays, the *A. thaliana* plants were grown in controlled conditions of 12 h light/ 12 h dark at 21 °C in soil. The soil was composed of 3-parts 'Einheitserde SP ED63 T' (Einheitserdewerke Werkverband e.V.) and 1-part perlite.

For protein production and to perform segregation tests, the *A. thaliana* plants were grown in half MS, 1% sucrose, MES and 1% plant agar medium, which was poured in square plates (13 cm (W) x 13 cm (H) x 3 cm (D)). The plants grew at 21 °C in 16 h light/8 h dark.

2.5.1.2. *Seed sterilization and vernalization*

The seeds were sterilized by Vapor-Phase Sterilization. Due to the toxic nature of the chlorine gas, the protocol was performed in the hood. The seeds were put in eppendorfs, which were put in a vessel along with a beaker containing 100 mL of bleach and 3 mL of HCL (37%). The vessel was then sealed. The seeds were incubated with the gas for 1 h. After opening the vessel, the tubes were quickly closed and taken to the laminar flow hood where water was added to them.

Seeds were vernalized for 2 days in the dark at 4 °C.

2.5.1.3. *Agrobacterium-mediated transformation using the floral dip method*

The *A. thaliana* plants previously transformed with the construct pGG-DR5::GFP - HYGR/SpecR were dipped with the constructs: pGG-35s:XVE-dummy-Mcherry-Myc-Ubq10-BastaR (XVE::mCherry) and pGG-35s:XVE-dummy-IAS8-Mcherry-Myc-Ubq10-BastaR (XVE::IAS8) by Martin Darino, PhD as described by Zhang and collaborators in 2006.

2.5.1.4. *Segregation tests*

The transformed seeds were sown in soil and sprayed with BASTA two times for two weeks. The surviving plants were grown for seed production. Once a high number of siliques appear, the plants were bagged for seed collection. After 8-10 weeks after sowing, once the siliques were brown

and the plants are dried, the seeds were harvested. The seeds were left to dry for a week at 37 ° C. Then the seeds were sterilized and stratified as described in section 2.5.1.2.

The T1 generation was screened for single insertions by plating 50 seeds of each line in half MS, 1% sucrose, MES, 1% plant agar medium and 30 mg/L of BASTA plates. The plates were left horizontal for 7 days at 21 °C in 16 h light/8 h dark. The line segregation was concluded from applying the chi-squared test. The lines segregating 3:1 were carried over to the next generation.

This process was repeated until homozygotes lines were found for XVE::IAS8 and for XVE::mCherry lines. For all further assays, only homozygotes lines were used.

2.5.1.5. *Estradiol-induced-protein production*

For protein production, the *A. thaliana* plants were grown in half MS, 1% sucrose, MES and 1% plant agar medium, which was poured in square plates (13 cm (W) x 13 cm (H) x 3 cm (D)). The plants grew vertically on a 100 µm nylon mesh (SEFAR) for 7 days, at 21 °C in 16 h light/8 h dark. After 7 days, tissue was collected, and the remaining plants were transferred to a plate with 5 µM of β-estradiol and kept in the same light and temperature conditions. After 24 h, the tissue was collected. The protein was extracted as described in section 2.4.1.

2.5.1.6. *DR5 induction assay*

The evaluation of the induction of auxin signalling by the IAS8 was achieved performing DR5 induction assay. The DR5 promoter is composed by seven to nine copies of a synthetic and highly active auxin response element (AuxRE) fused upstream of a minimal promoter derived from the cauliflower mosaic virus 35S promoter (Wells et al., 2013). The synthetic promoter was fused to coding sequences for YFP or GFP, so the fluorescence could be measured.

In this study, leaf discs of 4-week-old plants were incubated at RT on benchtop in a 10 µM of β-estradiol solution to induce protein production and in a mock solution. To evaluate the induction of the DR5 promoter, the $OD_{485\text{-}528}$ was measured after 24 h and 48 h, and the fold change was calculated according to the equation:

$$Fold\ change = \frac{\mathrm{A}_{485\ -\ 528}\ \mathrm{sample} - \mathrm{A}_{485\ -\ 528}\ \mathrm{blank}}{\mathrm{A}_{485\ -\ 528}\ \mathrm{negative\ control} - \mathrm{A}_{485\ -\ 528}\ \mathrm{blank}}$$

To both XVE::mCherry-myc and XVE::IAS8, the blank, *A. thaliana*, Col-0, was removed. Then the XVE::IAS8 was divided by the negative control, XVE::mCherry-myc.

2.5.2. *Nicotiana benthamiana*

2.5.2.1. *Growth conditions*

N. benthamiana was grown in controlled short-day conditions (8 h light/16 h dark) at 22 °C. The used soil was composed of 3-parts 'Einheitserde SP ED63 T' (Einheitserdewerke Werkverband e.V.) and 1-part perlite. The tobacco plants were watered by flooding for 15 min every two days.

2.5.2.2. *Transient expression in Tobacco leaves mediated by Agrobacteria*

The transient expression in *Nicotiana benthamiana* leaves was used for DR5 assays and for protein production for the co-immunoprecipitations. The *Agrobacterium tumefaciens* cultures were inoculated in LB (MES AS) (Table 22) with Rifampicin, Gentamycin and Spectinomycin, overnight, at 28 °C, while shaking at 180 rpm. The OD at 600 nm was measured, the cells were pelleted by centrifuging for 10 min at 3000 *g*. The supernatant was discarded, and the cells were resuspended in Agrobacterium resuspension medium (ARM) (Table 22) to an OD_{600} of 0,2. The culture was incubated for 5 h at room temperature. Since, co-infiltration, with either the DR5::YFP or the effector, was necessary the cultures were mixed to a final OD_{600} of 0,1. The mix was used to infiltrate 4-6 stage tobacco leaves of 4-week-old plants. The plants were kept in short-day conditions at 22 °C.

Table 22. Solutions used in *Agrobacterium tumefaciens* transformation process.

Medium	Reagents
LB (MES AS)	10 mM MES NaOH, pH 5,6 0,02 mM Acetosyringone
ARM	10 mM $MgCl_2$ 10 mM MES NaOH, pH 5,6 0,15 mM Acetosyringone

2.5.3. *Marchantia polymorpha*

2.5.3.1. *Growth conditions*

Marchantia polymorpha plants were grown in aseptic culture, in continuous light at 22 °C. The medium was composed by half strength Gamborg's B5 with vitamins, Sucrose (1% w/v) and (1% w/v) plant agar. Its pH was set to 5,2 with KOH and then it was autoclaved Autoclave

5 min 125 °C. It was poured about 1 cm thick medium is set in disposable round plastic dishes (9 cm in diameter, 2 cm in depth) taped with breathable tape 3M Micropore Surgical Tape 2 cm.

2.5.3.2. *Transformation mediated by Agrobacteria*

M. polymorpha, accession Takaragaike-1 (Tak-1), gemmae were spread on CM^M (Table 23) plates and grown as described in section 2.5.3.1. Under the laminar flow hood, 14 day-old-plants were cut into bits of approximately 0,5 cm^2 in size. 5 to 10 g of tissue was inoculated with 20 mL Inoculation Medium (Table 23), 1 mL *Agrobacterium*-suspension OD_{600} 2,0 for 30 min. The agrobacterium suspension was discarded, and the tissue was transferred onto a sterile filter paper placed to dry for 30 min. The plant material was plated on Co-Culture Medium (Table 23) and grown for 3 days at 21 °C in the dark. The tissue was then moved to CM^M incl. 300 mg/L Timentin for 3 days at 22 °C under in continuous light. After 3 days, the tissue was transferred onto the Selection Medium (Table 23) and re-plated every 10-14 days, being always kept under in continuous light at 22 °C.

Table 23. Mediums used to grow *Marchantia polymorpha* in aseptic culture.

Medium	Components
Inoculation Medium pH 5,2	1,5 g/L ½ Gamborg B5 basal salt mixture 0,5 g/L MES 10 g/L Sucrose 100 µM Acetosyringone
Co-Cultur Medium	1,5 g/L ½ Gamborg B5 basal salt mixture 0,5 g/L MES 10 g/L Sucrose 100 µM Acetosyringone 10 g/L Plant Agar
Selection Medium	1,5 g/L ½ Gamborg B5 basal salt mixture 0,5 g/L MES 10 g/L Sucrose 100 µM Acetosyringone 10 g/L Plant Agar

To assess if IAS8 induced any auxin related phenotype in *M. polymorpha*, Tak-1 was transformed, as previously described with the following constructs: pGG103-EF2-Omega-mCherry-3myc-UbqTer-2x35S-HygR-35ST (EF2::mCherry-3myc) and pGG103-EF2-Omega-IAS8-3Myc-UbqTer-2x35S-HygR-35ST (EF2::IAS8-3myc).

3. Results

3.1. *UBPs are present in several plant species*

Several of the hits in the mass spec results were UBP proteins. Among them, UBP6 and its homologs in *N. benthamiana* UBP 1, 2, 3, 4 and 5 were found. A protein blast, using the NCBI platform, showed that the NbUBP6 has several orthologs. Through the distance-based method in CLC workbench, a phylogenetic tree of the orthologs was obtained (Figure 10). This predicts the evolutionary relationship between proteins. The most distant relative of NbUBP6 is *M. polymorpha's* ubiquitin carboxyl-terminal hydrolase 7 (MpUBP7). In the host of *U. maydis*, *Z. mays* ubiquitin carboxyl-terminal hydrolase (ZmUBP) was found. In *A. thaliana*, the partially redundant proteins AtUBP12 and AtUBP13 were found.

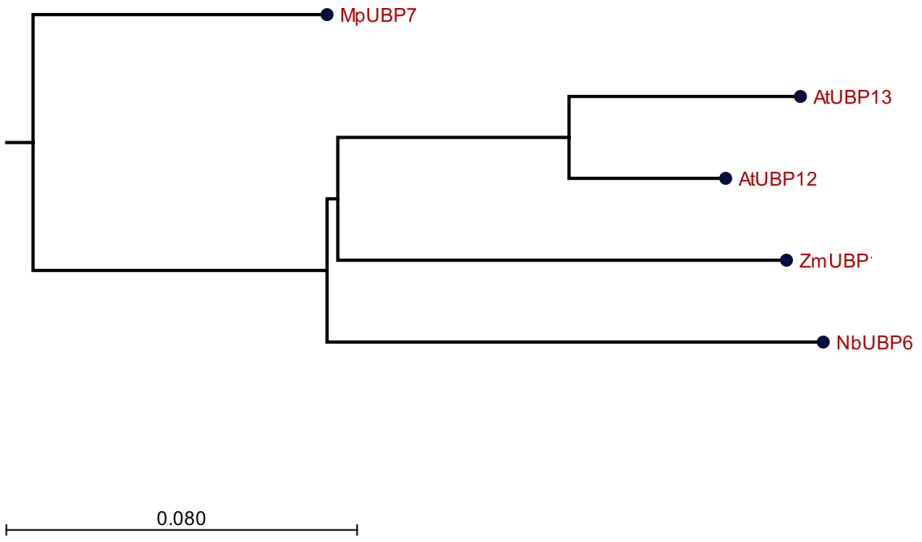

Figure 10. Phylogenetic tree of NbUBP6. To uncover possible orthologs, a protein blast in the NCBI platform was performed. A phylogenetic evaluation the highest hits was performed by the distance-based method, in the CLC workbench.

The analysis of these proteins with the Pfam software revealed that they all have three domains: MATH, which has no known function in plants, UCTH, which has the catalytic domain, and the USP7 domain, ubiquitin specific protease 7 (Figure 11**Erro! A**

Figure 11. Identified domains in NbUBP6 and its orthologs. NbUBP6 were identified by a protein blast ran in the NCBI. The domains of each protein were identified using the sequence search function in the Pfam software. All proteins have in common three domains: MATH, which has no known function in plants, UCTH, which has the catalytic domain, and the USP7 domain, ubiquitin specific protease 7.

origem da referência não foi encontrada.).

To identify which domains of the NbUBP6 are required for the interaction with IAS8, each domain and the combination of them were cloned and C-terminally fused to 3HA tag to perform a Co-IP together with IAS8 in *N. benthamiana* leaves. The same was performed with ZmUBP.

3.2. *IAS8 interacts with the orthologs of NbUBP6 in* A. thaliana*, At UBP12 and 13, in a co-immunoprecipitation assay*

It was previously shown that IAS8 induces auxin signalling in *N. benthamiana* in a DR5 induction assay. It was also shown that *A. thaliana* closest orthologs of NbUBP6, AtUBP12 and 13, interact with IAS8 (Figure 12). To verify the interaction between IAS8 and the closest orthologs of NbUBP6, AtUBP12 and 13, each was transiently expressed in *N. benthamiana* with the IAS8 and a Co-IP was performed. All proteins used in this assay were tagged in the C-terminal, the IAS8 was cloned with a MYC tag and used as bait. While the AtUBP12, AtUBP13 and mCherry were tagged with a HA tag. The Co-IP was followed by the western blot shown in Figure 12.

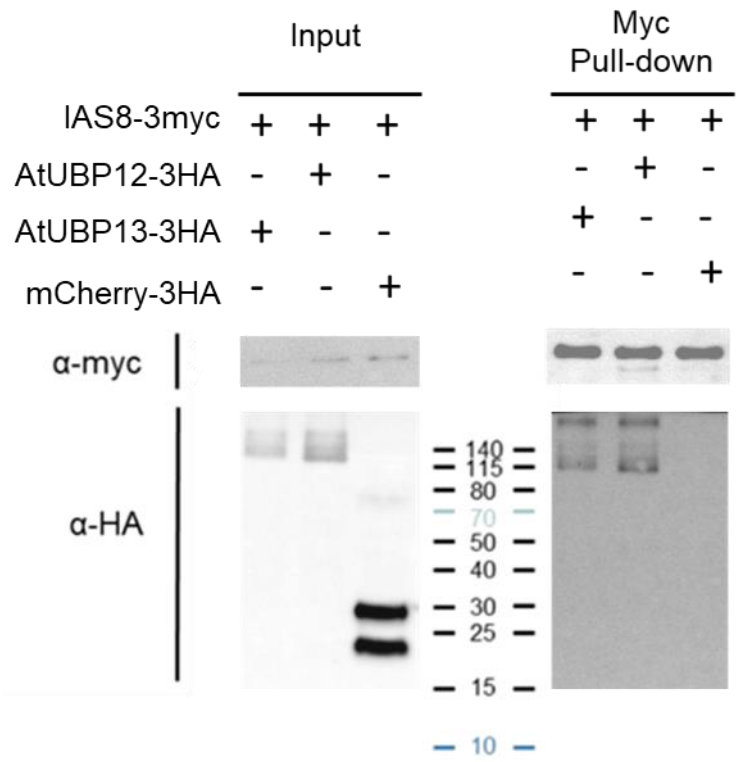

Figure 12. AtUBP12 and AtUBP13 interact with IAS8 in Co-IP assay. AtUBP12-3HA and AtUBP13-3HA were each co-expressed with IAS8-3Myc in *N. benthamiana*. mCherry-3HA was co-infiltrated with IAS8-3myc and used as a negative control. Three days after agroinoculation, the tissue was collected. The proteins were pulldown by an anti-myc antibody and immunodetected by both anti-myc and anti-HA antibodies.

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As observed in the anti-myc and anti-HA input all the proteins are present. However, only AtUBP12 and AtUBP13 were pulldown with the effector. Thus, verifying the interaction between

IAS8 and both UBPs of *A. thaliana* (IP α-HA). No interaction was observed between IAS8 and the negative control, mCherry. These results were consistent over the 3 replicates.

3.3. *IAS8 induces auxin signalling in* A. thaliana *in a DR5 assay*

To evaluate if IAS8 is also able to induce auxin signalling in *A. thaliana*, we decided to perform a DR5 assay (see section 2.5.1.6). The double transgenic lines of DR5::GFP with an estrogen receptor-based trans-activator, XVE, associated to the 35S promoter driving the expression of IAS8-mCherry-3myc and, as a negative control, mCherry-3myc were produced, selected and tested for protein expression (see sections 2.5.1.1, 2.5.1.2 and 2.5.1.4) To analyse protein expression of the transgenic lines, tissue was collected before and after transferring the 7-day-old *A. thaliana* seedlings to plates with 5 µM of β-estradiol for 24 h (see section 2.5.1.5). An Amido Black assay was performed to evaluate the protein concentration of the samples. 15 µg of protein were loaded onto an SDS PAGE and the immunodetection was made with the anti-mCherry antibody (Figure 13).

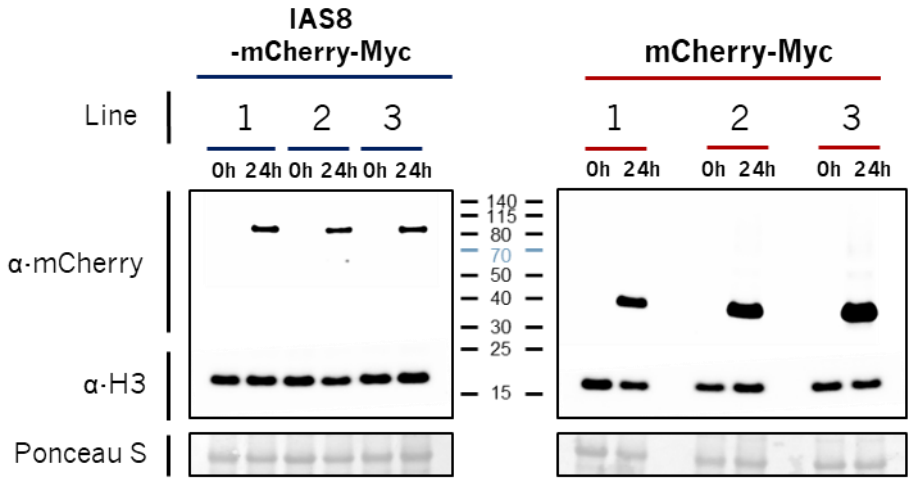

Figure 13. Protein expression in β-estradiol-inducible-*A. thaliana*-lines of IAS8 and mCherry verified by western blot after induction with 5 µM of β-estradiol. *A. thaliana* β-estradiol inducible lines of IAS8 and mCherry were grown for 7 days in half MS, 1% sucrose, MES and 1% plant agar medium 21 °C in 16 h light/8 h dark. Tissue was collected before and 24 h after induction by transferring to 5 µM of β-estradiol, half MS, 1% sucrose, MES and 1% plant agar medium. Before induction, there is no ectopic protein expression in any of the lines. All lines show protein after induction with 5 µM of β-estradiol Ponceau S and anti-H3 antibody show equal protein loading.

None of the lines showed ectopic protein expression before being induced. The three chosen effector lines seem to produce equal amounts of protein. Since all the mCherry lines seem to produce protein, line one was chosen as a negative control for the DR5-induction assay.

Three-independent-homozygote-lines of XVE::IAS8 were evaluated through a DR5 assay. The *A. thaliana* plants were grown in controlled conditions of 12 h light/ 12 h dark at 21 °C in soil. Four Leaf discs were cut from 4-week-old XVE::IAS8, XVE::mCherry and Col-0 plants. Ten plants per construct were used. The leaf discs were incubated either on a 10 µM of β-estradiol solution or on DMSO solution (Mock) on the bench top. The $OD_{485-528}$ was measured after 48 h. The fold change in DR5 activity was calculated as mentioned in section 2.5.1.6. The results of the three replicates are shown in Figure 14.

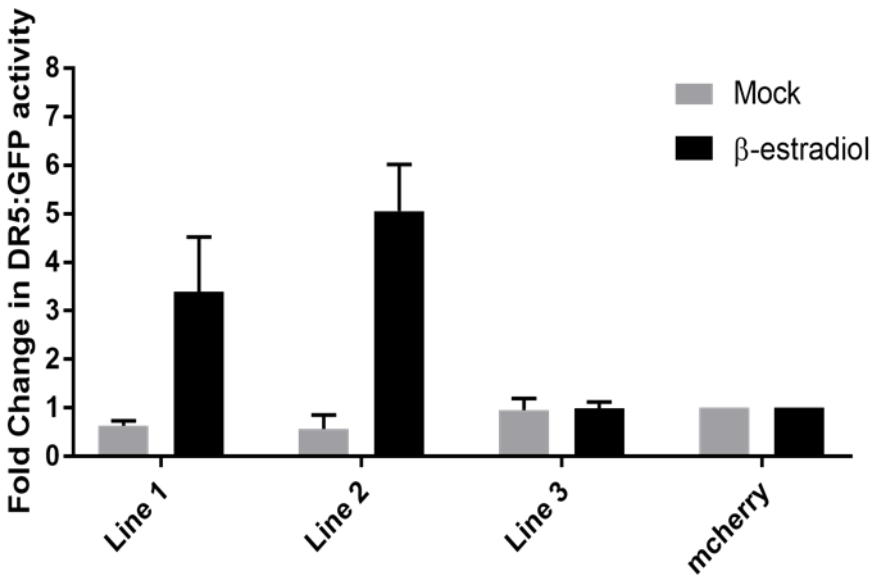

Figure 14. IAS8 induces auxin signalling in *A. thaliana*. The plants were grown under 12 h light/ 12 h dark in 21°C for 4 weeks. Leaf discs were cut and incubated in mock (DMSO) and 10 µM of β-estradiol solution on the benchtop for 48 h. The $OD_{485-528}$ was measured. The Col-0 values were removed. The fold change was then calculated by dividing the samples by the negative control, mCherry. IAS8 induces DR5::GFP in lines 1 and 2. The values are mean ± SD, n=3.

After 48 h of incubation, the fold change in mock-treated-samples was line 1, 0,639 ± 0,0991 SD, Line 2, 0,573 ± 0,283 SD, Line 3, 0,958 ± 0,240 SD (n=3). In contrast, the fold change in β-estradiol-treated-samples line 3,394 ± 1,128 SD, Line 5,054 ± 0,959 SD, Line 3, 0,996 ± 0,125 SD (n=3). In summary, in lines 1 and 2 IAS8 highly induced auxin signalling. However, in line 3 we see no DR5 induction.

The fluorescence data for the DR5 induction assay was analysed by a two-way ANOVA and by the Sidak's multiple comparisons test (Figure 15).The differences between lines (F=25,52,

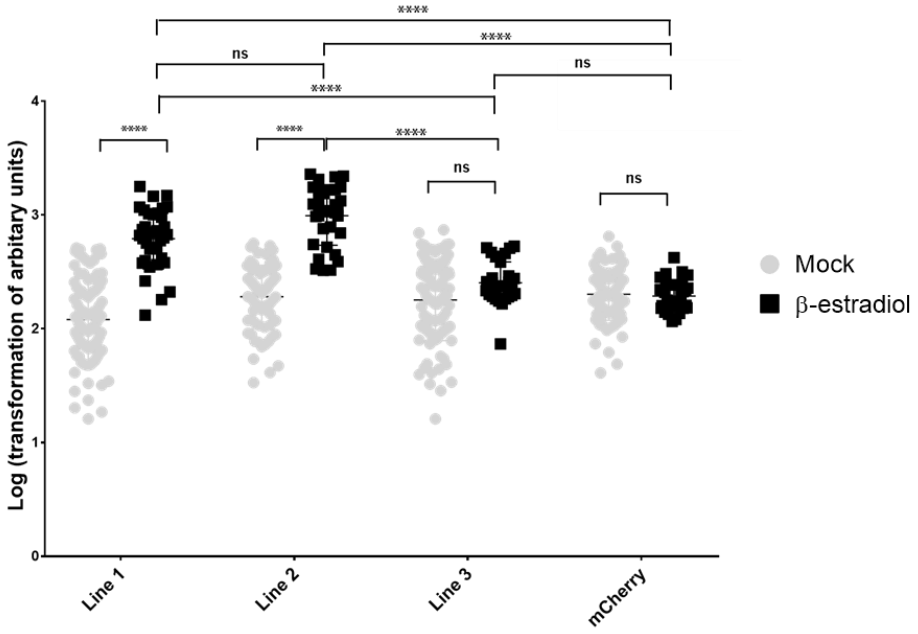

Figure 15. Lines 1 and 2 are significantly different from the mCherry control. The plants were grown under 12 h light/ 12 h dark in 21°C for 4 weeks. Leaf discs were cut and incubated in mock (DMSO) and 10 µM of β-estradiol solution on the benchtop for 48 h. The $OD_{485-528}$ was measured. The Col-0 values were removed. A two-way ANOVA was performed. The differences between lines (F=25,52, P<0,0001), treatment (F=167,5, P<0,0001) and influence of treatment on the lines (F=39,77, P<0,0001) were all significant. Significance difference between mock and treatment and between effector lines and control was calculated by Sidak's. ns, p>0.05; *, p<0.05; **, p<0.01; ***, p<0.001.

P<0,0001), treatment (F=167,5, P<0,0001) and influence of treatment on the lines (F=39,77, P<0,0001) were all significant. Sidak's test showed that there is a high significance between lines 1 and 2 mock and respective treated samples. Additionally, there is also a high significance between treated lines 1 and 2 and the negative control, mCherry. However, line 3 seems to not have responded to the treatment in the same way. In line 3, no difference was found between mock and β-estradiol treatment as well as in between its β-estradiol treatment and the negative control, mCherry. In conclusion, the treatment had a strong effect on line 1 and 2. However, it did not affect line 3 or the negative control, mCherry.

3.4. *IAS8 has an effect in Marchantia polymorpha*

To assess how the IAS8 affects *M. polymorpha,* the liverwort was transformed with EF2::IAS8 and EF2::mCherry, which allow for the constitutive expression of both genes. Two months after agrobacteria-mediated transformation, the transformants were photographed (Figure 16).

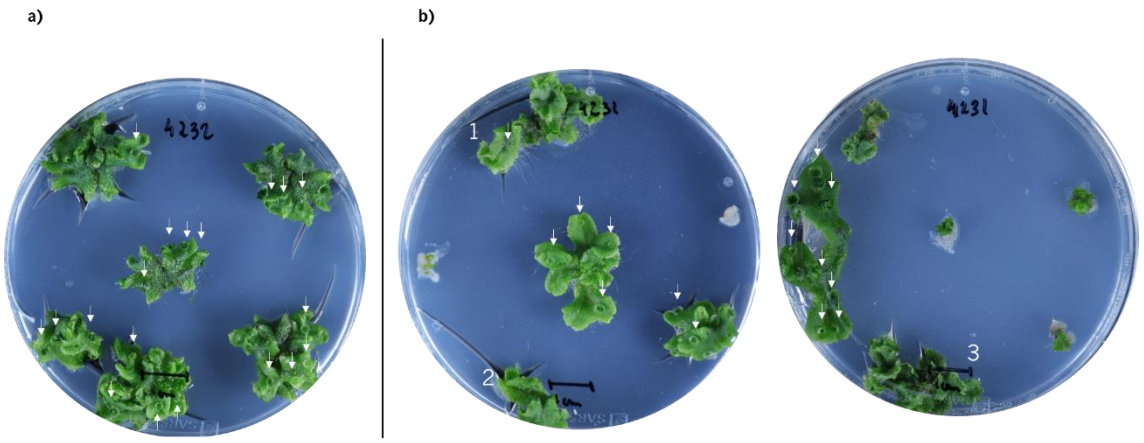

Figure 16. IAS8 has an effect on *M. polymorpha*. After transformation, the plants were grown at 22°C under continuous light conditions half strength Gamborg's B5 with vitamins, Sucrose (1% w/v) and (1% w/v) plant agar. Two months after transformation, the plants were photographed. a) EF2::mCherry-3myc. b) EF2::IAS8-3myc. Arrow is marking the presence of gemma cups.

The mutants in Figure 16 resulted from the transformation procedure, however, they are not technically the same age since agrobacterium transformation could have occurred at any time during a three-day-period. This should be kept in mind when comparing the plants. Even so, comparing to the mCherry transformants, it appears that most effector transformants have a growth defect. The small transformants grew into undifferentiated small balls uncapable of forming rhizoids (data not shown). However, the most noteworthy difference found between the overexpression lines of the IAS8 and mCherry lines, is the absence of gemma cups.

The transformants with the most severe phenotype could not be analysed due to the lack of tissue. The 3 transformants marked in Figure 16b were analysed through an immunoblot and a RT-PCR (Figure 17) to see if they were expressing the construct.

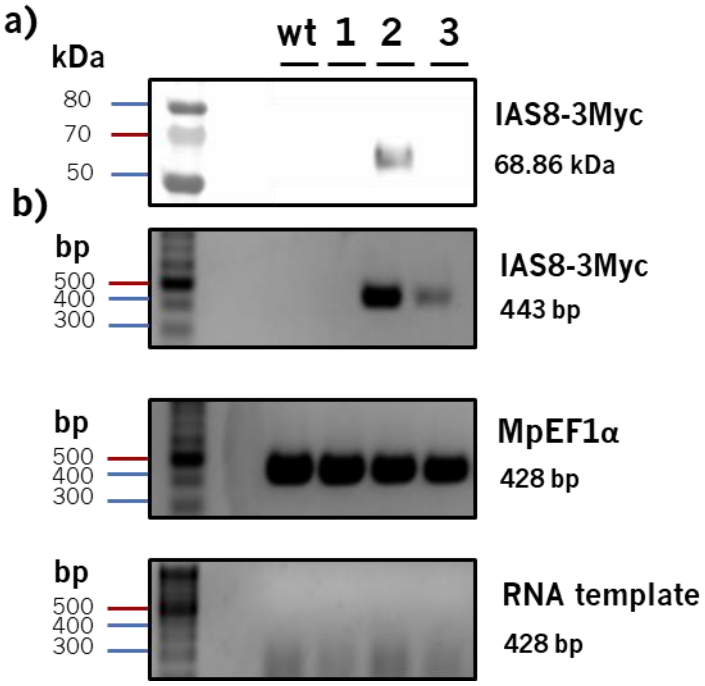

Figure 17. IAS8-3myc is present in *M. polymorpha* transformants. The plants, EF2::IAS8-3myc and wild type, were grown in half strength Gamborg's B5 with vitamins, Sucrose (1% w/v) and (1% w/v) plant agar, under continuous light at 22 °C. Protein and RNA were isolated from the thallus of the plants. a) Immunodetection with an α-myc antibody of IAS8-3myc in *M. polymorpha* mutants. IAS8 was only found in mutant 2. b) Reverse transcription polymerase chain reaction (RT-PCR) of *M. polymorpha* mutants and wt. Mutant 2 and 3 were found to be expressing IAS8. The *M. polymorpha* ELONGATION FACTOR1-α (MpEF1α) was used as loading control.

The immunodection only revealed protein in one mutant (Figure 17a). In RT-PCR analysis we observed that the IAS8 was successfully amplified in two of the transformants (Figure 17b). The amount of protein obviously differs between mutant 2 and 3, which might account for the difference in the severity of their phenotype. The *M. polymorpha* ELONGATION FACTOR1-α gene (MpEF1a) was amplified at similar levels in all samples, thus demonstrating the integrity of the RNA preparation (Figure 17b). The RT-PCR made with the RNA templates shows no bands, indicating that there is no genomic DNA contamination (Figure 17b).

3.5. *IAS8 interacts with NbTPR3 and NbUBP6.1 in a co-immunoprecipitation assay*

To verify the interaction between IAS8 and NbUBP6 and between IAS8 and NbTPR3, each was transiently expressed in *N. benthamiana* with the IAS8 and a Co-IP was performed. All proteins used in this assay were tagged in the C-terminal, the IAS8 was cloned with a MYC tag and used as bait. While the NbTPR3, NbUBP6 and mCherry were tagged with a HA tag. The Co-IP was followed by the western blot shown in Figure 18.

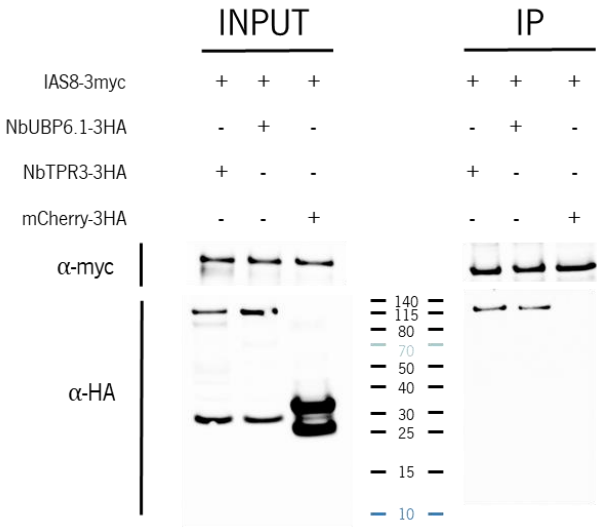

Figure 18. NbTPR3 and NbUBP6 interact with IAS8 in Co-IP assay. NbUBP6-3HA (120 kDa) and NbTPR3-3HA (114 kDa) were each co-expressed with IAS8-3Myc in *N. benthamiana*. mCherry-3HA was co-infiltrated with IAS8-3myc and used as a negative control. Three days after agroinfiltration, the tissue was collected. The proteins were pulldown by an anti-myc antibody and immunodetected by both anti-myc and anti-HA antibodies. In contrast with mCherry, both NbTPR3 and NbUBP6 are present in the pulldown fraction. Immunoprecipitation (IP) buffer with 10 mM DTT.

As observed in the anti-myc and anti-HA input all the proteins are present. However, only NbTPR3 and NbUBP6 were pulldown with the effector. Thus, verifying the interaction between IAS8 with NbTPR3 and IAS8 and NbUBP6 (IP α-HA). No interaction was observed between IAS8 and the negative control, mCherry. These results were consistent over the 3 replicates.

3.6. *IAS8 interacts with NbMATHUCTH domain of NbUBP6 in a co-immunoprecipitation assay*

The NbUBP6 has three identified domains: MATH, UCTH AND USP7. To understand which of these domains is targeted by the IAS8, each of the domains and their combinations were cloned

into vectors (2.3.3) that allowed for their transient expression in *N. benthamiana*. After two days the tissue was collected. The effector was tagged with a myc tag and used as bait. All prey proteins were cloned with an HA tag. All the proteins used in this assay were tagged in C-terminal end. The NbUBP6 was used as a positive control. A close homologue of NbUBP6, the NbUBP26, which has only one known domain, the UCTH domain, was also included in the experiment. The pulldown was followed by the western blot shown in Figure 19.

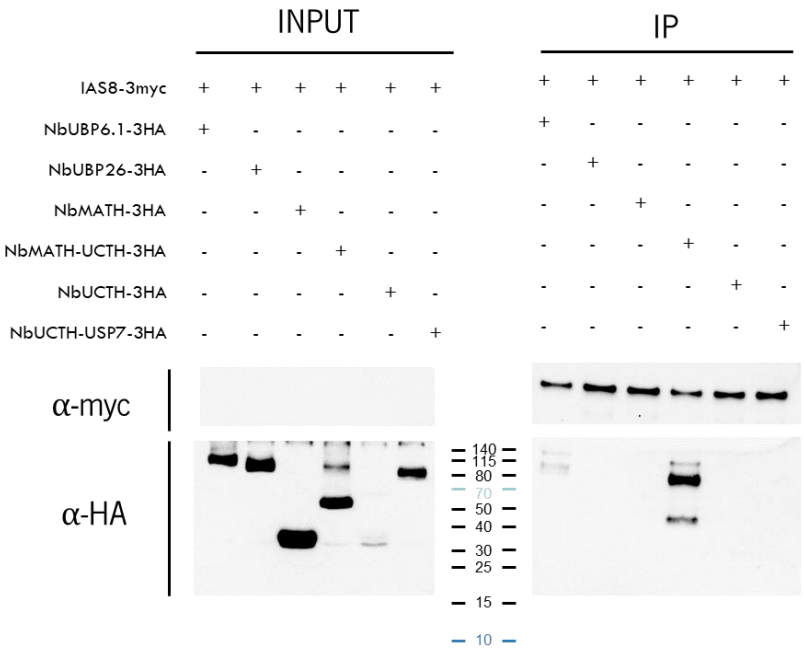

Figure 19. IAS8 interacts with NbMATH-UCTH of NbUBP6.1 in a Co-IP assay. NbUBP6-3HA (120 kDa), NbUBP26-3HA (114 kDa), NbMATH (28,48 kDa), NbMATHUCTH (58,96 kDa), NbUCTH (38,61 kDa) and NbUCTHUSP7 (103,4 kDa) were each co-expressed with IAS8-3Myc in *N. benthamiana*. mCherry-3HA was co-infiltrated with IAS8-3myc and used as a negative control. 3 days after agroinoculation, the tissue was collected. The proteins were pulldown by an anti-myc antibody and immunodetected by both anti-myc and anti-HA antibodies. Unlike the NbUBP26 and the NbUCTHUSP7, the NbUBP6 and the NbMATHUCTH were pulled down.

As observed in the anti-myc and anti-HA input all the proteins are present. However, only NbUBP6 and NbMATHUCTH were pulldown with the effector. Since none of the other domains is pulled down, it seems that only the NbMATHUCTH is required for interaction with the IAS8 (IP α-HA). No interaction was observed between IAS8 and the negative control, NbUBP26. These results were consistent over the 3 replicates.

3.7. *IAS8 interacts with ZmUBP through its ZmMATH domain in a Co-IP assay in* N. benthamiana

The ZmUBP also shares three identified domains: MATH, UCTH AND USP7. It's also the closest ortholog in *Z. mays* of NbUBP6. To understand which of these domains is targeted by the IAS8, each of the domains and their combinations were cloned into vectors (2.3.3) that allowed for their transient expression in *N. benthamiana*. The effector was used tagged with a myc tag and used as bait. All prey proteins were cloned with an HA tag. All the proteins used in this assay were tagged in C-terminal end. The mCherry protein was used as negative control. The Co-IP was followed by the western blot shown in Figure 20.

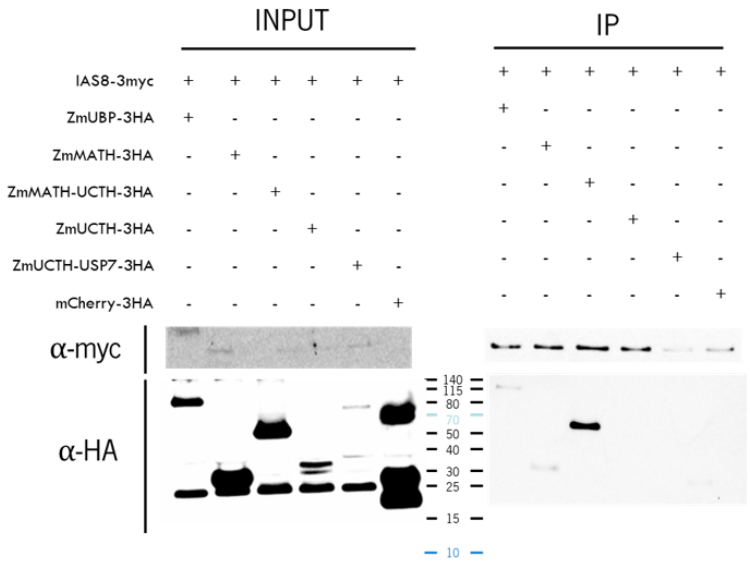

Figure 20. IAS8 interacts with and ZmMATH and ZmMATHUCTH of ZmUBP in a Co-IP assay. *ZmUBP*-3HA (127,82 kDa), ZmMATH (31,02 kDa), ZmMATHUCTH (67,65 kDa), ZmUCTH (46,64 kDa) and ZmUCTHUSP7 (110,99 kDa) were each co-expressed with IAS8-3Myc in *N. benthamiana*. mCherry-3HA was co-infiltrated with IAS83myc and used as a negative control. 3 days after agroinoculation, the tissue was collected. The proteins were pulldown by an anti-myc antibody and immunodetected by both anti-myc and anti-HA antibodies. Contrasting with mCherry protein, ZmUBP and ZmMATH and ZmMATHUCTH were pulled down.

As observed in the anti-myc and anti-HA input all the proteins are present. However, only ZmUBP, ZmMATH and ZmMATHUCTH were pulldown with the effector. Since none of the other domains is pulled down, it seems only the ZmMATH is required for interaction with the IAS8 (IP α-HA). However, the band MATHUCTH is much stronger, hinting that the combination might conformationally stabilize the interaction. No interaction was observed between IAS8 and the negative control, mCherry. These results were consistent over the 3 replicates.

4. Discussion

4.1. *IAS8 induces auxin signalling in* A. thaliana *in a DR5 assay*

The induction of DR5::GFP was three-folds higher in 2 out of 3 XVE::IAS8 lines than in the control after treatment with β-estradiol. The two-way ANOVA showed that the two inducing lines responded to the treatment in the same way. Furthermore, it showed that line 3 and the mCherry control reacted to the treatment in the same way. However, these results do not negate the hypothesis that IAS8 induces DR5 and thus auxin signalling. Our results clearly prove that IAS8 induces auxin signalling in *A. thaliana*.

Auxin is often a target of plant-pathogens and it's easy to see why. Specially for biotrophic fungi, such as *U. maydis*, because auxin has been described as a major antagonist of SA signalling, the hormone that regulates the defence response against biotrophic fungi (Yan & Dong, 2014). Furthermore, IAA was shown to induce cell wall loosening in rice (Fu et al., 2011), which would also be advantageous in the infection process.

The tumour-inducing-ability of a quadruple-mutant *U. maydis* for the four enzymes involved in the fungus' IAA biosynthesis, was unaltered even though the IAA concentration in the tumour was much lower (Reineke et al., 2008). This is strong evidence that, somehow, *U. maydis* induces auxin signalling without inducing auxin production. Our results validate this hypothesis, by showing that at least one effector, IAS8, induces auxin signalling in 3-fold in *A. thaliana* in a DR5 assay. Perhaps by interacting with the NbTPR3 ortholog in *A. thaliana* and preventing from repressing auxin signalling. The fact that this effector induces auxin signalling in both angiosperms, *N. benthamiana* and *A. thaliana*, is a strong indication that it might do so in *U. maydis*' host, *Z. mays*.

4.2. *IAS8 has an effect in* M. polymorpha

In *M. polymorpha*, growth defects have long been associated with auxin. For instance, it was shown that auxins severely inhibited growth of gemmalings by growing them on medium with 1 and 10 μM of NAA and. Less severe results were obtained with IAA (Ishizaki et al., 2012). Moreover, through the generation of different mutants in the auxin biosynthetic pathways and it was observed that, similarly to the phenotype caused by the constitutive expression of IAS8, the

mutants: $_{pro}$EF1::iaaL, $_{pro}$SHI::iaaL, $_{pro}$SHI::amiR-YUC2^{miR160}, and all TAA, $_{pro}$EF1::amiR-TAA^{miR160}, and $_{pro}$EF1::amiR-YUC2^{miR160} were impaired in growth and did not produce any, or only rarely produced, gemmae or gemma cups (Eklund et al., 2015). Auxin signalling has been shown to be involved in gemma cups development. At the bottom of developing and mature gemma cups, it was observed high GH3::GUS activity, indicating that auxin signalling might play a critical role in developmental regulation of vegetative-propagating-tissue in *M. polymorpha* (Ishizaki et al., 2012).

As shown previously, IAS8 interacts with *N. benthamiana's* Topless related protein 3 (TPR3) (see Figure 21). *M. polymorpha* has a single Topless (Flores-Sandoval et al., 2015). A protein blast of NbTPR3 in the *M. polymorpha*'s genome data based showed it aligned with MpTPL (E=0,00). This opens the possibility that IAS8 is interacting with MpTPL thus causing the lack or a reduce number of gemma cups in the mutants. Furthermore, the link between Topless and gemma cup development has brought forward before. In one study, the authors expressed VENUS with MpTPL's native promoter and documented its activity in gemma and gemma cups during their development. In contrast, in the EF1::MpTPL lines they observed a disruption in gemma cup formation in 80% of the cases, suggesting that the TPL overexpression results in an inhibition of auxin transcriptional response (Flores-Sandoval et al., 2015). However, in this study the MpTPL protein or transcript levels of this overexpressing mutant were never verified. So, we can postulate that there might have been a gene silencing or a negative feedback event that caused this phenotype. On the other hand, the effector might be causing TPL to be degraded or preventing it from acting, and this provokes the plant to over produce TPL thus silencing auxin signalling.

Since no gemma were available from IAS8 mutants, a proper assessment of a phenotype defect caused by the effector in *M. polymorpha*, should be done in the future using thalli tissue or by establishing a working inducible system in *M. polymorpha*. Furthermore, due to the similarity between our mutants and the ones altered in auxin biosynthesis, this phenotype might be caused by alterations in auxin levels in the plant as well. To determine if this is a direct result of lower or higher auxin biosynthesis, performing growth experiments after applying auxin exogenously and auxin inhibitors might be useful. The hypothesis was raised that lack of gemma cups is caused by the activation of auxin signalling due to the inactivation of TPL by IAS8. To examine the veracity of this hypothesis, first the interaction between IAS8 and MpTPL should be verified by either a Co-IP or a Y2H. Then, we propose to assess how IAS8 affects the auxin signalling in *M. polymorpha* by transforming with two constructs: XVE:EF2::IAS8-3myc and a DR5::GUS and evaluate the growth

and check for the presence/absence of gemma cups. Additionally, through microscopy and staining we can evaluate what is happening to auxin signalling upon induction of the effector.

4.3. *IAS8 targets TPR3 in* N. benthamiana

Topless has a pivotal role in auxin signalling. In low concentrations of auxin (Figure 21a), TPL forms a complex with Aux/IAA and ARFs, recruits the chromatin remodelers and repressing gene expression. In a three-times repeated experiment, TPR3 was shown to interact with IAS8 (Figure 18).

The interaction of the IAS8 and NbTPR3 might be the reason why the effector is able to induce auxin signalling. One of the mechanisms might be, preventing the formation of the repressive complex (Figure 21c) by outcompeting the binding to the Aux/IAA. It is also possible that IAS8 is forming a complex with NbTPR3 and the NbUBP6 (Figure 21d), and preventing topless from deubiquitinated, thus keeping it on track for degradation. Another possibility is that the effector is stabilizing the interaction between NbUBP6, which, like its orthologs in *A. thaliana*, is thought to be substrate specific, and NbTPR3 this way preventing NbUBP6 from deubiquitinating as much NbTPR3 as possible.

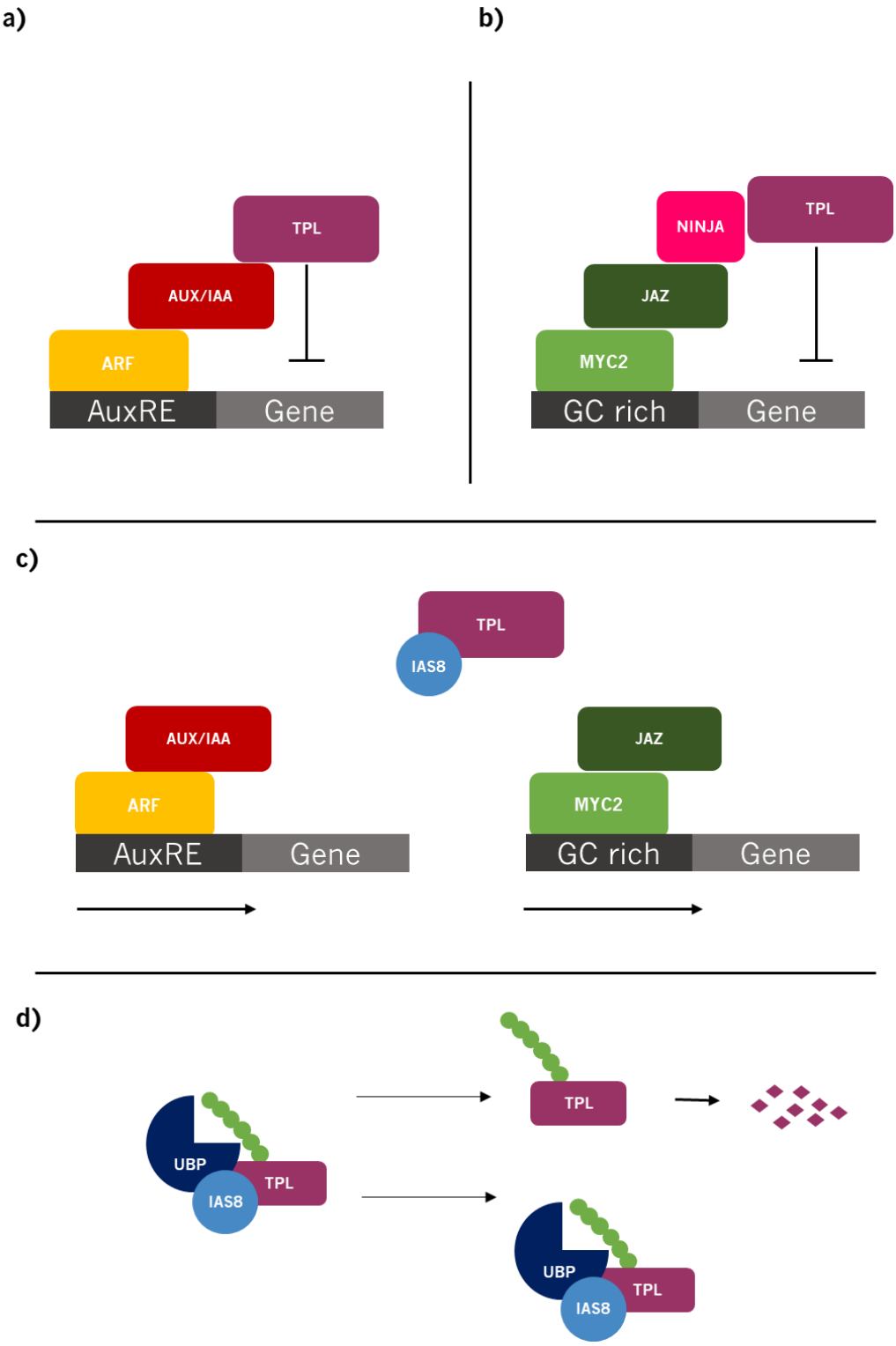

Figure 21. Models of interaction between IAS8 and TPR3. a) When the concentration of auxin, the auxin/IAA (Aux/IAA) proteins function as repressors by recruiting Topless (TPL), which in turn recruits cromatin remodelers, inhibiting gene expression. b) MYC2 recognizes elements in the DNA, recruits JAZ proteins. The JAZ proteins recruit the negative regulator, NINJA, that Topless interacts with. Then, topless recruits chromatin remodelers and inhibits JA signalling. c) IAS8 binds to TPL and prevents its binding to Aux/IAA, so there is no repression of

expression and auxin signalling is upregulated. Also, the binding of IAS8 to TPL prevents its binding to NINJA, thus upregulation of JA signalling occurs. d) IAS8 either prevents TPL from being deubiquitinated and thus it is degraded. Another possibility is that the effector is stabilizing the interaction between NbUBP6, which, like its orthologs in *A. thaliana*, is thought to be substrate specific, and NbTPR3 this way preventing NbUBP6 from deubiquitinating as much NbTPR3 as possible.

The question of which of these is the correct model wasn't addressed by the experiments performed in this study. However, we propose how to answer it in the future. One of the problems with a Co-IP *in planta* is that the interaction observed might not be direct. So, complementing this approach with a Yeast-2-Hybrid (Y2H) would tell us if the interaction is direct or not. And to test if the effector is interacting with the two proteins at the same time, a Yeast-3-Hydrid (Y3H). If through a complex, is how these proteins are interacting, expressing them together in *N. benthamiana*, and immunodetect as well with anti-ubiquitin antibodies. Furthermore, we propose to inhibit the proteasome by applying MG132 and check if topless is more degraded in the presence of the effector or in its absence.

Auxin isn't the only hormone that Topless regulates. TPL is also a key regulator of JA signalling (Figure 21b). Like in auxin signalling, a transcription factor, MYC2 in this case, recognizes elements in the DNA, recruits JAZ proteins. JAZ proteins recruit the negative regulator, NINJA, that Topless interacts with. Then, topless recruits chromatin remodelers and inhibits JA signalling. The similarities between these repression systems might mean that IAS8 also interferes with JA signalling in the same way. An upregulation of auxin and JA signalling would be a great advantage for the fungus, since the plant's resources would be rewired from defence against biotrophic invaders to necrotrophic and plant growth, due to the antagonistic relationship between JA/Auxin and SA signalling. Thus, making the host a more auspicious environment for *U. maydis* to grow in.

4.4. *IAS8 targets NbUBP6.1 and its* Z. mays *ortholog, ZmUBP, through their MATHUCTH domain*

NbUBP6 has orthologs in *A. thaliana*, *M. polymorpha* and *Z. mays.* Through Co-IP assays, IAS8 was shown to interact with NbUBP6 (Figure 18 and Figure 19), AtUBP12 and 13 (Figure 12)and ZmUBP (Figure 20). The effector was unable to interact with the paralog of NbUBP6, the NbUBP26. This UBP has no MATH domain. This, along with the Co-IP where we were able to pull

down the ZmMATH, hints that only the MATH domain is required for the interaction with the effector.

The MpUBP7 is the only UBP in *M. polymorpha* with a MATH domain, however it's not the only protein with a MATH domain. The fact that the MATH domain has been present in land plants even before liverworts emerged and remains present with such high conservation in all these species hints that there is a biological purpose for it yet to be uncovered.

The best characterized of the UBP6 orthologs are AtUBP12 and 13. These to enzymes share 91% similarity and are partially functionally redundant (Ewan et al., 2011), besides that they are unable to self or heterodimerize (Jeong et al., 2017). Similarly to the binding between IAS8 and NbUBP6/ZmUBP (Figure 19 and Figure 20), AtUBP12 and 13 were shown to interact *in vitro* through the MATH domain with MYC2, a known transcription factor of JA signalling (Jeong et al., 2017). The same study proved that polyubiquitinated MYC2 is specifically deubiquitinated by AtUBP12 and 13 and hinted at the specificity of MYC2 as a substrate for AtUBP12 and 13. These results strongly indicate that AtUBP12 and 13 are positive regulators of JA signalling in *A. thaliana*. This hints also to the possibility that the effector, even though it doesn't interact with the catalytic domains of UBP, is deubiquitinated by NbUBP6 and its orthologs. This hypothesis could also be tested *in vitro* by performing a Ubiquitination/deubiquitylation assay. This assay would also answer a more prominent question, is IAS8 a substrate of these UBPs? If not, is it mimicking a co-factor of this protein or perhaps outcompeting its natural substrate (Figure 22)?

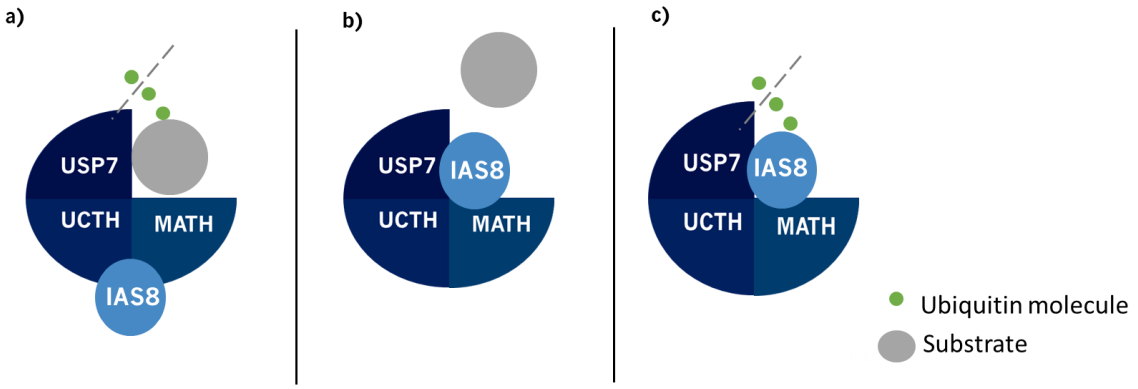

Figure 22. Possible models of interaction between IAS8 and NbUBP6/ZmUBP13. a) IAS8 micks a co-factor of the UBPs, aiding in target recognition or b) it binds to the MATH Domain and prevents interaction with target proteins or c) it competes with other proteins to be deubiquitinated by the UBPs.

In light of these results, a number of hypothesis emerge. Considering that JA signalling has long been correlated with resistance to necrotrophic fungi and to antagonize SA signalling, which is responsible for resistance against biotrophic fungi. Could IAS8 be to manipulate this crosstalk? Either, as previously mentioned by interacting with topless, or interfering with MYC2 deubiquitylation. Could the effector be competing with MYC2 for the MATH domain of these UBPs, thus, by preventing it from being deubiquitinated, MYC2 ends up on being degraded by the 26S proteasome? In the future, we hope to test if there is competition between MYC2 and IAS8 through a Y3H approach.

5. Concluding remarks

In this study, we confirmed the interaction between the effector and NbUBP6, and its orthologs, AtUBP12 and 13, and ZmUBP. Our results indicate that IAS8 targets the MATH domain, which is highly conserved among all these orthologs. In the future, it would be interesting to test if the effector is able to interact with other plant proteins that contained this domain. Further assays to uncover the mechanism of this interaction are needed.

Our results also show that the effector IAS8 alone induces auxin signalling in *A. thaliana* by three-fold and drastically impairs the morphology of *M. polymorpha*. Its interaction with NbTPR3 and NbUBP6 hints at that it might be able to manipulate other hormones and signalling. Moreover, our results allude that the pathway through which IAS8 induces auxin signalling is highly conserved. We hope to elucidate the extension of the IAS8 manipulation through an RNA-seq using the lines examine test in this study.

The effector IAS8, here partially characterized, seems to be one of the reasons why *U. maydis* is a master manipulator of hormones and their signalling.

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