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Studying Effectors from *Ustilago maydis* - Interaction Networks and Screening for UPR Interference

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You can't play God without being acquainted with the Devil.

Dr. Robert Ford

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Contents

Abstract

Pathogens secrete small molecules, termed effectors, to manipulate their hosts. There is enormous co-evolutionary pressure for rapid adaptation of pathogenic effectomes, which results in low homology of effector proteins, even among closely related species. Therefore, tools generally used in biology to infer protein function, such as functional domain prediction, have limited use in effector studies. In this thesis, some overarching features of effector biology were investigated. These are: the ability of effectors to interact with each other to increase the versatility of the effectome, and the development of a method to identify molecules that interfere with plant Unfolded Protein Response (UPR).

Plants rely on intricate interaction networks that perceive invading pathogens and activate signaling cascades to coordinate immune responses. Effectors have evolved alongside plant immune systems, yet most effector research focuses on the characterization of these molecules in isolation. In this work, the possibility of effectors to interact with other effectors was tested. A systematic yeast-two-hybrid (Y2H) screen showed an unexpected abundance in effector-effector interactions within the effectome of *Ustilago maydis*, a model biotrophic pathogen. After analyzing how the interaction network can change through the infection process, a few evolutionary drivers that may stabilize effector-effector interactions were discussed.

Another aspect of effector biology studied here involved the central role of protein secretion in disease. During a pathogenic invasion, plants rapidly synthesize defense-related proteins that can overload the folding machinery from the secretory pathway. This activates UPR, which in turn leads to the upregulation of host defense-related genes, making this mechanism a prime target for pathogenic effectors. To investigate that, a high-throughput method to identify proteins with a role in plant UPR was developed. A pilot screen of 35 putative effectors from *U. maydis* led to the identification and validation of one protein, UMAG_05927, which reduces UPR signaling *in planta*.

ZUSAMMENFASSUNG

Krankheitserreger sezernieren kleine Moleküle, sogenannte Effektoren, um ihre Wirte zu manipulieren. Es besteht ein enormer koevolutionärer Druck auf die schnelle Anpassung pathogener Effektome, was zu einer niedrigen Homologie der Effektorproteine führt, selbst bei eng verwandten Arten. Daher haben Werkzeuge, die allgemein in der Biologie verwendet werden, um die Proteinfunktion abzuleiten, wie z.B. die Vorhersage funktioneller Domänen, nur begrenzten Einsatz in Effektorstudien. In dieser Arbeit wurden weitere übergreifende Merkmale der Effektorbiologie untersucht. Diese sind: die Fähigkeit von Effektoren, miteinander zu interagieren, um möglicherweise die Vielseitigkeit des Effektoms zu erhöhen, und die Entwicklung eines Verfahrens zur Identifizierung von Molekülen, die die ungefaltete Proteinreaktion (UPR) der Pflanze stören.

Komplexe Proteininteraktionsnetzwerke sind Grundlage des effizienten Immunsystems der Pflanzenum die Immunantworten zu koordinieren und eindringende Krankheitserreger wahrnehmen und Signalkaskaden aktivieren zu können. Die Effektorforschung konzentriert sich jedoch hauptsächlich auf die Charakterisierung dieser Moleküle in der Isolierung. In dieser Arbeit wurde die Möglichkeit der Interaktion von Effektoren mit anderen Effektoren getestet. Ein systematischer Hefe-Zwei-Hybrid (Y2H)-Screen zeigte eine unerwartete Fülle von Effektor-Effektor-Interaktionen, zumindest innerhalb des Effektoms von *Ustilago maydis*. Nachdem analysiert wurde, wie sich das Interaktionsnetzwerk durch den Infektionsprozess verändern kann, wurden einige evolutionäre Treiber diskutiert, die die Effektor-Effektor-Interaktionen stabilisieren können.

Ein weiterer Aspekt der hier untersuchten Effektorbiologie war die zentrale Rolle der pflanzlichen Proteinsekretion als Teil der Immunabwehr. Während der pathogenen Invasion überlasten steigende Proteinsekretion die faltende Maschinenfabrik aus dem sekretorischen Weg. Dies aktiviert die UPR, was wiederum zur effizienten Faltung im endoplasmatischen Retikulumn und effizienter Proteinsekretion führt die mit der Wirtsabwehr zusammenhängen, was wiederum diesen Mechanismus zu einem primären Ziel für pathogene Effektoren macht. Um dies zu untersuchen, wurde eine Hochdurchsatzmethode zur Identifizierung von Proteinen mit einer Rolle in der pflanzlichen UPR entwickelt. Ein Testexperiment mit 35 mutmaßlichen Effektoren von *U. maydis* führte zur Identifizierung und Validierung eines Proteins, UMAG_05927, welches die UPR *in planta* beeinträchtigt.

PREAMBLE

Plant Pathogen Research Contextualized

The Food and Agriculture Organization of the United Nations (FAO) estimated that hunger affects approximately one out of every nine people in the world (FAO, IFAD, & WFP, 2015). Even though there is a trend towards the reduction in undernourishment prevalence, food demand is expected to double by 2050 and current increase rates in crop production will not be able to satisfy it (Ray *et al.*, 2012). Therefore, it is of paramount importance to develop crop varieties that produce better yields and to reduce the losses caused by environmental factors.

Crop yields are significantly limited by stresses that affect their development (Suzuki *et al.*, 2014). Environmental stresses such as heat, drought, salinity, lack of nutrients, and others, lead to severe limitations in crop production (Boyer, 1982; Qin *et al.*, 2011). Additionally, plant diseases can cause devastating effects, especially in crops with low genetic variability (Hajjar & Hodgkin, 2007; McClure *et al.*, 2014). In fact, it is estimated that plant pathogens can lead to up to 43% in yield losses worldwide (Savary *et al.*, 2012). A common and efficient strategy to control pests is the use of pesticides, but numerous off-target effects with a high impact on the environment and biodiversity have been extensively documented (Aktar *et al.*, 2009; Yang *et al.*, 2011). Therefore, understanding the mechanisms of infection is crucial to develop strategies for limiting the impact of plant diseases in crop yields and increase productivity to meet expected food demands.

Plant Biotic Interactions

Environmental cues profoundly affect the way plants develop and grow, allowing them to adapt their genetic programming to their surroundings. In addition to abiotic conditions, plants live in complex communities with a wide range of organisms that can be beneficial, neutral, or harmful to their development and survival. Most plants establish symbiotic interactions with other organisms that can increase their nutrient uptake and even improve resistance to several stresses, thus leading to increases in biomass (Parniske, 2008; Saia *et al.*,

2014). For instance, symbioses with arbuscular mycorrhizal fungi improve water and nutrient uptake, most notably of phosphate and nitrogen forms that are not readily accessible to the plant (Maclean *et al.*, 2017; Martin *et al.*, 2016). Another example involves legumes that establish symbiotic structures with nitrogen-fixing soil rhizobia, where nitrogen is fixed and provided to the plant in exchange for sugars to the bacteria (Poole *et al.*, 2018; Udvardi & Poole, 2013).

In addition to symbiotic organisms, the general composition of soil microbiota has been shown to have an impact on plant development and physiology (Li *et al.*, 2019; Lu *et al.*, 2018; Wei *et al.*, 2019). Thus, plants developed the ability to promote the proliferation of soil microorganisms that are perceived as beneficial (Haichar *et al.*, 2008; Olanrewaju *et al.*, 2019). However, all these interactions create opportunities for pathogens to exploit plant vulnerabilities and facilitate their colonization. For that reason, plants have developed complex surveillance systems to distinguish organisms that are beneficial from those that are pathogenic. In turn, successful pathogens have developed equally intricate molecular tools to avoid plant perception and subvert their development and metabolism, to create optimal conditions for their proliferation. That is the topic of this thesis.

Plant Immunity

Plants constantly monitor their environment and integrate multiple cues to better respond and adapt to the surrounding conditions. When interacting with microorganisms, it is crucial that they can distinguish friends (*i.e.* symbiotic species) from foes (*e.g.* parasitic organisms). The first protection layer against microbes is physical. Thick cuticles and stomata that close in response to diverse stimuli, provide an efficient barrier and isolate plant cells from the outside world (Ziv *et al.*, 2018). Additionally, plants produce and release chemicals that are toxic to microorganisms and therefore inhibit their growth (Pretorius *et al.*, 2003; Tam *et al.*, 2015). However, well adapted pathogens have evolved to overcome these obstacles, which in turn led to plants evolving complex mechanisms to perceive invasions and respond accordingly.

Surface receptors and pattern-triggered immunity (PTI)

To integrate multiple signals from their environment, plant cells have numerous receptor proteins in their cytoplasmic membrane. These usually fall in one of two types: proteins that consist of a transmembrane domain and an extracellular domain (ED) that binds to specific ligands, called receptor-like proteins (RLPs); and those with an additional intracellular kinase domain, called receptor-like kinase (RLKs). The genome of *Arabidopsis thaliana* encodes for 57 RLPs and more than 600 RLKs (Shiu & Bleecker, 2003; Wang *et al.*, 2008), which highlights their relevance in several physiological processes. RLK families are divided according to differences in their EDs, where the largest group has leucine-rich repeat (LRR) sequences. This family contains 233 proteins that can be further divided into 15 sub-families (Shiu & Bleecker, 2003). Upon ligand recognition, RLKs and RLPs typically oligomerize and mediate the initiation of a mitogen-activated protein kinase (MAPK) signaling cascade, which in turn triggers a response (Meng & Zhang, 2013). In addition to the specificity of the receptor-ligand interaction, it is its association to specific proteins on the membrane surface and/or in the cytosol that integrates multiple signals and results in a fine-tuned response to perceived stimuli. The complexity of these receptor networks was only recently systematically addressed in a study that found more than 500 possible interactions between EDs of about 200 LRR-RLKs (Smakowska-Luzan *et al.*, 2018). This intricate network results in two major

functional yet antagonistic responses: those that influence growth and development, and those that modulate immunity (He *et al.*, 2018). Here, the latter group is reviewed.

Microbial pathogens cause disruptions to cellular structures which release molecules in the apoplastic space, generally termed damage-associated molecular patterns (DAMPs, sometimes also referred to as danger-associated molecular patterns). Examples of DAMPs include cuticle or cell wall fragments, free extracellular adenosine triphosphate (ATP), and extracellular deoxyribonucleic acid (DNA; de Lorenzo *et al.*, 2018; Tang *et al.*, 2017). Additionally, invading pathogens carry what is usually referred to as microbe or pathogen associated molecular patterns (MAMPs or PAMPs, respectively). These can be of different nature, where the two most commonly cited examples are: flagellin peptides from bacterial flagella and the polysaccharide chitin from fungal cell walls. RLPs and RLKs that recognize DAMPs, MAMPs or PAMPs are called pattern recognition receptors (PRRs) and trigger an immune response termed PTI.

Many receptor-ligand binding pairs that lead to PTI have been identified and studied. Probably the most prominent example is the recognition of flg22, a conserved 22 amino acid peptide from bacterial flagellin, by the LRR-receptor kinase (RK) FLAGELLIN SENSITIVE2 (FLS2; Gómez-Gómez & Boller, 2000). Differences in flagellin amino acid sequences between bacterial species can lead to reduced affinity in FLS2 binding and, therefore, weaker immune responses (Felix *et al.*, 1999). Another example of a conserved PAMP from bacteria is the elongation factor Tu (EF-Tu), which cannot be detected by *Nicotiana benthamiana* plants but binds to the LRR-RK EF-TU RECEPTOR (EFR) in *A. thaliana* (Zipfel *et al.*, 2006). Upon ligand binding, both FLS2 and EFR converge to the BRASSINOSTEROID RECEPTOR1-ASSOCIATED RECEPTOR KINASE1 (BAK1), which is another LRR-RLK (Chinchilla *et al.*, 2007). The formation of these heterodimers leads to the almost immediate phosphorylation of the receptors and initiates a signaling cascade to induce an immune response (Schulze *et al.*, 2010). Receptor-ligand interactions between plants and fungi have also been identified. More notably, PTI induced by chitin and β-glucan was discovered to depend on the CHITIN-ELICITOR RECEPTOR KINASE1 (CERK1), a lysine motif (LysM) RK (Mélida *et al.*, 2018; Miya *et al.*, 2007). Further research showed that CERK1 had low affinity to chitin, and that it relied on the association to CHITIN ELICITOR-BINDING PROTEIN (CEBiP) in rice, or to LYSM-CONTAINING RECEPTOR-LIKE KINASE (LYK) 4 and 5 in *A. thaliana*, for efficient PAMP detection (Cao *et al.*, 2014; Kaku *et al.*, 2006). Altogether, these examples show how

complicated PAMP recognition can be and how different ligands converge to the same hubs to induce immune responses.

On the onset of PTI, a series of reactions occur that lead to the activation of proteins and mediate the mechanisms that result in immunity. These responses are highly intricate and involve numerous feedback loops that regulate them. While the understanding of each of these components is constantly expanding, there are contradictory reports on their timing and regulation (Bigeard *et al.*, 2015; Yu *et al.*, 2017). Nonetheless, several key elements have been extensively characterized. The recognition of DAMPs and MAMPs typically leads the PRR receptors to interact with other RLKs and cytoplasmic kinases, which in turn lead to a series of phosphorylations that result in an immune response. Most, if not all, facets of PTI seem to be dependent on MAPK signaling cascades, since the presence of kinase inhibitors stop most immune responses upon stimulation with DAMPs and MAMPs (Meng & Zhang, 2013). MAPK signaling has been implicated in Ca^{2+} influx, stomatal closure, reactive oxygen species (ROS) burst, activation of transcription factors, hormone production, among other immune responses (Bigeard *et al.*, 2015; Yu *et al.*, 2017). One of the earliest physiological changes is the quick and transient increase in cytoplasmic Ca^{2+} (Ranf *et al.*, 2011), which functions as further signaling and activates some physiological responses. These include the activation of calcium dependent protein kinases (CDPKs), and the opening of other ion transporters that depolarize the membrane and alkalify the apoplast (Jeworutzki *et al.*, 2010). Just after calcium influx, there is a ROS burst which is primarily mediated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD). RBOHD leads to the accumulation of reactive oxygen species in the apoplast that is converted into H_2O_2 by superoxide dismutases (Daudi *et al.*, 2012; Kadota *et al.*, 2015). H_2O_2 production has two main effects: toxicity to the invading pathogens and activation of a positive feedback loop by leading to the increase of Ca^{2+} uptake in adjacent plant cells (Fones & Preston, 2012; Petrov & van Breusegem, 2012). Another result of PAMP elicitation is the activation of transcription factors to upregulate defense genes. Among them, phytohormones are produced to activate systemic defense responses. While jasmonic acid (JA) and ethylene (ET) have been implicated in the defense against necrotrophic pathogens (*i.e.* organisms that proliferate in dead host tissues; Moffat *et al.*, 2012; Rahman *et al.*, 2019; Wang *et al.*, 2015), salicylic acid (SA) was found to be involved in defense against biotrophic and hemibiotrophic pathogens (*i.e.* species that colonize living tissues at least in part of their lifecycle; Ullah *et al.*, 2019; Yang *et al.*, 2015). In fact, SA and JA

are known to have antagonistic effects in signaling, gene expression, and immune responses (Gimenez-Ibanez & Solano, 2013; Kunkel & Brooks, 2002; Niki *et al.*, 1998). Interestingly, SA has been shown not only to promote defense responses during a pathogenic attack but also to confer enhanced resistance during a secondary attack, in what is usually referred to as systemic acquired resistance (SAR; Fu & Dong, 2013; Ryals *et al.*, 1994).

All the signaling mechanisms ultimately result in responses that aim to neutralize and kill the invading pathogen. These include: closing of stomata to prevent the entry of more pathogenic microbes (Melotto *et al.*, 2006; Zeng *et al.*, 2010); cell wall thickening and callose deposition to difficult microbe movement and penetration (Luna *et al.*, 2011; Voigt, 2014); restriction of nutrient transport to the apoplastic space to prevent microbial proliferation (Moore *et al.*, 2015; Yamada *et al.*, 2016); release of cytotoxic ROS molecules (Daudi *et al.*, 2012; Kadota *et al.*, 2015); production of antimicrobial compounds (Breen *et al.*, 2017; Tam *et al.*, 2015); among others.

Intracellular receptors

PTI stops non-adapted microbes from proliferating in plant tissues. However, pathogens have evolved a set of molecular tools, termed effectors, to dampen or completely evade plant immunity. Therefore, selective pressure on plants led to the development of a second immunity layer based on the recognition and response to effectors, termed effector triggered immunity (ETI; Jones & Dangl, 2006). In this case, intracellular nucleotide-binding/leucine-rich repeat receptors (NLRs) detect the presence of effectors and lead to the repetition and amplification of PTI responses. This often culminates in a hypersensitive response (HR) that ultimately leads to programed cell death (PCD; Coll *et al.*, 2011; Heath, 2000). A neat example of this defense involves the *Pseudomonas syringae* effector HopAI1, which stops the MAPK signaling cascade upon flg22 perception (Zhang *et al.*, 2007). This disruption of the signaling cascade is perceived by the SUPPRESSOR OF MKK1 MKK2 2 (SUMM2) NLR, which in turn activates ETI (Zhang *et al.*, 2012).

In plants, NLRs represent a large family of resistance genes. Shao *et al.* (2016) surveyed their abundance in the genomes of 22 angiosperm species and found that it ranged between 88, in *Thellungiella salsuginea*, and 571, in *Medicago truncatula*. The stereotypical structure of an NLR protein consists of three parts: a C-terminal LRR, which usually confers specificity

(Cesari, 2018); a central nucleotide binding domain (NB), that changes the receptor's conformation depending on the substrate it is bound to (Hu *et al.*, 2013; Wang, Wang, *et al.*, 2019); and an N-terminal domain with either a toll–interleukin 1 receptor (TIR) or a coiled-coil domain (CC), both of which have been associated with HR (Collier *et al.*, 2011; Swiderski *et al.*, 2009). However, alternative structures and working mechanisms have recently been characterized.

Differences in acting mechanisms start at the effector recognition phase. Some NLRs bind directly to their effector targets and start the downstream signaling (Césari *et al.*, 2014; Varden *et al.*, 2019). This is the mechanism that better fits the gene-for-gene plant resistance model proposed by Flor (1971). However, mechanisms of indirect effector recognition were found to be more abundant (Cesari, 2018; Kroj *et al.*, 2016), which highlights its increased versatility. Indirect effector surveillance by NLRs generally fall in one out of three models: guard, decoy, or integrated decoy. In the guard model, effectors bind to other host proteins and cause modifications that can be perceived by NLRs. An example of this mechanism is the HR response caused by the recognition of AvrPphB, a protease from *P. syringae*. Cleavage of the serine/threonine-protein kinase AVRPPHB SUSCEPTIBLE1 (PBS1) by AvrPphB leads to the exchange of adenosine diphosphate (ADP) for ATP in the RESISTANCE TO PSEUDOMONAS SYRINGAE5 (RPS5) NLR, activating it and inducing to ETI (Ade *et al.*, 2007). Alternatively, plants produce proteins that mimic the targets of effectors, termed decoys, which upon effector binding activate NLR signaling. Decoys have been found as single proteins, but also as part of the structure of some NLR proteins (*i.e.* integrated decoys; Kroj *et al.*, 2016; Maqbool *et al.*, 2015). In addition to these variations, the presence of truncated NLR/NLR-like genes in plant genomes has been suggested to have a role in resistance, not only to pathogens but also to other environmental conditions (Cesari, 2018; Cui *et al.*, 2015; Wang *et al.*, 2013; Zbierzak *et al.*, 2013).

NLR signaling activation and downstream transduction mechanisms are still largely unknown, but a few components have been discovered. In some cases, interaction between NLRs was deemed relevant for ETI activation. Ade *et al.* (2007) showed that multiple domains from RPS5 could interact with each other and activate immune responses. Another study showed that the homodimerization of the TIR domain of L6, an NLR from *Linum usitatissimum*, is irrelevant for effector recognition but is essential for immunity (Bernoux *et al.*, 2011). In other cases, effector recognition relies on the interaction between different

NLRs. For instance, in RESISTANCE TO PSEUDOMONAS SYRINGAE4 (RPS4) and RESISTANCE TO RALSTONIA SOLANACEARUM1 (RRS1) TIR homeodomain interaction is required for effector recognition (Williams *et al.*, 2014). Alternatively, sensor NLRs are responsible for the effector recognition, while the signal transduction requires helper NLRs. This might result in the multifunctionalization of NLRs, like in the case of the activated disease resistance (ADR) family which, in addition to ETI, are also involved in PTI signaling (Bonardi *et al.*, 2011). In specific cases, more complex NLR structures can be formed to either boost downstream signaling events or directly mediate cell death (Jubic *et al.*, 2019; Wang, Hu *et al.*, 2019). All of these examples show very complex recognition and signaling mechanisms that confer robustness and versatility to plant immune systems, which are a direct result of co-adaptive pressures from plant-pathogen interactions.

Effectors and Plant Manipulation

Pathogens secrete effectors and use them as tools to manipulate their hosts. Many effectors have a role in regulating plant immunity but they can also be involved in creating a favorable environment for the pathogen's proliferation and development. Indeed, some biotrophic pathogens can cause significant changes in the host's transcriptional programming that lead to changes in cellular and even tissue identity. Those changes can lead to increases in nutrient flow to the infection site and create structures for the successful completion of the pathogen's lifecycle (Lemoine *et al.*, 2013; Zuo *et al.*, 2019).

While effector molecules can be of different nature, such as small ribonucleic acids (RNAs), hormones, peptides, and proteins, this section will primarily focus on protein effectors. This research field is mostly constrained by limitations that are caused by the enormous adaptive pressure on molecules that mediate the infection process, both in pathogens and plants. Mutations that result in improved infection increase the fitness of the pathogen, while mutations that lead to improved resistance increase the fitness of the plant. This molecular arms race effectively results in rapid genome evolution, especially in molecules that mediate the pathogenic interaction (Dong *et al.*, 2015; Jiang *et al.*, 2008). In fact, a genomic comparison of 2 closely related fungi, *Ustilago maydis* and *Sporisorium reilianum*, revealed that protein conservation was much higher in proteins without secretion signal peptides (Schirawski *et al.*, 2010). Together with the observation that effectors from filamentous fungi tend to cluster in compartments with repetitive sequences and transposable elements, the concept of two-speed genome evolution has been proposed (Dong *et al.*, 2015). According to it, highly selective co-evolutionary pressures lead to rapid adaptation in genomic islands that are more relevant for the establishment of compatible interactions. Consequentially, the effector repertoire of a given pathogen is mostly specific for the interaction with its host range and bares low conservation to closely related species. This generally results in the absence of conserved motifs that could be used to identify effector proteins and provide hints towards their molecular function (Lanver *et al.*, 2017; Uhse & Djamei, 2018; Vleeshouwers & Oliver, 2014). However, it also opens opportunities to discover new protein functions that can be used as powerful tools to study plant cellular mechanisms (de Lange *et al.*, 2014; Schreiber *et al.*, 2019; Toruño *et al.*, 2016).

Despite the difficulties in identifying effector proteins, there are some features that can indicate genes as putative effectors. One factor to consider when studying effectors is their expression patterns. Generally, effector proteins are highly upregulated during infection. However, depending on the pathogen's lifestyle, different expression patterns can be expected. Typically, necrotrophs deploy a battery of effectors early in the infection stage to trigger PCD and consume the nutrients from the dead cells (Wang *et al.*, 2014). Biotrophs, on the other hand, express their effectors in waves to dampen immunity throughout infection, and influence their host's proteome, metabolome and physiology (Selin *et al.*, 2016; Toruño *et al.*, 2016). Hemibiotrophs, have an intermediate expression pattern, with early effectors interfering with immunity and other cellular processes to promote the pathogen's growth, and late effectors promoting plant cell death (Toruño *et al.*, 2016). Another factor to consider when doing effector research is the secretion mechanism by which effectors are delivered to their hosts. Pathogenic gram-negative bacteria rely on the type III secretion system (T3SS) to deliver effectors directly to their host's cytoplasm (Wagner *et al.*, 2018). Although there are no specific T3SS secretion signals for all effectors, there are conserved features within the first 25 amino acids – such as enrichment of polar amino acids, depletion of charged and hydrophobic ones, and absence of secondary structures – that can be used to predict new effector candidates (Samudrala *et al.*, 2009). In oomycetes, common N-terminal domains such as RxLR and crinkler (CRN) motifs have been identified, linked to effector secretion, and used to identify new effectors (Jiang *et al.*, 2008; Stam, Jupe, *et al.*, 2013). In fungi that produce haustoria, there has also been some evidence for conservation of a short Y/F/WxC motif among secreted effectors (Godfrey *et al.*, 2010). However, in other pathogenic filamentous fungi, conserved N-terminal sequences among effectors are yet to be discovered.

Integrating new patterns of effector biology with traditional effector characteristics has significantly improved effector mining in genomes of pathogens. Traditional effector candidate identification relied mostly in the presence of signal peptides in small proteins that have cysteine-rich sequences, which are considered hallmarks of proteins that remain stable in acidic apoplastic environments (Kämper *et al.*, 2006; Mueller *et al.*, 2008; Saunders *et al.*, 2012). With the increased availability of genomes, transcriptomes, and computational methods, recent effector prediction pipelines include more parameters. Examples of those include: gene expression profiles, similarity to other known effectors, sub-cellular localization signals, position in the genome, exclusion of domains with no relation to pathogenicity, among others (Godfrey et al., 2010; Saunders *et al.*, 2012; Schuster, Schweizer *et al.*, 2018).

Alternatively, experimental approaches to identify potential effectors. An example of these is the comparison between infected and healthy samples of isolated proteins from the apoplast or within plant vessels, followed by protein identification by mass spectrometry (MS; Bolton *et al.*, 2008; Delaunois *et al.*, 2014; van den Burg *et al.*, 2006). More sophisticated approaches have also been developed, including one from Guttman *et al.* (2002), where mutants of *P. syringae* with random transposon insertions of $AvrRpt_{281-255}$ were used to identify type III secretion signals from previously unknown effectors. The C-terminal part of AvrRpt causes an HR response in *A. thaliana* and by randomly inserting it in the genome of *P. syringae* the authors were able to identify 25 out of 75000 mutants that caused HR. Furthermore, the amino acid composition of the N-terminal part of those 25 effectors allowed to predict 15 new proteins that are secreted by the T3SS. Regardless of the methods used to identify putative effectomes, after compiling a list of candidates, validation, characterization, and effector functions can be studied.

Effectors that interfere with immunity

Several effectors have been implicated in controlling plant immunity, particularly in biotrophic and hemibiotrophic pathogens. There are several strategies to achieve that, including: avoidance of PAMP perception by PRRs, disruption of downstream PRR signaling, ROS burst inhibition, promotion of hormonal changes, among others. Additionally, pathogens often have effectors with redundant functions. This section focuses on examples of a few of these effector types.

The fungal pathogen *Cladosporium fulvum* has at least two effectors that prevent chitin detection by different mechanisms. Avr4 binds to chitin, preventing its degradation by plant apoplastic chitinases and its subsequent detection by PRRs (van den Burg *et al.*, 2006). Ecp6, on the other hand, competes with PRRs in binding to free chitin degradation products, thus avoiding their detection (Sánchez-Vallet *et al.*, 2013). Another mechanism to avoid PRR activity is to promote their degradation, and at least four effectors from the bacterial pathogen *P. syringae* were described to do that (Toruño *et al.*, 2016). The effector AvrPtoB was shown to promote the polyubiquitination of both FLS2 and CERK1 in *A. thaliana*, thereby promoting their degradation (Gimenez-Ibanez *et al.*, 2009; Göhre *et al.*, 2008). AvrPtoB, but also AvrPto and HopF2, were also shown to bind to BAK1, preventing its interaction with other PRRs and the subsequent activation of immune responses (Shan *et al.*,

2008; Zhou *et al.*, 2014). Another similar mechanism was described in the effector HopAO1, which interacts with the EFR and reduces its phosphorylation, thus preventing the activation of an immune signaling cascade (Macho *et al.*, 2014). This observed functional redundancy and conservation among other organisms highlights its importance in perception avoidance by plant PRRs.

One of the effects of avoiding PAMP recognition by PRRs is to reduce the ability for plants to trigger ROS burst. However, other effectors can achieve the same result by targeting different pathways. For instance, the effector HopN1 from *P. syringae* binds to a protein from the photosystem II (PSII), PsbQ, and contributes to its degradation. Silencing of PsbQ led to a reduction of ROS burst in tobacco plants, thus linking this complex subunit with immunity (Rodríguez-Herva *et al.*, 2012). A more direct way by which pathogens reduce the ROS burst was found in the biotrophic pathogen *U. maydis*. The conserved effector Protein essential for penetration 1 (Pep1, UMAG_01987) was reported to directly interact and inhibit POX12, a secreted peroxidase from maize (Hemetsberger *et al.*, 2012).

There are many other strategies that pathogens use to downregulate immune responses from their hosts. Effectors from many plant pathogens have been shown to interact with various classes of plant transcription factors involved in upregulating immune responses, such as NACs, TCPs and WRKYs (McLellan *et al.*, 2013; Sarris *et al.*, 2015; Stam *et al.*, 2013). Another highly conserved manipulation strategy is the control of hormones involved in immunity. A good example of its relevance in infection is the case of *P. syringae*, which has at least three independent ways to promote JA signaling. On one hand, it produces and secretes a coronatine, a JA analog that leads to the upregulation of JA related genes (Gnanamanickam *et al.*, 1982; Zheng *et al.*, 2012). Additionally, it has two effectors, HopZ1a and HopX1, which independently inhibit transcriptional repressors of JA-responsive genes (Gimenez-Ibanez *et al.*, 2014; Jiang *et al.*, 2013). By deploying this combination of effectors, the pathogen promotes the downregulation of the JA antagonist, SA, which is involved in defense against biotrophic pathogens (Kunkel & Brooks, 2002; Niki *et al.*, 1998; Ullah *et al.*, 2019; Yang *et al.*, 2015). However, by altering hormone balances other developmental and physiological changes can be affected.

Effectors that interfere with other biological processes

In addition to downregulating SA responses, pathogens that upregulate JA signaling influence innumerous other mechanisms in which these hormones are involved. Moreover, many other plant processes ranging from photosynthesis to respiration, growth, flowering, senescence, and gravitropism are impacted by these hormonal changes and exploited by pathogens (Huang *et al.*, 2017; Rivas-San Vicente & Plasencia, 2011). In fact, other hormone signaling pathways are targeted by effectors from plant pathogens, such as auxin, abscisic acid (ABA), and cytokinins (Chen *et al.*, 2007; Goel *et al.*, 2008; Hann *et al.*, 2014). However, hormones are only one of many ways that pathogens use to manipulate their host's growth and development.

An alternative way to influence global changes in the plant's development and physiology is to directly influence gene expression. Some pathogens have a class of effectors capable of binding to DNA and promote transcription, called transcription activator-like effectors (TALEs; Mak *et al.*, 2012). This type of effector has been found in a few bacterial species, with the most detailed examples reported in *Xanthomonas oryzae*. In addition to upregulating proteins involved in immune suppression (Cai *et al.*, 2017), TALEs from this pathogen have been reported to promote the expression of sugar transporters, and therefore increase the export of carbon sources to the apoplast (Wang *et al.*, 2018). It has been speculated that the accumulation of sugars in the extracellular space likely results in an osmotic tension that leads to water flow from the plant towards the apoplast, which represents an additional benefit to the pathogen (Macho, 2016).

Some effectors have been shown to localize in chloroplasts (Li *et al.*, 2014; Rodríguez-Herva *et al.*, 2012). While chloroplasts have a direct role in immunity (Caplan *et al.*, 2015) and take part in the biosynthesis of JA and SA, some effectors that target this organelle abd specifically suppress photosynthesis. In turn this leads to changes in redox capacity and plant metabolism, which might play a role in the source to sink tissue transition that some pathogens are known to promote (Lemoine *et al.*, 2013; Matei *et al.*, 2018; Xu *et al.*, 2019).

Effector interplay

The plant immune system is composed by an intricate network of protein interactions with hubs where different signaling pathways converge to produce a robust response to a

pathogenic attack (Cao *et al.*, 2014; Chinchilla *et al.*, 2007; Kaku *et al.*, 2006; Smakowska-Luzan *et al.*, 2018). Pathogen effectors evolved alongside plant immunity, yet most effector research has focused on studying the impact of single effectors in plant cells.

There are however a few examples in the literature of effectors with antagonistic or synergistic mechanisms in host tissues (Cain *et al.*, 2008; Kleemann *et al.*, 2012; Kubori *et al.*, 2010; Urbanus *et al.*, 2016), some of which were mentioned above. More interestingly, there are also a few examples of effectors that directly influence other effectors *in vivo*. One of the earliest examples of that was described in *Agrobacterium tumefaciens*, the bacterium commonly used as a vector to genetically manipulate several plant species. This bacterium uses virulence proteins (Vir) to transfer T-DNA from the pathogen to the plant (Nester, 2015). Tzfira *et al.* (2004) showed that VirF localizes to the plant's nucleus where it interacts with the VirE T-DNA import protein and the plant protein VIRE2-INTERACTING PROTEIN 1 (VIP1), causing their targeting for proteasomal degradation. It was later found that VirD5 is able to interact with VirF and prevent its activity (Magori & Citovsky, 2011). Other examples of effector-effector interaction and its relevance in pathogenicity were also found in oomycetes and fungal species. For instance, in *Phytophthora sojae* the homodimerization of the effector PsCRN63 was shown to be necessary for the suppression of PTI in its host (Li *et al.*, 2016). A surprisingly contradictory example of this was found in *Fusarium oxysporum* f. sp. *lycopersici*, where the heterodimerization of Avr2 and Six5 *in planta* was found to activate I-2 NLR-mediated ETI. Single gene knockouts (KOs) of these effectors enabled ETI evasion in tomato plants (Ma *et al.*, 2015). This example illustrates how some interactions between effectors can be detrimental for pathogenicity. Systematic host-pathogen interactome characterizations have reported that many effectors from different pathogens target the same hubs from host immune networks (Crua Asensio *et al.*, 2017; Mukhtar *et al.*, 2011; Weßling *et al.*, 2014). It is possible that the Avr2-Six5 interaction, one of the effectors can serve as a hub and its interaction with other effectors is monitored by I-2, which then activates ETI. However, only systematic effector-effector interactome studies can shed light on the role of hubs from pathogen effectomes.

A few systematic studies of protein-protein interactions in pathogen proteomes have been described. The proteome of the causal agent of malaria, *Plasmodium falciparum*, was queried for interactions in a high-throughput yeast two-hybrid (Y2H) screen (LaCount *et al.*, 2005). However, the analysis of the interactome was mainly focused on the pathogen's cellular

processes (Suthram *et al.*, 2005), presumably in an attempt to find new therapeutic targets, and no conclusions about effector-effector interactions were drawn. A different study explored the concept of effector hubs and their relevance to pathogenicity using six proteins from *Salmonella typhimurium* (Cain *et al.*, 2008). A functional association screen to find effector interplay after delivery into the host revealed multiple interactions between effectors, which resulted in synergistic or antagonistic effects depending on the protein combination. A more systematic screen for "metaeffectors" (*i.e.* "effectors of effectors") in *Legionella pneumophila* revealed ten effector pairs that were able to interact in Y2H and have an antagonistic effect in yeast growth (Kubori *et al.*, 2010; Urbanus *et al.*, 2016).

In summary, there is increasing evidence that effector proteins can not only interact with each other, but also that the outcome of those interactions has a direct effect on pathogenicity. Therefore, effector research should consider the possibility that the models presented in single effector studies may not represent a complete picture. One way to tackle that is to test an effector of interest for interactions with the effectome of the pathogen. Alternatively, systematic screens for effector-effector interactions can provide useful frameworks in the study of host-pathogen interactions and help better understanding the disease processes.

Finding new effector functions

Effector functions are intrinsically difficult to study. Most agriculturally relevant plant-pathogen interactions involve organisms that lack significant experimental resources, such as annotated genomes, genetic manipulation techniques, growth of pathogen cultures in axenic conditions, readily available antibodies for native proteins, *etc.* Furthermore, because of the functional redundancy observed in the effectomes of pathogens, effectors that have relevant roles in pathogenicity might not be identified in single gene KO strains. Often, these limitations lead to the use of heterologous systems that generally limit scientific discoveries to conserved effector targets. However, it also presents as an opportunity to: streamline effector research by expanding experimental toolkits; accelerate research by using organisms with shorter lifecycles; and find molecular interactions that potentially extend to closely related species.

A good example of the use of heterologous systems to screen for effector functions was published by Petre *et al.* (2015). The authors applied what they called an "effectomics pipeline" to determine the subcellular localization of 20 putative effectors from the rust pathogen *Melampsora larici-populina*. The coding sequences of the effector candidates were cloned to produce protein fusions with the green fluorescent protein (GFP), which were then expressed in *N. benthamiana*. Effector candidate localization was visualized by confocal microscopy and the effector interacting proteins were identified after co-immunoprecipitation (co-IP) of the GFP tag, followed by MS. This approach led to important hints towards the function of almost the entirety of this pathogen's predicted effectome, which were then studied in more detail. However, this holistic strategy was only possible due to the reduced number of effector proteins predicted in this pathogen's genome. *Colletotrichum higginsianum*, on the other hand, was predicted to have over 100 putative effectors (Kleemann *et al.*, 2012). When exploring the effectome of this species, only a subset of these effector candidates were tagged for localization experiments, which led to the discovery of 16 proteins with specific subcellular localization *in planta* (Robin *et al.*, 2018).

A different functional screen tested the ability of the 30 effectors from *P. syringae* to interfere with *N. benthamiana* immunity (Gimenez-Ibanez *et al.*, 2018). By expressing these effectors in *N. benthamiana* and monitoring its Ca^{2+} levels, ROS burst, plant immunity-related gene expression, and HR, the authors were able to dissect the ways by which that pathogen can suppress plant immunity. Specifically, it was found that *P. syringae* has 3 types of immunity-suppressing effectors: broad-range, inhibitors of PAMP-induced transcription activation, and Ca^{2+}-independent ROS-burst inhibitors. Similarly, screening for HR responses after expression of a subset of effector candidates from *Colletotrichum higginsianum* in *N. benthamiana*, uncovered proteins with antagonistic effects that were expressed at different lifecycle stages from this hemibiotrophic pathogen (Kleemann *et al.*, 2012).

Another relevant example of a method to find effector functions was published by Janik & Schlink (2017), who described a Y2H screen to identify targets of putative effectors from *Candidatus Phytoplasma mali*. This pathogen is an obligate bacterial parasite that proliferates in the phloem of woody plants. Its lifestyle makes it impossible to cultivate in axenic conditions, making this method a powerful tool to better understand this disease.

All these examples highlight how versatile and useful heterologous experimental systems are. Despite their limitations – target conservation, gene expression levels, protein stability,

etc. – functional effector screens provide useful hints in the study of plant-pathogen interactions. Y2H approaches between effector candidates and genes from the infected plants allows not only to study difficult pathogens, but also to find targets from most cellular processes. On the other hand, screens in *N. benthamiana plants* can be adapted and expanded from the discovery of effectors that interfere with immunity to other processes. With the establishment of robust reporters, any cellular processes of interest can be queried for effector interference, and further characterization.

U. maydis, the model plant biotrophic pathogen

Smuts are basidiomycete fungi that can cause plant disease on many agriculturally relevant crops from the *Poaceae* family, such as maize, sugar cane, wheat, and others (Godfray *et al.*, 2016; Wennström, 1999). Typically, these pathogens infect seedlings, grow within the plant's meristematic tissues, and only cause characteristic symptoms in reproductive organs. *U. maydis*, the causal agent of corn smut, belongs to this group of pathogens and is its most studied model organism (Brefort *et al.*, 2009; Dean *et al.*, 2012). Under appropriate conditions, diploid teliospores from this species germinate and go through meiosis to form haploid cells. After detecting a compatible mate, these sporidia form conjugation tubes that lead to cell fusion and filamentous growth of a dikaryotic hypha. The hyphal tip then develops an appressorium to penetrate plant cells and establishes a biotrophic interaction interface. A few days after continuous growth and branching the fungus induces the formation of galls, commonly referred to as tumors, where it promotes the development of a gelatinous matrix with optimal conditions for karyogamy and spore development. The rupture of these structures restarts its lifecycle (Feldbrügge *et al.*, 2004; Lanver *et al.*, 2017).

Although not economically threatening, its ability to cause visible symptoms in all aerial tissues of maize plants within one week after infection makes it ideal for streamlining research in this area. For that reason, many tools were developed to better understand its biology and how it interacts with its host. After the discovery of the loci that define its mating types (Bölker *et al.*, 1992; Gillissen *et al.*, 1992) and the description of increasingly sophisticated transformation methods (Brachmann *et al.*, 2004; Fotheringham & Holloman, 1990; Kämper, 2004; Schuster, Trippel *et al.*, 2018; Schuster *et al.*, 2016), the development of solopathogenic haploid strains (Bölker *et al.*, 1995; Brachmann *et al.*, 2001) dramatically streamlined genetic and developmental research studies in this species. Furthermore, the

genome of *U. maydis* has been fully sequenced (Kämper *et al.*, 2006), which provides valuable insights into its size and organization. It was found that this 20.5 megabase (Mb) genome encoded for 6902 predicted proteins, 82 of which were found in 12 clusters that encoded for small secreted proteins of unknown function. These clusters showed upregulation of gene expression during biotrophy and the deletion of individual clusters led to altered virulence, thus highlighting their relevance in the infection process. Further analysis of the genome revealed that the predicted secretome of *U. maydis* is composed of 554 proteins, where 168 had a predicted enzymatic function and the remaining 386 proteins did not show any conserved domains that could hint towards their function (Müller *et al.*, 2008). Recently, the genomes of pathogenic smut fungi were analyzed with more modern bioinformatic prediction tools (Schuster, Schweizer *et al.*, 2018). This reanalysis reviewed the number of secreted proteins in *U. maydis* down to 467, of which 264 had predicted functional domains and 203 did not. Another useful resource when studying *U. maydis* is the recently published RNA-seq data analysis from the fungus along its biotrophic development (Lanver *et al.*, 2018). This study discovered 14 different gene expression modules, three of which were considered "virulence modules" because they included mostly secreted proteins with an expression profile compatible with the 3 pathogenic developmental stages: plant invasion, biotrophic establishment, and gall induction. Tissue and cell type specific gene expression from *U. maydis* have also been measured, revealing differences in the fungal transcriptional programming that led to the identification of effectors with organ-specific functions (Skibbe *et al.*, 2010; Villajuana-Bonequi *et al.*, 2019). Lastly, another recent study developed a tool to identify mutants with altered virulence in a high-throughput manner (Uhse *et al.*, 2018). After generating a library of 195 single gene KO mutants of putative effectors, maize plants were infected with pools of those mutants and mutant-specific sequences were purified for next generation sequencing. This allowed for the discovery of 28 virulence factors, 23 of which had not been previously described. This method can be adapted to test different insertional mutagenesis libraries and infection conditions, thus improving our understanding of genes that are essential for pathogenesis.

Despite all these hallmarks in *U. maydis* research, the number of characterized effectors in this fungus remains relatively small. This reflects the difficulty of studying the interaction between plant and pathogen, where maize's relatively large genome with highly repetitive sequences (Schnable *et al.*, 2009) and long lifecycle creates difficulties for its genetic manipulation. Furthermore, the redundancy known to occur in pathogenic effectomes leads

to a limited number of effectors with a direct impact on virulence. This results in limited single gene KO mutants that show altered virulence (Uhse *et al.*, 2018) and often multiple KOs are required to connect gene clusters/groups with their role in pathogenicity (Brefort *et al.*, 2014; Kämper *et al.*, 2006).

Nonetheless, a few effectors from *U. maydis* have been successfully functionally characterized. Pep1 was found to accumulate in the apoplastic space and is required for pathogenicity (Doehlemann *et al.*, 2009). Further characterization revealed that this small effector inhibits the production of apoplastic ROS, therefore protecting the fungus from early plant defense responses (Hemetsberger *et al.*, 2012). Another apoplastic effector is the Protein involved in tumors 2 (Pit2, UMAG_01375), which was deemed as essential for virulence by interacting with maize cysteine proteases and reducing their activity (Doehlemann *et al.*, 2011; Mueller *et al.*, 2013). An effector that was found to translocate to the host's cytosol and to have a direct impact in virulence was the Chorismate mutase 1 (Cmu1, UMAG_05731; Djamei *et al.*, 2011). This protein reduces plant immune responses by interfering with the shikimate pathway, lowering the amount of precursor for SA synthesis. Tumor inducing 2 (Tin2, UMAG_05302) is another cytoplasmic effector that influences the phenylpropanoid pathway by stabilizing a kinase involved in this pathway (Tanaka *et al.*, 2014). This ultimately leads to the characteristic accumulation of anthocyanins and suppression of lignin biosynthesis in maize cells. The only fully characterized effector from *U. maydis* with a tissue specific role is the Seedling efficient effector 1 (See1, UMAG_02239), which is required for the SGT1-dependent reactivation of DNA synthesis, an essential process for the induction of galls in leaves (Redkar *et al.*, 2015). The Repetitive secreted protein 3 (Rsp3, UMAG_03274) was suggested to coat fungal hyphae and protect it from the antifungal activity of proteins from the DUF26-domain family (Ma *et al.*, 2018). Recently, two more effectors from *U. maydis* were characterized in detail, Jasmonate/ethylene signaling inducer 1 (Jsi1, UMAG_01236) and Merope1 (Mer1, UMAG_03753). Jsi1 interacts with topless/topless-related transcription factors and leads to the derepression of genes that facilitate biotrophy (Darino *et al.*, 2019). Mer1, on the other hand, belongs to cluster of functionally redundant proteins which inhibit PTI, and promotes the autoubiquitination of RFI2 which ultimately leads to the suppression of immune responses (Navarrete *et al.*, 2019). Other virulence factors, such as Apathogenic in B73 (ApB73, UMAG_02011; Stirnberg & Djamei, 2016) and Cysteine-rich core effector 1 (Cce1, UMAG_12197; Seitner *et al.*, 2018) were shown to be essential for *U. maydis* virulence but the inability of identifying their

interacting proteins limited the discovery of their acting mechanisms and their identification as true effectors.

In summary, *U. maydis* is an interesting organism to study biotrophic plant-pathogen interactions. The many tools and data collected over the last few years allowed for critical advances in this area both in the characterization of mechanisms by which single effectors influence pathogenicity, and the global transcriptional changes along the infection process and in specific tissues. Together with recent advances in fungal and plant genetic manipulation tools, such as the multiplexed gene editing in *U. maydis* (Schuster, Trippel *et al.*, 2018) and the transient expression of proteins in monocot plants (Bouton *et al.*, 2018; Fursova *et al.*, 2012; Lee *et al.*, 2015; Li *et al.*, 2009), significant breakthroughs in this area can be expected soon.

The Unfolded Protein Response (UPR) and Infection

Proteins play a central role in most cellular mechanisms and require specific conformations to function correctly. Often, correct folding involves post-translational modifications, such as glycosylation, which generally start in the endoplasmic reticulum (ER) and fully mature in the Golgi apparatus. The canonical protein secretion pathway takes this route before vesicles containing the mature proteins fuse with the plasma membrane and release the mature proteins to the extracellular space (Schekman, 2010). The import of proteins to the ER is a co-translational process, where nascent peptides with a secretion sequence are directed to the ER membrane and the remaining protein is directly translated into the ER lumen. During translation, glycans are attached to the nascent protein and LUMINAL BINDING PROTEINS (BiPs) assist their folding (Otero *et al.*, 2010).

In stress conditions, the ER can become overloaded with newly synthesized proteins that its folding machinery cannot correctly fold. Then, ER quality control (ERQC) machinery detects the accumulation of unfolded proteins and activates signaling mechanisms to restore homeostasis, in what is known as the unfolded protein response (UPR). The main components of UPR are conserved in most eukaryotic organisms and perturbations to its function lead to disease and cell death (Chakraborty *et al.*, 2016). Recent advances in UPR signaling in plants and fungi, and its role plant-pathogen interactions are reviewed in this section.

UPR signaling in plants

In plants, two main branches of UPR activation have been identified. The first is mediated by the INOSITOL-REQUIRING ENZYME 1 (IRE1), which is an ER transmembrane protein with an N-terminal protein binding domain in the ER lumen, and kinase and ribonuclease domains facing the cytoplasm. In the absence of stress, IRE1 monomers are bound to BiP proteins which prevent IRE1 oligomerization. In stress conditions, BiP proteins bind to the unfolded proteins that accumulate in the ER lumen, releasing IRE1 which can then form dimers and oligomers (Iwata & Koizumi, 2012). The interaction between IRE1 proteins leads to their phosphorylation, activating its ribonuclease domains. IRE1 ribonuclease activity unconventionally splices 23 nucleotides from BASIC REGION/LEUCINE ZIPPER MOTIF 60

(bZip60) transcripts, causing a frameshift close to the 3' terminal end of the transcript. This results in a nuclear localization signal, as opposed to a transmembrane domain that is translated from the full bZip60 messenger RNA (mRNA; Iwata & Koizumi, 2012). The bZip60 transcription factor moves then to the nucleus, where it binds to UPR responsive elements (UPRE, TGACGTGR) and/or ER stress elements (ERSE, CCAAT-N9-CCACG) and promotes the upregulation of UPR-related genes (Iwata & Koizumi, 2005). Alternatively, UPR can be triggered via two other bZip transcription factors, bZip17 and bZip28. Like IRE1, in the absence of stress these transcription factors are in the ER membrane and interact with BiPs. On the onset of cellular stress, BiP proteins preferentially interact with the increasing numbers of newly synthesized proteins that require folding, releasing the transmembrane bZip transcription factors 17 and 18, that are then shuttled to the Golgi apparatus for proteolytic cleavage (Srivastava *et al.*, 2013; Tajima *et al.*, 2008). The Golgi SITE 1 PROTEASE (S1P) and the cytosolic SITE 2 PROTEASE (S2P) release the transcription factors from the membrane, allowing their translocation to the nucleus where they associate with NUCLEAR FACTOR Y (NF-Y) subunits to upregulate UPR responsive genes (Liu *et al.*, 2007; Liu & Howell, 2010).

Despite its ubiquitous nature in stress response, differences in signaling and gene upregulation have been identified in a tissue- and/or stress-specific manner. For instance, a recent transcriptomic analysis in combinatorial double KO lines of the three bZip proteins involved in UPR revealed that bZip28 and bZip60 are the major activators of UPR-related gene upregulation (Kim *et al.*, 2018). Surprisingly, the same dataset showed that bZip17 and bZip28 modulated the expression of genes involved in cell growth and sustain it in ER stress conditions. This is a good example of the complex interplay between UPR and plant growth. IRE1 was also found to have different roles in ER stress conditions. The genome of *A. thaliana* encodes for two copies of this gene and IRE1a was shown to have a predominant role in SA-mediated UPR induction, while IRE1b is preferentially involved in bZip60 mRNA splicing when ER glycosylation is impaired (Moreno *et al.*, 2012). Therefore, *A. thaliana ire1a* mutants are more susceptible to pathogenic attacks, presumably partly because of impaired secretion of antimicrobial PATHOGENESIS RELATED (PR) proteins. This example illustrates the relevance of UPR in plant-pathogen interactions and hints towards the possibility of this process being a target for effector manipulation.

Current tools to better understand ER stress signaling have limited applicability and are, for the most part, laborious and relatively expensive. Chen & Brandizzi (2013), published a protocol where UPR marker genes can be measured by quantitative polymerase chain reaction (qPCR). This is the most widely used method to assess UPR and allows for sensitive measurements of ER homeostasis perturbations. However, it involves a fair amount of sample handling and becomes prohibitively expensive if used to screen gene libraries for new players in UPR signaling. McCormack *et al.* (2015) described a high throughput method to identify seedlings with altered sensitivity to the UPR-inducing chemical tunicamycin (Tm). This method streamlines the screening of numerous genotypes in a relatively short amount of time but is limited to the screen of seed collections. Other smaller scale techniques have been developed to address specific questions in genes that were known to influence UPR but were limited in the identification of new genes influencing this cell process (Hayashi *et al.*, 2013; Liu & Howell, 2010; Meng *et al.*, 2017; Nawkar *et al.*, 2017). For that reason, a quick, simple, and reliable assay to perform genetic screens for new actors in UPR signaling has the potential to significantly expand our knowledge of this essential mechanism.

UPR in *U. maydis*

In fungi, UPR has been better studied in yeast. Briefly, the only mechanism described for UPR signaling is IRE1p-dependent, which is very similar to the pathway described above. Upon ER stress, BiP proteins release the transmembrane IRE1p which dimerizes, promoting its trans-autophosphorylation and unconventional splicing of an mRNA encoding a transcription factor (Sidrauski & Walter, 1997). The UPR-mediated splicing of HAC1 mRNA leads to the translation of an active transcription factor that upregulates UPR-related genes (Mori *et al.*, 2000).

Not much is known about UPR in *U. maydis*, but recently a few components of this mechanism and their role in virulence were described. Conditional expression of the HAC1 homolog, named Clp1 interacting protein1 (Cib1, UMAG_11782), revealed that UPR is essential for suppression of defense responses in maize (Schmitz *et al.*, 2019). Cib1 stability and phosphorylation was previously found to be influenced by Clampless1 (Clp1, UMAG_02438), which in turn increased *U. maydis*' tolerance to ER stress (Heimel *et al.*, 2013; Pinter *et al.*, 2019). Cib1 was also found to bind to UPREs, including in promoters of known effectors such as Tin1 (UMAG_05294) and Pit2, leading to their upregulation

(Brefort *et al.*, 2014; Hampel *et al.*, 2016). Furthermore, deletion of genes related to UPR in *U. maydis* almost always leads to the complete abolishment of pathogenicity (Hampel *et al.*, 2016; Heimel *et al.*, 2013; Lo Presti *et al.*, 2016; Pinter *et al.*, 2019; Schmitz *et al.*, 2019). Altogether, these studies show that despite the limited data on UPR in *U. maydis*, this mechanism is central in the biotrophic interaction and merits further research.

Effectors that influence UPR

Effectors are known to perform a vast array of functions and affect multiple cellular processes. The secretion of both defense and pathogenic molecules defines interaction between host and pathogen, yet very few effectors that interfere with secretion and/or UPR have been studied. Examples of these include the Avrblb2 from *Phytophthora infestans,* which prevents protein secretion, specifically of the papain-like cysteine protease C14 that promotes proteolysis of proteins in the apoplast (Bozkurt *et al.*, 2011; Liu *et al.*, 2018). More recently, the PsAvh262 effector from *P. sojae* was shown to bind to their host's BiPs, leading to its stabilization and preventing degradation. This results in reduced UPR signaling and subsequent defense gene activation, which ultimately suppresses cell death and facilitates the pathogen's proliferation. Both these examples indicate that the manipulation of UPR can be beneficial to certain pathogens and may represent an important effector target but more research in this area is necessary.

AIMS

To better understand plant-pathogen interactions, particularly in the effector biology field, this thesis had two main goals. Firstly, to investigate how abundant effector-effector interactions are and how that might increase the versatility and robustness of a pathogen's effectome. The second objective was to develop a functional screen to identify effectors that interfere with the ubiquitous UPR in plants. To achieve these goals, a library of putative effectors from the model biotrophic fungal pathogen *U. maydis* was used

Abundance of Effector-effector Interactions and its Potential Role in Disease

A few effectors were reported to act synergistically, antagonistically, or require the interaction with other effectors to fully function. In that context, a systematic Y2H screen was performed to explore the abundance of effector-effector interactions in the predicted effectome of *U. maydis*. To understand how the interaction network might change throughout the infection process, publicly available data, including expression patterns and conservation, were integrated and analyzed. Based on the findings, models for effector-effector interaction outcomes were conceptualized.

Development of a Method to Identify Effectors that Interfere With UPR

Interference with plant defenses is the function attributed to most of the effectors studied to this date. Plant-pathogen interactions are known to activate UPR which is linked with the upregulation of genes related to plant immunity. However, very few effectors have been described to interfere with plant UPR. With the objective of streamlining the identification of molecules that interfere with this process, a new high-throughput screening method was developed. The assay was optimized by testing different reporter constructs, UPR induction conditions and relative *A. tumefaciens* strain suspension concentrations. After validation, a

pilot screen with 35 proteins was performed to find if effectors from *U. maydis* could interfere with conserved plant UPR signaling components.

PUBLICATIONS

List of Publications

Publication I

Alcântara, A., Bosch, J., Nazari, F., Hoffmann, G., Gallei, M., Uhse, M., Darino, M. A., Olukayode, T., Reumann, D., Baggaley, L., & Djamei, A. (2019). Systematic Y2H screening reveals extensive effector-complex formation. Frontiers in Plant Science, 10: 1437. doi: 10.3389/fpls.2019.01437.

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Contribution: study design, experimental work, data analysis, and manuscript writing.

Publication I

Systematic Y2H Screening Reveals Extensive Effector-Complex Formation

André Alcântara, Jason Bosch, Fahimeh Nazari, Gesa Hoffmann, Michelle Gallei, Simon Uhse, Martin A. Darino, Toluwase Olukayode, Daniel Reumann, Laura Baggaley, & Armin Djamei

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Systematic Y2H Screening Reveals Extensive Effector-Complex Formation

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During infection pathogens secrete small molecules, termed effectors, to manipulate and control the interaction with their specific hosts. Both the pathogen and the plant are under high selective pressure to rapidly adapt and co-evolve in what is usually referred to as molecular arms race. Components of the host's immune system form a network that processes information about molecules with a foreign origin and damage-associated signals, integrating them with developmental and abiotic cues to adapt the plant's responses. Both in the case of nucleotide-binding leucine-rich repeat receptors and leucine-rich repeat receptor kinases interaction networks have been extensively characterized. However, little is known on whether pathogenic effectors form complexes to overcome plant immunity and promote disease. *Ustilago maydis*, a biotrophic fungal pathogen that infects maize plants, produces effectors that target hubs in the immune network of the host cell. Here we assess the capability of *U. maydis* effector candidates to interact with each other, which may play a crucial role during the infection process. Using a systematic yeast-two-hybrid approach and based on a preliminary pooled screen, we selected 63 putative effectors for one-on-one matings with a library of nearly 300 effector candidates. We found that 126 of these effector candidates interacted either with themselves or other predicted effectors. Although the functional relevance of the observed interactions remains elusive, we propose that the observed abundance in complex formation between effectors adds an additional level of complexity to effector research and should be taken into consideration when studying effector evolution and function. Based on this fundamental finding, we suggest various scenarios which could evolutionarily drive the formation and stabilization of an effector interactome.

Keywords: protein–protein interaction network, effector proteins, *Ustilago maydis*, plant pathogen, yeast-two-hybrid

INTRODUCTION

Molecular interactions play a central role in the disease outcome between a pathogen and their hosts. In plants, pattern recognition receptors (PRRs) on the membrane surface recognize typical damage- or pathogen-associated molecular patterns (PAMPs). Examples of well characterized PAMPs include the flg22 peptide from bacterial flagella, and the cell-wall sugar chitin from fungi and insects, which upon receptor binding lead to the activation of PAMP triggered immunity (PTI) (Jones and Dangl, 2006). To overcome PTI, pathogens secrete small molecules, termed effectors, which have evolved to suppress the host's immune system and create a suitable environment for its development and reproductive success (Buttner, 2016; Toruño et al., 2016; Uhse and Djamei, 2018). However, some of these effectors can be recognized by nucleotide binding–leucine-rich repeat receptors (NLRs) triggering rigorous defense responses that lead to localized cell death in the infected region (Cesari, 2018). This effectively results in a molecular arms race between plants and their pathogens as they must rapidly adapt to increasingly intricate defense and infection strategies.

It is well known that proteins from both classes of the plant's immune system—PRRs and NLRs—rely on interactions between multiple host proteins to neutralize an invading pathogen. One of the most well studied examples of interaction between PRR proteins occurs between the membrane leucine-rich repeat (LRR) receptor kinases FLS2 and BAK1 which, upon flagellin perception, heterodimerize to trigger a rapid immune response through the initiation of a phosphorylation signaling cascade (Chinchilla et al., 2007). The complexity of the interactions that occur between membrane receptors was recently addressed in a study where many of the extracellular LRR domains tested were found to be able to homo and/or heterodimerize (Smakowska-Luzan et al., 2018). In the case of NLR proteins, there have been several effector recognition mechanisms that have been found or hypothesized (Cesari, 2018). However, evidence of an NLR interaction network and its importance for immune signaling has only recently been described with "sensor" NLRs recognizing pathogenic proteins and converging to "helper" NLRs that potentiate the signaling cascade and therefore immune responses (Wu et al., 2017). For example, the recognition of AvrAC from *Xanthomonas campestris* pv. *campestris* causes the uridylation of PLB2 which in turn binds to an NLR from *Arabidopsis thaliana*, ZAR1. This binding results in the pentamerization of PLB2 that ultimately leads to pathogen resistance (Wang et al., 2019). Altogether, the complexity of these protein receptor interaction networks resulted as a direct consequence of the diverse signals that plants integrate and coordinate to adequately respond to the challenges imposed by their environment.

Ustilago maydis is a biotrophic fungal pathogen able to infect all aerial parts of maize plants. Its lifestyle is supported by absorbing nutrients from sink tissues, where it induces the formation of galls and develops spores. Like other pathogenic organisms, *U. maydis* relies on effectors to perform a wide range of tasks, from host defense suppression to manipulation of plant metabolism and development to favor the pathogen's own growth and proliferation. Although hundreds of putative effector proteins are encoded in the *U. maydis* genome, only a few of these have been functionally characterized. Examples include Pep1, which reduces the accumulation of H_2O_2 in the apoplastic space (Doehlemann et al., 2009), Pit2, which inhibits apoplastic cysteine proteases (Mueller et al., 2013), Rsp3, which coats the fungal hyphae preventing the activity of antifungal proteins (AFP) 1 and 2 (Ma et al., 2018), and Cmu1 and Tin2, which were proven to interfere with the production of salicylic acid and lignin, respectively (Djamei et al., 2011; Tanaka et al., 2014). Other virulence factors, such as Stp1, ApB73, and Cce1 were shown to play a role during infection, yet their functions remain elusive (Schipper, 2009; Stirnberg and Djamei, 2016; Seitner et al., 2018). While these studies expanded our knowledge of the mechanisms of biotrophic pathogenesis in plants, considering that the *U. maydis* genome encodes for many putative effector proteins it is clear that the complexity of the host–pathogen interaction is still poorly understood.

The most recent analysis of the *U. maydis* genome identified 467 proteins that are predicted to be secreted, representing almost 7% of its total proteome. Of these, 203 (43%) lack predicted domains which could indicate their function (Schuster et al., 2018). A recent comprehensive transcriptome analysis of *U. maydis* throughout its biotrophic development showed three discrete, tightly regulated expression patterns of these secreted proteins (Lanver et al., 2018). Additionally, there are effectors that are known to have tissue-specific functions. For instance, See1 was linked to DNA synthesis reactivation in the host and is essential for gall formation in leaves but not in floral tissues, where cell division occurs regardless of the infection process (Redkar et al., 2015; Matei et al., 2018). Thus, the localized and temporal regulation of effector protein expression throughout the infection process is crucial for the fungal pathogen to successfully complete its lifecycle.

Considering their relatively limited number of effectors, it is astonishing that biotrophs can overcome the highly complex host immune system and regulate biotrophic virulence in a multicellular host. An interaction network between effectors could provide an additional level of complexity to create a versatile and robust effectome. In fact, few cases of functional characterization of effector homo- and heterodimers from bacteria, oomycetes, and fungi have been reported (Gürlebeck et al., 2005; Djamei et al., 2011; van Damme et al., 2012; Flayhan et al., 2015; Ma et al., 2015; Li et al., 2016). Some of these dimers have even been linked with pathogenicity. For example, the effector PsCRN63 from *Phytophthora sojae* is only able to suppress cell death associated with PTI upon dimerization (Li et al., 2016). Other cases of bacterial effector interplay in the context of function redundancy, antagonistic effects, and in host regulation have been reported and reviewed in Shames and Finlay (2012). Despite this, there have only been a few attempts to systematically characterize interactions within a pathogen's effector repertoire. A screen for metaeffectors (*i.e.* "effectors of effectors") in *Legionella pneumophila* revealed 23 effector pairs with antagonistic effects in yeast cell growth, 10 of which showed direct effector–effector interaction in a yeast-two-hybrid (Y2H) setting (Kubori et al., 2010; Urbanus et al., 2016).

The relevance of protein interactions in plant immunity, the rapid co-evolutionary arms race in plant–pathogen interactions, and the increased versatility that emerges from intermolecular networks suggest that effector dimerization and complex formation could have evolved to improve fitness in *U. maydis*. Using a systematic Y2H approach, we show that homo- and heterodimerization of putative effectors is not only possible but occurs abundantly in the *U. maydis* effectome. These interactions were found between more than a third of all effector candidates tested and analyzed in context of other publicly available data to speculate on how they can affect the functionality of an effectome. Our data shed new light on how fungal effectors can act *in planta* and future functional analyses will need to take into account inter- and intraspecies protein–protein interactions, to advance our understanding of how effectors shape the infection process.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions

DNA manipulation and plasmid generation were performed according to standard molecular cloning procedures (Ausubel et al., 1987; Sambrook and Russell, 2006). All DNA manipulations were performed using the *Escherichia coli* MACH1 strain (Thermo Fisher Scientific, Waltham, MA, USA). Plasmids and primers are compiled in **Tables S1**–**S3**. Some plasmids were generated using the GreenGate system (Lampropoulos et al., 2013). All vector maps containing detailed sequence information are available upon request.

The library of putative effectors was cloned based on the effector prediction analysis described in Mueller et al. (2008). The full list of genes used in this study is compiled in **Table S1**, which includes the gene specific primer sequences used to isolate the genes and the updated signal peptide prediction scores by SignalP v5.0 (Armenteros et al., 2019). All putative effectors were cloned without the predicted signal peptide encoding region into a modified pEntry4b vector either by BspHI–NotI or by NcoI–NotI restriction sites. Prior to pEntry4b cloning, native BspHI, NcoI, and NotI sites in putative effector coding sequences were mutated without affecting the encoded amino acid. The effector candidate-containing pEntry vectors were used to subclone by LR-reaction all putative effectors into the respective modified pGBKT7 and pGADT7 gateway destination vectors (Thermo Fisher Scientific, Waltham, MA, USA).

Yeast Work

Saccharomyces cerevisiae strain AH109 was transformed with pGBKT7 derivatives as previously described (Rabe et al., 2016), using standard protocols (Clontech/Takara Bio, Saint-Germain-en-Laye, France). Strains carrying N-terminal Gal4 DNA binding domain (BD) fusions with putative effectors were tested for autoactivity by growth in minimal synthetic defined (SD) dropout medium and spotted on SD plates depleted of tryptophan, adenine, and histidine (SD-Trp/Ade/His). SD-Trp plates were used as a control for strain viability. pGADT7 derivates with N-terminal Gal4 activation domain (AD) fused to putative effectors from *U. maydis* were transformed into the yeast strain Y187 from the Matchmaker™ GAL4 Two-Hybrid System (Clontech/Takara Bio, Saint-Germain-en-Laye, France).

Yeast strains AH109 containing one of the 274 non-autoactive pGBKT7-effectors were mated with a library of the Y187 yeast strains containing 297 AD-effector candidate fusions. Mating was performed according to the manufacturer's protocol. Diploids carrying both plasmids were selected on SD plates depleted of tryptophan and leucine (SD-Trp/Leu), and dimerization was tested by growth on intermediate (SD-Trp/Leu/His) and high (SD-Trp/Leu/Ade/His) stringency media. 710 colonies from SD-Trp/Leu/Ade/His plates were picked for prey identification and bait confirmation by Sanger sequencing, after colony PCR using standard protocols (Clontech/Takara Bio, Saint-Germain-en-Laye, France).

One-on-one screening was performed in liquid cultures using a Bravo Liquid Handling Platform (Agilent, Santa Clara, California, USA). *S. cerevisiae* strains carrying the 63 non-autoactive pGBKT7 strains that showed interactions in the first screen and all the pGADT7 constructs were grown in liquid SD-Trp or SD-Leu, respectively, before being co-inoculated in PD medium and left overnight to mate. This and all subsequent steps were performed in 96-well tissue culture plates (VWR, Radnor, Pennsylvania, USA). The cultures were moved to SD-Trp/Leu for 1 day to select for successful mating after which the cultures were inoculated in SD-Trp/Leu, SD-Trp/Leu/His, and SD-Trp/Leu/His/Ade and grown for 3–4 days. To determine culture growth, OD_{600nm} was measured with a Synergy 2 automated plate reader (BioTek, Winooski, VT, USA). Mating success was measured by growth on SD-Trp/Leu and successful interactions were defined as growth on all three auxotrophic media above a specified OD_{600nm} threshold: 0.1 for SD-Trp/Leu and 0.25 for the other media.

The workflow and the list of tested putative effectors for the Y2H work can be found in **Figure S1**, and **Tables S1** and **S2**, respectively.

Transient Expression in *Nicotiana benthamiana* and Co-Immunoprecipitation

Effector candidates from a small interaction subnetwork (in focus in **Figures 2** and **S2**) were cloned into plant expression vectors by golden gate cloning using the vectors and the methods described in Lampropoulos et al. (2013). Expression of the putative effectors was controlled by the strong 35S promoter and N-terminally tagged with either HA or C-myc triplicated sequences. All vector maps containing detailed sequence information are available upon request. The vectors were then electroporated into the *Agrobacterium tumefaciens* strain GV3101 and infiltrated into leaves of 4 to 5 week-old tobacco plants. Three days post-infiltration, the plant material was harvested, snap frozen in liquid nitrogen, milled using a Retch Mixer Mill MM 400 (Retsch GmbH, Germany) at 30 Hz for 1 min 30 s, and kept at −80°C until further processing.

The plant powder was resuspended in IP buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, 2% PVPP, and protease inhibitor cocktail) and centrifuged three times at 20,000×*g* to remove solid

debris. C-myc tagged proteins and their interacting proteins were isolated using the μMACS c-myc Isolation Kit (Miltenyi Biotec, Germany) following the manufacturer's manual, using the above described buffer without PVPP for the washes. For Western Blot protein detection, samples were resolved by SDS-polyacrylamide 4–20% gradient gel electrophoresis and transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, CA, USA). C-myc tagged proteins were probed with a mAB α-Myc-tag; clone 9E10 (produced by the Molecular Biology services from the GMI/IMBA/IMP service facilities) and detected by hybridizing with a sheep raised anti-mouse secondary antibody coupled to horseradish peroxidase (HRP; GE Healthcare, IL, USA). HA tagged proteins were detected using the HRP coupled anti-HA antibody raised in mouse (Miltenyi Biotec, Germany). HRP activity was visualized by using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, MA, USA) and imaged in either a ChemiDoc imaging system (Bio-Rad, CA, USA) or on Amersham Hyperfilms ECL (GE Healthcare, IL, USA), depending on protein amounts.

Data Handling and Network Analysis

Data from the various experiments were combined and analyzed using R scripts (R Core Team, 2014). The network analysis and visualization were done using the open-source, JavaScript-based graph library Cytoscape (Franz et al., 2016).

To check that the interactions were not occurring randomly, we calculated the number of interactions which would have been observed in triplicate given the number of interactions detected in each replicate of the screen if the growth were random. We then used the Fisher's exact test to compare the expected and observed number of interactions to see whether our results differed from random growth.

RESULTS AND DISCUSSION

Many Putative Effectors From *U. maydis* Are Highly Interconnected

To identify effectors able to homo- or heterodimerize and to estimate their abundance, we cloned 310 putative effectors of *U. maydis* without their predicted signal peptides into Y2H vectors encoding either an N-terminal Gal4 DNA binding domain (BD; pGBKT7 derivate) or an N-terminal Gal4 activation domain (AD; pGADT7 derivate). After excluding autoactive strains, each of the remaining 274 bait strains were mated against a pool of 297 AD-effector candidate prey strains and incubated on high selection plates. Colony growth was observed on 86 plates. Thus, at least 30% of the putative effectors tested were able to interact with each other in the Y2H. Despite sequencing the pGADT7 inserts from 710 yeast colonies we did not reach screen saturation, revealing that the effector candidate interaction network is more complex than previously thought.

To overcome this issue, we used a robotic system to perform individual matings in liquid medium between the 86 bait putative effectors that showed interactions on plate with each of the 297 AD-effector candidate fusions. This change in methodology led to a reduction in screened bait proteins to 63, either due to technical issues or because the strains exhibited some growth in high-selection liquid medium before mating. This change in autoactivation was most likely caused by the known difference of growth rates in liquid *vs* solid media (Herricks et al., 2017), and by having an OD_{600nm}-based threshold for the liquid cultures rather than a subjective visual inspection of growth on plates. With this final protein set, bait putative effectors were transformed and mated independently three times. Interactions with prey strains were only considered valid if they were reproduced in all three matings. This resulted in an interaction matrix of 126 putative effector proteins producing 867 unique potential interactions (**Figure 1**), representing a highly connected network of protein interactions between almost 40% of the tested proteins. The number of interactions per putative effector varies between 1 (for 26 putative effectors) and 63 (in the case of UMAG_03201), and 12 putative effectors showed the ability to form homodimers. In the few instances where interactions were tested reciprocally, we observed 26 interactions where the swap of bait and prey domains did not influence the observed interaction. Altogether, this highly connected effectome can lead to increased versatility and robustness in the context of the molecular arms race between plants and their pathogens.

By integrating publicly available datasets with the interaction matrix, we identified other emerging features of this interactome regarding genome clustering, relevance in infection, and conservation in closely related species. More than 18% of the putative effector encoding genes from *U. maydis* are clustered in the genome (Kämper et al., 2006) and it has been observed that co-localization in these genomic islands allows for transcriptional co-regulation and might implicate involvement in similar biological processes. However, our network has only 61 interactions between putative effectors encoded in the same chromosome that are on average approximately 350 kbp apart, therefore not showing a bias for effector candidates to interact with others within the same cluster. Uhse et al. (2018) established a next-generation sequencing-based screening method that enabled the identification of new virulence factors of *U. maydis*. Ten of the 28 virulence factors identified in that screen showed potential interactions with multiple other putative effectors (**Figure 1**). This could explain why these proteins are important for virulence as multiple other effectors might be in a complex with them to fulfil their roles during infection. Finally, when looking at conserved effectors between closely related pathogens (*i.e.* core effectors; Schuster et al., 2018) there was no overrepresentation of interactions between only core or non-core effectors (**Figure 1**). This means that effector–effector interactions are not abundantly conserved among smut fungi and, therefore, result from adaptation to specific host–pathogen interactions. Nevertheless, it would be interesting to address the relevance of the interactions between any given core effector pair by testing whether interactions are also formed between the orthologs and are therefore a conserved feature. This could help focus functional studies of effector–effector interactions on those with higher likelihood of biological significance.

Although Y2H assays have been widely used to identify host protein-effector interactions and enabled significant advances

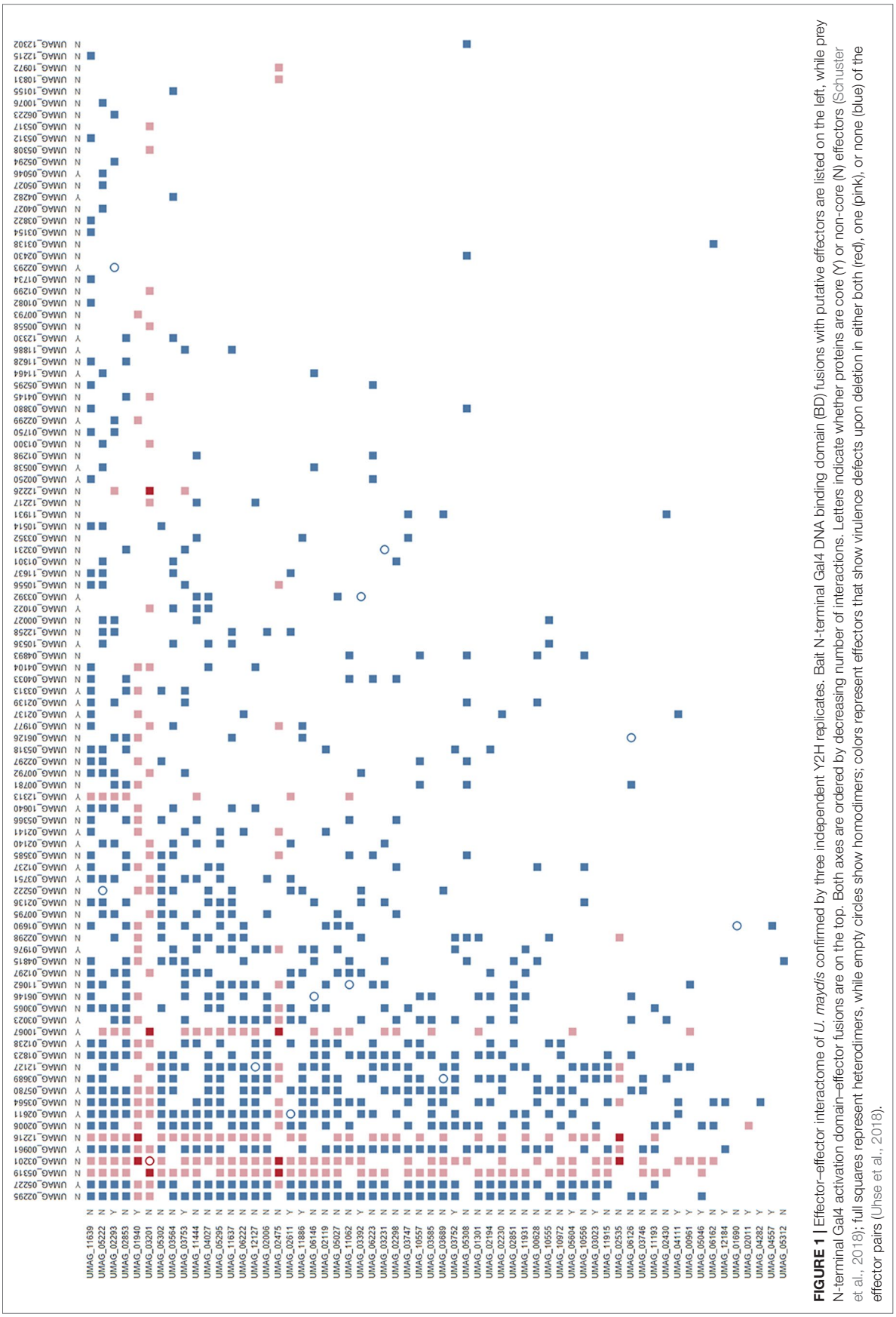

FIGURE 1 | Effector–effector interactome of *U. maydis* confirmed by three independent Y2H replicates. Bait N-terminal Gal4 DNA binding domain (BD) fusions with putative effectors are listed on the left, while prey N-terminal Gal4 activation domain–effector fusions are on the top. Both axes are ordered by decreasing number of interactions. Letters indicate whether proteins are core (Y) or non-core (N) effectors (Schuster et al., 2018); full squares represent heterodimers, while empty circles show homodimers; colors represent effectors that show virulence defects upon deletion in either both (red), one (pink), or none (blue) of the effector pairs (Uhse et al., 2018).

in the field of effector biology (Mukhtar et al., 2011; Weßling et al., 2014), the methodology has its limitations as it forces co-expression and co-localization of the two proteins tested for interaction. This limitation may result in some false positives among the interactions found. However, it is important to note that based on the number of interactions detected in each of the three screens, if the interactions were to occur purely by chance we would expect only 48 interactions to be confirmed across the three replicates. Instead, we found 867 interactions, which is significantly more than if the observed interactions were to occur randomly ($p = 1.16 \times 10^{-200}$). This increases the confidence in these results and indicates that interactions between proteins from the *U. maydis* putative effectome are seemingly highly complex (**Figure 1**). On the other hand, considering the exclusion of putative effectors with autoactivity from our screen and that the Gal4 activation and DNA-binding domains may interfere with the ability of some putative effectors to interact, it is likely that some meaningful interactions were not detected and the effector candidate interactome presented here might still be underestimated.

Attempts to confirm some of these interactions during infection proved to be extremely difficult due to the low concentration of specific putative effectors as a result of the relatively insignificant fungal biomass in comparison to the maize tissue. In fact, RNA-seq data from maize infected tissue showed that fungal RNA represents less than 5% of total transcript abundance at 8 days post infection (dpi; Lanver et al., 2018). In order to not disturb the fine balance of protein expression and therefore their interactions, our efforts focused on using knockout strains of specific putative effectors complemented by in-locus recombination of tagged versions under control of the endogenous promoters. Western blots from co-immunoprecipitation samples of infected tissues proved to be below the detection threshold for the specific interactions tested (data not shown). Therefore, an improved method of protein detection from infected tissue will be needed to independently validate the interactions in maize.

Regardless of the mentioned limitations, we took a subset of one of the subnetworks shown in **Figures 2** and **S2**, and tried to confirm 12 of its interactions using an alternative method. Protein pairs with either an HA or c-myc triple tag were transiently co-expressed in *N. benthamiana*, followed by co-immunoprecipitation (co-IP, **Figure S3**). We used the c-myc tagged proteins as bait and a c-myc tagged mCherry construct as a negative control to exclude the possibility of false positive interactions from technical constraints (samples 1, 2, and 3). Within the subnetwork, several interactions found previously by Y2H could be confirmed (namely for the interactions between UMAG_03201 and UMAG_03689, UMAG_05227 and UMAG_03564, UMAG_05780 and UMAG_03689, UMAG_05780 and UMAG_03201, and UMAG_03689 and UMAG_03564). The results for the remaining tested interaction pairs did not overlap with the Y2H data which could be due to inherent limitations in both techniques. For instance, in both cases, the proteins are not secreted as they would be in the native system and are expressed with tags that can differently interfere with their stability, solubility and function. While both methods rely on the heterologous expression of proteins, the yeast expression system is phylogenetically closer to *U. maydis*, which potentially influences expression of some proteins and could be one reason for the observed discrepancies between the results. The major conclusion from integrating the information obtained from both, the Y2H and the co-immunoprecipitation approach, is that a great number of effectors should not be characterized in isolation but instead in context with their respective potential interacting co-effectors. The characterization of all interactions would be well beyond the scope of this study, but the integration of the network we identified opens the possibility to further test single interactions and represents a valuable resource for future effector research.

Change in Expression Profiles Throughout Infection Reveals Network Dynamics

Effector expression is tightly regulated and commonly occurs in waves during the course of infection (Toruño et al., 2016). These expression profiles enable the pathogen to finetune the plant's defense and metabolism along its lifecycle to create a favorable environment for its development. By combining our data with the recently published RNA-seq data of infected maize leaves at different timepoints (Lanver et al., 2018) we were able to see how interactions might change during the infection process. As effectors are expressed at different levels during the infection process, they may form different dimers or even complexes with different functions throughout the biotrophic stages of the pathogen. **Figure 2** shows how expression levels change within the global network at two different infection stages: 1 and 6 dpi.

By focusing on specific proteins, this network plasticity becomes more obvious. For instance, UMAG_03065 interacts directly with 10 other putative effectors, with only two of them not being expressed in the earlier infection stage (**Figures 2** and **S2**). However, at the later timepoint, more protein coding sequences within this network seem to be downregulated, resulting in fewer possible interactions. In the case of UMAG_00628, the central protein in this network has lower expression at 6 dpi, opening the possibility for the peripheral proteins in this subnetwork to interact with each other (**Figures 2** and **S2**). Both examples highlight the changing interaction network and suggest an additional level of plasticity in the *U. maydis* effectome from the interplay between gene expression patterns and protein–protein interactions. In addition to gene expression, this plasticity can be further increased by the affinity of the interaction. Given different interaction partners, more dimers will be formed between the proteins that have a higher binding affinity. Finally, effectors that are translocated through different compartments are subjected to different conditions (*e.g.* pH) that can change the affinity of two proteins to bind to each other. Thus, depending on the subcellular localization of the proteins in the network their affinity, and therefore their function, can be affected.

It is important to note that this overlay of interaction with expression data can reveal observed interactions that might not relate to a biological function, in cases where a protein pair does not show co-regulation during the life cycle of *U. maydis*. However,

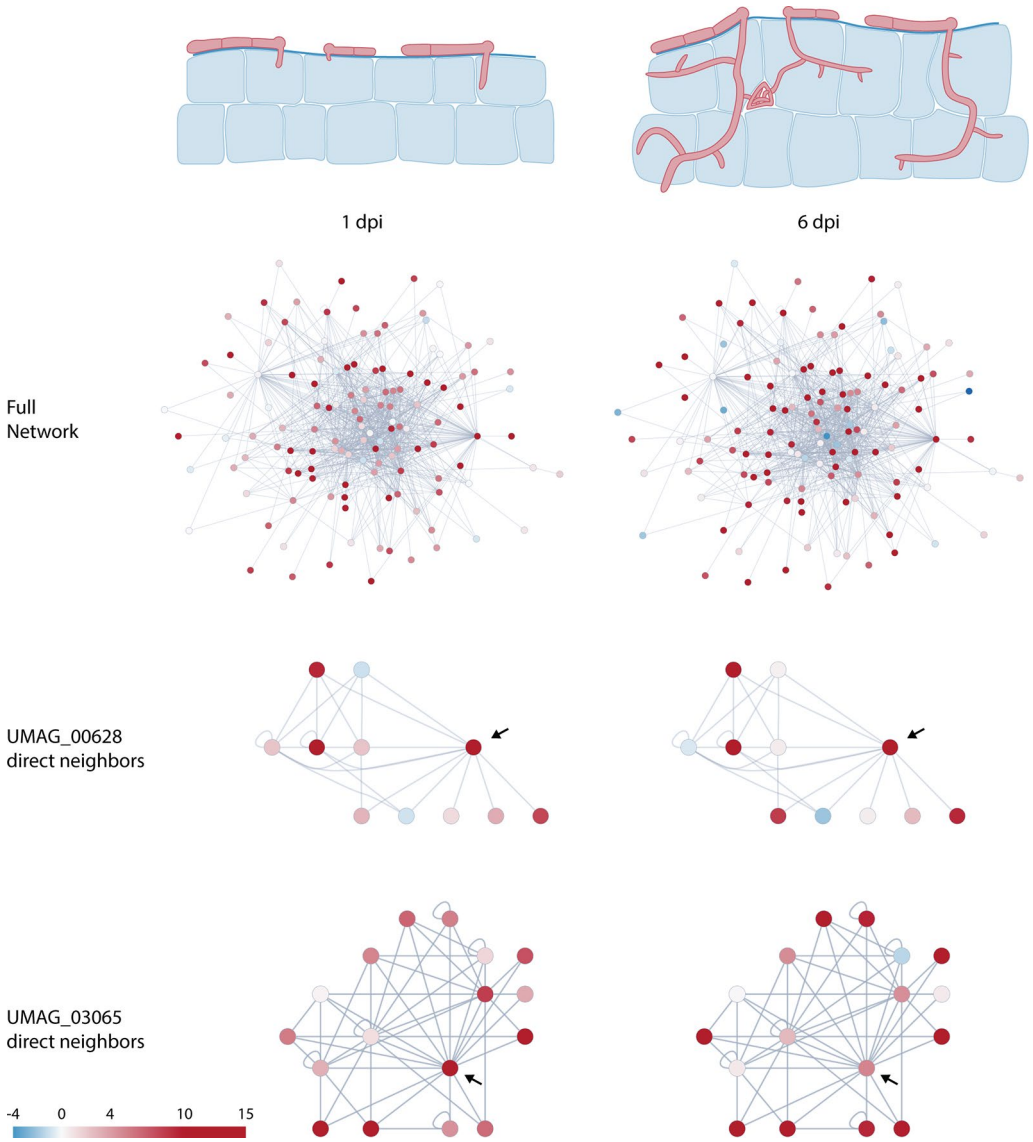

FIGURE 2 | Schematic representation of *U. maydis* infection at 1 and 6 days post infection (dpi) and changes in predicted protein–protein interaction network dynamics of putative effectors. Nodes are colored according to Log_2 fold change relative to axenic culture (Lanver et al., 2018). Arrows in the subnetworks indicate network centers.

it is equally relevant to mention that other factors influence effector gene expression, such as host tissue specificity. Using microarrays, it was found that at 3 dpi only 21% of upregulated *U. maydis* effector genes were expressed in three different maize tissues while 45% were expressed in only one type of tissue (Skibbe et al., 2010). Therefore, some of the interactions found here are probably relevant in a tissue specific context, rather than the infection stage.

Functional Models of Effector Interactions

There are many evolutionary driving forces that can lead to the stabilization of effector–effector interactions. In **Figure 3** we speculate on a few possible outcomes from interactions between effectors and propose three possible models. Plants evolved NLRs for direct or indirect effector recognition leading to effector-triggered immunity (ETI; Jones and Dangl, 2006; Macho and Zipfel, 2014; Wu et al., 2017). It is therefore feasible that effectors have evolved to interact and compete for receptor recognition sites that would activate ETI, in what is referred to as the "protector model" in **Figure 3**. In this example, effector 1 is recognized by a plant NLR and will trigger ETI. However, upon interaction with effector 2, the site that the plant NLR recognizes is blocked and the pathogen can continue the infection process. This mechanism could also lead to the protection of effectors from plant proteases or other possible protein modifications that would impede their function or target them for degradation.

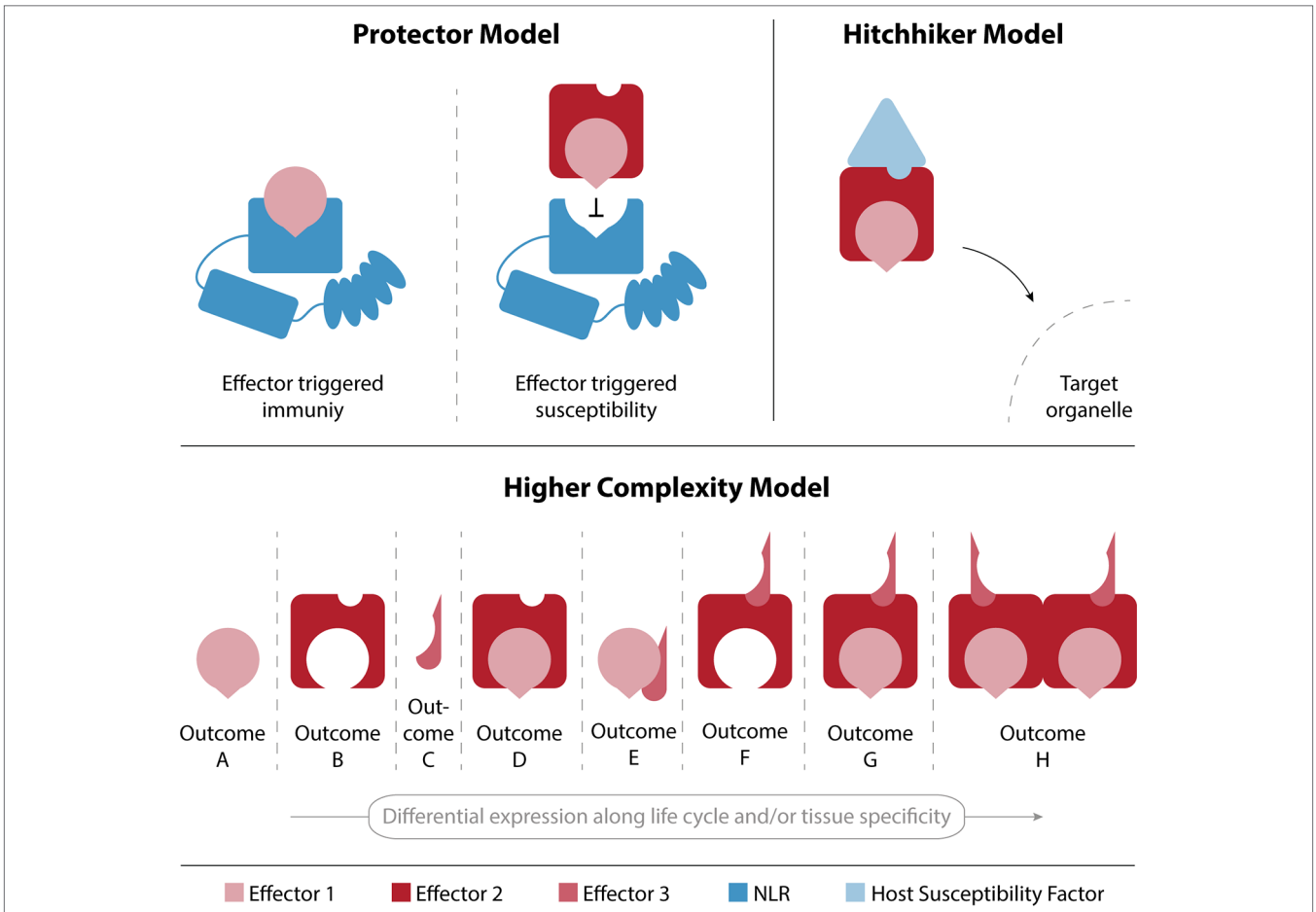

FIGURE 3 | Models for effector-effector interaction outcomes. The protector model describes an interaction between effectors 1 and 2, which results in the avoidance of recognition of effector 1 by the plant's immune system and therefore leads to a successful infection. In the hitchhiker model, effector 1 is able to shuttle to its target organelle by interacting with effector 2, which in turn interacts with a plant susceptibility factor that mediates the shuttling upon effector binding. The higher complexity model highlights the plasticity that can emerge by differential effector expression along the pathogen's lifecycle and/or in specific tissues.

Another possibility would be that an effector interacts with the host cellular machinery to shuttle with it to a specific subcellular compartment and fulfil its biological role. The "hitchhiker model" represents this effector as an interaction hub with other effectors that can shuttle with it to the same subcellular destination. In the representation given in **Figure 3**, the localization of effector 2 is determined by its interaction with a plant susceptibility factor, and its ability to bind to other effectors—such as effector 1—results in the shuttling of multiple effectors to a target organelle in host cells. The relevance of interaction-dependent protein localization to subcellular compartments is well known and has even been shown to play an important role in a similar interactome dataset of endosomal sorting complexes, required for transport (ESCRT) proteins in *A. thaliana* (Richardson et al., 2011). This mechanism would allow for the evolution of a very efficient transporter that acts as a hub for effector shuttling, rather than having localization signals in all effector proteins.

Finally, the presence of different effectors can lead to different outcomes depending on their spatial and temporal distribution. **Figure 2** shows the plasticity of interaction networks that can be created along the infection process as a direct consequence of effector expression patterns. It is reasonable to assume that the interactions between effectors described here, lead to an increased phenotypical complexity shown in the combinatory model of **Figure 3**. The illustration of the "higher complexity model" shows how outcomes can vary by changing the expression of three proteins either in different tissues or along the pathogen's life cycle. This model has recently been suggested by Thordal-Christensen et al. (2018), and the overlay of our data with the publicly available expression profile supports such a scenario. The exact nature of those outcomes remains to be determined and further research is needed to shed new light into the full extent of the plasticity that interactions between effector proteins can confer.

The network presented here is neither complete nor will all Y2H verified interactions play a biological role. Nevertheless, it provides a valuable framework for future *U. maydis* effector studies and widens our view on the consequences of the

co-evolution between the host immune system and the effectome of the pathogen. The extent of the interactome shows that effector biology is more complex and intricate than previously thought and the possibility of effector–effector interactions should not be neglected when studying plant–pathogen interactions.

CONCLUSION

Protein–protein interactions are crucial for diverse biological functions across all lifeforms. While increasing evidence suggests extended protein interaction networks among plant immune receptors, little focus has been put on protein interactions between virulence factors that have co-evolved with it. Here we show evidence of complex effector–effector interactions in *U. maydis* that seem to mirror the intricate networks found in plant immune systems. Despite the limitations of the Y2H methodology, the *U. maydis* effectome shows a surprisingly high number of interactions between secreted proteins. In combination with temporal and spatial regulation, future functional characterization of effectors will need to take into consideration the possibility of effector–effector interactions and their role in the infection process.

DATA AVAILABILITY STATEMENT

All datasets for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

AD conceived the original research plan and designed the experiment. AA, JB, FN, GH, MG, SU, MD, TO, DR, and LB contributed to the experimental work. JB coordinated the experimental work and tested the interactions. JB and AA did the data analysis. AA, AD, and JB wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01437/full#supplementary-material

TABLE S1 | List of putative effectors used as either bait and/or prey proteins. Gene specific primer sequences used to clone the coding sequences, signal peptide prediction scores, and peptide length are also listed.

TABLE S2 | Identity of bait proteins used in the liquid media screen. Baits were tested for autoactivity by growth in liquid culture and on plates. In the mating test (SD-Leu/-Trp/-His/-Ade) the bait strain was mated with yeast containing an empty pGADT7 plasmid. In liquid tests, strains were considered autoactive when OD600 was higher than 0.25 after 3 days of incubation at 28oC.

TABLE S3 | Primers used for sequencing of inserts (effectors) after gateway reaction.

FIGURE S1 | Workflow of Y2H work. Numbers represent coding sequences of putative effector proteins tested. See **Supplementary Tables S1, S2,** and **S3** for further details.

FIGURE S2 | Detailed description of the subnetworks represented in **Figure 2**. Core effectors were identified in Schuster et al. (2018), clusters were described in Kämper et al. (2006), iPool-Seq data was obtained from Uhse et al. (2018), and sequencing data was taken from Lanver et al. (2018). The centers of the networks are highlighted in bold; circles represent homodimers and squares represent heterodimers.

FIGURE S3 | Co-immunoprecipitation of 12 proteins from the UMAG_00628 subnetwork. Proteins were tagged with either 3x myc or 3x HA N-terminal tags, which was the same side of the activation and binding domains in the Y2H screen. Nicotiana benthamiana plants were transiently transformed and expressed the fusion proteins for 3 days before harvest. On the left, the interactions found by Y2H in the subnetwork subset are illustrated. Full blue boxes with white numbers represent expected interactions, empty boxes with black numbers represent protein pairs that are not expected to interact, and circles represent homodimers. On the right are the same interactions tested by Co-immunoprecipitation. The sample numbers from the Y2H matrix for each interaction pair are represented on top of the western blots.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publication II

A High-throughput Screening Method to Identify Proteins Involved in Unfolded Protein Response Signaling in Plants

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A High-throughput Screening Method to Identify Proteins Involved in Unfolded Protein Response Signaling in Plants

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Abstract:	Background The unfolded protein response (UPR) is a highly conserved process in eukaryotic organisms that plays a crucial role in adaptation and development. While the most ubiquitous components of this pathway have been characterized, current efforts are focused on identifying and characterizing other UPR factors that play a role in specific conditions, such as developmental changes, abiotic cues, and biotic interactions. Considering the central role of protein secretion in plant pathogen interactions, there has also been a recent focus on understanding how pathogens manipulate their host's UPR to facilitate infection.Results We developed a high-throughput screening assay to identify proteins that interfere with UPR signaling in planta. A set of 35 genes from a library of secreted proteins from the maize pathogen Ustilago maydis were transiently co-expressed with a reporter construct that upregulates enhanced yellow fluorescent protein (eYFP) expression upon UPR stress in Nicotiana benthamiana plants. After UPR stress induction, leaf discs were placed in 96 well plates and eYFP expression was measured. This allowed us to identify a previously undescribed fungal protein that inhibits plant UPR signaling, which was then confirmed using the classical but more laborious qRT-PCR method.Conclusions We have established a rapid and reliable fluorescence-based method to identify heterologously expressed proteins involved in UPR stress in plants. This system can be used for initial screens with libraries of proteins and potentially other molecules to identify candidates for further validation and characterization.
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Suggested Reviewers:	Kai Heimel, PhD Professor, Georg-August-Universität Göttingen kheimel@gwdg.de Dr. Heimel works on proteins from U. maydis that have a role in (fungal) UPR. Our pilot screen used a library of effectors from this organism to test for UPR interference.
	Juan Antonio Garcia Martin, PhD Group Leader, Centro Nacional de Biotecnologia ja.garcia@cnb.csic.es Dr Garcia published a paper in Plant Methods titled "Rapid fluorescent reporter quantification by leaf disc analysis and its application in plant-virus studies" (doi: 10.1186/1746-4811-10-22). This seems very much in line with what our manuscript tries to address.
Opposed Reviewers:	
Additional Information:	
Question	**Response**
Is this study a clinical trial?<hr>A clinical trial is defined by the World Health Organisation as 'any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes'.	No

A High-throughput Screening Method to Identify Proteins Involved in Unfolded Protein Response Signaling in Plants

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Abstract

Background: The unfolded protein response (UPR) is a highly conserved process in eukaryotic organisms that plays a crucial role in adaptation and development. While the most ubiquitous components of this pathway have been characterized, current efforts are focused on identifying and characterizing other UPR factors that play a role in specific conditions, such as developmental changes, abiotic cues, and biotic interactions. Considering the central role of protein secretion in plant pathogen interactions, there has also been a recent focus on understanding how pathogens manipulate their host's UPR to facilitate infection.

Results: We developed a high-throughput screening assay to identify proteins that interfere with UPR signaling *in planta*. A set of 35 genes from a library of secreted proteins from the maize pathogen *Ustilago maydis* were transiently co-expressed with a reporter construct that upregulates enhanced yellow fluorescent protein (eYFP) expression upon UPR stress in *Nicotiana benthamiana* plants. After UPR stress induction, leaf discs were placed in 96 well plates and eYFP expression was measured. This allowed us to identify a previously undescribed fungal protein that inhibits plant UPR signaling, which was then confirmed using the classical but more laborious qRT-PCR method.

Conclusions: We have established a rapid and reliable fluorescence-based method to identify heterologously expressed proteins involved in UPR stress in plants. This system can be used for initial screens with libraries of proteins and potentially other molecules to identify candidates for further validation and characterization.

Keywords

Unfolded protein response (UPR), high-throughput, *Nicotiana benthamiana*, transient expression, *Ustilago maydis*.

Background

The unfolded protein response (UPR) is a conserved mechanism across eukaryotic organisms for maintaining homeostasis in the endoplasmic reticulum (ER). Proteins from the secretory pathway are translated into the ER where they acquire their native folding and undergo posttranslational modifications. Then, the proteins are shuttled to other organelles, for further processing, or directly to their target compartment, to fulfil their functions. Due to their sessile nature, plants rely heavily on the secretory pathway to respond to changes in, and interact with, their environment. A change in environmental stimuli can lead to significant changes in a cell's transcriptional programing, which in turn cause an overloading of the ER with newly synthesized proteins. These overwhelm the chaperones within it, leading to the accumulation of unfolded proteins, which causes ER stress (Chakraborty et al., 2016; Nawkar et al., 2018; Strasser, 2018). Examples of environmental factors that can lead to UPR include temperature changes, ionic and osmotic stresses, high light, heavy metal toxicity, and biotic interactions (Gao et al., 2008; Liu et al., 2007; Meng et al., 2017; Moreno et al., 2012; Nawkar et al., 2017; Valente et al., 2009; Zhang et al., 2019). Together with changes in developmental programming, these deviations from cellular homeostasis can lead to protein oxidation and/or defects in protein glycosylation that lead to their denaturation and accumulation in different organelles, including the ER, leading to stress.

In plants, there are at least two different mechanisms by which ER stress can be perceived and activate a signaling cascade that triggers UPR. In the Inositol-requiring enzyme 1 (IRE1) pathway, luminal binding proteins (BiPs) interact with the ER-membrane protein IRE1 in the ER

lumen. When unfolded proteins accumulate, they are bound by BiPs, releasing IRE1 proteins that then form dimers which unconventionally splice basic leucin zipper (bZip) 60 mRNAs in the cytosol. The spliced mRNA translates into a functional transcription factor that shuttles to the nucleus and promotes the upregulation of genes that contain UPR responsive elements (UPREs) and ER stress elements (ERSEs) in their regulatory regions (Hayashi et al., 2013; Mori et al., 1996; Sun et al., 2013). The other UPR signaling pathway involves the ER-membrane bZips 17 and 28, which are also bound by BiPs. Upon their release, they are transported to the Golgi apparatus. There, two proteases cleave the full length protein – the site 1 protease (S1P) in the C-terminal region inside the Golgi and the site 2 protease (S2P) in the cytosol – releasing the transcription factor which then migrates to the nucleus and upregulates ER stress genes (Gao et al., 2008). Both signaling pathways ultimately lead to the upregulation of genes to either correctly fold or degrade misfolded proteins, and to regulate transcription and translation to restore ER homeostasis (Iwata et al., 2010a; Srivastava et al., 2018). Transient ER stress can be relieved by the UPR, while persistent ER stress may lead to programmed cell death (PCD; Moreno & Orellana, 2011).

Some of the downstream targets of UPR signaling include genes related to plant immunity. Biotic stresses cause dramatic changes in the host's transcriptional programing that lead to UPR (Moreno et al., 2012; Xu et al., 2019). Depending on their lifestyle, plant pathogens evolved mechanisms to either promote PCD – in the case of necrotrophic organisms – or to inhibit it and other immune responses – in the case of biotrophs. It is therefore not surprising that plant UPR components were recently reported as targets of the molecules pathogens secrete to control their host (i.e. effectors). For instance, after determining that the *Phytophthora sojae* effector Avh262 was required for full virulence, Jing et al. (2016) transiently expressed it in *N. benthamiana* fused to a green fluorescent protein. Co-immunoprecipitation followed by mass spectrometry revealed

that PsAvh262 binds to BiP proteins and further experiments showed that stabilization of this target dampens plant resistance. More recently, the *Phytophthora capsica* effector Avr3a12 was found to interact with FKBP15-2, a plant peptidyl-prolyl cis-trans isomerase which was found to be required for ER stress mediated immunity (Fan et al., 2018). However, the lack of a method for screening proteins that interfere with plant UPR has made it difficult to identify effectors in other pathogens that might play a role in this process.

Though the conserved pathways of UPR signaling in plants have been described, a number of factors involved in its regulation remain to be characterized. Due to its central role in various stress responses, methods for identifying UPR modulators in specific conditions are crucial to advance our understanding of this cellular mechanism. Chen & Brandizzi (2013) described different ways of inducing ER stress in *Arabidopsis thaliana* plants and measure their effects through quantitative polymerase chain reaction (qPCR) measurement of UPR target genes. Another method was described by McCormack et al. (2015) who developed a screening assay to test the sensitivity of *A. thaliana* seedlings to tunicamycin (Tm) – an N-glycosylation inhibitor that causes ER stress and triggers UPR – in response to different stimuli and/or with different genetic backgrounds. Additionally, other authors have adapted protocols to investigate the specific role of their proteins of interest in UPR (Hayashi et al., 2013; Liu & Howell, 2010; Meng et al., 2017; Nawkar et al., 2017) but a simple, reliable, high-throughput method to identify new proteins, and potentially other small molecules or environmental conditions, involved in this mechanism is yet to be reported.

Here we report a method for screening proteins, and potentially other molecules or conditions, that influence plant UPR. This method relies on fluorescence measurements of *Nicotiana benthamiana* leaf discs transiently expressing two genetic constructs. One of them expresses the protein of interest, while the second plasmid encodes an ER-stress responsive promoter

controlling the expression of enhanced yellow fluorescent protein (eYFP). By using a subset of proteins from a library of secreted proteins (*i.e.* putative effectors) from the maize pathogen *Ustilago maydis*, we were able to identify one protein that inhibits UPR signaling in plants. After validation by more classical, laborious methods, this simple approach allows for the screening and identification of new players in plant UPR that may have a role in specific conditions.

Results

A fluorescence-based assay to measure UPR stress

We developed a method that measures relative UPR stress and signaling in plants (Fig. 1). By co-expressing a reporter construct and a protein of interest, interference in UPR signaling can be assessed and new players in this cellular mechanism can be identified. Additionally, the same reporter plasmid could be used to assess the influence of other molecules or environmental conditions on UPR signaling.

In brief, candidate genes are cloned in an expression vector under the control of the CaMV 35S promoter (p35S). An mCherry (mCh) fluorophore coding sequence is cloned in frame with the candidate gene but is separated by the porcine teschovirus-1 2A (P2A) self-cleaving peptide (Kim et al., 2011). This results in strong expression of the proteins of interest with a small C-terminal tag – which minimizes interference with the native folding and function – and the separate expression of a fluorophore in equimolar amounts. mCh fluorescence is then used as a proxy for transformation efficiency and relative protein expression levels. A library of constructs with proteins of interest can easily be generated to efficiently test for UPR interference. A construct with a second mCh coding sequence instead of the gene of interest is used as a reference (*i.e.* a construct that does not interfere with UPR signaling). Each construct is

electroporated into *Agrobacterium tumefaciens* strains and co-infiltrated in *N. benthamiana* plants with a reporter construct expressing eYFP under the control of the ER stress inducible promoter pBIP1. Two days after infiltration, the same *N. benthamiana* leaves are infiltrated with either 0.5% DMSO, as a mock treatment, or 5 μg/mL of tunicamycin (Tm), to induce ER stress and UPR signaling. Approximately 24 hours after the second infiltration, leaf discs are sampled and floated on water in 96 well plates. eYFP and mCh fluorescence are then measured in a plate reader. By comparing eYFP fluorescence in the samples expressing the proteins of interest with eYFP fluorescence in the mCh-P2A-mCh reference construct, novel candidate factors influencing UPR signaling can be identified.

Reporter optimization

To establish the assay presented in Fig. 1, several conditions were tested and optimized to guarantee the reliability of the assay. First, a suitable UPR responsive promoter had to be identified which shows sufficient strength and high reproducibility in its response to UPR stress. We cloned the promoter regions from four genes that had been reported to be upregulated in ER stress conditions: S-phase kinase-associated protein 1 (SKP1; LOC107761682), bZIP60 (LOC109230966), BIP1 (AT5G28540), and BIP3 (AT1G09080; Iwata & Koizumi, 2005; Ye et al., 2013). These promoters were cloned into plant destination vectors regulating the expression of eYFP, electroporated into *A. tumefaciens*, and infiltrated into *N. benthamiana* leaves. Two days later, we infiltrated the same leaves with 5 μg/mL Tm to induce UPR and measured eYFP levels approximately 24 hours after the second infiltration (Fig. 2A). The regulatory region of SKP1 was the only one that did not lead to a significant increase in eYFP fluorescence after UPR induction. From the remaining promoters, bZIP60 showed the highest fold change of eYFP expression under ER stress conditions (6.03 ± 2.41), followed by BIP1 (5.57 ± 2.19), and BIP3

(4.27 ± 3.51). Considering the high variability observed for pBIP3 and the low fluorescence levels in samples with the bZIP60 promoter, we concluded that pBIP1::eYFP was the most suitable construct for this method. Therefore, all remaining optimization steps were performed using pBIP1::eYFP as the reporter construct.

The second factor we optimized was the measurement time after UPR induction. We tested samples at 6, 12, 24, and 48 hours after 5 µg/mL Tm infiltration and compared them to the mock treated samples (Fig. 2B). The timeseries shows a gradual increase in eYFP fluorescence after UPR induction, with the 48 hour timepoint showing overwhelming eYFP levels. In fact, the gain of the detector had to be reduced from 100 to 90 in order to avoid overflow of the signal in these samples, making the arbitrary fluorescence units not directly comparable to the earlier timepoints. However, by comparing the fluorescence fold change between mock and Tm treated plants, we established that there was no further relative induction of promoter activity between the 24 (5.11 ± 1.17) and 48 (5.14 ± 1.57) hour time point. Due to the lower variability in samples measured 24 hours after UPR induction, we decided to use this timepoint in all subsequent experiments.

After determining that the regulatory region of BIP1 displayed a good signal to noise ratio after 24 h of ER stress, we determined the optimal Tm concentration to induce promoter activity. By infiltrating different Tm concentrations in plants transiently expressing eYFP under regulation of the BIP1 promoter, we observed the highest eYFP fluorescence and lowest variation with 5 µg/mL Tm (Fig. 2C). Therefore, this concentration was used for all remaining experiments.

Next, we tested the influence of the ratio between the p35S::mCh-P2A-mCh expression construct and the pBIP1::eYFP reporter vector. Fig. 2D shows the influence of different optical densities at 600 nm ($OD_{600\ nm}$) culture ratios in eYFP expression upon ER stress induction. A 1:2 ratio of for pBIP1::eYFP to p35S::mCh-P2A-mCh ($OD_{600\ nm}$ = 0.1 and 0.2, respectively) showed the lowest eYFP expression induction. When compared to the other samples however, it showed

a similar fluorescence fold change and lower variation (4.40 ± 0.79). An equal ratio of both plasmids ($OD_{600\ nm}$ = 0.2) resulted in a 4.28 ± 1.52 fold change, while a 2:1 ratio of pBIP1::eYFP to p35S::mCh-P2A-mCh ($OD_{600\ nm}$ = 0.2 and 0.1, respectively) led to a 5.47 ± 1.27 fluorescence increase. Importantly, samples in which the reporter plasmid had a lower $OD_{600\ nm}$ relative to the expression plasmid had significantly higher mCh fluorescence (Fig. 2E). Thus, a 1:2 ratio of pBIP1::eYFP to p35S::mCh-P2A-mCh ($OD_{600\ nm}$ = 0.1 and 0.2, respectively) leads to similar eYFP induction, while allowing for higher expression of candidate genes. It is also important to note that eYFP induction upon UPR was lower in this assay when compared to the previous experiments. This is likely due to competition in the transient production of two proteins as opposed to one. Nonetheless, in these conditions, eYFP is more than four times more abundant in ER stressed plant leaves.

Confirmation of UPR induction and proof of principle

To confirm that the assay conditions tested in Fig. 2 and reporter fluorescence correlated with UPR onset, we measured the expression of marker genes by qRT-PCR (Fig. 3A). To that end, the control p35S::mCh-P2A-mCh construct was co-expressed with the pBIP1::eYFP reporter plasmid and the expression of bZIP60, CNX1, SKP1, and PR1 (Chen & Brandizzi, 2013; Hamorsky et al., 2015; Shen et al., 2017; Ye et al., 2011) were measured in both mock and Tm infiltrated leaves. Three of the four marker genes showed a statistically significant upregulation after Tm-induced UPR. In the case of PR1, there seems to be higher expression in UPR conditions but the variability in the dataset and low sample numbers likely led to the observed lack of statistical significance. Nonetheless, this more traditional qRT-PCR based UPR measurement confirmed that the conditions we optimized for our fluorescence-based method leads to ER stress.

Finally, we tested whether our conditions can detect UPR interference using proteins known to be involved in UPR signaling. We co-infiltrated the pBIP1::eYFP reporter construct with either: p35S::mCh-P2A-mCh, as a reference for unaltered UPR signaling; p35S::IRE1a (AT2G17520), which leads to the upregulation of UPR-related genes; or p35S::HY5 (AT5G11260), which is involved in the downregulation of ER stress genes (Fig. 3B; Iwata & Koizumi, 2005; Koizumi et al., 2001; Nawkar et al., 2017). In mock treated samples, we saw a significant induction of eYFP expression caused by the overexpression of IRE1a, showing that this method is capable of identifying proteins that induce UPR signaling. Co-infiltration of Elongated Hypocotyl 5 (HY5) led to a reduction in eYFP upregulation in both mock and Tm treated samples. Taken together, these data show that our method provides a good resolution for identifying proteins that interfere with UPR in plants.

Library screen and new UPR-interfering protein identification

After optimizing the method with proteins known to have a role in UPR, we aimed to identify novel proteins involved in ER stress signaling. Recent studies showed that some pathogenic effectors can interfere with plant UPR (Fan et al., 2018; Jing et al., 2016). We used a subset of 35 proteins from a library of putative effectors from the biotrophic fungal pathogen *U. maydis* to test whether our method could link any of them to UPR signaling (Fig. 4). In both mock and Tm treated samples, we observed relatively high eYFP fluorescence variation between samples. We therefore decided to apply a strict significance threshold of $p \leq 0.01$ in our ANOVA tests. In DMSO (mock) infiltrated plants, only the expression of UMAG_02826$_{23\text{-}399}$ – a putative effector expressed without its signal peptide – led to highly significantly increased eYFP fluorescence in *N. benthamiana* cells (Fig. 4A). On the other hand, under ER stress conditions, six putative

effectors downregulated eYFP expression, four of which were highly significantly different from the mCh control ($p \leq 0.001$; Fig. 4B).

To confirm these results, we repeated the fluorescence-based assay on the four putative effectors that showed highly significant downregulation of eYFP expression and UMAG_02826$_{23\text{-}399}$, which had the opposite effect. In the DMSO treatment, the fold change of eYFP fluorescence relative to the mCh control was relatively consistent in four of the five effectors retested. However, UMAG_02826$_{23\text{-}399}$ which significantly upregulated eYFP expression in the first experiment, showed only a slight tendency towards upregulation that was not significant in the second experiment (Fig. 5A). Similarly, variation between the two repetitions in Tm-treated samples was also observed (Fig. 5B). In trying to understand the source of this variation, we considered whether it could be due to changes in protein expression between the two replicates. Because the plasmids encoding the candidate genes also express mCh in equimolar amounts, we used this protein's fluorescence as an estimate for protein levels of the different constructs (Fig. 5C). We found that there was indeed variation in protein levels between the two replicates in some samples and this is a factor that should be considered when using this method. Nonetheless, the putative effector UMAG_05927$_{24\text{-}370}$ consistently downregulated pBIP1 activity to approximately half of what was measured in the mCh control sample (Fig. 5A and B). In Tm infiltrated leaves, qRT-PCR analysis of the same maker genes measured in Fig. 3A showed that expression of UMAG_05927$_{24\text{-}370}$ led to a significant decrease in CNX1, SKP1, and PR1 expression, but not bZIP60 (Fig. 5D). This indicates that UMAG_05927$_{24\text{-}370}$ can interfere with UPR, either downstream of bZIP60 or in a signaling pathway-specific manner.

There was one more observation we noted that might influence some of the variability of the data. When testing the effect of the 35 putative effectors, we infiltrated the p35S::mCh-P2A-mCh reference construct before, in the middle, and after the infiltration of constructs for effector

expression, and measured their fluorescence (Fig. 5E and F). Throughout infiltration, the average signal for both eYFP and mCh fluorescence tend to decrease both in intensity and variability. The only statistically significant decrease was observed in mCh between the first and last samples in Tm infiltrated leaves. Nonetheless, the linear regressions have a high r^2 fit to the average intensities in all samples. For simplicity, and considering the small scale of our pilot screen, the statistical analysis in Fig. 4 used only the mCh samples from the middle of the assay as a reference. However, if the number of proteins or plants to be tested is increased, a correction factor can be calculated based on the equation from the linear regressions.

Discussion

UPR is a cellular mechanism that restores homeostasis in stressed cells with highly active transcriptional machineries resulting from abiotic, biotic, or physiological stresses. Due to its importance and ubiquitous nature, the core components that regulate this mechanism are well conserved among eukaryotic organisms and have been characterized in detail (Chakraborty et al., 2016; Iwata & Koizumi, 2012; Strasser, 2018). However, recent studies have been focusing on proteins involved in UPR in specific conditions (Gao et al., 2008; Liu et al., 2007; Meng et al., 2017; Moreno et al., 2012; Nawkar et al., 2017; Pinter et al., 2019; Valente et al., 2009; Xu et al., 2019). This is especially relevant in plants, which rely on signals from their environment to finetune their responses and adapt to diverse changes in their growing conditions. We believe the development of a simple, high-throughput method to identify new factors involved in UPR in plants can lead to important discoveries in this field.

The most commonly used method to link proteins of interest with UPR is qRT-PCR for ER stress marker genes (Chen & Brandizzi, 2013). It requires RNA extraction, cDNA synthesis, and

PCR optimization, all prior to experimental testing. This is relatively time-consuming, laborious, expensive, and therefore not suitable to screen libraries of proteins or other molecules. McCormack et al. (2015) described a high-throughput method to screen for the sensitivity of *A. thaliana* to ER stress by growing seedlings in a Tm solution. While this method is simple, efficient, and involves little manipulation of the plant material, its use in identifying new proteins involved in UPR is limited to available seed collections. There are currently no methods available for screening libraries of proteins to identify those that influence UPR in plants.

When studying specific proteins, several studies developed and described small scale methods for specific uses (Hayashi et al., 2013; Liu & Howell, 2010; Meng et al., 2017; Nawkar et al., 2017). While investigating the competition of HY5 with bZip28 for the binding of ER response elements (ERSE), Nawkar et al. (2017) used a construct that upregulated luciferase expression upon ER stress. This enabled them to test the influence of co-expression of two additional proteins on UPR signaling. A similar approach had been described by Iwata & Koizumi (2005) when investigating the regulation of UPR by bZip60 in *A. thaliana*. We have modified and optimized this method to increase its throughput and allow for the simultaneous testing of a large number of proteins for effects on UPR signaling (Fig. 1).

In contrast to other commonly used reporters, fluorescent proteins can be measured directly in leaf discs, leading to minimal sample handling. This results in the reduction of errors that can be introduced in other reporter systems that require further sample preparation steps, such as pipetting inconsistencies, sample mix ups, etc. In addition, fluorescence measurement in leaf discs is fast, reliable, and relatively inexpensive, which dramatically increases the throughput of the method. Furthermore, by using a reference construct, the fold change in eYFP expression can be compared between multiple sampling days and mCh expression can be used as a proxy for transformation efficiency and protein levels. This is achieved by the use of the P2A sequence,

which allows for the translation of two separate proteins from the same mRNA molecule in equimolar amounts (Kim et al., 2011). However, the stability of the proteins of interest vary and mCh fluorescence should be used as more of an indicative rather than absolute measure. Nonetheless, antibodies for the P2A peptide are commercially available and a more precise quantification of the proteins can be performed if necessary.

The use of transient protein expression in *N. benthamiana* plants allows for the screening of many candidate genes in a relatively short timeframe, with a restricted growth chamber footprint, and circumvents the restriction of only testing available seed collections. Effectively, this overcomes the gene pool limitations from previous methods, allowing for proteins from virtually any biological source to be screened. However, it has the limitation of restricting the proteins that can potentially be identified to those with conserved targets in *N. benthamiana* UPR signaling. Additionally, inconsistencies in protein expression between samples, as seen in Fig. 5C, E, and F, and known phenotypic changes that occur between transient and stable protein expression have to be taken into account when analyzing data obtained by this method (Bashandy et al., 2015). Because of this, we recommend that an initial screen be used to short list proteins for a second round of testing. Proteins that show a consistent effect on eYFP expression across the two replicates can then be validated by qRT-PCR and further characterized.

Many genes have been reported to be differentially expressed during UPR (Iwata, et al., 2010a; Srivastava et al., 2018). Typically, conserved genes involved in UPR signaling have a basal expression level in most tissues and show a rapid upregulation upon ER stress. From the genes with that expression profile, we tested the regulatory region of 4 of them: SKP1, bZIP60, BIP1, and BIP3 (Fig 2A). In the case of SKP1, Fig. 3A shows that this gene is only moderately upregulated after Tm infiltration. It was therefore not surprising that we could not detect its upregulation in the fluorescence-based assay. This highlights a disadvantage of this method,

namely that it is limited to the discovery of proteins with a strong influence on UPR signaling. BiP proteins are essential for UPR and their expression is tightly regulated during this process. bZip60, on the other hand, has a role in early ER stress signaling events and its mRNA is transcribed in non-stress conditions so that it can be unconventionally spliced during UPR (Iwata & Koizumi, 2005; Nagashima et al., 2011). However, the bZIP60 construct tested in Fig 2A showed relatively low levels of eYFP fluorescence in both mock and Tm treated leaves. While the fluorescence fold change was comparable to the promoters of BIP proteins, we considered that the overall low expression could lead to a higher false discovery rate in the identification of proteins with a role in UPR. Regarding the remaining tested promoters, BIP1 has been described to be expressed in low amounts in non-stress conditions and to be upregulated after Tm treatment. On the other hand, BIP3 was found to only be expressed in ER-stress conditions (Iwata et al., 2010b; Maruyama et al., 2014; Nagashima et al., 2014). Surprisingly, fluorescence levels from the BIP1 and BIP3 promoter constructs in non-stress conditions were similar. This could possibly be due to our use of *A. tumefaciens*, which might lead to a small upregulation of UPR genes or to transcription of genes by the bacterium itself. The latter limitation can be overcome by introducing plant specific introns into the coding sequence of the genes, thus preventing their expression by the bacteria (Vancanneyt et al., 1990). Nonetheless, the promoter region of BIP1 showed a more than 4-fold increase in fluorescence after Tm treatment which was sufficient for further testing and proved to be adequate for the purposes of this method.

Another relevant aspect to consider is the induction of UPR itself. In initial experiments, we tested several factors, such as heat stress, ectopic salicylic acid (SA) application, dithiothreitol (DTT) infiltration, and Tm infiltration (data not shown). From these, DTT and Tm infiltrations were the most effective in inducing UPR, with DTT samples showing higher variability in fluorescence intensity. This was most likely due to changes in the cellular redox state which are

known to alter the fluorescence of these reporters (Avezov et al., 2013). Additionally, the changes in the redox balance caused by the infiltration of DTT would lead to cellular responses that were not specific to UPR. Therefore, induction of ER stress by Tm infiltration seems to be the most suitable to induce UPR signaling under the conditions tested. However, it is important to note the highly toxic nature of this chemical (Heifetz et al., 1979; Keller et al., 1979; Takatsuki & Tamura, 1971) and appropriate safety precautions should be followed to avoid any direct physical contact with the Tm solution, especially when infiltrating *N. benthamiana* leaves.

The co-expression of the known UPR inducer IRE1a or inhibitor HY5 with our reporter construct showed the expected correlation with eYFP expression following induction of UPR. Together with the measurement of UPR marker genes by qPCR, Fig. 3 shows that the optimal conditions determined in Fig. 2 effectively lead to UPR and that the method is suitable for discovering new proteins that influence this mechanism.

Our small screen with a set of *U. maydis* effectors (Fig. 4) led to the identification of a protein, UMAG_05927$_{24\text{-}370}$, which seems to interfere with this process. This effector consistently led to the down regulation of eYFP expression from the reporter construct (Fig. 5A and B) and 3 out of the 4 measured UPR marker genes (Fig. 5D). It is worth noting that the expression of UMAG_05927$_{24\text{-}370}$ did not influence bZIP60 transcription, which is commonly upregulated upon ER stress. It did however strongly downregulate pathogenesis related 1 (PR1) expression, which is widely reported to be upregulated upon SA signaling (Seyfferth & Tsuda, 2014). It is tempting to speculate that the influence of UMAG_05927$_{24\text{-}370}$ on UPR may be dependent on SA signaling, rather than a more generic UPR inhibition. However. further functional characterization of this protein is needed to better understand its role in UPR interference and pathogenesis. Nonetheless, our method led to the identification of this protein's involvement in UPR and provided useful hints on how it might function.

Conclusions

We developed a simple, reliable, and high-throughput method to identify proteins that interfere with plant UPR. Constructs encoding proteins of interest are co-transformed in *N. benthamiana* plants with a fluorescent UPR reporter. Fluorescence is then measured in leaf discs and by comparing control plants with those expressing the protein of interest, in mock or Tm treated samples, that protein's influence on UPR signaling can be assessed.

Our method enables the testing of gene, and potentially small molecule, libraries using relatively limited resources and time. By using fluorescence as the output of the assay, which can be measured from leaf discs in 96 well plates, many factors can be easily tested in parallel. In fact, our pilot experiment tested 35 proteins and identified one which influences UPR signaling. We anticipate that this reporter system will lead to the discovery of new players in plant UPR signaling, particularly those involved in biotic interactions or that play a role in specific environmental conditions. This will lead to a better understanding of this ubiquitous and very complex cellular homeostasis mechanism and its role in plant biology.

Methods

Plant growth conditions

Nicotiana benthamiana plants were grown on a 4:1 soil:perlite mixture, at 21^{o}C, 60% humidity and with an 8/16 h dark/light photoperiod in a controlled environment growth chamber. Throughout the growth period, the plants were watered twice per week. *Arabidopsis thaliana* plants for genomic DNA isolation were grown under the same conditions.

Genomic DNA isolation

Plant genomic DNA for promoter and gene cloning was isolated from leaves of 5 week old plants that were snap-frozen in liquid nitrogen and ground using a Mixer Mill MM 400 (Retsch GmbH, Germany) for 1 min 30 sec at 30 Hz. To the resulting powder, 500 µL of extraction buffer (5.5 M Guanidine Thiocyanate, 20 nM Tris-HCl, pH 6.6) was added and the sample was vigorously vortexed before centrifugation at 20,000 x *g* for 5 min. The supernatant was loaded into an EconoSpin® All-In-One Silica Membrane Mini Spin Column (Epoch Life Science, INC., USA) and centrifuged at 20,000 x g for 1 min. The membranes were washed twice with cleaning buffer (80% ethanol, 10 mM Tris-HCl, pH 7.5) and centrifuged at 20,000 g for 1 min. The DNA was eluted with 50 µL of purified water and stored at -20ºC until further use.

Vector construction

DNA manipulation and plasmid assembly were performed according to standard molecular cloning procedures (Ausubel et al.,1987; Sambrook & Russell, 2006), using the GreenGate vector set and cloning conditions (Lampropoulos et al., 2013). All DNA manipulations were performed using the *Escherichia coli* MACH1 strain (Thermo Fisher Scientific, USA). Cloned genes and promoter sequences were blunt-end ligated into the pJet vector (Thermo Fisher Scientific, Waltham, MA, USA) before further golden gate cloning procedures. The plasmids used from the GreenGate vector set have the following Addgene IDs:48815, 48820, 48828, 48834, 48841, 48848, and 48868. Primers used in this study are listed in Table 1. Whenever necessary, BsaI restriction sites native to the coding sequences of the promoters or putative effectors were mutated. Silent mutations were introduced by site directed mutagenesis (Liu & Naismith, 2008) to preserve the native amino acid sequence and maintain the efficiency of the Golden Gate

cloning method (Engler et al., 2008). In the case of the fluorophores, eYFP was re-cloned from a different vector system using primers with adaptors to enable its compatibility with our cloning strategy (Table 1). Nested PCR from the Addgene vector 48828 was performed to create the P2A-lifeact-mCh CD module compatible with the GreenGate vector set (forward primer 1 – atatggtctcatcagctGGTTCTGGAGCTACTAACTTCTCTCTCTTGAAGCAAGCAGGAGATGT GGAAGAAAACCCTGGTCCAATG, forward primer 2 – AAGAAAACCCTGGTCCAATGGGTGTCGCAGATTTGATCAAGAAATTCGAAAGCATCT CAAAGGAAGAAGTGAGCAAGGGCGAGGA, and reverse primer – atatggtctctgcagctaCTTGTACAGCTCGTCCA). The lifeact sequence, which attaches the fluorophore to actin filaments, was originally planned for the effector library used here. Because mCh fluorescence was merely used for estimating protein expression, we refer to this part of the construct as "P2A-mCh" for simplicity.

The library of putative effectors was cloned based on the effector prediction analysis described in Mueller et al., 2008. Genes, specific primer sequences used to isolate them, and the updated signal peptide prediction scores calculated in SignalP v5.0 (Armenteros et al., 2019) was recently described in Alcântara et al. (2019). All putative effectors were cloned without the predicted signal peptide.

Agrobacterium tumefaciens infiltration and UPR induction

Plasmids were transformed into *A. tumefaciens* strain GV3101 (pSoup) by electroporation (Holsters et al., 1980; Lampropoulos et al., 2013) Transformed cells were selected on Luria broth (LB)-agar media supplemented with antibiotics (50 μg/mL rifampicin, 100 μg/mL spectinomycin, 50 μg/mL gentamycin) and grown at 28°C for 2 days. Colonies were then grown overnight in liquid LB medium supplemented with the same antibiotics, 20 μM acetosyringone,

and 10 mM 2-(N-morpholino)ethanesulfonic acid (MES, pH 5.6). When necessary, glycerol stocks of the strains in liquid culture were done by adding glycerol to a final concentration of 40% v/v and freezing at -80ºC until further use. Liquid cultures were pelleted at 3000 x g for 10 min and resuspended in 10 mM MES, pH 5.6, 10 mM Magnesium chloride, and 0.15 mM acetosyringone. $OD_{600\ nm}$ was measured and the cultures were diluted and mixed with the strain carrying the reporter construct to the final target $OD_{600\ nm}$. The suspensions were then left at room temperature for a minimum of 3 hours to allow for the expression of virulence genes. Finally, each bacterial mixture was co-infiltrated in the first two fully developed leaves from two tobacco plants (4 leaves/suspension in total). After 2 days, either DMSO (mock treatment) or tunicamycin (Tm; UPR induction) were infiltrated into the same leaves. Tm stock solutions were dissolved in DMSO to a concentration of 1 mg/mL and frozen at -20ºC until further use. Mock treatments were typically infiltration of a 0.5 % DMSO solution, the same as the final 5 μg/mL Tm solution.

Fluorescence measurements

One day after the second infiltration step, four discs from each infiltrated leaf were collected with a disposable 4 mm biopsy punch (Integra York PA, Inc, USA), and floated on 100 μL of water in 96 well black plates. Leaf disc fluorescence was measured in a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Inc, USA). eYFP was excited at 485 nm and measured at 528 nm, while mCh was excited at 570 nm and measured at 610 nm. Autofluorescence was measured in uninfiltrated leaves and the averaged value was subtracted from all fluorescence measurements.

Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was performed as described in Rabe et al. (2016). Briefly, RNA was extracted from infiltrated tobacco leaves in 3 independent replicates, using the RNeasy Plant Mini Kit following the manufacturer's protocol (QIAGEN Inc., Germantown, MD, USA). DNA was removed with the RapidOut DNA Removal Kit, and reverse transcription was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR measurements were performed with the Roche LightCycler® 96 system according to manufacturer's instructions (Roche Diagnostics, Rotkreuz, Switzerland). Relative expression values were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). All primers used are listed in Table 1.

Statistical analysis

Statistical significance was tested in GraphPad Prism 8.0.2 (2019). T-tests, one-way or two-way analysis of variance (ANOVA) followed by a multiple comparison Tukey hypothesis testing were used when appropriate. In each sample, two leaves of two plants were infiltrated twice and each infiltration spot (8 in total per sample) was considered a technical replicate.

List of abbreviations

BiP: luminol binding protein; bZIP: basic leucin zipper; DTT: dithiothreitol; ER: endoplasmic reticulum; ERSE: ER stress elements; HY5: Elongated Hypocotyl 5; IRE1: Inositol-requiring enzyme 1; LB: Luria broth cell culture medium; mCh: mCherry; MES: 2-(N-morpholino)ethanesulfonic acid; $OD_{600\ nm}$: optical density at 600 nm; p35S: CaMV 35S promoter; PCD: programmed cell death; qRT-PCR: quantitative real time polymerase chain reaction; S1P: site 1 protease; S2P: site 2 protease; SA: Salicylic acid; Skp1: S-phase kinase-

associated protein 1; Tm: tunicamycin; UPR: unfolded protein response; UPRE: UPR responsive elements; eYFP – enhanced yellow fluorescent protein.

Declarations

Ethics approval and consent to participate

Not Applicable

Consent for publication

Not Applicable

Availability of data and materials

Vectors and vector maps containing detailed sequence information necessary to use this method are available from Addgene (Massachussetts, USA; Table 2). Detailed information on the remaining plasmids is available upon request.

Conflict of interest statement

The authors declare that there is no conflict of interest in this research.

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Authors' contributions

AA designed the study; AA, DS, and FN did the experimental work; AA analyzed the data and wrote the manuscript. AD supervised the project and provided critical feedback. All authors read and approved the final manuscript.

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Fig. 1. Graphical protocol to screen proteins for influence on unfolded protein response (UPR) signaling. Candidate genes are cloned into a plant expression vector and co-infiltrated with the reporter plasmid at different 600 nm optical densities ($OD_{600\ nm}$), into *N. benthamiana* leaves. Two days post-infiltration (dpi), the same leaves are infiltrated with either tunicamycin (Tm) or DMSO (mock) to assess inhibition or induction of UPR signaling, respectively. At 3 dpi, leaf discs are sampled and floated on water in 96 well plates. Fluorescence intensity is measured in a plate reader. pBIP1 – regulatory region of the BiP1 protein from *A. thaliana*; eYFP – enhanced Yellow Fluorescent Protein; mCh – mCherry; p35S – CaMV 35S promoter; P2A - porcine teschovirus-1 2A "self-cleaving" peptide

Fig 2. Reporter choice and optimization of unfolded protein response (UPR) induction **(A)** Four reporter constructs were tested for enhanced Yellow Fluorescent Protein (eYFP) upregulation after UPR induction by 5 μg/mL tunicamycin (Tm) infiltration. Subsequent tests were carried out using the pBIP1::eYFP construct. **(B)** Sampling at 6, 12, 24, and 48 hours after Tm infiltration was tested. In the 48 h samples, the gain value of the fluorescence detector was lowered from 100 to 90. The 24 h timepoint was used for further tests. **(C)** Different Tm concentrations were tested for UPR induction. All future tests were done with 5 μg/mL Tm. **(D)** Three optical density at 600 nm ($OD_{600\ nm}$) ratios of *A. tumefaciens* strains were tested for optimal eYFP induction after Tm infiltration, and **(E)** candidate protein expression levels, using mCherry (mCh) as a reference. In both **(D)** and **(E)**, the *A. tumefaciens* strain carrying the pBIP1::eYFP reporter construct was co-infiltrated with an *A. tumefaciens* strain carrying a p35S::mCh-P2A-mCh control construct. Error bars represent standard deviation. Error bars represent standard deviation, asterisks represent statistically significant differences (one-way ANOVA or t-test) between samples: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, and n.s. – not significant. Lower case letters represent

differences between treatments among samples infiltrated with the same *A. tumefaciens* suspension, while capital letters represent differences within the same treatment among samples infiltrated with different *A. tumefaciens* suspensions in a two-way ANOVA test ($P \leq 0.05$). a.u. – arbitrary units.

Fig. 3. Proof of principle. **(A)** Conditions determined to be optimal for enhanced Yellow Fluorescent Protein (eYFP) upregulation upon tunicamycin (Tm) infiltration were confirmed by measuring unfolded protein response (UPR) marker genes by qRT-PCR. **(B)** Reporter construct expression after co-infiltration with either mCherry (mCh), a UPR signaling component, IRE1a, or the UPR signaling inhibitor, HY5. In both cases, eYFP fluorescence was measured with and without Tm treatment. Error bars represent standard deviation, asterisks represent statistically significant differences (one-way ANOVA or t-test) between samples: * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$, when statistical analysis was performed. n.s. – not significant.

Fig 4. Pilot screen to identify proteins that influence in unfolded protein response (UPR) signaling using a subset of an effector library from the biotrophic plant pathogen *U. maydis*. enhanced Yellow Fluorescent Protein (eYFP) fluorescence in **(A)** DMSO (mock) treated samples to identify proteins that induce UPR signaling, and in **(B)** samples where ER stress was induced (Tm) to identify proteins that inhibit UPR signaling. In both cases, samples of plants expressing mCherry (mCh) were used as a reference. Grey lines represent the average fluorescence (full line) and standard deviation (dashed lines) in mCh samples. Error bars represent standard deviation, asterisks represent statistically significant differences (one-way ANOVA or t-test) between samples: * $P \leq 0.01$, ** $P \leq 0.001$. a.u. – arbitrary units.

Fig 5. Reproducibility, sources of variability, and confirmation of a protein that interferes with unfolded protein response (UPR) signaling. Variability in enhanced Yellow Fluorescent Protein (eYFP) fluorescence from 2 independent replicates in both **(A)** DMSO (mock) and **(B)** tunicamycin (Tm) treated leaves. **(C)** Estimated variation between replicates of protein expression based on mCherry (mCh) fluorescence in Tm infiltrated samples. **(D)** Relative gene expression of UPR marker genes in samples expressing UMAG_05927$_{24\text{-}370}$. **(E)** eYFP and **(F)** mCh fluorescence decrease as a function of sample number. Error bars represent standard deviation, asterisks represent statistically significant differences (one-way ANOVA or t-test) between samples: * * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$, when statistical analysis was performed. n.s. – not significant. a.u. – arbitrary units.

Fig. 1.

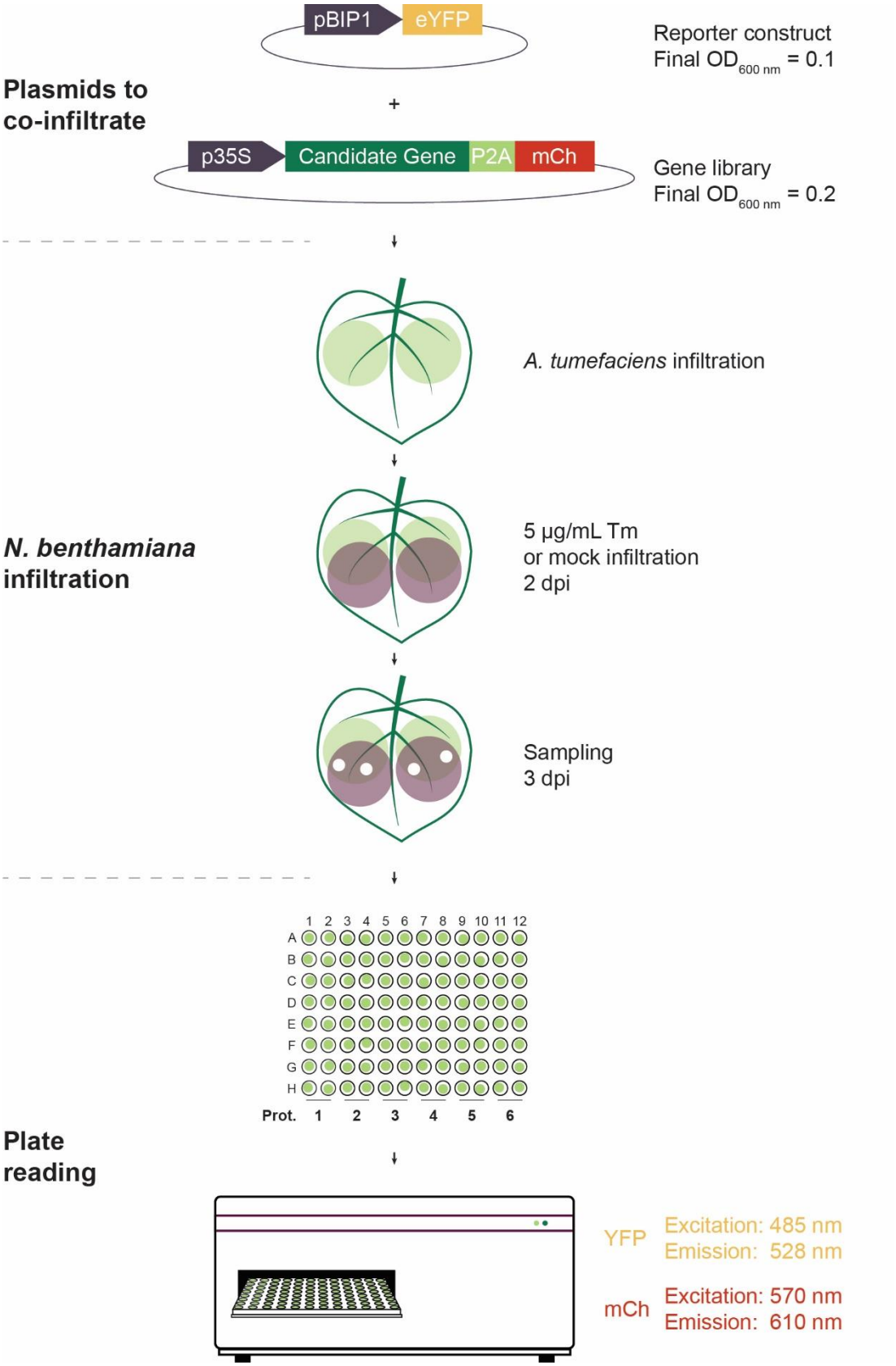

pBIP1
eYFP
Reporter construct
Final OD600 nm = 0.1
Plasmids to co-infiltrate
+
p35S
Candidate Gene
P2A
mCh
Gene library
Final OD600 nm = 0.2
A. tumefaciens infiltration
N. benthamiana infiltration
5 µg/mL Tm
or mock infiltration
2 dpi
Sampling
3 dpi
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Prot. 1 2 3 4 5 6
Plate reading
YFP
Excitation: 485 nm
Emission: 528 nm
mCh
Excitation: 570 nm
Emission: 610 nm

Fig. 2.

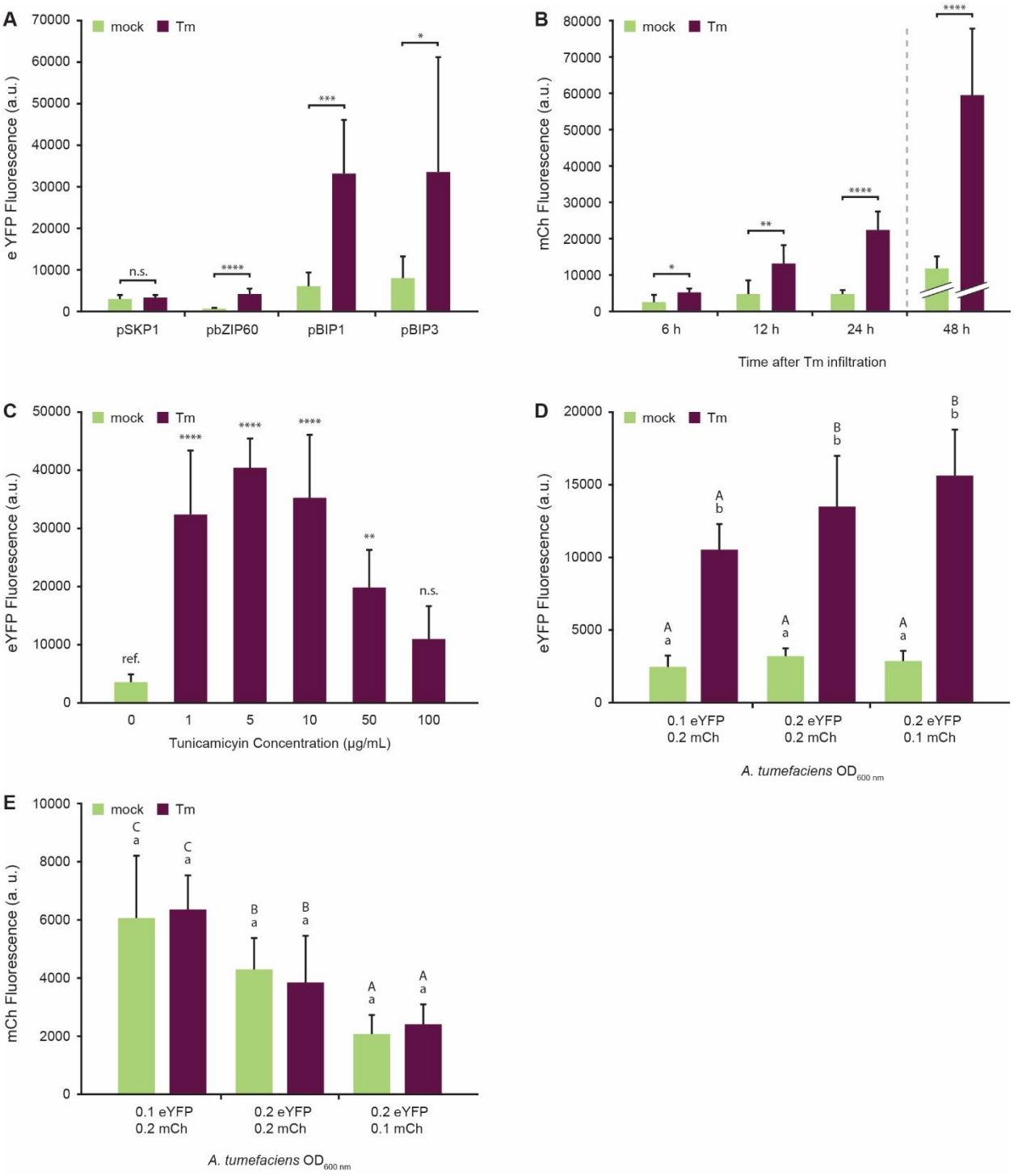

A
mock
Tm
e YFP Fluorescence (a.u.)
70000
60000
50000
40000
30000
20000
10000
0
n.s.
****
***
*
pSKP1
pbZIP60
pBIP1
pBIP3
B
mock
Tm
mCh Fluorescence (a.u.)
80000
70000
60000
50000
40000
30000
20000
10000
0
*
**
****
****
6 h
12 h
24 h
48 h
Time after Tm infiltration
C
mock
Tm
eYFP Fluorescence (a.u.)
50000
40000
30000
20000
10000
0
ref.
****
****
****
**
n.s.
0
1
5
10
50
100
Tunicamicyin Concentration (µg/mL)
D
mock
Tm
eYFP Fluorescence (a.u.)
20000
15000
10000
5000
0
A a
A b
A a
B b
A a
B b
0.1 eYFP
0.2 mCh
0.2 eYFP
0.2 mCh
0.2 eYFP
0.1 mCh
A. tumefaciens OD600 nm
E
mock
Tm
mCh Fluorescence (a. u.)
10000
8000
6000
4000
2000
0
C a
C a
B a
B a
A a
A a
0.1 eYFP
0.2 mCh
0.2 eYFP
0.2 mCh
0.2 eYFP
0.1 mCh
A. tumefaciens OD600 nm

Fig. 3.

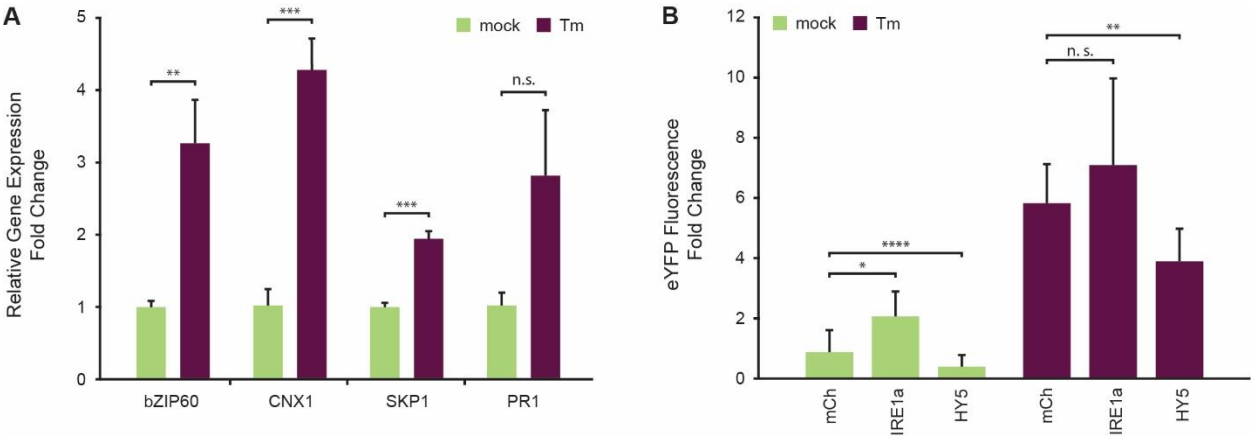
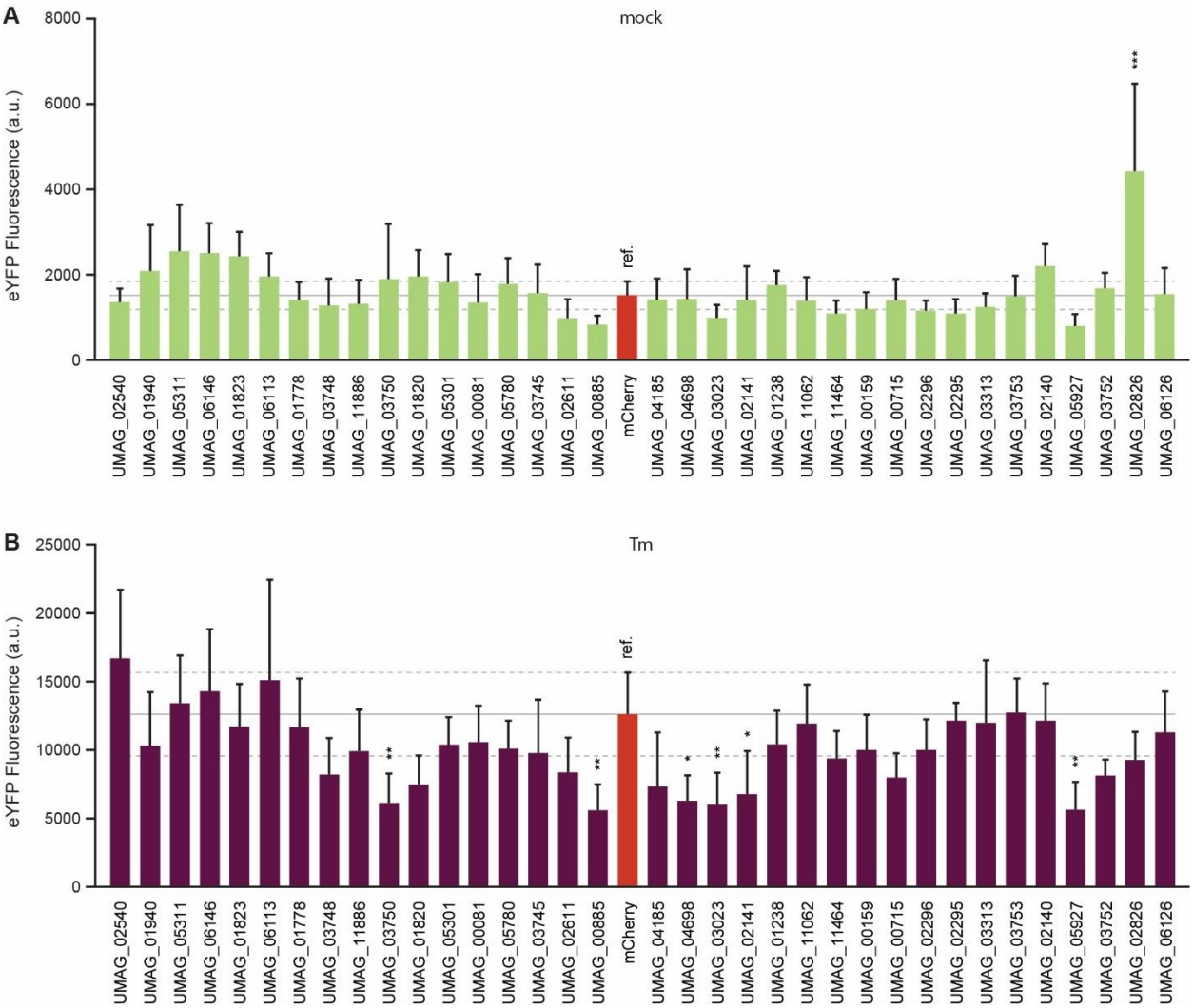

A
mock
Tm
Relative Gene Expression
Fold Change
bZIP60
CNX1
SKP1
PR1
n.s.
B
eYFP Fluorescence
Fold Change
mCh
IRE1a
HY5
n. s.

Fig 4.


A
mock
eYFP Fluorescence (a.u.)
ref.
mCherry
B
Tm
UMAG_02540
UMAG_01940
UMAG_05311
UMAG_06146
UMAG_01823
UMAG_06113
UMAG_01778
UMAG_03748
UMAG_11886
UMAG_03750
UMAG_01820
UMAG_05301
UMAG_00081
UMAG_05780
UMAG_03745
UMAG_02611
UMAG_00885
UMAG_04185
UMAG_04698
UMAG_03023
UMAG_02141
UMAG_01238
UMAG_11062
UMAG_11464
UMAG_00159
UMAG_00715
UMAG_02296
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UMAG_03752
UMAG_02826
UMAG_06126

Fig. 5.

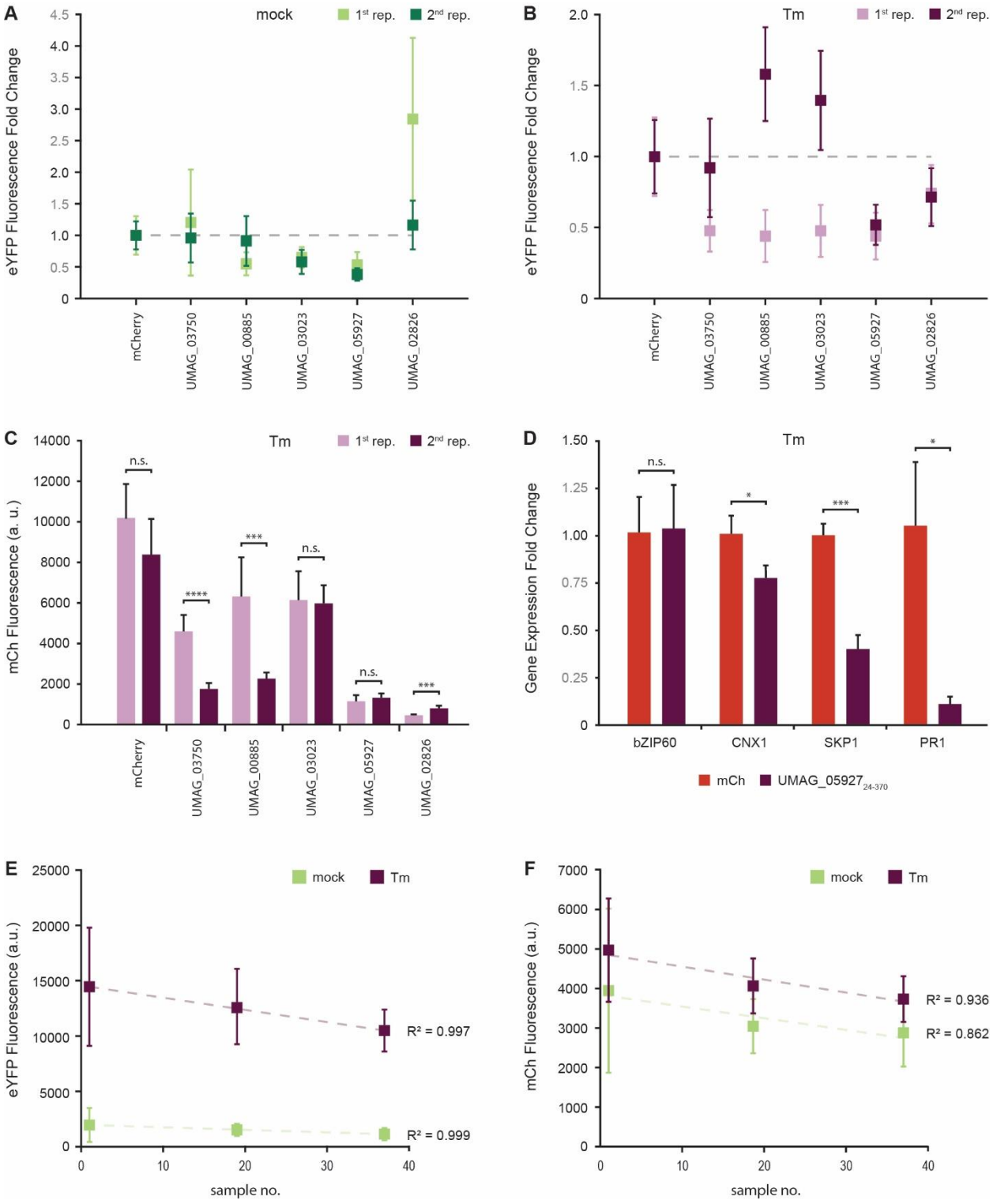
A
mock
1st rep.
2nd rep.
eYFP Fluorescence Fold Change
mCherry
UMAG_03750
UMAG_00885
UMAG_03023
UMAG_05927
UMAG_02826
B
Tm
1st rep.
2nd rep.
eYFP Fluorescence Fold Change
C
Tm
1st rep.
2nd rep.
mCh Fluorescence (a. u.)
n.s.
****
***
n.s.
n.s.
***
D
Tm
Gene Expression Fold Change
n.s.
*
***
*
bZIP60
CNX1
SKP1
PR1
mCh
UMAG_05927 24-370
E
mock
Tm
eYFP Fluorescence (a.u.)
R² = 0.997
R² = 0.999
sample no.
F
mock
Tm
mCh Fluorescence (a.u.)
R² = 0.936
R² = 0.862
sample no.

Table 1. List of primers used for promoter and gene isolation, and relative gene expression measurement by qRT-PCR. Small letters in the primer sequence represent adapters for golden gate cloning, compatible with the GreenGate vector set (Lampropoulos et al., 2013). F and R represent forward and reverse primer sequences, respectively.

Purpose/name		Primer sequence	Reference
Promoters			
pNtbZIP60	F	aacaggtctcaacctGGTAAGGTTGCCGTAGTAAAAG	N/A
	R	aacaggtctcatgttCGCCTATTCTACAACCCAGA	
pNtSKP1	F	aacaggtctcaacctCGACACGTTTGGTAGACTCATC	N/A
	R	aacaggtctcatgttCGTAGCAACACTAACCCTAG	
pAtBIP1	F	aacaggtctcaacctAGAGGAGGTTGAGAGAGAAGATAGAC	N/A
	R	aacaggtctcatgttATCGGAAACTTTTGCGTACGAT	
pAtBIP3	F	aacaggtctcaacctTGCATCGGGAAATCTTGTTT	N/A
	R	aacaggtctcatgttTTTTCGTTGTTGAGAACTCTTCTT	
Genes			
eYFP	F	atatggtctcaggctctATGGTGAGCAAGGGCGAGGA	N/A
	R	atatggtctcactgaCTTGTACAGCTCGTCCATGCCGAGAG	
AtIRE1a	F	atatggtctcaggctccATGCCGCCGAGATGTCCT	N/A
	R	atatggtctcactgaTTAGATGATGTCGCATTTGAAGTACTTTC	
AtHY5	F	atatggtctcaggctccATGCAGGAACAAGCGACTAGC	N/A
	R	atatggtctcactgaTCAAAGGCTTGCATCAGCATT	
qRT-PCR			
Nt18S	F	ATGGCCGTTCTTAGTTGGTGGAGC	Ye et al., 2011
	R	AGTTAGCAGGCTGAGGTCTCGAAC	
NtbZIP60	F	CCTGCTTTGGTTCATGGGCATCAT	Ye et al. (2011)
	R	AGAAGACCGTGGTTTCTGCTTCGT	
NbCNX1	F	ATCTTTGGCGGGAAGAAGC	N/A
	R	TCCTCTGTAGCTCCTTGGCTGT	
NbSKP1	F	GGCTGCCAACTATTTGAACA	Shen et al., 2017
	R	CATTCTCCCTCCTGACTTCTT	
NtPR1F	F	CCGTTGAGATGTGGGTCAAT	Hamorsky et al., 2015
	R	CGCCAAACCACCTGAGTATAG	

Table 2. Publicly available vector set to use this method.

Name	Brief description	Bacterial resistance	Plant resistance	Addgene ID
pBIP1::eYFP	UPR signaling reporter (upregulates eYFP expression under UPR conditions)	Spectinomycin	Basta	135231
p35S::IRE1a	Constitutive expression of IRE1a (induces UPR signaling)	Spectinomycin	Basta	135232
p35S::HY5	Constitutive expression of HY5 (downregulates UPR signaling)	Spectinomycin	Basta	135233
p35S::mCh-HA-P2A-lifeact-mCh	Constitutive expression of 2 mCherry molecules (control plasmid)	Spectinomycin	Hygromycin	135234

DISCUSSION

Effector research is instrumental to understand how pathogens manipulate their hosts and successfully promote disease. With a better understanding of plant-pathogen interactions new strategies to limit yield losses caused by biotic stresses can be developed. Furthermore, some effectors can be used as tools to study specific biological processes (de Lange *et al.*, 2014; Schreiber *et al.*, 2019; Toruño *et al.*, 2016).

The two publications in this thesis focus on effector research in two distinct ways. The first publication, titled *Systematic Y2H screening reveals extensive effector-complex formation*, addresses the hypothesis of effectors interacting with each other to facilitate the infection process. Analogous to the interaction networks found in the plant immune system (Smakowska-Luzan *et al.*, 2018; Wu *et al.*, 2017), the data presented show that effectors can interact with other effectors, forming a complex interaction matrix. The manuscript named *A High-throughput Screening Method to Identify Proteins Involved in Unfolded Protein Response Signaling in Plants* describes a method by which an effector that can interfere with plant UPR was identified. In this section, further analysis of the advantages, pitfalls, and impact of these studies on effector research is presented.

Effector Complexes in Plant-Pathogen Interactions

Protein-protein interactions are fundamental for virtually all biological processes and the correct functioning of complex cell machinery. In fact, multiple genetic diseases are caused by mutations that disrupt binding between specific proteins (Gonzalez & Kann, 2012; Sahni *et al.*, 2015). Research in pathogenic diseases has focused either on interactions between host and pathogen proteins (Crua Asensio *et al.*, 2017; Tekir & Ülgen, 2013; Mukhtar *et al.*, 2011; Zuo *et al.*, 2019) or within proteins from the host (Bigeard *et al.*, 2015; Tang *et al.*, 2017; Yu *et al.*, 2017), but little attention has been directed towards interactions among proteins of pathogenic origin. The publication *Systematic Y2H screening reveals extensive effector-complex formation* addresses this knowledge gap, shows how abundant interactions between effectors can be, and discusses their potential roles in disease.

This screen revealed that 126 out of the 310 putative effectors tested were able to interact with at least one other effector candidate. More importantly, these proteins formed 867 interactions, showing that effector candidates from *U. maydis* are highly interconnected. While effector proteins from plant pathogens have been described to form dimers and oligomers (Djamei *et al.*, 2011; Q. Li *et al.*, 2016; L. Ma *et al.*, 2015; Magori & Citovsky, 2011), this dataset highlights how the extent and role of effector-effector interactions in disease has mostly been overlooked. These data provide a useful framework to better understand effector mechanisms, and how they might function over the course of an infection process. The combination of these data with other publicly available datasets, such as transcriptomes at different timepoints or specific tissues, and further biochemical characterization of the proteins, becomes crucial to form a comprehensive overview of the interaction between maize and *U. maydis*.

There are however a few limitations that need to be considered when analyzing these data. The three main factors that influence the outcome are: the reliance in bioinformatic tools to predict which proteins are likely to be effectors, the necessity to express the proteins of interest in a heterologous system, and the reliance on fusion proteins to detect the interaction. Due to their nature, effectors are under high selective pressure and do not typically display known domains that might hint towards there function. While some pathogens have short motifs that are involved in protein translocation into plant cells (R. H. Y. Jiang *et al.*, 2008; Stam, Jupe, *et al.*, 2013), there are no known conserved motifs among

effectors from filamentous fungi. Therefore, effector prediction in these species is based on more generic features, such as secretion signal peptides, protein size, cysteine content, *etc.*, which changes the predicted effectome depending on the tools that are used (Mueller *et al.*, 2008; Schuster, Schweizer, et al., 2018). This means that the some of the proteins tested in the screen may not be effectors and that some effectors might not be in the putative effector library tested. The second issue to consider relates to the fact that protein coding sequences from *U. maydis* were inserted in yeast expression vectors to perform the interaction screen. While these proteins are still expressed in a fungal organism, which is expected to have mostly similar protein translation and folding machinery, requirements for the correct function of some of the putative effectors might be absent. These could be, for instance, specific chaperones that are required for the folding of specific effector candidates (Lo Presti *et al.*, 2016; Lohou *et al.*, 2013; Page & Parsot, 2002; Yi *et al.*, 2009). Furthermore, effector proteins are typically cysteine-rich and go through the secretion pathway to acquire the correct folding and maintain their function when secreted to the apoplastic space (Kämper *et al.*, 2006; Mueller *et al.*, 2008; Saunders *et al.*, 2012). However, the proteins expressed in the Y2H screen do not have their secretion signal peptides and are therefore synthesized in the cytosol, which can influence their functionality and the ability to bind to other proteins. Additionally, this method expresses the proteins regardless of their expression pattern in native conditions. Thus, it is dependent on additional expression information from other experiments to understand the relevance of the interactions found. One final factor to consider are the tags that are fused to the proteins of interest, which can interfere with their expression, function, and stability (Costa *et al.*, 2014; Paraskevopoulou & Falcone, 2018). In the case of the Y2H dataset, no information to that regard was collected. However, in the few interactions that were re-tested by co-immunoprecipitation after transient expression in *N. benthamiana* plants, the western blots show clear differences in protein amounts (Fig. S3). This is likely one of the causes of the discrepancy between the data obtained from the two methods. However, and despite all the pitfalls, the overwhelming amount of interactions found merits the investigation of the role of effector-effector interactions during pathogenesis in future studies.

Based on previously described mechanisms that emerge from protein interactions, the publication also speculates about what might be the role of effector-effector interactions in disease. From those, the "Higher complexity model" highlights the plasticity that the interaction network can confer to a pathogen's effectome. It also alludes to how those

interactions can be relevant in a context-specific manner, depending on the various expression patterns that can be found or measured. Very much like the plant immune system, which depends in a complex network of receptors to finetune it's response to diverse external stimuli (Cesari, 2018; He *et al.*, 2018; Smakowska-Luzan *et al.*, 2018), the effectome of *U. maydis* reflects a similarly complex protein interaction network. Based on this, studying effector functions should take into account context-specific interactions to fully understand the role and contributions of specific effectors to the infection process.

Altogether, these data shed new light in the complex world of effector biology. As long as the limitations of the method are considered, this dataset provides a useful resource for future characterization of effectors from *U. maydis* and their role in maize infection. For other pathogens, similar experiments should be performed to understand the evolution and conservation of these interactions and, ultimately, to better understand disease.

Fluorescence-Based Detection of Plant UPR

The arms race between pathogens and hosts leads to the rapid evolution of the molecules that mediate their interaction. Because of that, the effector repertoire of a pathogen is typically highly specific for the successful colonization of their host, which leads to poor protein sequence conservation, even between closely related species (Schirawski *et al.*, 2010). Unsurprisingly, this leads to the absence of known domains that could be used to infer protein function. To overcome that, functional screens are often used to identify proteins that are involved in specific cellular processes (Gimenez-Ibanez *et al.*, 2018; Guttman *et al.*, 2002; Petre *et al.*, 2015; Stam, Jupe, *et al.*, 2013).

Recent research has shown how UPR plays a relevant role in plant-pathogen interactions. In *U. maydis*, the main components of UPR have been identified and deemed essential for pathogenic infection (Hampel *et al.*, 2016; Heimel *et al.*, 2013; Pinter *et al.*, 2019). In plants, components of UPR were recently shown to directly regulate immunity towards pathogens (Moreno *et al.*, 2012; Xu *et al.*, 2019). Therefore, it is not surprising that a few effectors were found to interfere with plant UPR, to ultimately limit the immune responses that are upregulated as a consequence of ER stress. Specifically, in two *Phytophtora* species, effectors have been identified to interfere with the host's UPR and facilitate infection (Fan *et al.*, 2018; Jing *et al.*, 2016). The function of these effectors was discovered after linking their expression with virulence and identifying their interacting proteins, by either Y2H or MS after co-IP. To identify other effectors that interfere with this cellular mechanism, a functional screen would probably be the most straightforward approach. However, the lack of a simple and efficient method to identify proteins involved in UPR led to the work reported in the manuscript titled *A High-throughput Screening Method to Identify Proteins Involved in Unfolded Protein Response Signaling in Plants*. In it, a method was developed and optimized to transiently co-express a reporter construct with proteins of interest and analyze their interference in plant UPR signaling, in a high-throughput manner. In a pilot screen, out of the 35 *U. maydis* proteins tested, at least one was confirmed to lead to the downregulation of UPR-related genes in *N. benthamiana*.

The identification of one effector candidate from *U. maydis* that is able to interfere in plant UPR encourages the expansion of the screen to the remaining putative effectors of this pathogen. In fact, based on the data published by Uhse *et al.* (2018), the KO of UMAG_05927

is not expected to cause a virulence defect, which may indicate that other effectors from *U. maydis* might have a similar function. While it is difficult to predict the number of proteins that can be involved in this pathway, redundancy is known to occur (Navarrete *et al.*, 2019; Selin *et al.*, 2016; Tan *et al.*, 2015) and more effectors with a role in this pathway can be expected. More broadly, the method presented can be used to identify proteins from other organisms with a role in conserved components of plant UPR. Potentially, libraries of other molecules could also be used, although that has not been tested yet.

The pitfalls of the method were thoroughly discussed in the manuscript, some of which overlap with the ones discussed in Publication I. It is important to note that while *U. maydis* is used as a model organism for smuts and other plant biotrophs, maize displays less than ideal characteristics for research in molecular biology and protein engineering. Despite recent developments in transient expression of proteins in monocot species (Bouton *et al.*, 2018; Fursova *et al.*, 2012; J. F. Li *et al.*, 2009), *N. benthamiana* remains a far better model plant for large-scale and high-throughput screens. The major limitation of using this heterologous expression platform is to limit the discovery of proteins with influence in UPR that have conserved targets. Once identified, further validation is needed to confirm the role of those proteins in the native system. Furthermore, the folding limitations described above also apply to this assay. By removing the secretion signal peptide and expressing the proteins in a different organism, some of them might display folding and/or stability aberrations. This can be inferred by the mCherry fluorescence, which was considerably different between the proteins tested. It is also important to note that by removing secretion peptides from protein coding sequences, it is mostly the impact on signaling components of plant UPR that is assessed. Proteins that need to locate to the ER to function properly might not be identified by this method. However, proteins that translocate to the ER by different methods can still be identified. This was the case of the PSAvh262 effector, which is translated in the pathogen, migrates to host cells where it ultimately localizes to the ER and interacts with BiP proteins to suppress PCD (Jing *et al.*, 2016). It is worth noting that the 35 proteins tested didn't have any known ER retention motifs. These are usually HDEL, KDEL, or KKxx – where xx is any amino acid – motifs which are can be found in the C-terminal ends of proteins. In the case of UMAG_05927, its sequence has a secretion peptide and is upregulated during fungal biotrophic development. All these characteristics increase the likelihood of this protein to be a true effector that is uptaken by the host, where it interferes with its UPR signaling.

Nonetheless, further experiments are needed to understand the mechanism by which this protein contributes to pathogenesis.

In the context of the plant effectome this method assesses the contribution of single putative effectors in plant UPR. As it was addressed in Publication I, many effector candidates can interact between each other. While it would be impractical to test all of the effector pairs and potential larger complexes found, the information from a functional screen can be overlapped with the published interactome. For instance, UMAG_05927, which had an effect on the expression of the reporter construct, did not show any interactions with other effectors. This means that this particular effector might interfere with plant UPR without interacting with other effectors. However, if for instance UMAG_03023 had been confirmed to consistently downregulate UPR in *N. benthamiana*, its further characterization would be less straightforward as it was found to interact with 26 other proteins. In that case, the impact of combinatorial protein expression would have to be assessed and it is possible that its full impact on virulence is dependent on some of those interaction partners. This highlights how both studies complement each other and their impact in effector research.

Epilogue

This thesis presents two studies that address different aspects of effector research, using the model biotrophic fungal pathogen *U. maydis*. The first publication shows a surprising abundance of interactions between effector candidates. While previous studies have shown the role of a few effector dimers in pathogenesis, the data presented highlights the need to study effector complexes in more detail to fully understand pathogenesis. In the second manuscript, a method was developed that allows to screen for the influence of proteins, and potentially other molecules, in plant UPR. By using libraries of genes from pathogens, effectors that interfere with their host's UPR can be identified. The overlap of both datasets permits for a better overview of effector biology and their role in host manipulation. Altogether, these data represent a small but significant step in understanding plant disease and might become instrumental in the search for new strategies to improve plant immunity, thus reducing yield losses caused by disease.

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Appendix

Abbreviations

ABA – abscisic acid
ADP – adenosine diphosphate
ATP – adenosine triphosphate
BiP – luminal binding protein
CC – coiled-coil domain
CDPK – calcium dependent protein kinase
co-IP – co-immunoprecipitation
CRN – crinkler motifs
DAMP – damage-associated molecular pattern
DNA – deoxyribonucleic acid
e.g. – *exempli gratia* (Latin, meaning: 'for example')
ED – extracellular domains
EF-Tu – elongation factor Tu
ER – endoplasmic reticulum
ERQC – endoplasmic reticulum quality control
ERSE – ER stress element
ET – ethylene
et al. –et alia (neuter plural; Latin, meaning: 'and others')
etc. – *et cetera* (Latin, meaning: 'and more')
ETI – effector triggered immunity
GFP – green fluorescent protein
HR – hypersensitive response
i.e. – *id est* (Latin, meaning: 'in other words')
JA – jasmonic acid
KO – knockout
LRR – leucine-rich repeat
LysM – lysine motif
MAMP – microbe-associated molecular pattern
MAPK – mitogen-activated protein kinase
Mb – megabase
mRNA – messenger ribonucleic acid
MS – mass spectrometry
NB – nucleotide binding domain
NADPH – nicotinamide adenine dinucleotide phosphate
NLR – nucleotide-binding/leucine-rich repeat receptor
PAMP – pathogen-associated molecular pattern
PCD – programed cell death
PRR – pattern recognition receptor
PSII – photosystem II
PTI – pattern triggered immunity
qPCR – quantitative polymerase chain reaction
RK –receptor kinase
RLK – receptor-like kinase
RLP – receptor-like protein
RNA – ribonucleic acid
ROS – reactive oxygen species
SA – salicylic acid
SAR – systemic acquired resistance
T3SS – type III secretion system
TALE – transcription activator-like effectors
TIR – toll–interleukin 1 receptor
Tm – tunicamycin
UPR – unfolded protein response
UPRE – UPR responsive element
Vir – virulence proteins
Y2H – yeast two-hybrid

Supplementary Material from Publication I

Table S1 – List of putative effectors used as bait and/or prey proteins

Gene specific primer sequences used to clone the coding sequences, signal peptide prediction scores, and peptide length are also listed.

Gene	Forward Primer	Reverse Primer	SignalP 5.0 Score	Signal Peptide Length (aa)	pGBKT7 (bait)	Baits with growth after pooled mating	Used as bait in liquid media	pGADT7 (prey)
UMAG_00027	GATCCGCTCCAGTGCCAC	AGACTTGTTCTCCTCGTT	0.998	21	Yes	No	No	Yes
UMAG_00041	CAGCTGACATCATCCTCC	CTCCTGCACAGAGGTGCG	0.632	40	Yes	No	No	Yes
UMAG_00054	AGCTTGCAGCACTCGTGG	TGGCTTCACGGGATGAGG	0.899	26	Yes	No	No	Yes
UMAG_00081	GCTCCGAAGCTCGAGATT	GAGCAGGATGGTGAGACC	0.985	22	Yes	No	No	Yes
UMAG_00159	GCGATGCCATGGTTGCCG	AAGGATGCCTAGTTGCTG	0.979	20	Yes	No	No	Yes
UMAG_00250	TCGTCCCACGCCTCGAAC	GGAGTCAGCCTGCTGCTG	0.979	22	Yes	No	No	Yes
UMAG_00309	CAGCAGCCTACCGGCACC	GAGAGCAAACGCCGCAGC	0.998	21	Yes	No	No	Yes
UMAG_00326	CAGAGCAGCACTACCGCT	GAGCATGGCAGCACCGGC	0.980	22	Yes	No	No	Yes
UMAG_00420	TCGGTCCAGTATGCGGAG	AAGCGACGCAGATTCACG	0.936	20	Yes	No	No	Yes
UMAG_00466	GCACCCACTGGTAATGAT	GTAGTCGTGGTGACTCCA	0.999	20	Yes	No	No	Yes
UMAG_00480	GACGGTCTGCAGGCCGAC	AAGGAGAACCGAGTTGCT	0.889	30	Yes	No	No	Yes
UMAG_00492	CGGTCCCCCTTCGCCCCC	GAGAACCAACGCGATCAG	0.940	21	Yes	No	No	Yes
UMAG_00538	TACCACCTGTCACCTACT	ACGAGCAGCGACTGTTTT	0.825	29	Yes	No	No	Yes
UMAG_00558	GAATGGCTGAAGGTCTCA	GACATTGTCCGGGAGATT	0.991	22	Yes	No	No	Yes
UMAG_00598	TGGGATTGGCTCCCAACT	CAAAGCGCTAAAACCAAG	0.993	23	Yes	No	No	Yes
UMAG_00628	GCTCCTGTTGTCGGCGAT	ACCGCGGAACCAAGAAGG	0.875	31	Yes	Yes	Yes	Yes
UMAG_00664	GCTCCCTTGACTCAAATC	GACGAGCCCTCCGTTTCC	0.998	19	Yes	No	No	Yes
UMAG_00692	GTGCCCCGCCACGCTAAA	GACCAGCATAGATCCGGC	0.919	24	Yes	No	No	Yes
UMAG_00715	GACAATCCGCAGATCACA	AGCAGAAGTGCTGTCGAT	0.998	24	Yes	No	No	Yes
UMAG_00781	GCTCCAGTGCCGCTACAG	CGACTTGGCCACACCATG	0.988	22	Yes	Yes	No (Autoactive)	Yes
UMAG_00792	GCGGTCAATGCGGGCTTT	ATCGTCTTTTGATACGGG	0.977	30	Yes	No	No	Yes
UMAG_00793	GAAGAGACGGCCACACAC	CTTGTTGCAGAAGCAGTT	0.991	24	Yes	No	No	Yes
UMAG_00795	GCTAGACGTGACTCGGGT	TCGCCAAAGAGGCAGGAG	0.823	31	Yes	No	No	Yes
UMAG_00822	CTGCCCAACTTCGGCGCC	AGCGCATTTGAAGGTAGC	0.991	22	Yes	No	No	Yes
UMAG_00885	GCACCTCTGGAAAAGCGT	GATAACGATTGTCTCGCT	0.917	24	Yes	No	No	Yes
UMAG_00961	GCCTACGACCCCGCTCAC	AGGCTCCTCCTCCCACTC	0.998	19	Yes	Yes	Yes	Yes
UMAG_01022	GCCTCCGTCGACACGAAC	GTTCTTGGTGAAGAGCTT	0.994	18	Yes	No	No	Yes
UMAG_01061	GATCAACGATCCCTACGG	GTCAGTCACAGTCTTGGC	0.449	N/A	Yes	No	No	Yes
UMAG_01082	CAAATCGCATCGCTTGAT	CACACCCATAACGAGTGC	0.958	24	Yes	No	No	Yes
UMAG_01130	AAGTCAACGTCGTTCTGG	CGAGCTGTTATCAGGGGC	0.972	20	Yes	No	No	Yes
UMAG_01234	TCAGGTGGGACTCGCCGA	GGTGTTTATCAACAAGAT	0.311	N/A	Yes	No	No	Yes
UMAG_01235	AGTCCTCCTTTTGAAGAC	GGGCAGATGTCTTTGAGC	0.924	27	Yes	No	No	Yes
UMAG_01236	ACTCCGCCTTCGAAACTG	TGGAAGGTTGGTCATACT	0.871	24	Yes	No	No	Yes
UMAG_01237	TCCCCATTTCCACCCCCA	TGGAAGTCCAAGCATGTT	0.982	21	Yes	No	No	Yes
UMAG_01238	GCTCCTCCCACTTCGCAA	TGGAGAGTAGAGAAATAG	0.997	20	Yes	Yes	No (Autoactive)	Yes
UMAG_01239	CAGCCTCTGCTGCGACTT	GGTACTGTAGTAAACGTC	0.997	18	Yes	Yes	No (Technical issue)	Yes
UMAG_01240	CAGCCTCTGCTGCGACTT	AATAGCATCAATCCTCTG	0.996	18	Yes	No	No	Yes
UMAG_01241	CGCGGGGGCGGTCATAGC	AACTGCTGGGCGCGGCGC	0.858	24	Yes	No	No	Yes
UMAG_01297	TTGCCCACCTCTGACTCG	CAGCTTGATGGGCTGATT	0.991	19	No	No	No	Yes

Gene	Forward Primer	Reverse Primer	SignalP 5.0 Score	Signal Peptide Length (aa)	pGBKT7 (bait)	Baits with growth after pooled mating	Used as bait in liquid media	pGADT7 (prey)
UMAG_01298	ATGACACTGCATACGCGC	GAGTACGATTGGGTGGGA	0.885	20	Yes	No	No	Yes
UMAG_01299	GCTTTAGATAGCGGGATT	CAAAGTGAAGATTTTCCG	0.957	22	Yes	No	No	Yes
UMAG_01300	GGTTGGGATACGCCTTCC	GGGAAGGGAGATAGCTGC	0.999	20	Yes	No	No	Yes
UMAG_01301	GGATGGAACCCCGAGCAG	CTTTGGAATCGCAGGAAC	0.996	22	Yes	Yes	Yes	Yes
UMAG_01302	AATTTTTTGAAGGTTTCG	GGCAGAGACGATGTAGTC	0.996	21	No	No	No	Yes
UMAG_01375	AAGCAGATTCCGGTGCGT	TTCCCAGATGACCACATC	0.989	25	Yes	No	No	Yes
UMAG_01513	CATCAGCTTAACTCCCGA	AGCCCAGTCCCACTTGAT	0.949	27	Yes	No	No	Yes
UMAG_01553	CATGTGCAGACCGGCGGC	ACTGAGCCTCTTAGCACC	0.951	21	Yes	No	No	Yes
UMAG_01632	CAGTCCGCCGCCACCAAC	GAGAGCTACGAGAGCAAC	0.955	29	Yes	No	No	Yes
UMAG_01690	ATTGCAGTGCCCCAACCT	GGCGTCCGGCTTGGGCTC	0.996	21	Yes	Yes	Yes	Yes
UMAG_01705	GTGCCTCACGTCCAAGTC	CGACGATGCATCGACCGT	0.598	20	Yes	No	No	Yes
UMAG_01725	AGCGTTCAGCCTACGCCG	CAAAGCAAAGACACCAGC	0.998	20	No	No	No	Yes
UMAG_01734	AGTCAGCATCGAATGGTC	GGCCTTGCTGGCGAGATC	0.653	23	Yes	No	No	Yes
UMAG_01750	AGACCACTTCTCTCAGCA	CAACTGATGCTCACTGGG	0.969	22	Yes	Yes	No (Autoactive)	Yes
UMAG_01778	GCGACTCTACCCGCCCAC	CTCCTGTTTGGCCTCGGC	0.942	26	Yes	No	No	Yes
UMAG_01779	AAGCCTGTCGCTCAAGAT	GGCGTGGCAGGTGTTGCT	0.982	27	Yes	Yes	No (Technical issue)	No
UMAG_01786	TATTTTCCGCACAAGAAC	AGCCTCTACCCAATCCTT	0.583	19	Yes	No	No	Yes
UMAG_01820	GCCGTTGACATCACTTTT	ACCCACGGTGTTGATGTC	0.995	21	Yes	No	No	Yes
UMAG_01823	GCAGCGACAAGTGCAACT	GTTGGTGGTGGAGGTGGT	0.971	22	Yes	No	No	Yes
UMAG_01851	ACCATCGTTGTCACCAAA	GAGTAAAGCGGCACCAAC	0.951	17	Yes	No	No	Yes
UMAG_01854	CTCGCTCCTCTCGAGCGA	GTTCTTATCCACGAATGC	0.906	21	Yes	No	No	Yes
UMAG_01855	GCTTCCATTCGCACTCGC	AGCAAGACCAAGGCGAGA	0.880	21	Yes	No	No	Yes
UMAG_01858	AGCCCGCATTCTCCCGGA	CAGTTCGTCCCCATGATT	0.991	24	Yes	No	No	Yes
UMAG_01940	GGTGGCCCTCACAACTCC	GGTGGAAGCCGCTGGCGT	0.836	23	Yes	Yes	Yes	Yes
UMAG_01970	CAGCTGGTGGATTCGGTT	CAAGGTGATCATCGCCGA	0.977	21	Yes	No	No	Yes
UMAG_01976	AAGTCGCTCAGCAACAGG	GGTGTAAGAGAAGGGATA	0.973	26	Yes	No	No	Yes
UMAG_01977	GCGAATATTTCACCTACG	GAGGATGTCGGCTTTCAC	0.997	20	Yes	No	No	Yes
UMAG_01987	GATGCTGCGGGTGCGGTA	CATGCCAAACATGCTACC	0.997	26	Yes	No	No	Yes
UMAG_01997	CAGACCATCTCGCAGCCA	GACGAGCAGAGTGAGGAG	0.988	21	Yes	No	No	Yes
UMAG_02006	TCGCCGCTCCCCACCAAG	GAGtAGACCGCCGTCGTTTCTG	0.978	21	Yes	Yes	Yes	Yes
UMAG_02011	GAATTCTTACGAGAACAT	ACTGGTAGAGAAAGTAGA	0.999	19	Yes	Yes	Yes	Yes
UMAG_02119	GCTGCGGCACCGAAGCTG	GTGAGCTGCACGGAAACG	0.998	24	Yes	Yes	Yes	Yes
UMAG_02136	AGACCCGCGGGTTCGGAT	CTTGTGCATGCGACGAAC	0.773	21	Yes	Yes (Autoactive)	No	Yes
UMAG_02137	TATCCAGCTGGCAATGAA	GATGTGCACTCGAGACCC	0.963	21	Yes	No	No	Yes
UMAG_02138	TCGTCAGGCAATGTAGGC	GCCATGCAGATGTGCTGC	0.951	24	No	No	No	Yes
UMAG_02139	GCGCCAGCCGGCCCTGCT	CAACCCTTTCAGCAATGG	0.992	16	No	No	No	Yes
UMAG_02140	GCCCCGGCCGGCCCTGGT	CAATCGAAGCTCGGCTGC	0.992	16	Yes	No	No	Yes
UMAG_02141	GTTCCAATGGACGGTGCA	TATGTGAGACGAAGATAG	0.967	22	No	No	No	Yes
UMAG_02192	GGGTTCATCAACTCGGAA	CGGAAGTCTGCGTTGACC	0.675	22	Yes	No	No	Yes
UMAG_02193	GCCTCGATTCCCCTTTAT	GAAAGCTCTTTGCGGTAT	0.969	21	Yes	No	No	Yes
UMAG_02194	GCCCCGCTCAGCCCTTAT	CTGGCGAGTTCGAGAGGT	0.990	20	Yes	Yes	Yes	Yes
UMAG_02196	AAGCCGATGGGTGTCGCA	CAAGATGTGATGGAGAAC	0.987	23	Yes	No	No	Yes
UMAG_02229	CAGATGACTGAGTTGGAT	GCCTCGAAGGACCTCGCC	0.893	22	No	No	No	Yes
UMAG_02230	TATGCTTCACTGGTAACA	GATTGGTCGAATTCGGGA	0.904	21	Yes	Yes	Yes	Yes
UMAG_02231	CAGCCTTTGACAGGTAAA	CACCACCGGGTGTACCTT	0.931	24	Yes	No	No	Yes
UMAG_02239	CATCCTCTACAATCGTTT	CGTCGTCGGCCCAAATTT	0.987	21	No	No	No	Yes
UMAG_02293	CACAAGGCCAAGGACGAT	TAAGTGCCATTTCGGAAG	0.839	27	Yes	Yes	Yes	Yes
UMAG_02294	TCGAGACTCCCTATCGGA	GACTTTTTGCATTGGCGC	0.995	22	Yes	No	No	Yes
UMAG_02295	GCAGGCTTGCCGCTTGGT	TGTTTGTGAATGTGGGAG	0.683	23	Yes	Yes (Autoactive)	No	Yes
UMAG_02296	GCGTCGATCGCACTGGAC	CGTGATCATAGGCTGCCC	0.967	26	Yes	No	No	Yes
UMAG_02297	ACGTCGATCTCACTGGAC	CTGTGAGTCTAATACGGG	0.978	26	Yes	Yes (Autoactive)	No	Yes
UMAG_02298	CGGCCGGGCGACAATTTC	CCTTGCAGGTTGGACGTA	0.997	20	Yes	Yes	Yes	Yes

Gene	Forward Primer	Reverse Primer	SignalP 5.0 Score	Signal Peptide Length (aa)	pGBKT7 (bait)	Baits with growth after pooled mating	Used as bait in liquid media	pGADT7 (prey)
UMAG_02299	GTATTTCCGGCTCGCTTG	GGGGATCCTCCTCCCACC	0.860	23	Yes	Yes (Autoactive)	No	Yes
UMAG_02430	CAGCTTGATCCTTTGCTC	CAGGAGGGTGAGTGCGGC	0.973	21	Yes	Yes	Yes	Yes
UMAG_02466	CTGCCTCTCTATTCGTAT	GCGGTTGAACCTAGATGG	0.813	21	Yes	No	No	Yes
UMAG_02473	GCAATGGCGCTAGAGCGA	GAATTGTTTGGCGCACGA	0.984	21	Yes	No	No	Yes
UMAG_02474	GGACCTGCCGTTCAACGT	CATGGCTTCGTTGTCCAT	0.984	18	Yes	No	No	Yes
UMAG_02475	AACGGCTCGATCTCGAAC	ACGAGAAGGAGGAGGTGC	0.848	28	Yes	Yes	Yes	No
UMAG_02533	GCTGCAAGTGAGTTGAGC	ATGGACGTGCTCAAGACC	0.969	22	Yes	No	No	Yes
UMAG_02535	CTAGCCCCTTCGCTTCCG	GCTTGAGCCACCTATTGT	0.962	21	Yes	Yes	Yes	Yes
UMAG_02537	ATGCCAGTGAACCCCATG	CGAGCGTGGACGCTTGCT	0.987	21	Yes	No	No	Yes
UMAG_02538	ACACTGCCAGAGATCATC	TCCATGGCTGCTTTCGGC	0.995	22	Yes	Yes	Yes	Yes
UMAG_02540	GTCTTGCCCCACGTCATG	GGACTGGTTTCTGCGGGA	0.863	20	Yes	Yes	No (Autoactive)	Yes
UMAG_02611	GCCAGTCTGCGCCAAAGC	CTTGGGTAGATATCTGAT	0.975	20	Yes	Yes	Yes	Yes
UMAG_02620	GCCCTTGTTGCAGCGCAG	CTTGCGGGGCTTTCCGCG	0.996	24	Yes	No	No	Yes
UMAG_02640	GCCTCGGACGCATGTATC	TGGACCCTCTTCGGCGGT	0.983	25	Yes	No	No	Yes
UMAG_02756	TTCATGACTGCCGTGTGT	TCTGGCAAGTAGCTCGTC	0.528	22	Yes	No	No	Yes
UMAG_02826	GATCCGCCCAAAGCCGCG	GAAATGAACTCCTGCCAA	0.968	22	Yes	No	No	Yes
UMAG_02851	GCTCCCACAACAGCATTT	TCCCCACGGTGACGGTTG	0.998	21	Yes	Yes	Yes	Yes
UMAG_02852	TATCTTCTCCCTAAAGAA	TCCAGCGACCAAAGAGCT	0.923	23	Yes	No	No	Yes
UMAG_02853	TACCAGATCGAAGCAGGA	GCTCCTGGATCCGGCGCG	0.982	23	Yes	Yes	Yes	Yes
UMAG_02854	GTGCCCATAGTTCCGGTA	TGCAGAATAGGATTTACC	0.868	29	Yes	No	No	Yes
UMAG_02921	CAGAAGAAGCCCACGTTC	CTTGAGAGGCTTAGCAAT	0.997	22	Yes	No	No	Yes
UMAG_02925	TCACCCACGGTCGACGTC	AAACAGTCGACGTCGCAC	0.996	22	Yes	No	No	Yes
UMAG_02981	CACATACCCAGCCTCAAT	TACGAAAGACCACTTGAT	0.991	19	Yes	No	No	Yes
UMAG_03023	GAGCCTCTCCTCGGCCAA	AGGCTTGGGGAGGTACCT	0.998	19	Yes	Yes	Yes	Yes
UMAG_03046	AGCAGCCAATGCGACAGT	GCGCCAAAATCCGCAGGC	0.998	20	Yes	No	No	Yes
UMAG_03065	GCTTCTGTTGACGAATCG	GGCAAGACTCTTAACTGA	0.996	19	Yes	No	No	Yes
UMAG_03112	CTGCCAAACCTCCACGCC	TTTGCACTTGAATTGGGC	0.989	22	Yes	No	No	Yes
UMAG_03138	CACCCTATCTCGGCCGAC	CATCTCACCACCTTTCTT	0.973	25	Yes	No	No	Yes
UMAG_03154	AGGCCGACGCTGTCTCGC	TACAGGATTGTCACGGCT	0.822	25	Yes	No	No	Yes
UMAG_03201	TACCCGTTCCGCGTCGCA	CAAATCGGCGAGGGGGAC	0.986	19	Yes	Yes	Yes	Yes
UMAG_03202	GCTGCAGTCGATCGCCCT	GTGCTCGGCATTGGCGCA	0.999	22	Yes	No	No	Yes
UMAG_03223	TGGCCAGCGCCCACCATG	CTCCTTTACATGAATGTG	0.995	24	Yes	No	No	Yes
UMAG_03231	GCGCCGCTTCCACCTCGA	TCCAATCTGGTTGGTCAA	0.983	26	Yes	Yes	Yes	Yes
UMAG_03232	ATGCCTCTGCCGGCACGC	TAGGCCTGGGAATCTTGC	0.941	32	No	No	No	Yes
UMAG_03313	GCCCCAGCCGGCCCGGGT	CAATCGAGTCTCGGCTGC	0.001	N/A	Yes	Yes (Autoactive)	No	Yes
UMAG_03314	GCCCCAGCCGGCCCGGGT	TGGTCGGGTTCCAGCTGC	0.998	19	Yes	No	No	Yes
UMAG_03349	CAGACCTCGGTGTCGGCC	CACCAACCAAGTCCCGCT	0.987	19	Yes	No	No	Yes
UMAG_03352	GCCTCTAAGCAGACCCTG	TATGGGCTCATATGCTCC	0.792	36	Yes	No	No	Yes
UMAG_03382	CTGTGGCCACATACCCAG	CAGGTAGCAATTCTTCCA	0.938	18	Yes	No	No	Yes
UMAG_03392	CTTCCAAGCGACAGTGCC	TTCGCAGATATTGTTGCA	0.998	19	Yes	Yes	Yes	Yes
UMAG_03397	CCTGACGTGCCTCTGGAC	AGTACGTTTCTGATTCGA	0.979	19	Yes	No	No	Yes
UMAG_03564	CAAGACACGGCATCGACG	GAGAATGAGGAAGTCGGA	0.996	26	Yes	Yes	Yes	Yes
UMAG_03585	AGCCCTTTGAAAGAGCGT	TACCGACTGTGACTGGAA	0.988	21	Yes	Yes	Yes	Yes
UMAG_03586	GTCCAAGTTTCGATGACG	AGAAAGAACCGAGGTCCG	0.972	21	Yes	No	No	Yes
UMAG_03614	GATCGTGTATCTTTCGGT	GGCAAGCACGACGAGGGC	0.964	28	Yes	No	No	Yes
UMAG_03615	TCCCTGGAACCCTCTTTG	CTCATCGTCGTCCTCGCC	0.972	19	Yes	No	No	Yes
UMAG_03689	AGGGAGGACATTCACCCC	CAGCTCAATGTGAAGTGG	0.962	18	Yes	Yes	Yes	Yes
UMAG_03744	GCTGAAGGCGAAGCAGAT	ACGACGACGACGACGACG	0.983	33	Yes	No	No	Yes
UMAG_03745	GCCCCGGTAGGCACACCG	GATGGGTCTTTGGATGTG	0.997	18	Yes	No	No	Yes
UMAG_03746	ATCGGGGAGAAGTCCTTG	GTCGCCATGCATTCTTCC	0.902	26	Yes	Yes	Yes	Yes
UMAG_03747	CTACCGCTGCCGATGTAC	AGGGCCACGAATGTACCT	0.779	20	Yes	Yes	Yes	Yes
UMAG_03748	GCCGTTCGACCCGAAACT	GTGTGCGAGGTGAATTAC	0.991	16	Yes	No	No	Yes
UMAG_03749	ACGTCTACCTCCACATCT	ATGGATGTGGCTGAAGAA	0.998	21	No	No	No	Yes

Gene	Forward Primer	Reverse Primer	SignalP 5.0 Score	Signal Peptide Length (aa)	pGBKT7 (bait)	Baits with growth after pooled mating	Used as bait in liquid media	pGADT7 (prey)
UMAG_03750	GCCCCGGTAGGCACACCG	GATGGGTCTTTGAACATA	0.997	18	Yes	No	No	Yes
UMAG_03751	AAGCAGGTGTCGGAACAA	CAAGTGAGGCATCACCTC	0.949	31	Yes	No	No	Yes
UMAG_03752	GCCGACAATGAAGGCCAA	TACTGGCAGGTGCAACCG	0.981	27	Yes	Yes	Yes	Yes
UMAG_03753	GCACGAAACGACCAGGAA	TACTGGCAAGTGTAACTT	0.980	22	Yes	Yes	Yes	Yes
UMAG_03807	GCAACAGTAGCACCGCGC	GCAGAACTGGACGTTCCA	0.977	21	Yes	No	No	Yes
UMAG_03818	TCCACTACTGCTGATGCA	CCTCGGAGCAGCGGCGAG	0.983	31	Yes	Yes	Yes	Yes
UMAG_03822	CAGGATAGCTCGGTCGCT	GAGAGCAAAGGCACCGGC	0.996	21	Yes	No	No	Yes
UMAG_03880	GTTCCGCATCCCGTTTTG	GGCCAGCGTCGATGCAAG	0.985	23	Yes	No	No	Yes
UMAG_03923	GCTCCAGCAGCAGTAGCT	CATGCCAGCTTGAGCAAA	0.972	19	Yes	Yes (Autoactive)	No	No
UMAG_04027	GCTACGGTTCCGGCGACA	AATAATACAGTATCCCGG	0.978	20	Yes	Yes	Yes	Yes
UMAG_04033	GCACCAACTCTTTCTTCA	CCGTCTGATCGTATCGAG	0.969	22	Yes	No	No	Yes
UMAG_04035	GCTCCACCTCAGCACCTG	GAAATGCGGCATGAAGCG	0.997	24	Yes	No	No	Yes
UMAG_04038	GGCCCTATCAAGCCACCT	AAGACGCGAGAGAACACT	0.992	18	Yes	No	No	Yes
UMAG_04039	ATGCCCACGTTTGGTCAG	CATTGGCCGAGATAGGTC	0.980	26	Yes	No	No	Yes
UMAG_04057	TCTCCTGCCAACTACAAG	GTTCTTCCAGCCAGGACG	0.288	N/A	Yes	No	No	Yes
UMAG_04096	GTTCCAGCTTTCAGCGAA	AATAGGAGTAGACGATGT	0.951	19	Yes	No	No	Yes
UMAG_04104	CAGACGCAAAACCCGGAA	ATAGCCACTGGCATCAAT	0.989	30	Yes	No	No	Yes
UMAG_04111	CATCCCGGTGGCCACGCG	GGAAGGAGGAGCTCGACC	0.990	20	Yes	Yes	Yes	Yes
UMAG_04114	GCCCCGAACAACGGCGCC	GAGCTGCTGACGACGAGC	0.985	22	Yes	No	No	Yes
UMAG_04145	TCCAATGACGATCCTCGC	GTCGCTCTGTTGCAGCCT	0.999	23	Yes	No	No	Yes
UMAG_04185	GCGGATTCATCCGCCCAA	GAAGAGCGTCCAGACACC	0.995	27	Yes	No	No	Yes
UMAG_04248	AACTCCGCTATCGCCACC	AGCCGAGACAATGGGCGC	0.994	19	Yes	No	No	No
UMAG_04282	GCTGTCTTGTTGCCTCGC	ATAGACAGTACCGTTTCG	0.833	20	Yes	Yes	Yes	Yes
UMAG_04514	GCATCCAACCAACCAAAT	ATCCCACACCAACCTGCC	0.265	N/A	Yes	No	No	Yes
UMAG_04533	GTTCCCAGCGATCTCGTC	AAACGTGGGGCCCTGTTG	0.996	21	Yes	No	No	Yes
UMAG_04557	ACCACGGTGGCGCAAGTG	GCTACTGTAGGCGTTAGC	0.990	20	Yes	Yes	Yes	Yes
UMAG_04696	AGTCCTCTGCCTGCGATT	AGAGGGGCAAGTGCCGTT	0.990	17	No	No	No	Yes
UMAG_04698	GCACTCACCATCGAAACA	CGATTTCGGAGCAATGGC	0.637	27	Yes	No	No	Yes
UMAG_04815	CTTCCAATGTTGAGCACA	GCCTTTTAACAAATCGAT	0.917	23	Yes	No	No	Yes
UMAG_04893	CTCAGCGTATCGTCCTCG	GGCGGGCGTCTCCTTGGT	0.944	23	Yes	No	No	Yes
UMAG_05027	AACGAAAGGCCCAGCAAT	CCAACAATGGTGCTCATA	0.987	28	Yes	Yes	Yes	Yes
UMAG_05036	AGTCCAGAAGCTATGATG	GGATCGACGAGAGGTCTG	0.988	20	Yes	No	No	Yes
UMAG_05046	ATGAGTCTTCCAGCCGGC	CATCTTGGTGAAGCTGAC	0.989	27	Yes	Yes	Yes	Yes
UMAG_05104	GGCGATGACACTTGTGCT	GGCCACGACGTTGACCGT	0.999	20	Yes	No	No	Yes
UMAG_05222	ACGCCTCTGGCATTCAAC	GAACAAGTGGACAAACAC	0.989	23	Yes	Yes	Yes	Yes
UMAG_05227	TCGGTCTTGCCTCGAGTG	AGCGAGCTTGAGGAAGTA	0.986	20	Yes	No	No	Yes
UMAG_05294	GCTCGCAGTGCGAGGGTT	AAGGAACAAAGCGGCTCG	0.991	24	No	No	No	Yes
UMAG_05295	GAGCCATCTGGTGCTCAA	CCAGTTGCGATCCCAATG	0.998	20	Yes	Yes	Yes	Yes
UMAG_05300	CTGCCGAGCCAAAAGCCA	GCGGAGAATTTCGTGGAG	0.987	21	Yes	No	No	Yes
UMAG_05301	GTAGCAAAGCCTGGTAGT	GTGAATCGGGATCCCTCG	0.684	28	Yes	No	No	Yes
UMAG_05302	ACTGGCGGCTTTGACTAC	AAGAGGGAAGCGAGGGAG	0.966	25	Yes	Yes	Yes	Yes
UMAG_05303	GCAGGACTACTTCCATTA	ATGCAGCCAGTGCAAAGT	0.991	21	Yes	No	No	Yes
UMAG_05305	ACCGATGACGAAGAAGCT	GACACGACGAGTAAGGAA	0.951	27	Yes	No	No	Yes
UMAG_05306	CAGTTCAGCTGCCACACT	AGTAGGCGGTCTGTAAGT	0.977	28	Yes	Yes	No (Autoactive)	Yes
UMAG_05308	CAACCAATACCTCCGACA	GATAAGATTGACAGCCAG	0.889	22	Yes	Yes	Yes	Yes
UMAG_05309	AGATCCACTCGCTTTAGA	CACTTCTCGGACGATGCC	0.702	22	Yes	No	No	Yes
UMAG_05310	TCTAAATCAGCTCGTTCT	CACCTCACGGACGATGCC	0.967	22	Yes	No	No	Yes
UMAG_05311	GCGCCAGTTTCTGTAGAA	TCGTAACAAGCACGTGAT	0.911	25	Yes	No	No	Yes
UMAG_05312	GCGAGCACAAATCTCGAC	GACTGCAGGCGTGACGAG	0.924	22	Yes	Yes	Yes	Yes
UMAG_05314	GCACCTAGACAGGAAGGA	AGGATCTTCAATTCGTAA	0.971	23	Yes	No	No	Yes
UMAG_05317	GTTGAGCGTATCCAACCA	CGCGCCCAACAAGGGCAA	0.992	21	Yes	No	No	Yes
UMAG_05318	GCAGAGAGCGACGGTGGA	GAAGTGCAAAGCAAGTGA	0.989	27	Yes	Yes (Autoactive)	No	Yes

Gene	Forward Primer	Reverse Primer	SignalP 5.0 Score	Signal Peptide Length (aa)	pGBKT7 (bait)	Baits with growth after pooled mating	Used as bait in liquid media	pGADT7 (prey)
UMAG_05319	GCATTTTCCGAAGCGGCC	GATTTCTGGCACCTCGGG	0.995	24	Yes	Yes (Autoactive)	No	Yes
UMAG_05341	TCAATCCTACCTCACAAG	GCCGAGTTTTGGACAGAC	0.273	N/A	Yes	No	No	Yes
UMAG_05366	CACTTTACGCTCGACTAC	AATCAAGGATACAGCCCC	0.996	21	Yes	No	No	Yes
UMAG_05548	CTTCCGAGCTCGTCATCG	CAATACGGCGTTGACATT	0.877	25	Yes	No	No	Yes
UMAG_05562	GCTCCCGCTCAGAAGCGC	CGAAGAGAGCGCGGAGAT	0.802	18	Yes	No	No	Yes
UMAG_05604	ATGCCCACGCCTGCCGAG	GTCGAAAAGGACGTACTG	0.969	22	Yes	Yes	Yes	No
UMAG_05633	GAAGTGGCAAGAGTACGG	AAGCAAGACGGAAGCCCC	0.971	18	No	No	No	Yes
UMAG_05733	TCGCCAACGACGTCGGCG	GAGGACGAAGGTGGTGAT	0.971	27	Yes	No	No	No
UMAG_05780	GCGCCAGTAGGATCGAGT	GGCTCTGAGTTGTCGACG	0.892	24	Yes	No	No	Yes
UMAG_05781	ATCGAGGTGCCCACTTCA	TCGGGCAACGCCAGGAAT	0.574	21	No	No	No	Yes
UMAG_05819	GCGCCCTTGTCACCAGTC	CAGATCGTTGTAGTTGAC	0.923	23	Yes	No	No	Yes
UMAG_05824	GCACCCATCCCGCGTCCC	CGATGAACTGCTTCTGCG	0.997	23	Yes	No	No	Yes
UMAG_05861	TTTCCCGCCTCACTCGGT	CGCCGGCTCGACCTCGTT	0.997	20	Yes	No	No	No
UMAG_05927	GCCGGAAACAGCGGTCAG	TAGAGGACGGCCAAAAGT	0.985	23	Yes	Yes	Yes	Yes
UMAG_05928	ACAGATGGCACCCTTCCG	AACTGGTCGAAGCGAACG	0.980	22	Yes	No	No	Yes
UMAG_05930	ATCGACCAGTCGGGCTGG	CAGACTGTGAGCCTCGCT	0.992	21	Yes	No	No	Yes
UMAG_05931	GTGAAAATGCCAAAACTC	AACAATCTGCCAGCTGGC	0.982	19	Yes	No	No	Yes
UMAG_05932	GTGATGACTGAGCAGGAC	GTGAACATGCTTCATGAC	0.982	18	Yes	Yes	No (Autoactive)	Yes
UMAG_05953	AGCCTGCACAGACGAGAC	GACGACCACAAACGCTGC	0.959	22	Yes	No	No	Yes
UMAG_05988	CATGGCCCGGAAAGCAAG	GAGCTCCTCGTGTGCAGG	0.980	24	Yes	No	No	Yes
UMAG_06064	CAGCAAGGAGCGCCACCC	TAGCAGAAGAGCAGCACC	0.997	25	Yes	No	No	Yes
UMAG_06113	GCTCCTATTGCTGACCAG	GATGATACCGTCGAGGGG	0.963	20	Yes	No	No	Yes
UMAG_06119	GCACCGCTCGATGGCACG	ATGCGAGGACCACGCAGG	0.976	23	Yes	No	No	No
UMAG_06126	GGTCCGATGGGCGGTCGT	AGGGCACTGGAACCACTC	0.936	23	Yes	Yes	Yes	Yes
UMAG_06127	CCGAACCCAGTCGCTCTC	GGCTGCACGAGAATCGAG	0.894	26	Yes	No	No	Yes
UMAG_06128	CATCCTCCTCCTCCGACG	ACTAGAGCTACTCGGTGC	0.722	27	Yes	No	No	Yes
UMAG_06146	GCTCCTGCTCAGCAGCTT	GGCAAAGCTCACGTAGGT	0.998	21	Yes	Yes	Yes	Yes
UMAG_06158	TCGCTAGCAAAGTCGGAC	GGGCTGCACTTGGAACGC	0.981	26	Yes	No	No	Yes
UMAG_06162	CAGTCGGTCGCTCCTTTC	GAGGAGGTAAGCACCGAC	0.990	18	Yes	Yes	Yes	Yes
UMAG_06178	GTTCAGATCAATCTCAAC	AGGACAAGCGTAAGAGGG	0.684	26	Yes	No	No	Yes
UMAG_06179	AGTCCGATCAACGTCGAG	ATGGCACTTGAACTTTGG	0.959	26	Yes	Yes	No (Autoactive)	Yes
UMAG_06180	GGTCGGGCGAATGGCGAC	AGGACACGCGTAAAAGGG	0.860	25	Yes	No	No	No
UMAG_06181	GGTCGGATCGATCGCGGC	ATCCTGCGGCATCTGCGC	0.951	25	Yes	Yes	No (Autoactive)	Yes
UMAG_06221	ATGACGAGCCGACCACCG	GGGCCGATGAGACTGGGG	0.987	18	Yes	No	No	Yes
UMAG_06222	GCACCTATGGATTGGGTC	TTGCGTTTCGCGAAGCAG	0.997	18	Yes	Yes	Yes	Yes
UMAG_06223	GCTCCCACTCCCACGCCT	CCAATGGTATTCATGGTG	0.998	19	Yes	Yes	Yes	Yes
UMAG_06255	GCTCCTGTTGGACGCGTG	CGAACCTGGCTGGAATTG	0.805	29	Yes	Yes (Autoactive)	No	Yes
UMAG_06428	TCGGCCATCCCACAAAGA	CTGCCCGCAAGCAGCGGT	0.943	25	Yes	No	No	Yes
UMAG_06440	CTCCCTTTCCCCACCAAT	CGCTGGCCTGACGGGACC	0.996	24	Yes	No	No	Yes
UMAG_10024	ACCGGTCCTTCCCACCCA	CTGTGTCTTTTTATCGCC	0.960	19	Yes	No	No	Yes
UMAG_10030	TCGCTTGATTCTGTCGAG	GAAGATTGGGCCGTCCTG	0.881	24	Yes	No	No	Yes
UMAG_10067	TTGCAGCAAAAGCGACCC	GACGTGTGCAACATTGCC	0.905	25	Yes	No	No	Yes
UMAG_10076	GTAAAACAGCCATACGAG	GAAGTGTCGCAATACCGT	0.893	23	Yes	Yes	No (Autoactive)	Yes
UMAG_10115	GCCCCTACGGAGGGGCGA	GTGATACTTGGAGCCGAA	0.949	32	Yes	No	No	Yes
UMAG_10155	TTGCCGTCAGGCGTATCG	GTAGTCATTAGTGACGTG	0.984	17	Yes	No	No	Yes
UMAG_10221	TCAGCTACATTGGCAAAC	AGAGCACTTTCTCGGCCT	0.879	27	Yes	No	No	Yes
UMAG_10274	CGTTTCTTTGGCGCCAAC	GCGTCTCTTATTACCCTT	0.776	21	Yes	No	No	Yes
UMAG_10278	CGGCTCCTCCGCGTAAAG	CCACCGGTGCTGGTGTGT	0.779	27	Yes	No	No	Yes
UMAG_10317	CAACACCAGATTCGTGGT	CTGGTAGTGACCGTGTGG	0.971	20	Yes	No	No	Yes
UMAG_10403	GCTGCAGTCGAGCGCCCT	ACCAGTGCGCTGGTCGAG	0.997	22	Yes	No	No	Yes
UMAG_10418	AGCATCCTAGCTGGCGGT	CGATTGGAACAAATCCTT	0.978	21	Yes	No	No	Yes
UMAG_10474	TCATCCTCGGTCCCCGCA	GAGCATCATGGCGCCAGC	0.731	25	Yes	Yes	No (Autoactive)	Yes

Gene	Forward Primer	Reverse Primer	SignalP 5.0 Score	Signal Peptide Length (aa)	pGBKT7 (bait)	Baits with growth after pooled mating	Used as bait in liquid media	pGADT7 (prey)
UMAG_10514	TCAAGCATGGCCAATGTG	CGTCTGGCTGAGTTTGGC	0.417	N/A	Yes	No	No	Yes
UMAG_10536	GACACGCGCTTGTCGGAC	CAATTCGGCGTGTGCGGA	0.998	19	Yes	No	No	Yes
UMAG_10553	GTGAAACCATCAGTCGAG	ATGGTCCCCGTGTAGGAC	0.929	27	Yes	No	No	Yes
UMAG_10554	GCATCTTCTGTTCACACG	AACAGGATGATGTTCAAG	0.820	24	Yes	No	No	Yes
UMAG_10555	ACTCGTTCTGCGCCAGAG	AAAGCCATGGATTCGACC	0.931	23	Yes	Yes	Yes	Yes
UMAG_10556	GTGAAACTCCCTCTTATT	CGATGACGTTCCCTCATC	0.940	21	Yes	Yes	Yes	Yes
UMAG_10557	GCAATCGGCGTGCCTCAC	GAAACGGACGTGGCCGTA	0.870	24	Yes	Yes	Yes	Yes
UMAG_10640	GCTCCAGTAGAGAAGCGC	AGTCCACCAGTTGATGGG	0.996	19	Yes	Yes (Autoactive)	No	Yes
UMAG_10742	CTCACGCCAGCAGTGTCG	GCGAAGTCGTTCAAACGA	0.276	N/A	Yes	No	No	Yes
UMAG_10811	AAGCATCTCGAGATATGC	TCGCTGCCCCACCGGACT	0.066	N/A	Yes	No	No	Yes
UMAG_10816	TTCGAGATCACCTTTCCC	GACAAGCAGACCAGCGCC	0.987	19	Yes	No	No	Yes
UMAG_10831	TGGGAGACCAAGGAACAA	TTGAAATACTAGTGTTGG	0.877	31	Yes	No	No	Yes
UMAG_10881	GCTCCTCTTGAGCCGCGC	GTAGTCGCATCCAACAAA	0.999	17	Yes	No	No	Yes
UMAG_10972	GCCAAACAAGGCGAACAC	GACCTTTTTGAGTGCCAC	0.948	27	Yes	Yes	Yes	Yes
UMAG_11002	CAGGTGTATGCTGCGCAT	ACCCGAGCTGTGGCTGCC	0.996	21	Yes	Yes	No (Autoactive)	Yes
UMAG_11058	TTGGTTGTCCCGTCCACC	CGGGACAAGACCTTTCGT	0.889	20	Yes	No	No	Yes
UMAG_11060	CTACCACCTTCCAGCTCA	GGCCTTGTAGTTCCTGAA	0.990	19	Yes	No	No	Yes
UMAG_11062	GTTGGTACACCGCGAGCT	GAGGTGGAAGCCCTTCCC	0.978	20	Yes	Yes	Yes	Yes
UMAG_11094	GACAGTCCCAAGTCCAAC	GAGGCGAGAAGCGCGAGA	0.539	25	Yes	No	No	No
UMAG_11111	AAGACGGTACAGCATGAC	CACGAAAAGAGCCGGGTG	0.960	23	Yes	No	No	Yes
UMAG_11193	AACCCTGCGCCGCCATCC	CAAATGCTGCCAGATCTG	0.903	28	Yes	Yes	Yes	Yes
UMAG_11250	GGTCATATTGACCTCGAC	GGGACACATATAGGAGGC	0.874	26	Yes	No	No	Yes
UMAG_11305	GTCAGCTATGTACTTGAC	TGCAGAGTGAGAGTCGGG	0.826	20	Yes	No	No	Yes
UMAG_11362	TCAGCCCCGCGTTGTGAT	GGGAAGCGTCCAGTCGGA	0.999	21	Yes	No	No	Yes
UMAG_11377	TGGCCATATCCTTTTGGC	GGCATGCAGACGAGTTGC	0.942	23	Yes	No	No	Yes
UMAG_11403	GACTGGCTTCAGGATTTC	GAACTCATCCTTGGCACG	0.981	27	Yes	No	No	Yes
UMAG_11415	ACTTCTTACATGCCGGAG	GCTTCCACTTGTGCCGGC	0.993	18	Yes	Yes	No (Technical issue)	Yes
UMAG_11416	GCCACAAGCCTGTCAGAG	CTTTGGTGGGGCGTCCGG	0.946	24	Yes	No	No	Yes
UMAG_11417	GGCGACGACCTGGGAAGC	AGATTCCGAAACAGCGTC	0.938	17	Yes	No	No	Yes
UMAG_11443	GCACGCTCAGCCTACTGC	GCCATCGCTGTGGCTGCC	0.998	24	Yes	Yes	No (Autoactive)	Yes
UMAG_11444	GGCCATACAGCCTACTGC	GCCGTTGCTGTGGCTGCC	0.998	23	Yes	Yes	Yes	Yes
UMAG_11464	GCCTTCCACTCGAACAAC	AGGGGTATGCGATCTCTG	0.999	20	Yes	No	No	Yes
UMAG_11484	GTGCCGGCGGGTGACAAC	CCTGTGCCGGATAGTTGC	0.957	25	Yes	No	No	Yes
UMAG_11554	ATCTCGACCAGCATCCCG	GAGCAATGTGGAAGCAAG	0.984	23	Yes	No	No	Yes
UMAG_11586	CACATCACACTCACTGTG	TGGATACCGGATGCCAAA	0.253	N/A	Yes	No	No	Yes
UMAG_11628	GCTTCAGTCCGGCTGAGC	ACTTGAACCAAGCGTCCC	0.941	19	Yes	No	No	Yes
UMAG_11637	AAGCGCCTTGATGTCGTT	ATGCCCGGTGTAGGAATA	0.960	18	Yes	Yes	Yes	Yes
UMAG_11639	TGCTTTGCCAATATCGTG	CTCGGAAAATTCGTATTC	0.113	N/A	Yes	Yes	Yes	Yes
UMAG_11813	CTTTCGCCGTCACCTTGT	GGCCTGCGGCGAcACCTG	0.401	N/A	Yes	Yes (Autoactive)	No	Yes
UMAG_11886	GACATAACCAAACCTGTC	AGCCTGAAGCAGCTTGGG	0.979	23	Yes	Yes	Yes	Yes
UMAG_11910	GCCCCTGTTCGCGCTGGC	GTGGAACATTTCTTTGAT	0.820	20	Yes	No	No	Yes
UMAG_11915	GCATCCTCGCACAAAAGA	ACACCCCTGCAACTTCCA	0.706	34	Yes	Yes	Yes	Yes
UMAG_11931	GCCACTTTGACGTGGGAC	GACGAGAGCTTTGACAAT	0.999	22	Yes	Yes	Yes	Yes
UMAG_11940	GCACCGATCCCGAGCCCA	GGCGGTATTGCGTTGGGT	0.992	24	Yes	Yes (Autoactive)	No	Yes
UMAG_11980	GGAAACTGCGACGCCAAC	TGAAACCTTTGGTTCCAC	0.842	21	Yes	No	No	Yes
UMAG_12015	ACTCCCTCTGCACATGAT	CTTGCACGAAGCGAGAAT	0.974	27	No	No	No	Yes
UMAG_12101	ACCGCACCAGCGTTGATC	TACGACTTCGTACACGGC	0.669	26	Yes	No	No	Yes
UMAG_12127	GCGCTTCATCTGAACGAA	CAAGCGGGCAGGAACCTG	0.489	N/A	Yes	Yes	Yes	Yes
UMAG_12184	GCACAGCCAACCATCAGC	CTTGGGTCCCGCGCCGAT	0.966	24	Yes	Yes	Yes	Yes
UMAG_12197	GCGACGCAGAGGGTCGGG	GTGGTGTACTTGGGACCA	0.999	23	Yes	Yes	No (Autoactive)	Yes
UMAG_12215	GCCAAGAAACCTTTCTAC	CCGCCTGATCCTGATCAC	0.795	28	Yes	No	No	Yes

Gene	Forward Primer	Reverse Primer	SignalP 5.0 Score	Signal Peptide Length (aa)	pGBKT7 (bait)	Baits with growth after pooled mating	Used as bait in liquid media	pGADT7 (prey)
UMAG_12216	ATTGTTGAAGACGAGCCA	GATCCCACTACCATCGCT	0.793	27	Yes	No	No	Yes
UMAG_12217	ATTTGCATGCAGTGTGAT	GTTGCTGTCTTTTGTGTC	0.999	19	Yes	No	No	Yes
UMAG_12226	GCGTCCCAGTCCATGTGT	CGGCAGCCGCTTGCAAAT	0.999	23	Yes	No	No	Yes
UMAG_12238	ACTCCCTCTGCACATGAT	CTTGCACGAAGCGAGAAT	0.974	27	Yes	No	No	No
UMAG_12253	GAAAGCGTCGCCTGGAGC	TGACGCAAGTACATGGAT	0.971	25	Yes	No	No	No
UMAG_12258	GTGTACTTTGATCCGCAG	GAGAACGACGTGACGGTC	0.972	24	Yes	No	No	Yes
UMAG_12302	ACGAGGTATACTAATGTC	ATGAGGCCAGTCGGCTGG	0.980	19	Yes	Yes	No (Autoactive)	Yes
UMAG_12313	GCACCGTTCGAGCGCGCG	GACCACGGCATGAGTGCC	0.955	28	Yes	No	No	Yes
UMAG_12316	GAACCAGCTTCTCACCGT	GAGCAGCCCTATGCCAAA	0.953	33	Yes	No	No	Yes
UMAG_12330	CAACTTGGTAAACGCGAC	GGCGGTTCTGGGAACAAT	0.950	24	Yes	No	No	Yes
UMAG_15089	AAGAAGCAGTGCCGTGCC	CCTGCGGACAGGGACGAG	0.987	28	Yes	No	No	No

Table S2 – Identity of bait proteins used in the liquid media screen.

Baits were tested for autoactivity by growth in liquid culture and on plates. In the mating test (SD-Leu/-Trp/-His/-Ade) the bait strain was mated with yeast containing an empty pGADT7 plasmid. In liquid tests, strains were considered autoactive when $OD_{600\ nm}$ was higher than 0.25 after 3 days of incubation at 28ºC.

Effector	Autoactivity test		
	$OD_{600\ nm}$ in liquid media		on plate
	SD-Trp/-His/-Ade	SD-Leu/-Trp/-His/-Ade	SD-Trp/-His/-Ade
UMAG_00628	0.090	0.064	No
UMAG_00961	0.094	0.055	No
UMAG_01301	0.092	0.072	No
UMAG_01690	0.171	0.142	No
UMAG_01940	0.165	0.070	No
UMAG_02006	0.113	0.059	No
UMAG_02011	0.091	0.058	No
UMAG_02119	0.089	0.076	No
UMAG_02194	0.048	0.075	No
UMAG_02230	0.086	0.073	No
UMAG_02293	0.092	0.064	No
UMAG_02298	0.106	0.060	No
UMAG_02430	0.101	0.061	No
UMAG_02475	0.106	0.068	No
UMAG_02535	0.242	0.069	No
UMAG_02538	0.046	0.056	No
UMAG_02611	0.209	0.077	No
UMAG_02851	0.092	0.060	No
UMAG_02853	0.164	0.065	No
UMAG_03023	0.092	0.068	No
UMAG_03201	0.100	0.101	No
UMAG_03231	0.090	0.062	No
UMAG_03392	0.108	0.063	No
UMAG_03564	0.234	0.067	No
UMAG_03585	0.089	0.056	No
UMAG_03689	0.099	0.058	No
UMAG_03746	0.099	0.072	No
UMAG_03747	0.102	0.059	No
UMAG_03752	0.099	0.070	No
UMAG_03753	0.091	0.072	No

Effector	Autoactivity test		
	$OD_{600\ nm}$ in liquid media		on plate
	SD-Trp/-His/-Ade	SD-Leu/-Trp/-His/-Ade	SD-Trp/-His/-Ade
UMAG_03818	0.076	0.059	No
UMAG_04027	0.108	0.085	No
UMAG_04111	0.102	0.064	No
UMAG_04282	0.233	0.056	No
UMAG_04557	0.117	0.058	No
UMAG_05027	0.099	0.059	No
UMAG_05046	0.092	0.058	No
UMAG_05222	0.193	0.083	No
UMAG_05295	0.085	0.061	No
UMAG_05302	0.085	0.072	No
UMAG_05308	0.214	0.092	No
UMAG_05312	0.098	0.061	No
UMAG_05604	0.092	0.057	No
UMAG_05927	0.096	0.062	No
UMAG_06126	0.106	0.072	No
UMAG_06146	0.101	0.073	No
UMAG_06162	0.123	0.066	No
UMAG_06222	0.097	0.068	No
UMAG_06223	0.102	0.087	No
UMAG_10555	0.088	0.082	No
UMAG_10556	0.097	0.077	No
UMAG_10557	0.240	0.075	No
UMAG_10972	0.087	0.056	No
UMAG_11062	0.137	0.081	No
UMAG_11193	0.109	0.071	No
UMAG_11444	0.091	0.071	No
UMAG_11637	0.122	0.076	No
UMAG_11639	0.212	0.152	No
UMAG_11886	0.089	0.067	No
UMAG_11915	0.092	0.070	No
UMAG_11931	0.100	0.076	No
UMAG_12127	0.091	0.077	No
UMAG_12184	0.092	0.076	No

Table S3 – Primers used for sequencing of inserts (effectors) after gateway reaction

Primer	Sequence
pGADT7_Fw	ATCAAGTATAAATAGACCTGC
pGADT7_Rv	CTATAGATCAGAGGTTACATGG
pGBKT7_Fw	TATCAAGTATAAATAGACCTGC
pGBKT7_Rv	TAAATCATAAGAAATTCGCC

Figure S1 – Workflow of Y2H work

Numbers represent coding sequences of putative effector proteins tested. See Supplementary Tables S1, S2, and S3 for further details.

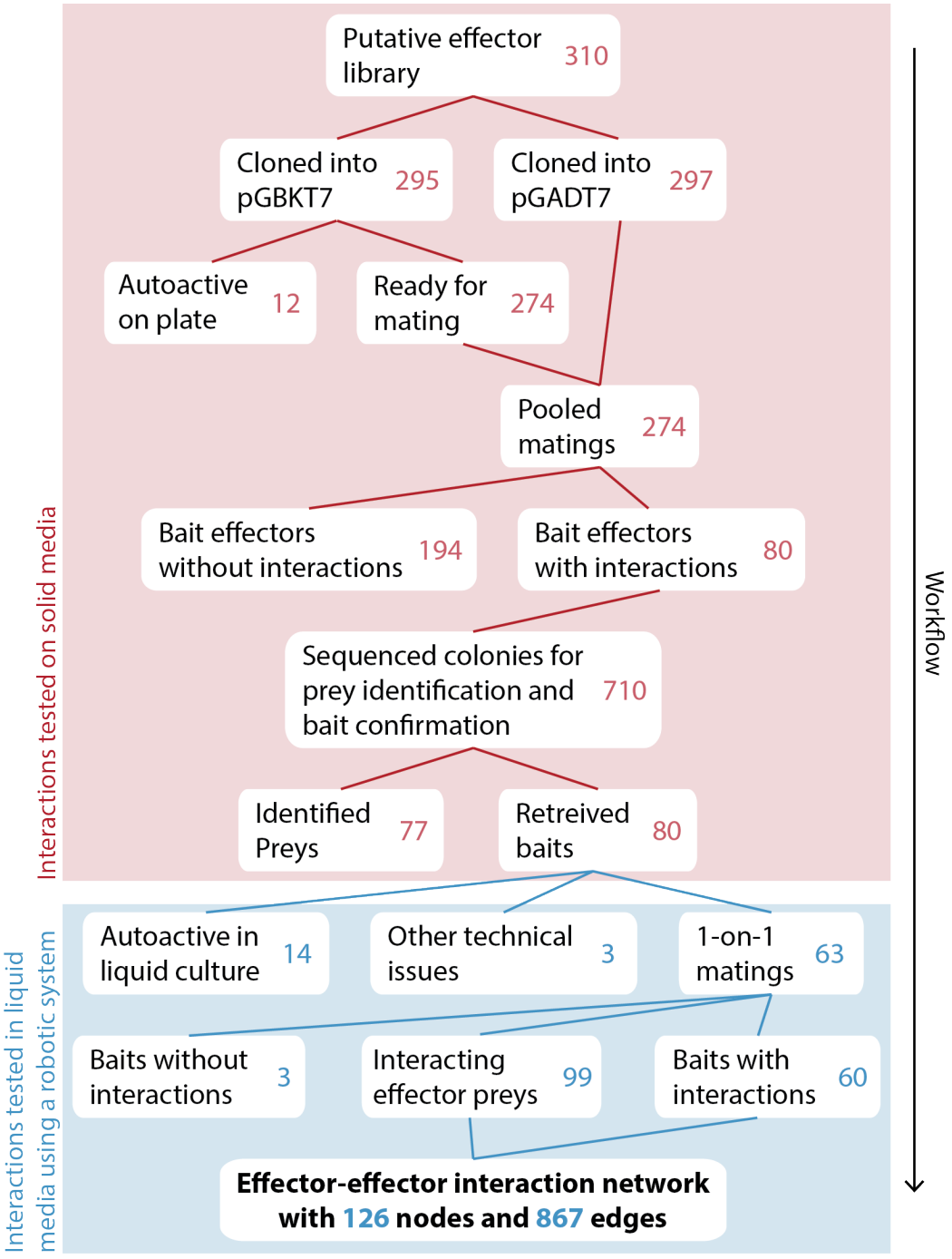
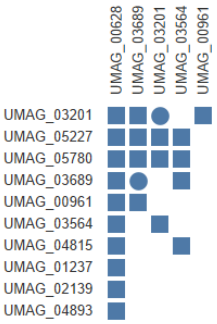


Figure S2 – Detailed description of the subnetworks represented in Figure 2

Core effectors were identified in Schuster et al. (2018), clusters were described in Kämper *et al.* (2006), iPool-Seq data was obtained from Uhse *et al.* (2018), and sequencing data was taken from Lanver *et al.* (2018). The centers of the networks are highlighted in bold; circles represent homodimers and squares represent heterodimers.

Gene	Core	Cluster	iPool seq Depleted	0.5 dpi		6 dpi	
				log_2FC	adj. P val.	log_2FC	adj. P val.
UMAG_00628	N	-	N	9.51	2.04E-83	12.32	4.26E-142
UMAG_00961	Y	-	N	-0.06	0.81	-2.00	2.36E-26
UMAG_01237	Y	2A	N	2.95	8.02E-06	9.33	3.26E-235
UMAG_02139	Y	-	N	1.65	5.29E-10	2.68	3.52E-39
UMAG_03201	N	8A	Y	8.99	4.96E-50	12.71	1.52E-106
UMAG_03564	N	-	N	2.87	0.03	0.60	0.67
UMAG_03689	N	-	N	2.76	8.41E-15	-0.65	0.24
UMAG_04815	N	-	N	0.78	0.18	8.38	3.29E-151
UMAG_04893	N	-	N	-0.09	0.95	0.54	0.70
UMAG_05227	Y	-	N	-0.13	0.77	0.44	0.12
UMAG_05780	Y	-	N	6.06	3.41E-46	10.92	1.58E-172



Gene	Core	Cluster	iPool seq depleted	0.5 dpi		6 dpi	
				log_2FC	adj. P val.	log_2FC	adj. P val.
UMAG_02006	N	-	N	7.62	1.10E-48	4.63	1.31E-15
UMAG_02119	N	-	N	4.86	6.35E-10	9.84	1.14E-67
UMAG_02293	Y	-	N	3.90	1.89E-11	9.49	1.38E-226
UMAG_02475	N	5B	Y	8.14	2.91E-89	11.73	2.85E-195
UMAG_02611	Y	-	N	1.92	3.20E-23	2.77	7.28E-59
UMAG_02851	N	-	N	9.36	1.09E-43	10.81	2.41E-61
UMAG_02853	N	-	N	8.74	1.68E-39	11.09	3.72E-67
UMAG_03065	N	-	N	12.27	1.50E-61	5.10	1.14E-10
UMAG_03231	N	-	N	3.50	1.04E-04	9.38	4.06E-76
UMAG_03747	N	10A	N	5.88	4.24E-12	12.86	5.68E-100
UMAG_04027	N	-	N	5.97	9.35E-54	4.75	1.16E-35
UMAG_05222	N	-	N	1.35	0.01	-1.36	0.01
UMAG_05295	N	19A	N	4.73	2.59E-06	13.46	2.70E-101
UMAG_05302	N	19A	N	5.53	3.52E-10	10.31	4.70E-58
UMAG_06146	N	-	N	1.54	3.34E-03	0.73	0.17
UMAG_11193	N	-	N	1.82	7.22E-07	0.70	0.06
UMAG_11639	N	-	N	0.30	0.77	0.14	0.89

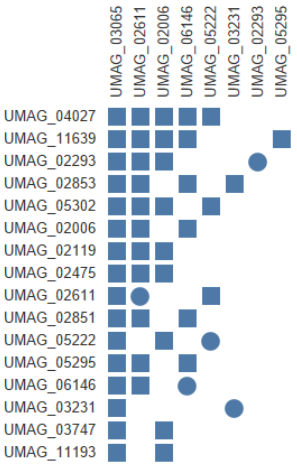


Figure S3 – Co-immunoprecipitation of 12 proteins from the UMAG_00628 subnetwork

Proteins were tagged with either 3x myc or 3x HA N-terminal tags, which was the same side of the activation and binding domains in the Y2H screen. *N. benthamiana* plants were transiently transformed and expressed the fusion proteins for 3 days before harvest. On the left, the interactions found by Y2H in the subnetwork subset are illustrated. Full blue boxes with white numbers represent expected interactions, empty boxes with black numbers represent protein pairs that are not expected to interact, and circles represent homodimers. On the right are the same interactions tested by Co-immunoprecipitation. The sample numbers from the Y2H matrix for each interaction pair are represented on top of the western blots.