T.R.
EGE UNIVERSITY
Graduate School of Applied and Natural Science

TARGETED Y2H SCREENING TO IDENTIFY NOVEL EFFECTOR-HOST PROTEIN INTERACTIONS

MSc THESIS

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2022

Here I certify that this thesis entitled " TARGETED Y2H SCREENING TO IDENTIFY NOVEL EFFECTOR-HOST PROTEIN INTERACTIONS" conducted, prepared, and presented by Maaz ANWAR for the degree of Master of Science has been recommended for acceptance and approval for oral defence as it complies both with the "Ege University Graduate School policies" and "Ege University Graduate School of Natural and Applied Sciences" rules and regulations. This thesis for the degree of Master of Science is sufficient in scope and quality, and the Candidate was found successful and the thesis was approved by the thesis committee unanimity voting on 06 / 09 / 2022.

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DECLARATION

I hereby declare that this thesis study entitled as "**Targeted Y2h Screening To Identify Novel Effector- Host Protein Interactions**" represents my own work which has been done after registration for the degree of MSc at Ege University and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications. I also declare that all the information in this document has been obtained and presented in accordance with Ege University academic rules and regulations as well as ethical conduct. Lastly, I declare that, as required by these rules and conduct, I have fully cited and referenced all material and results, which are not original to this work.

06/09/2022

Maaz ANWAR

ÖZET

HEDEFLENMİŞ Y2H TARAMASI İLE YENİ EFEKTÖR-KONAK PROTEİN ETKİLEŞİMLERİNİN BELİRLENMESİ

ANWAR, Maaz

Yüksek Lisans, Department of Seed Science and Technology
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Eş Danışman: Prof. Anders KVARNHEDEN
Eylül 2022, 43 pages

Mantar hastalıkları bitkilere ciddi zararlar vermektedir ve bitki hastalıklarının neden olduğu toplam verim kayıplarının %70-80'ininden sorumludur. Patojen olarak başarılarının nedenlerinden biri, bitki savunmasını engelleme yetenekleridir. Mısır patojeni *Ustilago maydis* gibi biyotrofik mantarlar, konakçı genomu modüle edebilen, bitkilerin bağışıklığını zayıflatan ve patojenlerin kendi üreme başarısını destekleyen efektör adı verilen bir dizi salgı proteini salgılar. Mantar efektörlerinin ana hedeflerinden biri, savunma sinyalizasyonu ile ilgili bitki sinyal yolaklarıdır. Sinyal moleküllerinin büyük bir sınıfı kinazlardır; örneğin, reseptör benzeri kinazlar, MAPK ve kaalsiyum bağımlı kinazlar gibi.

Bu çalışmada, varsayılan efektör proteinlerinin hedefi olarak Glikojen sentaz kinaz 3/SHAGGY benzeri kinaz (GSK3) süper ailesi odaklanılmıştır. Bitki GSK'ları, bitki bağışıklığında yakın zamanda tanımlanan rolleri de dahil olmak üzere çeşitli işlevlere sahiptir. Belirlenen 200 mantar efektör adayının, potansiyel bir hedef konak ile olası etkileşimlerinden oluşan yeni bir set, büyük bir Yeast-2-Hybrid (Y2H) test siteminde taranmıştır. Sonuçlar, GSK3 kinazlarından biri ile etkileşime giren üç varsayılan mantar efektör molekülünü ortaya çıkarmıştır. Daha sonra, aday efektör protein-konak etkileşimleri, *Nicotiana benthamiana* bitkilerinde Split Venus Assay (bimoleküler tamamlama testi) kullanılarak doğrulandı.

Daha sonra, varsayılan efektörlerin hücre altı lokalizasyonları da konfokal mikroskopi kullanılarak tespit edilmiştir. Ayrıca, varsayılan efektörlerle genetik olarak dönüştürülmüş bitki yaprakları da fenotiplendirilmiştir.

Bu tez çalışmasında; *U. maydis*'in konak metabolizmasını nasıl yönlendirdiğini ve savunma sinyal yolaklarını kendi lehine olacak şekilde nasıl hedeflediğini anlamak amacıyla Maya-2-Hibrit Sistemi, Golden Gate Klonlaması, *Agrobacterium* aracılı bitki transformasyonu ve konfokal mikroskopi dahil olmak üzere bir dizi modern ve son teknoloji moleküler yöntemler kullanılmıştır. Sonuçlar, birkaç efektör molekülünün sıklıkla aynı konak proteini hedeflediğine dair *U. maydis* efektome üzerine literatürdeki gözlemi mevcut desteklemektedir.

Bu çalışma, bağışıklık sistemi ile ilişkili kinazlar olarak bitki GSK'larının önemini daha da öne çıkarmakta ve efektörler-GSK'lar arası etkileşimlerin diğer bitki türlerinde de korunmuş olabileceğini öne sürmektedir. Bu ön çalışmanın sonuçları, konakçı hedef–efektör protein etkileşimlerinin doğası üzerine daha detaylı moleküler çalışmaların başlangıcı olarak hizmet edebilir. Tarım bitkilerinin savunma sistemlerini geliştirmek için yeni koruma stratejileri geliştirmek amacıyla patojen efektörler tarafından bitki GSK'larının hangi mekanizmalar ve yolaklarla hedef alındığını aydınlatmak için daha fazla araştırmaya ihtiyaç vardır.

Anahtar Kelimeler: Maya ikili hibrit sistemi, bitki-patojen etkileşimi, bitki savunması, *Agrobacterium*, *Ustilago maydis*, *Zea mays*, Split Venus Assay

ABSTRACT

TARGETED Y2H SCREENING TO IDENTIFY NOVEL EFFECTOR- HOST PROTEIN INTERACTIONS

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Master Thesis, Department of Seed Science and Technology
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Fungal diseases are responsible for causing serious damage to plants and account for 70-80% of the total yield losses caused by plant diseases. One of the reasons for their success as pathogens is their ability to hamper the plant defense. Biotrophic fungi, like maize pathogen *Ustilago maydis*, release a set of secretory proteins called effectors which can modulate the host genome, weakening the plants immunity and ensuring pathogens' own reproductive success. One of the major targets of fungal effectors are plant signaling pathways related to defense signaling. A major class of signaling molecules are kinases; for example, receptors like kinases, MAPK and Calcium dependent kinases.

In this study, Glycogen synthase kinase 3/SHAGGY-like kinase (GSK3) superfamily was focused as putative effector targets. Plant GSKs have diverse functions including their recently identified role in plant immunity. A novel set of 200 fungal effectors candidates' possible interactions with a potential host target were screened in a massive Yeast-2-Hybrid (Y2H) assay. The results revealed three putative fungal effectors interacting with one of the GSK3 kinases. Later, the candidate effector protein-host interactions were confirmed in the *Nicotiana benthamiana* plants using Split Venus Assay (a bimolecular complementation assay).

Afterwards, the subcellular localizations of the putative effectors have also been unraveled using confocal microscopy. Moreover, the plant leaves, genetically transformed with the putative effectors, have been phenotyped.

In this thesis study; a set of modern and state of the art molecular tools including Yeast-2-Ybrid System, Golden Gate Cloning, *Agrobacterium* mediated plant transformation and confocal microscopy have been employed to shed some light on the understanding of how *U. maydis* diverts the host metabolism and targets defense signaling pathways in its own favor. Supporting the observation for the *U. maydis* effectome, several effectors frequently target the same host protein.

This study outshines the importance of plant GSKs as immune kinases and suggests that effectors-GSKs interactions could be conserved in other plant species. The results of this preliminary study may serve as the onset for more detailed molecular studies on the nature of the host target–effector protein interactions. Further research is needed to elucidate the mechanisms and the pathways by which plant GSKs are being targeted by pathogen effectors in order to develop novel protection strategies for crop plants.

Keywords: Yeast two hybrid system, plant-pathogen interaction, plant defence mechanism, *Agrobacterium*, *Ustilago maydis*, *Zea mays*, Split Venus Assay

PREFACE

Plant pathogenic fungi can cause a devastating damage to plants which can lead to serious economic losses. Depending upon their need, fungi can interact with the plant differently and this interaction is facilitated on a molecular level. The host-pathogen cross talk is highly complex, where the pathogen releases different molecules called effectors that are designed to interact with host protein for diminishing the plant immunity. Therefore, studying their host pathogen interactions is inevitable to devise ideas for the effective control of plant fungal pathogens.

To study the host pathogen interactions, we thought of an advanced molecular approach Yeast 2 Hybrid Assay which is top notch and is used to study protein-protein interactions. Therefore, the thesis project was designed that revolves mainly around Y2H screening to identify novel effectors-host protein interactions. The project also encompasses validation of Y2H results by some *in plant* assays like split Venus assay and unraveling the subcellular localization of interaction partners.

Ustilago maydis is a biotrophic fungus which naturally attacks maize plant and causes gall formation in all aerial parts of the plants. After the sequencing of Ustilago genome, the fugus has extensively been used as a model for studying host-pathogen interactions on a molecular level. We have also employed the same strategy to answer our research questions. The effectors proteins extracted from Ustilago are being tested for their interaction against a potential target from maize e,g zm GSK2 kinase. ZmGSK2 kinase is potentially a plant defense related protein and studies have shown that the host proteins that are involved in plant defense are most likely the target of fungal effectors. Therefore, the above-mentioned extrapolation served in choosing the potential target of the study for the interactions assay carried out.

While working on this project, I sensed an immense satisfaction because I could see a plethora of research potential in this research area, and I really felt that I could discover something novel and unique. The research work, in the end, did not only bring some promising findings, but also teach me some cutting-edge

molecular techniques during the process. I am optimistic that my research findings will lay a strong foundation for future studies and would contribute towards satisfying a great cause e.g., effective control of plant fungal pathogens.

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1. INTRODUCTION

Plant pathogenic fungi are one of the most devastating groups of microorganisms that causes huge economic losses. Fungal pathogens alone account for 70-80 percent of the total yield losses caused by plant diseases. (Oerke, E.C. 2006). One of the major reasons behind such a significant number lies in their ability to modulate host genome in a highly specialized manner. Depending upon their own need, fungal pathogens interact with their hosts differently. How and when to kill a plant is determined by a specific mode of infection. For instance, a necrotrophic infection is based on killing the host cells to obtain nutrients, while a biotrophic fungus requires a living cell for completing its lifecycle. These infection strategies are also characterized by differential interactions with the host on a molecular level and respective modulation of its gene and protein functions in a way that ensures pathogen's reproductive success (Horbach *et al*, 2010).

Plants, on the other hand, have also developed defense mechanisms against pathogens during co-evolution. The attacking pathogen is often recognized by the Plant plasma membrane residing specialized receptors, followed by the initiation of a suitable defense response (Beck and Heard 2012). The type of defense response is determined greatly by the strategy of the invading fungi. For example, a biotrophic infection leads to a Salicylic acid SA dependent defense response while a Nectrophic pathogen initiates a Jasmonate or ethylene dependent defense response (Glazebrook, 2005). Plant immune responses are often very complex and involve the role of different protein kinases for activation of a targeted defense response (Innes RW., 2001). Kinases are usually a part of a bigger signaling cascade involved in defense related hormonal signaling and the kinase activity is required for transferring the stress response signal downstream to the genomic level and activation of a subsequent defense mechanism (Chen *et al.,* 2021). One such group of kinases is the Glycogen synthase kinase 3/SHAGGY-like kinase (GSK3) superfamily. There are 10 GSK3 kinases identified in Arabidopsis and are divided in 4 different clades (Dornelas *et al*., 1998; Jonak & Hirt 2002; Charrier *et al*., 2002). Plant GSKs have diverse roles including a recently identified function in plant immunity (Saidi *et al.,* 2012; Stampfl *et al.,* 2016).

The highly organized basal defense response by the plants is still prone to interruption by the pathogen. Plant pathogenic fungi have developed ways to

weaken the plant immunity by secreting specialized protein molecules called effectors that can target specific signaling kinases and disturb a defense pathway the signaling kinases are involved in (Jones & Dangl, 2006). Since we already have discussed the newly identified role of GSK3 kinases in plant defense, it is also most likely that they are also targeted by the pathogen effectors. The effector-host protein interactivities consist of complex and not entirely understood modulation of host genome. So, to meet increasing food requirements and to devise effective control measures against pathogens, the host-pathogen interaction mechanisms need to be further elucidated.

This thesis study entails the use of modern and state of the art molecular techniques to identify and study novel effectors-host protein interactions. We have taken *Ustilago maydis (U. maydis)* and its interaction with its host (maize) as a model system to discover host-pathogen interactions on a molecular level. A library of 200 effector proteins, extracted from *U. maydis*, is tested for its interaction against zmGSK2. ZMGSK2, a GSK3 Kinase, is a homologue of Arabidopsis AtSK21 (BIN2) in maize. AtSK21 belongs to the clade II of the Arabidopsis GSKs and has a known function in Brassinosteroids (BR) signaling (Hou *et al.,* 2022). We thought that zmGSK2 might possibly have a role in plant defense and is thus targeted by effectors. A massive Yeast-2-Hybrid Screening (Y2H) is conducted to discover the interaction partners. A set of putative effector proteins targeting zmGSK2 has been discovered. The subcellular localization of putative effectors and BIN2 has also been unraveled in Tobacco using mCherry fusions to identify their corresponding positions in cell and guess their chances of lying and interacting with each other. The interaction is finally confirmed in *planta* using Split-Venus Assay, a bimolecular fluorescent complementation assay (BiFC). The split Venus assay is performed through transient expression of effector and zmGSK2constructs in Tobacco, followed by confocal microscopy. Moreover, to identify the effect of the novel effectors on plant phenotype, changes in leaf phenotype of tobacco plants, transformed with identified effectors, have been observed. In addition to this, I have also performed floral dipping of Arabidopsis plants to generate stable lines for phenotyping of effectors induced Arabidopsis plants. The top effectors are finally subjected to some *in silico* analysis to unravel the structure and possibly learn the function of the identified effectors.

2. LITERATURE REVIEW

2.1 Plant Defense Responses

Many plants upon infection, trigger different degrees of defense responses. The type of defense is largely determined by the strategy of infection. For example, defense response to a necrotrophy involves Ethylene and Jasmonate dependent signaling, while a biotrophic infection leads to Salicylic acid associated defense response (Glazebrook, 2005). Moreover, the magnitude of defense is also greatly dependent on the stringency of attack.

2.2 Pattern Triggered Immunity (PTI)

The basal plant defense in all cases, against any type of pathogen, is initiated upon the recognition of pathogen derived molecular patterns (PAMP). PAMP are conserved microbial molecules like fungal chitin that are not present in the host and are perceived as foreign unrecognized molecules (Segonzac & Zipfel, 2011). At the host plasma membrane, resides these pattern-recognition-receptors (PRRs), which recognize PAMPs and initiate PAMP-triggered Immunity (PTI) (Beck & Heard 2012). PTI is a series of defense responses that on one hand helps plant withstand the microbial attack such as elevation of cytosolic Ca2+ levels which helps reinforce the plant cell wall and on the other restricts microbial colonization. The later encompasses reactions such as ROS burst - production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), and transcriptional reprogramming (Boller & Felix, 2009).

2.3 Effectors Triggered Immunity (ETI)

PTI only provides a first layer of defense against microbial invasion, so it is suppressed by molecules secreted from the microbe called effectors. These effectors help suppress the plant immune responses and thus are vital to continue the invasion (Jones & Dangl, 2006). Microbial effectors can also be recognized by the cytoplasm residing nucleotide binding leucine-rich repeats (NB-LRR) proteins. The NB-LRR proteins are encoded by R genes and the LRR domain of NB-LRR can provide

specificity for interaction with the effectors (Bernoux *et al,* 2011). The recognition of effectors by R genes initiates a second level of defense called effector triggered immunity (ETI). This mainly involves a hypersensitive response by the host leading to cell death of a localized region. (Greenberg & Yao, 2004).

2.4 GSK 3 Kinases and Their Potential Role in Plant Defense

Shaggy like kinases (GSK3 KINASES) are conserved group of kinases that were first identified in animals for their role in glycogen biosynthesis pathway (Cohen, P. *et al*. 1982). Later, they were also identified in land plants for performing a diverse range of functions. Arabidopsis GSK3s are known for regulating important plant growth and developmental pathways like flowering and cell expansion (Claisse *et al*., 2007). They have also been identified for their roles in abiotic stress response in plants (Koh, *et al*., 2007). Recently, one of the GSK3 kinases has also been identified for biotic stress response and regulating plant immunity (Stampfl *et al.,* 2016). AtSK11/ASKa is an Arabidopsis GSK3 kinase from clade I of the Arabidopsis GSK3s and regulates PTI responses in plants (Stampfl *et al.,* 2016). The discovery of ASKa function in plant defense, proposes that other GSKs from the remaining GSK3 clades might also be playing some part in plant defense responses. Investigating this idea in detail suggests that CLADE II OF the GSK3 kinases consists of Brassinosteroid signaling genes and since BR already have a proven role in plant defense, the clade 2 kinases might also be involved in regulating plant defense (Saidi *et al.,* 2012; Kong *et al.,* 2012). The clade II exclusively consists of Brasssinostroids insensitive (BIN2) and its close homologues BIN2-LIKE1 (BIL1) and BIL2. BIN2 is a negative regulator of Brassinosteroids (BR) signaling and its homologues have the functional redundancy with BIN2 (Yan *et al.,* 2009). Prior to predicting if BIN2 is a potential plant defense regulator and could be targeted by effectors, the BR signaling pathways and the role of BIN2 in regulating BIN2 signaling needs to be understood.

2.5 Brassinosteroids Signaling Pathway

Brassinosteroids are steroids hormones, and its signal transduction pathways has been explicitly unraveled in Arabidopsis and now is one of the best understood

signaling pathways. BR is perceived by the plasma membrane localized receptor BASSINOSTEROID INSENSITIVE1 (BRI1) (He *et al.,* 2000: Kinoshita *et al.,* 2005) (Figure 1). The perception of BR by BRI1 dissociates BRI from its inhibitor BRI1 KINASE INHIBITOR 1 (BKI1) and promotes BRI1 binding with its co-receptor BRI1-ASSOCIATED RECEPTORKINASE 1 (BAK1) (Li *et al.*, 2002: Wang & Chory, 2006). After complete activation, BRI1 phosphorylates two cytoplasmic kinases BR SIGNALING KINASEs (BSKs) and the CONSTITUTIVE DIFFERENTIAL GROWTH1 (CDG1) proteins (Tang *et al*., 2008; Kim *et al*., 2011). Phosphorylation of BSKs and CDG1 make them bind to and activate phosphatase BRI1-SUPPRESSOR 1 (BSU1). BSU1 belongs to the kelch-like domain family of protein phosphatases and identified as a positive regulator of BR signaling (Kim *et al.*, 2011; Mora et al, 2004). BSU1 dephosphorylates and inhibits the activity of the key negative regulator of BR signaling, BRASSINOSTEROID INSENSITIVE2 (BIN2) (Peng *et al.,* 2008). In the absence of BR, BIN2 phosphorylates the key regulators of BR induced transcriptional changes, BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1) (He *et al*, 2002; Yin, Y. *et al*. 2002). BZR1 and BES1 Phosphorylation diminishes their DNA binding activity and make them stay in the cytoplasm by 14-3-3 proteins (Gampala *et al*., 2007). Upon BR perception followed by downstream inactivation of BIN2, BZR1 and BES1 are dephosphorylated by PROTEIN PHOSPHATASE 2A (PP2A) which in turn release them from the 14-3-3 proteins. The freed BZR1 and BES1 can finally move to the nucleus and induce massive transcriptional reprogramming (Sun *et al*., 2010: Tang *et al*., 2011: Yu *et al*., 2011) (Figure 2.1).

2.6 BIN2, a GSK3 Kinase and A Potential Target of Effectors

BR is a plant growth promoting hormone and regulates important stages of the plant life cycle such as seed germination, vegetative growth, flowering, and stomatal development etc (Hussain *et al.,* 2020). The role of brassinosteroids in regulating plant defense has extensively been studied since the discovery of the components of BR signaling pathways being involved in defense responses (Kohli *et al.,* 2019). For example, BAK1, a leucine-rich repeat receptor-like kinase and an important component of BR signaling pathway works as an immune kinase and is

involved in initiating the defense related responses against pathogens (Chinchilla *et al.,* 2007). We know that the defense related kinases are also the potential targets of pathogen effectors so interestingly, BAK1 has also been reported for being targeted by a core effector NIS1(Necrosis Inducing Secreted Protein 1) from a diverse range of plant pathogens (Irieda *et al.,* 2019). Therefore, we suspect that other kinases involved in BR signaling might not only be involved in plant defense but are also targeted by pathogen effectors to impair plant immunity. Downstream of BAK1 in BR signaling, works BIN2, a GSK3 kinase and a negative regulator of BIN2 signaling. Since we have already discussed the potential role of GSK3 kinases in plant defense, we suspect that BIN2 might also be involved in plant defense and is targeted by pathogen effectors. BIN2 makes itself potential effectors target not only for being a GSK3 kinase but also for being a major regulator of BR signaling (He *et al*., 2002).

2.7 Ustilago-Maize, A Model System to Study Host-Pathogen Interactions

In order to identify and study novel effectors-BIN2 interactions, we have taken *Ustilago maydis (U. maydis)* and its interaction with its host as a model system which is now widely used in molecular pathology studies. *U. maydis*, a member of smut fungi is a biotrophic fungus and is known to cause tumors in all arial parts of corn. The plant infection is facilitated by the infectious dikaryotic hyphae which is formed by the fusion of two haploid sporidia. The infection occurs by a penetration phase which is mainly mediated by the activity of cell wall degrading enzymes secreted by the appressorium. The invading hyphae is then surrounded by the host plasma membrane resulting in the formation of an interaction zone. The host-pathogen cross talk and exchange of molecules between them is facilitated by the interaction zone. The effector proteins from *U. maydis* employ this interaction zone to enter maize cells and cause massive changes in plant's primary and secondary metabolism (Banuett, 1995; Brefort *et al*., 2009).

Sequencing of *U. maydis* genome and transcription profiling led to the discovery of Ustilago's effectors proteins that shape an interaction with the host genome (Mueller *et al.,* 2008). At Djamei Lab we already have a library of 200 effector

proteins extracted from *U. maydis*. We also amplified Shaggy-like kinases from maize and cloned them in suitable vectors. We will be employing a diverse range of advanced molecular techniques to test the mentioned hypothesis in the previous sections and answer our research questions. Following is a brief introduction of the main molecular techniques used in this study

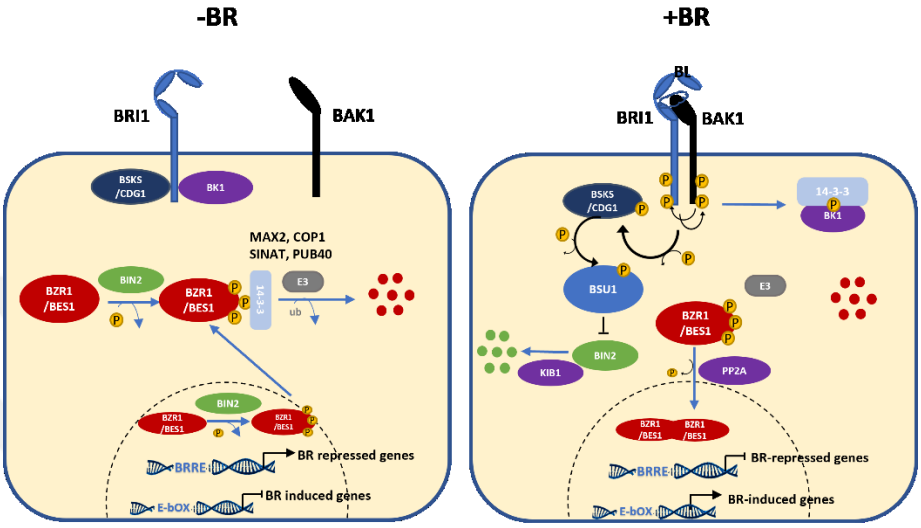

Figure 2.1. **BR Signaling pathway in Arabidopsis; On the left**: In the Absence of BR, BIN2 phosphorylates BZR1 and BES1 which promotes their binding with 14-3-3 and make them stay in the cytoplasm. No transcriptional activation of BR responsive genes is happening. **On the Right:** In the Presence of BR, BSU1 inhibits BIN2 activity and promotes its degradation. BZR1 and BES1 can enter the nucleus and induce transcriptional changes.

2.8 Yeast 2 Hybrid System (Y2H)

Y2H is a molecular biology technique used extensively in studying protein-protein interactions (Young, 1998). The physical interaction between proteins is confirmed by the activation of reporter gene(s). The reporter gene has Upstream Activating sequences UAS, a promotor region, which requires the binding of transcription factor for the activation of reporter gene. The transcription factor GAL4 is split into 2 parts called GAL4 DNA binding domain (BD) and GAL4 activation domain (AD) (Figure 2.2). The GAL4BD binds with UAS region, and it needs GAL4AD for transcriptional activation of the reporter gene (Giniger *et al.,* 1985).

The 2 potential interacting proteins are termed bait and prey proteins. The bait is fused with the GAL4 BD, while the prey is attached with the GAL4 AD (Figure 2.2). The GAL4 BD and AD constructs fused with bait and prey proteins respectively are cloned into suitable vectors and transformed into 2 sexually opposite strains of yeast. The BD-Bait combination is usually cloned into PGBKT7, while AD-prey fusion is cloned into pGAD. The two opposite yeast strains Y187 and AH109 which are mated and in case there is an interacting to be happened, the prey protein comes closer to the Bait and brings in close proximity the GAL4 AD And BD. This promotes the transcriptional activation of the reporter gene (Figure 2.2). Depending upon the stringency of interaction, more than one reporter genes could be activated individually. The reporter genes in Y2H screening are vital hormones like Histidine (his) and Adenine (ad) required for the yeast to survive. The readout in Y2H screening is the active growth of yeast (Young 1998; Matchmaker® Gold Yeast Two-Hybrid System User Manual).

2.9 Split Venus Assay

Split Venus assay or Biomolecular fluorescent complementation assay is also a widely used molecular technique to investigate protein-protein interactions. Unlike Y2H split Venus provides the evidence of interaction within the plant itself (in planta), but the principle of split Venus assay is very much like Y2H. The fluorescent protein VENUS (a yellow fluorescent protein) is split into 2 non fluorescent halves (Kodama & Hu 2012). Each half is combined with the 2 potentially interacting proteins and their interaction recombine the broken halves of VENUS and the fluorescence is restored (Figure 2.3). The readout in this case is the fluorescence which is the confirmation of interaction and can be seen under confocal microscope Bracha-Drori *et al.,* 2004; Horstman *et al.,* 2014.

2.10 Subcellular Localization of Proteins

Discovering the subcellular location of the protein of interest is an extensively used concept in cell biology as it can help predict the protein function, The protein is attached with a known fluorescent marker like mCherry (red Fluorescent protein) or GFP (Green Fluorescent Protein) etc. and is transformed into plant. The

transformed plant cell can show the fluorescence in specific compartments or in all parts of the cell depending upon the function and properties of protein of interest. The fluorescence is observed under the confocal microscope (Tanz *et al.,* 2013).

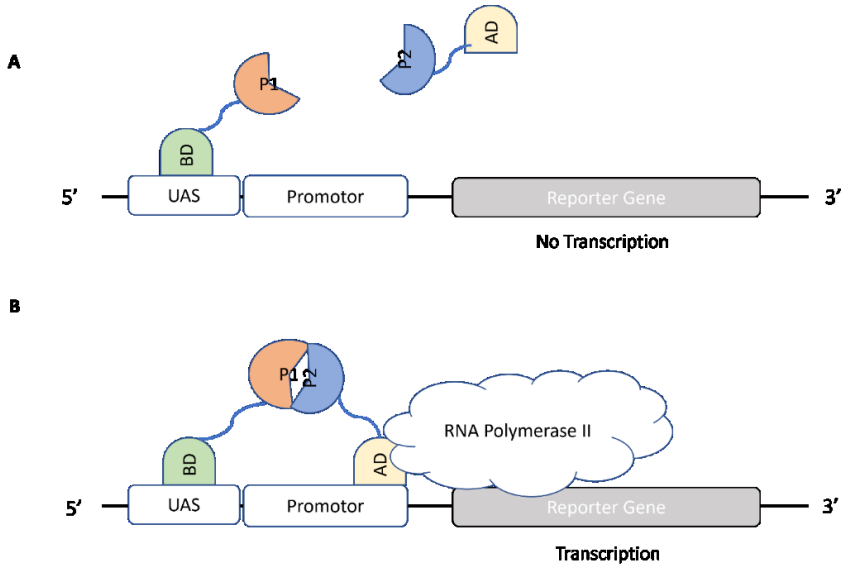

Figure 2.2. **Pictorial representation of Yeast 2 Hybrid System (Y2H): A.** The two potential interacting proteins (P1 & P2) are fused with Activation and Binding Domains (AD and BD) of the GAL4 transcription factor. There is no interaction between P1 and P2. Thus, AD and BD are far apart from each other, and no transcription of the reporter gene is happening. **B.** Interaction of P1 and P2 brings the AD and BD closer which promotes the recruitment of transcription factor and activation of reporter gene.

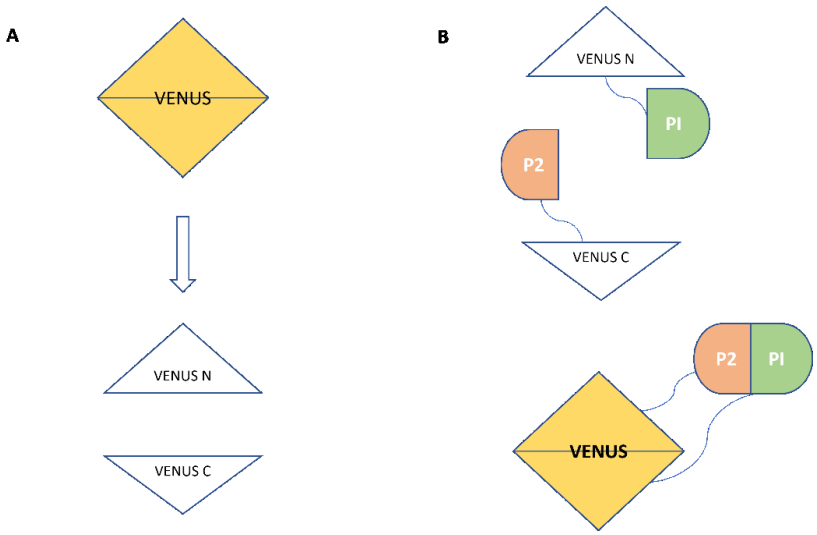

Figure 2.3. **Pictorial representation of Split Venus**: **A**. The fluorescent Protein Venus is split into two non- fluorescent halves e.g., N and C terminal parts of Venus (Venus N and Venus C) **B**. The two parts of Venus are fused with the interacting proteins P1 & P 2. In case of an interaction, P1 and P1 come closer and rejoin the broken halves of Venus and the yellow fluorescence of Venus is restored.

3. MATERIALS AND METHODS

3.1 Cloning BIN2 and Effectors into Suitable Vectors

For conducting Y2H screening, BIN2 and *Ustilago*'s effectors were cloned into suitable vectors. BIN2 was cloned into the bait plasmid PGBKT7. PGBKT7 has a GAL4BD and reporter gene i.e., gene for tryptophan synthesis. On the other hand, each of the 200 effector protein genes from *U. maydis* were cloned separately in into the prey plasmid pGAD. The prey plasmid is equipped with GAL4 AD and gene for leucine biosynthesis as a reporter.

3.2 Transformation of Yeast Strains

The PGBKT7-BIN2 (plasmid) was transformed into AH109 yeast strain using Polyethylene glycol (PEG) mediated yeast transformation. Similarly, the pGAD-effectors (200 effector constructs) were also transformed separately into Y187, a yeast strain sexually compatible to AH109. This yeast effectors library was already generated and stored at Djamei Lab (University of Bonn) before the project started.

3.3 Preparation of Liquid Yeast Cultures

The effectors yeast library (Y187) was stored in the solid form. The inoculum from the original stock was taken and inoculated in the minimal liquid medium (SD-LEU) in 48 wells plate with the help of tooth picks. Each well contains yeast colonies with different effector. A total of 192 wells (4 plates and each containing 48 wells) were inoculated with effector yeast colonies. Meanwhile, the inoculum from the original BIN2 yeast culture from the solid medium was taken and inoculated on a selective liquid medium (SD-trp). Prior to mating, the effector and BIN2 liquid cultures were placed in a shaker at 180 rpm and 30 C for 18-24 hours, to obtain a considerable growth.

3.4 Yeast Mating

The grown AH109 and YI87 yeast cultures were mixed with each other to accomplish mating. 150 ul of AH109 and 150 ul of Y187 liquid yeast cultures were taken and added together into the wells of the 48 wells plate containing 500 ul of yeast extract peptone dextrose (YEPD), YEPD is a complete medium for yeast growth yeast growth medium YEPD. The plates were sealed and incubated for 24 hours at 80 RPM and 28 C for 24 hours:

3.5 Confirmation of Yeast Mating and Interaction

The Effector-BIN2 yeast culture after an overnight growth was inoculated on 3 different types of solid minimal mediums for following purposes;

1. SD-LEU-TRP medium: for confirmation of mating,
2. SD-LEU-TRP-HIS medium: for confirmation of interaction,
3. SD-LEU-TRP-HIS-trp medium; for confirmation of strong interaction.

3.6 Independent Transformation of Top Interactors and Their Autoactivation Test

The top effectors identified for interacting with BIN2 in the initial screening were traced back to original stock and transformed cloned into the PGAD vectors and transformed into Y187. The effectors yeast colonies (y187) were mated one time with BIN2 yeast colony and another time with empty BD yeast colonies. The later was done to check the autoactivation of putative effectors. The top effectors-BIN2 and Top Effectors-Empty BD yeast colonies were inoculated on three selection media mentioned separately for confirming their mating and interaction reassurance.

3.7 Golden Gate Cloning

Several plasmid constructs that were used in different molecular experiments in this study were generated by using golden gate cloning. The entry vectors termed as pJET, containing our genes of interest in different modules were already generated at Djamei lab. For each golden gate reaction, all the entry vectors along with destination vector were added in the same PCR tube. The reaction tube was also added with restriction enzyme BSa1 and Ligase enzyme along with ligase buffer. The golden gate reaction was run on the PCR machine using a defined golden gate program. After a PCR reaction, the reaction mixture was transformed in E. coli for the initial selection of successful golden gate recombination. The actual sizes of the golden gate constructs were confirmed later by doing colony PCR followed by Gel Electrophoresis (Figure 3.1).

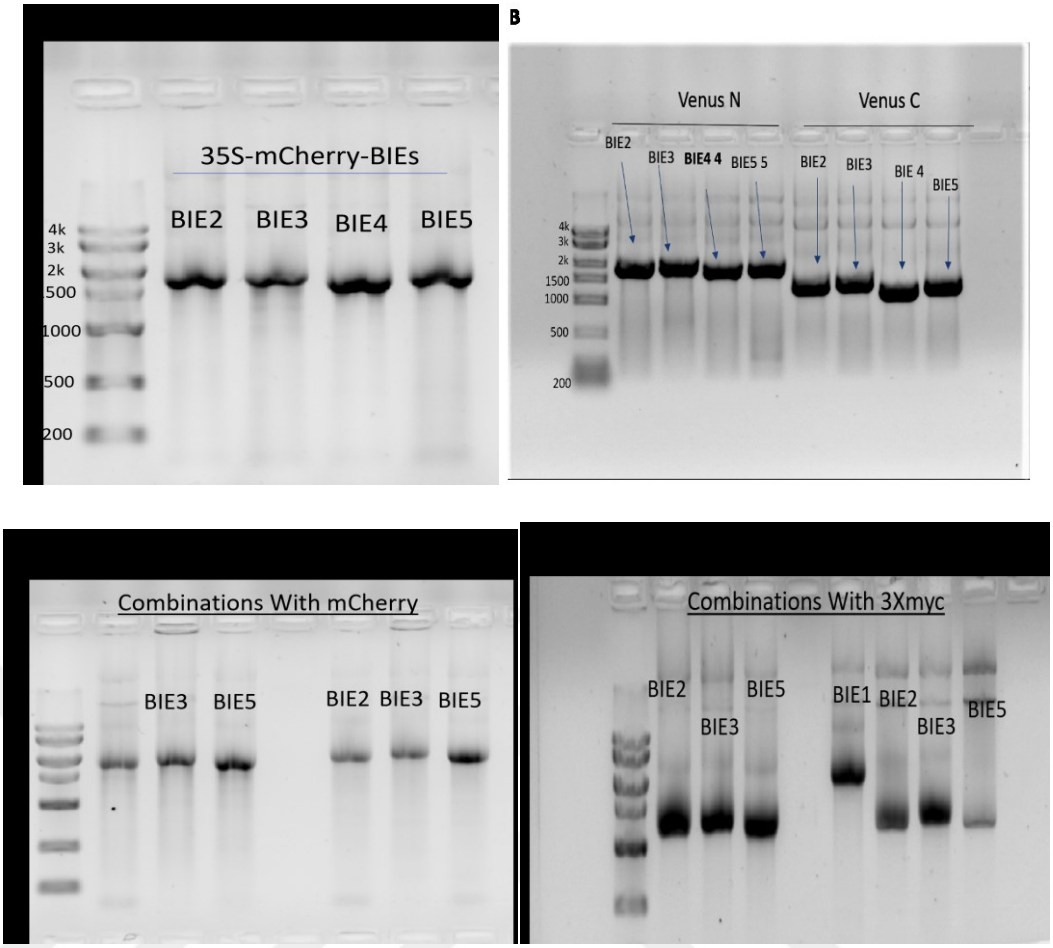

Figure 3.1. **Confirmation of Cloning by PCR followed by Gel Electrophoresis: A**. BIE constructs with mCherry for Localization experiment: Containing mCherry-BIE2 under 35S promotor. All BIE constructs have sizes between 1500 Bp and 2000 Bp. 35S Pro-Forward UBQ10-term-Reverse primers were used. **B.** Split Venus Constructs: All BIEs fused with N and C terminal parts of VENUS under 35 s Promotor. N terminally Fused BIEs have sizes between 1500 and 2000 bp, while C terminal fusions led to the Band Sizes between 1000 and 1500 Bp. **C.** BIE Constructs with mCherry for floral Dipping: Constructs containing the expected sizes e.g. between 1500 and 2000 bp. **D.** BIE constructs with 3X myc.

Depending on the need of the experiments, Golden Gate constructs containing BIEs in different combinations were generated and their sizes were confirmed using Polymerase chain reaction PCR followed by Gel Electrophoresis.

The simplified plasmid maps of the constructs are given below, under the subheadings 3.8, 3.9 and 3.10.

3.8 Constructs for Subcellular localization (Figure 3.1 B)

For deciphering the subcellular localization of the potential interaction partners, the interacting proteins (BIEs & BIN2) were cloned in the golden gate plasmid vectors and were fused downstream of mCherry protein tags using golden gate cloning. The interacting proteins were cloned and expressed as the C-D modules of the golden gate vector.

Following is representation of the cloning combinations used for unravelling the subcellular localization of interacting proteins.

1. Destination vector (A-G) 35S (A-B) _mCherry (B-C) BIE2 (C-D) Dummy (D-E) Terminator (E-F) Basta (F-G)
2. Destination vector (A-G) 35S (A-B) _mCherry (B-C) BIE3 (C-D) Dummy (D-E) Terminator (E-F) Basta (F-G)
3. Destination vector (A-G) 35S (A-B) _mCherry (B-C) BIE4 (C-D) Dummy (D-E) Terminator (E-F) Basta (F-G)
4. Destination vector (A-G) 35S (A-B) _mCherry (B-C) BIE5 (C-D) Dummy (D-E) Terminator (E-F) Basta (F-G)
5. Destination vector (A-G) 35S (A-B) _mCherry (B-C) BIN2 (C-D) Dummy (D-E) Terminator (E-F) Basta (F-G)

3.9 Constructs for Split Venus Assay (Figure 3.1 B)

Split Venus plasmid constructs were also generated using golden gate cloning. The 2 halves of venus protein ($Venus^N$ & $Venus^C$) were cloned as D-E modules in 2 different plasmid vectors. The BIEs were cloned as C-D modules into the golden gate vectors containg $Venus^N$. On the other hand, BIN2 was cloned as C-D module into the $Venus^C$– containing golden gate vector.

The golden gate constructs for conducting Split Venus Assay are given below.

1. Destination vector (A-G) 35S (A-B) _omega elements_ (B-C) BIE2 (C-D) $Venus^N$ (D-E) Terminator (E-F) Basta (F-G)

2. Destination vector (A-G) 35S (A-B) _omega elements_ (B-C) BIE3 (C-D) $Venus^N$ (D-E) Terminator (E-F) Basta (F-G)
3. Destination vector (A-G)35S (A-B) _omega elements_ (B-C) BIE4 (C-D) $Venus^N$ (D-E) Terminator (E-F) Basta (F-G)
4. Destination vector (A-G)35S (A-B) _omega elements_ (B-C) BIE5 (C-D) $Venus^N$ (D-E) Terminator (E-F) Basta (F-G)
5. Destination vector (A-G) 35S (A-B) _omega elements_ (B-C) BIN2 (C-D) $Venus^C$ (D-E) Terminator (E-F) Basta (F-G)

3.10 Constructs for Agroinfiltration (Figure 3.1 C & D)

Golden gate construct designed for conducting future effectors-induced phenotyping in Arabidopsis and for co-immunoprecipitation assay are given below. These constructs were transformed into *A. thaliana* using agroinfiltration. Here, the effector proteins (BIEs) were fused with either mCherry tags or with 3xmyc protein. The BIEs were cloned as the C-D modules, while mCherry and 3xmyc were cloned as B-C and/or D-E modules in the golden gate plasmid vector.

(mCherry B-C)

1. Destination vector (A-G) _estradiol promotor (A-B) _mCheryy _ (B-C) _ BIE2 (C-D) _Dummy (D-E) _Terminator (E-F) _Basta (F-G)
2. Destination vector (A-G) _estradiol promotor (A-B) _mCheryy _ (B-C) _ BIE3 (C-D) _Dummy (D-E) _Terminator (E-F) _Basta (F-G)
3. Destination vector (A-G) _estradiol promotor (A-B) _mCheryy _ (B-C) _ BIE4 (C-D) _Dummy (D-E) _Terminator (E-F) Basta (F-G)
4. Destination vector (A-G) _estradiol promotor (A-B) _mCheryy _ (B-C) _ BIE5 (C-D) Dummy (D-E) Terminator (E-F) Basta (F-G)

(mCherry D-E)

1. Destination vector (A-G) _estradiol promotor (A-B) omega elements _ (B-C) BIE2 (C-D) mCherry (D-E) Terminator (E-F) Basta (F-G)
2. Destination vector (A-G) _estradiol promotor (A-B) omega elements _ (B-C) BIE3 (C-D) mCherry (D-E) Terminator (E-F) Basta (F-G)

3. Destination vector (A-G) _estradiol promotor (A-B) omega elements _ (B-C) BIE4 (C-D) mCherry (D-E) Terminator (E-F) Basta (F-G)
4. Destination vector (A-G) _estradiol promotor (A-B) omega elements _ (B-C) BIE5 (C-D) mCherry (D-E) Terminator (E-F) Basta (F-G)

(3xMYC B-C)

1. Destination vector (A-G) estradiol promotor (A-B) _3XMYC _ (B-C) BIE2 (C-D) Dummy (D-E) Terminator (E-F) Basta (F-G)
2. Destination vector (A-G) estradiol promotor (A-B) _3XMYC _ (B-C) BIE3 (C-D) Dummy (D-E) Terminator (E-F) Basta (F-G)
3. Destination vector (A-G) estradiol promotor (A-B) _3XMYC _ (B-C) BIE4 (C-D) Dummy (D-E) Terminator (E-F) Basta (F-G)
4. Destination vector (A-G) estradiol promotor (A-B) _3XMYC _ (B-C) BIE5 (C-D) Dummy (D-E) Terminator (E-F) Basta (F-G)

(3xMYC B-C)

1. Destination vector (A-G) estradiol promotor (A-B) omega elements _ (B-C) BIE2 (C-D) 3XMYC (D-E) Terminator (E-F) Basta (F-G)
2. Destination vector (A-G) _estradiol promotor (A-B) omega elements _ (B-C) BIE3 (C-D) 3XMYC (D-E) Terminator (E-F) Basta (F-G)
3. Destination vector (A-G) _estradiol promotor (A-B) omega elements _ (B-C) BIE4 (C-D)3XMYC (D-E) Terminator (E-F) Basta (F-G)
4. Destination vector (A-G) _estradiol promotor (A-B) omega elements _ (B-C) BIE5 (C-D) 3XMYC (D-E) Terminator (E-F) Basta (F-G)

3.11 Electroporation Mediated Bacterial Transformation

The plasmid constructs were transformed into electro competent GV3101 *Agrobacterium* cells by electroporation using a defined protocol.

3.12 Agroinfiltration

Transformed GVE101 cells were grown overnight in LB medium containing appropriate antibodies for selection (Rifampicin for chromosomal, Gentamycin for Ti plasmid and Spectinomycin for binary vector selection). The bacterial cells were resuspended into *Agrobacterium* Resuspension Buffer (ARM Buffer) and optical density of the culture was checked. We set a target OD of 0.4 for agroinfiltration. The target OD was obtained by adding suitable amount of ARM buffer in bacterial cell. The volume of ARM buffer required to obtain the targeted OD of 0.4 was calculated by following formula:

$V_{ARM} = OD_{target} \cdot V_{Total} / OD_{culture}$

Here,

V_{ARM} = The volume of ARM buffer required to obtained the targeted OD

OD_{target} = The Desired OD of the final culture (0.4)

$OD_{culture}$ = The initial OD of the culture measured using OD meter

V_{Total} = The desired volume of the final culture

ARM Buffer contains:

- 10 mM MES-NaOH pH 5.6
- 10 mM MgCl 2

1. 0.15 mM Acetosyringone.

After the desired OD was achieved, the bacterial solution was infiltrated gently into the abaxial side of the leaf of 1-2 weeks old *Nicotiana benthamiana* (*N. benthamiana*) plants with the help of syringe.

3.13 Floral Dipping for Arabidopsis Phenotyping

Arabidopsis plants (COL0) were grown until they started flowering. Meanwhile, transformed GV3101 cells were grown overnight in liquid LB medium with antibiotics. The cells were harvested from the medium and resuspended into 5 % sucrose solution. The sucrose solution was also added with acetosyringone (0.02 %). We dipped for each plasmid construct, 4 Arabidopsis plants and for each Arabidopsis pot, 200 ml of Agro-Sucrose solution was prepared. Before dipping, Silwet L-77 to a concentration of 0.05% (500 ul/L) was also added. The above ground parts of the plant were gently dipped into the solution for 10-20 seconds. The dipped plants were covered for 16 to 24 hours to maintain high humidity and then later transferred to the growth chamber (Bechtold *et al.,* 1993). The plants were kept in the growth chamber until they started producing seeds. The seeds were then harvested and later steps for plant phenotyping are still to be performed.

Following Y2H screening, the putative BIN2 interacting effectors were subjected to multiple experiments which involved cloning of effectors with reporter genes in different combinations.

4. RESULTS

4.1 Testing BIN2 for Autoactivation

BIN2, prior to be tested against a pool of *U. maydis* effector proteins, was first tested for auto-activity. The test is important to ensure that the bait is not capable of independently activating the reporter gene and thus not giving the false positive results. The bait vector yeast (AH109) was mated with Y187 containing empty AD vector. The mated colonies were grown on Histidine lacking yeast growth medium. No autoactivation of BIN2 was observed (i.e., No growth of the yeast on the selection medium) (Figure 4.1).

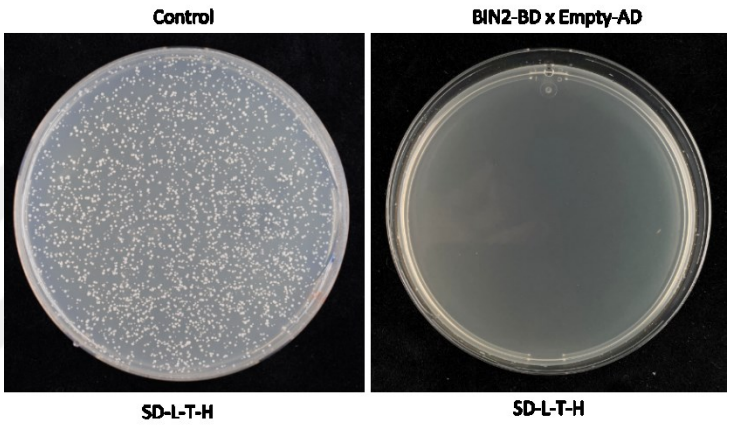

Figure 4.1. **BIN2 Autoactivation Test**: On the left is control: BIN2-BD is mated with TIP-AD. TIP is a protein having an expected interaction with BIN2. The growth readout can be seen. On the right, empty AD is mated with BIN2-BD. Three days post inoculation on the minimal medium (SD -leucine -Tryptophan -Histidine), no yeast growth is observed.

4.2 Testing BIN2 for Toxicity

The bait cloned into a BD vector can sometimes happen to be toxic for yeast. Thus, a yeast strain containing toxic bait plasmid, produces smaller-sized colonies as compared to a control, containing yeast strain with empty BD vector (Matchmaker® Gold Yeast Two-Hybrid System User Manual). Upon independent transformations of BIN2-PGBKT7 and empty-PGBKT7 into AH109 yeast strain, no real toxicity was observed in both BIN2 containing yeast and yeast containing empty PGBKT7. SD-TRP plates, after 3 days of culture spreading and incubation

at 28°C, depict same sized colonies with handsome growth for both control and BIN2 containing yeast (Figure 4.2).

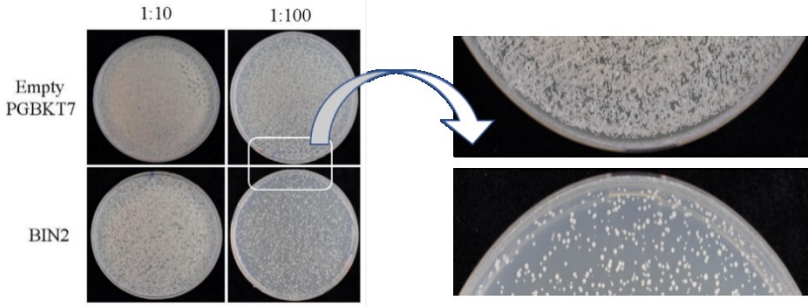

Figure 4.2. **BIN2 toxicity**: BIN2 shows no toxicity in reference to the control PGBKT7. In both serial dilutions, BIN2 containing colonies has almost the same size as control. The arrow shows a zoomed in section of the picture, depicting colonies

4.3 BIN2 Mating with Effectors Library

The Y187 yeast-effector library of 200 effectors was mated with AH109 yeast strain containing BIN2-pGBKT7 and plated on solid SD -leu -trp. agar plates. The yeast colonies appeared 3 days after incubation at 28°C, evidencing the completion of mating. 163 out of 192 effectors grew and mated successfully with BIN2, while 29 could not grow (Figure 4.3).

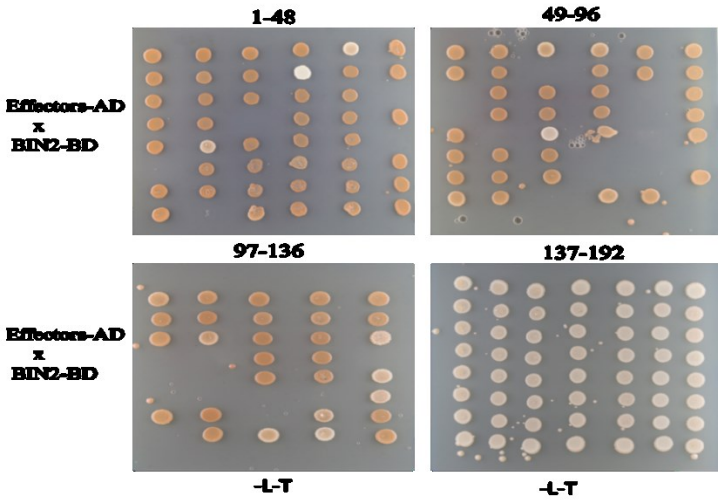

Figure 4.3. **Effectors-BIN2 mating on SD-Leu-Trp**: BIN2-AHI09, mated with Effector-Y187 showed normal growth on SD-leu-trp. There are 4 separate plates, each containing 48 BIN2-Effector colonies. Each plate has 6 lanes, and each lane entails 8 colonies grown independently of each other. The missing spots represent the colonies that could not complete mating and thus did not show any growth.

4.5 Confirmation of Interaction

The effector protein, fused with GAL4 AD, if interacts with its interaction partner BIN2 which is attached with GAL4 BD, also brings closer the GAL4 AD to the GAL4 BD. Depending upon the stringency of interaction, the GAL4 can quantitatively express the reporter genes (Young KH 1998; Matchmaker® Gold Yeast Two-Hybrid System User Manual) (Figure 2.2 in Section 2). In our case, the reporters are histidine and adenine, the vital growth components required for the yeast to survive.

The effectors-BIN2 colonies, that completed the mating, were then screened for interaction on a less stringent selection medium (SD-LEU-TRP-HIS), 47 out of total 192 mated colonies, showed growth on the less stringent selection, while 145 could not grow (Figure 4.4).

On the other hand, rather small number of colonies could grow on more stringent selection (SD-leu-trp-his-ade). A total of 19 interacting colonies could grow on quadruple selection medium. The growth of these colonies was consistent across all 3 selection mediums (Figure 4.5).

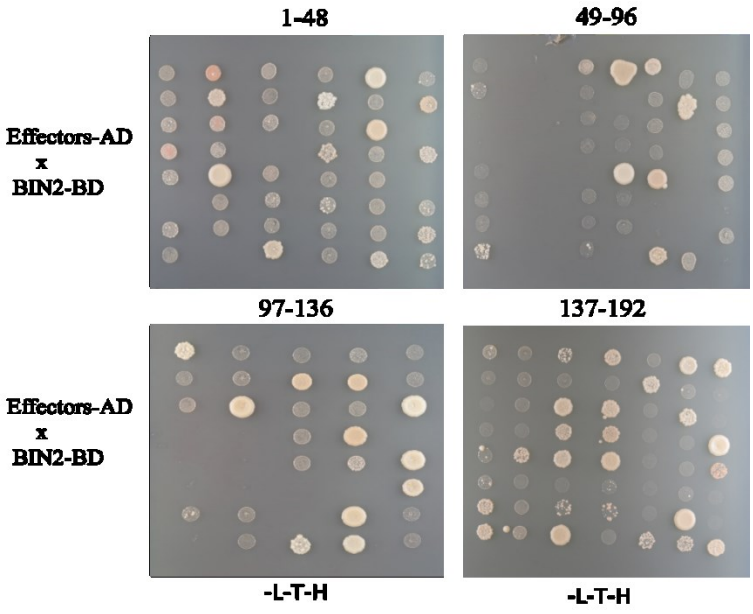

Figure 4.4. **Interaction Screening on Less Stringent Selection:** -L-T-H represent minimal growth medium lacking leucine, Tryptophane and histidine. The bright yellowish/whitish colonies represent clear growth and the proof of some interaction.

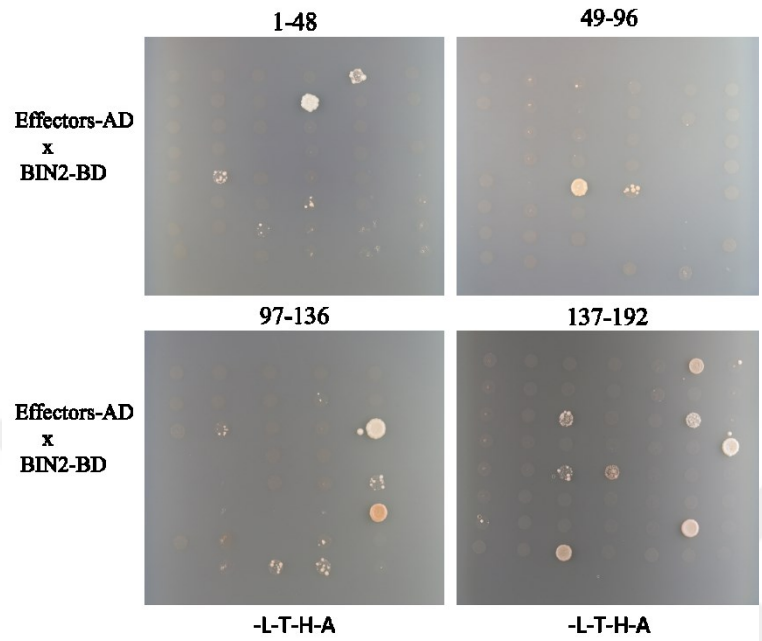

Figure 4.5. **Interaction Screening on stringent selection:** -L-T-H-A represent minimal growth medium lacking leucine, Tryptophane and histidine and adenine. The bright yellowish/whitish colonies represent clear growth.

4.6 Independent Transformation of Top Interactors and Their Autoactivation Test

The 19 initially identified effectors is a high number and it seemed very difficult to validate the interactions with BIN2 for all 19 effectors, since the time duration to complete the thesis study was limited. Therefore, we proceeded with 9 promising interacting effectors for later validations. At first, it was imperative to repeat the Y2H screening for the identified effectors to ensure the quality of initial screen and rule out any artifacts. The nine selected effector colonies were independently mated with BIN2 yeast and with empty PGBKT7. This is necessary to not only have the confirmation of interactions but also to check the effectors for auto-activity, so that false positive results can be avoided. Out of nine independently mated effectors, four showed a clear autoactivation while, five of them were still interacting with BIN2 (Figure 4.6). The ones interacting with BIN2 were given unique names e.g., BIN2 INTERACTING 1-5 (BIE 1-5).

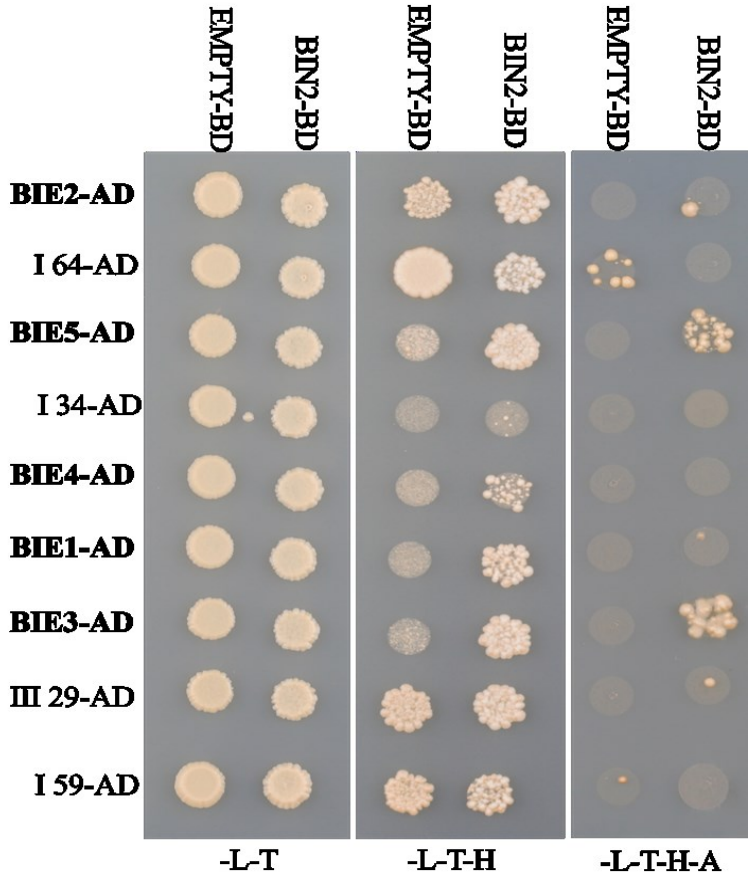

Figure 4.6. **Independent confirmation of Interaction:** 5 out of 9 independently transformed interaction partners show no autoactivation. On the left, are the names of 9 potential interacting proteins fused with AD. The 9 effector colonies are one time mated with empty BD yeast and another time with BIN-BD yeast colonies. On -L-T, all colonies are growing, showing successful mating. On -L-T-H, some of the effector colonies are also completing their growth while mated with EMPTY BD, meaning they are autoactivating. The ones, not autoactivating are in bold and given names (BIEs). BIE2 was also later proved to be autoactivating as its growth can be seen with empty BD as well. Out of 5 BIEs, BIE3 and BIE5 are growing on quadruple selection as well.

4.7 Subcellular Localization of BIEs and BIN2

The BIEs and BIN2 were fused with fluorescent proteins and were transformed into *N. benthamiana* leaves. Three days post infiltration (3 DPI) the infected leaf sections were taken, and the subcellular location of the proteins were unraveled using confocal microscopy, BIE1 and BIE2 were eliminated from the screening as they were autoactivating. The rest 3 BIEs were successfully transformed into plants and their subcellular locations were identified. mCherry-BIE3 was localized in all parts of the plant cell including nucleus and cytoplasm

but not the nucleolus. Similarly, mCherry-BIE3 & mCherry-BIE5 was localized in all compartments of the cells except nucleolus (Figure 4.8 a).

We then checked the cellular location of BIN2-GFP to have comparison between the locations of BIEs and BIN2. BIN2-GFP was cytosolic and nuclear in its localization (Figure 4.8 b).

4.8 Split Venus Assay

After deciphering the subcellular localization of potential interactors, we then looked for the real possibility of interaction between them. All three BIEs were fused with the N-terminal parts of the Venus. BIN2 on the other hand was fused with the C terminal part of the Venus using golden gate cloning. The complementary constructs were co-infiltrated into the *N. benthamiana* leaves. Three days post infiltration, the leaves infiltrated with "BIE3 $^{\text{Venus N}}$. BIN2 $^{\text{Venus C}}$" were shinning and depicting interaction in all parts of the cells. Similarly, "BIE4$^{\text{VenusN}}$ & BIN2$^{\text{VenusC}}$" constructs showed restoration of fluorescence, imitating a strong interaction in all cellular compartments (Figure 4.9 a and 4.9 b). Interestingly, "BIE5$^{\text{VenusN}}$ & BIN2$^{\text{VenusC}}$" was only expressed in the nucleus and the interaction was confined only in a specific region within the nucleus (Figure 4.9 c).

While confirming the interactions in planta, it was also imperative to ensure if the interaction with BIN2 is specific or the fluorescence is being restored because of some random interactions happening at subcellular level. Therefore, we co-infiltrated all BIE constructs with a Luciferase construct, which is fused with C terminal part of Venus as a negative control. In all "BIEs$^{\text{VenusN}}$ & Luciferase$^{\text{VenusC}}$" co-infiltrations, no fluorescence/interaction was observed under confocal microscope (Figure 4.9).

4.9 Leaf Phenotyping of the Transformed *N. benthamiana* Plants

All the putative BIN2 interacting proteins were checked for their ability to induce any defense related phenotype in plants. The purpose was also to look for any BIN2 related phenotypes. e.g., an impaired plant growth mimicking the *bin2*

mutant phenotype. The BIEs cloned with 35S promotor were infiltrated into leaves of 2 weeks old *N. benthamiana* plants and the change in the phenotype was observed in reference to a control leaf. Not BIE3 and BIE5 showed any change in leaf phenotype, but BIE4 showed a strong Hypersensitive (HR) response and necrosis in the localized area. Moreover, the wilting was also observed in BIE4 expressingleaves (Figure 4.10).

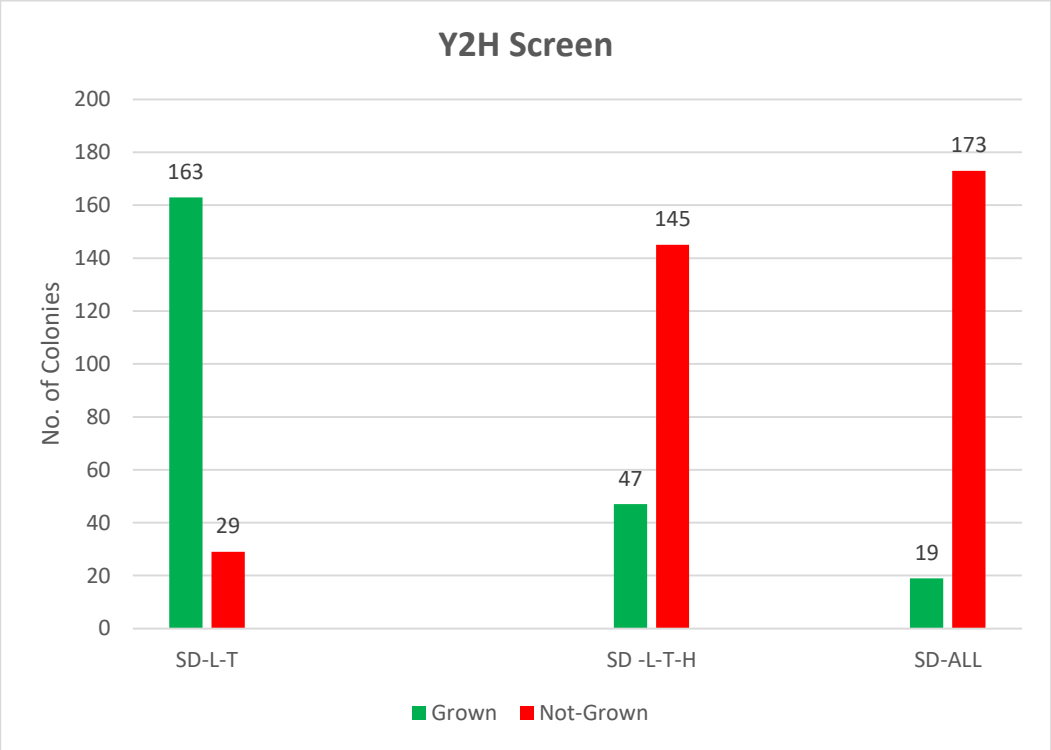

Figure 4.7. **Graphical Summary of Y2H Screening**: X-axis represent the total number of Colonies grown in different selection medium. The green bars depict the colonies with successful growth and the red bars show the colonies with no growth.

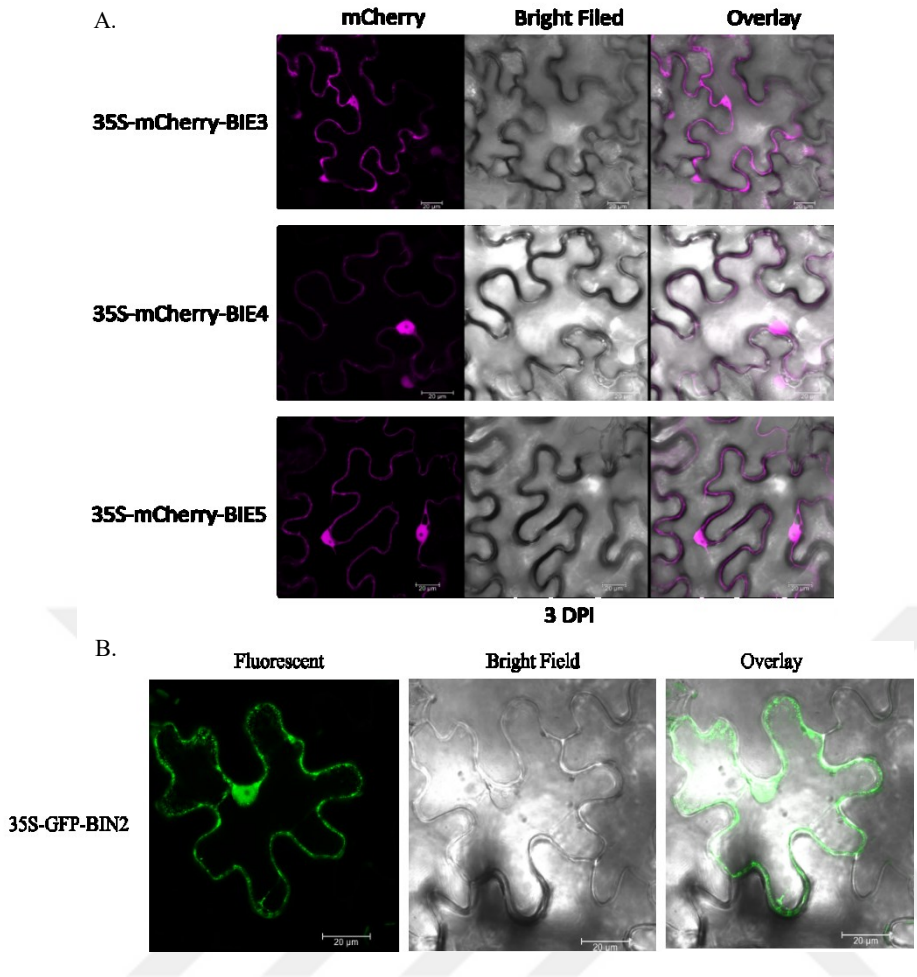

Figure 4.8. **Subcellular Localization of BIEs. A**. All three mCherry-BIEs are being expressed under 35S constitutive promotor and showing fluorescence in all parts of the plant cell under confocal microscope. The microscopy is done 3 days after infiltration. **B**. Subcellular Localization of BIN: BIN-GFP expressed under 35S constitutive promotor is localized in all cellular compartments

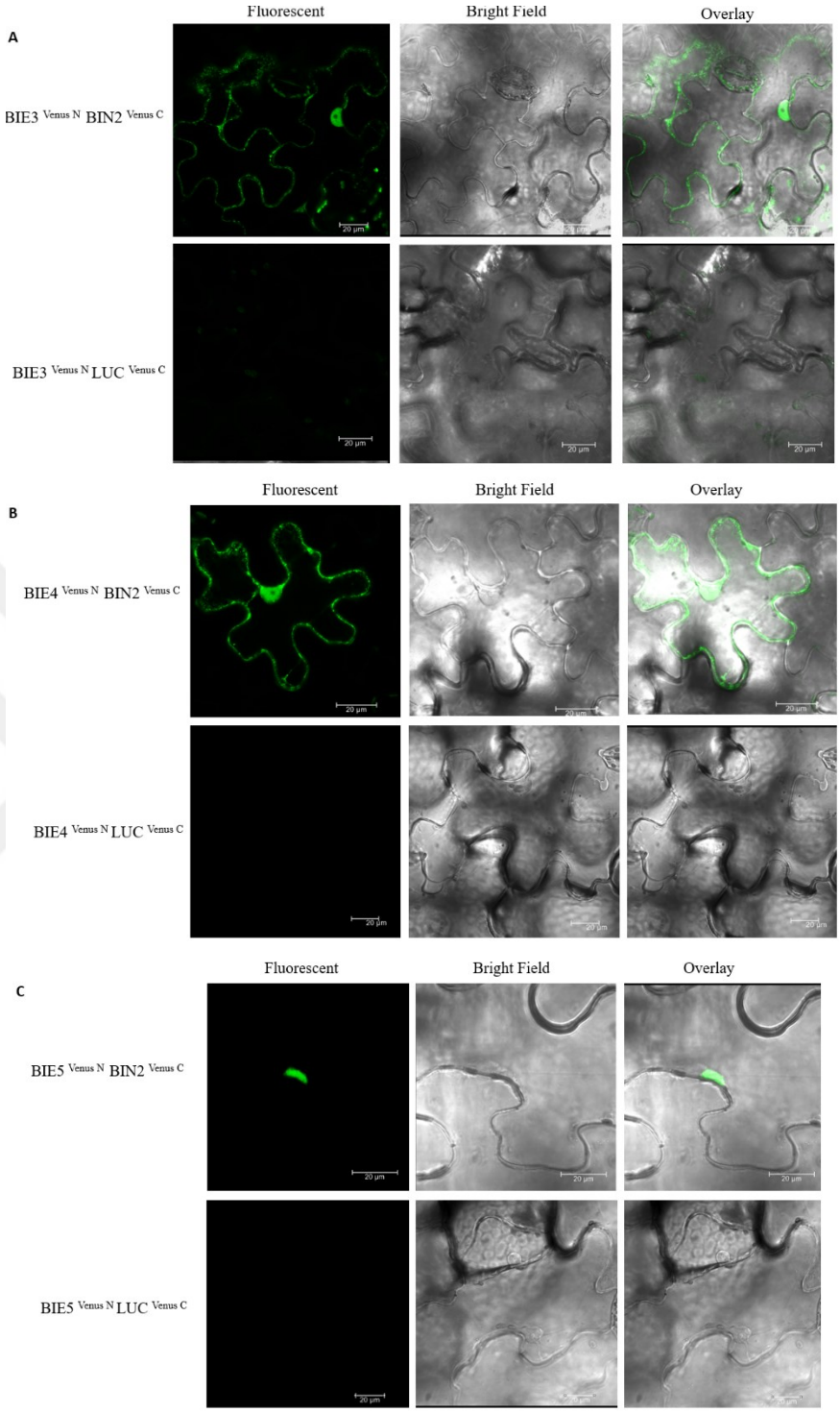

Figure 4.9. **Bimolecular Fluorescence Complementation Assay (Split Venus Assay):** A. "BIE3 $^{Venus\ N}$. BIN2 $^{Venus\ C}$" is being expressed constitutively under 35S promotor in all parts of the cell. As they can be seen under the confocal microscope it is indicating interaction, while BIE3 $^{Venus\ N}$. Luciferase $^{Venus\ C}$ is not shinning under the microscope, hence interaction could not be confirmed B. BIE4$^{Venus\ N}$. BIN2$^{Venus\ C}$ is showing fluorescence in all parts of the plant cell. On the other hand, the negative control, BIE4$^{Venus\ N}$. Luciferase$^{Venus\ C,}$ is not producing any fluorescence. **C.** BIE5$^{Venus\ N}$. BIN2$^{Venus\ C}$ is showing interaction exclusively in the nucleus and not in other cellular compartments. The negative control BIE5$^{Venus\ N}$. Luciferase^{VenusC} is not shinning under the microscope·

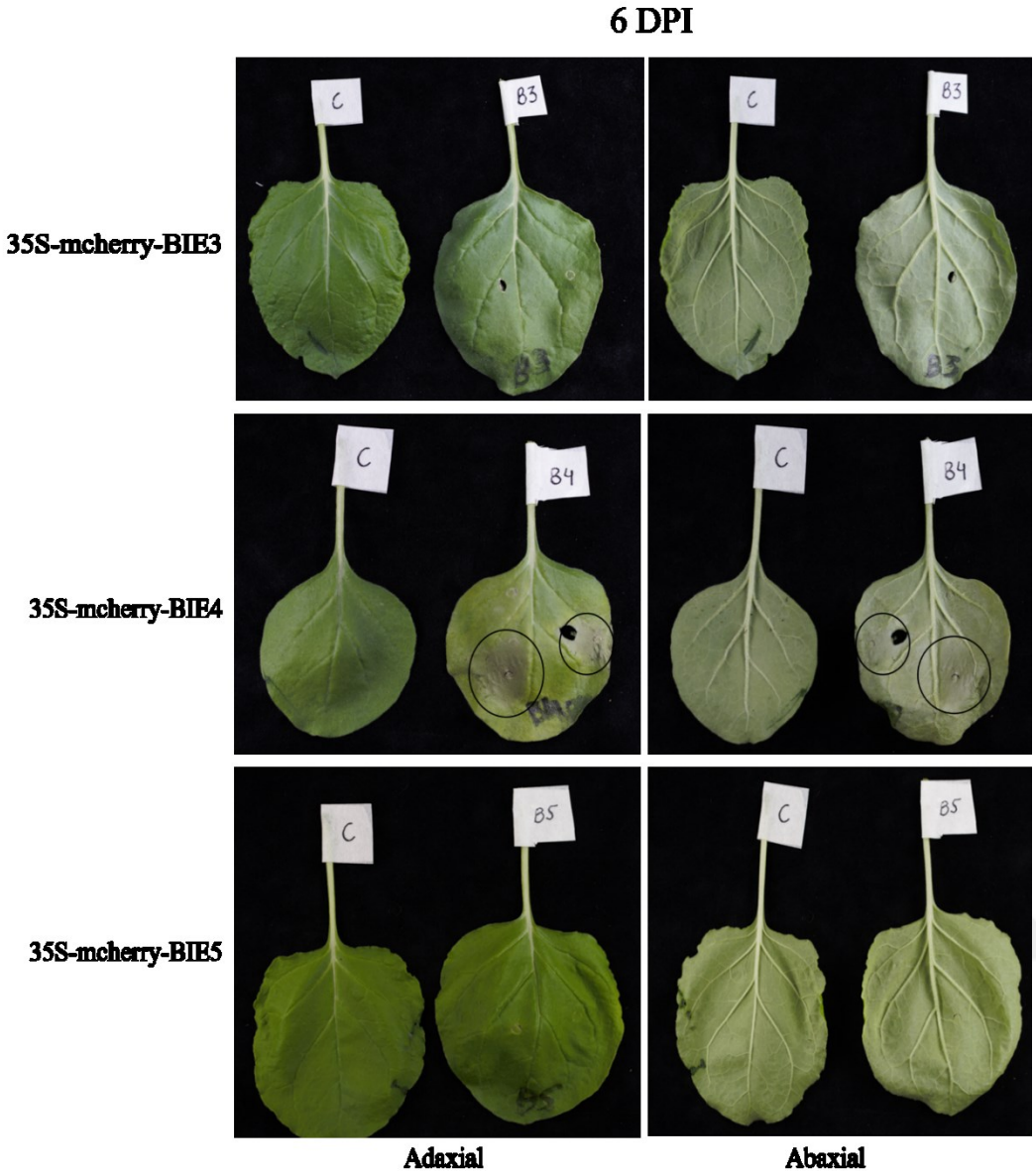

Figure 4.10. **Leaf phenotyping of the transiently transformed *N. benthamiana* plants:** On the left is the Adaxial side of leaves, while the right side encompasses Abaxial sides of transformed leaves. 35S:BIE3 and 35S:BIE4 do not have any change in the phenotype even 6 days after inoculation, while 35S:BIE4 is inducing cell death in the infiltrated area. Moreover, Necrotic lesions can be seen on both sides of the leaf (marked in circles)

5. DISCUSSION

Plant pathogens have developed highly specialized ways to modulate host genome and weaken the plant immunity. Pathogens release a set of secretory proteins called effectors which can target specific hormonal or defense related proteins of plants (Jones & Dangl, 2006). One such group of protein kinases that is possibly targeted by effectors is the GSK3 kinases Superfamily. We have identified a GSK3 kinase being targeted by a set of putative effectors extracted from *U. maydis.* We have taken *U. maydis*, a biotrophic pathogen, as a model system to study effectors-host proteins interactions.

5.1 Putative Effectors-BIN2 Interactions

ZmGSK2 (putative homolog of AtBIN2) is a GSK3 kinase and a homologue of Arabidopsis AtSK21 in Maize. This member of GSK3 kinase superfamily has a well-known function in Brassinosteroids (BR) signaling in *A. thaliana*. BRs perform diverse functions in plants including a role in defense related signaling (Saidi *et a*l., 2012; Kong *et al.,* 2012). So, BIN2 for being a part of a defense related signaling cascade, might also be involved in plant defense directly or indirectly and thus could be a potential target of effectors. A library of 200 effector proteins extracted from *U. maydis* was screened against maize- BIN2. A diverse range of molecular tools have been employed to conduct this screening starting from a massive Yeast 2 Hybrid Assay, followed by unraveling the subcellular localizations of identified effectors and then a split Venus assay to confirm the interactions *in planta*. We could confirm, by the end of our screening three novel effectors interacting with BIN2 termed as BIN2 Interacting proteins (BIEs).

5.2 Artifacts and False Positivity in Y2H Results

In an initial screening using Y2H system, we identified 19 novel effector proteins interacting with BIN2. The number appeared to be very high and was way bigger than our expectations. Only one protein being targeted by 19 effectors seemed unlikely. The reasons for such a high number of interactions could be due to the false positive results in the Y2H system. Yeast's cellular machinery differs

greatly from the plants, thus the plant proteins transformed into yeast might not be accepted by the yeast cells and thus could lead to genetic mutations. Such mutations might lead to the overgrowth or undergrowth of the yeast cells and thus the Y2H system can produce false positive or false negative results respectively (Serebriiskii & Golemis, 2001; Brückner *et al.,* 2009).

5.2.1 False positivity in y2h

The Y2H essay is prone to false positivity and one of the reasons for that is the possibility of yeast to mutate itself and grow even on a minimal growth medium lacking essential hormones. Moreover, yeast is also susceptible to contamination by other microbes like Bacteria which can colonize the yeast and occupy its living space and form colonies with or within the yeast. So, on a nutrient medium, yeast and bacteria might grow together and appear as genetic chimera. A genetic chimera involves a single organism composed of cells from 2 distinct organisms. The chimeric mutations in Y2H system could be hard to differentiate from the normal yeast growth and thus provide false signs of interaction (Serebriiskii & Golemis, 2001; Brückner *et al.,* 2009)

5.2.2 Broader binding specificity between effectors and the target in Y2H

In Y2H system, the effectors' target i.e., BIN2 could have a broader binding specificity which means that more than one effector can interact with the same target. The reason for this could be that the target might contain a conserved domain which could be recognized by multiple effectors in an artificial system like Y2H (Uchikoga *et al*., 2016). For example, BIN2 contains a conserved serine/threonine kinase domain which is also present in other GSK3 kinases and the effectors targeting the GSK3 kinases bind the highly conserved kinase domain. So, when all the effectors and the potential target are brought closer to each other in an artificial system like Y2H, more than one effector show binding affinity with the target. The 19 effectors interacting with BIN2 in the initial Y2H screen might possibly bind in the natural system to various GSK3 like kinases depending on their time of expression and specific affinity and subcellular localization.

5.3. False Negative in Y2H System

In a natural system e.g., plants, the interaction between 2 proteins is facilitated by the prior posttranslational modification of the targeted protein. Such modifications occur naturally in the plant system with the help of enzymes like kinases that phosphorylate its substrate. In yeast system, no plant machinery is available to induce the required modifications and therefore, false negative results appear. Taking the idea of false negativity, a bit further, the interacting proteins also need to be correctly folded 3-dimensionally for developing a stable interaction, but since the interacting proteins are fused with the reporter proteins (i.e., BD & AD of GAL4), the proper folding of the polypeptide chains does not occur. This cause steric hindrance between the proteins and thus blocking the interaction (Brückner *et al.,* 2009; Xing *et al.,* 2016).

5.4. Confirming The Interactions i*n Planta*

Since Y2H is prone to error, therefore it was indispensable to obtain another level of confirmation about the interactions. We transformed the interacting proteins in a comparatively familiar system for the protein e.g., *N. benthamiana* plants. At first, the subcellular localization of BIN2 and the interacting proteins was revealed to look for their possibilities of lying in the same compartment and interacting with each other. Both BIN2 and the interacting proteins (BIEs) were localized all over in the cellular area which makes it difficult to deduce whether and where in the cell they are interacting. But we could at least identify that the interacting proteins were lying in the different compartment and are not far from each other. Thus, it's most likely that they have some binding affinity with each other. We then, finally confirmed the interactions in planta using split Venus Assay. At the end of the screening, we could identify 3 putative effectors interacting with BIN2.

5.5 Induction of Defense Related Phenotype by Bies

The validation of Effectors-BIN2 interactions was can also be obtained on another level by directly transforming the effector proteins in the plants and look

for the change in phenotypic characteristics especially the ones BIN2 is involved in. Therefore, we transformed with BIEs, the *N. benthamiana* and *A. thaliana* plants. During phenotyping of *N. benthamiana* leaves, we did not see any specific defense related changes for any of the effectors except for BIE4 which induced leaf wilting followed by HR response. The induction of defense related phenotype does not categorically mean that BIN2 is responsible for that, thus to get a clearer idea, we planned to generate Arabidopsis plants that are transformed with effectors. The phenotyping of the transformed plants would not only help to confirm if BIN2 is being targeted by effectors but also would aid in unravelling the pathway or the mechanism by which effectors target BIN2. As the thesis study was entailed with limited time duration, the phenotyping of transformed Arabidopsis plants is still in the pipeline. Therefore, it's hard to get the phenotypic validation of interaction for now. But, since our screening still suggests that there are 3 novel effector proteins interacting with BIN2, we would try to learn the mechanisms by which the newly identified effectors can target BIN2.

5.6 Growth-Defense Tradeoff

A Plant possesses limited number of resources and whether to invest these resources for plant growth or for plant defense is a major dilemma for plants (Bethany *et al.*, 2014). Such a delicate resource allocation between plant growth and plant immunity is regulated by different hormones. Recently, the role of Brassinosteroids (BR) in maintaining this sensitive balance, is discovered (Lozano & Zipfel, 2015). BR is a plant growth promoting hormone and regulate important stages of plant life cycle such as seed germination, vegetative growth, flowering, and stomatal development etc. (Clouse, 2011: Zhu *et al.*, 2013). Studies have shown that the activation of BR signaling increases plant susceptibility to microbial pathogens (Belkhadir *et al*., 2012: Kim. *et al.,* 2013). The role of BZR1, a BR signaling gene and a major regulator of BR-induced transcriptional changes, has been identified in regulating the tradeoff between plant defense and plant immunity. Activation of BZR1 leads to impaired PTI responses (Lozano *et al.*, 2013).

6. SPECULATION AND FUTURE DIRECTIONS

The literature study suggests that BIEs disturb the growth-defense balance and decrease plant immunity by an upstream modulation of BZR1 activity. It is quite ubiquitous from the BR signaling pathway that BZR1 activity is regulated by the upstream negative regulator BIN2 (He *et al.*, 2002; Yin *et al.,* 2002). To maintain the growth defense tradeoff, plant also regulates the internal BR biosynthesis (Lozano & Zipfel, 2015). We would try to learn, from the following scenarios, that how this growth-defense balance is being interrupted by BIEs.

In a growth promoting situation, the BR level is enhanced leading to the BIN2 obstruction and BZR1 activation (Lozano & Zipfel, 2015). It is suspected that BIEs might obstruct this growth promotion by interacting with BIN2 and causing a hypermorphic, a gain of function mutation in BIN2. This leads to no activation of BR responsive genes and thus the plant shows impaired growth

On the hand, during an invasion by the pathogen, plant focuses more on the defense and stops its growth by a reduction in local BR biosynthesis (Lozano & Zipfel, 2015). So, in the absence of BR, BIN2 is activated and hampers BZR1 activity. It is most likely that BIEs interact with BIN2 at this stage and cause a hindrance in its activity by inducing a posttranslational modification in BIN2. So, BIN2 cannot restrict BZR1 activity, and this leads to the BR induced growth promotion of the plants. The BZR1 activation would lead to decrease PTI and this would make it easier for pathogens to continue the invasion.

In this study, 3 novel effector proteins from *Ustilago*, interacting with BIN2 are identified. This finding would help not only to realize the important role of BIN2 and Brassinosteroids in plant defense but also provide ideas to design effective control measures against pathogens. This study also outshines the importance of plant GSKs as immune kinases and suggests that effectors-GSKs interactions could be conserved in other plant species. Of course, further research is needed to further elucidate the mechanisms and the pathways by which plant GSKS are being targeted by effectors, but the thesis findings would still make a strong foundation and would pave paths towards future research.

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Maaz Anwar comes from Pakistan, and he has completed his bachelor's degree from his home country as a gold medalist in Agriculture, majoring Biotechnology with the CGPA 3.99/4. As soon as he completed his bachelors, he commenced a Joint master's degree program in Plant breeding on ERASMUS MUNDUS Scholarship. The joint degree started in Swedish university of Agricultural sciences Uppsala, Sweden (SLU, Sweden), where the student completed his first year. The 2nd year of the master's degree started at Ege University where the first 6 months involved a course work and then later a master thesis internship abroad at University of Bonn Germany.