

## ABSTRACT

Title of Dissertation: REGULATION OF THE BROAD-SPECTRUM DISEASE RESISTANCE PROTEIN RPW8.2 BY PHOSPHORYLATION AND 14-3-3 IN ARABIDOPSIS

Harlan King, Doctor of Philosophy, 2017

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Ascomycete powdery mildew (PM) fungi belonging to the order of Erysiphales cause diseases on more than 10,000 plant species including economically important staple food crops and numerous horticultural plants. The Arabidopsis protein RPW8.2 confers broad-spectrum resistance against all infectious powdery mildew pathogens. RPW8.2 is unique among characterized plant R proteins in that it activates broad-spectrum resistance to PM fungi, and the protein is specifically targeted to the extra-haustorium membrane (EHM). However, how RPW8.2 is regulated to exert haustorium-targeted defenses remains poorly characterized.

To understand how RPW8.2 is regulated, I first performed a thorough site-specific mutagenesis of potential serine or threonine residues in RPW8.2 and identified two residues, threonine at 64 and serine at 138 to be critical for RPW8.2's function. While the T64A mutation makes RPW8.2 auto-active, the S138A mutation abolishes RPW8.2's ability to activate cell death and defense, with S138A being dominant over T64A. This suggests that RPW8.2 is negatively and positively regulated by (de)phosphorylation at T64 and S138, respectively. One candidate phosphatase and two kinases were genetically and biochemically tested for a potential role in (de)phosphorylation of RPW8.2.

I also investigated how an RPW8.2-interacting protein 14-3-3 $\lambda$  regulates RPW8.2's function. To this end, I developed a novel, divalent 14-3-3-sequestering protein named RYC to circumvent likely functional redundancy among different 14-3-3 isoforms. Our results demonstrate that RYC can effectively sequester multiple 14-3-3 isoforms from plants and human. When expressed in guard cells of Arabidopsis, RYC sequestered 14-3-3s away from H<sup>+</sup>-ATPases, thereby inducing stomatal closure, which in turn increased drought tolerance of transgenic Arabidopsis. When expressed in powdery-mildew-invaded cells, RYC abrogated RPW8.2-mediated resistance to powdery mildew, yet did not grossly affect its EHM-specific localization, suggesting that the C-terminus of RPW8.2 may exert a self-inhibition function which can be relieved when 14-3-3 $\lambda$  binds to the C-terminus during fungal infection. Taken together, our results lead to a better understanding of the molecular mechanisms regulating RPW8.2.

REGULATION OF THE BROAD-SPECTRUM DISEASE RESISTANCE  
PROTEIN RPW8.2 BY PHOSPHORYLATION AND 14-3-3 IN ARABIDOPSIS

by

Harlan Joseph King

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Professor Illarion Turko

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## Acknowledgements

Every morning between 6:30 and 7:30 am I can expect Mario to come through our lab and empty the trashes. We've grown accustomed to each other. On Mondays he sweeps and mops the floors. Once a month he checks our glass disposal box. The lights are off so I can more clearly see my monitor, but I hear his shuffling footsteps and plastic bangs of the bins emptying their contents into larger containers and moving on into the next lab.

I have the feeling he was doing this before I could spell the word 'science'. No matter how early I get into the lab to write or perform experiments, Mario and his crew always seem to arrive earlier. We don't talk much, mostly because we speak different languages. Mine: science. His: cleanliness. My poorly pronounced "No es basura." remind him and me that although my bench looks like basura we probably shouldn't touch it in case a positive result wants to spontaneously self-announce. He smiles, nods his head, and moves on.

My early morning hours help me catch a glimpse into his routine. If I didn't see him I might think that my bench trash receptacle just gets emptied. I don't see a hundredth of what Mario does to keep our lab and other labs operating in clean and safe conditions. Mario's diligence and daybreak visits to me are representative of all behind-the-scenes efforts by dozens of personnel devoted to the smooth operation of the Institute for Bioscience and Biotechnology Research (IBBR) in Rockville, MD.

We have different tasks, but we're united in our pursuit of scientific excellence.

Mario's excellence begins early every morning.

Other early morning heroes include Mark Leser. He's my go-to man when I require general information about the operation of a piece of equipment or need him to repair something. Dan Wilson timely responds to my service tickets requesting repairs of leaky faucets or malfunctioning autoclaves. Jim Johnson keeps everything humming and in tune.

Our IT manager, Christian Presley, made sure my 65 GB of images and raw data found backup and versioning. He and his team effortlessly orchestrate an incessant stream of bits and bytes to and from my computer connecting me to other researchers and providing the means for expression. Christian and crew provide one of the few IT shops where software, hardware and expertise are all abundant.

Frank Coker works tirelessly to grow my plants healthy and pest-free. Frank spends weekends and holidays in growth chambers and greenhouse zones wearing a white full-body suit and respirator spraying insecticides. He gives up his personal time to minimize the chance of chemical exposure to other researchers. His sacrifice is partially represented in the form of a dozen, green plant species that might all be award winning if state fairs allowed transgenic entries. His *Nicotiana benthamiana* plants are the envy of every Materials and Methods sections.

I'm indebted to all IBBR personnel. I'm grateful our interactions have offered me a glimpse into the massive complex of a modern state-of-the-art science facility. In some ways these buildings are macroscopic emblems of the complex microscopic

cells we study, continuously moving and buzzing with activity, a warehouse where every interaction has significance and every worker catalyzes a non-energetically favorable reaction. A lot of reactions are required to move against the strum-steady-beat of the entropic drum.

I love the friends and colleagues with whom I associate and work with at IBBR. Jennifer Tullman has made it her mission to help me expound upon biochemistry concepts I first learned as an undergraduate. Kyle Anderson spent a day preparing and testing my samples at NIST with the mass spectrometer. I've enjoyed conversations with Kinlin Cao who helped me interpret protein binding on the size exclusion column.

No longer do scientists make breakthrough discoveries working alone at home. Biological science has become more complicated requiring tissue culture rooms, software updates and thriving plants. Additionally, experiments and hypotheses become more refined with exposure to and critique from other scientists. Science is a community effort, and I'm immensely grateful for this community. No science bulge happens in a vacuum of service, and many labor tirelessly behind the scenes actively removing the burden from scientists to see and study farther than anyone before them.

Scientists continue to stand on the shoulders of giants. Some giants sweep the floors so that others may discover calculus.

## America

During the course of my studies I've seen the breakdown of society emanating from a protracted civil war in Syria. I've also witnessed the Arab revolutions in Tunisia, Egypt, Yemen, Syria and Iraq. I've watched civil unrest and economic depression unfold in Venezuela. Political disruption causes scientific disruption, and as much as scientists strive to remain indifferent to politics, at the end of the day they need predictable shipping routes to deliver reagents and dependable sources of electricity to power their centrifuges. I'm grateful to have lived and conducted science in a country where these were never a concern. I know there are billions in the world who don't have my opportunity. I'm thankful.

I'm also grateful for funding agencies like the NSF and NIH, both of which have supported me, to fund investigations, investigators and their students in asking the harder questions. The burden of student debt makes me thankful that mechanisms enabled me to secure loans in the first place.

## Maryland

I'm also grateful to live in a state that values scientific progress and research and commits funds to do the same. The IBBR building is resplendent in its manufacture and materials. A new biomedical sciences building is being erected to the west. I'm proud this state makes confident investments in life science research.

## University of Maryland

I'm grateful for the University of Maryland exhibiting patience and risk taking in admitting me as a student. I'm grateful for awards like the graduate summer stipend, T32 and others that helped fuel my career, and for researchers like Dave O'Brochta and Leslie Pick for sponsoring them. I'm grateful for teaching arrangements that were made by the dedicative staff consisting of Michelle Brooks and Gwen Warman. They performed the herculean task of keeping everyone on the same page and prodding us to anticipate our next steps. In many ways a graduate program takes on the identify of its administrators, and we're lucky our program has Michelle and Gwen pulling a thousand daily strings on our behalf. I've loved working with professors like Sandra Fox-Moon, Nancy Noben-Trauth and Caren Chang in delivering engaging, compelling science content to undergraduates. I count myself among the students they inspired.

## My In-Laws

There aren't many 30-year-old students with a growing family who can drop everything and follow their science passions in the pursuit of a PhD. We hear it endlessly in graduation speeches, to follow our dreams, but at the end of the day we have to put bread on the table. Robert "Bud" Barnes and Mike and Susie Weiler put bread on my table while I followed my dreams. They're financial support made this pursuit possible.

## My Parents

My first laboratory was a dairy barn. My first science words were *somatic cell counts*, *fat content* and *lactation*. My early life provided an ideal environment for an unending chain of questions that my father was always willing to answer. My father showed me that science can sometimes be smelly and extremely rewarding. I'm thankful he taught me to be a science man and a good man. I once asked him why he was so good. He replied that it was because his father was good to him. I hope to continue this unending chain of goodness with my own sons.

My dad fanned the flames but my mom added the wood. With her my ideas found undiminished encouragement and landed on fertile ground. She was co-author of one my earliest experiments in hypothesizing whether or not alfalfa sprouts would grow under an oak tree in our front yard. I believed that there was not enough water to quench the dry Texas soil enough for seeds to grow. My mom proved me otherwise. The sprouts grew and the negative result produced an indelible impression in my five-year-old mouth. I continued to grow with mom's nourishment of astronomical wallpaper art, science books and engineering toys. She never resented raising a nerd. In fact, I think she enjoyed it. In all my exploring she showed me that I could never be outside the boundaries of her love.

I owe everything to my angel mother.

## My Committee

No committee has probably been better selected at Maryland. From insightful discussions to words of caution to careful encouragement I felt my committee imbued the best qualities of every professional group. I'm fortunate to have been the beneficiary of their million-watt intellects and cumulative decades research experience which I continue to feel even after our meetings have concluded.

## Xiao Lab Members

If I had Wenming Wang's steady, industrious hands I could have finished this degree in three months. I'm very much indebted to him for so freely sharing his RPW8.2 data and results with me. Dr. Wang gave and permitted and authorized without ever expecting acknowledgement, a testament to his rigor and character. I'm also thankful for Dr. Xianfeng Ma's willingness to share his data. His drought-tolerant results found welcome place in chapter 3 and provide an exciting crescendo to my data. His data and analysis are also peppered throughout this work. Xianfeng was quick to assist me with cloning and other routine lab work, making his long, busy days longer and busier. In his assisting me we discovered that we share a love of abundant Arabidopsis, the Science Friday radio program and well-designed, discounted outdoor gear. Yi Zhang and I worked closely together on several projects. I'm thankful for his HA-fused RPW8.2 lines and *kin11* data. Yi provided steady support to my biochemical techniques. Lab legends like Robert Berkey showed me

how to stay organized and juggle a hundred biological hypotheses simultaneously while also playing in-lab football. Even from a distance he continues to inspire.

I'm also thankful to current Xiao lab members who encouraged, demonstrated and delighted. Their indefatigable pace and fervor contributing to our lab esprit de corps and to my sanity when all positive results defied me. Qiong Zhang is a walking textbook of cell biology. She's as comfortable discussing SNARE proteins as she is Chinese performing artists. She never complained or chastised me for my 8pm texts asking for her help in turning off the microscope I left on, or inoculating the culture I forgot or autoclaving the media I just run out of time to do. She was first to convince me I could finish my writing or perform just one more experiment when I wasn't convinced myself. I've enjoyed conversations and discussions with Bruce Levine. He is a walking master of many topics among them global geopolitics and chestnut fungi. Ying Wu reminds me that there's always time to be cheerful and to laugh especially at ourselves. I'm thankful for the many Wootton high school students and USG and UMCP undergraduates who have been comfortable spending time at our benches and sinks, cloning and washing dishes to aid us in collecting data they may never use.

Xiao lab members show me that we can be excellent scientists and also good human beings.

Dr. Shunyuan Xiao

Shunyuan Xiao walks faster than anyone else I know. He's like a cheetah with a backpack, if that cheetah were on its way to a meeting or to catch a bus to teach its



next class. If Xiao wore a Fitbit it would probably break down in protest because he would earn his 10,000 steps award every 15 minutes. Xiao is comfortable with a quick pace. He needs to be because the demands for his time are immense. Research, mentorship, teaching, faculty activities, grant applications are all priorities and are all important. His calendar is separated by two minute intervals. Yet, somehow, he finds unbounded intervals to daily support me.

He will drop everything to visit with me or read one of my chapters or help me refine my presentation slides. I've sent emails to him and received 2:15am responses. On several occasions I worked late in the lab convinced I was the only member present. I was surprised to see Xiao walking through the door asking me how everything was going and if I needed help. This attendance repeated itself on holidays, snow days, and sick days. He's a workhorse, and many of those work hours have been spent helping me.

Every advisor takes a risk with their student, and Shunyuan Xiao took a big risk with me. I'm just now beginning to understand the true value of mentorship in that the tireless hours I've spent writing and researching are only surpassed by him on my behalf. If there's any readable portion of this work or clever insight please know that it belongs to a man who unwaveringly expressed confidence in my feeble hands to make it meaningful and bring it to light.

I'm thankful for his example. His unbridled passion for science and research are contagious. He's one of the few PIs I know to still keep a bench in the lab. And he uses it! He taught me to think in publications. Put the figures together first. Add

placeholders for the data that has yet to be collected. I've caught the vision and methodology of science thanks to Shunyuan Xiao. He is an ideal mentor, possessing grant writing skills, cooking skills and an enviable bowling score. I always expected a mentor, but I was surprised to also find a friend.

## Wendy

As much as I have given to this work Wendy has given more. Behind every hypothesis, experiment, and result she has been there quietly applauding, supporting and encouraging. The diploma may have my name but the honor is hers. She sacrificed much so that I could stay in school. That I finished this work is a testament to her patience and enduring faith that I could complete it.

While her peers purchased new cars, new homes and new ring upgrades, Wendy could only hope for a day when her husband could offer more than liquid nitrogen ice cream, an occasional DIY home cleaner made with 70% ethanol and an endless supply of soiled latex gloves. Perhaps she's hoped to one day experience a budget-friendly birthday dinner not ending with me announcing we need to stay another year because her January birthday unfortunately coincides with the registration of spring semester. She's undoubtedly dreamt of a day when her husband's "...just 20 more minutes" pleas don't mutate into two more hours at the beginning of a family event. She'll probably be delighted when holidays no longer need to be shared with immunoprecipitated samples and western blots.

She may hope for these things, but she never let it prevent the happiness and joy we both experienced as a result of my protracted graduate studies. We've been co-writers, co-explorers, and co-creators. And though many partners would harbor begrudgement or complaint, Wendy instead resiliently met our challenges with bravery by believing that our family shouldn't wait until I finished my degree. She gave birth to the remaining three of our four children while I was a student. She never showed her frustration when I excused myself from her hospital bedside to return to the lab, sometimes the very next day because an experiment couldn't wait.

She was the sequence, the purification, the central dogma focusing me a singular task: to expand mankind's horizons by exploring a little more. My daily "what ifs" and "I'll bets" were only possible because she answered "why yes!".

For all the experiments I've performed and the data I've collected, for none have I so unequivocally discovered truth as this:

Love is eternal.

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## List of Abbreviations

<b>ACD</b>	
Accelerated cell death.....	10
<b>Avr</b>	
avirulent factor.....	4
<b>BAK1</b>	
BRI-associated receptor kinase 1 .....	11
<b>BIK1</b>	
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# Chapter 1 Introduction

## ***Food Security***

The world population is expected to reach almost 10 billion in 2050 putting increased strain on planetary resources (United Nations Department of Economic and Social Affairs Population Division (2017), 2017). Hence, food security has become a greater global challenge. Today, one billion people are estimated to be malnourished, and widespread dependence on single crops like cassava and rice especially in developing countries increases a population's susceptibility to losses (Fletcher et al., 2006). This is in part because plants are not only food for humans; they're also meals for all kinds of microorganisms from every kingdom including viruses, bacteria, oomycetes, nematodes and fungi that have evolved to attack and/or co-survive with plants. In the United States alone, an estimated 50,000 pathogens attack plants and account for 10-15% of crop losses (Fletcher et al., 2006; Madden, 2001; Madden & Wheelis, 2003). Increasing temperature changes and climate disruption may increase susceptibility as spatial and temporal disruptions aid the development of some pathogens (Rosenzweig, 2001).

Historically, there are numerous examples of large-scale crop destruction by aggressive pathogens. The potato late blight disease caused by *Phytophthora infestans* devastated potato crops in 1845 in Ireland, triggering the Great Famine (Saville et al., 2016). This oomycete pathogen still troubles potato production around

the world today (Fry et al., 2015). The chestnut blight fungus, *Cryphonectria parasitica*, destroyed ~4 billion American chestnut trees in the first half of the 20th century after its introduction to the US in 1904 (Grunwald, 2012). Ug99, a new aggressive strain of wheat stem rust fungus (*Puccinia graminis* f. sp. *tritici*) capable of overcoming resistance of most commercial cultivars, has spread from Africa to the Middle East and parts of South Asia, posing serious threat to global wheat production (Singh et al., 2011, 2015). The most recent example is the citrus greening disease caused by *Candidatus liberbacter asiaticus*, an intracellular bacterial pathogen. This disease has rapidly spread to most citrus orchards in Florida and is now seriously affecting the citrus industry in Florida (da Graça et al., 2016; Manjunath, Halbert, Ramadugu, Webb, & Lee, 2008). The above examples demonstrate the importance of pathogen detection, disease spread and control, and particularly the understanding of the mechanisms of host resistance and pathogenesis in order to better protect crop plants against various pathogens.

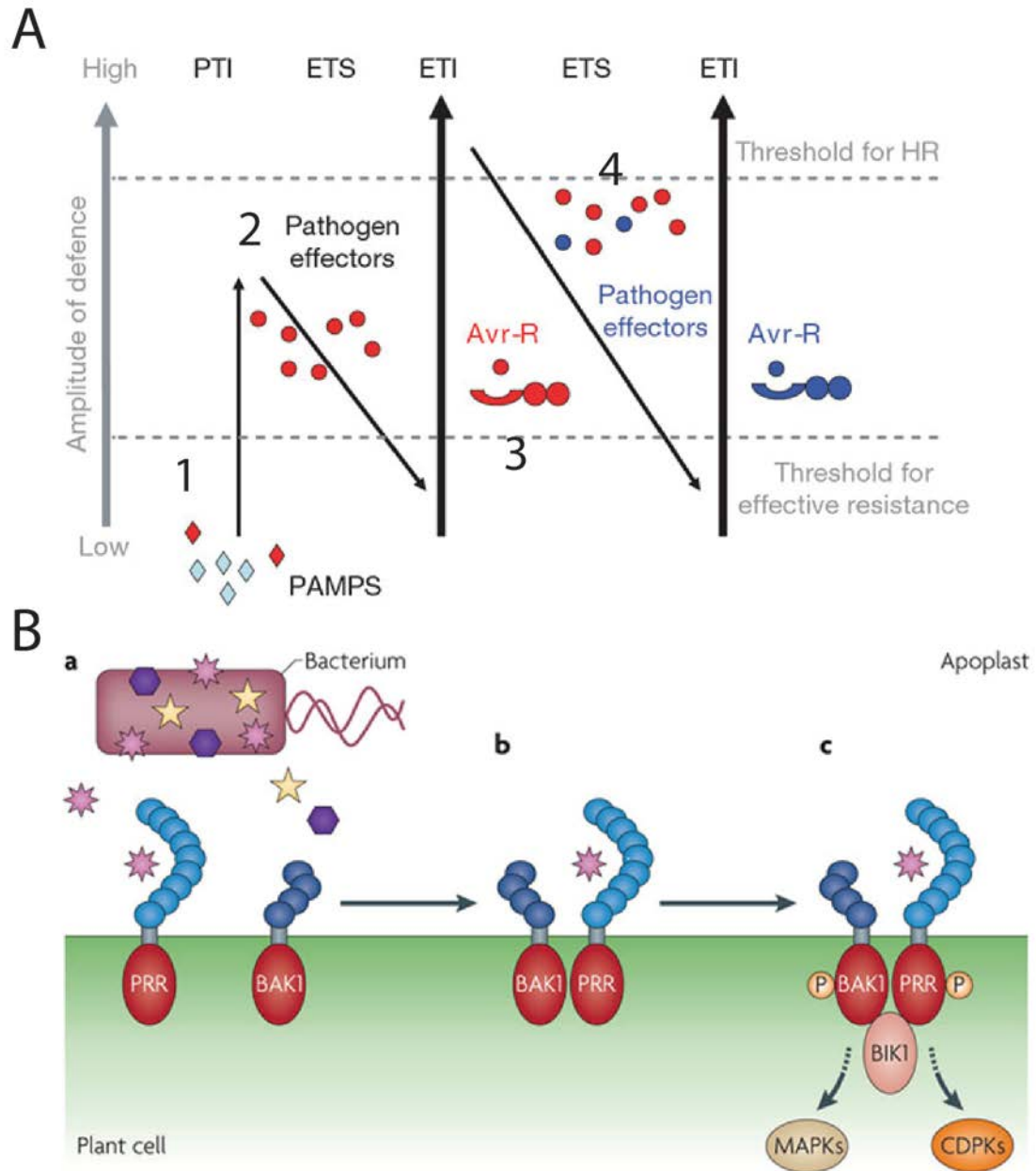
Plants are sessile; they cannot run away from their invaders, and unlike animals, they lack an adaptive immune system. Consequently, every plant cell has to rely on its own activation of defense to fight against pathogen invasion. Yet, in nature despite the presence of a myriad of microorganisms, most plants are resistant to most potential pathogens. This is because, apart from preformed physical barriers such as the rigid cell wall, the cuticle epidermis, and leaf hairs (trichomes) (Hülkamp, 2004; Malinovsky, Fangel, & Willats, 2014; Serrano, Coluccia, Torres, L'Haridon, & Métraux, 2014), plants have evolved a robust innate immune system to effectively

protect themselves from attack by the vast majority of potential pathogens. The innate immune system of plants consists of two layers that are evolutionarily interrelated and mechanistically interconnected (Chisholm, Coaker, Day, & Staskawicz, 2006a; Jones & Dangl, 2006; Nürnberger, Brunner, Kemmerling, & Piater, 2004). The first layer is called PAMP-triggered immunity (PTI), which is activated upon perception of conserved pathogen molecules by plant cell-surface localized pattern recognition receptors; The second layer is called effector-triggered immunity (ETI), which is activated when pathogen effectors are recognized by plant intracellular immune receptors.

### ***PAMP-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI)***

As briefly mentioned above, instead of trying to avoid provoking PTI by mutating conserved PAMPs (which often results in fitness cost), most pathogens secrete effector proteins into the host cells and use them to interfere with and disrupt PTI, and subsequently establish ETS (Janjusevic, Abramovitch, Martin, & Stebbins, 2006; Xiang et al., 2008) (Chisholm et al., 2006) (Jones and Dangl, 2006). To protect themselves against such adapted pathogens, plants have evolved intracellular immune receptors often called resistance (R) proteins that contain a nucleotide-binding site (NB) and leucine rich-repeats (LRRs) to detect the presence or virulence activity of specific pathogen effectors. This specific recognition activates the R protein and then a conserved SA-dependent downstream signaling pathway leading to defense gene

expression and in most cases the hypersensitive response (HR) (Cui et al., 2015; Harris et al., 2015). This so-called effector-triggered immunity (ETI) is mechanistically connected to PTI with some key signaling components being engaged in both layers of defenses (Bigear, Colcombet, & Hirt, 2015; Y. Kim et al., 2014; Tsuda, Sato, Glazebrook, Cohen, & Katagiri, 2008). and to some extent mechanistically resembles the innate immunity activated by animal NB-LRR receptor (NLR) proteins upon recognition of intracellular PAMPs (Lukasik & Takken, 2009; van Ooijen et al., 2008). Though the structure for any full-length plant NB-LRR R protein has yet to be resolved, the LRRs of NLRs located at the C-terminus of are believed to have ideal characteristics for ligand-specific binding. LRRs' slender conformation maximizes surface area and their bent shape mediates flexible binding; Such structural properties confer LRRs the capacity to tolerate high levels of variability (Padmanabhan, Cournoyer, & Dinesh-Kumar, 2009). For historical reasons, an effector known to be recognized by a cognate NLR R protein is termed avirulent factor (Avr). R-Avr recognition occurs at the top of the ETI signaling cascade and this step is subjected to natural selection in pathogens for evading recognition and in plants for regaining recognition, resulting in the host-pathogen arms-race (Maor & Shirasu, 2005; Ravensdale, Nemri, Thrall, Ellis, & Dodds, 2011; Tiffin & Moeller, 2006; Yunzeng Zhang et al., 2015). The evolutionary struggle can be represented by a zigzag model of immunity (Jones and Dangl, 2006) (**Figure 1-1A**).



**Figure 1-1 Models of PTI and ETI in Plants**

(A) Four phases of plant-pathogen co-evolutionary interaction. In phase 1, plants evolved PRRs to detect pattern associated molecular patterns (PAMPs) (diamonds). PAMP-triggered immunity (PTI) effectuates plant resistance. In phase 2, pathogens evolved effectors (red

circle) which act to subvert PTI. This results in effector-triggered susceptibility (ETS). In phase 3, plants evolve resistance (R) proteins which interact directly or indirectly with effectors (Avr once identified), leading to effector-triggered immunity (ETI) that is often accompanied by hypersensitive response (HR). In phase 4, pathogens mutate the recognized effectors (blue circles) thereby escaping detection and subverting ETI. Figure modified from (Jones & Dangl, 2006). (B) Bacteria like *Pseudomonas syringae* possess PAMPs like flagellin and EF-Tu (pink and purple shapes) that are detectable by pattern recognition receptors (PRR)s. A PRR recognizing a PAMP like flagellin associates with protein BAK1. Transphosphorylation of the PRR-BAK1 complex recruits BIK1 and other immune signaling distributors like MAP-kinases (MAPKs) and calcium-dependent protein kinases (CDPKs). Figure adapted from (Dodds & Rathjen, 2010a).



Based on this model depicted in Figure 1-1A, the amplitude of defense during ETI is higher than PTI and in most cases, the hypersensitive response (HR), i.e. programmed cell death (PCD) around the infection site ensues. ETI also often results in systemic defense signaling, leading to systemic acquired resistance (SAR) that protects plants from subsequent infection by a broad range of virulent pathogens (Dodds & Rathjen, 2010b; Jones & Dangl, 2006).

Plant resistance from ETI is often race-specific (with a very narrow spectrum). Based on early genetic studies of plant-pathogen interactions, Flor proposed the “gene-for-gene” hypothesis which predicts that the product of a plant *R* gene functions as a receptor that specifically interacts and recognizes a ligand encoded by an *Avr* gene from the pathogen, triggering defense responses (Flor, 1971). However, experimental evidence for R-*Avr* direct interaction is the exception rather than the rule. The “gene-for-gene” hypothesis has been replaced by a more mechanistically accommodative theory called the “guard hypothesis”. According to the new model, pathogen effectors target and manipulate key host proteins for increased virulence. R proteins associate with host target proteins and activate defense when detecting a modification or perturbation of a “guarded” host protein, or “modified-self”. Thus, R proteins recognize cognate *Avr* proteins by detecting their virulence activity rather than their physical presence in the plant cells (Dangl & Jones, 2001; Van Der Biezen & Jones, 1998). The “guard hypothesis” has been supported by many studies where R and *Avr* do not show direct interaction but both are associated with a host protein (Axtell & Staskawicz, 2003; Mackey, Holt, Wiig, & Dangl, 2002; Ntoukakis, Saur,

Conlan, & Rathjen, 2014; F. Shao, 2003). This new hypothesis offers a more rational explanation for the phenomenon that plants with a limited number of *R* genes are capable of protecting themselves against numerous pathogens: this is because watching for modifications to only a limited number of key host proteins by various effectors reduces the number of *R* proteins required to adapt and respond to attacks from multiple pathogens each with a distinct repertoire of effectors.

Plant NB-LRR *R* proteins belong to a superfamily that can be further divided into three sister subclasses based on their N-terminal domains. The first subclass possesses an N-terminal Toll and human interleukin receptor (TIR) domain. The second subclass possesses an N-terminal coiled-coil (CC) domain, and the third subclass contains a domain that shows homology to RPW8 (which is an atypical *R* protein identified in *Arabidopsis*; see later sections for details) (Zhong and Cheng, 2016; Qian et al., 2017). Intriguingly, while these three subclasses of *NB-LRR R* genes are ancient and present in angiosperms, only the *CC-NB-LRR* subclass *R* genes are found in monocots (Marone, Russo, Laidò, De Leonardi, & Mastrangelo, 2013; McHale, Tan, Koehl, & Michelmore, 2006; Z.-Q. Shao et al., 2016). Functionally characterized NB-LRR proteins, regardless of subclasses, are able to activate immune responses, i.e. ETI, but their immediate downstream signaling is transduced via dichotomous pathways (Eitas & Dangl, 2010). While TIR-NB-LRR *R* proteins require EDS1 (a lipase-like protein; (Falk et al., 1999)) for downstream signaling, CC-NB-LRR *R* proteins require NDR1 (an integrin-like protein; (Falk et al., 1999)) for signal transmission (Aarts et al., 1998). The signaling events from both types of *R*

proteins converge at the step of elevated biosynthesis of SA and regulation by the downstream component NPR1 before leading to the same or similar defense response featured with expression of pathogenesis-related protein (PR) genes, oxidative burst, and ultimately, the HR (Glazebrook, 2001; Yan and Dong, 2014). Most *R* genes activate narrow-spectrum resistance to one or a few strains of a particular pathogen and their resistance can be overcome by pathogens in a relatively short period of time. Thus, *R* genes conveying broad-spectrum resistance against many different species of adapted pathogens are valuable (Büschges et al., 1997).

### ***The HR and SHL Responses***

The hypersensitive response (HR) is manifested during ETI in most cases. Although the HR is believed to create a physical barrier for plants to block further aggression of biotrophic pathogens (Staskawicz, Ausubel, Baker, Ellis, & Jones, 1995) (Figure 1-3A), its precise role in defense signaling and plant resistance remains unclear. While the HR shares similar features of mammalian programmed cell death (PCD), it is probably mechanistically distinct from animal PCD, as caspases necessary for PCD in mammalian cells are dispensable for HR development (Coll, Epple, & Dangl, 2011). However, it was noted that in certain cases, caspase-like enzymes such as vacuolar processing enzymes were found to be involved in activating HR (Mur, Kenton, Lloyd, Ougham, & Prats, 2008; Rojo et al., 2004). It has also been established that both chloroplasts and mitochondria are critical to the production of reactive oxygen species like H<sub>2</sub>O<sub>2</sub> and nitrous oxide during the HR

(Zago, 2006). Because the HR is often associated with activation of ETI, spontaneous HR-like cell death (SHL), or accelerated cell death (ACD), or lesion mimic cell death has been used as a visual marker for identification of mutations that either results in auto-activation of NB-LRR receptor proteins (Zhang et al., 2003) (Dong et al., 2016), or inappropriate functioning of ETI signaling components (Lorrain et al., 2003) (Yao and Greenberg, 2006) (Lu et al., 2003), leading to a better understanding of the molecular mechanisms regulating ETI .

### ***Protein Phosphorylation and Plant Immunity***

A single phosphate attached to a protein can change all properties of that protein (P. Cohen, 2000). A phosphate is covalently attached to a protein by a kinase at physiological pH; phosphatases evolved to remove this post translational modification (PTM). The presence or absence of a phosphate activates or inactivates, allows or disallows a protein's location or functionality through surface charge augmentation (Karin & Hunter, 1995). Even though protein phosphorylation is one among ~200 protein modifications, its extraordinary evolutionary success especially in network signaling is in part attributed to its reversibility (Hunter, 2012; Milo & Phillips, 2016; Minguéz et al., 2012; Pawson, 2004).

Threonine, serine, and tyrosine and six other amino acids have the potential to be phosphorylated (Hunter, 2012). In plants and animals, amino acids serine and threonine are typically phosphorylated (Li et al., 2007; Lu et al., 2010), although recent studies are beginning to discover a greater abundance of tyrosine

phosphorylation in both kingdoms (Molina, Horn, Tang, Mathivanan, & Pandey, 2007; Olsen et al., 2006; Sugiyama et al., 2007).

Proteins can be quickly activated or deactivated in seconds to milliseconds via (de)phosphorylation (Humphrey, James, & Mann, 2017; Kennedy, 1983). Moreover, a protein's phosphostatus is reversible; and a network of proteins can be tuned, mitigated and switched off through phosphorylative changes to any of its members. These characteristics make phosphorylation a critical post-translational modification necessary for transducing a multitude of physiological signals including defense signals. Consequently, many immune-related proteins are phosphorylated in the course of PTI and ETI.

Flagellin-sensitive 2 (FLS2) is a PRR that recognizes flagellin, a basic protein needed for assembling flagella. A 22-residue peptide, flg22, from flagellin is able to elicit an immune response (Gómez-Gómez & Boller, 2000). In as few as 20 minutes, cells respond to treatment and begin to alkalinize as a preemptive defense strategy. In fact, Navarro et al. (Navarro, 2004) estimate about 3% of 8200 Arabidopsis genes are transcriptionally altered upon flg22 introduction in suspension cultures leading to reactive oxygen species (ROS) production, alkalinization, and activation of mitogen-activated protein (MAP) kinases (Felix, Duran, Volko, & Boller, 1999; Navarro et al., 2004, 2004; T S Nühse, Peck, Hirt, & Boller, 2000).

FLS2-initiated signaling depends on phosphorylation. After FLS2 binds the flg22 ligand it interacts with BAK1 (BRI-associated receptor kinase 1) in as little as 15 seconds (Chinchilla, Zipfel, Robatzek, Kemmerling, Nürnberger, et al., 2007;

Schulze et al., 2010). The co-receptor BAK1 is also a LRR-RLK (J. Li et al., 2002; Nam & Li, 2002) that heterodimerizes with many other immune proteins (Antje Heese et al., 2007; Roux et al., 2011) and is itself regulated by phosphorylation (Schwessinger et al., 2011; X. Wang et al., 2008). The association with BAK1 causes both BAK1 and FLS2 to transphosphorylate, and is sufficient for a signaling complex to further regulate downstream immune proteins. BAK1 is shown to be critical for initiating PTI in the presence of biotrophic pathogens (Roux et al., 2011).

Upon recognition of flagellin, activated FLS2 and BAK1 phosphorylate BIK1 (BOTRYTIS-INDUCED KINASE 1), which links flg22 binding with downstream intracellular signaling (Lu et al., 2010b). BIK1 belongs to a family of receptor-like cytoplasmic kinases (RLCKs) that function downstream of different PRRs. Recent studies have identified several additional RLCKs including PCRK1, PBL1, PBL13, and PBL27 to be key signaling components downstream of PTI (Lin et al., 2015; Sreekanta et al., 2015; Kong et al., 2016; Yamada et al., 2016). Activated RLCKs appear to further phosphorylate downstream components such as specific MAPKKKs, resulting in activation of a MAPK phosphorylation cascade. This PRR-RLCK-MAPK signaling module seems to be highly conserved in different plant species and perhaps across plant and animal kingdoms (Yamada et al., 2017)(Couto and Zipfel, 2016).

Not only are PTI proteins the instigators of downstream phosphorylation, NB-LRR R-proteins interacting with effectors are also regulated by phosphorylation. The NB-LRR protein RPM1 “guards” RPM1-INTERACTING PROTEIN4 (RIN4)

targeted by *Pseudomonas syringae* effectors AvrB and AvrRpm1. Interestingly, RIN4 is hyperphosphorylated in the presence of AvrB and AvrRpm1 (Chisholm, Coaker, Day, & Staskawicz, 2006b; Mackey et al., 2002). This modified state of RIN4 activates RPM1, which triggers plant resistance (Boyce, Nam, & Dangl, 1998).

PTI and ETI associated phosphorylation eventually leads to the phosphorylation of WRKY transcription factor (TF) proteins. The WRKY TF family consists of 74 members in Arabidopsis (Eulgem & Somssich, 2007) and some contain at least one DNA-binding domain which binds to the W-box in the promoters of defense related genes and initiate transcription of these gene during PTI and ETI. Several WRKYs have been confirmed to contain a conserved motif known as the “D” motif, which is a site for phosphorylation by MAP kinases (C. Y. Kim & Zhang, 2004; Popescu et al., 2009).

### ***14-3-3 Proteins Play a Role in Immunity***

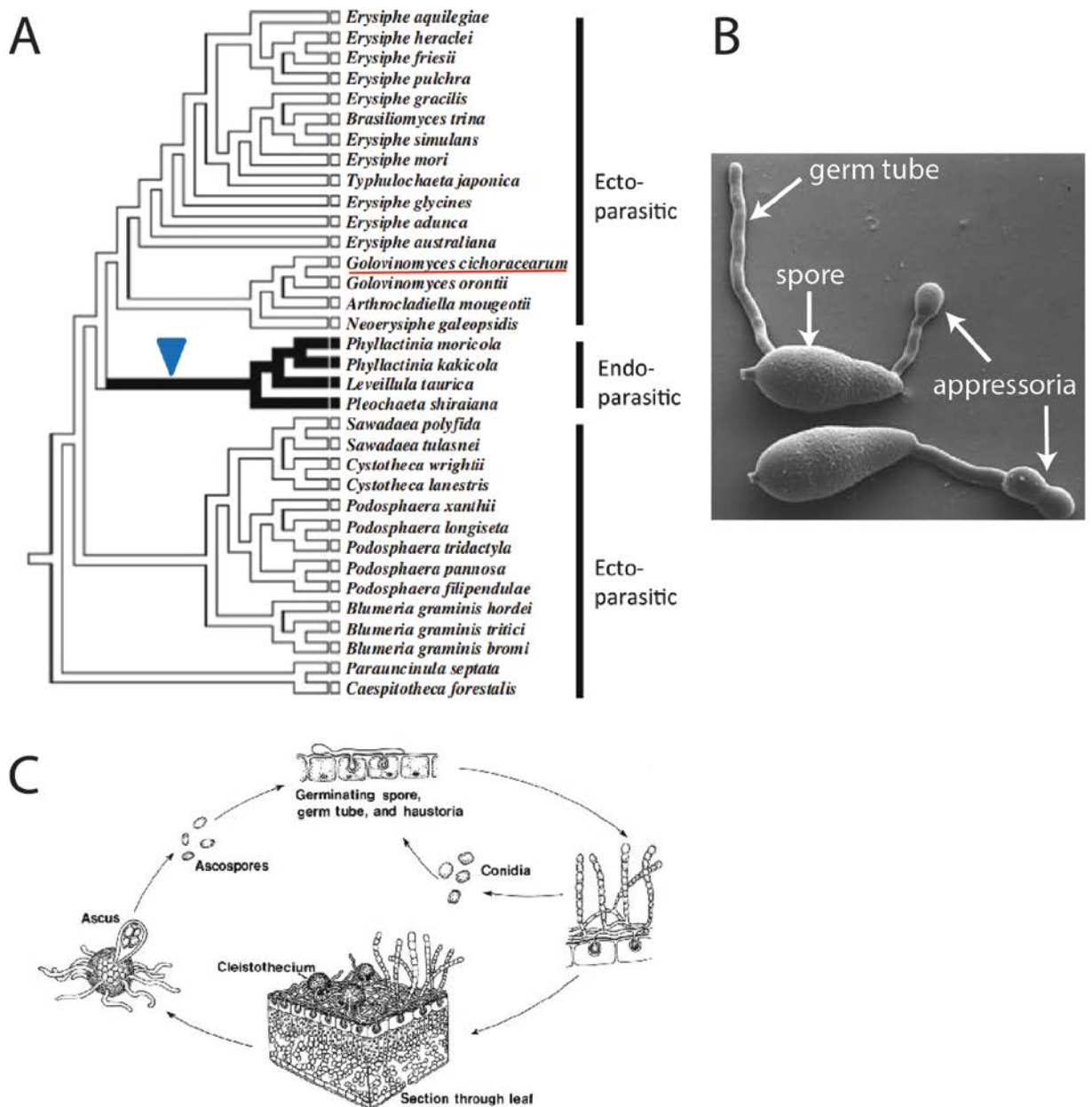
14-3-3 proteins are conserved, eukaryotic proteins that interact with typically-phosphorylated proteins involved in diverse cellular networks (Dreze et al., 2011). 14-3-3s have been implicated in the regulation of plant immunity (Oh & others, 2010). For example, 14-3-3 $\omega$  seems to associate with BAK1 and 7 WRKY TFs (I.-F. Chang et al., 2009). Tomato 14-3-3 protein 7 positively regulates immunity-associated programmed cell death by enhancing protein abundance and signaling ability of a MAPKKK (Oh et al., 2010; Oh and Martin, 2011). A rice 14-3-3 protein OsGF14e positively regulates panicle blast resistance in rice (Liu et al., 2016). Not

surprisingly, 14-3-3s have been shown to be host proteins targeted by pathogen effectors for interfering with plant defense activation and increased virulence. For instance, *Pseudomonas syringae* Effector HopQ1 interacts with 14-3-3 proteins for its subcellular localization and stability in the host cells (W. Li, Yadeta, Elmore, & Coaker, 2013). HopM1, another *P. syringae* effector, binds 14-3-3k from Arabidopsis to suppresses stomatal closure and ROS production (Lozano-Durán, Bourdais, He, & Robatzek, 2014).

### ***Powdery Mildew***

Powdery mildew (PM) fungi as a pathogen group make the list of the top 10 fungal pathogens based on science and economic importance (Dean et al., 2012). Nine-hundred species of PM exist in sixteen genera (Takamatsu, 2013). PM is one of the most common diseases of crops caused by Ascomycete fungi in the order of *Erysiphales* (Göllner, Schweizer, Bai, & Panstruga, 2008). The PM disease reduces yield and quality of agronomically-important crops like wheat and barley and various vegetable and fruit crops such as tomato, cucumber, strawberry and grape. Ornamental crops like roses and crape myrtle are also susceptible. PM fungi are a ecto-parasitic, obligate biotrophs, strictly requiring living hosts to complete their life cycle (Takamatsu, 2013) (**Figure 1-2A**). White, dust-like mycelia of any PM fungus develop on the leaves, stems, flowers and fruits of their hosts, thus earning ‘powdery mildew’ its name. PM pathogens do not kill their host, but they steal nutrients from host cells causing impaired growth and development of infected plants.





**Figure 1-2 Powdery Mildew Phylogeny and Characteristics**

(A) *Golovinomyces cichoracearum* is one of the causal agents of powdery mildew. 900 species of powdery mildew exist in 16 genera. *G. cichoracearum* can colonize Arabidopsis, cucurbits, and many other dicot plants. Not all members are ecto-parasitic. A

monophylogenetic clade evolved endo-parasitism (blue arrow). Figure adapted from (Takamatsu, 2013). (B) Within an hour a conidium (spore) landing on a suitable surface begins growing a primary germ tube. From this germ tube extends a specialized hypha, the appressorium. Figure adapted from (Zeigler, Leong, & Teng, 1994). (C) Powdery mildews have both sexual and asexual lifestyles. Powdery mildew propagation and host colonization can be completed by conidia as a result of conidiogenesis in the asexual cycle. To overwinter, powdery mildew may produce sexual spores in a fruiting body, cleistothecium. Here, haploid cells fuse (karyogamy) to form diploid cells. Diploid cells in an ascus undergo meiosis to form haploid ascospores which germinate on a suitable host forming appressoria and haustoria. Figure from (Schumann, 1991).

## ***Life Cycle of Powdery Mildew***

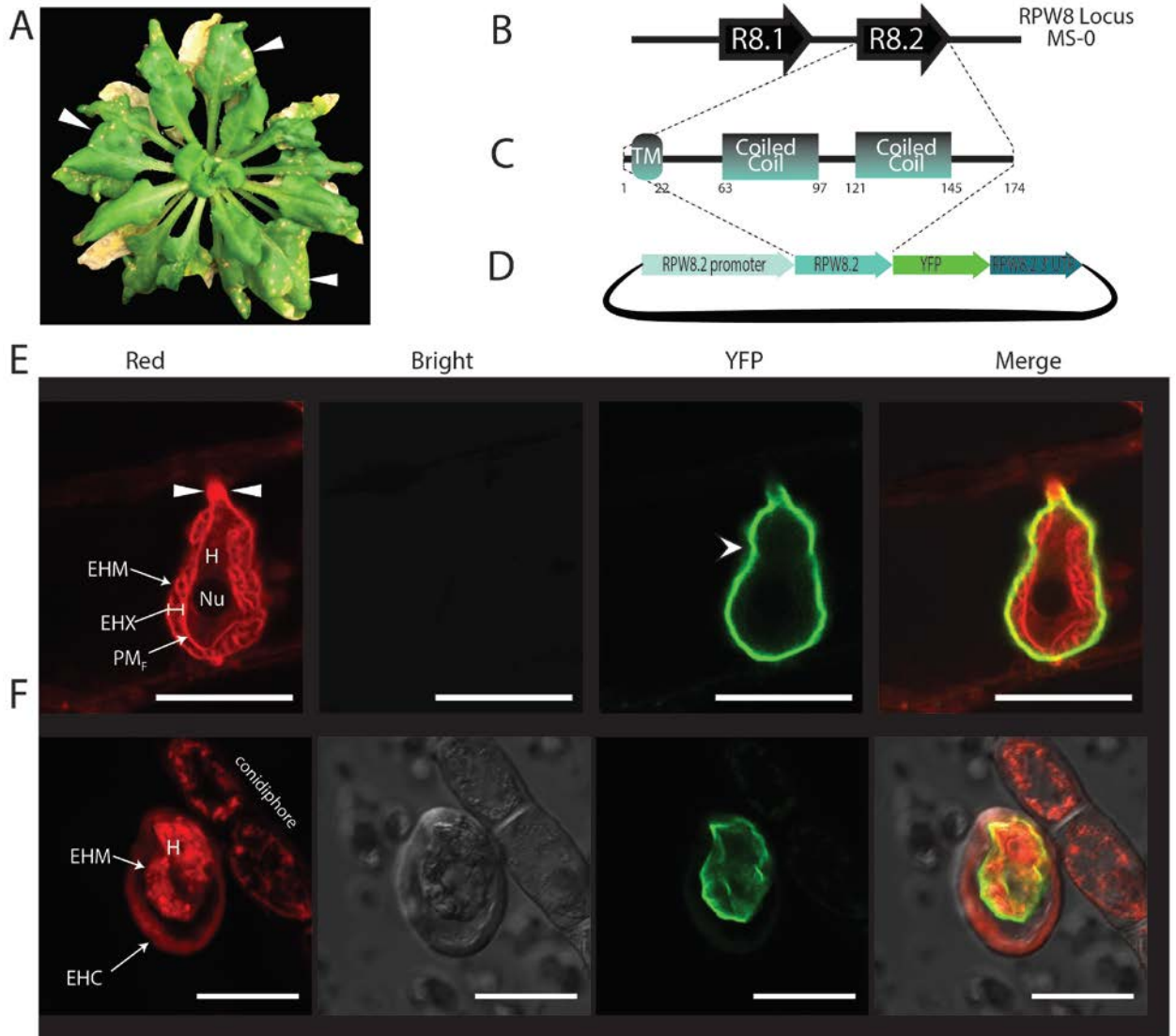
Powdery mildew has both a sexual and asexual life cycle. The sexual cycle produces ascocarps (cleistothecia), or fruiting bodies, which contain asci and ascospores (**Figure 1-2C**). This cycle is initiated late in the growing season as ascospores are resistant to drought and low temperatures in the winter. The asexual cycle produces conidia, or asexual, ovoid spores about 40µm in length. Successful sporulation and growth of the fungus generates more conidia. A square millimeter on a leaf surface containing successfully growing powdery mildew can generate tens of thousands of conidia usually with a few days of establishment. A single colony can generate 200,000 conidia (Z. Zhang et al., 2005). The exponential growth of conidia can lead to area epidemics (Pscheidt, 2015).

A conidium begins to grow a primary germ tube 30-180 minutes after landing, usually aurally, on a potential host (Wright, Thomas, & Carver, 2002). After 10 hours the primary germ tube forms an appressorium which penetrates the cuticle and cell wall with a penetration peg (Edwards, 2002) (**Figure 1-2B**). The penetration peg is a specialized hypha capable of puncturing and rupturing the cell wall and cuticle barriers of a plant cell probably through a combination of enzymatic and mechanical methods like lytic enzymes and turgor pressure (PRYCE-JONES, CARVER, & GURR, 1999). The plant resists penetration with the papilla, or an apoplastic, callosic-cell wall buildup (apposition), around the penetration site (WR Bushnell & Bergquist, 1975). Successfully avoiding or burrowing through the papilla, the penetration peg swells and differentiates to form a bulb-like feeding structure called

the haustorium. The haustorium is a fungal feeding appendage uptaking and transporting host nutrients to the fungus.

### ***The Haustorium***

Pre-invasion resistance strategies like the plant wall and cell-wall appositions are sufficient for warding off non-host pathogens. For an adapted PM fungus such as *Golovinomyces cichoracearum* on Arabidopsis, it can avoid or suppress the defensive mechanisms of its hosts and develop a functional haustorium. The role of the haustorium in establishing pathogen-host interactions includes secreting effector into the host cell to subvert host defenses and direct nutrient flow to the fungus. Despite its critical importance for successful fungal pathogenesis, the haustorium is still a poorly understood, enigmatic feature of host-pathogen tête-à-tête (WR Bushnell, 2002)(O'Connell & Panstruga, 2006).



**Figure 1-3 RPW8-Mediated HR and RPW8.2's Specific Localization to the EHM**

(A) The hypersensitive response (HR) (white arrows) is often an ETI response to PM pathogens and is typically characterized by lesions indicating local fungus-induced cell death.

(B) The RPW8 locus from Arabidopsis accession "MS-0" contains two genes, tandemly located, RPW8.1 and RPW8.2. (C) RPW8.2 is predicted to contain a putative transmembrane domain (TM) at the C-terminus and two coiled-coils. (D) When expressed by the native promoter, RPW8.2 in fusion with YFP at the C-terminus localizes to the extra-haustorial

membrane (EHM). (E) A haustorial complex stained with lipophilic FM4-64 dye shows the haustorium (H), nucleus (Nu), extra-haustorial matrix (EHX), extra-haustorial membrane (EHM), and fungal plasma membrane (PM<sub>F</sub>). The narrow neckband delineated with white arrows. The EHM can also be observed in the YFP-channel due to localization of RPW8.2-YFP (white arrowhead). (F) Another image showing an FM4-64 stained haustorial complex with an encasement of the haustorial complex (EHC). Note, two immature conidia attached to a conidiophores are visible on the right. Scale bars = 20μm.

The PM haustorium is a specialized fungal cell that maintain its own nucleus, cell wall, mitochondria and organelles (William. Bushnell, 2012; Coffey, Palevitz, & Allen, 1972) and is both a dock and a launching pad. Transporters and H<sup>+</sup>-ATPases dock here to import plant-derived glucose and nutrients (Voegelé, Struck, Hahn, & Mendgen, 2001b). Effector proteins are launched from the haustorium across the EHM---the host-pathogen interface, targeting immune-related proteins (Petre & Kamoun, 2014; Whisson et al., 2007). Thus, the haustorium is a biological hive of activity, each action and interaction impacting fitness. With the development of functional haustoria, rapid conidiogenesis typically occurs within 3-7 days in a fungal network indicating successful colonization (WR Bushnell, 2002).

Similar to the interaction between plants and other pathogens, to fight against PM invasion, any given plant host has evolved both pre- and post-invasion resistance strategies to stop the fungal penetration and /or restrict the fungal growth, respectively (Thordal-Christensen, 2003). Adapted PM fungi overcome PTI and are able to establish functional haustoria, leading to successful colonization. In response, host plants employ post-invasive resistance strategies most likely in the form of ETI to constrain the haustorium (W. Wang, Wen, Berkey, & Xiao, 2009; Wen et al., 2011). Thus, concerning host defense, the haustorium is the Achilles Heel of the PM fungi because for most PM fungi the haustorium is the only fungal structure in the host cells.

## ***The Extra-Haustorial Membrane***

Microscopic examinations reveal that the PM haustorium is surrounded by an interfacial membrane called the extra-haustorial membrane (EHM) (Gil, F., Gay, 1977; C. O. Micali, Neumann, Grunewald, Panstruga, & O'Connell, 2011). The EHM provenance is unknown. Two models of biosynthesis are proposed by Koh et al. (Koh, André, Edwards, Ehrhardt, & Somerville, 2005). First, the membrane may be derived from invagination of the plasma membrane at the time of haustoria formation. Gradually, the membrane is changed and modified through exocytosis and microdomain augmentation (Assaad, 2004). Second, the membrane may result from de novo synthesis instantiated through EHM-specific vesicles. The second mode explains the unique molecular feature of the EHM, which is unlike the PM (C. O. Micali et al., 2011; O'Connell & Panstruga, 2006). Recent evidence supports a de novo synthesis model for the EHM (Berkey et al., 2017). The extracellular space between the haustorial cell wall and the EHM is the extra-haustorial matrix (EHX). Collectively, the EHM, EHX and the haustorium comprise the haustorial complex.

It is believed that molecular exchange must occur across the EHX, as recent transmission electron microscopy images showed that the EHX was filled with plant-derived vesicles in a plasm of plant cell wall polymers and membrane-bound, fungal-derived vesicles (C. O. Micali et al., 2011). With each organism attempting to gain an advantage over the other, the EHM and the EHX as the host-pathogen interface serves as the critical battleground of bioactivity. Unfortunately, despite the high importance of the host-pathogen interface to the success of the host's defense and the pathogen's



pathogenesis, very little is known about the molecular interactions. and the origin and biogenesis of the EHM.

### ***RPW8.1 and RPW8.2 are Unique Resistance Proteins***

Aiming to understand how plants fight against haustorium-forming pathogens, Xiao et al. identified a gene locus in *Arabidopsis thaliana* accession Ms-0 that confers resistance to multiple powdery mildew isolates belonging to four different PM species (S Xiao et al., 2001). This locus contains two functional, tandemly-located genes named Resistance to Powdery Mildew (*RPW*)8.1 and *RPW*8.2. Both *RPW*8.1 and *RPW*8.2 are predicted to encode small (~20kDa), basic proteins containing a possible N-terminus transmembrane domain (TMD) and two coiled-coil domains (Xiao et al., 2001). While *RPW*8.1 and *RPW*8.2 are dissimilar to other R-proteins in that they confer broad-spectrum, rather than race-specific, resistance to powdery mildew pathogens, they are similar to R-proteins in two ways: 1) they activate defense via the conserved salicylic acid (SA)-dependent signaling pathway, and 2) their expression is linked to hypersensitive (HR) cell death at the site of pathogen invasion (Shunyuan Xiao et al., 2005)(Xiao et al., 2001). Intriguingly, *RPW*8.1 and *RPW*8.2 show homology to the N-terminal domain of one subclass of NB-LRRs (Collier et al., 2011), raising the possibility of its evolution through gene fission of R8-NB-LRR (Zhong and Cheng, 2016).

Transgenic plants expressing *RPW*8.1 and *RPW*8.2 display typical HR at 3-4 days post-inoculation (dpi) with powdery mildew (S Xiao et al., 2001; Shunyuan

Xiao, Brown, Patrick, Brearley, & Turner, 2003). Despite being an atypical R proteins in terms of protein structure, RWP8.1 and RPW8.2 activate defense and the HR via the EDS1- and SA-dependent pathway (Xiao et al., 2005). When overexpressed from their native promoters, RPW8.1 and RPW8.2 are capable of activating spontaneous HR-like (SHL) cell death (Shunyuan Xiao, Brown, et al., 2003), so are some RPW8.2 mutants (W. Wang et al., 2013).

Interestingly, expression of both RPW8.1 and RPW8.2 has recently been shown to be positively upregulated by PTI (Y. Li et al., 2017; W. Wang et al., 2009). In addition to rendering resistance to powdery mildew and oomycete pathogens in *Arabidopsis* (X. F. Ma et al., 2014), RPW8.1 was recently shown to also respond to bacterial pathogens as its expression was correlated with PTI signaling in the presence of bacterial pathogens *Pseudomonas syringae* in *Arabidopsis* and *Xanthomonas oryzae* pv. *oryzae* in rice (Y. Li et al., 2017). RPW8.1 localizes to the chloroplast in mesophyll cells (Y. Li et al., 2017; X. F. Ma et al., 2014). Because the chloroplast plays an important role in the HR (Zago, 2006), the association of RPW8.1 with the chloroplast and its localization in puncta bodies near the chloroplast may serve to prime and activate defense responses via a connection with an unknown regulator (Y. Li et al., 2017).

### ***RPW8.2 is Localized to the Plant-Pathogen Interface***

How RPW8.2 functions to confer resistance to PM fungi has been a research focus in the Xiao laboratory for the past 10 years. Interestingly, a fusion of RPW8.2

with yellow fluorescent protein YFP “RPW8.2-YFP” is specifically targeted to the EHM within 16-20 hours after inoculation (W. Wang et al., 2009) (Figure 1-3B-F). Additional analysis discovered that the localization of RPW8.2-YFP at the EHM correlates with two haustorium-directed defenses: the formation of a cell wall-like callosic encasement of the haustorial complex (EHC) and the accumulation of H<sub>2</sub>O<sub>2</sub> in the host-pathogen interface.(W. Wang et al., 2009). These results provide a physical explanation for the broad-spectrum nature of RPW8-mediated mildew resistance. However, how RPW8.2 is specifically targeted to the EHM and how it activates haustorium-targeted defenses is still unknown. Revealing RPW8.2-interacting partners may provide a clue to its molecular function and highly specific localization to the EHM.

### ***RPW8.2 Might Be Regulated by Phosphorylation and 14-3-3s***

To understand how RPW8.2 functions to activate haustorium-targeted defense, a previous yeast-two-hybrid screen identified two RPW8.2-interacting proteins, a protein phosphatase type 2C (PAPP2C) and 14-3-3λ. Genetic analyses indicate that RPW8.2 is negatively regulated by PAPP2C (Wang et al., 2012), while positively regulated by 14-3-3λ (Yang et al., 2009). Additionally, genetic evidence suggests that RPW8.2 is negatively regulated by EDR1, a putative MAPKK kinase. Given that 14-3-3s often interact with phosphorylated proteins, these early protein-protein interaction studies suggest that RPW8.2 is regulated by (de)phosphorylation

and 14-3-3s. However, the physiological relevance of these interactions vis-à-vis RPW8.2 functionality remains elusive.

The goals of my thesis project are to (1) determine if RPW8.2 is indeed regulated by (de)phosphorylation, and to test putative phosphatase and kinase interacting proteins. (2) Elucidate the mechanisms by which 14-3-3 $\lambda$  regulates RPW8.2's defense and/or EHM-specific localization.

## Chapter 2 RPW8.2 Regulation by Phosphorylation

### *Introduction*

Phosphorylation is a critical post-translational modification (PTM) of proteins in response to environmental stimuli. The phosphate group acts like a molecular switch endowing proteins new functionality or inactivating their functionality. Proteins can also be phosphorylated in multiple sites providing greater flexibility and regulation for kinases, which are proteins that phosphorylate, and phosphatases, which are proteins that dephosphorylate (Hunter, 1995).

Plant immune proteins need to be quickly activated, responsive and tunable. Consequently, the phosphorylation of plant immune proteins is essential, and is the most common PTM in signal transduction. For example, in Arabidopsis cell cultures, the introduction of xylanase and flg22 caused 472 proteins to be phosphorylated creating 1,168 phosphopeptides (Joris J. Benschop et al., 2007).

Pattern recognition receptors (PRRs) respond to conserved pathogen morphologies like flagella and chitin on potential pathogens. Recognition of pathogen-associated molecular patterns (PAMPs) by PRRs activates an innate layer of immunity, PAMP-triggered immunity (PTI) (Schwessinger & Ronald, 2012). Other receptor-like kinases (RLKs) interact with PRRs and relay signals typically through phosphorylative means. Reactive oxygen species and other defensive measures can be generated by plants in response to PTI (Segonzac & Zipfel, 2011).

PTI-related proteins are phosphorylated. For instance, two PRRs, flagellin sensitive 2 (FLS2) (AT5G46330) and chitin elicitor receptor kinase 1 (CERK1) (AT3G21630) become phosphorylated in response to bacterial flagellin and fungal chitin, respectively (Wan et al., 2008; Zipfel, 2009). FLS2 perceives flagellin or flagellin-peptide, flg22, and oligomerizes with BAK1. This complex is transphosphorylated by BIK1 which is itself activated via phosphorylation at T237 (Lu et al., 2010a). Flg22 activates MAPK cascades but BIK1's role in this is currently unknown (Asai et al., 2002a; Meng & Zhang, 2013).

Insect exoskeletons, crustacean shells and fungal cell walls contain chitin, a polymer of N-acetyl-D-glucosamine. In Arabidopsis chitin is detected by PRR CERK1 (Miya et al., 2007; Wan et al., 2008). CERK1 contains a lysine motif (LysM), a subfamily of receptor-like kinases, that interacts with chitin, dimerizes and phosphorylates (Liu et al., 2012; Petutschnig, Jones, Serazetdinova, Lipka, & Lipka, 2010). Phosphorylated CERK1 feeds into the MAPK cascade by phosphorylating MAPKKK5 via PBL27 to activate disease resistance (Yamada et al., 2016).

*Golovinomyces cichoracearum* strain UCSC1 (*Gc* UCSC1) is one of four powdery mildew fungi that can evade PTI and have successfully adapted Arabidopsis as a host (C. Micali, Göllner, Humphry, Consonni, & Panstruga, 2008; W. Wang et al., 2009; S Xiao et al., 2001). Colonization includes epidermal-cell insertion of a penetrating specialized hypha that develops into a parasitic organelle called the haustorium (Gil, F., Gay, 1977). The haustorium is a unicellular body that diverts to

itself host cell nutrients and glucose through its panoply of transporters (Voegelé, Struck, Hahn, & Mendgen, 2001a).

Although pre-invasion strategies are largely ineffective against *Gc* UCSC1, post-invasion strategies like effector-triggered immunity (ETI) are successful for some *Arabidopsis* ecotypes like MS-0 (Orgil, Araki, Tangchaiburana, Berkey, & Xiao, 2007). A broad-spectrum powdery-mildew resistant locus, *RPW8*, was isolated from this ecotype (S Xiao et al., 2001). One of these two proteins, RPW8.2, encoded by the two tandemly linked homologous genes in this locus, is an atypical resistance protein because it does not possess a canonical leucine rich repeat but instead possesses two coiled-coils and a transmembrane domain (Göllner et al., 2008; W. Wang et al., 2009; Shunyuan Xiao et al., 2005). RPW8.2 localizes to a membrane separating the haustorium from the plant cytoplasm, the extra-haustorial membrane (EHM). The ectopic expression of RPW8.2 increases *Arabidopsis* post-invasive resistance via generation and accumulation of H<sub>2</sub>O<sub>2</sub> in the haustorium-complex and formation of the encasement of the haustorial complex (W. Wang et al., 2009). However, how RPW8.2 is activated and regulated to induce these defenses is unknown. This work explores the possibility of phosphorylation as a PTM essential for RPW8.2 functionality.

### ***Evolutionary Data Suggests T64 May be Phosphorylated***

The *RPW8* locus confers broad-spectrum resistance to powdery mildew species (Göllner et al., 2008; Jorgensen & Emerson, 2008; S Xiao et al., 2001). The

locus contains two homologous genes, *RPW8.1* and *RPW8.2*, both of which act in a dominant or semi-dominant manner and are highly polymorphic among different *Arabidopsis* accessions (Xiao et al., 2001; Orgil et al. 2007). Analysis of intraspecific allelic polymorphism revealed that two sites, i.e. amino acid positions 64 and 116, in *RPW8.2* from 51 different *Arabidopsis* accessions are statistically associated with resistance to *Gc* UCSC1 an adapted powdery mildew pathogen (Figure 2-1) (Orgil et al., 2007).



### **Figure 2-1 Allelic Diversity of RPW8.2 in Ten Arabidopsis Ecotypes and Resistance to Powdery Mildew**

Orgil et al.(Orgil et al., 2007) surveyed 51 Arabidopsis ecotypes. In this modified figure, nine of ten Arabidopsis ecotypes show resistance(R). Can-0 displays intermediate resistance (I) with two polymorphisms at positions 190 and 475. Dots indicate identical nucleotides and dashes represent gaps. Intronic substitutions are shaded. Vertically-written numbers at the top indicate the nucleotide position relative to the start codon of the Ms-0 allele. Amino acid replacements caused by nucleotide substitutions are indicated at the bottom. Arrowheads depict significant nonsynonymous substitutions statistically correlated with disease resistance phenotype. “Accession” is the Arabidopsis ecotype and protein category groups polymorphic proteins according to the most closely related protein. For instance, the RPW8.2 amino acid sequence in accession “Ei-5” is most closely related to the RPW8.2 sequence from MS-0. Thus, this is one group, the first group labeled “(1)”.

The two T64S and D116G substitutions are found in 34 Arabidopsis accessions of which 27 displayed intermediate resistance or susceptibility compared to the resistant accession Ms-0 from which the RPW8 locus was characterized (Orgil et al., 2007). The mutation from threonine to serine at residue 64 suggests the increased susceptibility may result from inhibition or augmentation of phosphorylation at site T64.

## ***Results***

### ***Expression of $RPW8.2^{T64S}$ accelerate spontaneous HR-like cell death (SHL)***

To assess if the T64S substitution indeed is important for  $RPW8.2$ 's functionality both in terms of defense activation and protein localization,  $RPW8.2$  alleles carrying a T64A, T64E or T64S substitution were fused with YFP at the C-terminus, and the fusion genes were expressed from the  $RPW8.2$  native promoter in Col-0, a mildew-susceptible accession in which the  $RPW8$  locus is absent (Xiao et al., 2001). Because alanine is never phosphorylated it is frequently used to test a putative phosphorylation site (Sun, Enslin, Myung, & Maurer, 1994; Traenckner et al., 1995). Serine is phosphorylatable but at this position may not be modified due to nonrecognition by its cognate kinase. Glutamate is a phosphomimetic and closely mimics the structure and charge at neutral pH (Dephoure, Gould, Gygi, & Kellogg, 2013).

A population of T1 transgenic lines for each of the  $RPW8.2$  variant alleles, along with the wild-type (wt) allele (from accession Ms-0) were phenotypically examined. First, spontaneous HR-like lesions (SHL), which indicates constitutive defense activation, were found in ~2.4% of T1 lines expressing the  $RPW8.2$  wt allele; by contrast, 37% and 47% of T1 lines respectively expressing  $RPW8.2^{T64S}$  and  $RPW8.2^{T64A}$ , were found to have developed SHL (Table 1). This result indicates that  $RPW8.2^{T64S}$  has similar functional consequence as  $RPW8.2^{T64A}$ , implying that serine

at T64 cannot be phosphorylated. This is in an agreement with the prediction from NetPhos2 (see later text).

**Table 1 Resistance and Cell Death Phenotypes for Four RPW8.2 Mutants Compared to Wildtype**

Mutant	Before Inoculation		After Inoculation	
	SHL Lines/Total	SHL(%)	R+HR Lines/Total	R+HR(%)
R82 <sup>MS-0</sup>	3/124	2.4	28/110	25.5
R82 <sup>D116G</sup>	0/121	0	3/121	2.5
R82 <sup>T64S</sup>	43/116	37.1	59/114	51.8
R82 <sup>T64A</sup>	21/45	46.7	24/45	53.3
R82 <sup>T64E</sup>	13/119	10.9	22/104	21.2

Consistent with this, only 11% of T1 lines expressing RPW8.2<sup>T64E</sup> showed SHL.

Combined, these results suggest that phosphorylation at T64 may negatively regulate RPW8.2's function in activating defense and cell death in the absence of any pathogen and that the RPW8.2<sup>T64S</sup> substitution probably abolishes this capacity.

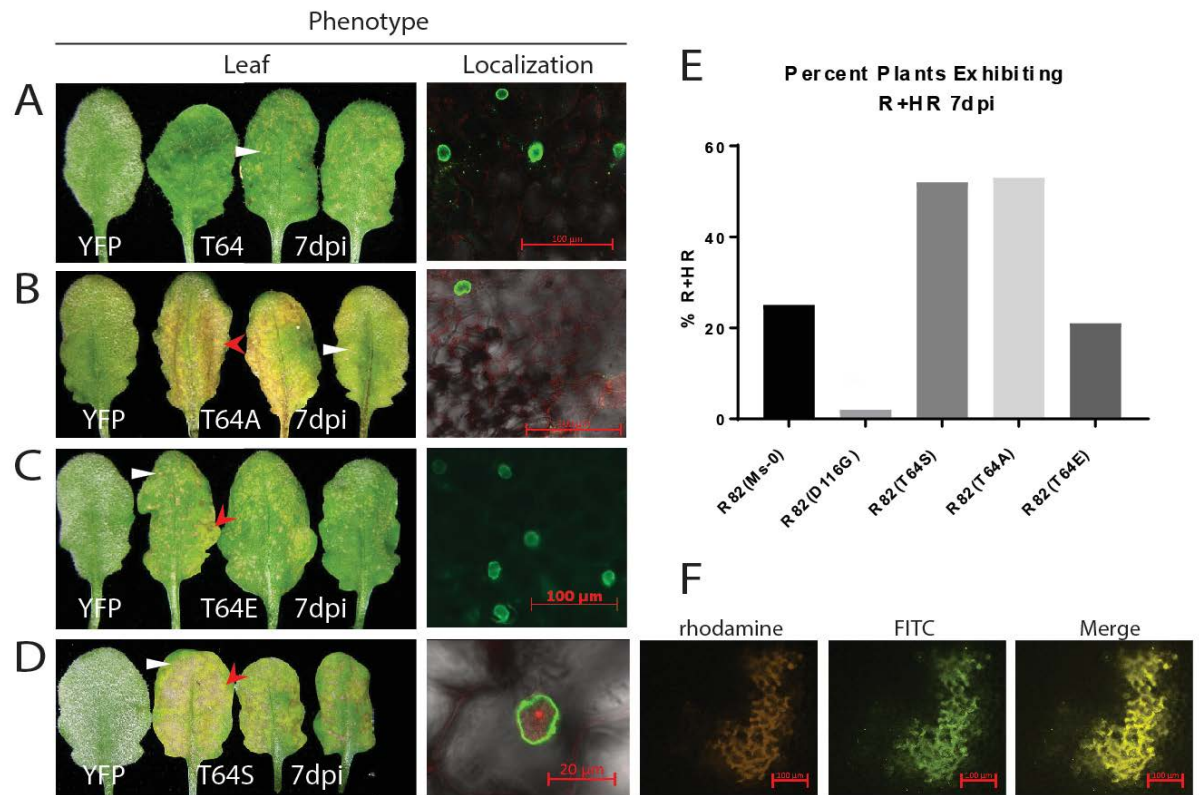
Second, the same T1 populations were inoculated with spores of *Gc* UCSC1, and examined for the development of hypersensitive response (HR) and resistance (R) (Figure 2-2A-D). The HR is a defense response manifested as localized programmed cell death at the site of infection that may prevent spread of the pathogen and is therefore often correlated with resistance to pathogens (Coll et al., 2011)(Hammond-Kosack & Jones, 1997).

RPW8-mediated resistance to powdery mildew features with a typical HR that is preceded by production and accumulation of ROS (such as H<sub>2</sub>O<sub>2</sub>) in the invaded epidermal cells (Xiao et al., 2003, 2005). Interestingly, the frequency of HR and resistance in the T1 lines expressing each of the four *RPW8.2* alleles correlated that of

the SHL (Table 1, Figure 2A-D), supporting the speculation about the functional consequences of the three substitutions at the T64 site.

Mutations at T64 did not change the localization pattern of RPW8.2. Similar to wildtype, all T64 mutant proteins were found to be correctly targeted to the EHM. YFP-expressed leaves were included as susceptible controls, which showed no signs of HR or resistance. HR is twice as frequently observed in RPW8.2<sup>T64A</sup> and RPW8.2<sup>T64S</sup> T1 lines compared to WT and the phosphomimetic RPW8.2<sup>T64E</sup> (Figure 2-2E). Phenotypic data from plants expressing RPW8.2<sup>D116G</sup> are included in the figure as a susceptible comparison. Microscopically, HR manifests as autofluorescing foci as shown here with a representative sample from plants-expressing RPW8.2<sup>T64A</sup> (Figure 2-2F)(Yu, Parker, & Bent, 1998).

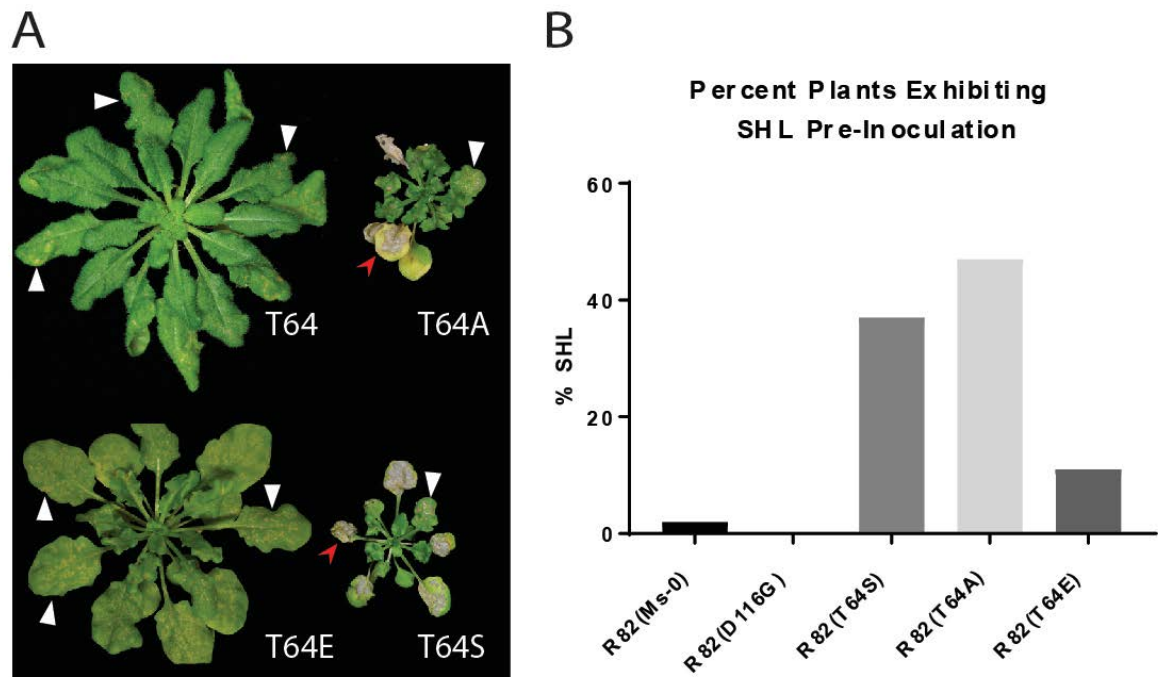
Taken together, the genetic and phenotypic data suggest the threonine at position 64 is phosphorylated and this phosphorylation is important for resistance vis-à-vis powdery mildew, consistent with the inference from a previous study on the intraspecific polymorphism at *RPW8.2* (Orgil et al., 2007).



**Figure 2-2 Increased Hypersensitive Response in Three RPW8.2 T64 Mutants**

(A) Resistant RPW8.2 wildtype leaves display HR (white arrowheads) 7 days post inoculation (dpi) with *Gc* UCSC1. Leaves expressing RPW8.2wt are resistant (R) to *Gc* UCSC1, but leaves expressing YFP show susceptibility. The localization of YFP-tagged RPW8.2 is to the plant-haustoria interface, the EHM. (B-D) Plants expressing RPW8.2<sup>T64A</sup>, RPW8.2<sup>T64E</sup>, or RPW8.2<sup>T64S</sup> also show HR and necrotic cell death (red arrowheads) and appear mostly resistant to *Gc* UCSC1 similar to wildtype. All mutant proteins were correctly targeted to the EHM, as shown by a representative confocal image concerning the localization of RPW8.2<sup>T64S</sup> in cells invaded by haustoria stained by propidium iodide (PI) 7dpi after inoculation. These percentages include plants identified with SHL in Figure 2-3

wildtype and phosphomimetic T64E mutants. (E) Table 1 data in columnar form displaying R and HR in T1 lines. (F) Merged fluorescence in two filters (rhodamine and FITC) is used to verify autofluorescence indicative of cell death in leaves expressing RPW8.2<sup>T64A</sup>. Scale bar distances indicated in each figure.



**Figure 2-3 Three RPW8.2 T64 Mutants Show Increased SHL**

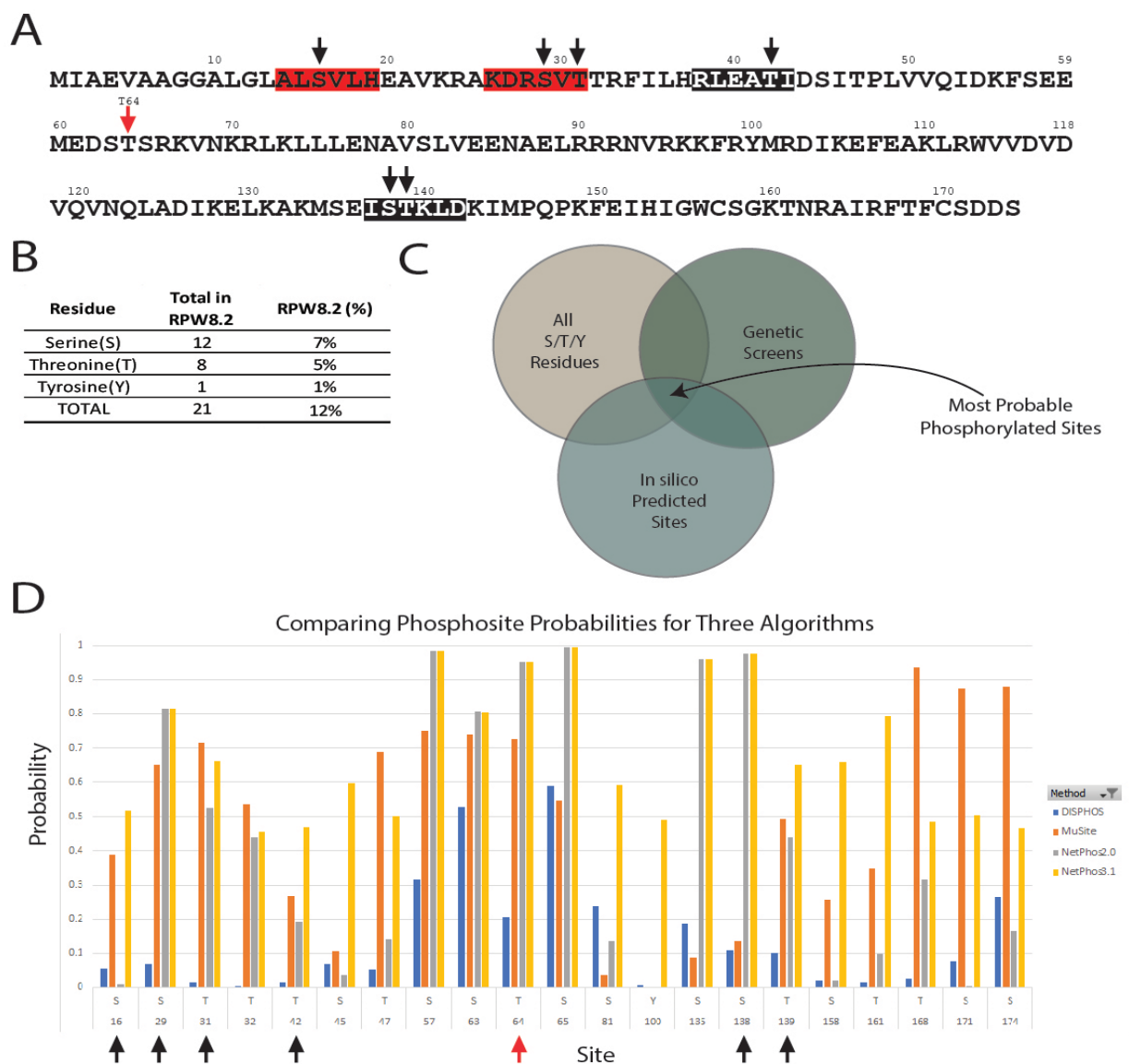
(A) SHL lesions (white arrowheads), an indication of protein toxicity, are evident on leaves of unchallenged plants. SHL is observed in RPW8.2 wildtype and mutant plants. Red arrows indicate necrotic cell death in mutants T64A and T64S. Both of these mutants display the SHL-phenotype with greater frequency in T1 populations. (B) The percentage of plants displaying SHL in the T1 generation, which are a visual presentation of the data shown in Table 1.



### ***NAAIRS-Replacement Mutagenesis Suggests Other Sites May Be Phosphorylated***

A NAAIRS-replacement mutagenesis was tiled across RPW8.2 to discover functional residues important for EHM targeting. The six-residue NAAIRS motif is frequently found in  $\alpha$ -helices and  $\beta$ -sheets and has been used to replace the indigenous residues of a protein for functional analysis. NAAIRS-replacement mutants minimize conformational changes while revealing functional residues (Marsilio, Cheng, Schaffhausen, Paucha, & Livingston, 1991). Plants transgenic for a mutated NAAIRS site containing RPW8.2(T64) showed normal EHM localization consistent with results from site-directed mutagenesis with T64.

However, no EHM localization or fluorescence was observed in other transgenic plants expressing NAAIRS-replacement RPW8.2 alleles containing potentially phosphorylated sites like T42, S138 and T139 (Figure 2-4A) (Wang, et al., 2013). Undetectable YFP fluorescence at the EHM or anywhere in the haustorium-invaded cells suggests that mutations at these sites may contribute to protein instability or degradation perhaps through phosphorylation or other mechanisms. Missense mutations and phosphorylation abnormalities are known to accelerate protein degradation (PJ, 2001). Thus, the NAAIRS study may suggest other functionally relevant, potentially phosphorylated RPW8.2 residues for further analysis.



**Figure 2-4 Scanning RPW8.2 for Probable Multisite Phosphorylation**

(A) NAAIRS mutagenesis tiled across RPW8.2 reveals two sites without any detectable fluorescence (black boxes) and two sites with significant reduction in EHM localization (red boxes). The red arrow indicates the position of T64. Black arrows indicate potentially phosphorylatable residues within NAAIRS mutants. (B) Twelve serine and eight threonine residues account for 12% of 174aa in RPW8.2. (C) Most probable sites for RPW8.2

phosphorylation are discovered taking into account several approaches including in silico prediction and previous polymorphism studies and genetic screens e.g. NAAIRS. (D) The RPW8.2 amino acid sequence was subjected to three phosphorylation prediction algorithms, DISPHOS, MuSite and NetPhos (two versions). Probability predictions were compared to each other at each site. Arrows mean the same as in (A).

### ***In Silico Analysis of RPW8.2 Candidate Phosphorylation Sites***

Natural polymorphism-guided mutagenesis and NAAIRS replacement suggest that RPW8.2 might be regulated through (de)phosphorylation. There are twelve serine and eight threonine residues in RPW8.2, accounting for about 12% of the total amino acids (Figure 2-4B). Previous genetic screens and phosphorylation predictive algorithms were invoked to narrow down likely candidates (Figure 2-4C) as computational methods can inform and direct biological hypotheses (Brodland, 2015). To understand which site or if multiple sites could potentially be phosphorylated in RPW8.2, I used three prediction algorithms: NetPhos, Musite, and DISPHOS.

NetPhos uses a neural network or machine learning to predict probable phosphorylation sites using proteins cataloged in large-scale phosphoproteomic databases.(Blom, Gammeltoft, & Brunak, 1999) Two NetPhos versions were available. NetPhos 3.1 adds kinase-specific predictions to NetPhos 2.0's generic predictions. Musite's algorithm utilizes a training set composed of several large scale phosphorylation studies from multiple organisms (Gao, Thelen, Dunker, & Xu, 2010). DISPHOS uses protein ordered and disordered regions to help differentiate between phosphorylatable and non-phosphorylatable sites (Iakoucheva et al., 2004). The probabilities for each site were stratified by algorithm (Figure 2-4D). All twenty-one phosphorylatable amino acids are represented in the prediction algorithms. That is, every site had some probability of being phosphorylated.

Tyrosine phosphorylation was excluded from analysis as it is rarely phosphorylated in planta (Hrabak et al., 2003). The probabilities were summed and ranked highest to lowest (Table 2).

A probability equal to 4 ( $p=4$ ) represents a 100% probability of phosphorylation predicted from all four algorithms. Bias exists in the sum as two different versions of NetPhos were included in the results. The top eight sites were selected for site-directed mutagenesis. Six residues are serine. Two are threonine. All sites were mutated to alanine.

Table 2 RPW8.2 Phosphorylation Probabilities from Three Prediction Sites

Number	Residue	Probabilities				$\Sigma$ (Prob)
		DISPHOS	MuSite	NetPhos2.0	NetPhos3.1	
1	65S	0.588	0.5467	0.995	0.995	3.12
2	S57	0.317	0.7521	0.985	0.985	3.04
3	63S	0.528	0.7393	0.805	0.804	2.88
4	64T	0.206	0.7254	0.95	0.95	2.83
5	29S	0.069	0.6514	0.814	0.814	2.35
6	138S	0.109	0.1365	0.975	0.975	2.20
7	135S	0.187	0.0879	0.959	0.959	2.19
8	31T	0.014	0.7133	0.525	0.662	1.91

***RPW8.2 Containing Seven S/T-Site Mutation Appears to Be Dominant Negative***

Plant immune proteins can be multiply phosphorylated in response to pathogens (Benschop et al., 2007). The possibility of multiple RPW8.2 phosphorylation sites were also investigated. Using the data from Table 2, an RPW8.2 mutant was created in which serine or threonine residues were mutated to alanine at seven sites. RPW8.2<sup>T64</sup> was not mutated. The RPW8.2 mutant was in-frame fused with YFP at the C-terminus (the fusion gene is named MUT7) and stably expressed from the *RPW8.2* native promoter in Arabidopsis accession “Col-0” containing the glabrous mutation (*gl*) (i.e. Col-*gl*). One homozygous line transgenic for RPW8.2(wt)-YFP (named R2Y4 as in (W. Wang et al., 2009)) was used as control (Figure 2-5A). (Note, R2Y4 is also depicted as “R82(MS-0)” in Table 1). R2Y4 was

compared to T1 lines transgenic for MUT7 (Figure 2-5B). MUT7 leaves showed no abnormalities in growth and development (Figure 2-5C) compared to Col-*gl* wt plants, whereas leaves of transgenic plants expressing RPW8.2<sup>T64A</sup> showed diminished size, chlorosis and necrosis (Figure 2-3A). MUT7 T1 leaves showed no difference in resistance compared to R2Y4.

MUT7 was also introduced into a Col-*gl* transgenic line named S5 line. S5 is transgenic for a genomic fragment containing *RPW8.1* and *RPW8.2* under control of their native promoters (Shunyuan Xiao et al., 2005). This homozygous line exhibits stable, broad-spectrum resistance to powdery mildew.

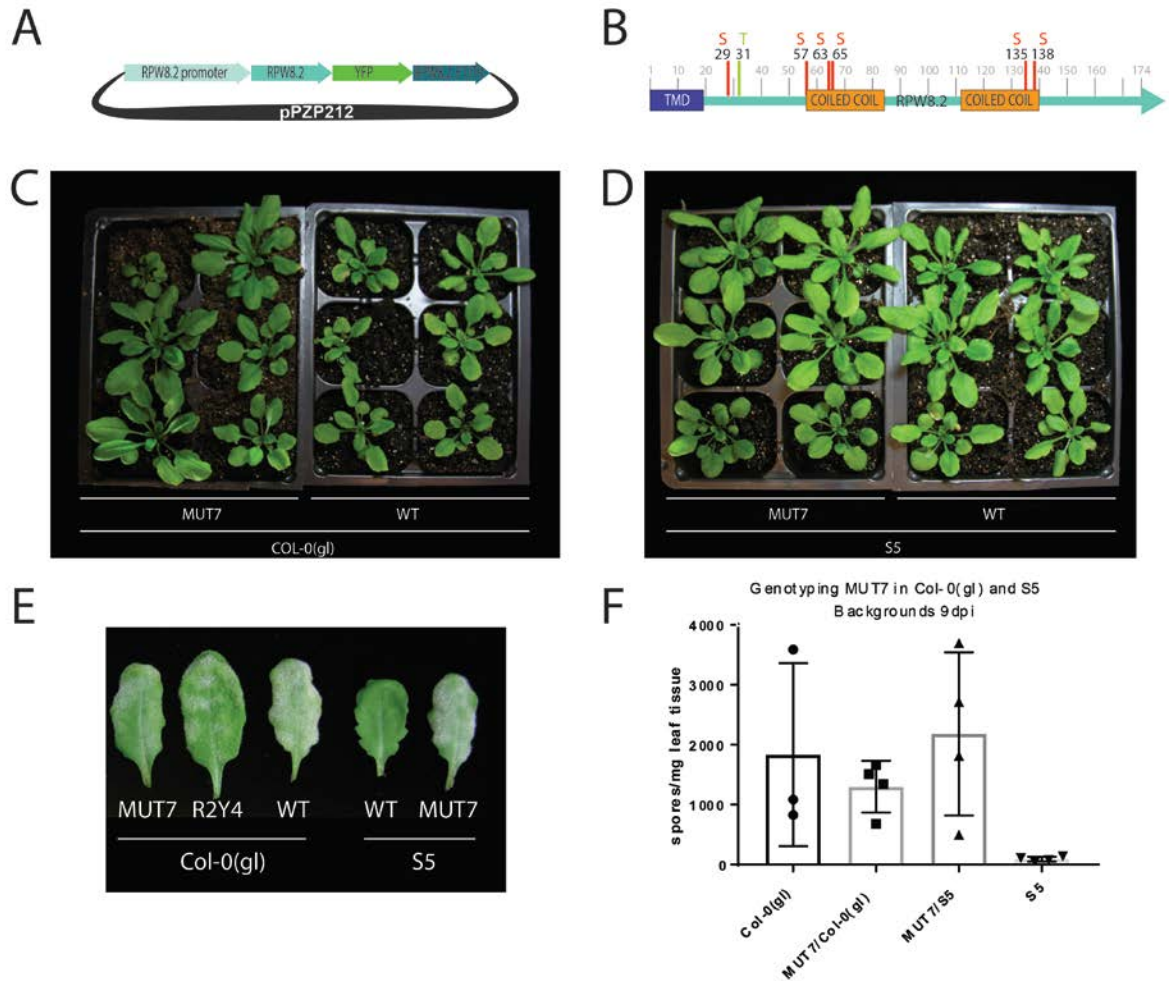
MUT7 T1 lines in S5 background displayed no SHL in the absence of powdery mildew inoculation. Remarkable, at 9dpi, these same lines showed significant susceptibility comparable to untransformed Col-0(*gl*) (Figure 2-5E-F), indicating loss-of-function for the *RPW8.2* wt gene. This result suggests that MUT7 is not only unable to activate defense but also can exert a dominant negative effect over the wt RPW8.2 and possibly wt RPW8.1 proteins, resulting in loss of resistance in S5 transgenic plants.

How MUT7 exerts a dominant negative effect over the functional RPW8 proteins is unknown. In this particular case, an apparent reason may be the absence of key phosphorylation sites in RPW8.2 that are essential for its functions. Two functional arms have been characterized for RPW8.2; these are (i) activation of SA-dependent defense responses, and (ii) mobilization of such defenses to the host-pathogen interface through localization of RPW8.2 to the EHM. Obviously, MUT7

fails to activate defense, which was evidenced by the observation that none of the twelve T1 transgenic plants was resistant to *Gc* UCSC1 (Figure 2-5E). However, whether the YFP-tagged RPW8.2 mutant protein is correctly targeted to the EHM is not known. Both *Col-gl* lines transgenic for MUT7 and S5 lines transgenic for MUT7 were used for localization analysis. Since the wt RPW8 proteins in S5 are not fluorescently labeled, all YFP fluorescence detectable at the EHM and/or elsewhere in this background must derive exclusively from MUT7. At 9dpi, leaves of T1 MUT7 transgenic plants inoculated with *Gc* UCSC1 were examined using confocal microscopy.

RPW8.2wt-YFP is mostly found in the EHM with occasional punctate distribution in the invaded epidermal cells especially when overexpressed, but is never found in the plasma membrane (PM) (W. Wang et al., 2009). Interestingly, in both S5 and *Col-gl* backgrounds, apart from typical EHM localization, MUT7 was also found in the PM, which is in a sharp contrast to the RPW8.2wt-YFP control (Figure 2-6A-B). This result suggests that lack of proper phosphorylation not only abrogates RPW8.2's ability to activate defense but also affects its EHM-targeting. It is conceivable that non-functional proteins loaded into the same vesicles may negatively impact the transport of the functional residents. Indeed, a YFP-tagged truncated RPW8.2 fusion protein exerts dominant negative effect on RPW8.2wt-YFP and render S5 susceptible to *Gc* UCSC1 (Q. Zhang et al., 2015). Taken together, these results indicate that one single or multiple mutations contained in MUT7 disrupt(s) the localization of RPW8.2 from the EHM to the PM.

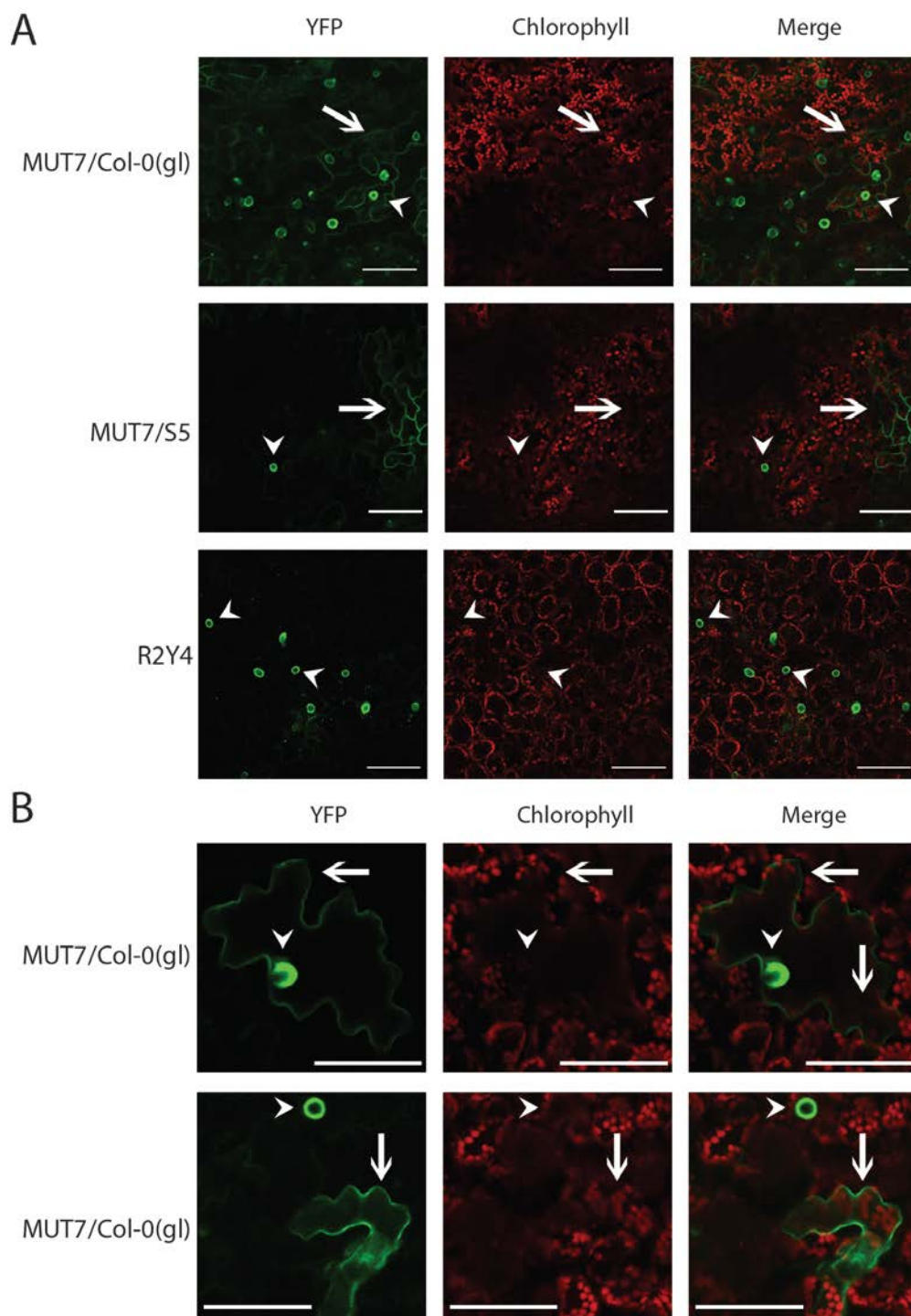




**Figure 2-5 Phenotyping Plants Expressing RPW8.2 "MUT7" Pre- and Post-Inoculation**

(A) The *RPW8.2* genomic sequence with 1262bp of its native promoter and 216bp of its 3' untranslated region (UTR) was in-frame fused with YFP at the C-terminus. The fusion gene was cloned into vector pPZP212. (B) *RPW8.2* in the fusion construct "MUT7" contains Ala mutations for seven most likely phosphorylated Ser or Thr as indicated. (C) T1 lines of *Col-0(gl)* transgenic for MUT7 showed no phenotypic differences compared to wildtype at 8 weeks before inoculation. (D) Similarly, no difference in growth phenotypes were observed for MUT7 T1 lines in the S5 background which contain a single copy of both *RPW8.1* and

*RPW8.2* compared with S5 alone. (E) At 7dpi MUT7 T1 lines were moderately susceptible, whereas T5 generation plants of R2Y4 (expressing *RPW8.2wt-YFP*) were moderately resistant (Note, T5 plants of R2Y4 were not as resistant as T1 plants due to transgene silencing). Similarly, MUT7/S5 lines were more susceptible than S5. (F) Spore counts demonstrate MUT7/S5 background is more susceptible to powdery mildew than S5 alone.



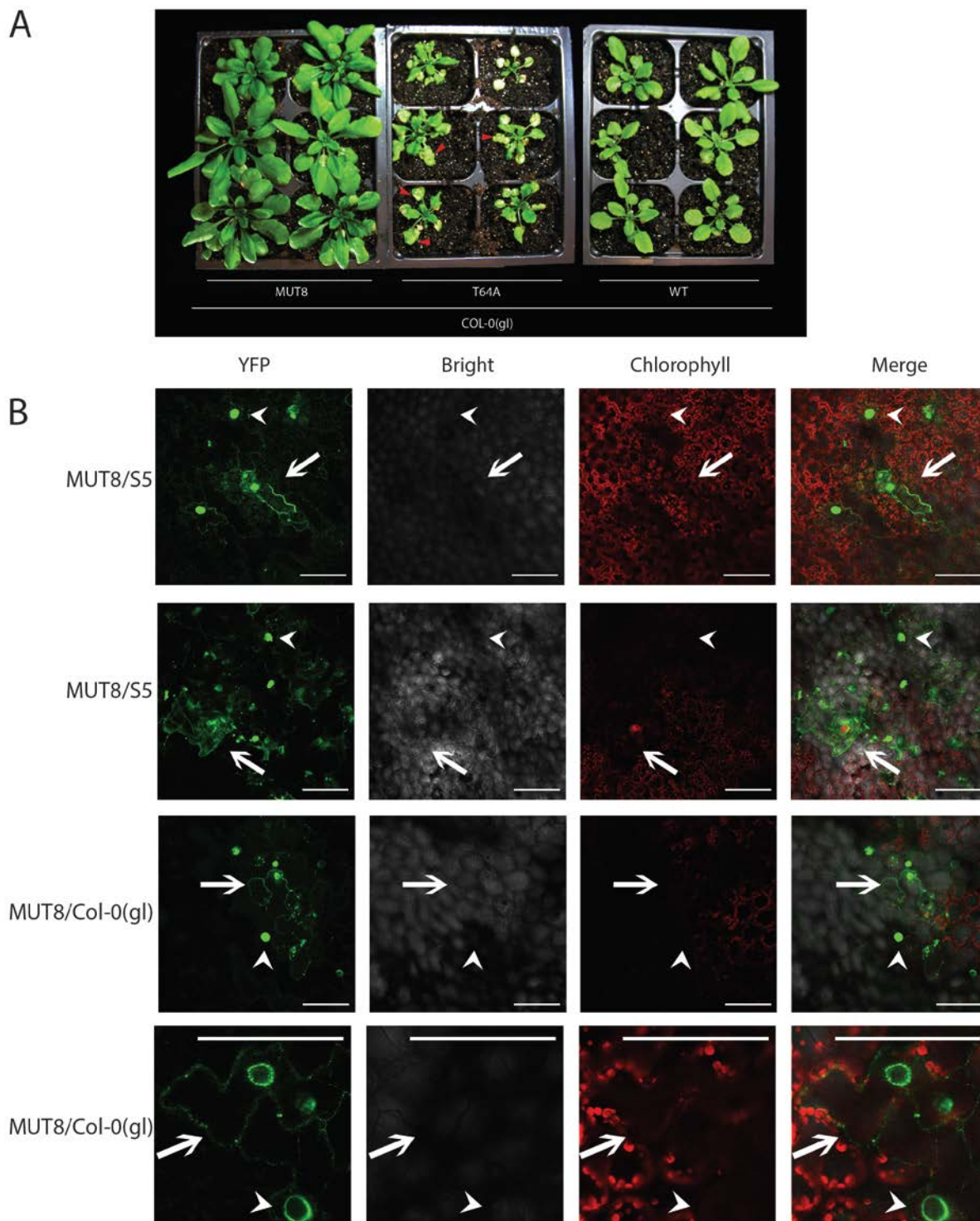
**Figure 2-6 Confocal Microscopy Showing PM Localization of MUT7 in Col-0(gl) and S5 Backgrounds**

(A) Representative confocal images showing that while the MUT7 RPW8.2 mutant protein was found in the EHM (white arrowhead), it was also detected in the plasma membrane (white arrow). Scale bars are 100 $\mu$ m. (B) Closeup images showing both EHM-localization and PM-localization of MUT7 in the same or neighboring cells. Scale bars are 50 $\mu$ m.

### ***MUT8-Containing T64A Is Mistargeted to the PM and Does Not Activate Cell Death***

RPW8.2<sup>T64A</sup> (or RPW8.2<sup>T64S</sup>) appear to be super-active in activation of cell death and defense, whereas seven Ser/Thr to Ala mutations in MUT7 render RPW8.2 nonfunctional (unable to activate HR). To determine if any of the seven phosphorylation sites may be required for T64A- or T64S-conferred cell death activity, an RPW8.2 variant containing all eight Ala mutations including T64A was made and in-frame fused with YFP (named MUT8) (Figure 5B).

Typically, transformed plants expressing the T64A mutation are diminutive with focal lesions of necrosis and SHL (Figure 2-3A). By contrast, none of the twenty-four T1 lines transgenic for MUT8 appear phenotypically indistinguishable from those transgenic for RPW8.2wt in the absence of powdery mildew infection (Figure 2-7A), indicating one or more of the seven Ser/Thr to Ala mutations abolished T64A-mediated SHL and reduced plant stature. Subcellular localization of MUT8 in cells invaded by powdery mildew was then examined. Intriguingly, while the mutant protein showed typical localization to the EHM in some cells, it was also mis-targeted to the PM (Figure 2-7B) in both Col-0(gl) and S5 backgrounds. These observations suggest that (i) RPW8.2 needs to be phosphorylated at T64 to prevent it from being super-active, (ii) activation of RPW8.2 by dephosphorylation of T64 or non-phosphorylation (e.g. in the case of T64S) requires phosphorylation of one or more Ser/Thr residues in RPW8.2.



**Figure 2-7 MUT8 Displays Both EHM and PM Localization**

(A) T1 Arabidopsis plants transformed with the MUT8 construct appear similar to untransformed WT, Col-0(gl). MUT8 plants have average stature and do not have SHL or necrosis (red arrows) despite containing the same T64A mutation. (B) Multiple images show that after 9dpi with powdery mildew MUT8 localizes to the EHM (arrowhead) and to the PM (arrow) in both S5 and Col-0(gl) backgrounds.

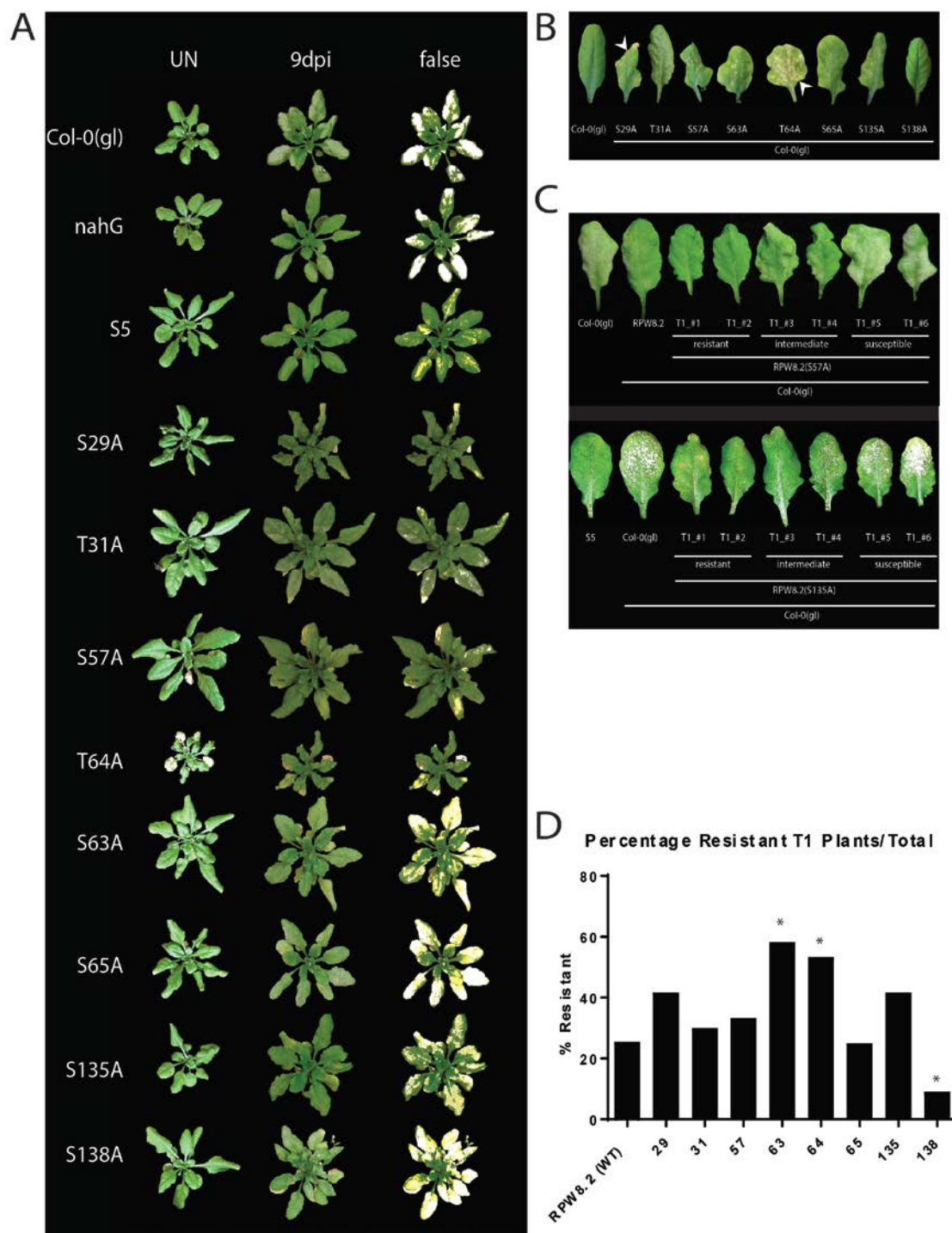
### ***S138 plays an important role for RPW8.2 defense function and EHM-targeting***

To further identify which Ser/Thr residue(s) among the seven assayed sites is required for T64A-mediated cell death and defense activation, seven RPW8.2 mutants containing a single Ala mutation in each of the seven Ser/Thr sites were made and in-frame fused to YFP and stably expressed from the *RPW8.2* native promoter in *Col-gl*. Eight-week-old T1 plants were inoculated with *Gc* UCSC1 and examined for disease infection phenotypes and subcellular localization of the respective RPW8.2-YFP proteins.

T1 transgenic plants from a population of 12-24 for each construct were photographed before and after inoculation of *Gc* UCSC1 (Figure 2-8A). At 9 dpi all mutants but one, RPW8.2<sup>S138A</sup>, showed comparable resistance or increased resistance to RPW8.2wt-YFP. Specifically, T1 lines of seven mutants RPW8.2<sup>(S29A, T31A, S57A, S63A, T64A, S65A, or S135A)</sup> displayed SHL in the absence of any pathogens with a frequency between 0-10% which is comparable to RPW8.2wt-YFP (Figure 2-8B). Resistance to *Gc* UCSC1 varied among individual T1 lines of the same population and among different populations, conforming with the notion that RPW8.2 is semi-dominant and its mediated resistance is dosage dependent (Shunyuan Xiao et al., 2005). Such variations are illustrated by leaves from T1 lines transgenic for RPW8.2<sup>S57A</sup> and RPW8.2<sup>S135A</sup> (Figure 2-8C). . As shown in Figure 8D, except for RPW8.2<sup>S138A</sup>, all other RPW8.2 mutant constructs containing a single Ser/Thr to Ala mutation showed either similar or even better resistance to *Gc* UCSC1 compared to RPW8.2wt.



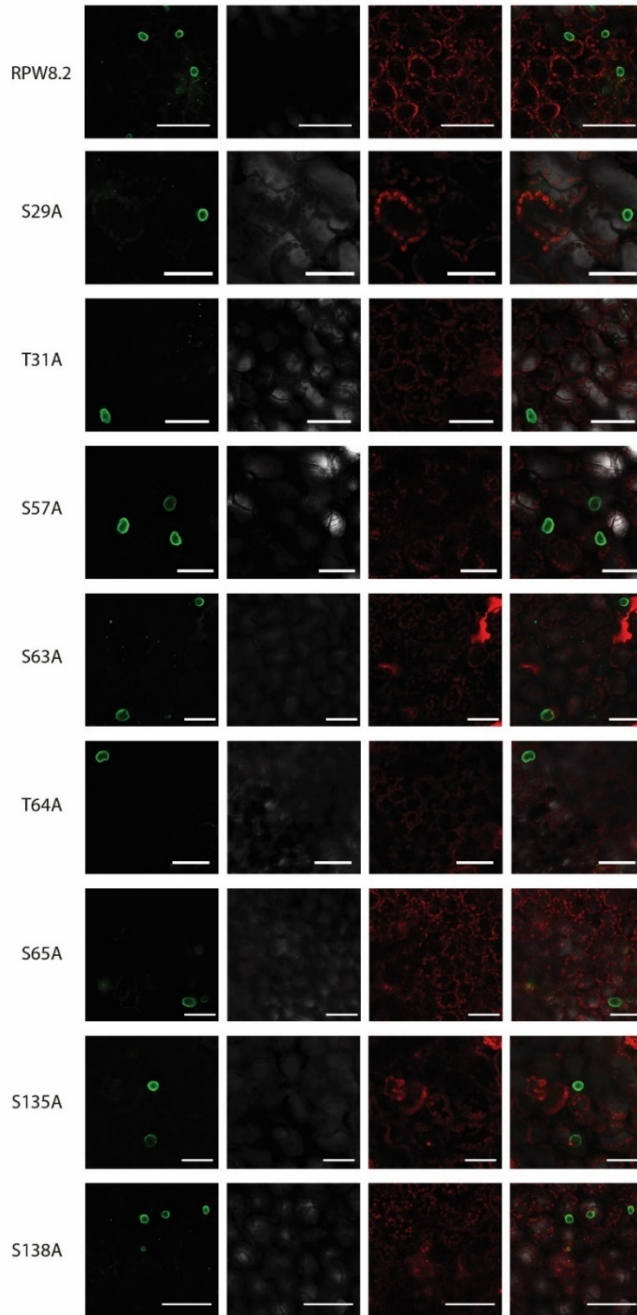
P-values refute the null hypothesis claiming no difference in resistance between wildtype and mutant phenotypes using a one-tailed binomial test (Wasserstein & Lazar, 2016). P-values less than 0.05 comparing wildtype with mutants are denoted with an asterisk. Based on the disease infection phenotypes and statistical data in Figure 8D, one may infer that while five single Ala mutations (29, 31, 57, 65, 135) had no significant impact on the disease resistance function of RPW8.2, the RPW8.2<sup>S63A</sup> and RPW8.2<sup>T64A</sup> single mutations each can enhance the resistance function of RPW8.2. By contrast, the RPW8.2<sup>S138A</sup> mutation appears to compromise the defense function of RPW8.2. This result suggests that RPW8.2<sup>S138A</sup> and its likely phosphorylation play an important role in RPW8.2's defense function.



**Figure 2-8 Functional assessment of the Seven Individual Ser/Thr mutations in RPW8.2**

(A) Uninoculated (UN) plants are compared to 9dpi (9dpi) inoculated T1 plants. The “false” column of 9dpi plants has been false colored to show powdery mildew (white) and cell death (yellow). (B) RPW8.2 defense functionally inferred by presence of SHL (arrowheads) in six T1 lines. No HR was apparent in RPW8.2<sup>S138A</sup> lines. (C) Leaves from T1 Arabidopsis plants with a single RPW8.2 mutation, RPW8.2<sup>S57A</sup>, and RPW8.2<sup>S135A</sup>, demonstrate spectrum of resistance from resistant leaves displaying HR to intermediate to susceptible. These leaves are from 9dpi Arabidopsis plants RPW8.2<sup>S57A</sup> and 15 dpi RPW8.2<sup>S135A</sup>. (D) Percentage of resistant lines from T1 populations. Significant resistance or susceptibility defined as  $p < 0.05$  was calculated using one-tailed binomial test.

RPW8.2's phosphorylation is hypothesized to impact its defense functionality, localization or both. In addition to resistance phenotype, EHM localization was also surveyed. All YFP-tagged RPW8.2 mutants with a single mutation showed normal EHM targeting, as evidenced by homogenous YFP signal in the EHM encasing haustoria (Figure 2-9). EHM-targeting efficiency may vary among these RPW8.2 mutant proteins, but this was not determined because the quality and age of haustoria and the expression level of the RPW8.2 mutant proteins all affect our ability to detect and quantify true YFP signal at the EHM.

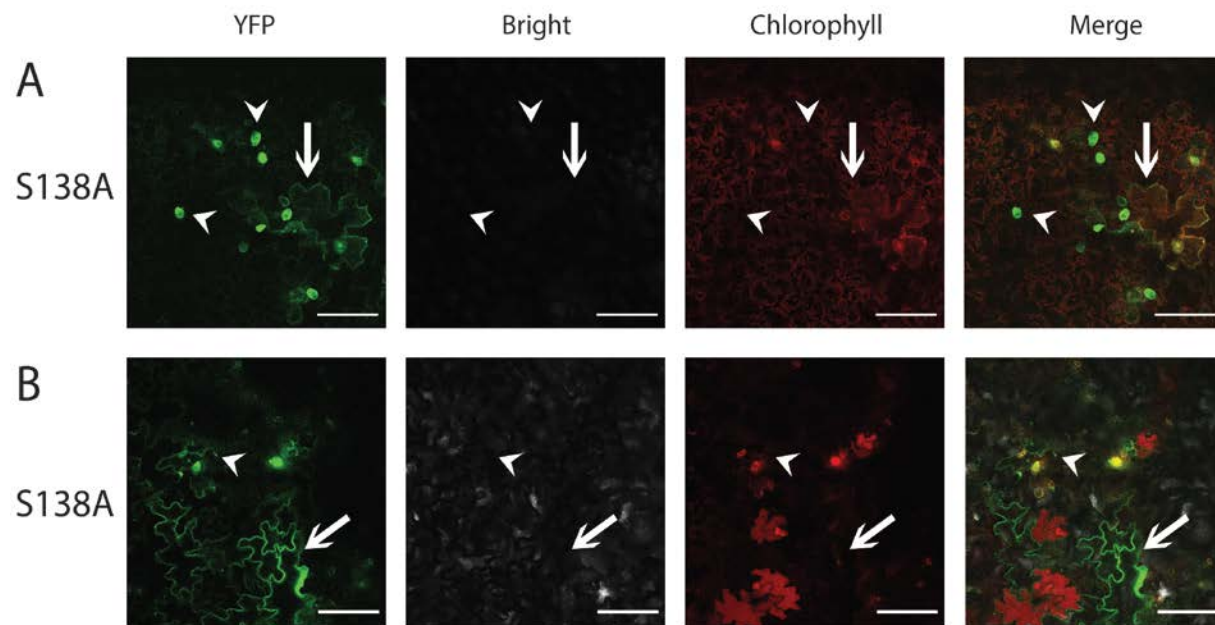


**Figure 2-9 Eight Rpw8.2 Mutants with A Single Ser/Thr Site Mutation Show Normal EHM Localization**

Individual mutants Rpw8.2(wt), Rpw8.2<sup>S29A</sup>, Rpw8.2<sup>T31A</sup>, Rpw8.2<sup>S57A</sup>, Rpw8.2<sup>S63A</sup>, Rpw8.2<sup>S65A</sup>, Rpw8.2<sup>S135</sup>, Rpw8.2<sup>S138</sup> (top to bottom) show normal EHM localization.

### ***RPW8.2<sup>S138</sup>-YFP Is Aberrantly Localized to the Plasma Membrane***

Despite showing normal localization to the EHM (Figure 2-10), RPW8.2<sup>S138A</sup>-YFP also appeared to be mis-targeted to the PM. Incorrect targeting of RPW8.2<sup>S138A</sup>-YFP may be attributable to this particular mutation or it may be due to overexpression of this mutant protein. However, given that (i) all the fusion constructs were expressed from the *RPW8.2* native promoter, and (ii) none of the remaining seven YFP-tagged RPW8.2 mutant proteins showed PM localization, and (iii) both MUT7 and MUT8 containing S138A were also found in the PM, it can be speculated that the S138A mutation somehow affects RPW8.2's EHM targeting, and consequently also compromises RPW8.2's ability to activate haustorium-targeted defenses.



**Figure 2-10 Localization of RWP8.2<sup>S138A</sup>-YFP to the EHM and the PM**

RPW8.2<sup>S138A</sup> shows typical localization to the EHM (arrowheads) and aberrant localization to the PM (arrow) after inoculation with *Gc* UCSC1. (B) Another image from the same line showing more PM localization (arrow) and punctate (arrowhead).

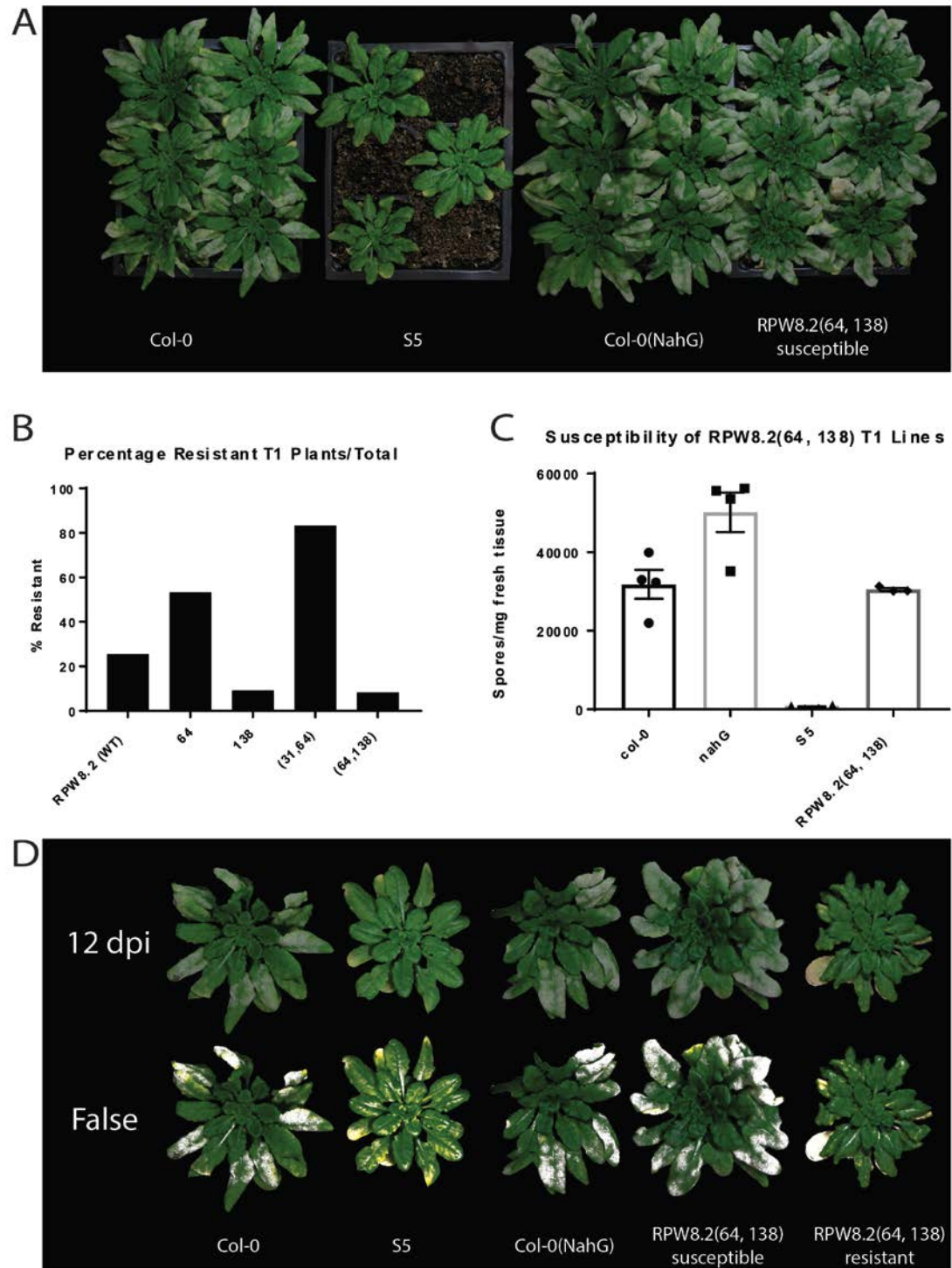
### ***S138A Suppresses T64A-Mediated Cell Death and Defense Activation***

The RPW8.2<sup>S138A</sup> mutation compromises RPW8.2's ability to activate resistance against powdery mildew not only in T1 plants expressing RPW8.2<sup>S138A</sup> but also in T1 plants expressing all the eight mutations including RPW8.2<sup>S64A</sup>. Given that expression of RPW8.2<sup>S64A</sup> activates massive SHL and enhances HR and resistance, one would speculate that RPW8.2<sup>S138A</sup> may exert an intragenic epistasis over RPW8.2<sup>T64A</sup>. To further test this, I created RPW8.2<sup>S64A/S138A</sup>-YFP and generated stable transgenic T1 plants. Unfortunately, disease data was not collected at this time, but will be in the near future. Disease tests showed that only 8.3% (3 of 36) of the T1 lines transgenic for RPW8.2<sup>S64A/S138A</sup>-YFP showed weak HR and resistance, whereas 26% (28 of 110) and 53% (24 of 45) of T1 plants respectively transgenic for RPW8.2wt-YFP and RPW8.2<sup>S64A</sup> displayed HR and resistance (Figure 2-11A and B).

To exclude the possibility that any other Ser to Ala mutation could also produce similar impact due to mutation additive effects (Parera & Martinez, 2014), a RPW8.2<sup>T31A/T64A</sup> double mutant was created as control to demonstrate that RPW8.2<sup>S138A</sup> is the functionally dominant mutation. As expected, T1 plants transgenic for RPW8.2<sup>T31A/T64A</sup>-YFP showed similar HR and resistance compared to those transgenic for RPW8.2-YFP (Figure 2-11B). To further evaluate disease resistance function of these mutant RPW8.2 proteins, number of spore per milligram of infected leaf tissue was counted at 14 dpi. The average spore count from four pooled leaf samples for T1 plants expressing RPW8.2<sup>S64A/S138A</sup>-YFP was more than 6x higher than that for resistant control S5. No significant difference was observed



between T1 plants expressing RPW8.2<sup>S64A/S138A</sup>-YFP and un-transformed control; however, the former were less susceptible than SA-pathway-defective susceptible Col-0(*nahG*) plants (Figure 2-11C). Only about 1% of the T1 plants transgenic for RPW8.2<sup>S64A/S138A</sup>-YFP exhibited some limited resistance (Figure 2-11D). Taken these data together, it can be concluded that RPW8.2<sup>S138A</sup> largely suppressed RPW8.2<sup>S64A</sup>-conditioned enhanced activity of RPW8.2.



**Figure 2-11 Susceptibility of Mutant RPW8.2(64, 138) to Powdery Mildew**

(A) The mutant RPW8.2<sup>T64A/S138A</sup> compared to Col-0, Col-0(*NahG*) and S5 (RPW8 locus) at 12dpi with powdery mildew *Gc* UCSC1. (B) Mutant RPW8.2<sup>T64A/S138A</sup> is less resistant (8%) compared to RPW8.2 wt (25%). Double mutant RPW8.2<sup>T31A/S138A</sup> is resistant while double mutant RPW8.2<sup>T64A/S138A</sup> is not, reflecting the functional impact of site RPW8.2<sup>S138A</sup>. This result is also corroborated by compromised resistance of the RPW8.2<sup>S138A</sup> single mutant. (C) Spore counts demonstrate fungal growth on RPW8.2<sup>T64A/S138A</sup> no different than Col-0 and 6x less resistant than S5 plants. (D) False colored imaging of inoculated plants show resistant plants like S5 demonstrate HR (yellow) while susceptible plants like Col-0 and most of the double mutant RPW8.2<sup>T64A/S138A</sup> T1 population support hyphal growth (white).

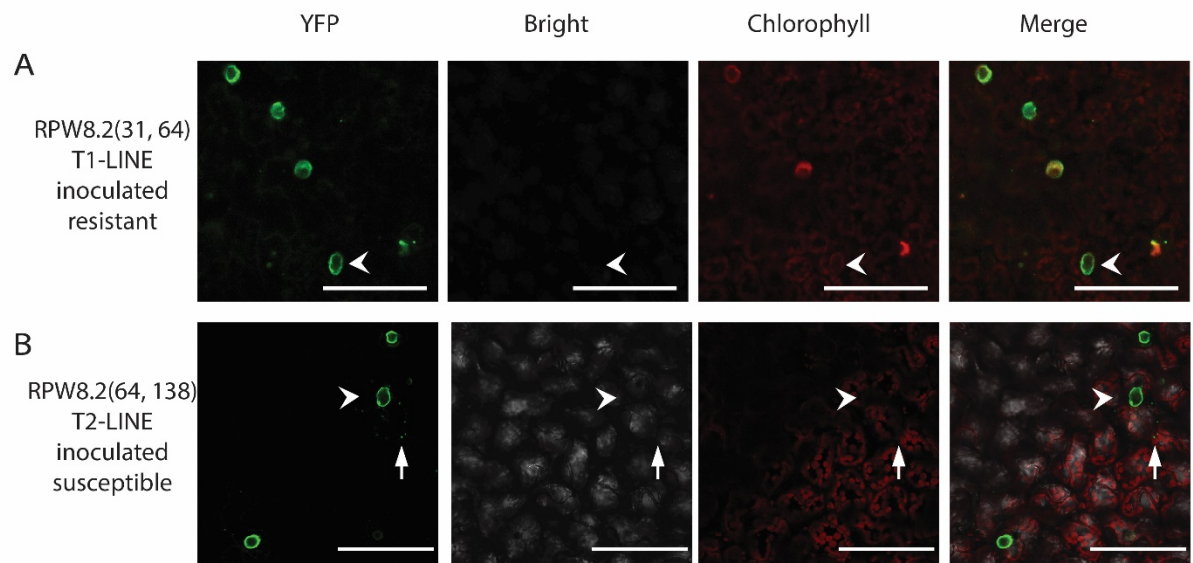
T1 lines expressing double mutants RPW8.2<sup>T31A/ T64A</sup> or RPW8.2<sup>T64A/ S138A</sup> displayed localization of the respective mutant proteins to the EHM (Figure 2-12A). T2 lines show similar EHM targeting for RPW8.2<sup>T64A/ S138A</sup> (Figure 2-12B). However, only the double mutant RPW8.2<sup>T64A/ S138A</sup> was found both in the EHM and in the PM. RPW8.2<sup>T64A/ S138A</sup> was observed in both susceptible and resistant T1 and T2 lines (Figure 2-13A-C). One resistant line displayed mesophyll localization in the same frame as EHM localization (Figure 2-13D), whereas a susceptible line displayed PM localization (Figure 2-13E). Taken together the data suggest that the RPW8.2<sup>S138A</sup> mutant protein results in abnormal fluorescence localization to the PM, and that this mislocalization is persistent in both susceptible and resistant T1 and T2 lines.

#### ***RPW8.2<sup>S64A/S138A</sup>-YFP Accumulates to Detectable Levels in Uninoculated Plants***

Arabidopsis plants transgenic for *RPW8.2-YFP* under control of the native *RPW8.2* promoter only accumulate RPW8.2-YFP to a level detectable by confocal microscopy in powdery mildew infected leaves. Interestingly, YFP signal was readily detectable in uninoculated T1 and T2 lines transgenic for RPW8.2<sup>S64A/S138A</sup>-YFP under control of the same native promoter (Figure 14A-B). This suggests that the mutant protein may be more stable compared to the wt protein. To further test this, a western blot was performed using the microsomal fraction of the total protein extracted from leaves of different genotypes (Figure 2-14C). As expected, RPW8.2-YFP was hardly detectable in uninoculated leaves but induced by ~2x in inoculated leaves (Figure 2-14D). This observation further suggests that the RPW8.2 wt protein

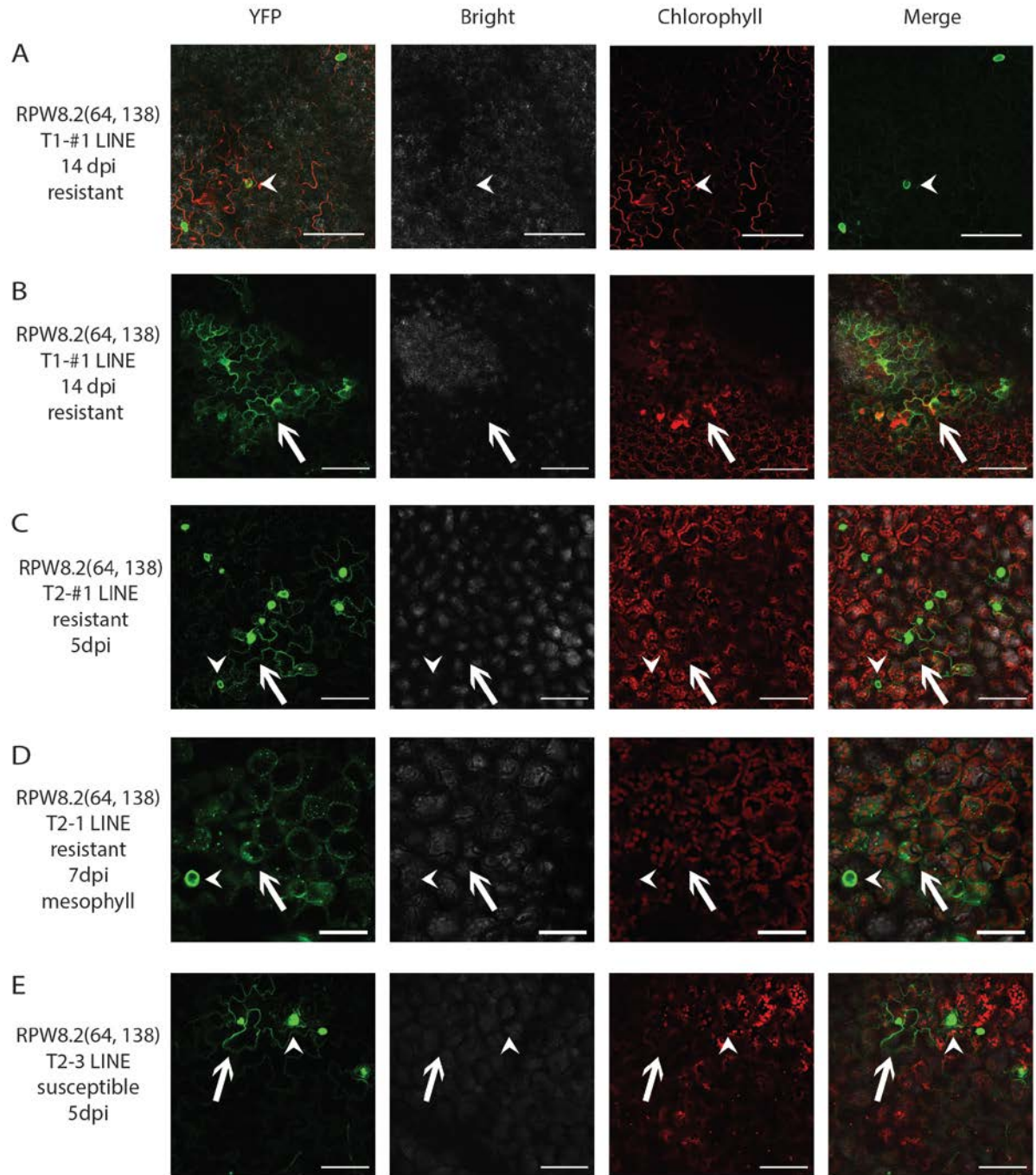
may be constitutively expressed but removed in the absence of any pathogen infection, however the RPW8.2<sup>S138A</sup> mutation renders the mutant protein more stable, hence the accumulation before inoculation. However, since the RPW8.2<sup>S138A</sup> mutant protein largely loses its defense function, there is little or no SA-dependent transcriptional amplification (as seen for the wt protein; (Shunyuan Xiao, Brown, et al., 2003)) for the mutant protein, and there may be even powdery mildew-induced degradation of the mutant (and wt) RPW8.2 protein, explaining the relatively higher level accumulation of RPW8.2<sup>S64A/S138A</sup>-YFP in uninfected plants.

Taken together, my results from site-directed mutational analysis on predicated phosphorylation sites in RPW8.2 demonstrate that at least two residues, i.e. RPW8.2<sup>T64A</sup> and RPW8.2<sup>S138A</sup>, play important roles in negative (RPW8.2<sup>T64A</sup>) and positive (RPW8.2<sup>S138A</sup>) regulation of RPW8.2, with the RPW8.2<sup>S138A</sup> mutation being dominant over RPW8.2<sup>T64A</sup>. These results further suggest that RPW8.2 is most likely phosphorylated and regulated by a kinase(s) and/or a phosphatase(s).



**Figure 2-12 RPW8.2 Double Mutants Are Correctly Localized to the EHM**

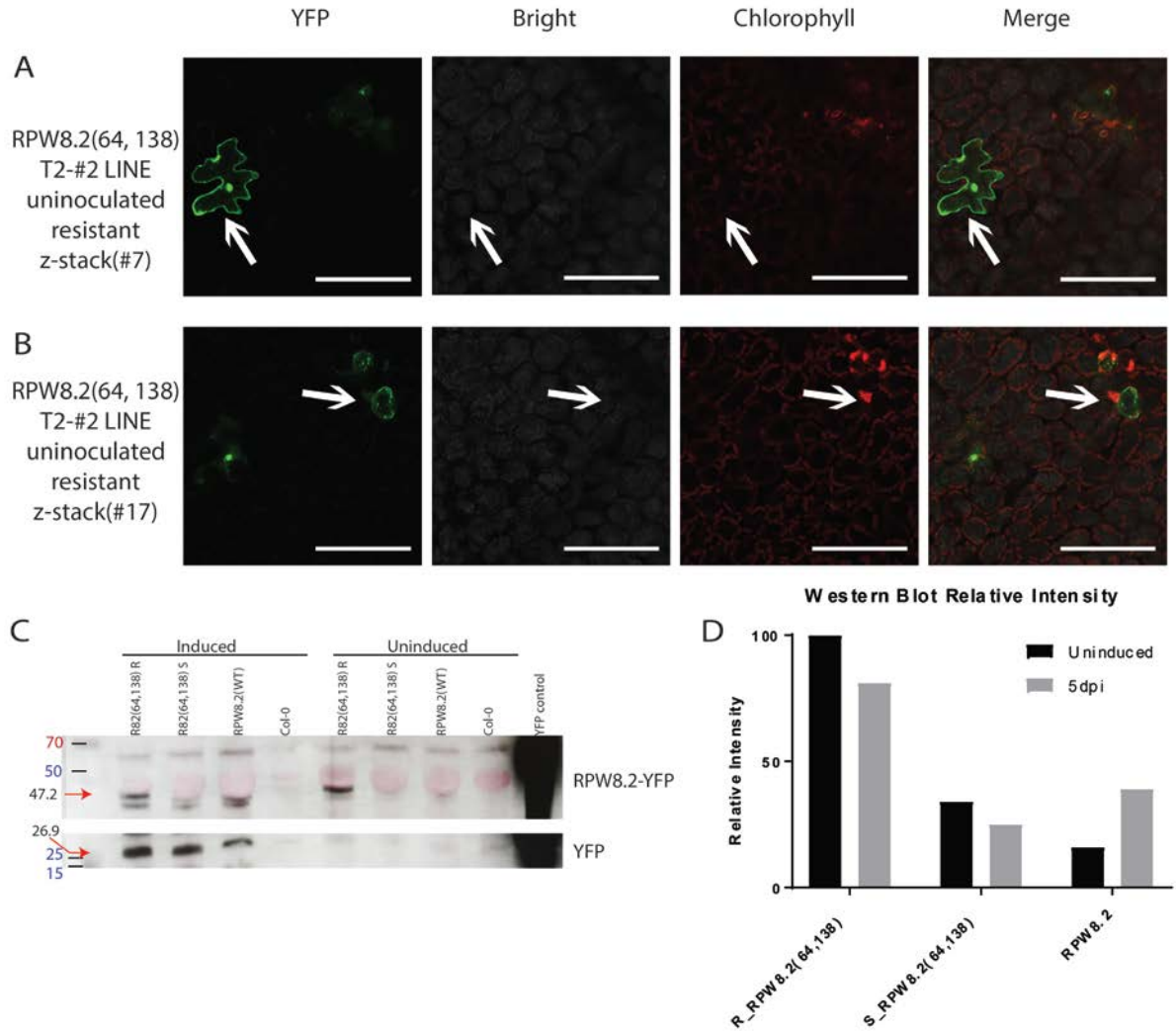
(A) The double mutant  $\text{RPW8.2}^{\text{T31A/T64A}}$  is targeted to EHM (white arrowhead). (B) EHM localization was also observed in  $\text{RPW8.2}^{\text{T64A/S138A}}$  T1 lines and subsequent T2 lines. The mutant protein in puncta (white arrow) is also visible in this image.



**Figure 2-13 PM Localization of RPW8.2<sup>T64A/S138A</sup> in Both Susceptible and Resistant Lines**

(A-C) The double mutant  $\text{RPW8.2}^{\text{T64A/S138A}}$  showed correct EHM localization (white arrowheads) and aberrant PM-localization (B) in resistant T1 transgenic lines, with similar results observed with T2 lines (C). (D) Expression of  $\text{RPW8.2}^{\text{T64A/S138A}}$  was also detected in mesophyll cells (white arrow) adjacent to the haustorium-invaded cells. (E) The  $\text{RPW8.2}^{\text{T64A/S138A}}$  mutant exhibited similar PM and EHM localization.





**Figure 2-14 RPW8.2<sup>S64A/S138A</sup> Accumulates in Uninoculated Arabidopsis Plants**

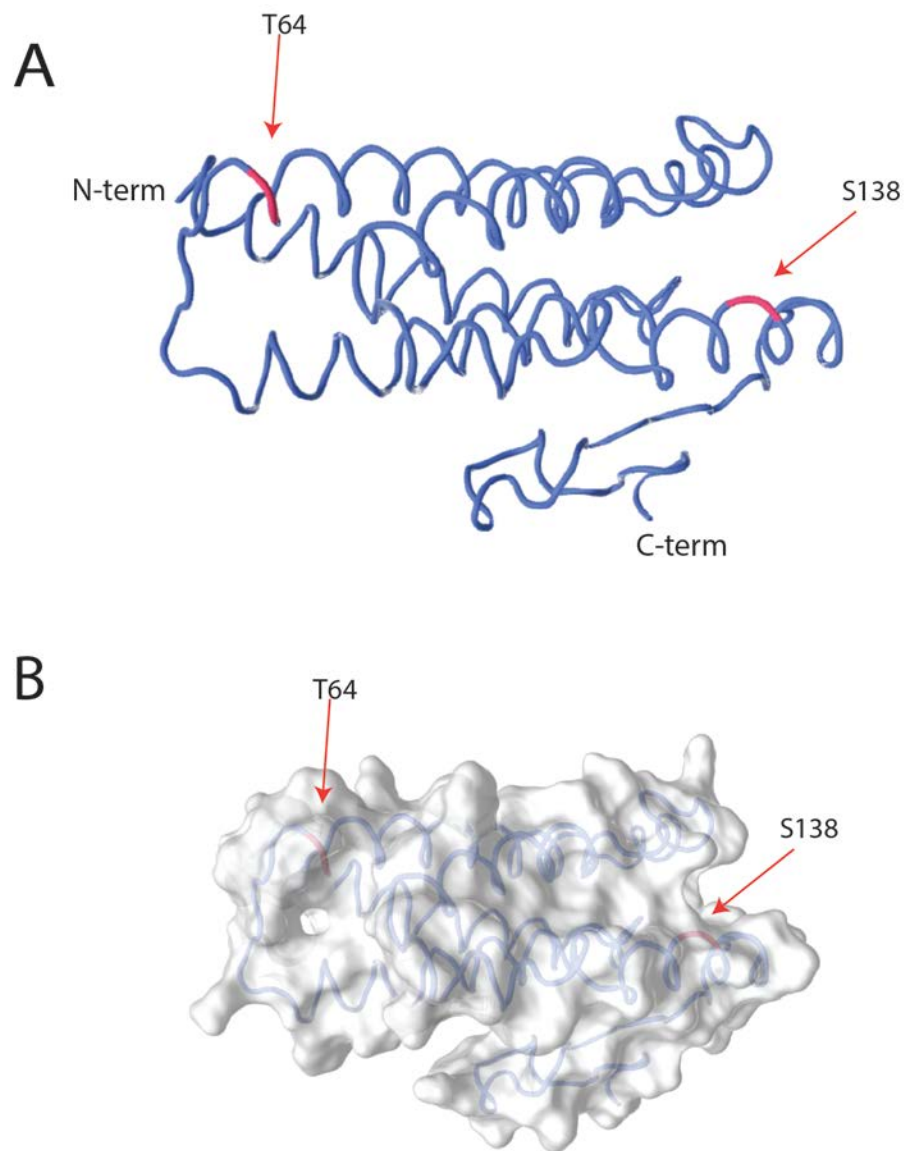
(A-B) The RPW8.2<sup>T64A/S138A</sup> mutant protein was detected in the PM (arrow) in epidermal cells (A) and mesophyll cells (B) of uninoculated plants. (C) A Western blot confirmed expression of RPW8.2<sup>T64A/S138A</sup> in leaf tissues of uninoculated (uninduced) plants and inoculated (induced) plants. The YFP band served as control. (D) Semi-quantification of the relative levels of expression of RPW8.2<sup>T64A/S138A</sup> detected in the Western blot in (C) using ImageJ.

### ***In Silico Model of RPW8.2 Predicts Surface Exposure of T64 and S138***

Because phosphorylation is a protein-surface PTM, I sought to understand the location of T64 and S138 in the RPW8.2 protein structure. Discovering cytosolically-oriented residues provides additional evidence the residues can be phosphorylated by a kinase. Internally phosphorylated residues are possible and can help stabilize conformational states (Johnson, 2009), but internal phosphorylation is rare. To wit, an analysis of phosphorylated-regulated mechanisms in the *Saccharomyces cerevisiae* protein cyclin-dependent kinase (cdk1) found that more than 90% of sites are predicted to be in cytosolically-exposed loops and disordered regions (Holt et al., 2009). My efforts in expressing and purifying soluble RPW8.2 to sufficient purity were unsuccessful. Efforts in folding RPW8.2 via Rosetta were also unsuccessful (Darrell Hurt, private communication). We used I-TASSER to provide a RPW8.2 structure.

I-TASSER(Roy, Kucukural, & Zhang, 2010; J. Yang et al., 2015; J. Yang & Zhang, 2015; Yang Zhang, 2008) is a folding prediction algorithm that identifies structural similarities to solved protein structures using primary sequences. Generating a fold using a protein's primary sequence has biological precedent as most tertiary protein folds available in the protein database are considered structurally complete (Garma, Mukherjee, Mitra, & Zhang, 2012).

Mapping T64 and S138 onto the structure generated by I-TASSER shows that both residues are cytosolically-oriented and capable of phosphorylation (Figure 2-15).



**Figure 2-15 I-TASSER Model Predicts That T64 and S138 Are on RPW8.2**

### **Surface**

(A) RPW8.2 was folded with in silico method I-TASSER. A ribbon model shows the placement of residues T64 and S138 within the predicted four alpha helices. (B)

Space-filling model shows that both are cytosolically oriented for possible phosphorylation by kinase.

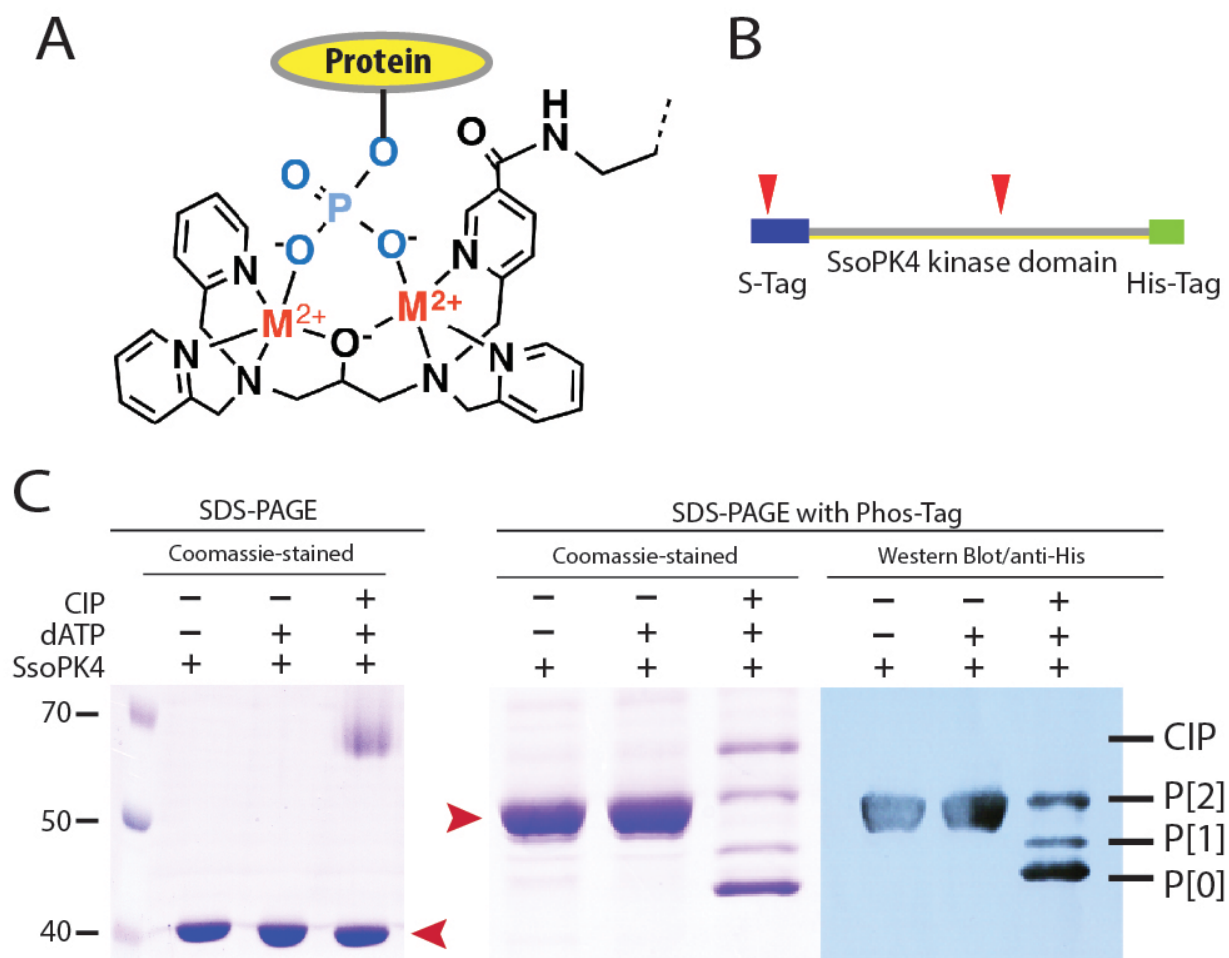
### ***Phos-Tag Detects Phosphorylated Proteins***

The Phos-Tag system can be used to interrogate the phosphostatus of proteins using an SDS-PAGE gel. The system requires no radioactive or chemical labels. Also, costly phospho-specific antibodies are not needed. Furthermore, not only phosphorylation status can be interrogated but also phosphomultiples can be detected as well. Some reports suggest phos-tag can differentiate site-specific phosphorylation (E. Kinoshita et al., 2008, 2009).

Phos-tag is a molecule that forms complexes with two bivalent cations like  $Mn^{2+}$  or  $Zn^{2+}$  to chelate phosphate groups covalently attached to proteins (Figure 2-16A). (E. Kinoshita, Kinoshita-Kikuta, Takiyama, & Koike, 2006) Bound Phos-tag retards a phosphorylated protein's migration through an acrylamide gel. Phosphorylated proteins are detected as a size shift compared to nonphosphorylated controls. Multiple bands represent different phosphoforms of phosphorylated proteins.

An archaeal kinase, SsoPK4 (kind gift from Peter Kennelly), from *Sulfolobus solfataricus* known to autophosphorylate was tested on an acrylamide gel doped with Phos-tag. (Ray, Potters, Haile, & Kennelly, 2015) SsoPk4 phosphorylates at two positions; once in the fused S-tag and another in the kinase domain (Figure 2-16B). Both positions were accessible and dephosphorylatable by calf-intestinal phosphatase (CIP). Both phosphorylated and dephosphorylated isoforms were detectable on Coomassie-stained SDS-PAGE and western blot (Figure 2-16C). The

Phos-tag system was used to determine if RPW8.2 is indeed (de)phosphorylated and analyze putative RPW8.2-interacting phosphatases and kinases.



**Figure 2-16 Phos-tag Acrylamide Gel Detects SsoPK4 Phosphorylation**

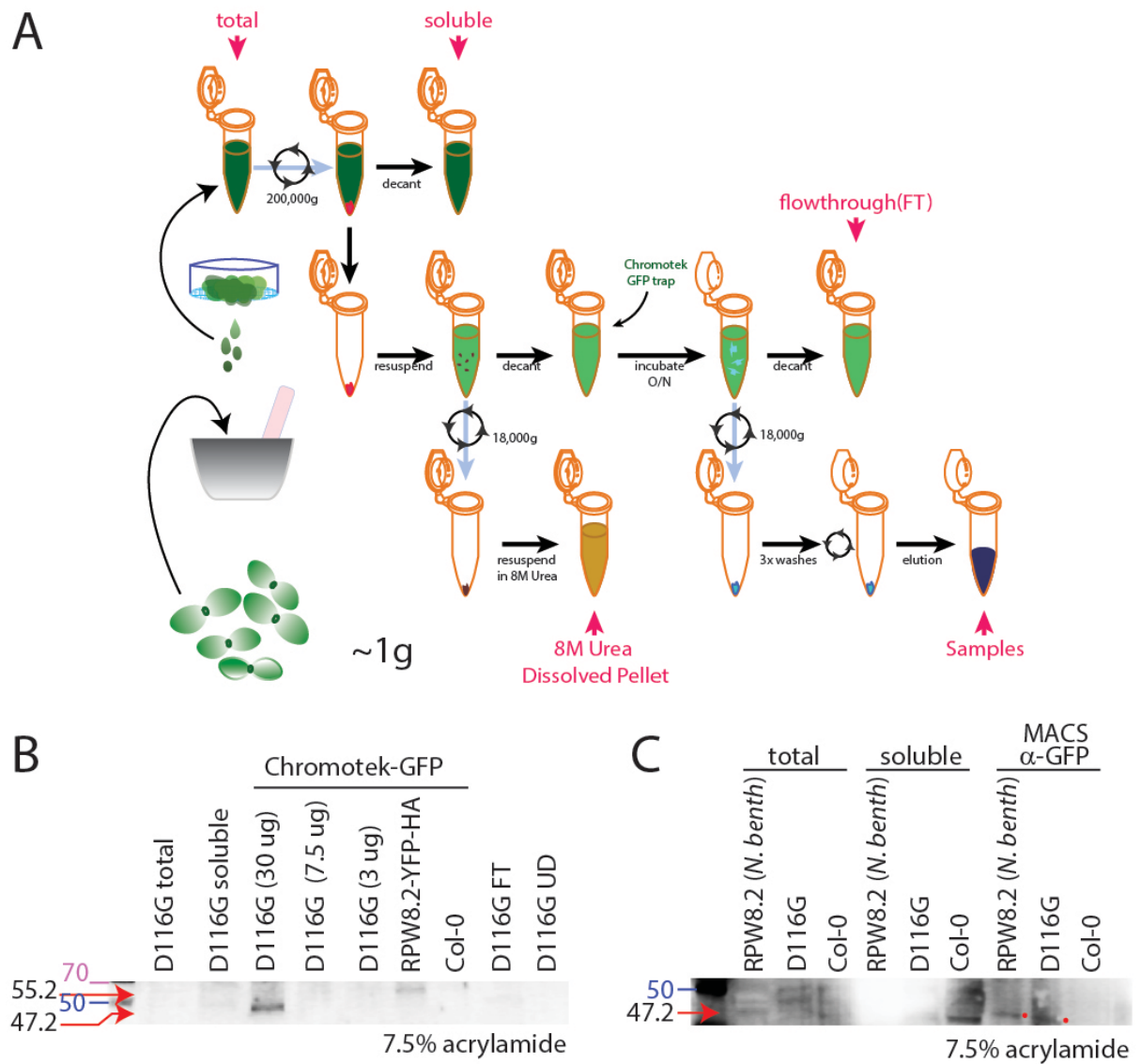
(A) Phos-tag molecule preferentially binds phosphorylated proteins. (B) SsoPK4 protein was used to test phos-tag's ability to detect phosphorylation and multiply-phosphorylated proteins. (C) Un-doped acrylamide cannot distinguish between three SsoPK4 phosphoforms using a Coomassie stain. Acrylamide gels with Phos-tag show all SsoPK4 isoforms with one and two phosphates. The addition of dATP in a kinase reaction is dispensable as sites on SsoPK4 are phosphorylated before purification and addition to the kinase assay. Calf-intestinal phosphatase (CIP) successfully dephosphorylates SsoPK4.

### ***Immunoprecipitated RPW8.2 is Detectable in Microsomal Pellets***

We wished to purify the RPW8.2 protein and assay its phosphostatus using a phos-tag-doped acrylamide gel. The RPW8.2 protein comprises a putative hydrophobic transmembrane domain at its N-terminus. Low-abundant, membranous proteins are challenging to purify.(Y. Qi & Katagiri, 2009) Therefore, I explored several purification strategies for RPW8.2 using an anti-YFP immuno-pulldown strategy. Powdery mildew infected leaves of plants expressing RPW8.2-YFP-HA were collected between 5 to 12 dpi and ground in liquid nitrogen, and a microsomal pellet was resuspended and incubated with anti-YFP immune-complexes as shown in Figure 2-17A. RPW8.2-YFP-HA was incubated with GFP nanobody (Chromotek) or anti-GFP magnetic beads (Miletenyi Biotec) and detected with anti-YFP or anti-HA antibodies.

To see if phosphorylation is indeed important for the defense function of RPW8.2, the RPW8.2<sup>D116G</sup> mutant was used as control, as this mutant protein is defective in defense activation even though it is correctly targeted to the EHM(W. Wang et al., 2013). Stable expression in Arabidopsis as well as Agrobacterium-mediated transient expression in *N. benthamiana* were explored. Although both RPW8.2<sup>D116G</sup>-YFP-HA and RPW8.2-YFP-HA bands are detectable When anti-YFP antibody is used, neither the Chromotek nor the magnetic beads provided a homogenous or abundant sample (Figure 2-17B-C).





**Figure 2-17 Detecting RPW8.2 in a Western Blot Using anti-GFP Nanobodies and Beads**

(A) Schematic overview of process for removing RPW8.2 from solution using Chromotek's anti-GFP nanobody. (B) Chromotek-GFP successfully immunoprecipitates RPW8.2<sup>D116G</sup>-YFP (47.2kDa) and RPW8.2-YFP-HA(55.2kDa) in microsomal fractions as detected with western blot and αYFP antibody. (C) Magnetic anti-GFP beads (Miltenyi Biotec) also

immunoprecipitate RPW8.2-YFP (47.2kDa) (red dots) when transiently expressed from *N. benthamiana* and *Arabidopsis* RPW8.2<sup>D116G</sup>-YFP.

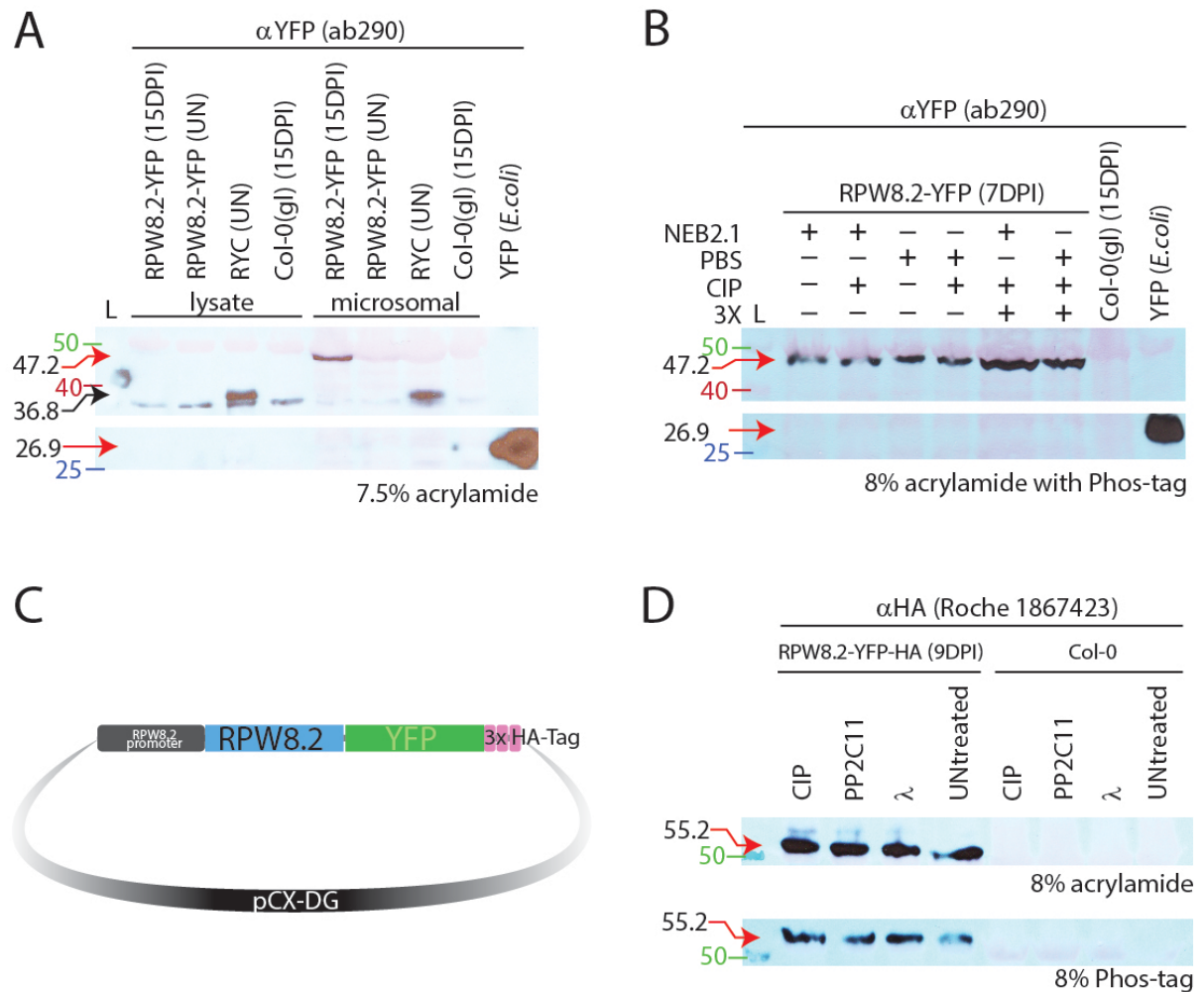
***Immunoprecipitated RPW8.2 Protein from Inoculated Arabidopsis Leaf Samples is Not Phosphorylated***

Immunoprecipitations from lysates using Chromotek and Miltenyi Biotec  $\alpha$ YFP resin was too impure for downstream analysis like mass spectrometry. Instead of further optimization with these two methods, I opted to use a more recently published technique using microsomal pellets (Avila, Lee, & Toriia, 2015).

Purifications with RPW8.2-YFP was purified from 15 dpi Arabidopsis plants. RPW8.2-YFP bands were detectable in the microsomal portion but not the lysate (Figure 2-18A). Another protein, R18-YFP-CT59 (RYC), which is a chimeric protein containing a 20aa peptide sequence that binds to 14-3-3 proteins (see Chapter 3 for details) known to express well in stable Arabidopsis and transient in *N. benthamiana* was used as control.

The reconstituted microsomal pellet was subjected to calf intestinal phosphatase (CIP) treatment in two different buffers, a buffer containing  $MgCl_2$ , BSA and other additives (NEB 2.1) and phosphate buffered saline (PBS, pH 8.0). CIP possesses strong, non-specific phosphatase activity and can dephosphorylate proteins (Swarup, Cohen, & Garbers, 1981).

The treated and untreated eluates were run on a phos-tag-doped acrylamide gel. CIP treatment failed to produce a band shift in RPW8.2-YFP from inoculated, Arabidopsis samples (Figure 2-18B). This suggests that either CIP failed to dephosphorylate RPW8.2-YFP or RPW8.2-YFP is not phosphorylated, or it is phosphorylated in vivo but dephosphorylated during extraction.



**Figure 2-18 Detection of RPW8.2-YFP by Western Blotting**

(A) RPW8.2-YFP was detected in microsomal portions but not in lysates. RYC was used as a positive control as this fusion protein is abundantly expressed cytosolically and in the PM. L = Spectra protein ladder (Thermo Fisher, cat. No. 26634) (B) Calf intestinal phosphatase (CIP) incubated with purified RPW8.2-YFP from Arabidopsis microsomal pellets did not show band-shifting after treatment. Two buffers were attempted, NEB2.1 and PBS. 3X= 3 time mass of starting material used, ~3g. (C) The RPW8.2-YFP-HA construct cloned in a binary vector, pCX-DG. (D) Three phosphatases incubated with immunoprecipitated protein

RPW8.2-YFP-HA from Arabidopsis did not detect phosphorylation. Phosphatases: CIP = calf intestinal phosphate (NEB 0290S), PP2C11 = recombinantly purified PAPP2C11 (At1G22280),  $\lambda$  = lambda phosphatase (NEB P0753S)t

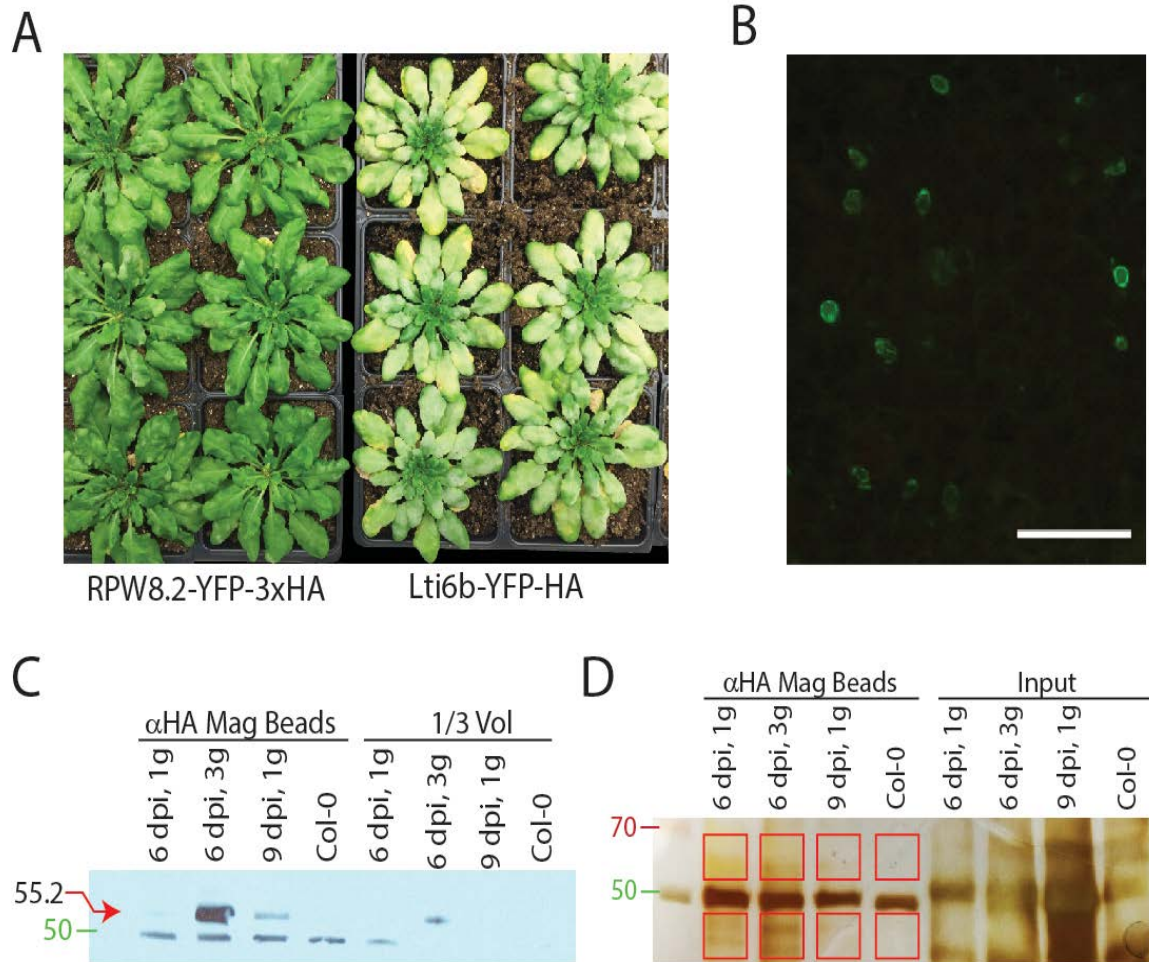
It is possible that the microsomal samples were impure, which might have prevented CIP from functioning properly. To exclude this possibility, RPW8.2-YFP was further fused with a triplicated human agglutinin (HA) epitope tag (Figure 2-18C). Homozygous T3 lines were generated and inoculated with powdery mildew. Microsomal pellets were incubated with anti-HA magnetic beads to pull down RPW8.2-YFP-HA. In addition to CIP, two additional phosphatases, Lambda phosphatase (NEB) and phytochrome-associated protein phosphatase type 2c (PAPP2C, AT1G22280) (which is a protein phosphatase type 2C that has been shown to interact with RPW8.2<sup>3</sup>) were also incubated with the immunoprecipitated RPW8.2-YFP-HA. Lambda phosphatase is a nonspecific phosphatase recommended for the dephosphorylation of proteins. However, none of the three phosphatases produced a detectable band shift (Figure 2-18D). These results suggest that RPW8.2 prepared from leaves infected by powdery mildew is unlikely phosphorylated. This, however, does not exclude the possibility that RPW8.2 is dephosphorylated by endogenous phosphatases during protein extraction.

### ***No RPW8.2 Phosphorylated Fragments Identified using Mass Spec***

Unable to identify phosphorylation via acrylamide-doped-phos-tag, a mass spectrometry approach was used. Mass-spec is a popular technique for the detection of phosphorylated protein in plants including plasma membrane proteins. (Mattei, Spinelli, Pontiggia, & De Lorenzo, 2016; Thomas S Nühse, Stensballe, Jensen, & Peck, 2004; Umezawa et al., 2013).

Leaves from Arabidopsis T3 lines were used. The same was homozygous for *RPW8.2-YFP-HA*. A T2 control line was used expressing *Low Temperature Induced Protein 6B* (Lti6B, AT3G05890). Lti6B was also fused with YFP-HA at its C-terminal end (Earley et al., 2006). Lti6B was chosen as a negative control for mass spectrometric analysis because it is localized to the PM but not to the EHM (Thompson & Wolniak, 2008).

Figure 2-19A shows the resistance of Arabidopsis plants transgenic for *RPW8.2-YFP-HA* compared to the susceptibility of plants expressing *Lti6B-YFP-HA*. *RPW8.2-YFP-HA* localizes at the EHM (Figure 2-19B). The presence of *RPW8.2-YFP-HA* was confirmed with a western blot (Figure 2-19C). Another gel run concurrently was stained with silver nitrate. Bands corresponding to the size of *RPW8.2-YFP-HA* were excised, purified and trypsinized (Figure 2-19D). Extracted peptides were dried and prepared for analysis using a LC MS/MS Agilent QTOF mass spectrometer. Sizes were searched against Mascot database using "Arabidopsis" taxonomy. The top scoring peptides are pooled for the *RPW8.2* samples and background and listed in Table 3. No phosphorylated peptides matching *RPW8.2* were discovered using mass spectrometry.



**Figure 2-19 Expression and purification of RPW8.2-YFP-HA for Mass Spectrometry**

(A) Arabidopsis plants transgenic for RPW8.2-YFP-HA are more resistant to powdery mildew compared to Lti6b plants. (B) RPW8.2-YFP-HA correctly localizes at the EHM. White scale bar is 100 $\mu$ m. (C) R82-YFP-HA can be immunoprecipitated with anti-HA magnetic resin, eluted, and probed with anti-HA antibody. (D) Silver stain of samples as in (C), but red squares indicate removed gel portions for mass spectrometric analysis.



**Table 3 Peptide Mascot Matches for Trypsinized RPW8.2-YFP-HA from Arabidopsis**

Sample	Accession	Score	Description
RPW8.2-YFP-HA	P56757	92	ATP synthase subunit alpha, chloroplastic OS=Arabidopsis thaliana GN=atpA PE=1 SV=1
RPW8.2-YFP-HA	P56757	73	ATP synthase subunit alpha, chloroplastic OS=Arabidopsis thaliana GN=atpA PE=1 SV=1
RPW8.2-YFP-HA	P56766	43	Photosystem I P700 chlorophyll a apoprotein A1 OS=Arabidopsis thaliana GN=psaA PE=2 SV=1
RPW8.2-YFP-HA	Q9LFE4	41	WE8 family protein At5g16730, chloroplastic OS=Arabidopsis thaliana GN=At5g16730 PE=1 SV=1
RPW8.2-YFP-HA	Q8GWV5	37	U-box domain-containing protein 3 OS=Arabidopsis thaliana GN=PUB3 PE=2 SV=2
RPW8.2-YFP-HA	Q9C977	35	Pentatricopeptide repeat-containing protein At1g80270, mitochondrial OS=Arabidopsis thaliana GN=At1g80270 PE=2 SV=1
RPW8.2-YFP-HA	Q9FG71	35	ER membrane protein complex subunit 8/9 homolog OS=Arabidopsis thaliana GN=EMB2731 PE=2 SV=1
RPW8.2-YFP-HA	Q0WNW0	34	Glycosyl hydrolase family protein OS=Arabidopsis thaliana GN=At5g04885 PE=2 SV=1
RPW8.2-YFP-HA	Q03042	32	Ribulose biphosphate carboxylase large chain OS=Arabidopsis thaliana GN=rbcl PE=1 SV=1
RPW8.2-YFP-HA	Q95MQ3	31	Myosin heavy chain-related protein OS=Arabidopsis thaliana GN=TSJ17.190 PE=4 SV=1
RPW8.2-YFP-HA	Q9LV33	29	Beta-glucosidase 44 OS=Arabidopsis thaliana GN=BGLU44 PE=2 SV=1
RPW8.2-YFP-HA	Q43295	26	Adenylyl-sulfate kinase 1, chloroplastic OS=Arabidopsis thaliana GN=APK1 PE=1 SV=1
Col-0	Q9LFE4	54	WE8 family protein At5g16730, chloroplastic OS=Arabidopsis thaliana GN=At5g16730 PE=1 SV=1
Col-0	B3H4I1	47	Calcium-dependent lipid-binding domain-containing protein OS=Arabidopsis thaliana GN=NTMC2T6.2 PE=4 SV=1
Col-0	Q7FK55	44	Protein kinase family protein OS=Arabidopsis thaliana GN=F18L15.130 PE=3 SV=1
Col-0	Q9FYK3	36	F21J9.27 OS=Arabidopsis thaliana GN=At1g24610 PE=4 SV=1
Col-0	Q6PQQ4	34	AP2-like ethylene-responsive transcription factor BBM OS=Arabidopsis thaliana GN=BBM PE=2 SV=2
Col-0	F4IJF0	30	Uncharacterized protein OS=Arabidopsis thaliana GN=At2g16015 PE=4 SV=1
Col-0	Q3E7C8	28	Uncharacterized protein OS=Arabidopsis thaliana GN=At1g77885 PE=2 SV=1
Col-0	B7ZWR7	28	At1g22460 OS=Arabidopsis thaliana GN=At1g22460 PE=2 SV=1
Col-0	Q9CA60	27	Cytochrome P450 98A9 OS=Arabidopsis thaliana GN=CYP98A9 PE=1 SV=1
Col-0	F4ITJ2	25	Phox (PX) domain-containing protein OS=Arabidopsis thaliana GN=At4g32160 PE=4 SV=1

### *Towards Identifying Kinases that phosphorylates RPW8.2: Kin11*

Phosphoproteomic studies have uncovered a plethora of phosphorylated proteins; yet, most cognate kinases remain unknown (Humphrey et al., 2017).

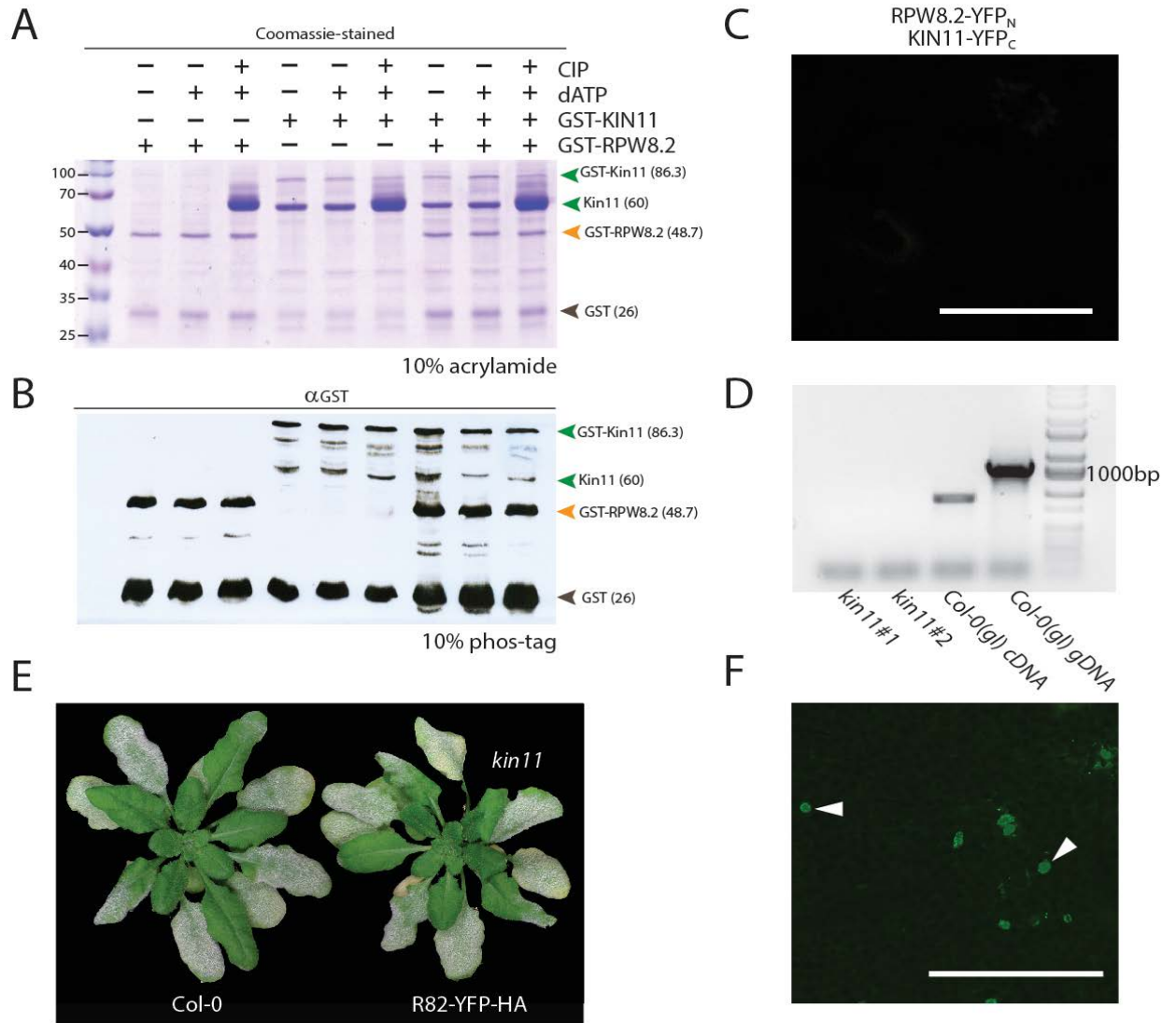
Understanding the role of RPW8.2 phosphorylation would be elucidated by identifying RPW8.2-interacting kinases.

A yeast two-hybrid screen revealed an association with Kin11 (AT3G29160) and Rpw8.2 KIN11 is a Snf1-related protein kinase (SnRK1). This conserved subfamily of serine/threonine kinases with yeast and mammalian orthologues restores homeostasis especially energy metabolism efficiency after abiotic and biotic stresses (A. Rodrigues et al., 2013). Despite several interacting partners like the 26s proteasomal subunit PAD1 and the SCF subunit SKP1/ASK1 (Farrás et al., 2001), and also knowing that KIN11 is presumed to have many downstream interactors (Polge &

Thomas, 2007), and knowing that itself is phosphorylated at a conserved T-loop T176, KIN11 has not yet been shown to phosphorylate any protein except the SAMS peptide (Fragoso et al., 2009).

Kin11 was cloned into the GST-4TI vector for *E. coli* expression under the T7 promoter. Purified KIN11 was incubated with recombinantly expressed RPW8.2 in kinase-specific conditions. After 1 hour, the samples were removed and run on a SDS-PAGE with and without phos-tag (Figure 2-20A-B). No noticeable size shift was observed with RPW8.2. This suggests KIN11 is unable to phosphorylate RPW8.2 or that the in vitro conditions were not optimized for phosphorylation to occur. This experiment was repeated three times with similar results.

We further tested for a possible interaction by performing BiFC between Kin11 and RPW8.2 in *N. benthamiana*. No interaction was observed (Figure 2-20). I attempted to validate this genetically by transgenically inserting RPW8.2-YFP-HA into a *kin11* background. The *kin11* knockout background was confirmed with RT-PCR. In this background Arabidopsis plants were susceptible even though RPW8.2-YFP-HA properly translocated to the EHM (Figure 2-20E-F).



**Figure 2-20 No Interaction Observed Between Kin11 and RPW8.2**

(A) Recombinant KIN11 and RPW8.2 were expressed and purified from *E. coli*. Purified products were co-incubated under kinase conditions and then run on a 10% acrylamide gel.

(B) No observed phosphorylation between KIN11 and RPW8.2 ON 10% acrylamide gel with phos-tag. (C) No BiFC between KIN11 and RPW8.2 transiently expressed in *N. benthamiana*. (D) RT-PCR confirms two lines are knocked out for Kin11. (E) RPW8.2-YFP-HA in *kin11* background. (F) RPW8.2-YFP-HA localizes at the EHM.

Future studies will investigate the phosphostatus of RPW8.2-YFP-HA in the *kin11-ko* background or better in a double *kin11/kin10* knockout background as both have been previously shown to act redundantly. (Baena-González, Rolland, Thevelein, & Sheen, 2007)

### ***Towards Identifying Kinases that phosphorylates RPW8.2: EDR1***

Preliminary genetic data suggests EDR1 and RPW8.2 may interact or function in the same pathway (Xiao et al., 2005). EDR1 (Enhanced Disease Resistance 1, AT1G08720) is a MAPKKK and negatively regulates salicylic acid (SA) responses. EDR1 has two domains, an C-terminal catalytic domain and an N-terminal recognition domain. The N-terminal domain interacts with mitogen activated protein kinase kinase 4 (MKK4) and MKK5 (Zhao et al., 2014). Acting redundantly, MKK4 and MKK5 target FRK1 to activate plant defenses (Asai et al., 2002b). The catalytic domain belongs to the “serine/threonine kinase (STK)-MAP3K” protein subfamily (Figure 2-21A).

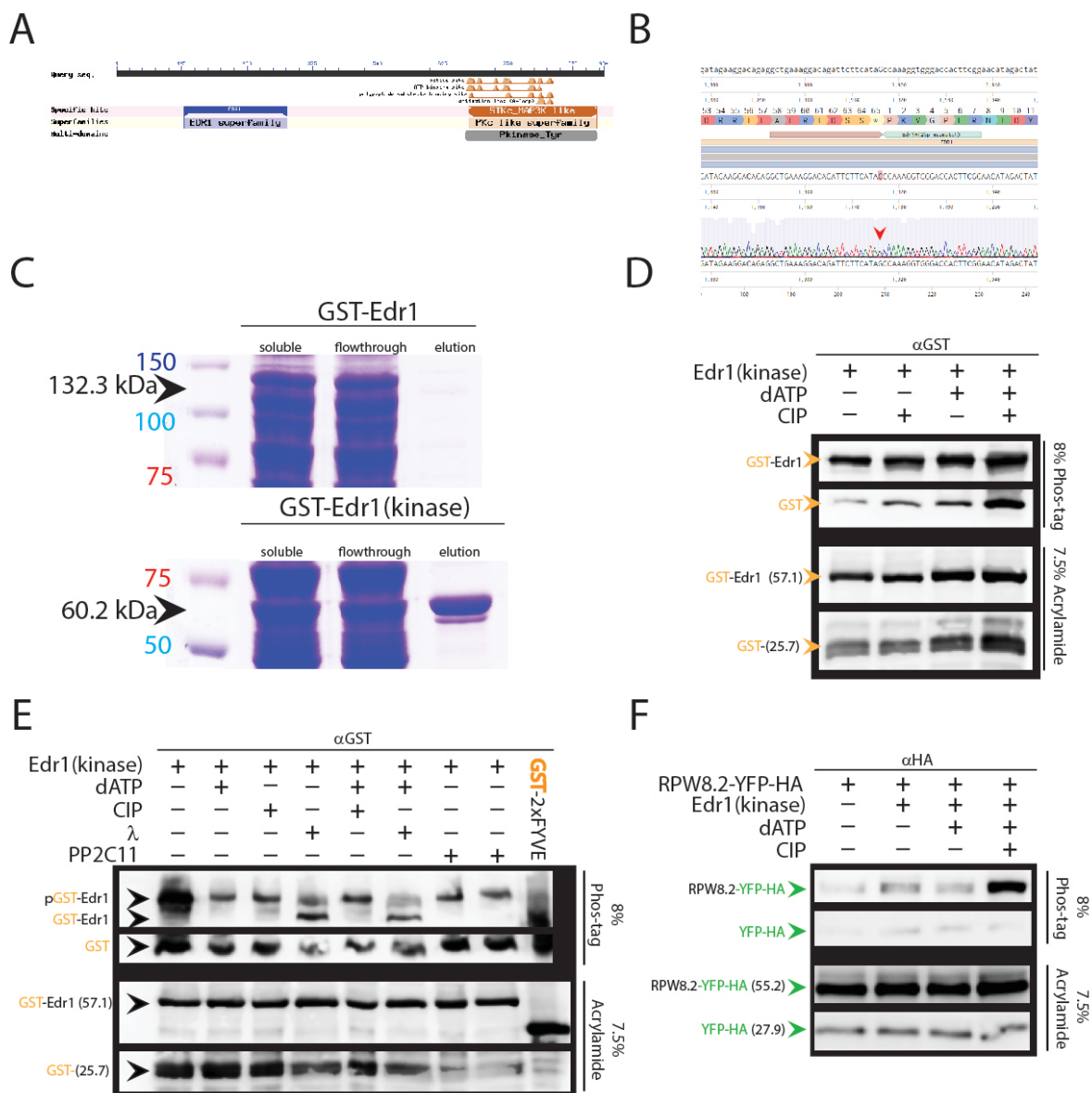
*Edr1* mutants are phenotypically indistinguishable from wildtype counterparts until inoculated with powdery mildew like *Gc* UCSC1; *edr1* mutant plants are more resistant to infection (Frye & Innes, 1998). The *edr1* mutant harbors a transversion (C→G) at nucleotide 1176 resulting in a premature stop codon and producing a truncated protein of 265 amino acids before the catalytic domain (Figure 2-21B) (Frye, Tang, & Innes, 2001). The EDR1 kinase domain autophosphorylates (Tang & Innes, 2002a).

RPW8.2 was also cloned into the same vector. The full length protein did not express consistent with previous results (Dingzhong Tang unpublished communication). However, the kinase domain was expressable in Novagen RosettaBlue(DE3)pLysS *E. coli* cells. Edr1's kinase domain autophosphorylates as demonstrated in the phos-tag-doped acrylamide gel. The autophosphorylation did not require incubation with dATP suggesting it autophosphorylated before incubation most likely in *E. coli*.

To verify the phosphorylation status of EDR1 as a prelude to validating its phosphostatus when incubated with RPW8.2 I performed an in vitro kinase assay with the mostly-soluble EDR1 kinase domain (Figure 2-21C). The coding sequences of full length Edr1 (kindly provided by Roger Innes) and the Edr1 kinase domain (aa658-934) (kindly provided by Ping He) were cloned into a Gateway-modified ligated pGEX-4ti vector (Kindly given by Shiv Kale). No band shift is observed for EDR1 in a western blot incubated with CIP (Figure 2-21D). I interpret the result of a single band shift in phos-tag-doped SDS-PAGE gel to mean that this kinase region is only phosphorylated once and that  $\lambda$  phosphatase, not CIP or PAPP2C11, is able to successfully dephosphorylate the autophosphorylated EDR1 kinase domain (Figure 2-21E).

To collect supporting data for the hypothesis that EDR1 and RPW8.2 interact I performed an in vitro kinase assay. The input was microsomal pellet resuspensions containing RPW8.2-YFP-HA from 9dpi Arabidopsis plants. No band shift is observed when resuspensions are incubated with EDR1 under conditions for kinase assays. CIP

also failed to dephosphorylate RPW8.2. This may be because CIP cannot recognize or dephosphorylate a RPW8.2 site or because RPW8.2 is not phosphorylated. Strangely, RPW8.2-YFP-HA band intensity increases when co-incubated with CIP and the EDR1 kinase domain. This might be a stabilizing interaction between EDR1 and RPW8.2.



**Figure 2-21 Edr1 Topology and Phosphorylation**

(A) Two domains comprise the MAPKKK, Edr1, a N-terminal recognition domain and a C-terminal catalytic domain. (B) Edr1 mutant at position 1176 results in a premature stop codon (TAC→TAG) insertion before the catalytic domain. (C) SDS-PAGE failed to observe soluble Edr1 expressed as a fusion peptide with GST (GST-Edr1). However, the kinase terminus

consisting of the last 278 amino acids of Edr1 fused with GST was partially soluble (GST-Edr1(kinase)). (D) The kinase domain of EDR1, if phosphorylated, is not dephosphorylated with calf-intestinal phosphatase (CIP) as observed on a phos-tag SDS-PAGE. (E) Lambda phosphatase and not CIP or PP2C11 are able to dephosphorylate the EDR1 kinase domain. The kinase domain is singly phosphorylated. GST-2x FYVE is a positive control. (F) Immunoprecipitated RPW8.2-YFP-HA from Arabidopsis leaves infected with powdery mildew at 9dpi was unaffected by incubation with the kinase domain.



We further probed the interaction between Edr1 and RPW8.2 using bimolecular fluorescence complementation (BiFC) in *N. benthamiana*. Both orientations of RPW8.2 were fused with the C-terminus of YFP and tested for interaction with EDR1. Agrobacteria containing the plasmids was infiltrated into 8-wk old *N. benthamiana* leaves. Three days post infiltration the leaves were microscopically observed to have no YFP fluorescence between YFP<sub>n</sub>-Edr1 and YFP<sub>c</sub>-RPW8.2 or RPW8.2-YFP<sub>c</sub> (data not shown). However, YFP<sub>n</sub>-Edr1 has been shown to successfully interact with a known interacting protein MMK4 (Zhao et al., 2014). Genetic crosses made between Edr1 and RPW8.2 are ongoing.

## ***Discussion***

### ***Genetic Data Suggests that RPW8.2 is Phosphorylated at More Than One Site***

Phosphorylation is a critical post-translational modification especially for immune-related proteins (L. Li et al., 2014a; Peck et al., 2001a; Tena, Boudsocq, & Sheen, 2011). A phosphorylation site can become fixed if it beneficially regulates a protein's interactions, localization and functionality (Hückelhoven, 2007; Jans, 1995; Ranjeva & Boudet, 1987). Given that RPW8.2 contains eight predicted phosphorylation sites, interacts physically with a phosphatase (PAPP2C) (W.-M. Wang et al., 2012) and genetically with EDR1 (Shunyuan Xiao et al., 2005), it is reasonable to hypothesize that RPW8.2 is functionally regulated by (de)phosphorylation. In this work I have collected extensive genetic data to suggest that RPW8.2 is likely phosphorylated in more than one site. Phosphorylation at RPW8.2<sup>T64</sup> may prevent RPW8.2 from autoactivation and triggering cell death, whereas phosphorylation at RPW8.2<sup>S138</sup> is likely required for RPW8.2 to activate defense.

The first piece of circumstantial evidence for a possible role of phosphorylation in RPW8.2's resistance function came from a study on the intraspecific allelic polymorphisms at RPW8.2 (Orgil et al., 2007). Most mildew-susceptible *Arabidopsis* accessions contain RPW8.2 alleles with the T64S nonsynonymous substitution. Interestingly, all these RPW8.2 variants also harbor the RPW8.2<sup>D116G</sup> mutation (Orgil et al., 2007). Coincidentally, while RPW8.2<sup>T64</sup> is

predicted to be phosphorylated (95% probability by NetPhos3.1), RPW8.2<sup>S64</sup> is not, (19%), raising the question whether Thr to Serine mutation at amino acid 64 is partially responsible for the mildew susceptibility phenotype. Surprisingly, I found that both RPW8.2<sup>T64S</sup> and RPW8.2<sup>T64A</sup> are likely auto-active in defense, respectively resulting in SHL in 37% and 46% T1 transgenic lines in the Col-0 background where RPW8.2 is absent. Agreeing with this result, the phosphomimetic RPW8.2<sup>T64E</sup> behaves just like RPW8.2 wt (Wang, et al., 2013). While these observations suggest that RPW8.2 is indeed regulated by phosphorylation at RPW8.2<sup>T64</sup>, the SHL and pronounced HR and resistance phenotypes are unexpected and prompted me to check if RPW8.2<sup>D116G</sup> could suppress RPW8.2<sup>T64S/A</sup>-mediated autoactivation of RPW8.2. Indeed, the RPW8.2<sup>T64A/D116G</sup> mutant allele did not activate SHL and showed reduced HR and resistance (Wang, et al., 2013). Taken together, these results confirm that the RPW8.2<sup>T64A</sup> site may perform a regulatory role in RPW8.2-mediated resistance rather than a role in localization.

I hypothesized that there might be additional RPW8.2 phosphorylation sites considering Arabidopsis proteins are known to multiply phosphorylate in response to phytohormones like salicylic acid (SA) and abscisic acid (ABA) (Takashi Furihata, Kyonoshin Maruyama, Yasunari Fujita, Taishi Umezawa, Riichiro Yoshida, Kazuo Shinozaki, 2006), and RPW8.2-mediated resistance is functionally dependent on SA pathways (Shunyuan Xiao et al., 2005; Shunyuan Xiao, Brown, et al., 2003). Also, Arabidopsis proteins from phosphoproteomic studies show differentially

phosphorylated proteins (J. J. Benschop et al., 2007). Consequently, I looked for additional phosphorylated sites that may play a role in resistance.

My work builds on previous work initiated by Wang et al (W. Wang et al., 2013). He used a NAAIRS replacement strategy (which minimize structural perturbations) to tile 6-amino-acid-mutants across RPW8.2. This analysis uncovered three potential phosphorylation sites as NAAIRS mutants substituted at these sites failed to produce detectable fluorescence. Combining this data and an in silico approach, an eight and a seven-site RPW8.2 mutant proteins, MUT7 and MUT8, were created and stably expressed in Arabidopsis. My work did not include tyrosine-mutated sites because evidence for tyrosine phosphorylation in Arabidopsis is limited.(Q. Xu, 1998)

MUT7 and MUT8 T1 transgenic lines were phenotypically characterized by resistance to powdery mildew and EHM localization. These lines were as susceptible as Col-0(gl) to powdery mildew verified by mycelia growth and spore counts (Figure 2-11C), indicating that these two RPW8.2 mutant proteins are functionally compromised. Unexpectedly, YFP fluorescence was also observed in PM of epidermal cells of these transgenic lines suggesting at least partial abnormal localization of MUT7 and MUT8. PM fluorescence has not been previously associated with RPW8.2 driven by the native promoter unless pharmacologically treated with actin inhibitors like cytochalasin E.(W. Wang et al., 2009). Homolog of RPW8 1 (HR1), HR2, HR3 and HR4, which are encoded by RPW8 family members located tandemly with *RPW8.1* and *RPW8.2*, also show PM localization when driven

by the 35S promoter (Berkey, 2013), but only when YFP was fused at the N-termini of these proteins (thus are not functional). Consequently, it is possible that the loss of either one or more particular phosphorylation sites or secondary structural perturbations resulting from cumulative mutations in MUT7 and MUT8 was responsible for reduced resistance.

Each serine (six) or threonine (two) in MUT8 was individually mutated to alanine and inserted into *Arabidopsis* via *Agrobacterium*. All T1 transgenic lines containing single-residue mutations correctly localized to the EHM 7-15 dpi.

Except for one mutant, RPW8.2<sup>S138A</sup>, all seven individual mutants displayed some resistance comparable to RPW8.2 wt. Transgenic plants expressing RPW8.2<sup>S138S</sup>, or RPW8.2<sup>T64A/S138A</sup> displayed susceptibility and showed PM-localization of the mutant proteins both before inoculation and post-uninoculation. Thus, my genetic data suggest that site RPW8.2<sup>S138A</sup> may be phosphorylated and that its phosphorylation is important for RPW8.2-mediated powdery mildew resistance.

That RPW8.2<sup>S138A</sup>, MUT7 and MUT8 confer susceptibility to the respective transgenic plants in Col-gl and S5 backgrounds (Figure 2-11C) suggests that the S138A mutation is a loss-of-function mutation and that RPW8.2<sup>S138A</sup> exert a dominant-negative effect over the RPW8.2 wt. It is possible that RPW8.2<sup>S138A</sup> might affect RPW8.2's interaction with other partners necessary for defense activation or accurate and timely targeting to the EHM.

### ***In Vitro Assays Fail to Find Evidence for RPW8.2 Phosphorylation***

I sought to biochemically uncover RPW8.2's interacting network by surveying known and putative kinases and phosphatases thought to interact with RPW8.2. Because RPW8.2 contains a putative transmembrane domain and because it localizes to the EHM and also because its concentration is limited I employed two techniques in assaying its phosphorylation. (1) A phos-tag-doped acrylamide capable of detecting a phosphorylated protein by pronounced band shift; (2) Mass spectrometry following trypsin digestion. Both techniques have been successfully used to detect phosphorylation (Bu et al., 2017; Kadota et al., 2014; Komis, Takáč, Bekešová, Vadovič, & Šamaj, 2014; L. Li et al., 2014b). I employed the first technique in testing known and putative RPW8.2 interacting partners.

Recombinantly purified PAPP2C was incubated with RPW8.2. No band shift resulted on a phos-tag gel suggesting PAPP2C cannot dephosphorylate RPW8.2. This might be because although they do interact their interaction is not phosphospecific. Instead, they could be assembled together as part of a complex or EHM-destination vesicle. Alternatively, the recombinantly-produced PAPP2C may not be active.

I attempted to purify other published known interactors of PAPP2C like phytochrome A (*PHYA*, AT1G09570) and *CPK28* (AT5G66210) to serve as controls. These proteins did not purify either. Consequently, without a positive control, I had no way of determining whether PAPP2C can dephosphorylate RPW8.2 but is improperly folded due to purification procedures or if PAPP2C does not dephosphorylate RPW8. 2.

I also tested two kinases EDR1 and KIN11 for possible phosphorylation of RPW8.2. KIN11 did not seem to phosphorylate RPW8.2-YFP judging from the lack of band shift in a phos-tag assay. No positive control was used with KIN11 because of previous reports demonstrating successful functionality of a recombinant KIN11 orthologue, AMPK, with the SAMS peptide (Neumann, Woods, Carling, Wallimann, & Schlattner, 2003; Sanders, Grondin, Hegarty, Snowden, & Carling, 2007).

The full length EDR1 protein did not express in *E. coli*, but the kinase domain was successful (kdEDR1). This domain autophosphorylated with a single phosphate consistent with previous reports (Asai et al., 2002c; Tang & Innes, 2002b). Three protein phosphatases were incubated with kdEDR1:  $\lambda$  protein phosphatase (P. T. W. Cohen & Cohen, 1989), CIP and PAPP2C11.  $\lambda$  protein phosphatase, with activity towards serine, threonine and tyrosine residues, successfully dephosphorylated kdEDR1, a finding not previously reported. Neither CIP nor PAPP2C11 had any phosphatase activity towards EDR1.

Thus, despite a lot of efforts, I found no evidence of RPW8.2 phosphorylation after incubating kdEDR1 and Arabidopsis-purified RPW8.2-YFP-HA protein determined by band shift in phos-tag acrylamide gel. The non-interaction between kdEDR1 and RPW8.2 may be attributed to the loss of the regulatory domain at the N-terminus of EDR1 which has been shown to be important for EDR1 functionality and protein-protein interactions.(Tang, Christiansen, & Innes, 2005; Zhao et al., 2014)

RPW8.2-YFP-HA samples were also immunoprecipitated from reconstituted microsomal pellets and separated by SDS-PAGE. Bands approximately RPW8.2-

YFP-HA's molecular weight were excised from the gel and submitted for LC MS/MS mass spectrometry analysis. No RPW8.2 peptides were detected. This may be partially due to difficulty in isolating and purifying RPW8.2 because of its relative low abundance and special EHM-localization. Future studies will need to purify greater quantities of Arabidopsis leaves or engineer the protein with a double affinity tag like the tandem affinity purification tag before subsequent MS/MS processing (R. B. Rodrigues et al., 2014; Stotz et al., 2014; Van Leene et al., 2014; Van Leene, Witters, Inzé, & De Jaeger, 2008). Additional purification using immobilized metal affinity chromatography (IMAC) like TiO<sub>2</sub> or Ni-NTA resin that preferentially binds to phosphorylated peptides may also aid discovery (Chen, Hoehenwarter, & Weckwerth, 2010; Lenman, Sörensson, & Andreasson, 2008; Roitinger et al., 2015).

### ***Reasons for not Detecting RPW8.2 Phosphorylation--RPW8.2 Protein Stability***

A dianionic phosphate attached to RPW8.2 and to other phosphorylated proteins may change its spatio-chemical properties and enhance or weaken its interaction with other proteins or with the environment. For instance, XA21, is a protein receptor-like kinase that confers resistance to *Xanthomonas oryzae pv. oryzae*, a bacterium that causes rice blight. XA21 autophosphorylates at three sites and interacts with a kinase at one site. Mutating each of the three autophosphorylation sites to alanine and the kinase site to the phosphomimetic causes degradation of XA21 in 1 hr. The mutant protein was also at a much lower concentration than the



wildtype protein (W.-H. Xu et al., 2006). The instability was noticed in both in vivo and in vitro applications.

The absence of phosphorylation can promote degradation but also the presence of phosphorylation can promote degradation. For instance, protein BZR1 is a positive regulator of the brassinosteroid signaling pathway in Arabidopsis. This phytohormone has important implications in Arabidopsis growth and regulation. BZR1 is positively regulated by brassinosteroid which keeps it in a unphosphorylated state. Phosphorylation by negative regulator kinase BIN2 causes BZR1 degradation via the proteasome (W.-H. Xu et al., 2006). Thus, in this case, phosphorylation targets a protein for degradation.

Analogously, RPW8.2's phosphostatus may be undetected in our assays due to rapid instability or degradation. Optimizing conditions specific for the protein in the extraction buffer or processing buffer may allow phosphodetection. For instance, proteasomal degradation may be inhibited by the addition of pharmacological agent MG132. Unstable protein may be stabilized by the addition of  $\beta$ -mercaptoethanol or detergents. The optimization of processing procedures may be critical for RPW8.2's PTM analysis.

### ***Limitations of the Phos-Tag Assay***

A protein can exist in multiple forms: phosphorylated vs unphosphorylated. A phosphorylated protein typically only represents 5-10% of protein's form in a sample (W.-H. Xu et al., 2006). A western blot is a sensitive technique capable of detecting

300 pg of protein (Schneppenheim, Budde, Dahlmann, & Rautenberg, 1991). Thus, although low abundant proteins are detectable with a western blot, their lower abundant phosphoforms can escape undetected (Mao et al., 2011). With bacteria protein SSoPK4 I showed both phosphorylated and non-phosphorylated proteins are simultaneously present and detected. I wished to overcome this low abundance limitation by identifying phosphatases and kinases to boost RPW8.2's phosphoforms and subsequent detection.

In its current embodiment, the phos-tag assay is unable to distinguish between a band representing completely phosphorylated RPW8.2 that is unable to be dephosphorylated by the three phosphatases I tested, or whether RPW8.2 is entirely unphosphorylated and the two kinases I attempted were non-phosphorylating. Tandem mass spectrometry is more sensitive and can detect femtomoles of a protein (Peck et al., 2001b; Shevchenko et al., 1996).

### ***Unoptimized Assay Conditions in Mass Spectrometry***

Tandem mass spectrometry (MS/MS) is a useful technique for analyzing PTM's on proteins. However, some PTM's like glycosylation and phosphorylation are labile and susceptible to loss before detection during the gas phase and especially during the process of collision activated dissociation, a technique of further fragmenting peptides by inducing collisions and breakage with neutral molecules (Chi et al., 2007; Ficarro et al., 2002; Udeshi, Shabanowitz, Hunt, & Rose, 2007). Thus, RPW8.2 peptides and their corresponding phosphates may be preserved by instead

using an electron-capture dissociation (ECD) or electron-transfer dissociation (ETD), a fragmentation technique different than collision activated dissociation and more conducive to detecting sensitive phosphorylated proteins (H. Zhang & Ge, 2011; Zubarev, Zubarev, & Savitski, 2008). ECD/ETD can then be coupled to an instrument like the ThermoFisher Orbitrap Fusion Lumos because of its high sensitivity (Parker, Mocanu, Mocanu, Dicheva, & Warren, 2010).

### ***Acknowledging the Null Hypothesis: RPW8.2 Isn't Phosphorylated***

The work here could be obfuscated by several variables including incorrectly operating phosphomimetics. Several of our candidate phosphorylation sites are two or three amino acids away from another phosphorylation site and adjacent phosphorylation has been shown to have a big impact on a protein's activity especially regulatory proteins (McDonald et al., 1998; Rabinovitz, Tsomo, & Mercurio, 2004). The regulation and differentiation of phosphorylation sites strongly contributes to phenotypic diversity in magnitude with transcriptional regulation (Studer et al., 2016). Thus, if a RPW8.2 mutant contributed to the mRNA stability or to its translation this might be interpreted to impute phosphorylation when none exists.

## ***Future Experiments***

### ***Is Phosphorylated RPW8.2 EHM-Targeting Efficiency Important for Resistance?***

Wang showed that RPW8.2 targeting depends on two internal motifs (R/K-R/K-x-R/K) (W. Wang et al., 2013). However, this report did not investigate the consequence of phosphorylation on targeting. Understanding how phosphorylation augments or complements these two internal localization motifs and other core residues shown important for targeting would be helpful in elucidating the enigmatic EHM-destined pathways. For instance, VAMP721 is an Arabidopsis soluble SNARE protein shown by Hyeran et al. to be indispensable for RPW8.2 trafficking (H. Kim et al., 2014). This data suggests that RPW8.2 is packaged into cargos carried by VAMP721 and other vesicles passing through the trans-golgi network. VAMP721 displays a ubiquitous expression pattern in plant tissue (Lipka, Kwon, & Panstruga, 2007) and quickly localizes to the site of fungal penetration as a first-response host defense (Yun, Panstruga, Schulze-Lefert, & Kwon, 2008). Perhaps the phosphorylation of RPW8.2 or other phosphorylation-dependent interacting proteins augment or moderate the targeting of vesicles containing RPW8.2 to the EHM instead of to other locations. This hypothesis has biological precedence as another defense protein, PEN1, requires phosphorylation for full defense via SNARE-mediated pathways. (Pajonk, Kwon, Clemens, Panstruga, & Schulze-Lefert, 2008)

My experiments did not measure the efficiency of the RPW8.2 mutant to target the extra-haustorial membrane (EHM) vs other location like the plasma

membrane (PM). Future studies could determine whether the mistargeting of RPW8.2 to the PM is consequential to the functionality of resistance. To accomplish this, haustoria could be stained with propidium iodide (PI) in RPW8.2<sup>+</sup> and RPW8.2<sup>S138A</sup> and other mutants transgenically selected in plants inoculated with powdery mildew. The number of RPW8.2-YFP-labeled EHM could be summed and divided by the total number of haustoria in a specific area size. Comparing EHM-targeting ratios would help determine if the functional cause of RPW8.2<sup>S138A</sup> susceptibility is a result of RPW8.2 mistargeting and provide evidence for a role of possible S138 phosphorylation in RPW8.2 functionality.

### ***Detecting Site Specific Phosphorylation***

Having generated eight RPW8.2 site mutants I envisioned a phos-tag assay in which Arabidopsis RPW8.2<sup>+</sup> samples would be simultaneously run with RPW8.2 mutants on the same acrylamide gel. Mutant band patterns matching no phosphorylation would be interpreted as site-specific phosphorylation. Thus, in one assay I could show the phosphorylation of RPW8.2 and also show it occurred at a specific site, hypothetically RPW8.2<sup>S138A</sup> and RPW8.2<sup>T64A</sup>. However, this assay will require first detecting the presence or absence of phosphorylation.

Site-directed mutagenesis like RPW8.2<sup>S138T</sup> and RPW8.2<sup>S138E</sup> will further clarify the phosphorylation role of S138. Should S138E partially restore S138 resistance this would add additional credibility to this site being phosphorylated and functionally relevant to RPW8.2-mediated disease resistance. A restorative mutant at

this residue would refute arguments that instead of being phosphorylated, S138 is critical for RPW8.2 structure. Confirming T1 plants are morphologically similar to RPW8.2<sup>+</sup> transgenic plants and also resistant to powdery mildew inoculation would be considered additional evidence that S138A is a loss of function mutation acting dominantly over its phosphorylated form.

### ***Repeat Studies to Confirm S138A-conferred Susceptibility***

Science is undergoing a reproducibility crisis (Begley & Ioannidis, 2014; Christie Aschwanden, 2016; Peng, 2015). The crisis is exasperated by small sample sizes, unreplicated experiments and over-reliance on p-values (Ioannidis, Chen, Kodell, Haug, & Hoey, 2005; Wasserstein & Lazar, 2016). I used binomial distributions, a pairwise test, to help calculate resistance significance among RPW8.2 mutant T1 populations. The RPW8.2<sup>S138A</sup> data was repeated twice in Col-0 background. All T1 results were duplicated except for RPW8.2<sup>S57A</sup>, RPW8.2<sup>S63A</sup>, RPW8.2<sup>S65A</sup> and RPW8.2<sup>S135A</sup>, which were performed once. Despite being performed once I believe the magnitude of the observed resistance, an indication of RPW8.2 functionality, will obviate multiple testing (Shrier, 2005). Permitted more time I would like to have triplicated resistance experiments and then tested for multiple variables and their interactions with ANOVA (Brady et al., 2015).

## ***Conclusion***

### ***Significance of Plant Resistance Protein Research***

Three criteria have been used to score food security in 118 nations for the previous five years: availability, affordability, and health and safety. Three-quarters of the nations report improvements. Technology and innovation can ensure increasing improvements despite challenges like climate change, soil erosion and pathogen susceptibility (The Economist, 2016). The rate of innovation in food crops will need to increase if food security is to increase among the earth's projected 10 billion inhabitants.

Technology improvements to crops can come in many forms including genetic improvements. For example, barley cultivars containing the resistant barley locus (mlo) to powdery mildew *Blumeria graminis f. sp. hordei* (Bgh) now account for more than 30% of the spring planting in western Europe (J. H. Jørgensen, 1992). Although breeding barley with the mlo locus may seem to be less technological and more traditional especially since it has been known for 80 years (I. H. Jørgensen, 1992) consider that only recently has an understanding of the recessive allele mlo- functionality been elucidated with modern technological methods like forward genetic suppressor screens (Acevedo-Garcia et al., 2013) and orthologous transient expression studies (Acevedo-Garcia, Kusch, & Panstruga, 2014; Holub & Cooper, 2004). In fact, only a few months ago was the barley genome been sequenced (Mascher et al., 2017). The high number of repetitive elements and lack of

recombination especially around the centromere made a full-genome assembly challenging requiring analysis of 3D chromosomal structure (Keller & Krattinger, 2017). Genome engineering tools like CRISPR continue to reveal functionality of the MLO gene family in barley, wheat, and other crops (Appiano et al., 2015; Pessina et al., 2016; Y. Wang et al., 2014).

Although mlo-mediated resistance to powdery mildew has been propagated by conventional breeding, some traits, like decoy engineering (S. H. Kim, Qi, Ashfield, Helm, & Innes, 2016) or natural insecticide resistance (Bravo, Gill, & Soberón, 2007) can never be achieved by conventional breeding. Thus, future resistance (R) proteins may be modified and customized from many sources and transformed into species. Transgenic crops have been around for 25 years and will undoubtedly become more popular (Giddings & Miller, 2016; National Academies of Sciences, 2016). Proteins conveying broad-spectrum and multi-pathogen resistance without reducing fitness or yield will be especially desirable.

### ***Utility of RPW8.2 Research***

Resistance proteins discovered in one species have been bred into other species to also confer similar protective benefits. For example, Haverkort et al. (Haverkort et al., 2008; Haverkort, Struik, Visser, & Jacobsen, 2009) used R-proteins from wild potato species germplasm to protect potatoes from *Phytophora infestans*. Traits introduced this way are cisgenic.



Transgenic traits have also been introduced. *Castanea dentata* (American Chestnut) was protected against increased oxalic acid production from the fungus *Cryphonectria parasitica* when transformed with the wheat protein oxalate oxidase (B. Zhang et al., 2013). The expression of sweet pepper protein “Hypersensitive response-assisting protein” (Hrap) in banana conferred resistance to gram negative bacteria *Xanthomonas campestris* pv. *musacearum*, which is the causal agent of Banana Xanthomonas wilt (Tripathi, Mwaka, Tripathi, & Tushemereirwe, 2010).

Whether acting cistronically in Arabidopsis (S Xiao et al., 2001; Shunyuan Xiao, Brown, et al., 2003) or acting transgenically (Shunyuan Xiao, Charoenwattana, Holcombe, & Turner, 2003), RPW8.2 has been shown to confer broad-spectrum resistance to powdery mildew species. Elucidating the mechanism of RPW8.2 functionality may allow RPW8.2 to be engineered to be constitutively active without fitness costs (Orgil et al., 2007), or to provide resistance to more than four powdery mildews capable of colonizing Arabidopsis like tobacco, grape, strawberry or tomato mildew. Further, because RPW8.2 is currently the only protein known to localize to the EHM, it may act as a targeting scaffold to deliver other pathogen-specific payloads like anti-fungal or anti-bacterial effectors or pharmacological agents.

### ***Two Phosphorylation Sites in RPW8.2***

Based on a comprehensive site-directed mutagenesis, I identified two residues T64 and S138 in the broad-spectrum disease resistance protein RPW8.2 that may be phosphorylated with consequential impact on RPW8.2’s function. While the

T64A mutation makes RPW8.2 autoactive implying negative regulation of RPW8.2 by phosphorylation of this site, the S138A mutation abolishes RPW8.2's ability to activate cell death and defense implying positive regulation of RPW8.2 by phosphorylation of this site. Interestingly, S138A suppresses T64A-mediated effect, suggesting distinct yet intrinsically connected phosphorylation mechanisms acting on RPW8.2. While phosphorylation at S138 appears to be required for efficient EHM-targeting of RPW8.2 as well as defense activation, further studies are required to determine how (de)phosphorylation at T64 and S138 by responsible kinases or phosphatases may results in functional changes of RPW8.2.

## ***Materials and Methods***

### *Arabidopsis and Nicotiana benthamiana Growth*

Arabidopsis plants and mutants (Col-0, Col-*gl*, Col-0(nahG), S5) were sown on MetroMix 360 or Sunshine Mix #1 (Maryland Plants and Supplies). Seedlings were stratified at 4°C for 2-4 days before placing at 22°C, 75% RH. Light strength was about  $\sim 125\text{-}170 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  for 10 hours and 14 hours dark cycle for 5-8 weeks. *N. benthamiana* seedlings were sown on the same soil but not stratified. Growing light was around  $125\text{-}150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  for 14 hour days (10 hour dark) cycles for 4-8 weeks before being used for transient *Agrobacteria tumefaciens* infiltrations.

### *DNA Construction*

RPW8.2 mutants were generated by overlapping PCR. Primers were generated by PrimerX (Bioinformatics.org, <http://www.bioinformatics.org/primerx/>), and are listed in table below. Ex Taq (Takara) polymerase was used to generate PCR fragments. Fragments were purified from 1% agarose gels using Promega Wizard SV Gel purification kits (Promega, A9281). Purified fragments were added to a new, overlapping PCR reaction at approximate equimolar concentrations. Whole-length fragments were again gel purified, digested with BamHI and ligated into same vector as that used in (W. Wang et al., 2009). Primers contained BamHI restriction sites.

**Table 4 Overlapping PCR Primers for RPW8.2 Mutant Generation**

Primer Name	Sequence
S29A-F	CAAAAGAGCAAAAGATAGAGCTGTAACCACAAGATTCATC
S29A-R	GATGAATCTTGTGGTTACAGCTCTATCTTTTGCTCTTTTG
T31A-F	GAGCAAAAGATAGATCTGTAGCCACAAGATTCATCTTACACC
T31A-R	GGTGTAAAGATGAATCTTGTGGCTACAGATCTATCTTTTGCTC
S57A-F	GTGGTTCAAATTGATAAGTTCGCTGAAGAAATGGAAGATTCAAC
S57A-R	GTTGAATCTTCCATTTCTTCAGCGAACTTATCAATTTGAACCAC
S63A-F	GTGAAGAAATGGAAGATGCAACATCGAGGAAAGTC
S63A-R	GACTTTCCTCGATGTTGCATCTTCCATTTCTTCAC
S65A-F	GAAATGGAAGATTCAACAGCGAGGAAAGTCAATAAAC
S65A-R	GTTTATTGACTTTCCTCGCTGTTGAATCTTCCATTTTC
S135A-F	GAACTCAAGGCCAAGATGGCTGAAATCAGCACTAAAC
S135A-R	GTTTAGTGCTGATTTTCAGCCATCTTGGCCTTGAGTTC
S138A-F	GATGTCTGAAATCGCCACTAACTTGAC
S138A-R	GTCAAGTTTAGTGCGGATTTTCAGACATC
BamR82F	CACCGGATCCATGATTGCTGAGGTTGCCGCA
BamR82RA	CGCGGATCCAGAATGCAGAACGTAAA

#### *Arabidopsis Agrobacterium-mediated Transformation and Seedling Selection*

Agrobacterium-mediated transformation of Arabidopsis was similar to that detailed here: (Clough & Bent, 1998). Seeds were sown on MS Agar and kanamycin antibiotic to select for positive transgenic plants. Positive plants were transplanted on soil.

#### *Pathogens strains, inoculation and phenotyping*

Powdery mildew isolate Golovinomyces cichoracearum UCSC1 (*Gc*-UCSC1) was maintained on live Col-0 or Col-NahG plants for generation of fresh conidia for inoculation purposes. Inoculation, visual scoring, photographing and quantification of disease susceptibility were done as previously described (Xiao et al., 2005).

### *Statistical Analysis of Resistance/Susceptibility Compared to RPW8.2 WT Plants*

The binomial test was performed with GraphPad Prism 6. The number of resistant/susceptible plants for each mutant was compared to the number of resistant/susceptible plants in wildtype background (no RPW8.2 mutation). The null hypothesis that no difference exists in the number of expected/observed resistant plants was rejected with  $p < 0.05$ . The binominal test was selected over a chi-square test because the binomial test is an exact test and the chi-square is an approximation. The binomial test is favored to the Fisher's exact test because of its ability to interpret smaller sample sizes.

### *Microscopy and Image Analysis*

Zen software version (Zen 2.3 lite, Zeiss) was used to capture images. Images were captured in the following way: LSM 710, AxioObserver, unidirectional scanning with 4x averaging and an Plan-Apochomat 20x/0.8 objective was used with beam splitter MBS 458/514/594. Laser power for 514nm laser was 5.6% and other settings as below:

Contrast Method	Track 1		Track 2
	Fluorescence		Fluorescence
Channel	Channel 1	Channel 2	Channel 3
Channel Name	Ch1-T1	T PMT-T1	Ch2-T2
Excitation			
Wavelength	514		561
Emission			
Wavelength	549		596
Detection			
Wavelength	519-578		544-648

Depth of Focus	1.72 $\mu$ m		
Binning Mode	1x1	1x1	1x1
Detector	PMT		PMT
Detector Gain	727.3	277.1	700.9
Detector Offset	0	0	0
Detector Digital Gain	1.5	1.1	1.0

Pixel time was 0.45 $\mu$ s and an image size of 2840. Total frame time was 68.74 sec with an average of 4. Specifics taken from image “S:\XiaoShared\Current Lab Members\Harley King\EXPERIMENTS\Exp703\N.benth.P2Y13.and.pm-rb\N.benth.7.dpi.pm-rb.and.Supp.fluor.image5”

### *Mass Spectrometry*

The resuspended microsomal pellets from 10dpi, UCSC1-inoculated Arabidopsis plants were incubated with anti-HA magnetic beads. The eluate containing potentially phosphorylated RPW8.2 protein was further purified on an acrylamide gel. Approximate locations were excised from the gel and digested with 0.5  $\mu$ g trypsin in 25 mM ammonium bicarbonate. Extracted peptides were dried and reconstituted in water containing 5% acetonitrile and 0.1% formic acid for analysis by mass spectrometry. Data-dependent acquisition of digests was performed by LC-MS/MS using an Agilent 6550 QTOF. Peptides were eluted from an Agilent ProtID C18 nanochip (75  $\mu$ m x 150 mm, 300 nm) over 120 min gradient from 3% to 35% acetonitrile containing 0.1% formic acid at a flow rate of 300 nL/min. Acquisition method in positive mode used capillary temperature 275 °C, fragmentor 180 V, capillary voltage 1950 V, a 300 m/z to 2000 m/z mass window at 8 spectra/s scan rate

for precursor ions, 1.3 m/z isolation window, and a 80 m/z to 1700 m/z mass window at 3 spectra/s scan rate for product ions. Peptide results scores are derived from MS/MS searches which is derived from the ions scores. Top scoring peptides (Mascot score >20) are kept.

#### *Highlighting Cell Death or Fungus in Arabidopsis Leaves*

Previous instances image analysis have been performed. For example, Schikura et al. previously developed an algorithm to quantify the symptoms of Salmonella virulence on Arabidopsis (Schikora et al., 2012). Photoshop CC Release 2017.0.1 (20161130.r.29x64) was used to highlight damage to leaves whether due to HR or fungal. HR appears yellow whereas fungal hyphae appears white. Selecting the image layer, “Select”>”Color Range”. In pop-up box, “Midtones” was used. Fuzziness = 62%, Range 140-211 > “ok”. Next, a new “levels” adjustment layer was added. To associate the color range selection with the levels layer “Image”>”Adjustments”>”Invert”. The image layer was selected, then color range was de-selected: “Select”, “Deselect”. Levels adjustment layer was selected. Again, “Image”>”Adjustments”>”Invert”. Input levels were modified from 0, 1.00 and 255 to 0, 1.00 and 72. This YouTube tutorial was followed, <https://www.youtube.com/watch?v=nqjUtwuemEo>.

For Individual Plants in RPW8.2(64, 138): each photograph had to be individually assessed when false colored especially due to factors like age, lighting and fungus age on plants. Layers were selected and grouped. Select->color fuzziness: 62% Range

1..92..211. The group layer blend mode changed from "Pass Through" to "Normal".  
New "levels Adjustment" layer added. This was clipped to the group layer below.  
Levels were adjusted to 0..1.00..85

#### *Western Blots*

A 40% acrylamide solution was used to make SDS-PAGE according to BioRad protocol (BioRad, n.d.). Prior to loading, samples were boiled at 100°C for 2.5 min in 1x Laemmli buffer. Antibodies used for detection on Antibodies: Anti-GST antibody (Amersham cat no. 27-4577-01). Anti-GFP (abcam 290). Anti-HA (Roche, 1815016). In Phos-Tag doped gels, manufacturer's procedure was followed.

#### *Western Blot Image Analysis*

RPW8.2-YFP expression increased 2x after inoculation. This expression increase is lower than previously reported for RPW8.2 levels, which discrepancy may be attributed to RPW8.2 protein from microsomal fractions in this assay and cDNA used as template in quantitative RT-PCR in other assays.(Shunyuan Xiao et al., 2005)  
The discrepancy may also be explained by the low amount (<50mg) of total leaf used and the also by the automatic band calculation technique by ImageJ and Photoshop. Consequently, total increase may be underestimated.



## Chapter 3 Investigating the Roles of 14-3-3s in Biotic and Abiotic Stress Responses with A Novel Divalent 14-3-3–Sequestering Protein

### *Introduction*

#### *14-3-3s, A Family of Conserved Regulatory Proteins in Eukaryotic Organisms*

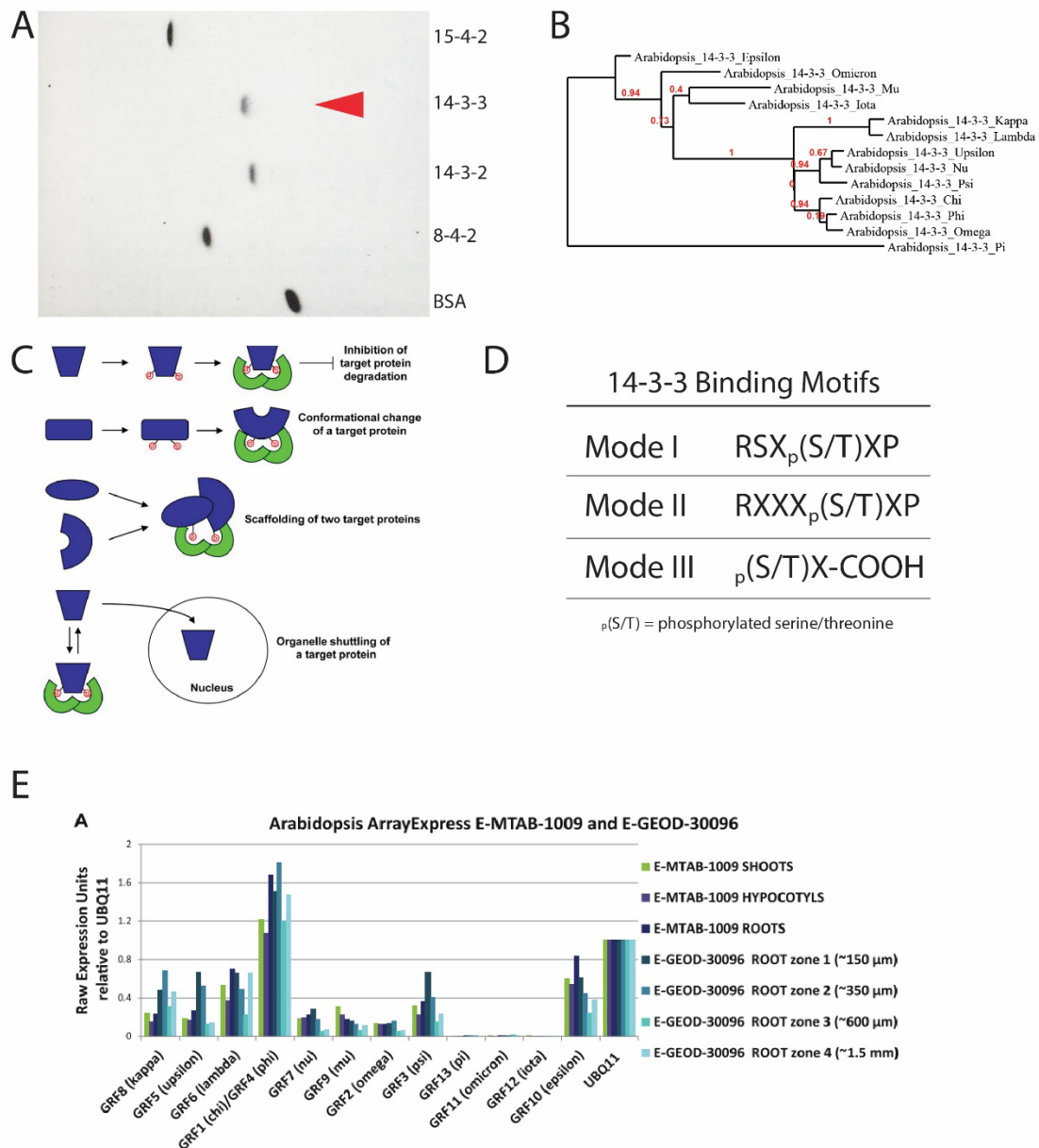
Moore and Perez (Moore & Perez, 1967) are credited with first identifying acidic proteins from bovine brain extracts after purifying with DEAE-cellulose chromatography and then separating with 2-D starch-gel electrophoresis. The “fourteen” in “14-3-3” refers to the 14<sup>th</sup> fraction tube from the chromatography, and 3-3 refers to the band identified after 2-D electrophoresis. Since their detection in bovine brain, 14-3-3 proteins have been detected in all eukaryotic organisms. Multiple 14-3-3 isoforms exist in a given genome. For examples, two isoforms are found in yeast and seven in mammals (G. P. H. van Heusden, 2009), and at least 13 isoforms are identified in the Arabidopsis genome (Rosenquist, Alsterfjord, Larsson, & Sommarin, 2001).

Arabidopsis and human 14-3-3 isoforms were originally distinguished by Greek letters. Then, Arabidopsis 14-3-3 proteins were discovered to interact with the G-box binding protein complex and were subsequently called G-box factor 14-3-3 homologs (GF14). When genomic clones were identified the 14-3-3 isoforms were labeled GRF for “General Regulatory Factor” (Wu, Rooney, & Ferl, 1997). Numbers designate the order in which the clones were identified. Today, Arabidopsis 14-3-3

proteins are known by the Greek and GRF nomenclature. For example, GRF6 is 14-3-3λ. The Greek names are kept throughout this document.

14-3-3 proteins typically bind phosphorylated target proteins within an amphipathic groove formed by homo- or heterodimers of 14-3-3s. The dimeric structure is resistant to perturbations and very rigid due to stabilizing interactions between its nine helices causing some to liken it to a “molecular anvil” (Yaffe, 2002). This allows the 14-3-3 protein family to act as an inviolable shape upon which a bound protein may be modified (Obsil & Obsilova, 2011; Yaffe, 2002).

Proteins that interact with 14-3-3s and are functionally impacted by the interaction are called 14-3-3 client proteins. The 14-3-3 family proteins have been shown to interact with many client proteins. For example, it is estimated that at least 300 *Arabidopsis* proteins may bind 14-3-3s (I. F. Chang et al., 2009). These proteins are found to be involved in a myriad of cellular functions including metabolism, hormone signaling and abiotic and biotic stress responses (Coblitz, Wu, Shikano, & Li, 2006; Denison, Paul, Zupanska, & Ferl, 2011; Christian Ottmann et al., 2007; Rosa Lozano-Durán, 2015; Uhart, Flores, & Bustos, 2016). Some 14-3-3-client proteins are conserved in *Arabidopsis*, yeast and human suggesting that these interactions and their underlying regulatory mechanisms are highly conserved (Paul et al., 2009). Given the conservation of interactions and the diversity of client proteins, 14-3-3 proteins act as nodes in signaling networks and connect functions of diverse pathways (Oecking & Jaspert, 2009).



**Figure 3-1 14-3-3 Discovery, Binding and Redundancy**

(A) The position 3-3 (red arrow) from the 14<sup>th</sup> fraction from bovine brain came to be the initial discovery of 14-3-3 proteins. Imaged modified from (Moore & Perez, 1967) (B) A phylogenetic tree of thirteen Arabidopsis proteins using *Phylogeny.fr* (Dereeper et al., 2008)

(C) 14-3-3 proteins bind client proteins for one of four reasons. i) Binding to phosphorylated proteins—blue shape with red knobs—14-3-3 proteins, green horseshoes,—prevent degradation. ii) 14-3-3 proteins can change the conformation of a interacting proteins. ii) 14-3-3 proteins can acts as scaffolding for two proteins. iv) 14-3-3 proteins can shuttle their proteins to organelle. Figure from (Oh & others, 2010) (D) Three canonical modes of 14-3-3 binding. Each mode requires phosphorylation. (E) Reproductive tissues in Arabidopsis Columbia show differentiable 14-3-3 expression levels using microarray data from ArrayExpress database. Figure from (Paul, Denison, Schultz, Zupanska, & Ferl, 2012).

### ***Actions Modes of 14-3-3 Proteins with Interacting Proteins***

Proteins interact with each other for many reasons. There are four reasons why client proteins have evolved to interact with 14-3-3 proteins (Figure 3-1C) (Oh & others, 2010). First, 14-3-3 proteins increase stability of the interacting protein by limiting their interaction with ubiquitinating proteins or proteases. Second, 14-3-3 proteins can change the conformation of an interacting protein. The change can induce activation or inhibition of a client protein's activities. Third, 14-3-3 proteins may serve their client proteins by acting as scaffolding proteins. The scaffolding can recruit other proteins necessary for completing a reaction. Finally, a 14-3-3 interaction may result in translocation of the client protein to another organelle (Aducci, Camoni, Marra, & Visconti, 2002; O'Kelly, Butler, Zilberberg, & Goldstein, 2002a).

In most cases, 14-3-3 proteins bind phosphorylated proteins (Moorhead et al., 1999; Petosa et al., 1998). Three phosphorylated motifs are considered canonical 14-3-3 binding motifs (Figure 3-1D). All canonical 14-3-3 binding motifs contain a phosphorylated serine or threonine denoted  $p(S/T)$ . Instances of non-phosphorylated motifs and non-canonical binding have also been noted (Liou et al., 2001; Tzivion & Avruch, 2002).

The later mode three binding is best characterized by the C-terminus of plant  $H^+$ -ATPase binding (Axelsen, Venema, Jahn, Baunsgaard, & Palmgren, 1999; Bartel, Schäfer, Stevers, & Ottmann, 2014) (Figure 3-2A). The plant plasma membrane  $H^+$ -ATPase is a proton pump that uses ATP to transport hydrogen ions out of the cell to

establish an electrochemical gradient that is required for the opening of a stoma, a pore created by two specialized plant cells called “guard cells” (Figure 3-2B). The binding of 14-3-3s to the C-termini of H<sup>+</sup>-ATPases activates the latter so that stomata remain open allowing for gas exchange and water transpiration (Figure 3-2C). The interaction between H<sup>+</sup>-ATPases and 14-3-3s is blue light-induced phosphorylation-dependent and reversible, rendering plants close their stomata during night to save water.



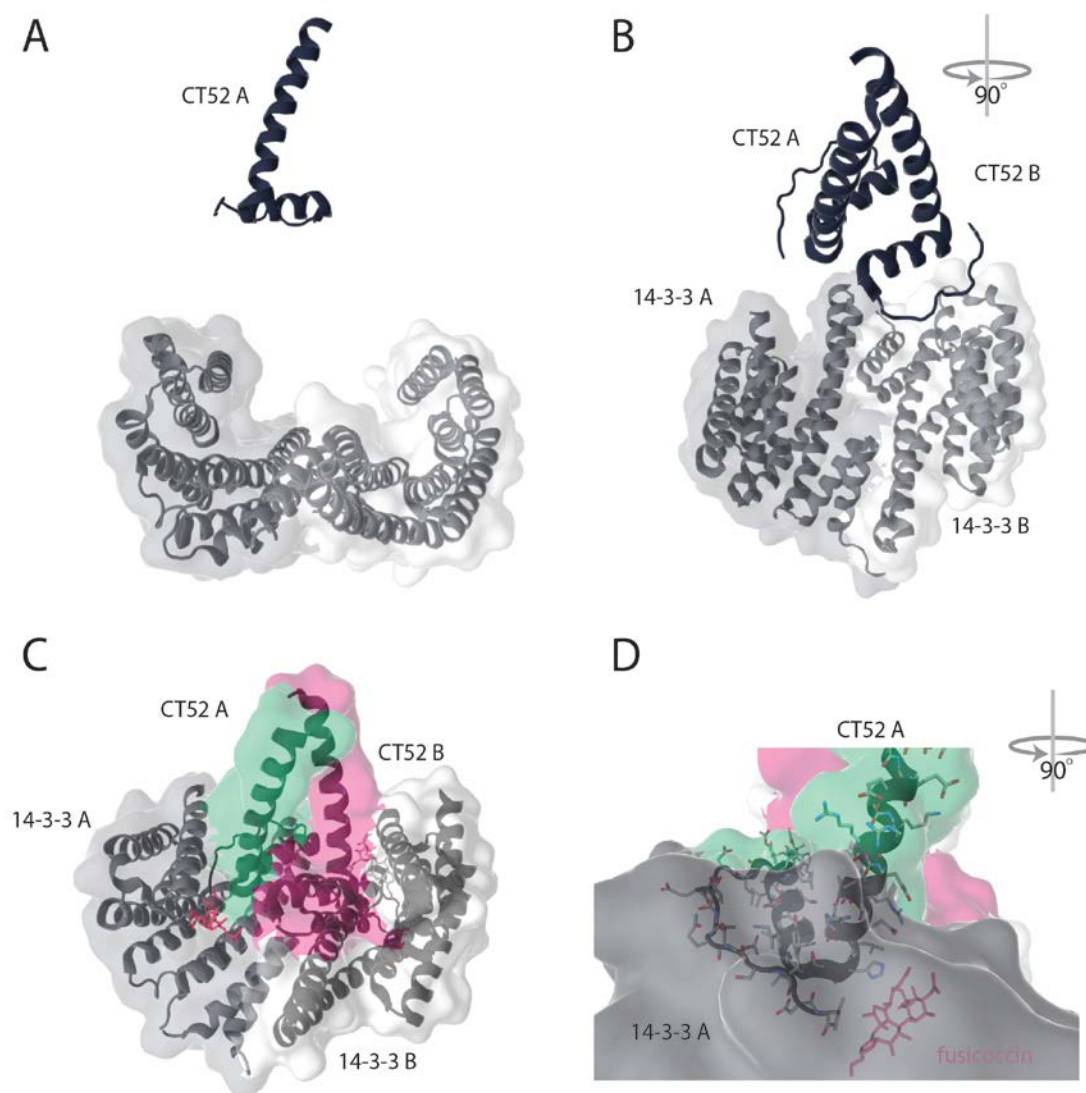
contributes to opening of stomata (pores on leaf surface), allowing for exchanges of gases including water vapor. (C) Stoma opening and closing is tightly regulated by CO<sub>2</sub>, water concentration, phytohormone abscisic acid (ABA) and small molecules like fusicoccin. (D) Fusicoccin, a fungal toxin, is known to interact with 14-3-3s and irreversibly lock up the binding between PMA2 and 14-3-3s, causing constitutive activation of PMA2.



Interestingly, it has been reported that *Fusicoccum amygdali*, a fungus on peach and almond trees (Ballio et al., 1964), produces a toxin capable of enhancing the binding between 14-3-3s and H<sup>+</sup>-ATPases, resulting in irreversible binding between 14-3-3s and H<sup>+</sup>-ATPases, which leads to the opening of stomatal pores and subsequent wilting of the infected plant (Marre, 1979).

The X-ray crystal structure for tobacco 14-3-3c binding to the C-terminal 52 amino acids (CT52) of the plant plasma membrane H<sup>+</sup>-ATPase (PMA2) from *Nicotiana plumbaginifolia* has been resolved (Christian Ottmann et al., 2007). The interaction between 14-3-3c and PMA2 is a typical mode III interaction (Figure 3-1D), the 14-3-3 interacting with a penultimate phosphorylated threonine (YpTV-COOH) on PMA2. This H<sup>+</sup>-ATPase and many other H<sup>+</sup>-ATPases are regulated by phosphorylation (Haruta, Gray, & Sussman, 2015), and PMA2 exhibits autoinhibition until phosphorylation and 14-3-3 binding to the C-terminus (Dambly & Boutry, 2001).

The crystal structure shows that one CT52 ligand interacts with a 14-3-3 monomer (Figure 3-3A). The 14-3-3 dimer coordinates two CT52 peptides in an anti-parallel manner with both exiting orthogonally from the 14-3-3 interface due to their unusual loop formation (Figure 3-3B) (Christian Ottmann et al., 2007). Twenty-seven amino acids from CT52 interact with each 14-3-3 monomer. These residues comprise two helices and a loop region that binds in the 14-3-3 groove (Figure 3-3C). Fusicoccin fits into the 14-3-3 groove and prevents the CT52 loop from exiting (Figure 3-3D).



**Figure 3-3 Crystal structures showing the binding between a 14-3-3c and a  $H^+$ -ATPase.**

(A) CT52 (black ribbon), the C-terminal 52 amino acids of an  $H^+$ -ATPase from *N. plumbaginifolia*, is used for co-crystallization with a 14-3-3 dimer. Crystal structure 2098. The images for were generated using Autodesk Molecule Viewer software, Version [0.2.1]. Copyright © 2017 Autodesk Inc., San Francisco, California, USA. Available at:

moleculeviewer.lifesciences.autodesk.com.) (B) Each CT52 binds to the groove of each monomer of a dimerized tobacco 14-3-3 protein. (C) Surface plots showing the interaction between Ct52 and a dimerized 14-3-3. (D) The same structure rotated 90° showing the C-terminal tail of CT52 inserted into the 14-3-3 binding groove with additional electron density provided by residues and the binding of fusicoccin (drawn in ball-and-stick mode) in the bottom of the groove, locking CT52 into binding groove

### ***Functional Redundancy Among 14-3-3 Isoforms***

14-3-3 proteins are highly conserved. The thirteen *Arabidopsis* isoforms share 77 identical sites in a total length of 322 amino acids. The average pairwise identity is 62% determined using Clustal Omega with Benchling (Biology Software. (2017). Retrieved from <https://benchling.com>). Inferring protein redundancy can't rely solely on protein sequence due to complexity (Devos & Valencia, 2000). However, multiple experiments demonstrate redundancy among the 14-3-3 proteins. For instance, primary root growth demonstrated overlapping function for six 14-3-3s in abiotic stress when plants contained 14-3-3 knockout mutations (Van Kleeff et al., 2014). Four *Arabidopsis* 14-3-3 proteins can individually rescue a lethal, double knockout of 14-3-3 genes in *Saccharomyces cerevisiae*, showing that variation in the non-conserved regions is tolerated and that heterologous proteins from the same family can perform redundant functions (G. P. van Heusden, van der Zanden, Ferl, & Steensma, 1996). Paul et al. used microarray data from untreated *Arabidopsis* (Col-0 accession) plants to show expression profiles for the 14-3-3 genes across tissues like roots, hypocotyls and siliques. They conclude that most isoforms are found in most tissues, and distinct localization of some individual 14-3-3 proteins may be due to their interaction with different client proteins (Paul et al., 2012; Paul, Sehnke, & Ferl, 2005). Ten 14-3-3 proteins are expressed in non-specific tissue and in a non-developmentally regulated manner (Keicher et al., 2017). Absence of noticeable phenotypes for single and combinatorial 14-3-3 T-DNA insertions is also evidence for the existence of functional redundancy among 14-3-3 isoforms (Krysan, Young,

Tax, & Sussman, 1996; W. Ma et al., 2016; Mayfield, Folta, Paul, & Ferl, 2007; Van Kleeff et al., 2014). A human protein-protein interaction phosphorylation network was resistant to perturbations when each of the 14-3-3 isoforms was systematically removed from the network. This was possible due to “redundant wiring”(Uhart et al., 2016).

### ***14-3-3 Proteins Involved in Plant Immunity***

Like in animals, innate immune proteins of plants are first-response defenders that must initiate and quickly ramp up plant responses to pathogenic threats. For instance, FLAGELLIN SENSING 2 (FLS2), recognizes flagellated bacterial pathogens and phosphorylates interacting BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1), a leucine rich repeat receptor kinase (Chinchilla, Zipfel, Robatzek, Kemmerling, N?rnberger, et al., 2007; A. Heese et al., 2007). This reaction happens in as little as 30-60 seconds after contact (Schulze et al., 2010). Plant innate immunity is a phosphorylation-driven event due to its speed and reversibility. Due to their interaction with phosphorylated proteins, 14-3-3 proteins act as phospho-sensors. Chang et al. used 14-3-3 $\omega$  fused with a tandem-affinity-purification tag to identify interacting partners with mass spectrometry. They discovered an interaction between 14-3-3 $\omega$ -BAK1 and BR1, which was missed in yeast two hybrid assays probably due to phosphorylation and 14-3-3 acting as a scaffold (I. F. Chang et al., 2009; Gampala et al., 2007). 14-3-3 proteins also undergo expression changes in response to pathogen attack. For example, an observed

decrease in expression of 14-3-3 $\lambda$  is due to its proteasomal degradation facilitated upon phosphorylation by a mitogen-activated kinase (MPK11), and a 14-3-3 $\lambda$ -null mutant is more resistant to potyvirus infection (Carrasco et al., 2014). This suggests that removal of 14-3-3 $\lambda$  is part of the infection strategy by potyviruses. In general, 14-3-3 proteins may be targeted by pathogens because of their strategic importance in phosphorylation networks and their servicing of a large client cadre (Mukhtar et al., 2011).

#### ***14-3-3 $\lambda$ Interacts with RPW8.2 for defense***

14-3-3 $\lambda$  is upregulated in response to powdery mildew infection (X. Yang et al., 2009), so is RPW8.2 (W. Wang et al., 2009). Yang et al. provided evidence for an interaction between 14-3-3 $\lambda$  and RPW8.2 and showed that the interaction depends on the C-terminus of RPW8.2 (X. Yang et al., 2009). Thus, 14-3-3 $\lambda$  may bind RPW8.2 in a mode III manner for trafficking or a masking function (O'Kelly, Butler, Zilberberg, & Goldstein, 2002b; Shikano, Coblitz, Wu, & Li, 2006). Yang et al. also demonstrated that a 14-3-3 $\lambda$  knockdown (*14-3-3 $\lambda$ -kd*) mutant (SALK\_129554) is slightly more susceptible to infection of a well-adapted powdery mildew pathogen *Gc*-UCSC1. When this *14-3-3 $\lambda$ -kd* allele was introduced into an Arabidopsis transgenic lines expressing both *RPW8.1* and *RPW8.2* (which act synergistically and cooperatively to protect against powdery mildew) (Ma et al., 2014, Plant Cell Physiol **55**, 1484-1496.), it compromised RPW8-mediated resistance

against powdery mildew *Gc*-UCSC1 (X. Yang et al., 2009). This suggests that the interaction with 14-3-3 $\lambda$  is important for RPW8.2's resistance function.

However, how 14-3-3 $\lambda$  regulates RPW8.2's resistance function is not known. Given RPW8.2's functional features, one may speculate the following two possible mechanisms by which 14-3-3 $\lambda$  regulates RPW8.2 via binding to the C-terminus of RPW8.2: (1) 14-3-3 $\lambda$ -binding is required for RPW8.2 to activate defense, and (2) 14-3-3 $\lambda$ -binding is required for efficient targeting of RPW8.2 to the EHM. Even though genetic data based on a *14-3-3 $\lambda$ -kd* mutant already support a positive and unique role for 14-3-3 $\lambda$  in regulation of RPW8.2, the residual expression of 14-3-3 $\lambda$  and/or potential functional redundancy between 14-3-3 $\lambda$  and other isoforms needs to be assessed in order to fully define the role of 14-3-3s in regulating RPW8.2-mediated resistance. More importantly, the underlying mechanisms for 14-3-3 $\lambda$ 's regulation needs to be clarified.

### ***14-3-3 Sequestration as An Alternative Strategy to Study 14-3-3 Functions?***

Engineered proteins have been previously described to bind and sequester metal and organic molecules (Drummond, Cundari, & Wilson, 2012; Krishnaji & Kaplan, 2013) and other proteins (Cooke, Prigge, Opperman, & Wickens, 2011; Kariolis et al., 2014; Karuppanan et al., 2017). It can be envisioned that an engineered protein capable of sequestering 14-3-3 proteins can (1) address likely functional redundancy among different 14-3-3 isoforms, (2) reveal mechanisms with a higher spatiotemporal resolution if the engineering protein is expressed in a controlled

manner. Hence, I explored the engineering of such a 14-3-3-sequestering protein and used it as a tool to elucidate 14-3-3-dependent regulatory mechanisms with a focus on biotic and abiotic stress responses.

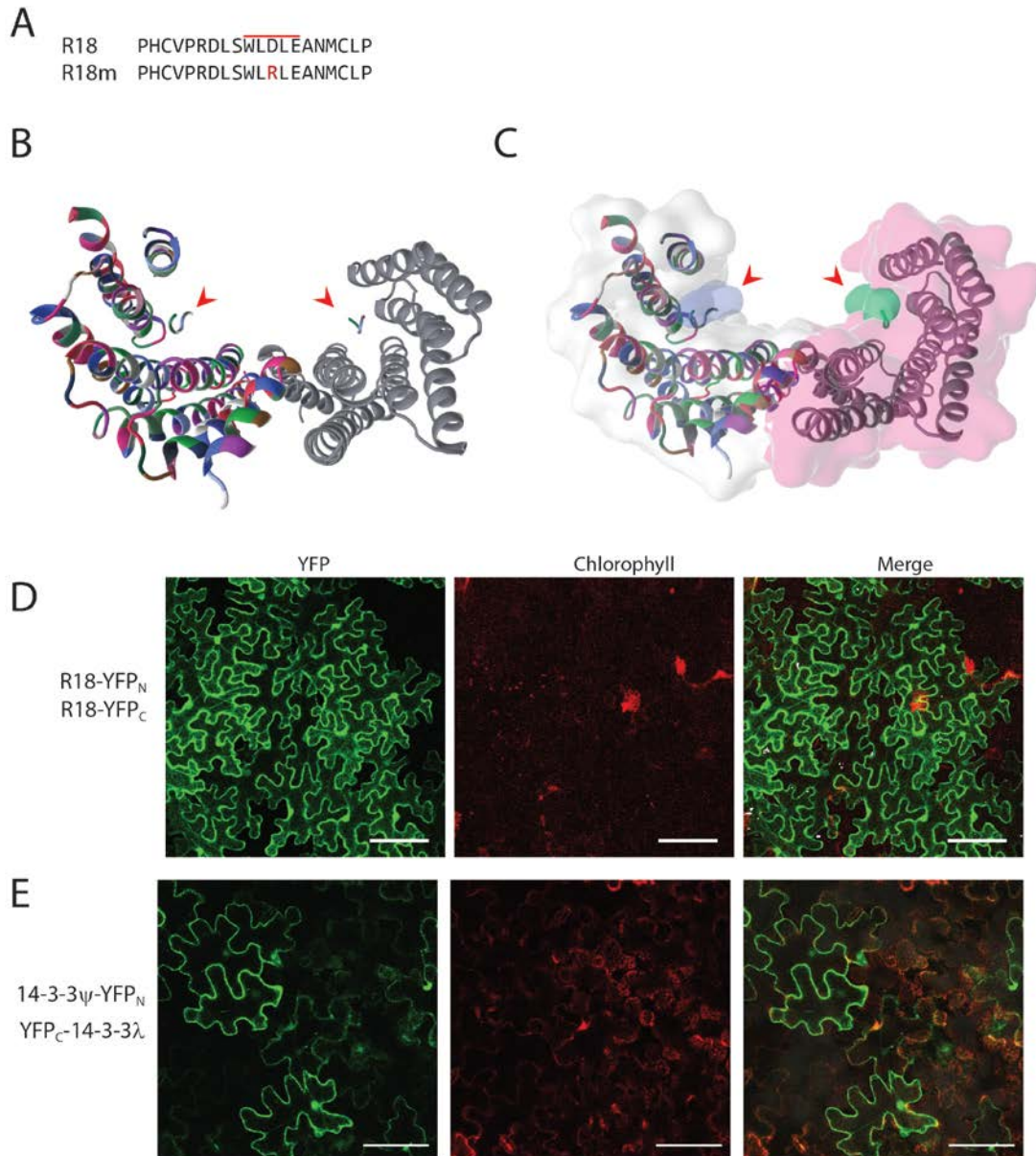


## ***Results***

### ***R18 Binds to Human 14-3-3 $\zeta$ and Six Arabidopsis Isoforms***

To further investigate how 14-3-3 $\lambda$  regulates RPW8.2-mediated resistance and circumvent the likely functional redundancy among different 14-3-3 isoforms, I sought to overexpress R18, a 20 amino acid peptide (PHCVPRDLSWLDLEANMCLP) in Arabidopsis plants because R18 has been shown to bind human 14-3-3 $\zeta$  with a high affinity (Wang et al., 1999) and inhibit 14-3-3s' binding with endogenous client proteins (Petosa et al., 1998; Du et al., 2005; Dong et al., 2007; Dong et al., 2008; Mu et al., 2008; Kent et al., 2010; Qi et al., 2010). A crystal structure shows that amino acids WLDLE of R18 tightly engages the 14-3-3 binding groove (Figure 3-4B-C) (Petosa et al., 1998). The acidic residues, glutamate and aspartate, of WLDLE mimic the serine/threonine phosphorylation of 14-3-3 ligands. A single mutation in R18 (D12R) abrogates binding with 14-3-3s probably due to electrostatic repulsion (Jin et al., 2004). Expression of R18 as a GFP-fusion protein also appeared to affect the subcellular distribution of 14-3-3s in trichome and guard cells of Arabidopsis (Paul et al., 2005). Thus, I speculated that R18, if expressed in Arabidopsis leaves, may be able to act as a competitive ligand to inhibit 14-3-3s' binding with RPW8.2, thereby compromising RPW8.2's resistance function.

Before stably expressing R18 as a transgene in Arabidopsis, I first used bimolecular fluorescence complementation (BiFC) to confirm if R18 indeed interacts with 14-3-3 $\lambda$  and other 14-3-3 isoforms *in planta*.

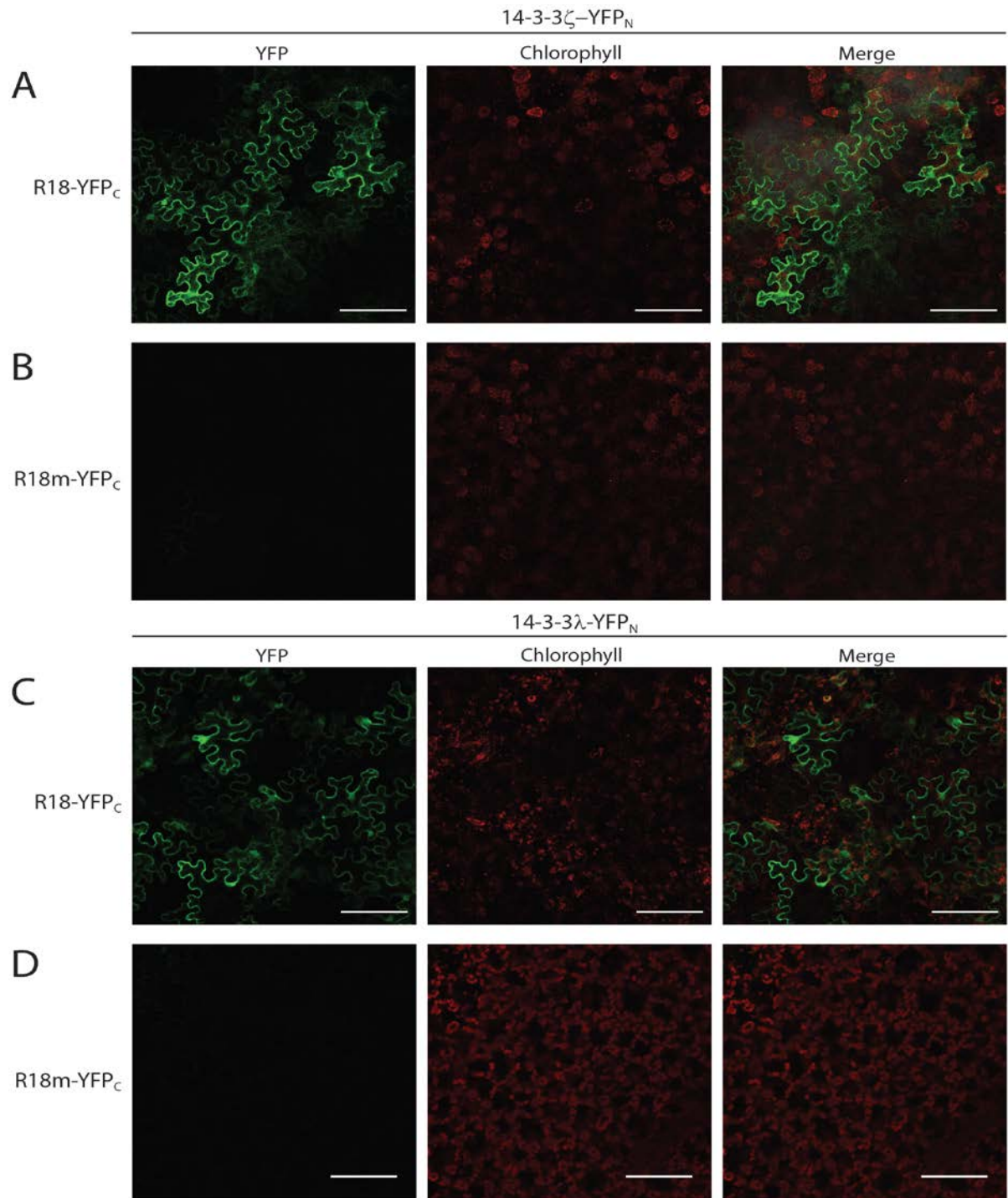


### Figure 3-4 R18 but not R18m Binds 14-3-3 $\lambda$ Protein

(A) R18 vs R18m sequences. A single amino acid change, D12R, renders R18 unable to bind 14-3-3 (B. Wang et al., 1999). Red line above peptide indicates residues WLDLE. (B) Crystal structure showing WLDLE residues (red arrows) in R18 protein bind in the 14-3-3 $\zeta$  binding groove of each monomer in the dimerized structure. Images created with MolViewer

(Autodesk). (C) Space filling model shows R18 in the cleft. (D) Two R18 molecules tagged with YFPc or YFPn are capable of reconstituting YFP when transiently expressed in tobacco leaf epidermal cells. (E) In vivo confirmation of 14-3-3 heterodimerization by transient expression.

Briefly, yellow fluorescent protein (YFP) was split into two halves. The N-terminus (YFP<sub>N</sub>) half contained amino acids 1 to 154. The C-terminus comprised amino acids 155 to 239. Each half was fused to an R18 peptide. Re-constitution of YFP demonstrates interaction between two separate R18 peptides mediated by their binding to the same dimerized 14-3-3 molecule in tobacco leaf cells (Figure 3-4D). BiFC was also used to confirm *in vivo* heterodimerization between 14-3-3 $\lambda$  and 14-3-3 $\Psi$  (Figure 3-4E) as a validation of BiFC with the 14-3-3 isoforms.



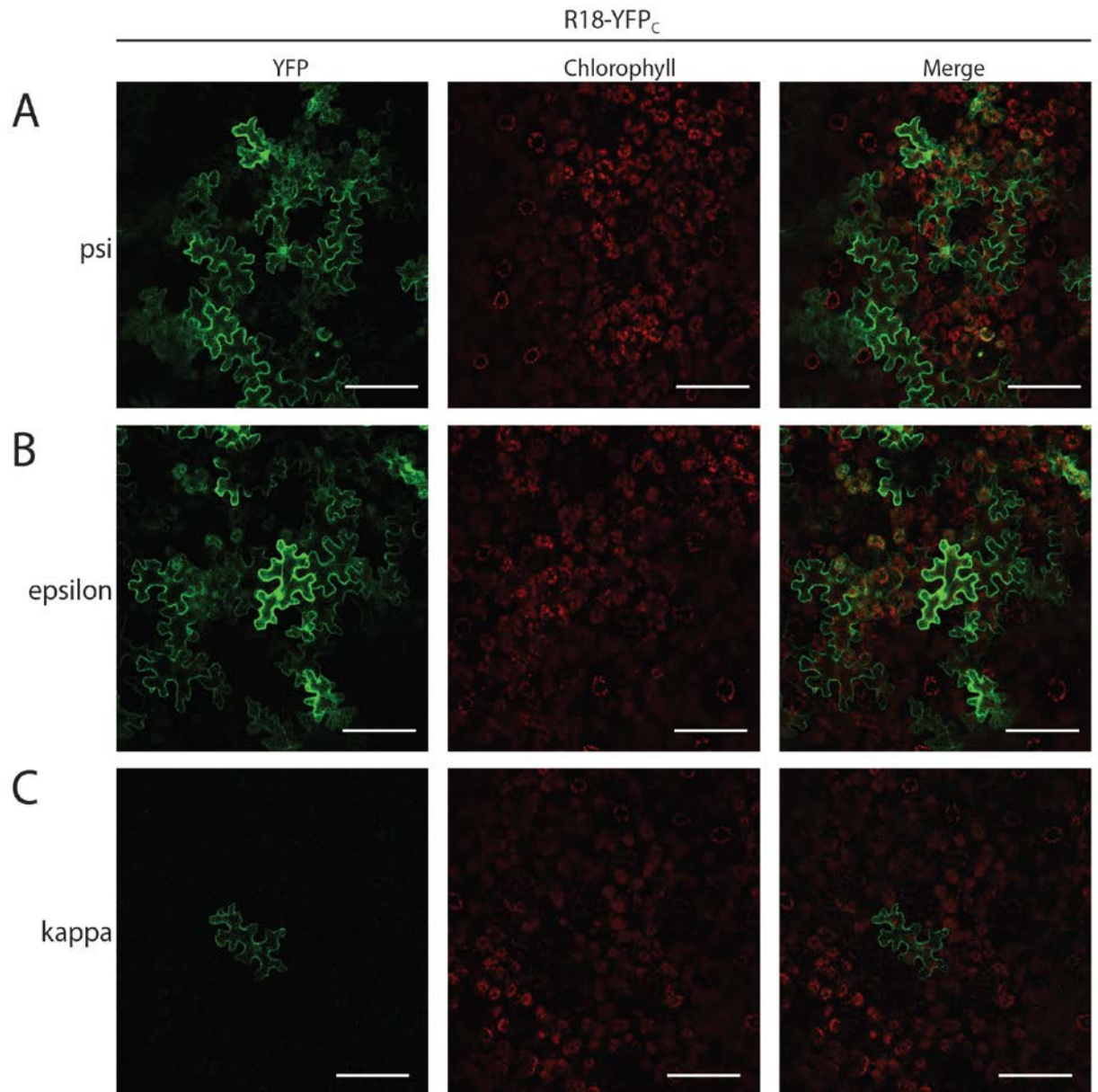
**Figure 3-5 14-3-3 $\lambda$  Interacts R18 but not R18m as shown by BiFC at Both Orientations**

(A) R18 interacts with human 14-3-3 $\zeta$  in tobacco epidermal cells. (B) R18m does not interact with 14-3-3 $\zeta$ . (C) R18 interacts with 14-3-3 $\lambda$ . (D) R18m does not interact with 14-3-3 $\lambda$ .

The interaction between R18 and 14-3-3 $\zeta$  has not been verified with BiFC. I fused YFP<sub>N</sub> to 14-3-3 $\zeta$  and confirmed its interaction with R18-YFP<sub>C</sub> in leaf epidermal cells of tobacco. An abundance of YFP fluorescence indicated a positive association between the two proteins (Figure 3-5A) compared to the lack of fluorescence when 14-3-3 $\zeta$ -YFP<sub>N</sub> or R18-YFP<sub>C</sub> was transiently expressed alone (data not shown). Moreover, no YFP fluorescence was detected between 14-3-3 $\zeta$ -YFP<sub>N</sub> and a YFP<sub>C</sub>-tagged mutant version of R18 (designated R18m) in which the 14-3-3 binding motif SWLDLE was mutated to SWLRLE in leaf epidermal cells expressing these two DNA constructs (Figure 3-5B), demonstrating that BiFC can faithfully report direct binding between R18 and 14-3-3 $\zeta$  in plant cells.

R18 has been previously employed as a potential competitor for 14-3-3–binding to release endogenous proteins bound with 14-3-3s in Arabidopsis (Paul et al., 2005). However, it has not directly been shown that R18 can indeed bind different Arabidopsis 14-3-3 isoforms *in vivo*. I thus tested if R18 binds different Arabidopsis 14-3-3 isoforms starting from 14-3-3 $\lambda$ . As shown in Figure 3-5C and 3D, and Figure 3-6A to 4C, R18 indeed interacts with all four isoforms (i.e.  $\lambda$ ,  $\Psi$ ,  $\epsilon$  and  $\kappa$ ) albeit seemingly at different levels with 14-3-3 $\kappa$  repeatedly having the weakest interaction as reported by the detection of only rare and sporadic YFP-expressing cells (Figure 3-6A-C).



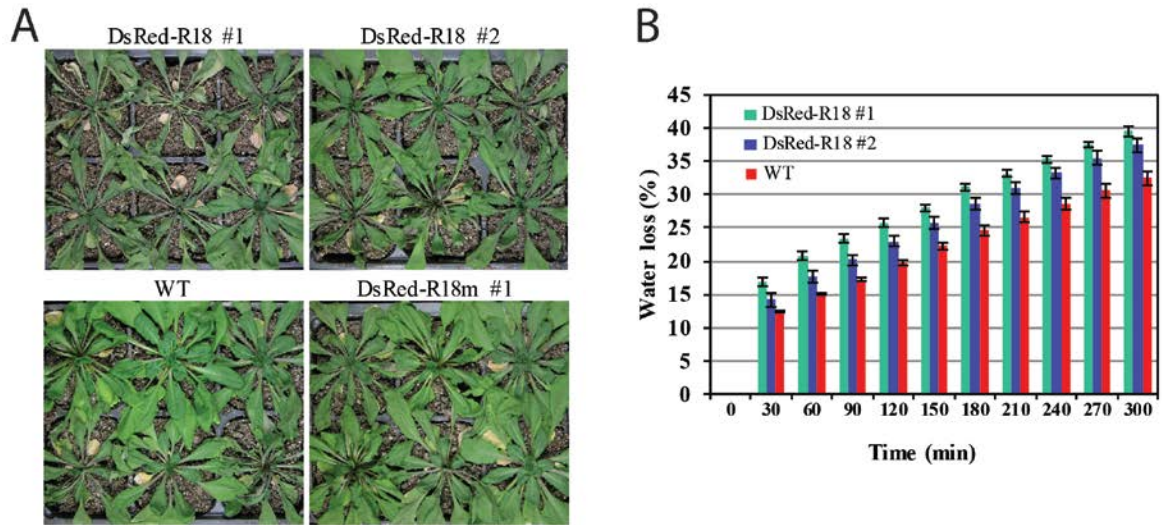


**Figure 3-6 R18 Binds Three Other 14-3-3 Arabidopsis Isoforms**

(A) R18 binds psi, (B) epsilon and (C) kappa.

### ***Overexpression of R18 renders plants more sensitive to drought stress***

In order to stabilize R18 and visualize its subcellular distribution in plant cells, I translationally fused an *R18* coding sequence (with codons optimized for plant expression) with that of DsRed and expressed the DsRed-R18 fusion protein from the 35S promoter. Confocal imaging of Arabidopsis plants transgenic for 35S::*DsRed-R18* showed that DsRed-R18 preferentially accumulated in guard cells and is ubiquitously distributed (data not shown). Infection tests with the adapted powdery mildew isolate *Gc* UCSC1 showed no difference between the transgenic plants and wild-type control (data not shown). Serendipitously, I found that plants of two homozygous transgenic lines expressing *DsRed-R18* in Col-0 were more sensitive to drought stress compared to the non-transgenic control plants (Figure 3-7A). Consistent with this, water-loss rate of the detached leaves from the two transgenic lines was significantly higher than that of the wild-type plants (Figure 3-7B). These results suggest that R18 might facilitate activation of H<sup>+</sup>-ATPases in guard cells, thereby promoting stomatal opening, which is opposite to what one would predicted based on H<sup>+</sup>-ATPase-14-3-3 interaction.



**Figure 3-7 DsRed-R18 Enhances Drought Sensitivity**

(A) Transgenic Arabidopsis expressing DsRed-R18 are more sensitive to drought compared to wildtype plants. R18m plants however are similar to WT. (B) Water loss calculated as a percentage over time shows DsRed-R18-expressing plants lose more water compared to WT control.

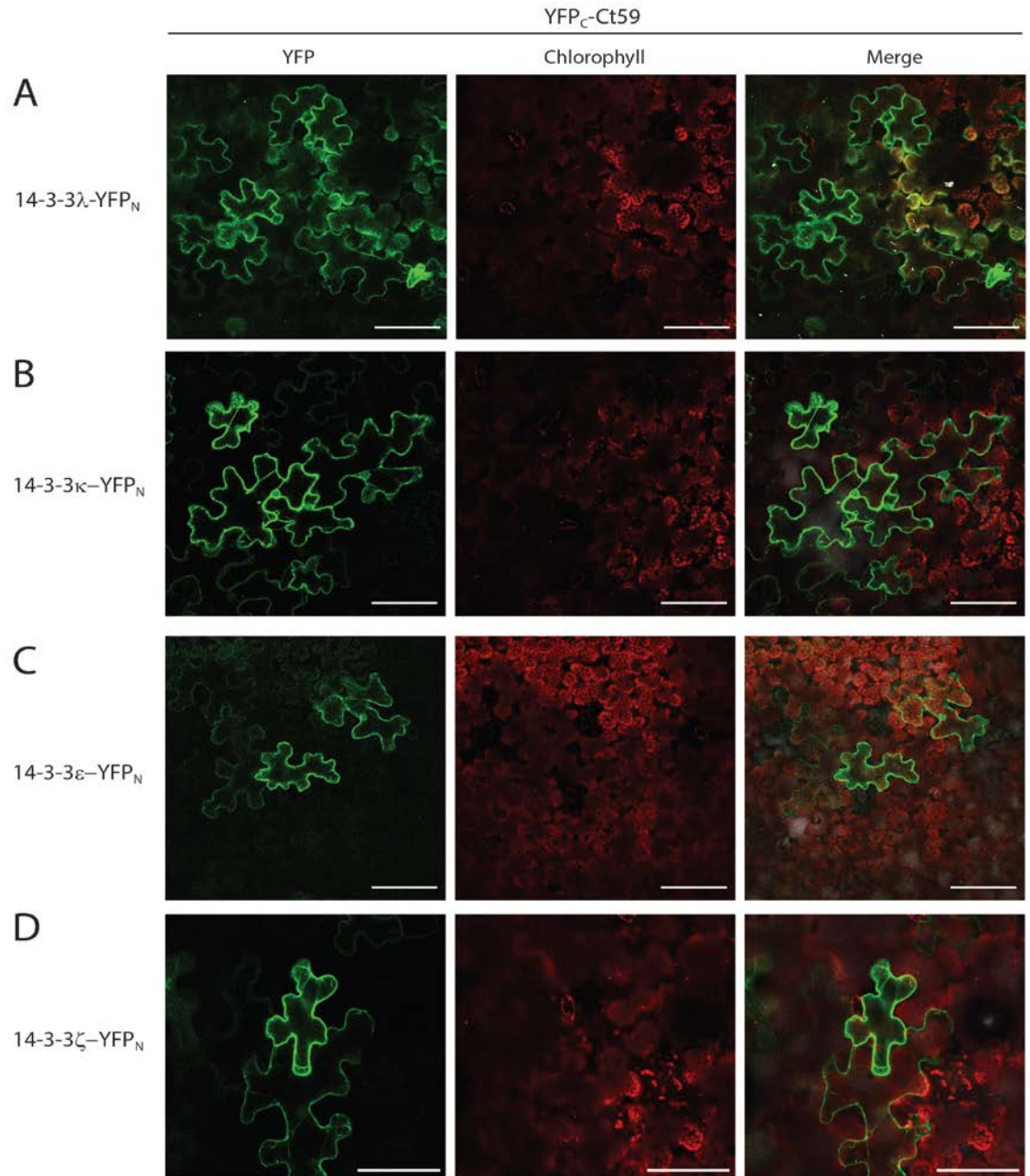
To test if the increased sensitivity to drought is indeed due to R18's interaction with 14-3-3s, I used R18m and fused it to fluorescent protein DsRed (*DsRed-R18m*) and stably expressed it in *Col-gl* from the *35S* promoter. Interestingly, transgenic plants expressing *DsRed-R18m* displayed similar performance during drought stress as WT plants (Figure 3-7A). Thus, our results, though puzzling, suggest that unlike in many other cases, R18's binding with 14-3-3s in guard cells may actually facilitate 14-3-3s' interaction with and activation of H<sup>+</sup>-ATPases as their client proteins, hence resulting in more stomatal opening when R18 is constitutively expressed in guard cells.

This mode of action is reminiscent of the mechanism by which the fungal toxin Fusicoccin (FC) induces stomatal opening. Structural studies showed that FC occupies the bottom of the binding grooves of a 14-3-3 dimer (Figure 3-3C-D), thereby stabilizing the otherwise reversible binding of the C-termini of H<sup>+</sup>-ATPases with the 14-3-3 dimer, which in turn results in activation of the H<sup>+</sup>-ATPases in guard cells, leading to irreversible stomatal opening and subsequent wilting of plants due to excessive loss of water (Korthout and de Boer, 1994; Oecking et al., 1994; Ottmann et al., 2001; Wurtele et al., 2003; Ottmann et al., 2007). A logical inference is that R18 might occupy a space in a 14-3-3 dimer in a manner, to some extent, similar to FC. As a result, R18 may be able to facilitate and/or stabilize 14-3-3's binding with H<sup>+</sup>-ATPases, thereby promoting stomatal opening

### ***Identifying an Additional 14-3-3-Binding Protein***

The C-terminus of PMA2 interacts with 14-3-3. In the presence of fusicoccin, the dissociation constant is measured to be 14nM (Christian Ottmann et al., 2007). Because the wildtype strength of this interaction is already quite strong but is subject to modulation via mutation or small molecule I decided to investigate the C-terminal portion of PMA2 for binding to 14-3-3. Specifically, I used PMA2's Arabidopsis orthologue, AHA2 (AT4G30190.2), rationalizing that an Arabidopsis 14-3-3 $\lambda$  isoform will have greater affinity for an Arabidopsis proton pump.

The C-terminal 59 amino acids (CT59) from AHA2 were fused with YFP<sub>C</sub>. Driven by the CaMV 35S promoter, YFP<sub>C</sub>-CT59 shows positive interaction with 14-3-3 $\lambda$ -YFP<sub>N</sub> (Figure 3-8A). Additionally, YFP<sub>C</sub>-CT59 successfully interacts with three other isoforms: 14-3-3 $\zeta$ , 14-3-3 $\kappa$  and 14-3-3 $\epsilon$  (Figure 3-8A-C).



**Figure 3-8 CT59 Interacts with 14-3-3 Isoforms**

(A) YFP<sub>C</sub>-CT59 interacts with 14-3-3 $\lambda$ , (B) 14-3-3 $\kappa$ , (C) 14-3-3 $\epsilon$ , and (D) 14-3-3 $\zeta$ . Bars = 100 $\mu$ m.

### ***CT59 Contains Multiple 14-3-3 Binding Sites***

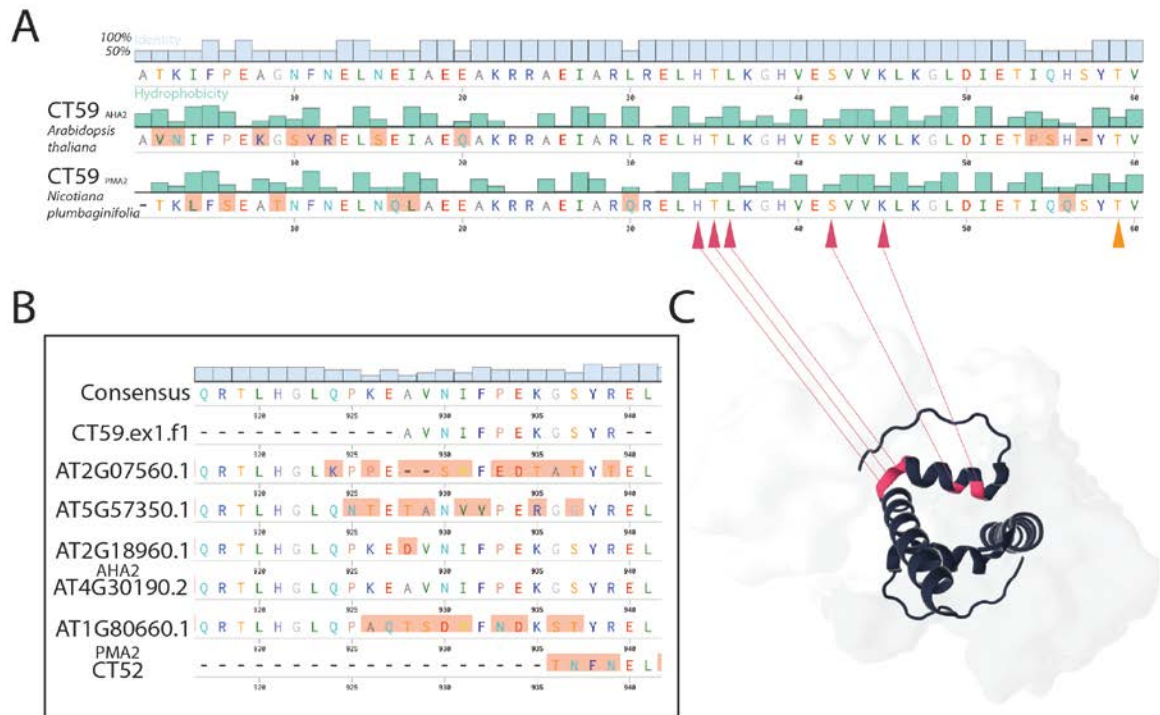
Structural studies with human 14-3-3zeta in complex with R18 showed that the five residues (WLDLE) involved in binding 14-3-3 occupies the bottom of the groove of each monomer (Petosa et al., 1998). Overlay of these two structures showed that the space WLDLE overlaps with that of FC in the groove of 14-3-3 but also likely interferes with the binding of the last two or three residues of the C-terminus of H<sup>+</sup>-ATPases with 14-3-3 (Figure 3-3A). Hence, based on the available protein structural information, it is rather difficult to infer if R18 would mimic FC in stabilizing H-ATPase or competing against its binding with 14-3-3 binding. However, our genetic data strongly indicate that R18 stabilizes H-ATPase-14-3-3 binding, since transgenic lines expressing Ds-Red-R18 were more sensitive to drought stress, whereas lines expressing the mutant version of R18 had no differences compared with untransformed control plants (Figure 3-7A-B). One possible explanation is that Ct59 contains 14-3-3-binding sites in addition to the C-terminal YDV-COOH (YpTV-COOH in endogenous H<sup>+</sup>-ATPases).

To test this hypothesis, I decided to make a series of deletion/truncation Ct59 mutants and test if they can still bind 14-3-3s. To facilitate this process, I first compared the Arabidopsis CT59 of AHA2 to that of C-terminal 59 amino acids of PMA2 using Benchling with the Clustal Omega algorithm. Alignment of these two sequences identified 41 identical sites (including the last three residues) with 65% identity (Figure 3-9A). Because the last three amino acids were shown to be critical for the mode III binding in *Nicotiana plumbaginifolia* PMA2 at T947 (Coblitz et al.,

2006; Fuglsang et al., 1999; Ganguly et al., 2005; Maudoux et al., 2000), I first deleted the three C-terminal residues including the phosphomimetic aspartic acid (Figure 3-9A, orange arrow). BiFC analysis with this new construct designated “CT56” showed that CT56 is still able to interact with 14-3-3 (Figure 3-10A-B). This indicates that there is at least one additional 14-3-3-binding site in CT59.

Ottmann et al. showed that individual mutation H930A, T931A, L932A, S938E, K943A in CT52 of PMA2 abrogate protein interaction with 14-3-3 (Figure 3-9A,C red arrows) (Christian Ottmann et al., 2007). These sites are conserved in CT59 and occur in the last small helix before the tail. Consequently, I mutated all three residues (H, T and L) to alanine and tested this new construct with an intact C-terminus. New construct was designated CT59m. I also truncated the last 3 residues and called this CT56m (Table 1Table 5). Interestingly, both CT59m and CT56m were still capable of interacting with 14-3-3 as reported by positive results from BiFC analysis. This suggests the existence of other 14-3-3-interacting site in CT56m (Figure 3-10C-D).



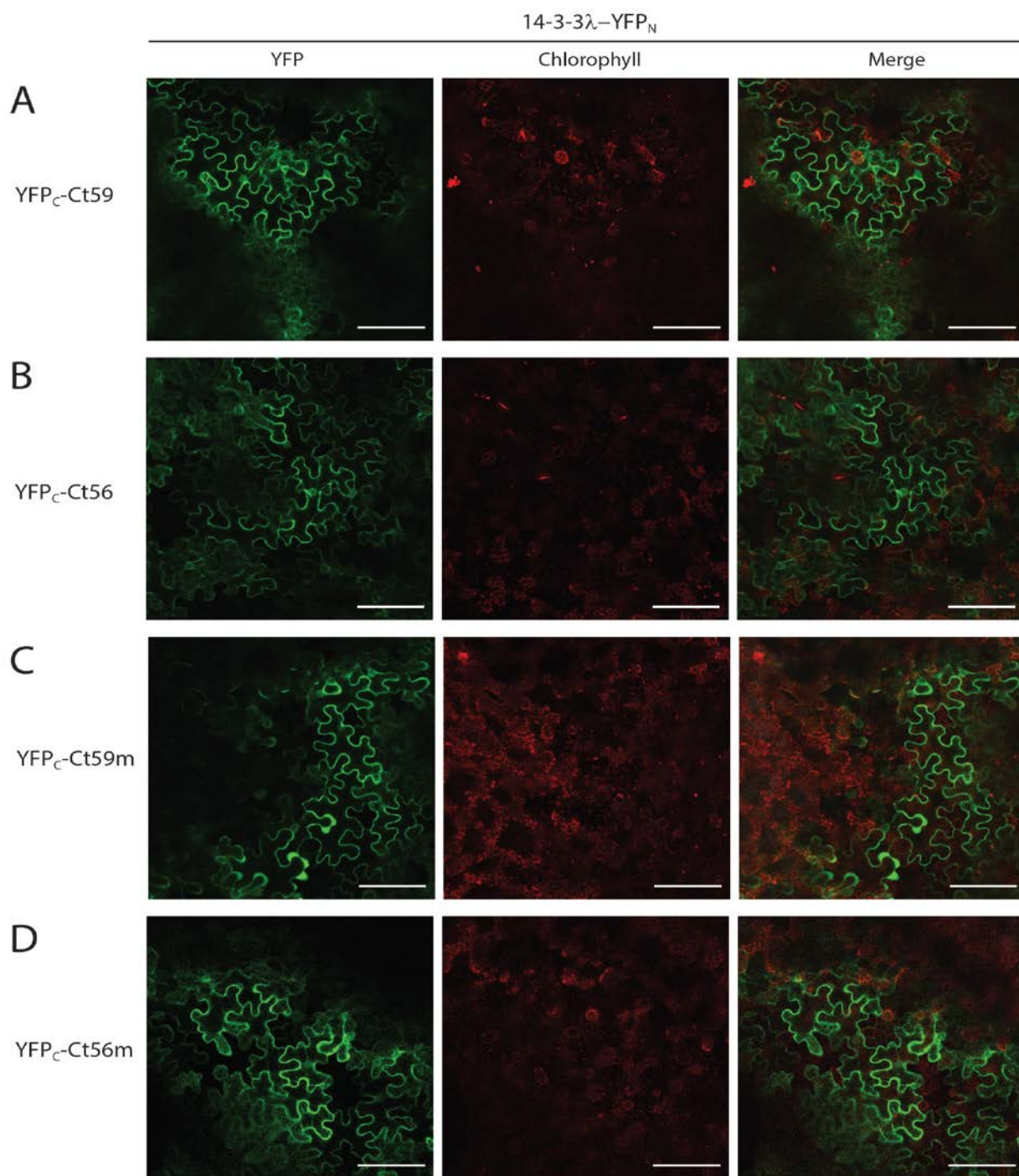


**Figure 3-9 Alignment and Mutations of Conserved Residues in C-termini of Some Plant H<sup>+</sup>-ATPases**

(A) Alignment of PMA2 orthologues from *Arabidopsis thaliana* and *Nicotiana glauca*. Overall pairwise identity is 68% with 41 identical sites. Sites with red arrows are mutations shown to be also critical for 14-3-3 interaction in a previous report (Ottmann et al., 2007). (B) Alignment of *Arabidopsis thaliana* H<sup>+</sup>-ATPase proteins with the first fragment encoded by exon1 of CT59, “CT59.ex1.f1” performed using clustal omega. (C) The CT52 structure showing positions of the mutations indicated by arrows in the pairwise alignment (A) .

To identify additional 14-3-3-interacting sites, mutants were created by splitting CT59 into two fragments based on its exonic sequence. “CT59.ex1” contains the N-terminal 29 amino acids of CT59. “CT59.ex2” contained the C-terminal 31 amino acids of CT59 (Table 5). The same arginine residue encoded by a codon in the exon-exon boundary was present in both fragments. The mutated residues are numbered with respect to the full length AHA2. Fluorescence was detected for both of these constructs in three independent BiFC analysis, suggesting an interaction between each of them with 14-3-3 $\lambda$  (CT59.ex1.f1 observed in Figure 3-14). Intriguingly, these two fragments also gave positive BiFC results when co-expressed with human 14-3-3 $\zeta$  (Figure 3-11A-C).

A shorter fragment CT59.ex1.f2 was further derived from CT59.ex1. This fragment contained 20 amino acids (AVNIFPEKGSYRELSEIAEQ, Table 5). When the YFP<sub>C</sub>-tagged construct was co-transiently expressed with 14-3-3 $\lambda$ -YFP<sub>N</sub>, YFP fluorescence was observable (Figure 3-11C), again suggesting an interaction with 14-3-3s. Eight amino acids were further removed from CT59.ex1.f2 to yield YFP<sub>C</sub>-CT59.ex1.f1, and surprisingly this 12 amino acid (AVNIFPEKGSYR) was also capable of interacting with 14-3-3 $\lambda$ -YFP<sub>N</sub> (Figure 3-12A-C). To further confirm the interaction between the 12 amino acid peptide and 14-3-3, I created YFP<sub>N</sub>-CT59.ex1.f1 and found that it also interacted with 14-3-3 $\lambda$ -YFP<sub>C</sub> (Figure 3-12A,C).



**Figure 3-10 Interaction between 14-3-3 $\lambda$  and CT59 derivatives**

YFP Fluorescence reporting in vivo interaction was observed between 14-3-3 $\lambda$ -YFPn and YFP<sub>C</sub>-CT59 (A), or YFP<sub>C</sub>-CT59 interacts with 14-3-3 $\lambda$  (B), or YFP<sub>C</sub>-CT56 in which three terminal residues of CT59 (Table 5) were deleted, also interacts. (C) CT59m contains a three-residue mutation (H, T, L) and interacts with 14-3-3 $\lambda$ . (D) The final three residues have been deleted and three residues (H, T, L) have been mutated to alanine (YFP<sub>C</sub>-CT56m) in which Bars = 100 $\mu$ m.

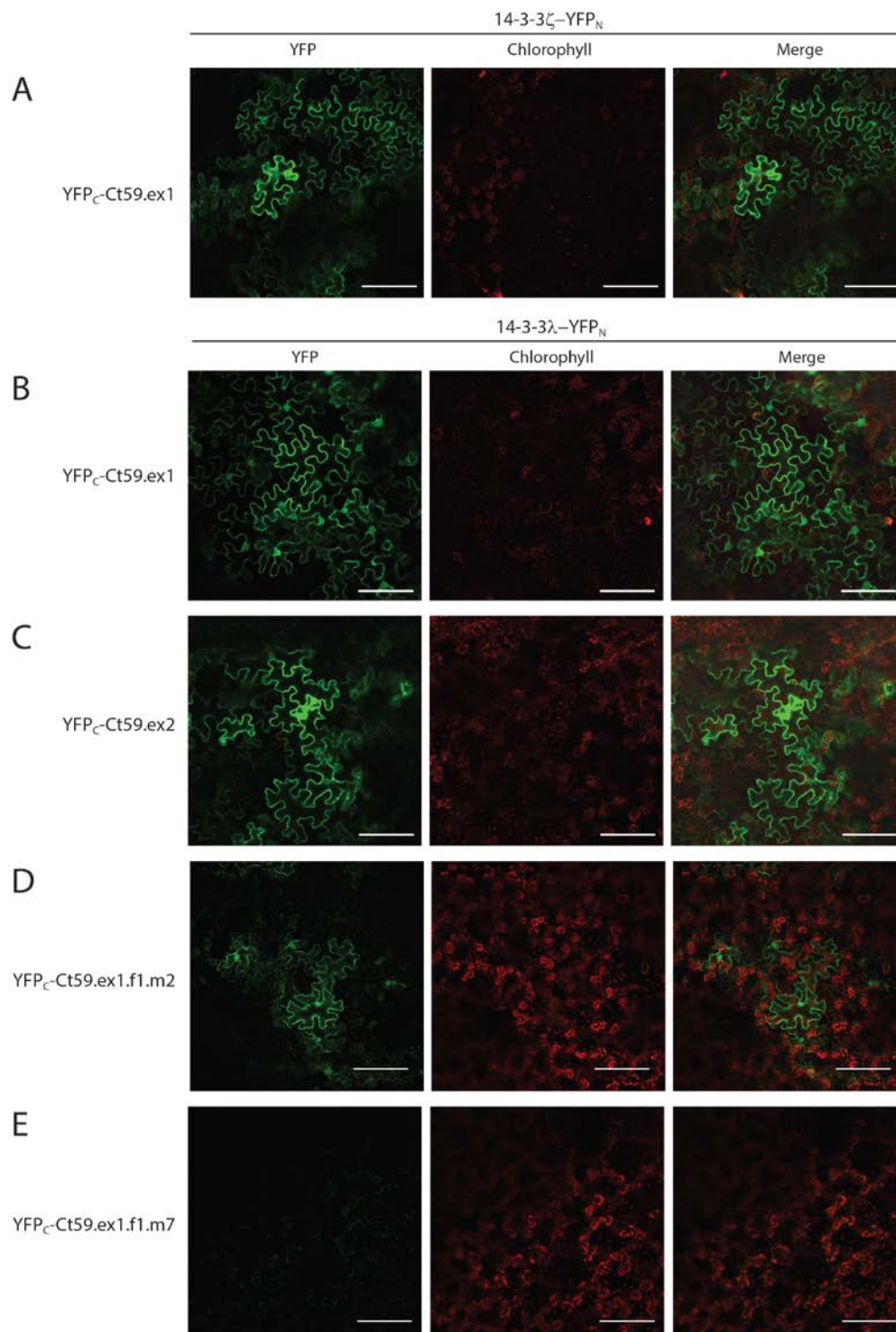
**Table 5 A Summary of Ct59-Derived DNA Constructs and Their Interaction with 14-3-3λ As Reported by BiFC Analysis**

Number	Name	YFPn or YFPc Fusion	Sequence	Mutation	Length(aa)	BiFC Interaction with YFPn-14-3-3λ	BiFC Interaction with YFPc-14-3-3λ
1	CT59	YFPc	AVNIFPEKGSYRELSEIAEQAKRRAEIARLRELHTLKGHVESVVKLGGLDIETPSHYDV	T980D	59	yes	
2	CT56	YFPc	AVNIFPEKGSYRELSEIAEQAKRRAEIARLRELHTLKGHVESVVKLGGLDIETPSH	Δ3aa(C-term)	56	yes	
3	CT59m	YFPc	AVNIFPEKGSYRELSEIAEQAKRRAEIARLRELAAAKGHVESVVKLGGLDIETPSHYDV	H956A, T957A, L958A	59	yes	
4	CT56m	YFPc	AVNIFPEKGSYRELSEIAEQAKRRAEIARLRELAAAKGHVESVVKLGGLDIETPSH	H956A, T957A, L958A Δ3aa(C-term)	56	yes	
5	Ct59.ex1	YFPc	AVNIFPEKGSYRELSEIAEQAKRRAEIAR		29	yes	
6	Ct59.ex2	YFPc	RLRELHTLKGHVESVVKLGGLDIETPSHYDV		31	yes	
7	Ct59.ex1.f2	YFPc	AVNIFPEKGSYRELSEIAEQ		20		
8	Ct59.ex1.f1	YFPc	AVNIFPEKGSYR		12	yes	
9	Ct59.ex1.f1	YFPn	AVNIFPEKGSYR		12		yes
10	Ct59.ex1.f1m2	YFPc	AVNIFPEKGSAA	Y933A, R934A	12	yes	
11	Ct59.ex1.f1m3	YFPc	AVNIGAAKGSYR	F927G, P928A, E929A	12	yes	
12	Ct59.ex1.f1m3	YFPn	AVNIGAAKGSYR	F927G, P928A, E929A	12		yes
13	Ct59.ex1.f1m7	YFPc	AANAAPAAAGSAA	V924A, I926A, F927G, E929A, K930A, Y933A, R934A	12	no	
14	Ct59.ex1.f1(VIK)	YFPc	AANAFPEAGSYR	V924A, I926A, K930A	12	yes	
15	Ct59.ex1.f1(K)	YFPc	AVNIFPEAGSYR	K930A	12	yes	

A surface plot of the 14-3-3-CT52 crystal structure (PDB:2O98) shows interaction between five residues (Thr898, Glu902, Leu903, Leu906 and Ala907) of the N-terminal regions of CT52 with human 14-3-3 $\zeta$  (Figure 3-13B-C) (Christian Ottmann et al., 2007). This is part of an autoinhibitory region of CT52 known for dimerizing (Dambly & Boutry, 2001; Jelich-Ottmann, Weiler, & Oecking, 2001). I also mutated two of the interacting residues Tyr933, Arg934 shown by the 14-3-3 $\zeta$ -CT52 crystal structure (Christian Ottmann et al., 2007) to Ala, and the resulting fragment “CT59.ex1.f1.m2” still retained interaction with 14-3-3 $\lambda$ -YFP<sub>N</sub> in BiFC (Figure 3-11D).

To identify other residues important for mediating the interaction between 14-3-3 $\lambda$  with CT59.ex1.f1, corresponding sequences of five Arabidopsis H<sup>+</sup>-ATPases were aligned. Three amino acids, F927, P928, E929, show the highest consensus score and were thus mutated to Gly, Ala and Ala, respectively (Figure 3-9B). This construct “CT59.ex1.f1.m3” when used for BiFC analysis also showed interaction with 14-3-3 $\lambda$  (Figure 3-12B). Interaction was still detected when CT59.ex1.f1m3 was fused with YFP<sub>N</sub> instead of YFP<sub>C</sub> and co-expressed with 14-3-3 $\lambda$ -YFP<sub>C</sub> (Figure 3-12D).



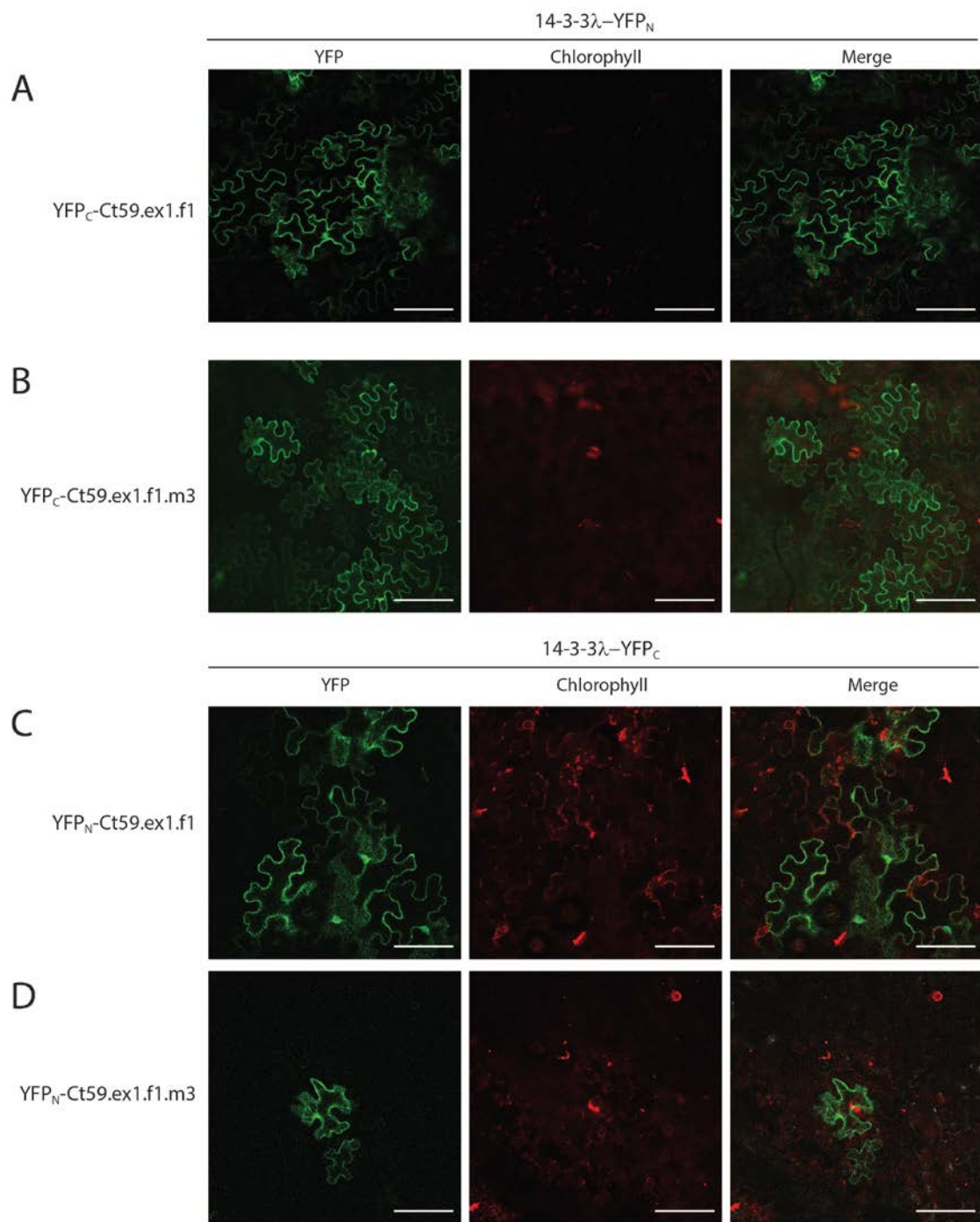


**Figure 3-11 Interaction of CT59 Mutants with 14-3-3**

All indicated DNA constructs were transiently co-expressed with *14-3-3λ-YFP<sub>N</sub>* or *14-3-3ζ-YFP<sub>N</sub>* in *N. benthamiana*. YFP Fluorescence indicates interaction. (A) *Ct59.ex1* (which encodes the first 29 aa of Ct59) + *14-3-3λ*; (B) *CT59.ex2* (which encodes the last 31 aa of Ct59) + *14-3-3λ*; (C) *Ct59-ex1.fl* (which encodes only 12 amino acids of Ct59) + *14-3-3λ*; (D) *Ct59-ex1.fl.m2* (which encodes only 12 amino acids of Ct59 with mutations in two residues) + *14-3-3λ*; (E) *Ct59-ex1.fl.m7* (which encodes only 12 amino acids of Ct59 with mutations in seven residues); (F) *CT59.ex1* + *14-3-3ζ*. Bars = 100μm.



To generate a CT59.ex1.f1 mutant that is unable to interact with 14-3-3, I made mutant constructs “*CT59.ex1.f1.K*” that encodes the 12 amino acids with only one mutation K930A , and “*CT59.ex1.f1.VIK*” that encodes the 12 amino acids with three mutations, V924A, I926A, K930A. However, fluorescence still resulted when transiently co-expressed with *14-3-3λ*. (Figure 3-13A-B). I then mutated seven of the 12 residues (V924A, I926A, F927G, E929A, K930A, Y933A, R934A) encoded by this construct to Ala, resulting in construct CT59.ex1.f1.m7. Transient co-expression of this YFP<sub>C</sub>-CT59.ex1.f1.m7 with 14-3-3λ-YFP<sub>N</sub> failed to produce fluorescence (Figure 3-12E), indicating this mutant fusion protein is unable to interact with 14-3-3λ, and further demonstrating that all other interactions are true and specific.



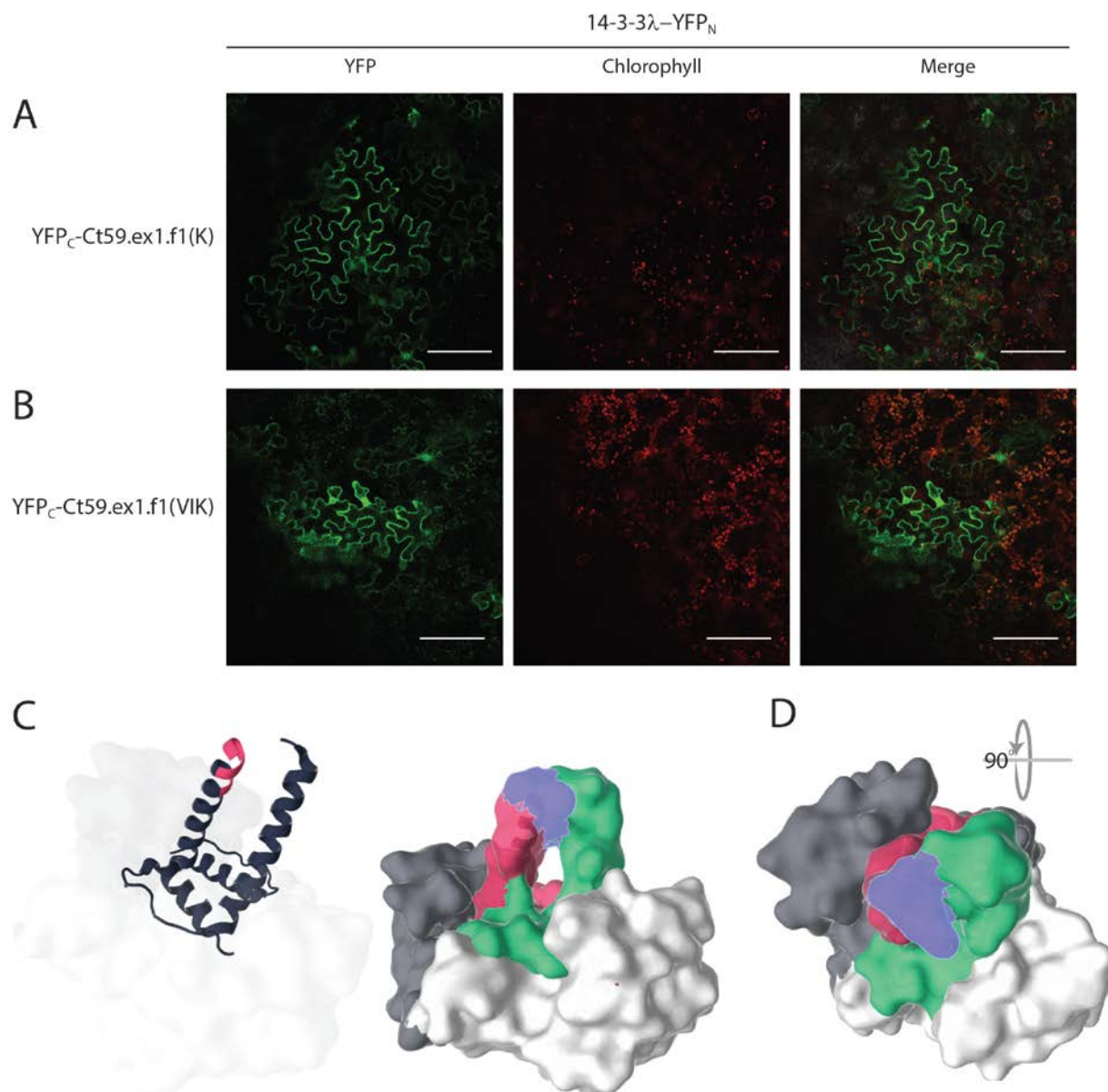
**Figure 3-12 Confirmation of BiFC for Two Ct59-derived Constructs by Switching the YFP Fragment in BiFC**

The two indicated DNA constructs were transiently co-expressed with *14-3-3λ-YFP<sub>N</sub>* (A and B) or *14-3-3λ-YFP<sub>C</sub>* (C and D) in *N. benthamiana*. YFP Fluorescence indicates interaction.

Bars = 100μm

### ***The Interaction of R18 and CT59 via 14-3-3 Proteins***

R18 and Ct59 were tested for their ability to interact with each other. BiFC was used to confirm this interaction transiently in *N. benthamiana*. The presence of fluorescence between R18-YFP<sub>N</sub> and YFP<sub>C</sub>-CT59 confirms their interaction (Figure 3-14A). 14-3-3 isoforms act as a scaffold for the interactions of R18 and CT59 because there is no evidence for these two proteins interacting directly, but multiple experiments confirming their interaction each with 14-3-3s. In addition to full length CT59, fluorescence was also generated between R18 and CT59.ex1, a 20-amino acid truncation of CT59 (Figure 3-14B). Because previous results showed truncated peptides of CT59 interacting with a PMA2 monomer, the fluorescence with R18 provides additional evidence that R18 and CT59 interact via a 14-3-3 dimer intermediary instead of with each other.



**Figure 3-13 CT59 Mutants Interact with 14-3-3 in BiFC Probably by Interacting with Autoinhibitory Region II of CT59**

(A-B) The indicated DNA constructs were transiently co-expressed with 14-3-3λ-YFP<sub>N</sub> in *N.*

*benthhamiana*. YFP Fluorescence indicates interaction. Bars = 100μm

(C) The autoinhibitory region contains CT52 helices that interact with each other in an antiparallel manner. Surface model shows contact between the two helices. (D) Model rotated 90° to show top and interacting helices.

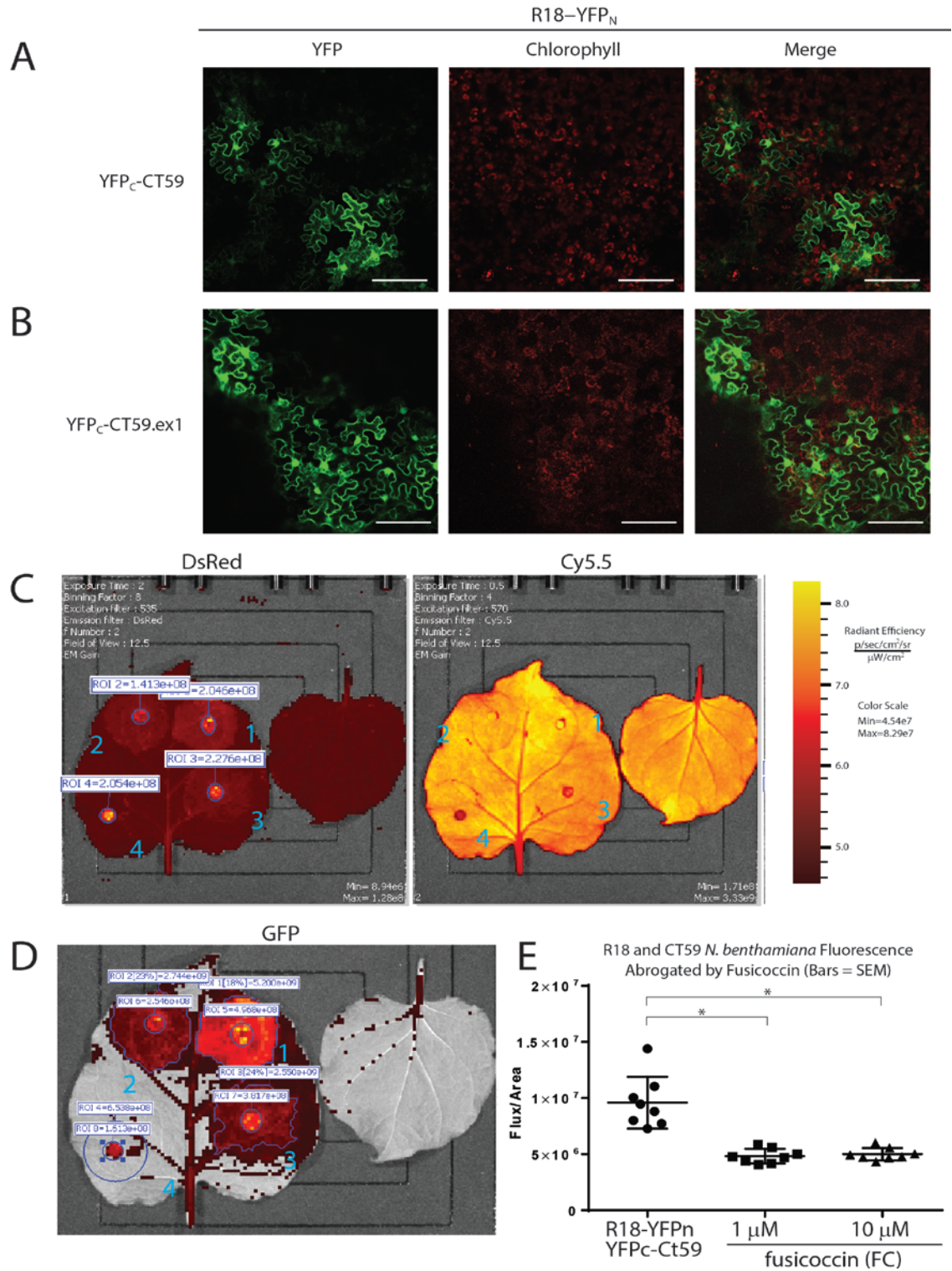
My results on Ct59 mutagenesis and the subsequent BiFC analysis of various Ct59 mutants with 14-3-3 demonstrate that there are indeed multiple 14-3-3 binding sites in Ct59. Although the number and precise topography of the binding from Ct59 that occur when Ct59 and R18 are co-expressed and occupy the same 14-3-3 groove remain unknown, these results provide a mechanistic explanation why R18 and Ct59 in R18-YFP-Ct59 fusion protein can sequester 14-3-3s away from H<sup>+</sup>-ATPases in guard cells, thereby inducing stomatal closure and drought tolerance.

### ***Can Fusicoccin Compete against R18 in Binding to 14-3-3s?***

Fusicoccin is a small molecule known to facilitate 14-3-3-PMA2 interaction by locking the C-terminus loop region of PMA2 into the 14-3-3 binding groove. To test if R18 occupies the same or similar space as Fusicoccin, and verify that the observed interaction between R18 and Ct59 in BiFC is via binding to the same 14-3-3 dimer (as scaffold), I used Fusicoccin as a competitor for 14-3-3-binding in BiFC. Two days after infiltration of *Agrobacterium* cells harboring R18-YFP<sub>N</sub> and YFP<sub>C</sub>-CT59, water, or fusicoccin dissolved in water at 1 or 10 $\mu$ m concentration was infiltrated into the same leaves of *N. benthamiana*. The following day fluorescence was measured using an IVIS Lumina XR (Caliper Life Sciences). The benefit of using IVIS is that whole-spot fluorescence may be quantified, a feature unavailable to confocal microscopy. Image analysis performed in Living Image software (v4.5, Perkin Elmer) allowed total area of fluorescing regions to be inscribed, and total fluorescence measured and autofluorescence from the same area subtracted. Two

filter sets, dsRed and Cy5.5, confirmed the presence of YFP fluorescence and the absence of autofluorescence (Figure 3-14C). Spots infiltrated with either 1 or 10 $\mu$ m fusicoccin showed significantly less YFP fluorescence compared to water treated spots, and there was no significant difference observed between leaf spots treated with 1 or 10 $\mu$ m fusicoccin (Figure 3-14D-E). Given that fusicoccin can facilitate the binding between C-terminus (e.g. Ct59) and 14-3-3s, these results suggest that fusicoccin may indeed compete against R18 for binding 14-3-3s, thereby disrupting the 14-3-3-mediated interaction between R18 and CT59.



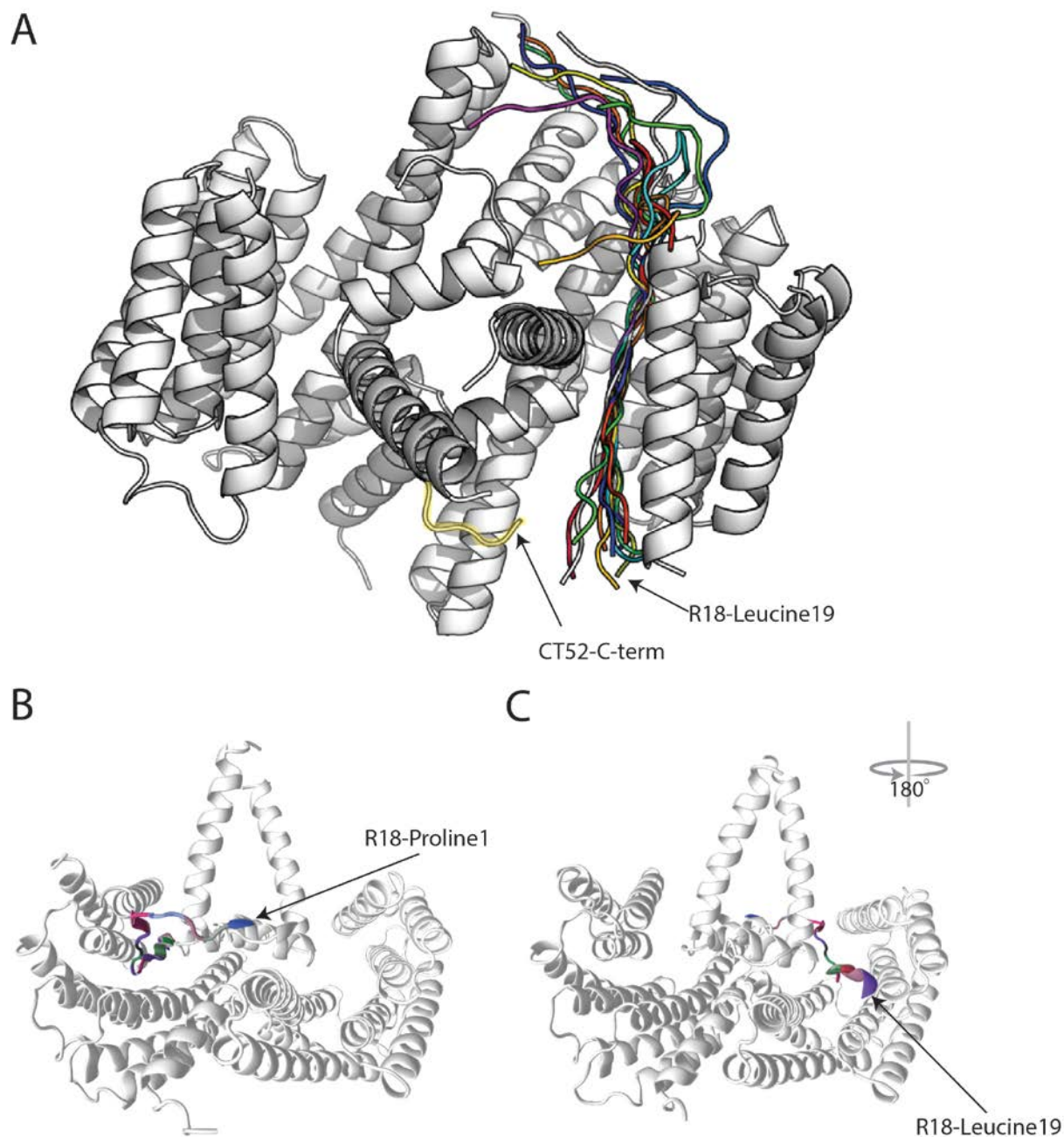


**Figure 3-14 14-3-3-Mediated Interaction between R18 and Ct59 Can Be Reduced by Fusicoccin Treatment**

(A-B) The indicated DNA constructs were transiently co-expressed in *N. benthamiana*. YFP Fluorescence indicates interaction. Bars = 100µm. (C) The IVIS system (Caliper Life Sciences) was used to quantitate fluorescence. No YFP fluorescence detected in dsRed filter set, whereas Cy5.5 detects far-red fluorescence and chlorophyll a. Numbered spots indicated infiltrations containing no fusicoccin (1), 1µM or 10µM FC (2, 3, respectively) or buffer(4) (D) Competition for 14-3-3 binding using fusicoccin. Fluorescence from BiFC and autofluorescence in the infiltrated leaf areas were measured and quantified (blue outline circles) one day after fusicoccin treatment using IVIS. Spots 1-4 were first infiltrated with agrobacterium cells harboring *R18-YFP<sub>N</sub>* and *YFP<sub>C</sub>-CT59*, and two days afterwards further infiltrated with 0, 1 or 10 µM fusicoccin, respectively. (E) A one-way ANOVA analysis shows significant difference ( $p < 0.05$ ) between areas 2 and 3 infiltrated with fusicoccin compared to area 1 infiltrated with water, and no significant difference between areas infiltrated with 1 or 10µM fusicoccin. Error bars are SEM.

### ***Can R18 Bind in the 14-3-3 $\lambda$ Groove in the Presence of the CT52 Peptide?***

A crystal structure exists showing the C-terminal 52 peptides from PMA2 interacting with tobacco 14-3-3c in the presence of fusaric acid (Christian Ottmann et al., 2007). I thus used this structure as a base model to explore whether the binding groove of a 14-3-3 monomer is able to simultaneously host CT52 and R18 peptides. Fusaric acid was first removed from the structure, then R18 and dimerized 14-3-3c were fitted using FlexPepDock (London, Raveh, Cohen, Fathi, & Schueler-Furman, 2011; Raveh, London, & Schueler-Furman, 2010). It was inferred that only when the CT52 loop region containing about the C-terminal 27 amino acids is removed from the model is R18 able to fit into the groove (Yizhou Yin (Moult Group at IBBR) personal communication, 2017). Removing the CT52 loop region has little perturbation to the 14-3-3 dimer, which is consistent with the rigidity previously noted with 14-3-3 dimers (Obsil & Obsilova, 2011; Yaffe, 2002).



**Figure 3-15 R18 Binding in the 14-3-3 Groove in the Presence of CT52**

(A) Ten overlaid models of R18 fitting into a 14-3-3c binding groove representing the lowest energy among 200 simulations from FlexPepDock. The entire 20 amino acid-long R18 peptide is too big for the groove but residues WLDLE (10-14) bind with high affinity

consistent with the crystal structure ( $\approx 80\text{nM}$ ) (Petosa et al., 1998). To test for R18 (1-20aa) groove-binding, the loop region of CT52 (27aa), previously engaged in the groove, needed to be removed from the structure. (B-C) Terminal residues of R18 like proline-20 (B) and leucine-19 (C) do not bind in the groove and do not have a fold.

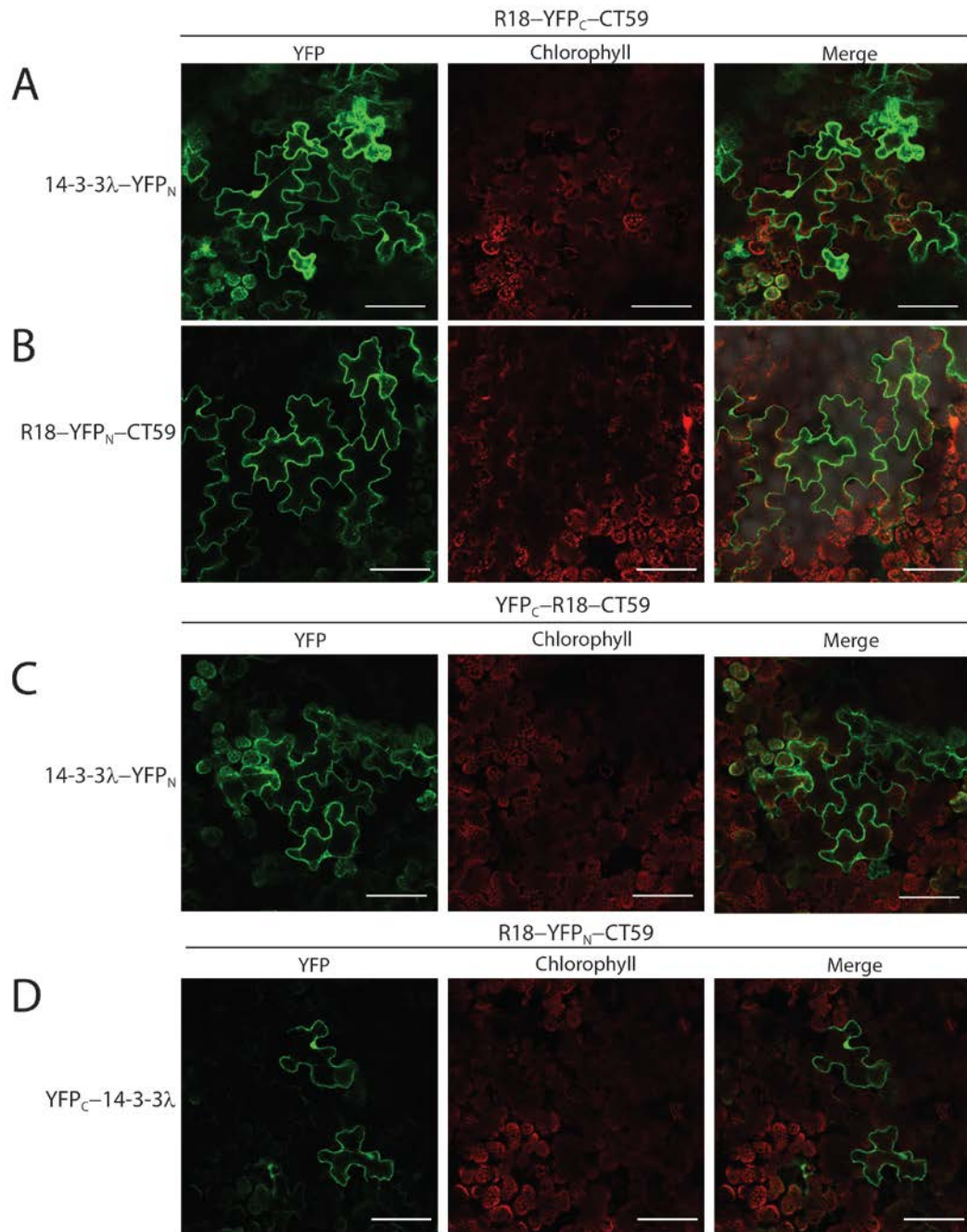
The top ten lowest energy models were overlaid on each other (Figure 3-15A). An alignment in the groove between the models shows little movement between the amphipathic sequence WLDLE and the 14-3-3 groove, suggesting multiple alignments in this space have the lowest energy. This is consistent with the crystal structure showing that these five residues also interact most tightly within the 14-3-3 groove. The groove cannot accommodate the entire R18 peptide thus terminal residues like proline-20 (Figure 3-15B) and leucine-19 (Figure 3-15C) do not bind with specificity. Hence, the C-terminal loop of CT52 and R18 are unlikely to co-occupy the 14-3-3 binding groove; however, the model leaves open the possibility that specific residues like WLDLE from R18 may partially bind while also accommodating residues in CT52 from PMA2 or in C-termini (such as Ct59) from other H<sup>+</sup>-ATPases in the 14-3-3 binding groove.

#### ***Chimeric R18-YFP-CT59 Binds and Other Derivatives Bind 14-3-3 Isoforms***

Given that both R18 and CT59 can bind 14-3-3 $\lambda$  and other isoforms, and that their binding position may be different, a divalent chimeric protein containing R18 and Ct59 in cis spaced by a linker may be able to effectively sequester 14-3-3s from their endogenous client proteins such as H<sup>+</sup>-ATPases. I thus made a chimeric DNA construct R18-YFP-Ct59 (designated RYC), considering that 1) YFP would act as a linker connecting both R18 and CT59 allowing each to bind a 14-3-3 monomer with flexibility, 2) the fluorescence generated by YFP would allow the expression of the fused protein to be conveniently monitored at the cellular and subcellular levels.

Before deploying RYC as a 14-3-3-trapping protein, I first assessed if such a protein can interact with multiple 14-3-3 isoforms as predicted. Briefly, I replaced YFP in the RYC or YRC with either YFP<sub>N</sub> or YFP<sub>C</sub> to enable BiFC assays. RY<sub>C</sub>C was first tested for its interaction with 14-3-3 $\lambda$ -Y<sub>N</sub>, which was positive in BiFC (Figure 3-16A). RY<sub>C</sub>C also interacted with RY<sub>N</sub>C (Figure 3-16B), consistent with the prediction that either or both these molecules can bind the same 14-3-3 dimer. This self-interaction was not tested with CT59. However, based on earlier confirming interactions with 14-3-3 $\lambda$ , and understanding that a crystallization model shows dimerized 14-3-3c interacting with two CT52 termini (Christian Ottmann et al., 2007), I hypothesize CT59 to also exhibit similar dimerization behavior like R18. I also showed that Y<sub>C</sub>RC interacted with 14-3-3 $\lambda$ -YFP<sub>N</sub> (Figure 3-16C) and RY<sub>N</sub>C, interacted with 14-3-3 $\lambda$ -Y<sub>N</sub> RY<sub>C</sub>C (Figure 3-16D). In addition, to determine if R18-YFP<sub>C</sub>-CT59 (RY<sub>C</sub>C) interacts with 14-3-3 in a non-isoform-specific manner, I tested RY<sub>C</sub>C with YFP<sub>N</sub>-tagged five different 14-3-3 isoforms including human 14-3-3 $\zeta$  and observed YFP fluorescence indicative of interaction (Figure 3-17A). Thus, these results indicate that *cis*-linked R18 and Ct59 with a spacer can bind (and sequester) the same 14-3-3 dimer and suggest that YFP can probably also act as a flexible linker to allow both R18 and CT59 to simultaneously engage a 14-3-3 dimer and report where and how much the fusion protein is expressed.



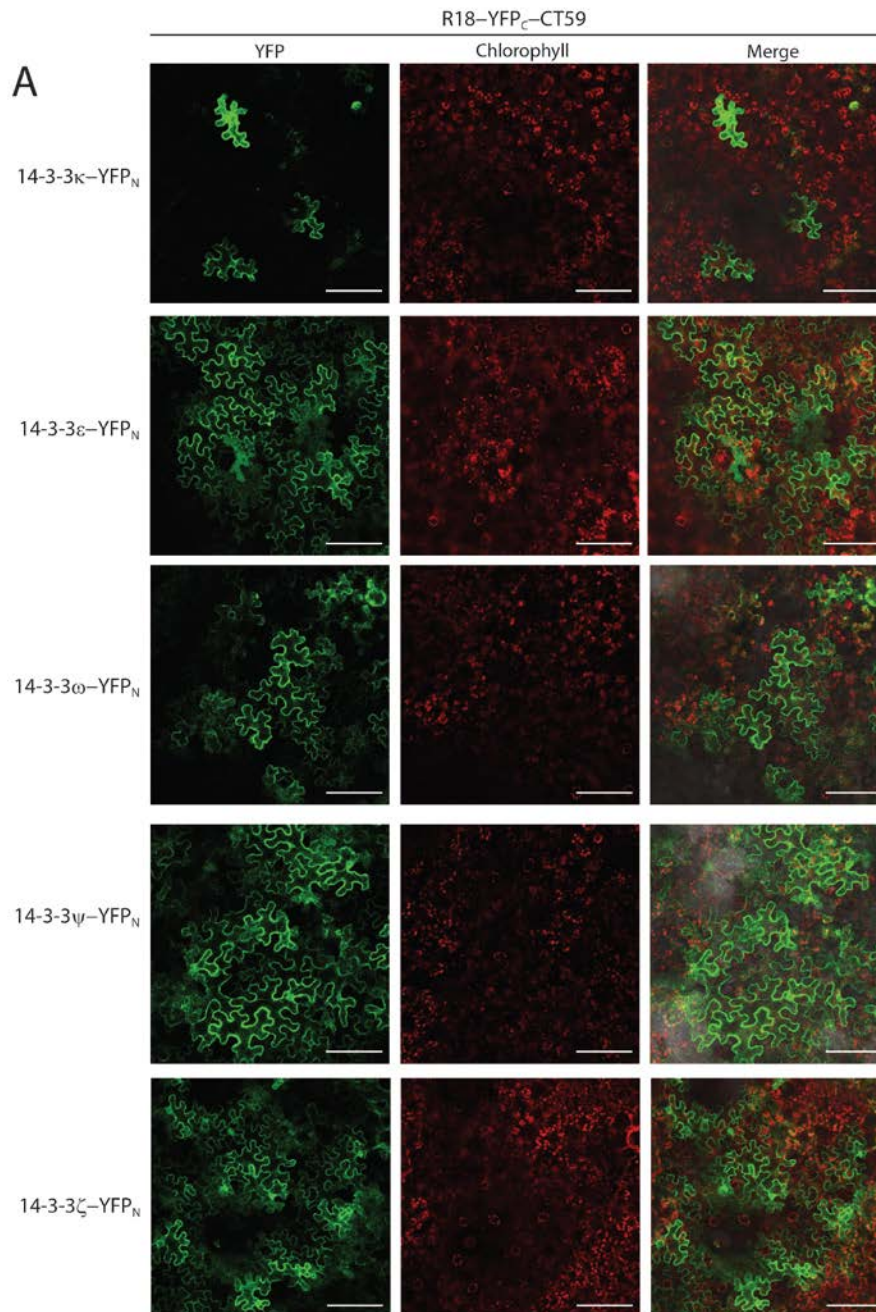


**Figure 3-16 Both RY<sub>C</sub>C and Y<sub>C</sub>RC Interact with 14-3-3λ**

The indicated DNA constructs were transiently co-expressed in *N. benthamiana*. YFP Fluorescence indicates interaction. Bars = 100μm (A) R18-YFP<sub>C</sub>-CT59 (RY<sub>C</sub>C) + 14-3-3λ-



YFP<sub>N</sub>. (B) RY<sub>C</sub>C + R18-YFP<sub>N</sub>-CT59 (RY<sub>N</sub>C). (C) YFP<sub>C</sub>-R18-CT59 (Y<sub>C</sub>-RY) + 14-3-3λ-YFP<sub>N</sub>. (D) RY<sub>N</sub>C + YFP<sub>C</sub>-14-3-3λ.



**Figure 3-17 RY<sub>C</sub>C Interacts with Five 14-3-3 Isoforms**

The indicated DNA constructs were transiently co-expressed in *N. benthamiana*. YFP Fluorescence indicates interaction. Bars = 100μm. Note, 14-3-3 $\zeta$  is from human and rest isoforms are from Arabidopsis.

### ***A synthetic promoter renders guard cell-specific expression of target proteins***

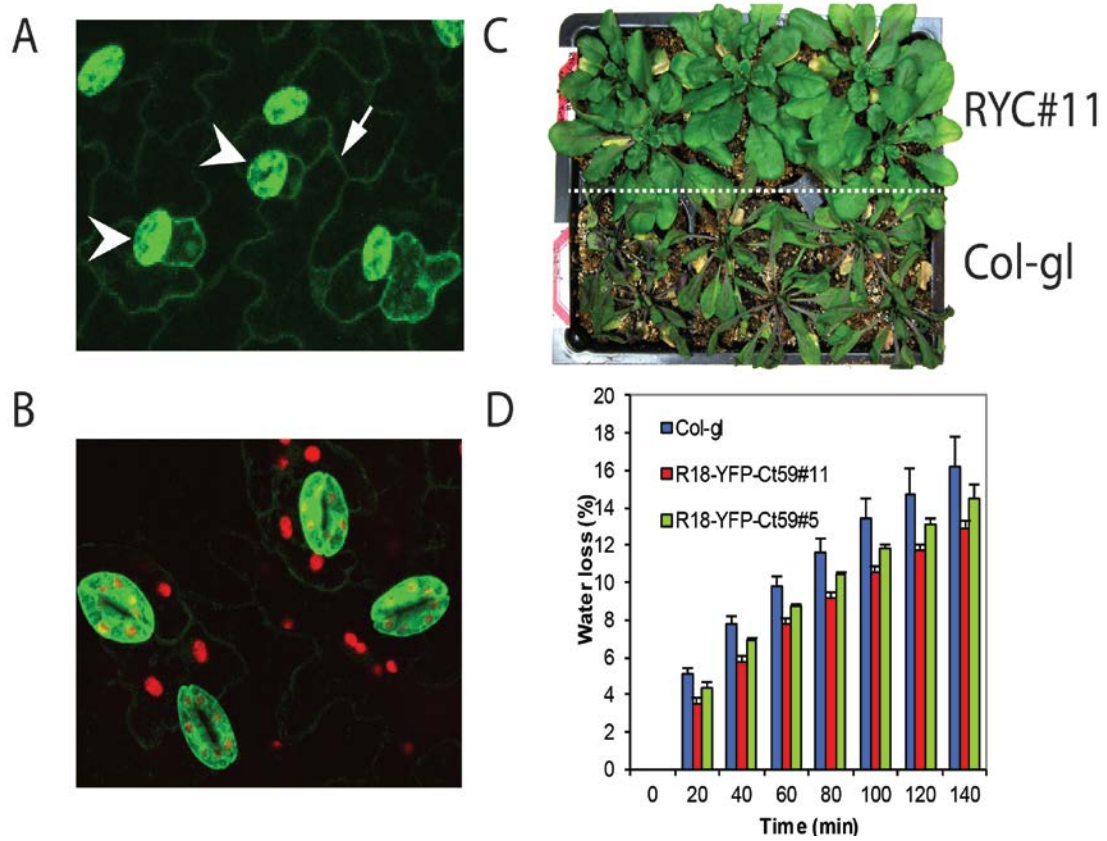
To minimize potential negative impact of constitutive expression of RYC on plant growth and development, I searched for promoters that could render drought-inducible and guard cell-specific expression of RYC. It was reported that a synthetic promoter combining a core element (~250bp) of the drought-inducible (and ABA-responsive) promoter of the Arabidopsis *rd29A* gene (Yamaguchi-Shinozaki and Shinozaki, 1994) and a core element (~250bp) of the guard cell-specific promoter of the potato *kst1* gene (which encodes a potassium inwardly rectifying channel) (Plesch et al., 2001) seemed to confer desirable drought-inducible and guard cell-specific expression of the GUS reporter (Li et al., 2005). Therefore, I made a similar chimeric promoter by combining a drought-inducible promoter element (261bp from -443 to -182bp upstream of the ATG start codon of *rd29A*, which contains 2x TACCGACAT, a drought-responsive element) and a guard cell-specific promoter element (253bp from -253 to -1 bp upstream of the ATG start codon of *Kst1*, which contains 2x TAAAG, a guard-cell-specific element). Stable expression of RYC from this chimeric sequence (named *DG* for simplicity) was barely detectable indicating low promoter activity before and after drought stress (data not shown). However, when placed downstream of the *35S* promoter (*35S-DG*), the resultant chimeric promoter appeared to possess high constitutive and guard cell-specific promoter activity in expressing RYC. YFP signal intensity from RYC in guard cells is ~100X fold higher than that in the neighboring pavement cells (Figure 3-18A-B) in about 80% of the independent transgenic lines. However, drought-stress (8 days without water supply) did not

seemed to further increase RYC expression, presumably because the basal-level expression is already too high.

### ***Expression of R18-YFP<sub>C</sub>-CT59 Increases Drought Tolerance in Arabidopsis***

Confirming R18-YFP<sub>C</sub>-CT59 (RYC) interacts with several 14-3-3 isoforms, I wondered if it could be used for increasing drought tolerance in plants by sequestering away 14-3-3 $\lambda$ . I made and stably expressed RYC in plants driven by the 35S promoter. The inclusion of the full-length YFP sequence gives a convenient means to assess expression levels and localization patterns of the fusion protein. Fluorescence microscopy showed that the fusion proteins are preferentially expressed in guard cells in all transgenic lines (>25) examined (Figure 3-18A-B), presumably because 14-3-3s accumulate at higher levels in guard cells compared to surrounding pavement cells (Leonhardt et al., 2004; Zhu et al., 2009), and 14-3-3-binding may stabilize RYC, resulting in its preferential accumulation in guard cells. Two independent lines with higher levels of guard cell-preferential expression of RYC were tested for drought tolerance. Excitingly, I found that plants of both transgenic lines expressing RYC showed enhanced drought tolerance (one line shown Figure 3-18C), whereas the two transgenic lines expressing YRC showed weak or no obvious enhanced drought tolerance (data not shown), suggesting that RYC may indeed sequester 14-3-3s from H<sup>+</sup>-ATPases, thereby promoting stomatal closure. I also monitored the growth and development of these plants and did not notice any abnormal morphological phenotypes from these transgenic lines (data not shown).

Water-loss measurement showed that detached leaves from two representative T3 homozygous lines transgenic for *35-DG::RYC* had significantly reduced water loss rate compared to the wild-type plants (Figure 3-18D). Thus, my results demonstrate that sequestration of 14-3-3s in guard cells by expression of a bivalent 14-3-3-interacting protein provides a novel and effective strategy to create drought-tolerant plants.



**Figure 3-18 RYC Accumulates in Guard Cells and Enhances Drought Tolerance**

(A) RYC localizes to guard cells (arrowhead) and the plasma membrane (arrow). (B)

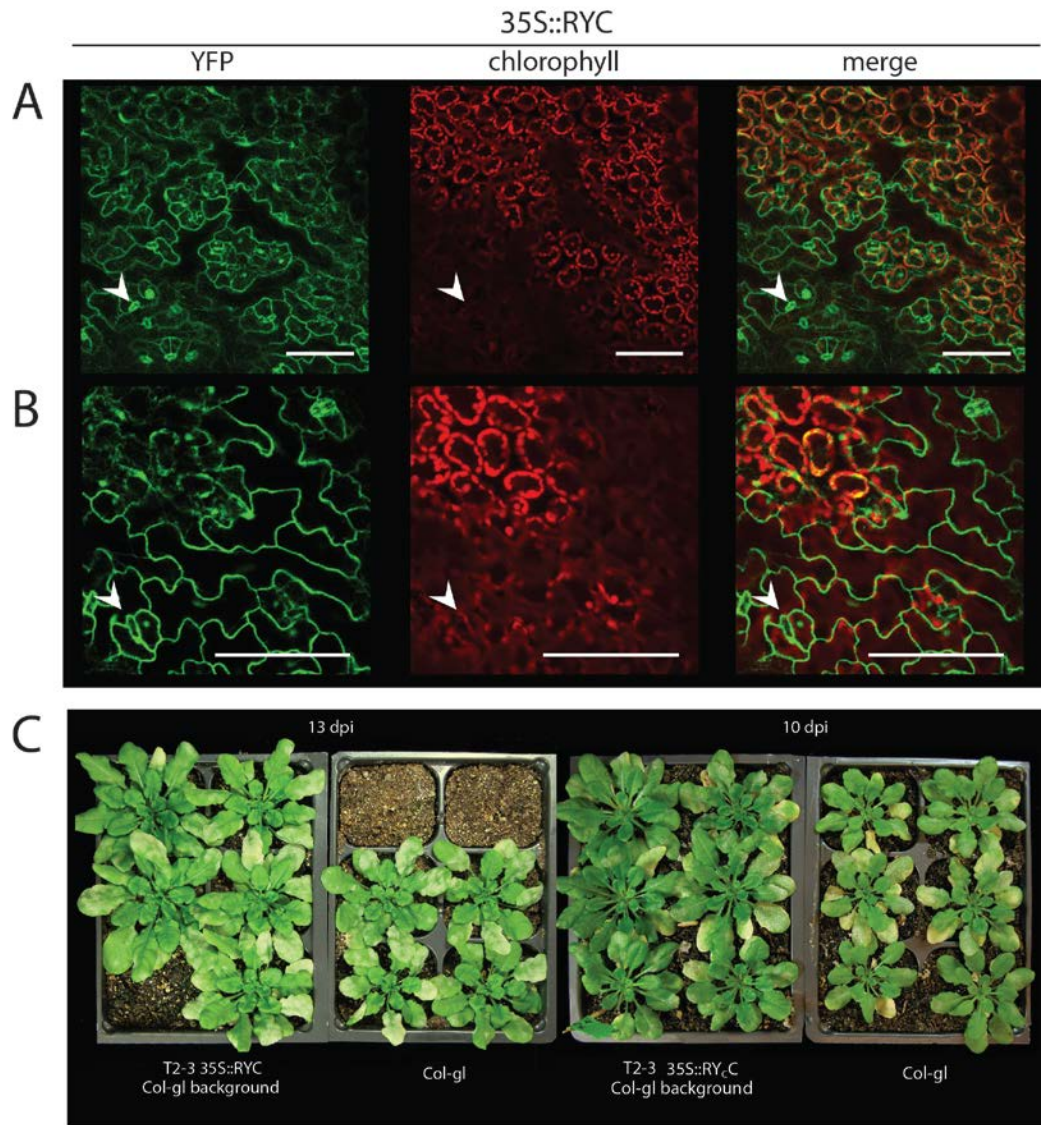
Increased magnification showing fluorescence is specific to guard cell localization. (C)

Transgenic plants expressing RYC are more resistant to control plants Col-gl. (D) Water loss in RYC plants is less than control plants.

***Overexpression of R18-YFP-Ct59 Has No Significant Impact on Basal Resistance to Powdery Mildew***

Because 14-3-3 $\lambda$  has been shown to interact with RPW8.2 (X. Yang et al., 2009), I assessed whether RYC could play a role in basal resistance against powdery mildew. I also generated Arabidopsis Col-gl T1 and T2 plants expressing RYC from the 35S promoter or the RPW8.2 promoter (as shown in the figure?). RYC was found to be expressed in pavement and preferentially guard cells in the leaf epidermis (Figure 3-19A-B). T2 plants from 3-5 selected independent T1 lines were inoculated with Gc UCSC1 and assessed for their disease susceptibility compared to Col-gl plants. No significant phenotypic differences were observed between any of these transgenic lines and WT controls (Figure 3-19C). I also made Col-gl transgenic lines expressing RY<sub>C</sub>C from the 35S promoter and found no significant phenotypic difference compared to controls (Figure 3-19C). Quantification of disease susceptibility by counting total number of fungal spores per mg fresh infected leaf tissue also did not show significant difference between transgenic plants and WT control (Figure 3-20D).





**Figure 3-19 Expression of RYC in Leaf Epidermal Pavement Cells of Arabidopsis Col-gi Plants**

(A) Expression of the chimeric protein R18-YFP-CT59 (RYC) from the constitutive 35S promoter in the PM of pavement cells. Guard cells (arrowhead) are also observed but RYC probably not guard cell specific due to constitutive expression. (B) 2x zoomed in image of RYC in the PM of pavement cells and guard cells (arrowhead). Scale bars are 100 $\mu$ M. (C) Examples of disease phenotypes of transgenic plants expressing RYC or RYC<sub>c</sub> from the 35S

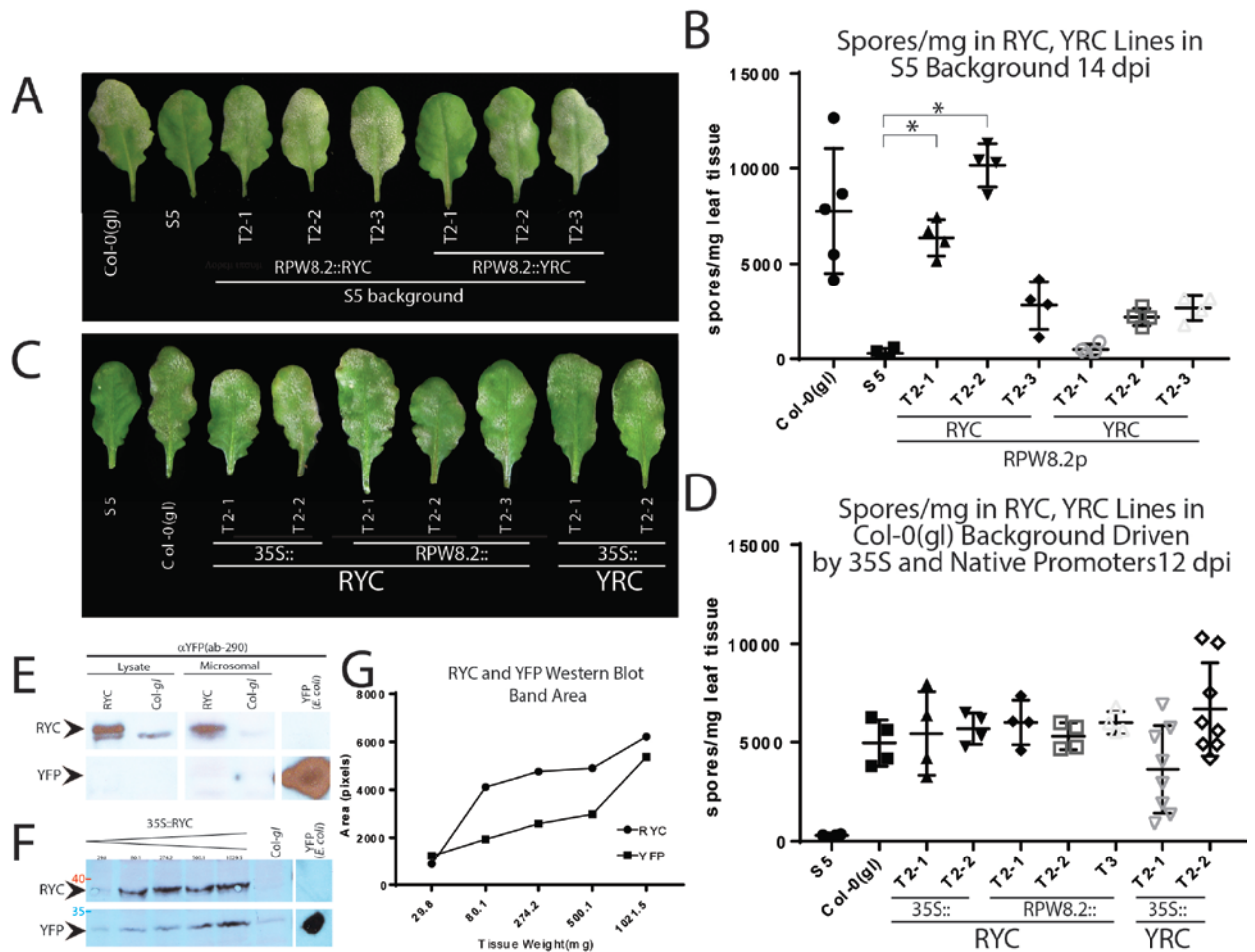


promoter and WT Col-*gl* plants. No enhanced disease susceptibility was seen in transgenic plants.

I thus introduced the RYC or YRC construct under control of the RPW8.2 promoter or the 35S promoter into line S5 that contains both RPW8.1 and RPW8.2. I obtained T2 transgenic lines and tested them along with S5 Wild-type plants for resistance to GC-UCSC1 (Figure 3-20A). Counting total number of fungal spores per mg fresh infected leaf tissues can be used to assess the severity of disease susceptibility or resistance especially in the later stages of the fungus's life cycle (Weßling & Panstruga, 2012). Therefore, I quantified spores from a pool of leaves collected from 12 plants for each selected transgenic T2 lines (two lines for each transgene) (Figure 3-20B). Ordinary one-way ANOVA analysis comparing S5 to all lines showed that two of the three representative RYC T2 lines supported significantly more spores (which is comparable to Col-gl) than S5, but the three YRC T2 lines did not show significant difference compared to S5 (Figure 3-20B). This may be because like the drought tolerance, RYC binds to 14-3-3s with greater affinity than YRC and our sample size did not have power to find a small effect. I also generated T2 plants expressing RYC or YRC from the 35S promoter in Col-gl and compared them with T2 Col-gl plants expressing RYC from the RPW8.2 promoter. I found no significant phenotypic difference between the transgenic lines and Col-gl (Figure 3-20C and Figure 3-20D).

Given that 14-3-3 $\lambda$  knockdown in the S5 background only moderately attenuated RPW8-mediated resistance (X. Yang et al., 2009), my results that RYC (to a lesser extent YRC) expression in S5 largely abolished RPW8-mediated resistance (Figure 18A and 18B) suggest the following: (1) RYC can effectively sequester 14-3-

3s in powdery mildew-invaded epidermal cells where RPW8.2 expression is induced upon infection; (2) 14-3-3 $\lambda$  indeed plays an important role in RPW8.2 (possibly RPW8.1 as well) mediated resistance to powdery mildew; and (3) other 14-3-3 isoform other than 14-3-3 $\lambda$  may also contribute to RPW8-mediated resistance.



**Figure 3-20 14-3-3 Sequestration by RYC or YRC Compromises *RPW8*-Mediated Resistance**

(A) Representative leaves from plants of the indicated genotypes showing infection phenotypes at 10 dpi of *Gc* UCSC1. Note the DNA constructs were under control of the *RPW8.2* promoter. (B) Quantification of fungal spores for the indicated transgenic lines and control plants. One-way ANOVA was used to test the statistical significance of the differences in susceptibility of the transgenic lines compared to that of the S5 controls. Asterisks indicate significance ( $p < 0.05$ ). (C) Representative leaves from *Col-0(gl)* lines expressing RYC or YRC from 35S or expressing RYC from the *RPW8.2* native promoter at 10 dpi of *Gc* UCSC1. (D) Spore counts for representative leaves from each sample show no

difference compared to *Col-gl* controls. (E) A Western blot showing RYC is distributed in the soluble (lysate) and membrane (microsomal) fractions prepared from leaves of a representative RYC transgenic line. *Col-gl* served as a negative control. An anti-YFP antibody (ABCAM ab-290) was used to detect YFP-containing proteins. (F) The intensity of the band corresponding to RYC increases with increasing leaf mass processed. (G) The area of cytoplasmic RYC (circles) and YFP (squares) band size increases commensurate with leaf mass. Size of error bars in (C) and (D) represent SEM.

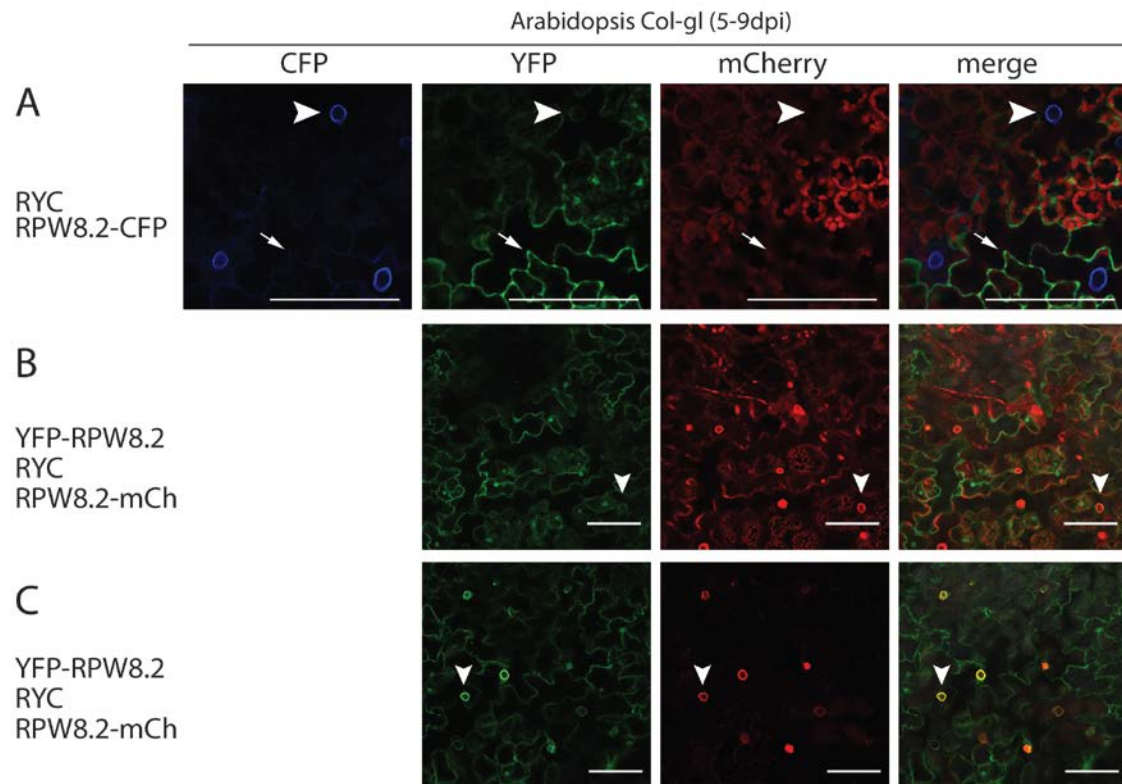
### ***RYC Expression Does Not Seem to Alter EHM-Specific Targeting of RPW8.2***

14-3-3-binding to RPW8.2 may be required for RPW8.2 to 1) activate SA-dependent defense responses (Xiao., et al., 2005) and/or 2) localize to the host-pathogen interface, the EHM. Several 14-3-3-interacting membrane proteins that contain mode III binding sites recruit 14-3-3 to their C-termini for efficient forward trafficking and correct membrane localization (Coblitz et al., 2006; Shikano et al., 2006). C-terminal recognition by 14-3-3 proteins for surface expression of membrane receptors (Shikano et al., 2006). Genetic isolation of transport signals directing cell surface expression (Heusser et al., 2006). A multimeric membrane protein reveals 14-3-3 isoform specificity in forward transport in yeast. Given that RPW8.2 is specifically localized to the EHM, I hypothesized that 14-3-3 $\lambda$  binding to the C-terminus (-SDDS-COOH) of RPW8.2 might change RPW8.2's conformation and/or masking potential ER retention motifs as 14-3-3-binding does for some other membrane proteins (O'Kelly et al., 2002b; Shikano et al., 2006), and thereby promoting its ER /Golgi export and EHM-oriented vesicle trafficking. To test this hypothesis, I generated Col-*gl* transgenic plants expressing RPW8.2-CFP (Earley et al., 2006) and RYC from the RPW8.2 native promoter. Confocal imaging showed that RPW8.2-CFP exhibited typical EHM-localization (Figure 3-21A), as previously observed for RPW8.2-YFP (Wang et al., 2009). This result seemingly suggests that 14-3-3 sequestration by RYC has no impact on RPW8.2's EHM localization. However, it is likely that fusion of CFP or YFP to the C-terminus of RPW8.2 may

abrogate the mode III binding of 14-3-3 $\lambda$  to RPW8.2's C-terminus (X. Yang et al., 2009), and that fusion of CFP (YFP) to the C-terminus of RPW8.2 may mimic the effect of mode III 14-3-3-binding, thereby obviating the binding of 14-3-3 to RPW8.2 for EHM-targeting. This would mean that results from RPW8.2-CFP (YFP) and RYC co-expression may not be informative or adequate in determining if mode III binding of 14-3-3 to the C-terminus of RPW8.2 is required for the EHM-localization of the latter. Given that YFP-RPW8.2, though not functional in defense, can still be targeted to the EHM (W. Wang, Berkey, Wen, & Xiao, 2010). Accurate and adequate spatiotemporal expression and localization of RPW8.2 is key to activation of resistance at the host-pathogen interface. I translationally fused mCherry to the C-terminus of RPW8.2 (the resulting DNA construct is designated *RPW8.2-mCh*) and generated stable Arabidopsis transgenic lines co-expressing two additional constructs, *RYC* and *YFP-RPW8.2* (Wang et al., 2010) from the *RPW8.2* promoter and use them for assessing if 14-3-3 sequestration can impact RPW8.2's EHM-localization. My rationale behind this experiment is as follows: 1) YFP-RPW8.2 has an intact C-terminus of RPW8.2. Hence its EHM-localization may require 14-3-3-binding to its C-terminus. RYC-mediated 14-3-3-sequestration may completely or partially affect YFP-RPW8.2's EHM-localization; 2) RPW8.2-mCherry should have normal EHM-localization in the presence of RYC due the reason explained above; 3) Confocal imaging of haustoria and their associated EHM for both YFP and mCherry should conveniently and accurately identify potential absence of YFP-RPW8.2 in the EHM where RPW8.2-mCherry is efficiently localized. Luckily, I obtained transgenic lines

that stably express YFP-RPW8.2, RPW8.2-mCh and RYC from the *RPW8.2* promoter by co-transformation. The T1 transgenic plants were inoculated with *Gc*-UCSC1 and subjected to confocal imaging. In order to readily identify haustoria and grossly assess the EHM-targeting efficiency of the two RPW8.2 fusion protein, I also stained the infected leaves with propidium iodide (PI) to label haustoria (Koh et al., 2005). Confocal imaging revealed that the EHM of most (70% of >40 haustoria) was clearly labeled by both YFP and mCherry (Figure 3-21C), indicating that both RPW8.2 fusion proteins are correctly and efficiently targeted to the EHM. Based on the above results, it appears that 14-3-3-binding is not required for RPW8.2's targeting to the EHM. As a corollary, it can be inferred that 14-3-3-binding is probably important for the RPW8.2 protein to activate defenses against powdery mildew pathogens.





**Figure 3-21 R18-YFP-CT59 (RYC) Expression Does Not Affect RPW8.2's EHM-Localization**

(A) Representative confocal images showing EHM-localization (arrowhead) of RPW8.2-CFP in Arabidopsis Col-0 T1 lines co-expressing RYC which is mainly found in the plasma membrane (PM) (arrow). Leave of transgenic plants were subjected to imaging at 2 dpi with *Gc* UCSC1. (B) RPW8.2 chimeric proteins, one fused at the N-terminus with YFP (YFP-RPW8.2), and the other at the C-terminus with mCherry (RPW8.2-mCh) appear to co-localize to the EHM (arrowhead). However, YFP fluorescence can't be distinguished by RYC that may also be localized here due to indiscriminate sloughing due to constitutive promoter, 35S. This leaf section was stained with propidium iodide. (B) Representative confocal images showing co-localization of YFP-RPW8.2 and RPW8.2-mCh to the same haustoria (arrow) in

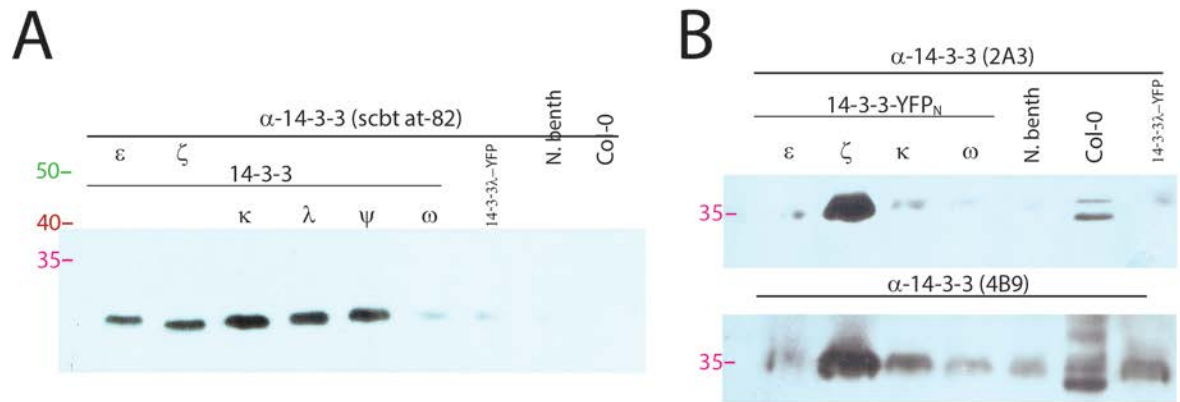
leaves of transgenic plant co-expressing RYC in addition to these two RPW8.2 fusion proteins. Bar = 100μm?

### ***Western Blot Unable to Resolve RYC Multiple- or Specific-14-3-3 Binding***

In addition to fluorescence confirmation, RYC-transformed lines were accessed for RYC expression via western blot. Cytoplasmic localized RYC was unknown as RYC was previously observed to localize to the plasma membrane and to guard cells when interacting with 14-3-3 $\lambda$  (Figure 3-19A-B). However, RYC expression was identified by western blot to both localize in the cytoplasm and in the PM (Figure 3-20E). To confirm cytoplasmic expression, different numbers of leaves were processed from 1 leaf (29 mg) to  $\approx$ 40 leaves ( $\approx$ 1000mg). Cytoplasmic RYC concentration increased commensurate with tissue weight processed (Figure 3-20F). RYC and YFP band size increased proportionally to tissue weight (Figure 3-20G). ] RYC binds multiple 14-3-3 isoforms in BiFC when tested individually. Whether RYC binds multiple 14-3-3 isoforms in vivo has not been tested. To address this question, I first tried to obtain commercially or lab-derived antibodies and test if they could distinguish individual 14-3-3 isoforms. Despite extensive Western blotting with ? different antibodies, I could not find one or a set of antibodies that could distinguish different Arabidopsis 14-3-3 isoforms endogenously or when they were transiently expressed in leaves of *N. benthamiana*.

**Table 6 Arabidopsis 14-3-3 Isoform Characteristics**

Species	Gene Name	Locus	Protein	Greek Letter	Wildtype		Fused with YFPn		Fused with YFP	
					Length (aa)	Size (kDa)	Length (aa)	Size (kDa)	Length (aa)	Size (kDa)
Homo sapiens	YWHAZ	HSU28964	zeta	ζ	245	27.745	399	45.052		
Arabidopsis	GF1	At4g09000	chi	χ	318	35.838				
Arabidopsis	GF2	At1g78300	omega	ω	259	29.161	413	46.469		
Arabidopsis	GF3	At5g38480	psi	ψ	254	28.477	408	45.784		
Arabidopsis	GF4	At1g35160	phi	φ	295	33.356				
Arabidopsis	GF5	At5g16050	upsilon	υ	268	30.181	--	--		
Arabidopsis	GF6	At5g10450	lambda	λ	249	27.975	402	45.283	488	54.962
Arabidopsis	GF7	At3g02520	nu	ν	265	29.824				
Arabidopsis	GF8	At5g65430	kappa	κ	248	28.028	402	45.336		
Arabidopsis	GF9	At2g42590	mu	μ	276	30.968				
Arabidopsis	GF10	At1g22300	epsilon	ε	251	28.556	405	45.864		
Arabidopsis	GF11	At1g34760	omicron	ο	255	29.121				
Arabidopsis	GF12	At1g26480	iota	ι	268	30.545				
Arabidopsis	GF13	At1g78220	pi	π	245	27.924				



**Figure 3-22 Detection of 14-3-3 Isoforms by Different Antibodies**

(A) Detection of endogenous 14-3-3 proteins from *N. benthamiana* by an anti-14-3-3 “at-82” (Discontinued antibody from Santa Cruz Biotechnology). (B) Detection of endogenous 14-3-3 proteins from *N. benthamiana* by two monoclonal anti-14-3-3 antibodies made by Dr. R. Ferl.

Most commercially-available antibodies recognize 14-3-3 proteins from humans. Three plant-specific antibodies were tested, one of them, anti-14-3-3 (Santa Cruz Biotechnology “at-82”, since discontinued) was commercially available. Two monoclonal antibodies, 2A3, 4B9, were produced in mouse ascites (kind gift from Robert Ferl) (Sehnke, Laughner, Cardasis, Powell, & Ferl, 2006). These antibodies were first tested for reactivity against Arabidopsis proteins transiently expressed in *N. benthamiana*. The same 14-3-3 isoforms were used as in previous BiFC experiments; thus, the 14-3-3 proteins were fused to YFP<sub>N</sub> at their C-termini. A list of 14-3-3 isoform molecular weights as wildtype and YFP<sub>N</sub> fusion products is available in Table 6.

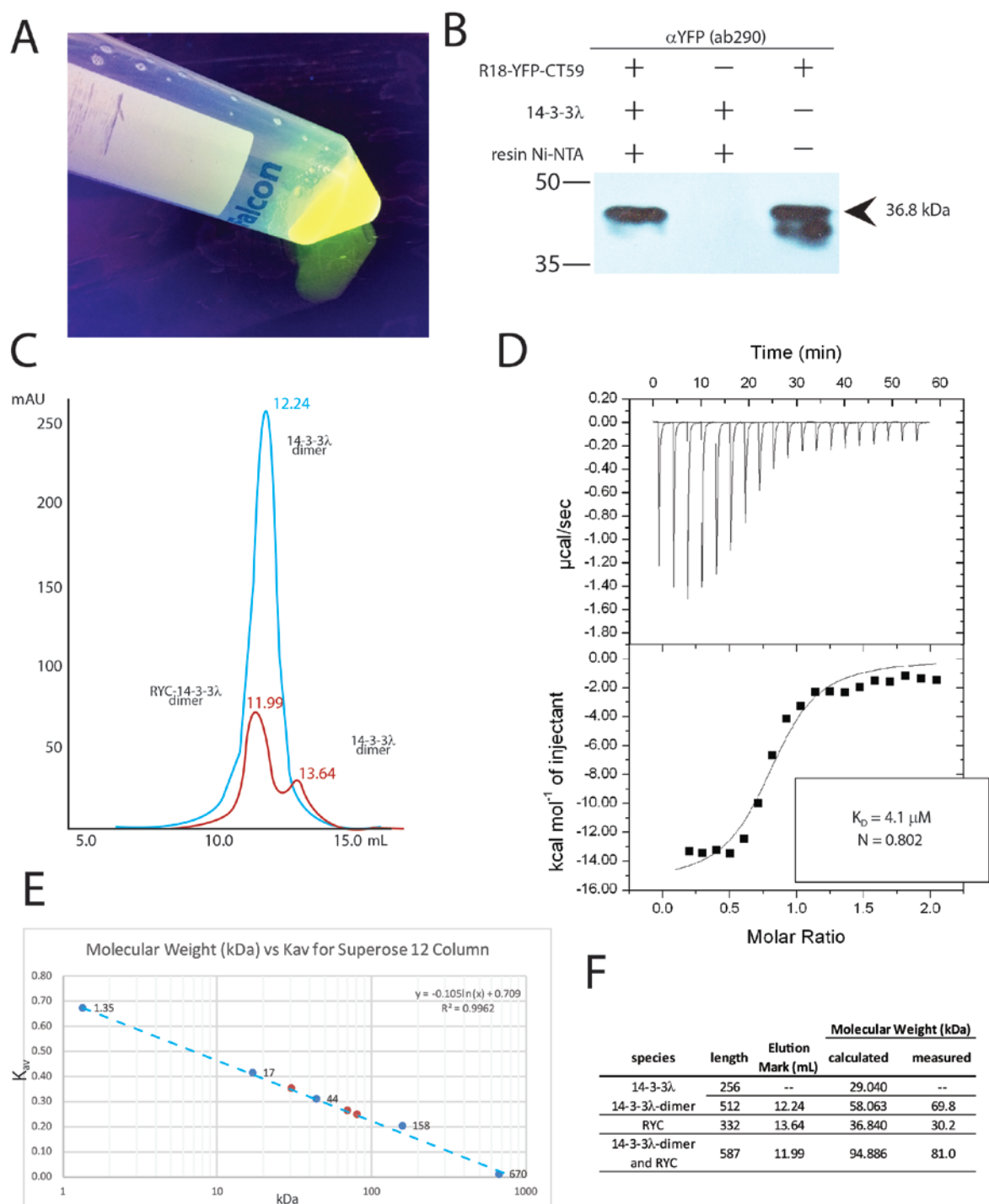
Three days post infiltration with *Agrobacteria* expressing their respective 14-3-3 isoform, *N. benthamiana* leaves were ground in liquid nitrogen. The lysates were purified, denatured, and probed by western blot using anti-14-3-3 antibodies. Bands around 35 kDa are visible on the blot using commercially available (anti-14-3-3 “at-82”). This suggests that endogenous 14-3-3 isoforms were detected (Figure 3-22A) rather than chimeric proteins. Band sizes were confirmed with monoclonal, 14-3-3 Arabidopsis-specific antibodies 2A3 and 4B9. Both monoclonal antibodies detected bands around 35kDa suggesting that rather than 14-3-3-YFP<sub>N</sub> proteins, the endogenous 14-3-3 proteins were detected (Figure 3-22B). Negative control Arabidopsis Columbia also shows bands around the same size consistent with detection of its endogenous 14-3-3 proteins.

Abundance may provide an explanation for the detection of *N. benthamiana* endogenous 14-3-3 isoforms over 14-3-3-YFP<sub>N</sub> isoforms. In Arabidopsis, 14-3-3chi and phi (35.8 and 33.3 kDa) were the most abundant isoforms in several tissues (Paul et al., 2012). Orthologous *N. benthamiana* 14-3-3 proteins may be similarly abundantly expressed. That no chimeric bands are detected at the 14-3-3-YFP<sub>N</sub> expected size ( $\approx$ 45kDa) suggests that the sample size was too little or that the heterologous isoform did not express well in host *N. benthamiana*. Localization may have been altered by fusion with YFP<sub>N</sub> and moved from soluble to insoluble consistent with BiFC data above. Steric hindrance may have also played a contributing factor as 4B9's epitope is at the C-terminus and fusion of YFP<sub>N</sub> may have prevented the antibody from interacting with its epitope (Sehnke et al., 2006).

Taken together, without first purification from endogenous proteins, 14-3-3 heterologous proteins expressed in *N. benthamiana* are unlikely to be detected and differentiated. Due to the similarity of 14-3-3 proteins, 14-3-3-specific antibodies are cross-reactive. Thus, other techniques like tandem affinity purification (R. B. Rodrigues et al., 2014; Stotz et al., 2014; Van Leene et al., 2014, 2008) followed by mass spectrometry may be more amenable for determining which 14-3-3 isoforms RYC binds. Low abundance of heterologously expressed 14-3-3 proteins may be boosted by co-expressing with a protein capable of host suppression like the coat protein of Turnip Crinkle Virus (Qu, Ren, & Morris, 2003).

### ***Quantitating the RYC-14-3-3 $\lambda$ Interaction In vitro***

How efficient is the 14-3-3 sequestration by RYC? To address this question, I used in vitro models to quantify the interaction between RYC and 14-3-3 $\lambda$ . RYC was cloned into pFN18A and purified using the HaloTag purification system (Ohana et al., 2009). 14-3-3 $\lambda$  cDNA was cloned into pET26b vector with a C-terminal 6xHis epitope tag. One advantage of using YFP as a linker protein is the ease of following it through the heterologous expression and purification process (Figure 3-23A).



**Figure 3-23 Quantification of RYC Interaction with 14-3-3**



(A) RYC purification aided by protein fluorescence. (B) The 6x-His epitope tag on 14-3-3 $\lambda$  is able to immunoprecipitate RYC (36.8kDa) when incubated with Ni-NTA resin as assessed by western blot using an anti-YFP antibody. The band below RYC is probably a nonspecific band this is removed with the addition of Ni-NTA resin. (C) Interaction of 14-3-3 $\lambda$  and RYC confirmed by size exclusion chromatography. In the absence of RYC, 14-3-3 $\lambda$  elutes at 12.24mL as a dimer (blue line). When RYC and 14-3-3 $\lambda$  were co-incubated, a 14-3-3 $\lambda$ -dimer-RYC complex eluted faster at 11.99mL. Smaller unbound RYC eluted slower at 13.64mL. (D) Isothermal titration calorimetry (ITC) reveals a dissociation constant between RYC and 14-3-3 $\lambda$ ,  $K_D$  of 4.1  $\mu$ M. Stoichiometry also reveals  $N = 0.7$ . (E) A range of globular proteins eluting from a size exclusion column can be used to measure (interpolate) the molecular weight of unknown eluting proteins. (F) The measured size can be evaluated against the calculated size for comparison.

Purified proteins RYC and 14-3-3 $\lambda$  were co-incubated together. Nickel resin was used to capture 14-3-3 $\lambda$  and any bound proteins. A western blot probing with  $\alpha$ YFP (Abcam ab290) identified RYC in eluates confirming in vitro binding between 14-3-3 $\lambda$  and RYC (Figure 3-23B). This binding was also validated on a gel filtration (size exclusion) column. By itself, 14-3-3 $\lambda$  was eluted as a dimer at 12.24 mL. However, after co-incubation with RYC and subsequent run on the same column, two species were observed. First, the heavier 14-3-3 $\lambda$ -dimer bound to RYC eluted earlier at 11.99mL. Second, the unbound RYC eluted at 13.64 mL (Figure 3-23C). A globular size-standard (BioRad #151-1901) with a size range from 1.35 to 670 kDa run on this column can be used to interpolate the sizes of the eluted peaks (Figure 3-23E). For instance, the 12.24 mL elution corresponds to a measured molecular weight of about 69.8 (Figure 3-23F). This value is consistent with previous reports of 14-3-3 elutions (Giles, Forrest, & Gabrielli, 2003; Sluchanko, Sudnitsyna, Chernik, Seit-Nebi, & Gusev, 2011). Calculated sizes for dimerized 14-3-3 protein bound to RYC and unbound RYC are also roughly estimated by interpolated measured sizes. The variability in size can be explained by the protein's Stokes radius, a characteristic of solute mobility (Erickson, 2009).

Isothermal titration calorimetry (ITC) was further used to evaluate the interaction between 14-3-3 $\lambda$  and RYC. RYC was subcloned into pET26b and purified and expressed from *E. coli*. Both RYC and 14-3-3 $\lambda$  proteins were purified to > 95% purity and dialyzed in the same buffer. RYC was slowly titrated into a solution containing 14-3-3 $\lambda$ . A dissociation constant of 4.1  $\mu$ M was calculated with a

stoichiometry of  $N = 0.71$ . A value less than 1 is possible depending on the shape of binding molecule (Dutta, Rosgen, & Rajarathnam, 2015). Taken together, ITC, size exclusion chromatography and immunoprecipitation experiments confirm that RYC and 14-3-3 $\lambda$  interact in vitro.

## ***Discussion***

I bioengineered a chimeric protein consisting of two 14-3-3 interacting proteins: a 20-residue peptide from a library and 59-C-terminal residues from an H<sup>+</sup>-ATPase. “R18”, the 20-residue peptide, was shown in this study to bind 14-3-3 $\lambda$  and five other Arabidopsis 14-3-3 isoforms including one human isoform, 14-3-3 $\zeta$ , using bimolecular fluorescence complementation (BiFC). To our knowledge, this is the first time BiFC was used to confirm the interaction between R18 and 14-3-3 isoforms. “CT59”, the 59-amino acid peptide from H<sup>+</sup>-ATPase, was also confirmed to interact with five Arabidopsis 14-3-3 isoforms, including 14-3-3 $\zeta$ , using BiFC. My CT59 truncation and mutation studies may have revealed a previously uncharacterized interaction with 14-3-3 $\lambda$ .

Both R18 and CT59 were fused in a single protein interspaced with yellow fluorescent protein (YFP) called “RYC”. I hypothesized this chimeric protein to bidentally engage 14-3-3 proteins such that its expression could be used to sequester 14-3-3 isoforms, which may prove useful in characterizing client proteins and their association within 14-3-3-networks.

Western blot assays failed to categorize the 14-3-3 proteins with which RYC interacts. Future studies will need to adopt other techniques like mass spectrometry to convincingly identify interacting 14-3-3 isoforms. Data collected in support of the 14-3-3-RYC sequestering hypothesis shows that plant backgrounds expressing the RPW8 locus, a locus conferring immunity to powdery mildew species in Arabidopsis,

are compromised in resistance when RYC is expressed. Preliminary data suggests that RYC doesn't affect RPW8.2, a protein in the RPW8 locus, in its trafficking to the extra-haustorial membrane (EHM). But future studies will be designed in a way as to provide greater resolving power in teasing apart this relationship.

In vitro experiments confirm in vivo observations: RYC and at least one isoform, 14-3-3 $\lambda$  interact with a  $K_D$  of 4.1  $\mu$ M.

### ***Is the CT59.ex1.f1 and 14-3-3 $\lambda$ Interaction in BiFC Real?***

This study confirms the interaction between the 59 C-terminal amino acids “CT59” from an Arabidopsis H<sup>+</sup>-ATPase, AHA2 (At4G30190.2), and an Arabidopsis 14-3-3 isoform, 14-3-3 $\lambda$  (At5G10450). CT59 truncated of the last three amino acids also generated fluorescence when previously shown to abolish an orthologous interaction in yeast due to disruption of the penultimate phosphorylate tyrosine residue (Christian Ottmann et al., 2007). The positive BiFC interaction was originally suspected to be background fluorescence, a common concern with in planta BiFC experiments (Horstman, Antonia, Tonaco, & Boutilier, 2014). To confirm the positive interactions, mutants should be used to disrupt the protein interactions (Kerppola, 2006). Ironically, it was in searching for a CT59 mutant incapable of binding 14-3-3 $\lambda$  that prompted the truncational and mutational analysis. Twelve more derivative truncations and mutants also generated fluorescence. Surprisingly, a 12-residue peptide called “CT59.ex1.f1” also fluoresced when co-expressed with 14-3-3 $\lambda$ , confirming an interaction. Six observations support the conclusion of an interaction:

- 1) Individual BiFC experiments were conducted multiple times. The interaction between 14-3-3 $\lambda$  and CT59.ex1.f1 was repeated 4x.
- 2) The abundance of fluorescence suggests this interaction is authentic. The fluorescence in truly positive interactions is much more robust than in false negatives.
- 3) I swapped split YPF domains between 14-3-3 $\lambda$  and CT59.ex1.f1 and recapitulated results.
- 4) More parsimonious to consider a previously uncharacterized interaction than false negatives in fourteen truncation/mutant experiments with replication.
- 5) Crystal structure supports hypothesis of CT59.ex1.f1 interaction with 14-3-3 $\lambda$ .
- 6) Small peptides (<21) including R18 in this study have been documented to produce fluorescence with BiFC (Lin et al., 2011; Speltz, Sawyer, & Regan, 2016).

Taken together, I believe the interaction between CT59.ex1.f1 and 14-3-3 $\lambda$  is authentic and replicable.



### ***The Mechanism of CT59.ex1.f1 and 14-3-3 $\lambda$ Interaction***

An orthologous interaction has been researched extensively using 14-3-3c and “CT52,” the 52 C-terminal amino acids from PMA2, a H<sup>+</sup>-ATPase from *Nicotiana plumbaginifolia*. The preeminence of this interaction in the literature is due to the unique role a fungal phytotoxin plays in strengthening the binding between PMA2 and 14-3-3c. A protein crystal structure for this interaction has been solved and was used to support my analysis (Corinna Ottmann, Weyand, Wolf, Kuhlmann, & Ottmann, 2009).

The structure shows that the terminal 27 residues of CT52, including the phosphorylated penultimate tyrosine residue, of PMA2 are critical for 14-3-3 groove-binding. CT52 forms two  $\alpha$  helices. One shorter helix coordinates loop binding in the 14-3-3 binding groove and spans the binding region between the two 14-3-3 monomers. The longer helix escapes from the center of the 14-3-3 dimer and also interacts with the other CT52 peptide helix at its N-terminus. This interaction among others may be responsible for observations that the PMA2 protein is known to dimerize and be inactive (Almeida, Martins, & Carvalho-Alves, 2006; Kanczewska et al., 2005).

CT52 contains known autoinhibitory regions, region I and region II. In the absence of 14-3-3 binding, the CT52 peptides anti-parallelize to create an autoinhibitory region that prevents PMA2 functionality. Region II is not exactly defined but includes portions of the CT52 helix region protruding from the 14-3-3



dimer. Two CT52 helices emanate from the 14-3-3 dimer in an antiparallel manner and interact as shown in the crystal structure. The autoinhibitory action of region II depends on these intermolecular contacts.

Peptide CT59.ex.f1 contains amino acids hypothesized to be important for these intermolecular interactions especially F907. In AHA2, the coordinating phenylalanine is a tyrosine, which could still maintain the hydrophobicity necessary for contacts with the opposite, antiparallel CT59 helix. The crystal structure identifies other upstream residues which may further coordinate region II auto-inhibiting cooperativity. Thus, this work provides additional clarification regarding the strength of this interaction and also suggests additional residues which may coordinate and interact together for the auto-inhibition of region II.

We propose a model in which the 12-residue peptide CT59.ex1.f1 and all mutants except CT59.ex1.m7 maintain enough identity to coordinate with at least one antiparallel, endogenous *N. benthamiana* PMA2 protein at the autoinhibitory region II. CT59.ex1.f1 interacts with 14-3-3 $\lambda$  when it binds to the C-terminal region of PMA2 upon phosphorylation to activate PMA2. This suggests a one-to-one ratio typically ascribed to BiFC. However, a cryoelectron microscopy (cryo-EM) model for the 14-3-3-PMA complex reveals that the 14-3-3 $\lambda$ -CT59.ex1.f1 ratio may be higher creating bias towards interaction and BiFC.

The cryo-EM model suggests that three PMA2 dimers hexamerize with three 14-3-3 dimers for the activation of PMA2. Hexamerization increases the probability that CT59.ex1.f1 and 14-3-3 $\lambda$  will interact, for the hexamer provides up to three

binding sites for at least one CT59.ex1.f1 to interact with another PMA2 in the autoinhibitory II region. The odds are similar for 14-3-3 $\lambda$ . Only one 14-3-3 $\lambda$ -YFP<sub>N</sub> protein need homodimerize or heterodimerize with another endogenous 14-3-3 isoform to be part of the complex and brought into close enough proximity with CT59.ex1.f1 to generate fluorescence.

Although distances of at least 100Å are needed for FRET with BiFC (Kerppola, 2008), no specific distance exists for BiFC, and two tethered, flexibly linked split-YFP particles can generate fluorescence (Kerppola, 2006). The cryo-EM hexamer shows a mostly-cylindrical shape with diameter 147Å and 120Å height. Thus, these dimensions clearly allow a close enough association for the generation of fluorescence. FRET may be troublesome but still probable due to the centrally-concentrated 14-3-3 $\lambda$ -PMA2 interactions.

The hexamerization of this complex and the *N. benthamiana* host in which it was expressed may have fortunately contributed to revealing this interaction.

### ***Why Does BiFC Show an Interaction While Yeast Studies do not?***

YAK2 is a specialized yeast strain in which previous 14-3-3-PMA2 interactions were performed. Two endogenous H<sup>+</sup>-ATPase genes have been removed YAK2 (De Kerchove d'Exaerde et al., 1995). The removal of both PMA1 and PMA2 allows survival testing of mutant, heterologous H<sup>+</sup>-ATPases like *Nicotiana plumbaginifolia* PMA2 (De Kerchove d'Exaerde et al., 1995). Wildtype *N.*

*plumbaginifolia* PMA2 permits yeast growth due to partial activation by endogenous 14-3-3 proteins (Piotrowski, Morsomme, Boutry, & Oecking, 1998).

The mutation of the penultimate residue (tyrosine) in CT52 was enough to abolish growth in yeast. Without the phosphorylated tyrosine T955A, PMA2 does not engage 14-3-3 to overcome the PMA2 autoinhibitory region II for activation and subsequent growth on glucose medium (Christian Ottmann et al., 2007).

Unexpectedly, CT56 ( $\Delta$ 3 terminal residues containing tyrosine) interacted with 14-3-3 $\lambda$  in *N. benthamiana* even though this reaction was confirmed not to occur in YAK2.

Deleting the terminal 27 residues did not restore the growth of yeast, suggesting the autoinhibitory domain was still engaged preventing growth. Deleting CT52 ( $\Delta$ 52) restored growth, and demonstrates that autoinhibitory region II is located between  $\Delta$ 27 and  $\Delta$ 52. Four residues, including one critical residue, F907, in  $\Delta$ 52 are found in CT591.ex1.f1. Mutating the last two residues in CT59.ex1.f1 to alanine did not abolish fluorescence with 14-3-3 $\lambda$ . CT59.ex1.f1 is seven amino acids longer than CT52 which may allow for interaction not able to be tested by Ottmann et al. Also, there may be orthologous differences between the autoinhibitory region II of AHA2 and PMA2.

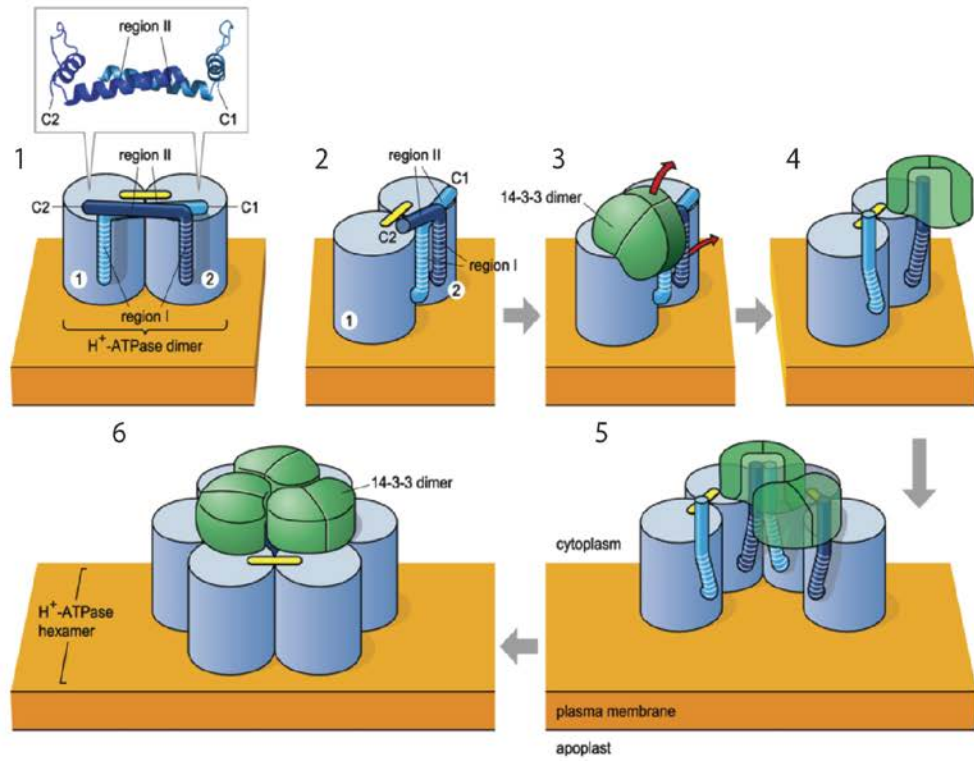
Given the data above, the discrepancy between positive BiFC in *N. benthamiana* and non-growth in YAK2 is due to CT56 and CT59.ex1.f1 and other variants interacting with endogenous H<sup>+</sup>-ATPases orthologues like PMA2 in *N. benthamiana*. In YAK2, orthologous interactions are impossible because yeast H<sup>+</sup>-ATPases PMA1 and PMA2 have been deleted. This confirms the functional

redundancy of 14-3-3 isoforms and AHA2, and the dimerization of the C-terminal region in forming autoinhibitory region II.

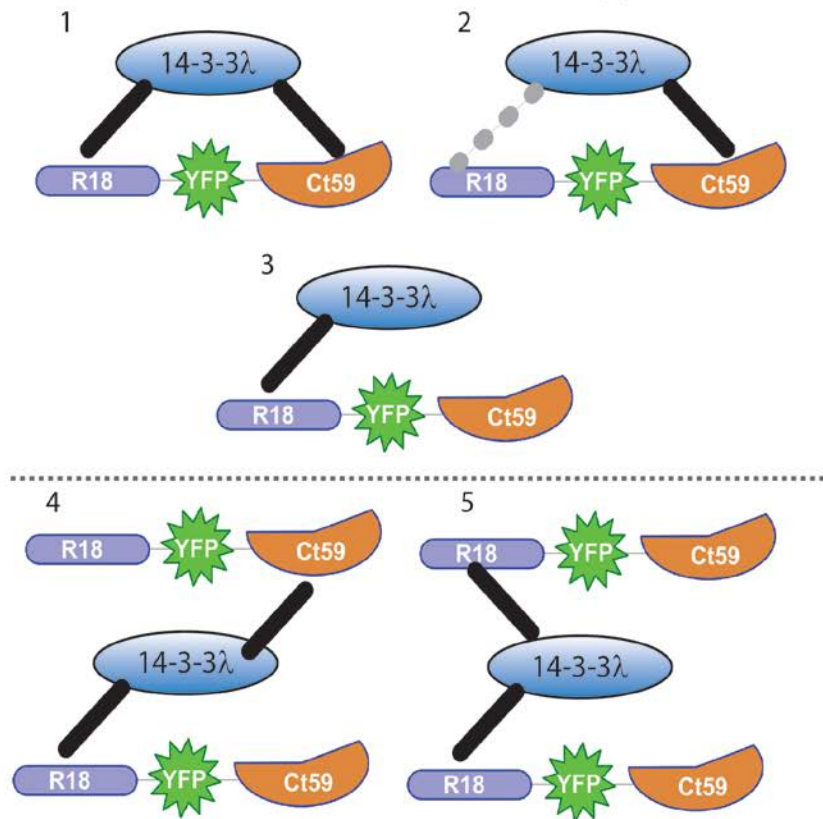
### ***Discrepancies Between $K_D$ in ITC vs SPR***

The dissociation constant, also affinity, is a measure of the binding strength of a complex to dissociate into a protein and ligand. This study used isothermal titration calorimetry to calculate a  $K_D$  of 4.1  $\mu$ M between RYC and 14-3-3. This was higher (lower affinity) than the  $K_D$  of 14nM calculated by Ottmann et al. using surface plasmon resonance spectroscopy (SPRS) (Christian Ottmann et al., 2007). SPRS was used to investigate CT52's affinity for 14-3-3 in the presence of mutations and small fusicoccin. The discrepancy between the two  $K_D$  values is the presence of fusicoccin, which has been shown to irreversibly lock the C-terminal tail into the binding groove of 14-3-3. Fusicoccin was not used in ITC, but the presence of fusicoccin in planta was shown to inhibit the interaction between R18 and CT59, both of which have been demonstrated to bind 14-3-3 proteins. The molecular basis for this is not known, though the presence of fusicoccin may act as a competitive inhibitor of R18 binding.

A



B



### **Figure 3-25 The Hexamerization of PMA2 and 14-3-3c and RYC Binding Possibilities**

(A) The hexamerization of PMA2 and 14-3-3c provides a model for the explanation of the 12-residue CT59.ex1.f1 interacting with 14-3-3 $\lambda$ . The C-terminal ends of PMA2 dimerize to create autoinhibitory region II (1-2). Upon phosphorylation and 14-3-3-binding, PMA2 undergoes hexamerization and subsequent activation (3-6). PMA2 residues in the C-terminal end continue to associate (5). Only one YFP<sub>C</sub>-CT59.ex1.f1 molecule is needed to interact with at least one other PMA2 endogenous full length H<sup>+</sup>-ATPase to be brought into contact with one molecule of 14-3-3-YFP<sub>N</sub> which may have homodimerized or heterodimerized with endogenous 14-3-3 isoforms. (B) The molecular binding between RYC and 14-3-3 $\lambda$  is unknown but could be cooperative(1), coordinated(2), preferred(3), heterodimeric(4) or homodimeric(5) binding.

## ***Future Experiments***

R18-YFP-CT59 (RYC) is a sequestering protein that in vivo engages six 14-3-3 isoforms. In vitro RYC binds 14-3-3 $\lambda$  with a  $K_D$  of 4.1  $\mu$ M. Despite confidence in its interaction the molecular mechanism of interaction is unknown. Does RYC interact with 14-3-3 isoforms in a mono- or bidentate manner? Do both 14-3-3 binding moieties bind equally, or does one bind preferentially due to different affinities? There are 5 possible states of RYC-14-3-3 $\lambda$  binding:

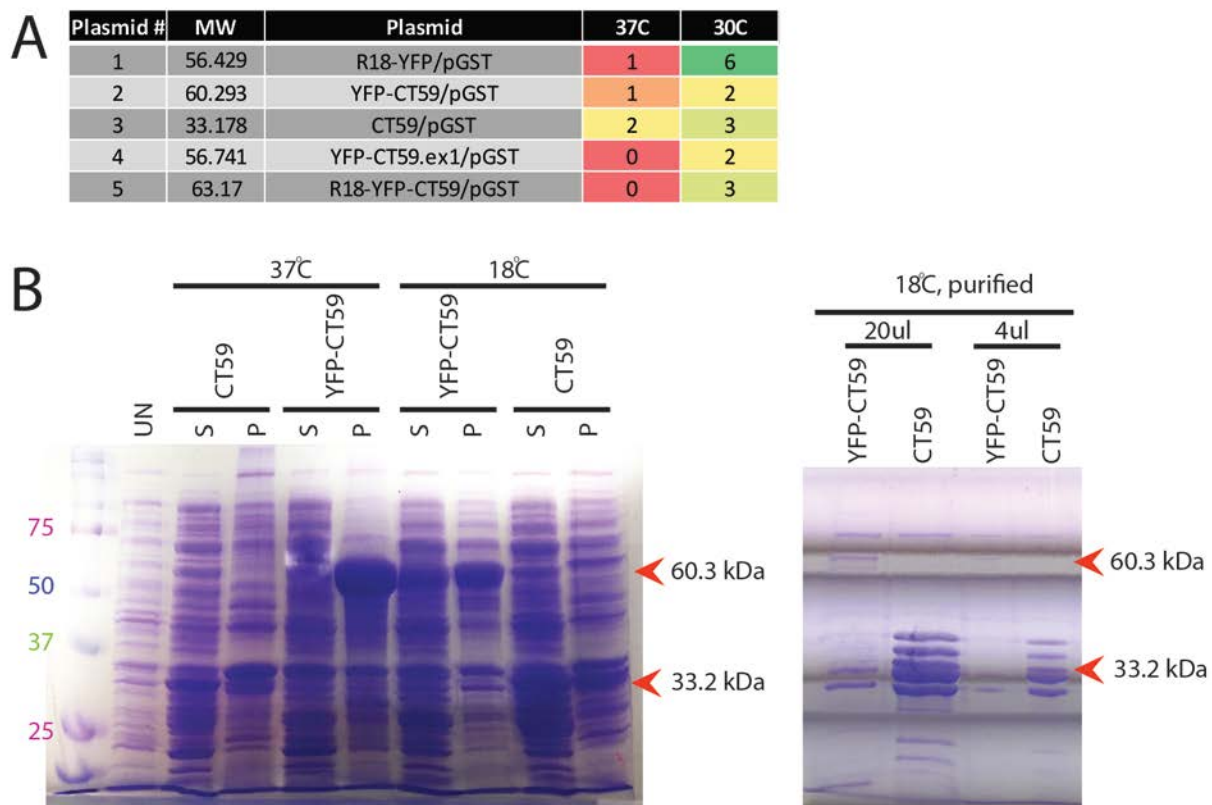
1. Cooperative Binding: One RYC molecule binds a single 14-3-3 $\lambda$  dimer.
2. Coordinated Binding: One moiety from RYC binds 14-3-3 $\lambda$  dimer while the other moiety partially binds or blocks groove.
3. Single Binding or Preferred Binding: Both moieties can bind, but only one does due to competitive binding or steric hindrance.
4. Heterodimeric Binding: Two different moieties from two RYC molecules bind a single 14-3-3 $\lambda$  dimer.
5. Homodimeric Binding: The same moiety from two RYC molecules bind a single 14-3-3 $\lambda$  dimer.

Two techniques can aid in clarifying this relationship:

1. Western Blot
2. Surface plasmon resonance (SPR) using Octet

Immunoprecipitation followed by western blots can clarify the interaction. R18 can be synthesized with N-terminal biotin. CT59 can be cloned with glutathione S-transferase (GST), and 14-3-3 can be cloned into a vector containing the 6xHIS tag. Following expression and purification from *E. coli*, 14-3-3 $\lambda$  can be incubated with each protein individually and together. Nickel resin can be incubated with the proteins. Once pulled from solution the bound proteins could be washed and eluted. I would expect to find both R18 and CT59 pulled out of solution by their association with 14-3-3 $\lambda$ . These associations could be confirmed by antibodies specific for R18, streptavidin, and CT59, anti-GST. As a control, proteins R18 and CT59 incubated together would not be expected to bind the Ni resin. This would confirm that both proteins need 14-3-3 $\lambda$  to bind, and that they do not bind to each other. Taken together, immunoprecipitations and western blots can confirm the R18 and CT59 interaction are specific for 14-3-3 $\lambda$ . However, western blot data can't be used to calculate dissociation constants.





**Figure 3-26 Recombinant RYC and Derivatives Expression in *E. coli***

(A) RYC and derivative fragments cloned into vector containing GST and N-terminus. Some fragments were also fused to YFP to more closely mimic the functionality of YFP as a fusion protein in RYC. 80% of the proteins did not express solubly in the lysate at 37°C. The lowered temperature, 30°C, also failed to enhance solubility. Protein solubility was ranked on a score of 0 – 10 with 10 being the most soluble. (B) The lysate expression profiles for two proteins at 37 and 18°C. Protease cleavage observed with GST-CT59. This same cleavage was also observed in a previous study (Jelich-Ottmann et al., 2001).

Measuring the affinity of each 14-3-3 binding moiety can be provided by SPR using an instrument like the Octet (Pall ForteBio). The Octet could be a useful platform for verifying the interaction of 14-3-3 binding moieties, R18 and CT59, for 14-3-3 $\lambda$ . These proteins could also be mixed and tested with 14-3-3 $\lambda$ .

I've cloned R18, CT59 and variants into a vector containing a N-terminal GST fusion tag with a TEV protease cleavage site. YFP was also fused to the proteins to act as a visual marker through expression and purification and also to more closely approximate the value of the YFP protein in "RYC" in its interaction with 14-3-3 $\lambda$  (Figure 3-26A). Eighty-percent of proteins were not found in the soluble lysate at 37°C. 30°C increased the solubility for some proteins slightly. A temperature of 18°C was used to express GST-CT59. A striated pattern was observed in the purified fraction. It was confirmed with western blot (data not shown) indicating protease cleavage (Figure 3-26B). This cleavage has been seen in previous purifications containing the C-terminus of PMA2 (Jelich-Ottmann et al., 2001).

The presence of an interaction between CT56 and 14-3-3 $\lambda$  in *N. benthamiana* but its absence between CT52 (with a penultimate, phosphorylation-null alanine mutation) and endogenous 14-3-3 proteins in yeast suggests that this interaction can't be explored with recombinant protein. CT56 binds to the autoinhibitory region II of an endogenous PMA2. The C-terminal of PMA2 is necessary for 14-3-3 interaction and subsequent hexamerization. Thus, without full length PMA2 capable of hexamerization, the CT59.ex1.f1 peptide would not be predicted to interact with 14-3-3 $\lambda$ . The Octet could be used to confirm this negative-interaction hypothesis.

***Producing CT59 Negative Interacting Protein and Testing with Multiple 14-3-3 Proteins***

Seven mutations were required to nullify the fluorescence between CT59.ex1.f1 and 14-3-3 $\lambda$ . However, this mutated peptide wasn't cloned into the larger CT59 fragment and observed for BiFC interreaction with 14-3-3 $\lambda$ . Thus, these same mutations made in the CT56 fragment may be enough to abolish interaction. Also, mutating residues G897, S898, Y899 and R900 to alanine may also abolish interaction. Previous analysis mutating Y899 and R900 to alanine failed to abolish interaction. The four amino acids overlap with CT52 from PMA2. The deletion of  $\Delta$ 52 removed the autoinhibition of autoinhibitory region II caused by dimerization of PMA2 C-terminus. Thus, mutating these four amino acids may also abolish interaction.

The CT59 fragment was confirmed to interact with other isoforms like 14-3-3 $\zeta$ . However, quantitative readings were not conducted. Maybe this could be done with IVIS. To show that CT59 preferentially interacts with certain 14-3-3 isoforms will show specificity among the isoforms and may be important for further characterizing 14-3-3 H<sup>+</sup>-ATPase interactivity. Transformed Arabidopsis plants could be used to substantiate this interaction.

## ***Conclusion***

### ***The Importance of Transgenics in Crop Improvement***

The Golden Banana reports at least 20  $\mu\text{g/g}$  of  $\beta$ -carotene (Paul et al., 2017). This amount represents  $\approx 7\text{x}$  increase over the wildtype banana and will administer an essential vitamin to 190 million preschool children (World Health Organization, 2011). The higher concentration of  $\beta$ -carotene is obtained by engineering phytoene synthase 2a (MtPsy2a) gene from a wildtype banana and driving it with a constitutively-active maize polyubiquitin promoter.  $\beta$ -carotene concentration was not as high with cisgenic promoters. The transgenic combination of promoter and gene produced the targeted amount  $\beta$ -carotene beginning at development and continuing until fruit ripening (Paul et al., 2017). Thus, only transgenics are able to deliver on increased  $\beta$ -carotene. Plant improvement and development can be enhanced with traditional breeding using germplasm (cisgenics), but some traits and characteristics like increased  $\beta$ -carotene concentration will only be attainable with transgenics (National Academies of Sciences, 2016).

### ***Transgenics in Drought Tolerance***

A requirement for all life on earth, including plants, is water (Benner, Ricardo, & Carrigan, 2004). Severe drought decreases survival and fruit development. Climate change is expected to create both wetter and dryer areas (IPCC, 2014), and future crops will both need to be thermally and drought tolerant (Howden et al., 2007).

Plants also need to defend themselves. In addition to drought tolerance, future crops will need to be more disease resistant as climate change affects pathogenicity of organisms including fungi (Karkowska-Kuleta, Rapala-Kozik, & Kozik, 2009; Pennisi, 2010). The introduction of resistance genes or modification to immune pathways via transgenic means will allow swift responses to plant pathogens (Jones et al., 2014).

### ***RYC and 14-3-3***

14-3-3 proteins are conserved Eukaryotic proteins involved in multiple cellular pathways including cell cycle division, apoptosis and biotic and abiotic stresses (Denison, Paul, Zupanska, & Ferl, 2011; Lozano-Durán, Robatzek, & Lozano-dur, 2015; Oecking & Jaspert, 2009; Van Kleeff et al., 2014). Thus, modification of the 14-3-3 proteins and their client proteins may be able to provide durable trait modification in plants (Jaspert, Throm, & Oecking, 2011).

Using in vivo and in vitro techniques I've demonstrated interaction between two 14-3-3 binding proteins: R18 and CT59. The mechanism of R18 binding 14-3-3 has been elucidated with a crystal structure (Ottmann et al., 2007). CT59 binding to 14-3-3 has also been elucidated and show C-terminal binding, but my in vivo BiFC experiments truncating CT59 do not recapitulate this data. The discrepancy is understood to be a result of the expression system in yeast where only one mutant copy is expressed versus *N. benthamina* in which multiple homologs exist. Bias

towards interaction may also result from hexamerization of CT59 and 14-3-3 proteins and suggest caution when using BiFC for membranes known to oligomerize.

A bidentate fusion protein capable of interacting with 14-3-3 proteins was created. With its expression in *Arabidopsis* I demonstrated drought tolerance and RPW8.2-mediated resistance to powdery mildew. Which 14-3-3 isoforms RYC binds and the exact nature of its interactions will be queued for future experiments.

## ***Methods and Materials***

### *Arabidopsis and N. benthamiana Growth*

Arabidopsis and *N. benthamiana* plants were grown as detailed above in Chapter 2.

### *DNA construction*

Derivative and truncated CT59 constructs were created based on R18-YFP-CT59 (RYC) sequence. RYC was created by overlapping PCR.

### *N. benthamiana infiltrations*

*Agrobacterium tumefaciens* (hereafter: Agro) strain carrying gene of interest were prepared in binary vector. The day before infiltration, growing colonies from a plate or glycerol stock were streaked into 5mL LB broth (half salt concentration with antibiotics (25 mg/mL Rifampicin, 25 mg/mL Gentomycin and 50mg/mL of kanamycin or 100 mg/ml spectinomycin) depending on the vector resistance. Following day, Agro cultures were spun down at 4500g for 10 to 20 minutes at RT in 5mL conical tubes. Tubes were decanted and pellets were resuspended in 5mL dionized water containing 10mM MgCl<sub>2</sub>. OD<sub>600</sub> using a Shimadzu UVmini 1240 spectrophotometer was between 1.8 and 2.5. Culture was aspirated into needless syringe with the air removed. Next, syringe was applied to abaxial leaf surface and infiltrated into leaf using steady force. Infiltration spots ranged between 0.5 cm<sup>2</sup> to > 5 cm<sup>2</sup>. Agro strains transformed with vectors were co-infiltrated with Agro containing

vector expressing turnip crinkle virus coat protein (TCV-CP) which has been shown to tamper down host immunity allowing greater ectopic expression (Qu et al., 2003; Thomas, Leh, Lederer, & Maule, 2003).

### *Protein Expression and Purification*

#### 14-3-3 Expression and Purification

14-3-3 cds was cloned into pET26 into NdeI and XhoI sites. Ligation into these sites granted C-terminal 6xHis tag and now pelB sequence. A 40mL culture overnight culture was inoculated the following morning into 800mL. Reaching OD600 0.8 to 1 in about 1.5 hours, broth previously incubating O/N in incubator at 37C. Culture was induced with 1mM IPTG for between 3.5-4 hours. Culture was pelleted and resuspended in 50mL buffer containing 20mM sodium phosphate pH7.5 and 150mM NaCl. Added 100ul of HALT phosphatase and protease inhibitor. A digital sonicate was used for lysis. Amplitude 30%, 10 sec on, 10sec off for 3min processing time. Sample was centrifuged for 30min at 10000g at 4C. 1mL Ni-NTA beads from Invitrogen were incubated with sample with end-over-end rotation for two hours and then O/N at 4C. The following morning, Ni\_NTA resin was washed with same buffer as lysis buffer but with 40mM imidazole. Protein was eluted with 500mM imidazole. Polishing step was done with Sephacryl S-200. 14-3-3 was dialyzed overnight with 20mM Tris pH7.5 and 20mM NaCl.



### *Ni-NTA Pulldown*

Purified proteins were incubated together overnight in 20mM Tris pH7.5 and 20mM NaCl. The following morning 50 ul of Ni-NTA beads were added to the samples and incubated for about 30minutes. Samples were washed several times with 150mM NaCl and 20mM phosphate buffer and eluted in 500mM imidazole in 25ul. 1x SDS was added to the sample and then boiled and run on 12% SDS-PAGE gel.

## ***Addendum: Exploring Divalent 14-3-3 Sequestration as A Potential Anticancer Therapy***

### ***Introduction***

Bidentate protein R18-YFP-CT59 (RYC) contains N- and C-terminal moieties capable of simultaneously engaging 14-3-3 proteins. 14-3-3 proteins are conserved eukaryotic proteins that serve phosphorylated client proteins in plants and humans (Oecking & Jaspert, 2009; Yaffe, 2002). 14-3-3 proteins play roles in cell-cycle regulation, apoptosis and proliferation (van Hemert, Steensma, & van Heusden, 2001). Consequently, many types of cancer like oral squamous cell carcinoma (J. T. Chang et al., 2005), breast (Ferguson et al., 2000; Umbricht et al., 2001) and lung (W. Qi, Liu, Qiao, & Martinez, 2005) note 14-3-3 misregulation. The upregulation of 14-3-3 $\zeta$  in cancers may act to antagonize proapoptotic protein Bad allowing cells to detach from the extracellular matrix without apoptosing increasing risk of metastasis (Cantley, 2002; Z. Li et al., 2008). Consequently, 14-3- $\zeta$  may potentiate targeted therapies or prognoses (Fan et al., 2007; Matta, Siu, & Ralhan, 2012).

Building on previous data demonstrating interaction between R18-YFP<sub>C</sub>-CT59 (RY<sub>C</sub>C) and 14-3-3 $\zeta$  using bimolecular fluorescence complementation (BiFC) in planta, I hypothesized that the bidentate modality of RYC may bind and sequester 14-3-3 $\zeta$ . Additional 14-3-3-binding proteins were also cloned to use as potential substitutes for Arabidopsis CT59 (AT4G30190.2, AHA2) should it not bind human 14-3-3 isoforms. “p85”, the regulatory subunit of PI3K shown to bind 14-3-3 $\zeta$  was

probed as a potential substitute (Neal et al., 2012). “CT54”, the 54 C-terminal residues from ExoS, a bacterial toxin from *Pseudomonas aeruginosa*, was also tested. ExoS has been shown to interact with 14-3-3 $\zeta$  through hydrophobic residues and not via one of the three 14-3-3-canonical binding motifs (Yasmin et al., 2006, 2010).

Using a combination of the 14-3-3 $\zeta$ -binding proteins in combination with R18, I hoped to show 14-3-3 $\zeta$  sequestration resulting in a remediation of 14-3-3 $\zeta$ -induced survival.

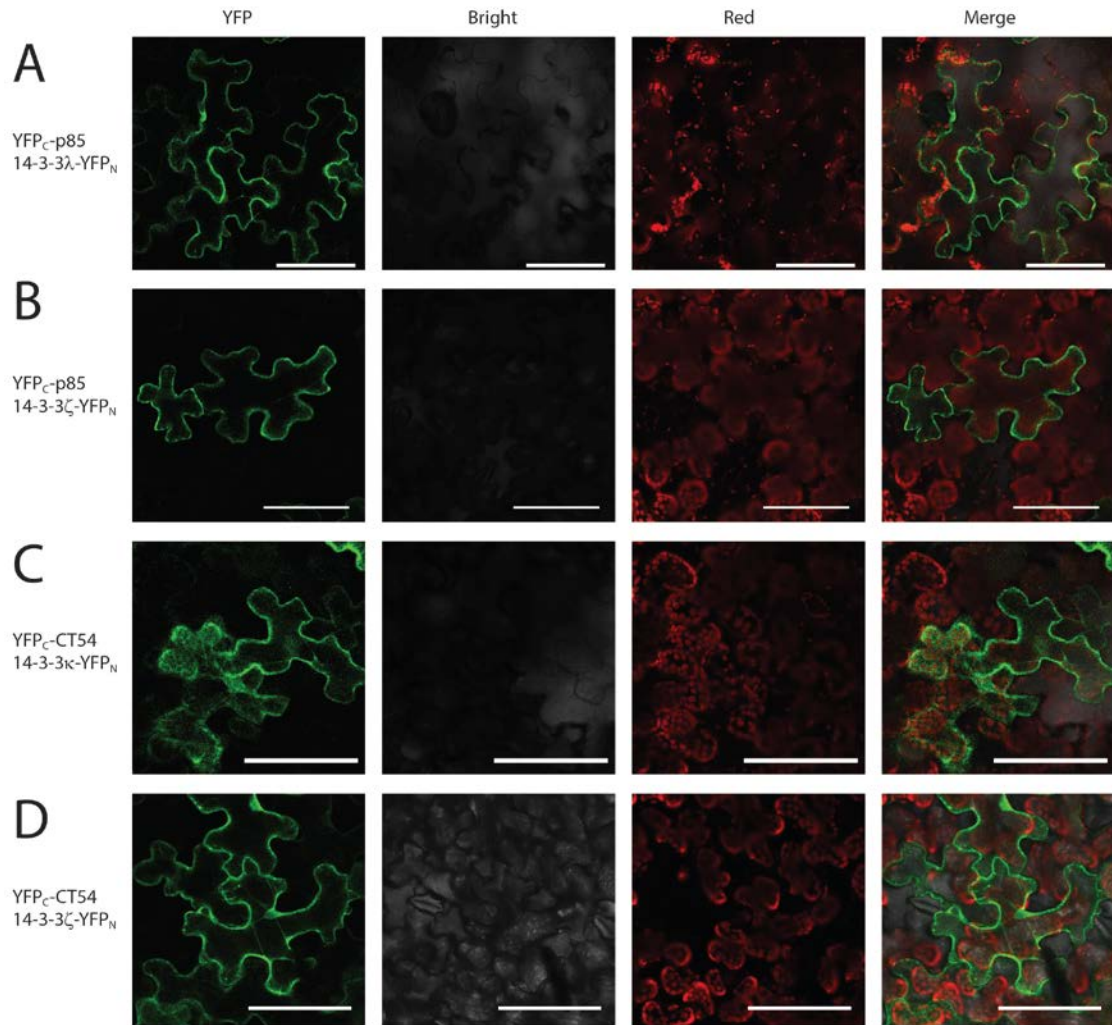
## **Results**

### *14-3-3 $\zeta$ -Binding Proteins Interact with 14-3-3 $\zeta$ In Planta*

The ORF of 14-3-3 $\zeta$ -binding protein R18-YFP-CT59 and variations were cloned into plant binary vectors. The N-terminus of YFP (YFP<sub>N</sub>) was fused to 14-3-3 $\zeta$ . The YFP C-terminus was fused to the 14-3-3 $\zeta$ -binding proteins. Previous data showed 14-3-3 $\zeta$  binding to both R18 and CT59. The same procedure was used to verify binding between 14-3-3 $\zeta$  and multiple constructs. p85 was verified to interact with 14-3-3 $\lambda$ , a plant 14-3-3 isoform, in addition to 14-3-3 $\zeta$  (Figure 3-27A-B). CT54 was observed to interact with 14-3-3 $\kappa$  along with 14-3-3 $\zeta$  (Figure 3-27C-D). 14-3-3 $\zeta$  and binding interactors were tested for interaction in cell lines.

Table 7 Size (kDa) of 14-3-3 $\zeta$ -Binding Proteins

Number	Plasmid	Size(bp)	size(aa)	kDa
1	YFP <sub>C</sub> -p85	6047	215	24
2	14-3-3 $\zeta$ -YFP <sub>N</sub>	6189	400	45
3	R18-YFP <sub>C</sub> -CT59	5584	168	19
4	YFP <sub>C</sub> -CT59	5430	147	16
5	R18-YFP <sub>C</sub> -CT54	5473	162	18
6	YFP <sub>C</sub> -CT54	5410	141	16
7	R18-YFP <sub>C</sub>	5310	107	12
8	YFP <sub>C</sub>	5247	86	10



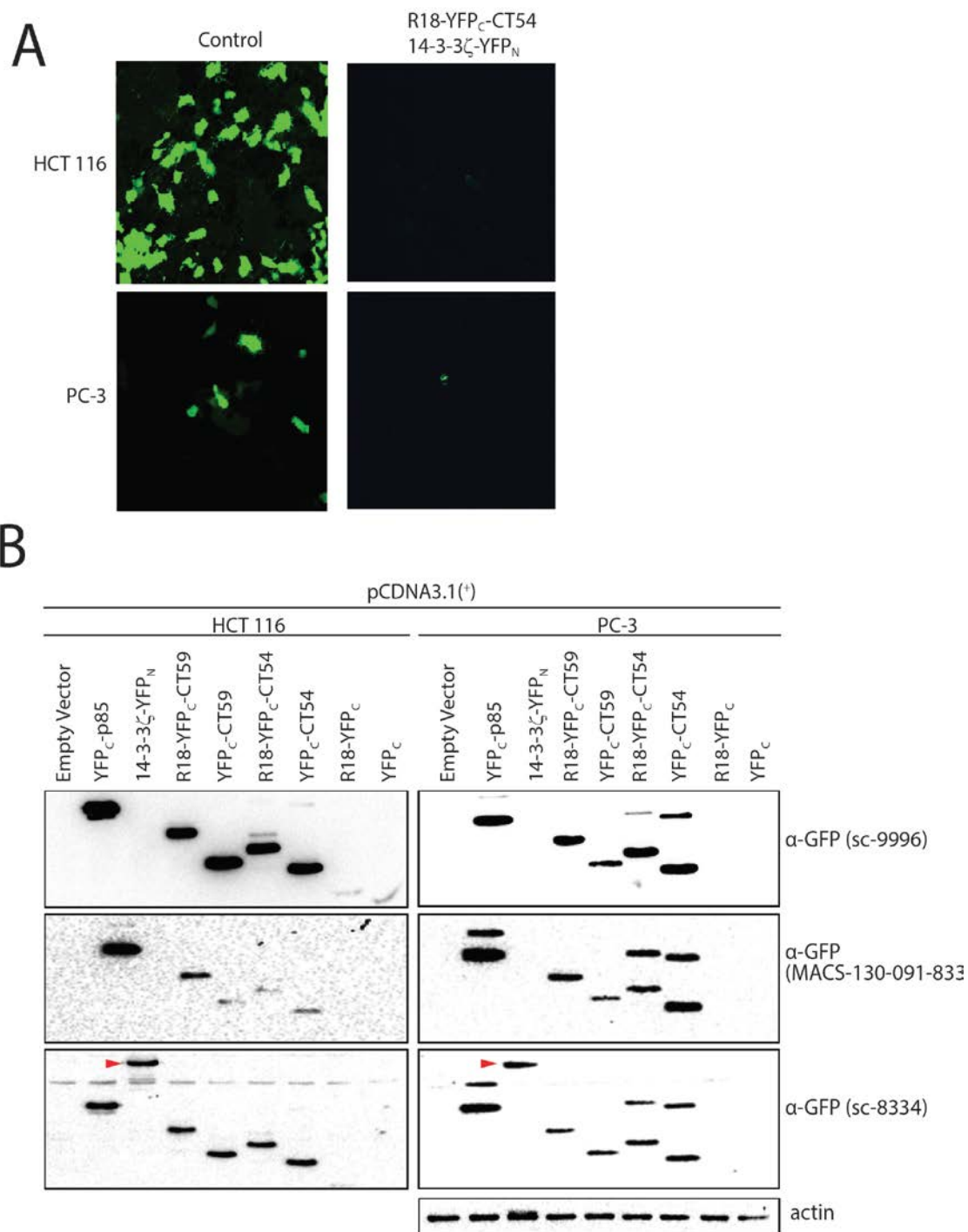
**Figure 3-27 14-3-3 $\zeta$  binds Both p85 and CT54 in BiFC**

(A) YFP<sub>C</sub>-p85 fluorescently interacts with 14-3-3 $\lambda$ -YFP<sub>N</sub>, an Arabidopsis 14-3-3 isoform in BiFC assay in *N. benthamiana*. (B) YFP<sub>C</sub>-p85 fluorescently interacts with 14-3-3 $\zeta$ -YFP<sub>N</sub>, a human 14-3-3 isoform. (C) The C-terminal fifty-four amino acids from a bacterial toxin from *Pseudomonas aeruginosa* CT54 successfully interacts with 14-3-3 $\kappa$ (D) 14-3-3 $\zeta$  also interacts with CT54.

### *14-3-3 $\zeta$ -Binding Proteins Express in Cell Lines*

We cloned RYC into mammalian-specific expression vector pCDNA3.1(+) (Thermo Fisher) containing constitutive promoter (CMV) from cytomegalovirus. The size and orientation of the 14-3-3 $\zeta$ -binding proteins can be seen in (Table 7). Three cancerous cell lines were used for testing. HCT 116 is a human colorectal carcinoma cell line. This line contains a mutation in the ras proto-oncogene at codon 13 (A549 ATCC) (R. Wang, Kobayashi, & Bishop, 1996). PC-3 is a human adenocarcinoma cell line derived from metastatic bone (PC-3 ATCC) (Su et al., 1996). A549 is an lung epithelial carcinoma cell line (A549 ATCC) (Lieber, Todaro, Smith, Szakal, & Nelson - Rees, 1976). 14-3-3 $\zeta$  co-transfected with R18-YFP<sub>C</sub>-CT54 generated very little fluorescence in PC-3 and HCT 116 lines (Figure 3-28A). A control, Monster Green® fluorescent protein (phMGFP, Promega), successfully transfected both cell lines.

The expression of 14-3-3 $\zeta$  and binding proteins was assayed in cell lines with a western blot and three different  $\alpha$ GFP antibodies (Figure 3-28B). Two antibodies did not recognize YFP<sub>N</sub> terminus of 14-3-3 $\zeta$ . However,  $\alpha$ GFP (SC-8334) did successfully detect confirming expression of 14-3-3 $\zeta$  in these two cell lines.



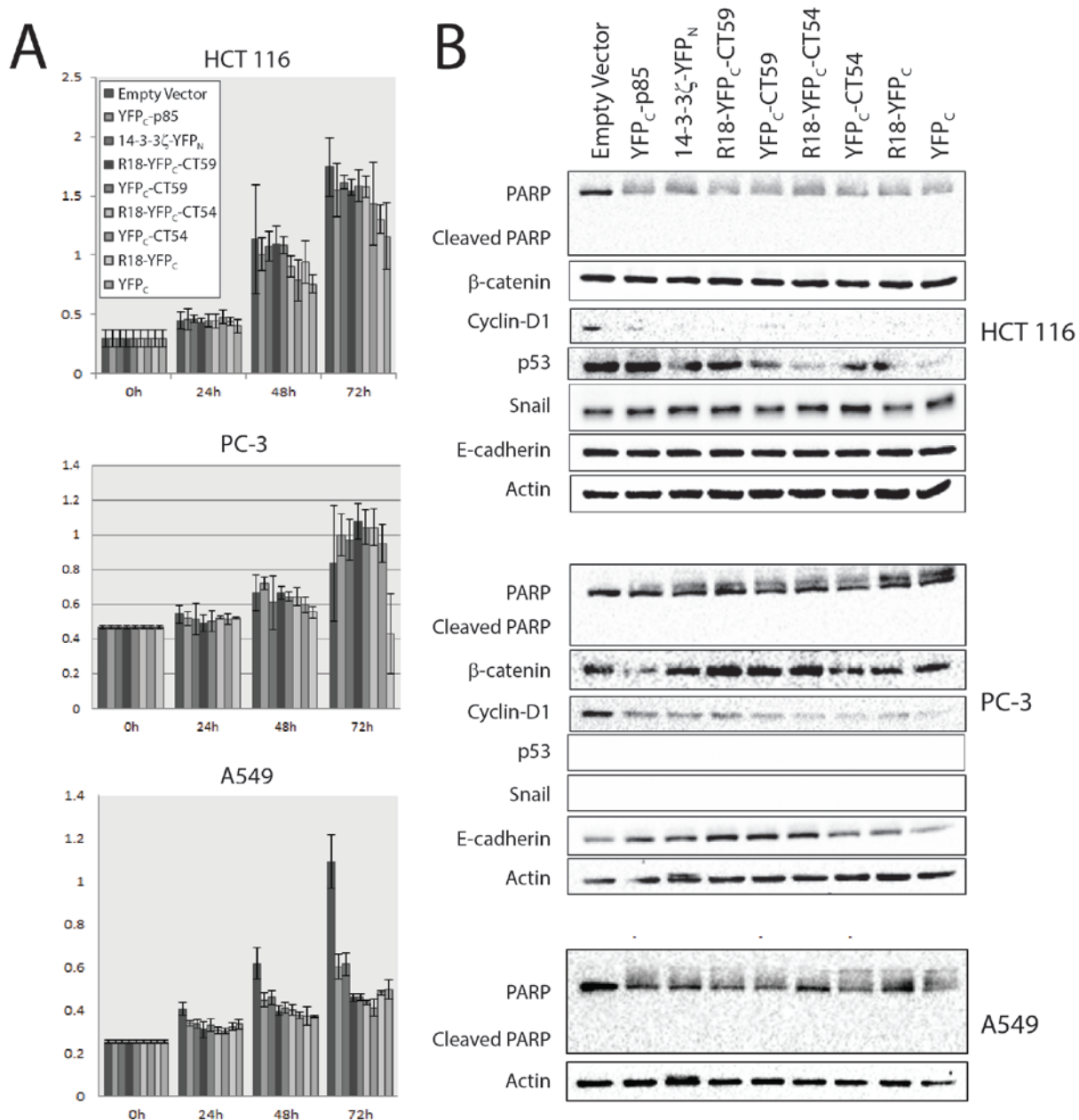
**Figure 3-28 14-3-3 Binding Proteins Express in Cells but Interact Weakly with 14-3-3 $\zeta$**

(A) 14-3-3 $\zeta$  does not interact or only interacts weakly with R18-YFP<sub>C</sub>-CT54 in HCT 116 or PC-3 lines compared to transfection control. Transfection control is Monster Green® fluorescent protein (pMGFP, Promega). (B) Testing expression of 14-3-3 $\zeta$ -binding DNA constructs in human cancer cells using different  $\alpha$ GFP antibodies. Eight DNA constructs were transiently expressed in two types of cancer cell lines, HCT-116 and PC-3. Total proteins were extracted from transfected cells, gel blotted and assayed with three different anti-GFP antibodies (Santa Cruz Biotech and MACS company).  $\alpha$ -GFP (sc-8334) could detect both YFP<sub>N</sub> and YFP<sub>C</sub>, whereas the other two antibodies could only detect YFP<sub>C</sub>. Based on the Western results, all the constructs appear to be expressed in the two types of human cancer cells. R18-YFP<sub>C</sub> (12kDa) and YFP<sub>C</sub> (10kDa) alone may be too small and ran out of gel, explaining the lack of a band in the gel blots. (B) Three  $\alpha$ GFP antibodies confirm expression of 14-3-3 $\zeta$ -binding proteins in cells on western blot. Only  $\alpha$ GFP (sc-8334) is able to detect YFPN terminus fused to 14-3-3 $\zeta$  (red arrow).



### *14-3-3 $\zeta$ -Binding Proteins Do Not Modify Cell Proliferation*

Cells that would normally undergo apoptosis are prevented by the upregulation of 14-3-3 $\zeta$  interfering with pro-apoptotic proteins (Cantley, 2002; Z. Li et al., 2008). Thus, removing 14-3-3 $\zeta$  should allow cells to resume apoptosis. Three cancer cell lines, HCT-116, PC-3 and A549, were transfected with 14-3-3 $\zeta$ -binding proteins. Cell proliferation was measured at 0, 24, 48 or 72 h after transfection. Next, cells were transfected and subjected to western blot to analyze six proteins implicated in apoptosis, cell cycle and EMT (epithelial mesenchymal transition). PARP, or poly(ADP-ribose) polymerase is a 116 kDa protein involved in DNA repair. When cells undergo apoptosis, this protein is cleaved by caspase-3 in a 89 kDa fragment (Lazebnik, Kaufmann, Desnoyers, Poirier, & Earnshaw, 1994). No significance difference in survival or protein cancer protein expression markers is observed.



**Figure 3-29 Post-transfection assays of treated human cancer cells using various protein markers.**

(A) Effects of 14-3-3 sequestering constructs on proliferation of cancer cell. HCT-116, PC-3 and A549 cells were plated on 96-well plate and transfected with different 14-3-3 constructs

for 0, 24, 48 or 72 h. (B) Cells were transfected with the indicated constructs and subjected to gel blotting assays. In the case of HCT-116 and PC-3 cancer cell lines, apart from actin (as an internal control), six proteins implicated in apoptosis, cell cycle and EMT (epithelial mesenchymal transition) were analyzed using specific antibodies. In the case of A549 cancer cell line, apart from actin, two indicated proteins markers were analyzed. No significance difference in protein expression was observed in this pilot study.

## ***Discussion***

The in planta interaction observed between p85 and CT54 for two Arabidopsis 14-3-3 isoforms confirms the amino acid conservation of 14-3-3 proteins across different kingdoms. A clustal omega alignment between 14-3-3 $\zeta$ , 14-3-3 $\lambda$  and 14-3-3 $\kappa$  shows 143 identical sites among 254 amino acids with 69% pairwise identity. This is the first study in which the binding of both CT54 and p85 to Arabidopsis isoforms has been demonstrated.

Three cancer cell lines transfected with 14-3-3 $\zeta$ -binding and sequestering constructs failed to reduce 14-3-3 $\zeta$  as measured by cell proliferation assays and gel blots probing for signature cancer proteins. The data can be explained with the following hypotheses. (i) The 14-3-3 $\zeta$ -binding proteins have successfully engaged and interacted with 14-3-3 $\zeta$ , but they binding did not prevent 14-3-3 $\zeta$  activation or interaction with pro-apoptotic proteins like Bad. (ii) 14-3-3 proteins are known to play redundant roles in human disease (Toyo-oka et al., 2003). Thus, although 14-3-3 $\zeta$  may have been successfully sequestered, other misregulated 14-3-3 isoforms were untouched. (iii) The expression of 14-3-3 $\zeta$ -sequestering proteins was not large enough compared to overall 14-3-3 $\zeta$  concentration. This is born out in the data noting that BiFC interactions in cell lines were weak compared to interactions observed in planta, yet western blots with three  $\alpha$ GFP antibodies confirm expression (Figure 3-28A-B). Future experiments will need to consider ectopic expression yield compared to 14-3-3 $\zeta$  concentration.



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