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Exploring Phosphorylation in the regulation of the WUSCHEL Protein Gradient and Transcription

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Dariush Ravandi Nejad

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Thesis Committee:
Dr. Venugopala Reddy Gonehal, Chairperson
Dr. Jaimie Van Norman
Dr. Morris Maduro

The Thesis of Dariush Ravandi Nejad is approved:

Committee Chairperson

University of California, Riverside

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Dedication

I feel an overwhelming gratitude for the people in my life who have supported me on this journey. I can't begin to express how remarkable it feels to be at this point in my career. Although it took an immense amount of hard work, determination and mental fortitude, I know that I could not have done this on my own.

I would like to dedicate this thesis to my family for all their love and support. I cannot express how thankful I am for the sacrifices my mother and father made to get me to where I am today but I hope that through my successes I can make that burden they took worth it. I want to thank my brother and sister, I love them more than they know and they inspire me every day to be someone they can look up to.

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Introduction:

Plant development vastly differs from animal development due to the fact that plants are able to maintain continuous organogenesis throughout their lifespan. This is possible because plants are able to maintain populations of undifferentiated stem cell located at the Shoot Apical Meristem (SAM) and Root Apical Meristem (RAM), which provide a constant supply of cells for tissue and organ development (Barton and Poethig, 1993, Sussex, 1989, Kaufmann et al., 2010). The SAM is responsible for all above ground organ formation producing rosette leaves during the vegetative stage transitioning to producing leaves, flowers and stem during its reproductive phase. On the other hand,the RAM produces all subterranean organs and in sustaining continuous root development and growth.

Structural Organization of the SAM

The SAM of the model organism Arabidopsis thaliana is a conglomeration of several functional domains organized into three clonally distinct layers of cells. The L1 is the outermost layer that generates the epidermis, the L2 is the subepidermal layer and the L3 is multilayered which is collectively referred to asthe corpus (Sussex, 1989)(Fig. 1.1A). The L1 and L2 layers divide anticlinally, perpendicular to the cell surface, generating clonally distinct monolayers (Fig. 1.1A). The L3 and below differ from the L1 and L2 in that it divides both anticlinally and periclinally, forming its own clonally distinct region (Fig. 1.1A).

Simultaneously, there are functional domains of the SAM that arise as cells divide and displace from the center of the L1-L3 outward. At the very center of the L1-L3 near the tip of the SAM there is a population of undifferentiated stem cells that form the Central Zone (CZ) (Reddy, 2008, Steeves and Sussex, 1989) (Fig. 1.1A). Divisions of these cells results in sustained maintenance of the CZ and the lateral displacement of daughter cells into the adjacent Peripheral Zone (PZ) where differentiation begins leading to the development of Organ Primordia (OP) (Fig. 1.1A). Below the CZ is a subset of cells called the Organizing center (OC) that has been shown to provide cues for stem cell specification (Fig. 1.1A). Encompassing the OC and the rest of the L3 and below is the Rib-meristem (RM).

Molecular Mechanisms Regulating the SAM

Maintenance of SAM organization, gene expression, and transitioning of slow diving stem cells to fast diving differentiating cells requires a wide array of signals and regulatory factors. Specifically, these mechanisms must coordinate to accomplish two basic tasks. First, establish stem cell identity and maintain a core population of stem cells aimed at replenishing themselves to sustain continual growth. Second, allow the timely passage of stem cell daughters into lateral regions and promote differentiation into organs. These two aspects are regulated by using a combination of factors raging from plant hormones to mobile transcription factors.

During the embryonic development, the transcription factors *WUSCHEL* (*WUS*) and *SHOOTMERISTEMLESS* (*STM*) expression is initiated in presumptive SAM cells. *WUS* can be seen during the 16-cell stage (Mayer et at., 1998). This is followed by the *STM* expression in presumptive SAM cells and in a small cluster of cells near the boundary of cotyledon primordia during the globular-stage (Long et al., 1996). Both transcription factors work to induce SAM identity and repress genes responsible for differentiation such as *AYSMMETRIC LEAVES2, YABBY3, KANADI1* and *KANADI2* (Lenhard et al., 2002, Yadav et al., 2013). Mutations in both *WUS* and *STM* lead to loss of SAM identity possibly due to a premature differentiation and the SAM fails to grow continuously, further cementing their vital role in SAM maintenance (Lenhard et al., 2002). Additionally, cytokinin works synergistically with these transcription factors by being involved in SAM development, maintenance and growth as depletion of the hormone leads to reduced SAM size while higher cytokinin levels leads to enlarged SAMs (Sablowski 2007, Werner et al., 2003, Werner & Schmülling, 2009). Cytokinin is perceived by histidine kinases (CRE1/WOL/AHK4, AHK2 and AHK3) which downstream will phosphorylate and activate two classes of Arabidopsis response regulators (ARRs) (Kakimoto 2003). The first is Type-B ARRs which contain a DNA binding domain and when phosphorylated work to activate cytokinin-induced genes (Gordon et al., 2009). The second are Type-A ARRs which are upregulated in cytokinin environments but work to negatively regulate cytokinin responses by preventing type-B ARR activity forming a

negative feedback loop to regulate type-B ARR activity (To 2004). It has been shown that cytokinin can activate WUS transcription and that WUS in turn represses type-A ARRs genes (Gordon et al., 2009, Leibfried et al., 2005). However, a recent study has shown that WUS expression remains unaffected in cytokinin receptor mutants while the WUS protein fails to accumulate showing that cytokinin stabilizes WUS protein (Snipes et al., 2018). The same study also revealed that cytokinin response pattern remains normal in wus-1 null mutants suggesting that WUS is not required for promoting cytokinin response in SAMs. Cytokinin biosynthesis has been shown to be activated by the *Class-I KNOTTED1-like homeobox (KNOX)* genes including *STM* and increasing the cytokinin biosynthesis has been shown to rescue rescue stm mutants (Jasinski et al., 2005, Long et al., 1996). Meanwhile, STM promotes the expression of genes involved in cytokinin biosynthesis (Yanai et al., 2005). Conversely, auxin allows cells in the PZ to differentiate into organ primordia. This is done by PIN1, an auxin efflux regulator protein, which promotes the accumulation of auxin in PZ organ primordia (Reinhardt et al., 2003). Auxin will then lead to the activation of *AUXIN RESPONSE FACTORS (ARFs)* ,such as *MONOPTEROS (MP)*, which is suggested to promote primordia formation (Hardtake et al., 1998). Mutations in either *PIN1* or *MP* lead to disruptions in lateral organ formation and developmental defects (Berleth et al., 1993, Bennett et al.,1995). Auxin is also attributed with negatively regulating the type-A ARR genes *ARR7* and *ARR15* needed for cytokinin signaling (Zhao et al., 2010). As a whole the basic

synergistic and antagonistic interactions between hormones, transcriptional regulators and target genes discussed here are essential for proper SAM maintenance and regulation.

Introduction of WUSCHEL and CLAVATA3 Regulation of the SAM

One of the well-studied regulatory mechanisms in plant stem cell maintenance, and the focus of this thesis, is the *WUSCHEL (WUS)* and *CLAVATA3 (CLV3)* feedback loop (Fig. 1.1B). WUS is a stem cell promoting transcription factor that is synthesized in the RM and diffuses into adjacent cells through plasmodesmata (PD), junctions between neighboring cells through a shared cell wall, to form a protein gradient across the stem cells from the site of synthesis to the outer layers (Yadav et al., 2011, Daum et al., 2014). WUS promotes stem cell identity by repressing key genes involved in cell-identity and differentiation found to be expressed in the cells of the PZ (Fig. 1.1B). (Yadav et al. 2013). In the case of several genes such as *AYSMMETRIC LEAVES2, YABBY3, KANADI1* and *KANADI2*, mentioned previously, WUS can directly bind to the promoter of these genes to repress their transcription (Yadav et al. 2013). In a similar manner, differences in cell-division rates between the CZ and PZ may be controlled by WUS as it accumulates at higher level in the CZ where cells divide relatively slowly than in the PZ where WUS accumulates at a lower level (Reddy 2005; Yadav et al. 2010) (Fig. 1.1B). WUS also activates the transcription of its own negative regulator CLV3 (Fig. 1.1B). CLV3 is a 96 AA protein that moves through the apoplastic space where it binds and activates

CORYNE/CLV1/CLV2, a family of membrane bound leucine-rich repeat receptor kinases (LRR-RKs). Activation of these LRR-RKs results in the negative regulation of WUS transcription (Ohyama et al., 2009, Rojo et al., 2002, Fletcher et al., 1999, Brand et al., 2000). This was confirmed with CLV3 genetic analysis which revealed mutant CLV3 and CLV3 RNAi knockdown both lead to over-proliferated SAMs and expansion of the WUS expression domain (Reddy and Meyerowitz, 2005, Schoof, 2000, Mayer et al., 1998). WUS has been shown to bind five closely spaced cis-elements (CRM) in order to activate CLV3 at lower WUS protein levels and repress it at higher WUS protein levels (Perales et al., 2016). Additionally, *in vitro* experiments show that WUS can also differentially bind the CRM as a monomer, at low protein concentrations, or as a dimer, at high concentration, suggesting that WUS operates in a concentration-dependent manner in order to regulate the SAM (Perales et al., 2016). The mechanisms that control how WUS can activate genes needed for regulating stem cells or repress genes needed for differentiation is largely unknown but it is clear the ability for WUS to diffuse and form a protein gradient is critical to this function.

Previous research has shown this by fusing 2xeGFP or a nuclear localization sequence to WUS which does not allow WUS to move out of the RM or rescue wus mutant lines (Yadav et al., 2011). Additionally, decreasing protein levels with a WUS RNAi makes WUS a better activator of CLV3 while increasing nuclear levels with a nuclear localization sequence makes WUS a better repressor (Perales et al., 2016). Since WUS is synthesized in the RM, nuclear

export or nuclear retention can entirely influence diffusion and stability of the protein. Nuclear retention can stabilize the protein but inhibit movement across the SAM, while nuclear export can increase the cytoplasmic WUS pool increasing diffusion but also decreasing stability. Much of the ability to control movement and diffusion of WUS are intrinsic to the protein. The N-terminal of WUS has been shown to mediate homodimerization and DNA binding that restricts WUS diffusion, the C-terminal contains the WUS-Box and EAR-like domain that antagonistically modulate nuclear-cytoplasmic partitioning, diffusion and protein stability as deletions of these domains lead to mis-accumulation of the protein (Rodriguez et al., 2016). Various external signals have also been shown to regulate WUS nuclear-cytoplasmic partitioning (Fig. 1.1C). CLV3 is suggested to stabilize WUS as it becomes highly unstable in the L1 of *clv3* mutants despite the protein accumulating at higher levels in the RM (Perales et al., 2016) (Fig. 1.1C). Additionally, cytokinin has been shown to act through the transcriptional regulatory domains, the acidic domain and WUSCHEL box, in order to stabilize the WUS protein in the deeper layers enhancing gradient formation (Snipes et al., 2018) (Fig. 1.1C). Finally, the GRAS family transcriptional regulators HAIRY MERISTEM (HAM) interact directly with WUS to promote indeterminacy (Zhou et al., 2015; Engstrom et al., 2011) (Fig. 1.1C). The mechanism utilized by these various signals to modulate the activity of these domains and regulate WUS protein gradient formation is still unclear. Due to the need for WUS levels to be fine-tuned, it is likely that protein complexes and post-

translational modifications may play a role in the function of these domains. The importance of protein complexes and post-translational modifications regulating transcription factors has already been seen in various systems. In animals, Nuclear factor of activated T-Cell (NFAT) transcription factors require phosphorylation from target kinases in order to properly regulate nuclear localization sequences (NLS) and nuclear export sequences (NES) that will then modulate nuclear-cytoplasmic partitioning depending on NFAT's phosphorylation state (Crabtree and Olson, 2002). In immune cells, NF-KB are a family of inducible transcription factors that are sequestered in the cytoplasm by IKB inhibitory proteins (Liu et al., 2017). Upon activation of IKB kinase (IKK) complexes, IKBa(alpha) is phosphorylated triggering the ubiquitin-dependent degradation of IKBs and subsequent nuclear translocation of NF-KB members (Beinke et al., 2004, Karin 2000, Hayden 2008). Protein complex mediated movement has even been seen in plant meristematic regulation in the case of the RAM with SHORT-ROOT (SHR). SHR is a transcription factor that is able to diffuse from its site of synthesis, the stele, into the adjacent Quiescent Center (QC) and endodermis layer where it is then sequestered in the nucleus by its co-factor SCARECROW (SCR) (Nakajima et al., 2001, Cui et al., 2007). These examples and concepts demonstrate the importance protein complexes and post-translational modification can have on regulating mobility, subcellular sequestration and other functions that are essential to WUS gradient regulation in the SAM.

Introduction to Protein Phosphorylation

Protein phosphorylation is one of the most common and widespread forms of post-translational modification across eukaryotic kingdoms (Frades et al., 2015). Protein phosphorylation was first identified in the protein Vitellin (Levene et al., 1906). Although the first instance of the enzymatic phosphorylation of proteins was not identified years later (Burnett et al., 1954). It is widely understood as the reversible covalent attachment of a phosphate group on to an amino acid residue which is catalyzed by kinases which utilize adenosine triphosphate (ATP) as the donor of the phosphate group (Fischer et al., 1955) (Fig. 1.2C). Once phosphorylated a protein typically changes form hydrophobic apolar to hydrophilic polar, as a result of phosphate groups having five outer electrons and high water solubility, allowing for conformational changes (Fatima et al., 2017; Hunter., 2009) (Fig. 1.2C). Addition of phosphate groups only occurs at amino acids with free hydroxyl groups that act as acceptors. Roughly 30% of phosphorylation events occur on serine (S), threonine (T) or tyrosine (Y) residues, although phosphorylation can occur on histidine (H) and aspartate residues (D) but it is much less stable than the others (Schwarts et al., 2011; Fukami et al., 1983).

Protein kinases are a family of proteins that are tasked with catalyzing the addition of phosphate groups. They are typically in their active forms or activated by phosphorylation which results in signal transduction and cascade events ending on the addition of phosphate groups on to target proteins (Alberts et al.,

2007). Activation or inhibition of kinases can be done by several different mechanisms, most commonly it is done through autophosphorylation, changes in sub-cellular localization or by binding to an inhibitor or activator protein (Roskoski., 2012). The catalytic domain of kinases, the N and C terminal, are held together by a peptidic stand that forms an active site or catalytic domain. This site contains a front pocket that contains catalysis residues and a back pocket (Fatima et al., 2017). Activation of the catalytic front pocket occurs through phosphorylation of an activation loop or through other allosteric mechanisms (Schwartz et al., 2011). Additionally, kinases contain non-catalytic domains that are meant to regulate docking and attachment of substrates, signaling proteins or docking to target proteins (Nishi et al., 2014).

Protein phosphorylation has been widely studied and is attributed with regulation of many biological processes. Specifically the cellular processes of protein synthesis, cell division, signal transduction, cell growth, development and aging have been found to be directly regulated by phosphorylation events (Fatima et al., 2017). Interestingly, the effects of phosphorylation on the dynamics transcription factors are of particular interest as it can be greatly altered aspects such as DNA binding, cellular localization, stability or oligomerization as a result of differential protein folding (Whitmarsh et al., 2000) (Fig. 1.2C). Therefore investigating the role of phosphorylation on WUS is critical since it could be vital in regulating the WUS protein gradient or function.

N-terminal Phosphorylation of the WUSCHEL Protein

In clv3-2 null mutant lines the WUS protein is produced at higher levels in the RM but is also exported at higher rates possibly resulting in increased degradation of the protein in the cytosol in the outer layers of the SAM (Perales et al., 2016). It is clear that CLV3 signaling acts in some way to stabilize the WUS protein in the higher levels in order for activation of CLV3. Currently, it is unknown what mechanisms CLV3 signaling uses to regulate WUS transcription and protein dynamics. Recent studies have shown that exogenous CLV3 peptide can activate the mitogen-activated protein kinase (MAPK), MPK3 and MPK6, via phosphorylation by the CLV1 and BAM1 receptors (Lee et al., 2019). Analysis of MAPK consensus sequences map docking of the MAPK kinases to the WUS-Box domain as it contains a RRTLPL motif (Dory et al., 2016) (Fig 1.2A-B). Biochemical analysis of these domains have shown that they are involved in subcellular localization, stability and transcriptional regulation as mutations to hydrophobic residues cause drastic changes to these aspects (Rodriguez et al., 2016). Intriguingly, these same hydrophobic residues are important to docking of these kinases as well. Additionally, in vivo analysis of the N-terminal residues (T108 and S112) have revealed them to be the target of the mitogen-activated protein kinase (MAPK) as co-transfection of MPK3 and WUS results in phosphorylation of WUS (Dory et al., 2016) (Fig 1.2A-B).

These findings suggest that CLV3 signaling may utilize phosphorylation to directly regulate the WUS protein gradient through kinases such as MAPKs.

Currently it is unknown how phosphorylation of the N-terminal residues of WUS can influence gradient dynamics despite the substantial evidence that these sites are actively modified.

Introduction to KIN10/KIN11

Previous work unveiled a vast protein network with which WUS interacts with identifying interactions between WUS and kinases (Snipes., 2016). Of all the proteins identified to directly interact with WUS a small subset of the protein identified had been previously characterized. One of these proteins is the SNRK1 (SNF1-Related Protein Kinase) protein kinases KIN11 (Snipes., 2016). KIN11, often associated with KIN10 due to functional redundancy, are a part of the evolutionary conserved family of energy sensor kinases which include the mammalian AMPK (AMP-ACTIVATED PROTEIN KINASE) and the yeast SNF1 (SUCROSE-NON-FERMENTATION1) (Celenza et al. 1986; Hardie et al., 2012; Hardie et al., 1998). The SnRK family comprises the SnRK1, SnRK2 and SnRK3 subfamilies. While the SnRK2 and SnRK3 subfamilies are associated with abscisic acid (ABA) and environmental stress signaling, SnRK1 proteins are responsible for regulating transcriptional networks as a result in metabolic or environmental changes (Dong et al., 2012; Hardie, 2004).

The SNRK1, SNF1 and AMPK kinases are highly conserved across all eukaryotic organisms and share the same αβγ heterotrimeric structure (Carling., 2004). The α-subunit contains a kinase responsible for phosphorylation and

regulatory domain containing an activation or T-loop (Hanks et al., 1995). The regulatory region of the SNRK1 (plants) differs from that of SNF1 and AMPKs in that it instead contains a ubiquitin-associated domain that promotes interactions with ubiquitinated proteins rather than an inhibitory domain (Farras et al., 2001; Pang et al., 2007). The α-subunit has also been shown to contain a kinase-associated 1 (KA1) domain needed for interactions between regulatory subunits and phosphatases (Rodrigues et al., 2013; Crozet et al., 2014). The γ-subunit has been shown to regulate kinase activity but has not yet been directly shown in plants (Jiang et al., 1996; Avila et al., 2012). B-subunit inherently act as a scaffold keeping the α- and γ-subunits together (Crozet et al., 2014). But has also been shown to function in regulating subcellular localization (Vincent et al., 2001). Activity of the SNRK1, SNF1 and AMPK family are all increased in response to any stress that results in depletion of ATP ,increasing the AMP:ATP ratio, and decreased in the presence of ATP (Mohannath et al., 2014). The specific mechanism that works to activate these kinases is not yet clear but it is known that upstream kinases will phosphorylate the activation loop located in the α-subunit leading to kinase activation (Sugden et al., 1999). It is also known that AMP allosterically stimulated kinase activity with the exception of SNRK1 in which AMP only functions to inhibit dephosphorylation of the activation loop (Sugden et al., 1999).

Previous work has revealed the role of SNF1 and AMPK as key metabolic sensors and master metabolic regulators (Polge et al., 2006). Recently, their

SNRK1 counterparts,KIN10/KIN11, have also been shown as sensors for changes in metabolism as well as their role in transcriptome reprogramming in response to metabolic changes as a result of stress, nutrient deprivation and/or darkness (Baena-Gonzalez et al., 2007). KIN10/KIN11 activity differentially express genes associated with biosynthetic pathways, catabolic processes, stress signaling, transcription factors, histone modifications and transporters resulting in changes of plant energy homeostasis, survival, reproduction, senescence and development (Baena-Gonzalez et al., 2008). Further supporting the possible role of KIN10/KIN11 in regulating the WUS protein. Previous work has implicated TOR (Target Of Rapamycin) in regulating the shoot apical meristem function. TOR is a protein kinase that acts as a master regulator by taking in hormone, light and metabolic signals to modulate developmental transition via differential gene expression and growth in the shoot and root apex (Li et al., 2017). SNRK1 and their AMPK/SNF1 homologs work antagonistically to TOR by repressing many of the same targets (Baena-González et al., 2008; Jossier et al., 2009). Which suggests that there may be overlap in the regulatory targets or processes of SNRK1 and TOR. Recently, it has been suggested that TOR plays a part in regulating WUS expression as a result of differential light and glucose signaling (Pfeiffer A et al,. 2016). There is currently no consensus for SNRK1 docking site but looking at AMPK alignments reveal that proper sites have a βΦβ motif, where β represents a basic amino acid residue and Φ represents a hydrophobic amino acid residue (Hardie 2011) (Fig 1.2A-B).

Located after the hydrophobic residue of this motif is a serine or threonine residue which then be phosphorylated by the kinase residue (Hardie 2011) (Fig 1.2A-B). Screening the WUS protein sequence with the AMPK consensus sequence reveals that the WUS-Box and EAR-Like domains contain sites of phosphorylation with similar βΦβ motifs suggesting that KIN10/KIN11 could very well dock to these regions (Fig 1.2A-B). Again, connecting KIN10/KIN11 and their regulation of WUS.

Aims of the Study

Maintaining the WUS gradient is critical to regulating the stem cell niche in the shoot apical meristem. Although the molecular mechanisms responsible for establishing proper gradient formation and modulating WUS are still largely unknown, phosphorylation appears to be a compelling answer to this question. In order to further understand these mechanisms the objectives of this thesis are as follows:

1. Determine the *in vivo* effects of phosphorylation of the N-terminus of the WUSCHEL protein.

2. Uncover the effect of SNRK1-WUSCHEL interactions on SAM maintenance.

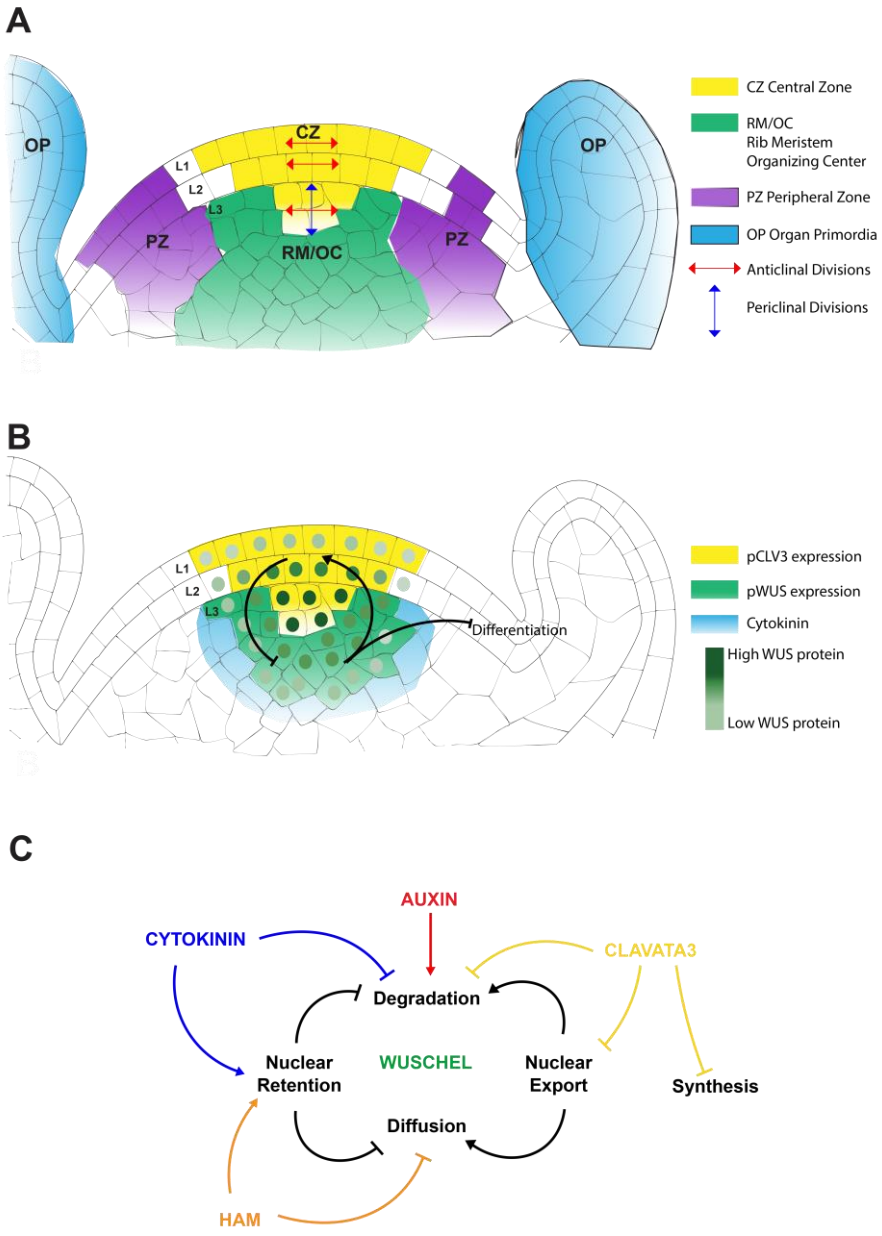


Fig 1.1 The shoot apical meristem and the signals and process regulating WUSCHEL protein levels

(A) Graphic representation of the side view of a SAM highlighting the cellular organization. The two outter layers (L1 and L2) undergo anticlinal (perpendicular to the surface) divisions forming a uniform cell layer. The inner layers (L3) undergo both anticlinal and periclinal cell divisions producing a multi-layered tissue. The SAM can then be futher divided into distinct functional domains. The CZ (yellow) starts at the top of the SAM harbors three stem cell layers. The RM/OC (green) is located directly underneath the CZ and functions to proliferate and maintain stem cells. The PZ (purple) suround the CZ and RM/OC and allows for stem cells to differntiate and begin to initiate OPs (blue). (B) A representation of the WUSCHEL-CLAVATA3 negative feeback loop within the context of the SAM. WUSCHEL (WUS) is expressed in the OC where cytokinin is also found working to stabilize the WUS protein. The WUS protein (green circles) moves from the OC forming a protein gradient with strongest accumulation near the site of synthesis as it tapers off as you move away from this area. WUS then activate CLAVATA3 (CLV3) in the CZ which will then signal back to repress WUS expression into the OC. (C) A represenation of the processes and signals needed to maintain the WUS protein gradient. In order to maintain proper protein levels degradation, diffusion and nuclear export/retention must be regulated, failure to do so leads to improper gradient formation. To do so cytokinin, auxin, CLV3 and HAM are anticipated to regulate these processes.

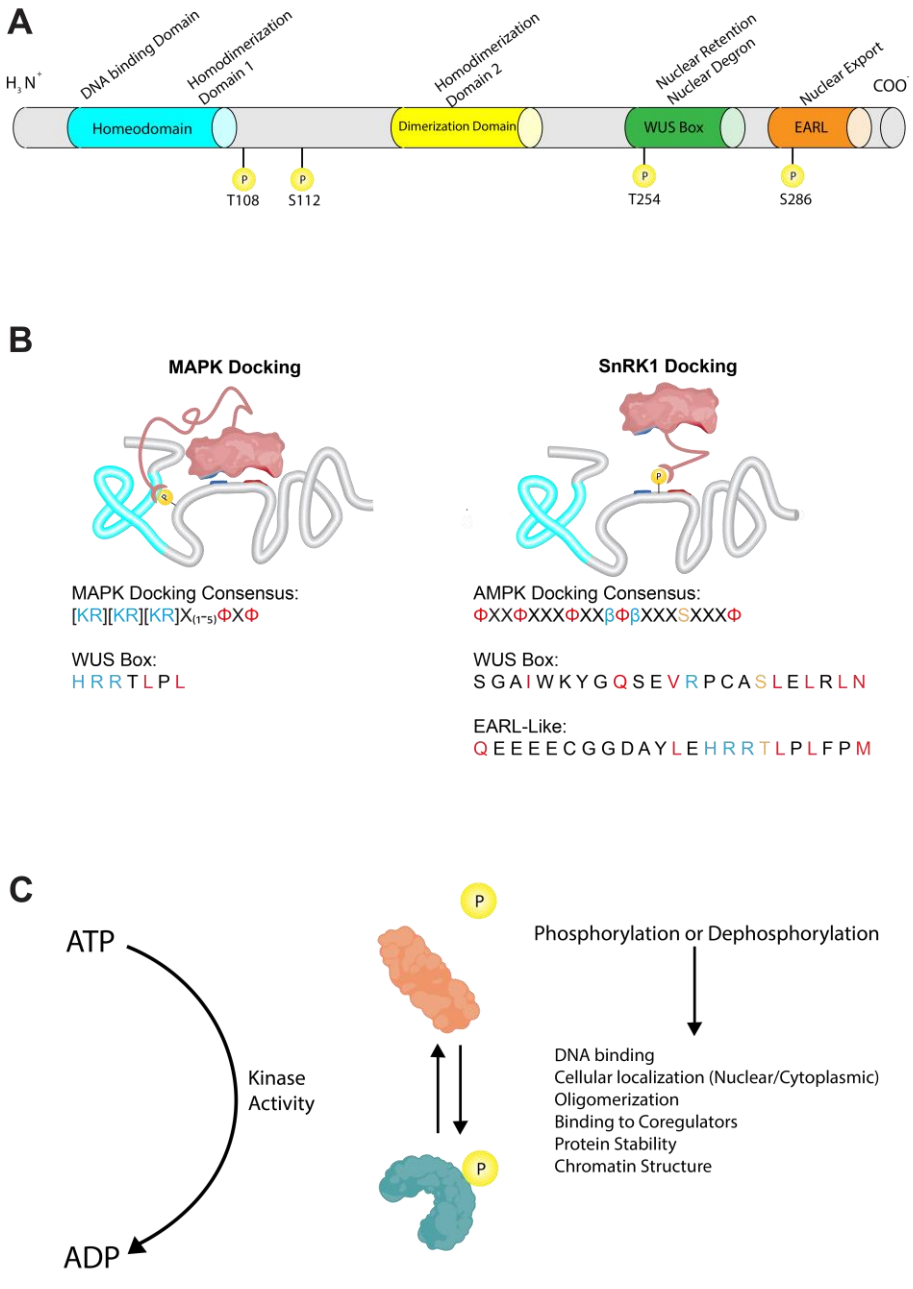


Fig 1.2 Phosphorylation as a regulatory mechanism for the WUSCHEL protein gradient

(A) A represenation of the WUS protein highlighting the location of key regulatory domains and the presence of sites of phosphorylation. (B) Graphic represenation of the kinase docking and phosphorylation dynamics. MAPK docking and phosphorylation of the N-termial site T108 and S112 has been well documented (Dory et al., 2016). On the other hand, there is no consensus for SnRK1 kinase docking. Due to the highly conserved catylitic domains of the SnRK1/AMPK/SnF1 family of kinases, it is possible to utilize AMPK consensus sequences to identify putative docking and sites of phosphorylation (T254 and S286) on to the WUS protein. (C) Once phosphorylated, conformational changes can lead altered transcription factor activity which may play a role in regulating the WUS protein gradient. Specifically, changes to DNA binding, cellular localization and protein stability can significantly alter WUS protein accumulation and in turn transcriptional function (Whitmarsh et al., 2000).

Chapter 2: Determining the *in vivo* effects of N-terminus phosphorylation of the WUSCHEL protein

Introduction:

In the shoot apical meristem (SAM) of *Arabidopsis thaliana*, a population of pluripotent stem cells is maintained at a balance of stem cell proliferation and differentiation. Characterization of the genes *WUSCHEL (WUS)* and *CALAVATA3 (CLV3)* have revealed it to be integral to the maintenance of this population of stem cells and a key regulatory mechanism. The WUS gene encodes a homeodomain containing transcription that is expressed in the rib meristem (RM) and is responsible for maintenance of the SAM as well as the activation of its own negative regulatory CLV3 (Laux et al., 1996; Mayer et al., 1998; Schoof et al., 2000). *CLV3* is a gene that encodes a singling peptide which, when expressed, will activated several transmembrane receptor kinases that will transcriptionally repress *WUS* in the RM (Fletcher et al., 1999; Clark et al., 1997; Brand et al., 2000; Kondo et al., 2006).

Although *WUS* expression is limited to just a few cells in the RM, the WUS protein has been shown to move into adjacent cells where it accumulates at a lower level forming a protein gradient (Yadav et al., 2011)(Fig 1.1 B). Proper spatial distribution of the protein requires regulation of movement across cell layers, proper nuclear/cytoplasmic accumulation and protein stability. Disruption of either of these processes leads to unregulated stem cell levels and in turn

improper plant development. Previous research has shown this by fusing 2xeGFP or a nuclear localization sequence (NLS) to WUS preventing movement outside of the RM as well as an inability to rescue *wus* null mutant lines (Yadav et al., 2011). Additionally, disruption of WUS protein levels has been shown to change its regulatory function as artificially decreasing protein levels makes WUS a better activator and increasing protein levels makes it a better repressor (Perales et al., 2016). The signals and modifications that target WUS for nuclear/cytoplasmic partitioning, degradation and diffusion still remain unclear. One proposed regulatory mechanism is through the post-translational modification, phosphorylation. Phosphorylation of transcription factors is already known to regulate processes such as DNA binding, cellular localization, stability or oligomerization as a result of differential protein folding, all of which can be vital to regulating the WUS protein gradient (Whitmarsh et al., 2000)(Fig1.2 C). Recent biochemical analysis have shown that hydrophobic domain located within the WUS protein are involved in subcellular localization, stability and transcriptional regulation (Rodriguez et al., 2016). One of these regions, the WUS-Box (WB), contains a docking site for the MITOGEN-ACTIVATIED PROTEIN KINASE 3 (MPK3) which has been shown *in vivo* to phosphorylate two phospho acceptor sites (T108, S112) located near the N-terminal of WUS (Dory et al., 2016) (Fig1.2 A-B). Interestingly, these phospho acceptor sites are with-in close proximity to the Homeodomain and Homodimerizaton domain suggesting that any misfolding in this region as a result of phosphorylation may have a

drastic effect on WUS function (Fig1.2A). Taken together, these observations suggest that phosphorylation of the WUS protein could influence protein accumulation and ultimately function, however the *in vivo* significance of WUS N-terminal phosphorylation has not been tested.

The following work presents an analysis of the function of the N-terminal phosphorylation sites for the regulation of the functional WUS protein gradient in addition to its subsequent effect on developmental patterns as a result of mutations to the sites of phosphorylation.

Results

In vivo WUSCHEL protein distribution upon mutation of phosphorylation sites

In order to understand the *in vivo* changes to WUS protein accumulation the phospho acceptor sites (T108 and S112) were mutated into a non-phosphorylatable (NP) version of WUS (T108A and S112A) or converted into a phospho-mimic (PM) version that is constitutively phosphorylated (T108D and S112D) (Fig 2.1). Both versions were then subsequently expressed from the WUS promoter and fused with a green fluorescent protein (*pWUS::eGFP-WUS*). Comparing wild-type WUS with non-phosphorylatable N-terminal residues resulted in increased nuclear protein accumulation whereas constitutively phosphorylated phospho-mimic versions result in lower protein accumulation

(Fig2.2). Additionally, analysis of WUS signal intensity reveals an overall increased signal intensity in non-phosphorylatable mutants and an overall decrease of WUS signal intensity in the phosphor-mimic mutant when compared to wild-type WUS protein (Fig2.2).

Alterations to WUS accumulation as a result of both mutant versions resulted in abnormal plant phenotypes due to changes in development. N-terminal non-phosphorylatable mutants result in SAM growth outside of the shoot axis as development of SAMs can be observed on leaf tissues (Fig2.3). Non-phophorylatable mutant plants also have a disruption of regular leaf pattern number and adaxial curling of leaves, likely due to a change in leaf growth rates (Fig2.3). N-terminal phospho-mimic mutants exhibit irregular and relatively enlarged SAMs found along the stem of the plant along with numerous ectopic underdeveloped flowers on the fasciated stem (Fig2.3). Plant stems were also much greater in thickness and exhibited curling. N-terminal phospho-mimic plants exhibited alterations to leaf phenotype as seen by the lateral curling of the leaves towards the stem resulting in a “pinwheel” phyllotaxy (Fig2.3).

To test changes to WUS ability to maintain SAM stability, both the non-phosphorylatable and phosphor-mimic version of WUS were introduced into the *wus-1* (null mutant) background. Non-phosophorylatable mutants are unable to rescue the *wus-1* phenotype as seen by the presence of plants with terminated SAMs (Fig2.4). These plants were then genotyped and tested positive for *eGFP-*

WUS confirming the presence of non-phosophorylatable WUS mutant. Phospho-mimic mutants were able rescue the *wus-1* phenotype as these lines could develop SAMs (Fig2.4). These plants were genotyped to confirm the *wus-1* genotype.

In vivo phosphorylation mutant protein distribution in the *clv3* null mutant background

In the *clv3-2* null mutants, the *CLV3* promoter is highly expressed in the central zone (CZ) and repressed in the rib meristem (RM) (Perales et al., 2016). Repression of p*CLV3* in the RM is likely due to the higher levels of *WUS* transcription and WUS protein observed in the *clv3-2* mutant. Meanwhile, p*CLV3* expression is increased in the CZ despite having lower levels of WUS protein when compared to wild-type WUS accumulation in the same regions. This suggests that the WUS protein is not being properly sequestered in the nucleus resulting in increased degradation and diffusion rates into adjacent cells as a result of losing CLV3-mediated signaling. Given that alterations to WUS phosphorylation states can mediate protein localization and stability it is critical to examine the accumulation of non-phosphorylatable and phospho-mimic versions of WUS in *clv3-2* mutants.

Phospho-mimic version of WUS in the *clv3-2* exhibit no florescence signal likely due to the extreme low levels and diffuse protein unable to adequately produce a signal (Fig2.5). This can be explained by inhibition of nuclear

sequestration of WUS as a result of loss of CLV3 regulation leading to increased WUS degradation rates.

Discussion

Regulation of the WUS protein gradient is critical for maintenance of stem cell populations and SAM proliferation. The post-translational modification, phosphorylation introduces an interesting mechanism for being able to regulate those processes. CLV3 mediated signaling has been shown to activate the MITOGEN-ACTIVATED PROTEIN KINASE (MAPK), MPK3 which is attributed with the phosphorylation of N-terminal residues on the WUS protein (Lee et al., 2019; Dory et al., 2016). In this study it is clear that phosphorylation of the phospho acceptor sites found near the N-terminus of the WUS protein is necessary for SAM maintenance and development as mutations inhibiting phosphorylation or constitutively phosphorylating these site leads to changes in protein accumulation and developmental patterns(Fig2.1-2.2). Phosphorylation of the N-terminus seems to cause instability of the protein as WUS is unable to accumulate in the nucleus effectively and is found at much lower levels in the phospho-mimic (PM) version than the wild-type while non-phosphorylatable (NP) versions of WUS lead to stability as seen by the increased nuclear accumulation and signal intensity (Fig2.2). This suggests that phosphorylation of the N-terminal residues, mediated by CLV3, mark the WUS protein for degradation and likely for nuclear export as opposed to stabilizing the WUS protein as previously thought.

While CLV3 also signals to stabilize small amounts of the protein in the outer layers. This is likely due to the fact that WUS at lower concentrations tends to act as activator as seen in previous studies which show that decreasing WUS protein concentrations allows it to highly express CLV3 (Perales et al., 2016). This phenomenon has been seen in other systems where transcription factor turnover can poise genes for transcriptional activation suggesting careful regulation of WUS phosphorylation states can be a mechanism to enhance the ability of WUS to activate its targets (Jones., 2012). CLV3 mediated phosphorylation of WUS may very be a mechanism to continuously cycle WUS in the outer layers of the SAM allowing WUS to activate *pCLV3* but to not accumulate into large enough levels that will inevitably push for repression of *pCLV3* and other genes associated with differentiation. This idea is supported by previous studies have found that WUS in a concentration-dependent manner can bind as a monomer (at low WUS concentrations) or a dimer (at high WUS concentration) to these cis-elements suggesting that the difference in dimerization state can modulate repression (dimer state) or activation (monomer state) of *CLV3* (Perales et al.,2016; Sloan et al., 2020). Perhaps it is the case that higher WUS accumulation in the RM results in enhanced binding to these cis-elements as a dimer complex, repressing *CLV3* and other differentiation associated gene. In the CZ, CLV3 mediated signaling activated kinases such as MPK3 to phosphorylation WUS resulting in increased nuclear export and degradation allowing for new WUS protein to bind to the regulatory cis-elements and activate

CLV3 transcription due to transcription factor turn over. This is supported by analysis of the functional significance of N-terminal phosphorylation is also observed in the *wus1-1* null mutants. Although the PM version of WUS is more unstable it is still able to rescue the *wus-1* phenotype and produce a SAM while the NP versions cannot (Fig.2.4). This shows that instability caused as a result of phosphorylation is essential for proper maintenance of the SAM while stability of the protein is associated with termination. Additionally, the changes to developmental phenotypes observed as plant expressing the PM version of WUS produce more and larger SAMs suggesting that this mutant version promotes indeterminacy which is associated with WUS function (Fig2.3). On the other hand NP versions of WUS lead to stability resulting in production of SAMs outside of the shoot axis, specifically on leaf tissues, showing a reversion of differentiated cells into undifferentiated cells (Fig2.3). This is likely due to the increased stability and nuclear retention of WUS causing it to be retained in cells as they move away from the growing tip allowing WUS to have residual function outside of the meristem context. Additionally, there is a curling phenotype associated with both mutant version. The phospho-mimic version results in thicker and curled SAMs with bilateral curling of leaves at early stages of growth, while the non-phosphorylatable version has consistent adaxial curling of leaves throughout the life of the plant (Fig2.3). This again can be associated with the change in WUS protein levels. Previous studies have linked WUS levels and cell division rates, as increasing WUS protein levels in turn increases cell division rates while

decreasing WUS protein levels decreases cell division rates (Yadav et al., 2010). It is possible that phosphorylation causes asymmetrical distribution of WUS protein in the leaf or stem and based on the phosphorylation state it can cause growth to occur at distinct rates. When the left and right axis of the leaf grow at different rates the leaves curl into the slower growing side leading to the 'pinewheel' phenotype observed. While the downward curling of the leaves could be due to increase in adaxial growth over the abaxial side. Furthermore, observations of PM versions of WUS in *clv3-2* mutants support the idea that CLV3-mediated signaling may also work to stabilize small amounts of the WUS protein in the nucleus. In the wild-type system small amounts of the WUS protein are kept stable in the outer layers by CLV3 to maintain activation of *pCLV3*. In *clv3-2* null mutant lines the WUS protein is produced at higher levels in the RM but is also exported at higher rates resulting in increased degradation of the protein in the cytosol preventing accumulation in the higher layers of the SAM suggesting CLV3 is needed to stabilize the protein in those layers (Perales et al., 2016). Here it is similarly seen that PM versions, which already accumulated at lower levels, cannot be seen *clv3-2* null mutant background again suggesting that CLV3 is needed to prevent complete degradation of the WUS protein pool in the outer layers of the SAM (Fig.2.5). Observation of the NP version of WUS in the *clv3*-2 null mutant line is still needed to fully understand what is occurring but if the proposed regulatory mechanism is correct we can anticipate the NP version will have similar or more protein accumulation than the wild-type. Because the

phosphorylation mark that seems to be tagging WUS for degradation cannot be added to the NP version the WUS protein may become stable even in the *clv3-2* background.

In order to fully understand the changes WUS function as a result of phosphorylation, it will be necessary to introduce the NP and PM version of WUS into *wus1*-1 null mutants carrying the reporter *pCLV3::H2B-mYFP*. This will show how phosphorylation states can alter the ability of WUS to activate or repress *CLV3* by observing changes to *CLV3* signal distribution and intensity. Due to the observation that NP version of WUS cannot rescue *wus1-1* and produce stable SAMs it will be impossible to observe changes to WUS transcriptional function under these conditions. It is likely that NP version of WUS are either non-functional or have enhanced function preventing proper maintenance of the SAM resulting in termination. Alternatively, changes to *CLV3* expression in the PM version may imply that is occurring in the NP version. It may be the case that in the PM version there is high levels of *CLV3* expression suggesting that the instability and turnover of WUS as a result of phosphorylation activates *CLV3*. Meanwhile, the stability and nuclear accumulation associated with the NP version represses *CLV3* expression preventing proper regulation of WUS levels resulting in termination.

In addition to altering protein accumulation phosphorylation may also work to modulate WUS transcriptional function. Since we can anticipate changes to

pCLV3 activity under each mutant condition it will important to understand how different phosphorylation states of WUS affect its ability to bind the various cis-elements. Previous research has shown that WUS has different affinities for each of the five cis-elements in the *CLV3* promoter and will bind to these site as monomers or dimers at different concentrations (Perales et al.,2016). In order to understand how phosphorylation states can affect WUS binding dynamics, electrophoretic mobility shift assays (EMSA) can done for the NP and PM version of WUS to see how binding to the different cis-elements may change. Given that the NP version of WUS seems to be more stable and cannot rescue the *wus1-1* phenotype, it may be the case that this version of WUS binds to the CRM as a dimer better. Conversely, the PM version of WUS may not bind as well to the CRM or may not form dimer complexes as well as the wild-type version.

Methods

Generation of WUS phosphorylation mutants

Mutant forms of WUS were created through PCR mutagenesis of phosphoacceptor residues described in (Dory et al., 2016). Wildtype WUS protein contains either a serine or threonine amino acid that contain available hydroxyl groups to covalently bond with a phosphate group. Non-phosphorylatable WUS versions converted these amino acids into alanines preventing addition of phosphate groups by using the primers 5'-TCTTCAGCACCCAACTCGGTTATGATG-3' and 5'-

TGGTGCGGTCATGTTTGTTCCGTTGAA-3'. Phospho-mimic version converted the phosphoacceptor residues into aspartic acid amino acids which have a highly negative change mimicking that of a phosphate group by using these primers 5'-TCTTCAGACCCCAACTCGGTTATGATG-3' and 5'-TGGATCGGTCATGTTTGTTCCGTTG-3'. The mutant WUS protein versions were then excited using AscI and StuI and introduced in the pCAMBIA2300 vector containing the wildtype WUS promoter and 3'UTR previously described in (Rodriguez et al., 2016).

Plant transformation and screening

Mutant constructs were introduced into heterozygous *wus1-1*, heterozygous *wus1-1/pCLV3::H2B-mYFP* and *clv3-2* lines via *Agrobacterium*-mediated floral dip transformation. *A. tumefaciens (GV301)* were transformed and grown at 28 ℃ for 2 days on LB(rifampicin 10 μg/mL, gentamycin 100 μg/mL and kanamycin 25 μg/mL) plates. A few small colonies were used to inoculate 5mL of LB(RGK) liquid and grown at 28 ℃ for 1 day with 250rpm agitation. This culture was then used to inoculate a large 300mL LB(RGK) liquid culture and grown for 1 day. The culture was then evenly distributed into 250mL bottle and centrifuged at 3500rpm for 10min. The supernatant was removed and the pellet was suspended in 50mL of dipping solution (5% sucrose and .8% Silwet-77 in DI H_2O). This mixture was poured into plastic containers and the flowers of plant were submerged into the mixture with gentle rocking for 30s. Dipped plant were

then covered in the dark for 1d and returned into continuous light growth conditions for 4-5 weeks.

Seeds were collected, dried and sterilized with NaOCl. Seeds were selected on 0.5% MS plates containing 50µg/mL kanamycin in continuous light for 10-12 days. T_1 positives were to soil and were imaged via fluorescent confocal microscopy.

wus1-1 protein and phenotypic analysis done in the T2 generation and confirmation of *wus1-1* and construct was done by genotyping. The *wus1-1* is based on the premise that the genomic *WUS* sequence carries two adjacent RasI restriction site and the *wus1-1* null mutant disrupts one of them. The PCR genotype disrupts the other restriction site allowing the PCR product to be digested and reveal characteristic banding patterns associated with either wild-type or *wus1-1*. The primers 5'- ACGTATATTAATATGTTTGAAGGGA-3' and 5'-TTGAATTAATGAATTATAGTTTGATACG-3' were used to amplify extracted genomic DNA. PCR product was then purified using isopropanol and RasI was added to the sample to digest. Identification of the *wus1-1* genotype was then scored based on the anticipated size of fragments if digested after running on a 2-3% agarose gel. Digested fragments with the size of 146bp, 64bp and 26bp indicate wild-type *WUS*. Meanwhile, undigested fragments with a size of 146bp, 98bp and 48bp indicate a *wus1-1* diagnostic. Presence of all five bands indicate a heterozygous line.

Fluorescent Confocal Imaging

Plastic boxes were filled with 20-30mL of 1.5% agar. Main or axillary stems were clipped 2cm below the SAM. Floral buds were removed with forceps and the stems were vertically inserted into the solidified agar. 200-300 µL of additional agar was pipetted to the base of the stem to further stabilize it. Stems were then submerged into DI H_2O were more precise removal of floral buds was done under a dissecting microscope. The DI H_2O was poured off and the SAM were dried with Kimwipes placed near the apex. A working solution of 10% w/v FM4-64x, 2% TE buffer, and 2% Silwet-77 was made for staining the apices. For each apex, 1-2µL of FM4-64x working solution was applied and incubated for 20-30mins. Samples were submerged in water once again and scanned using the Zeiss 880 upright LSM. Fluorescence of eGFP was excited at 488nm and emissions were captured at 500-530nm and FM4-64 dye was excited at 545nm and emissions were captured at 565-615nm. Confocal images were analyzed via the Zeiss LSM Image Browser.

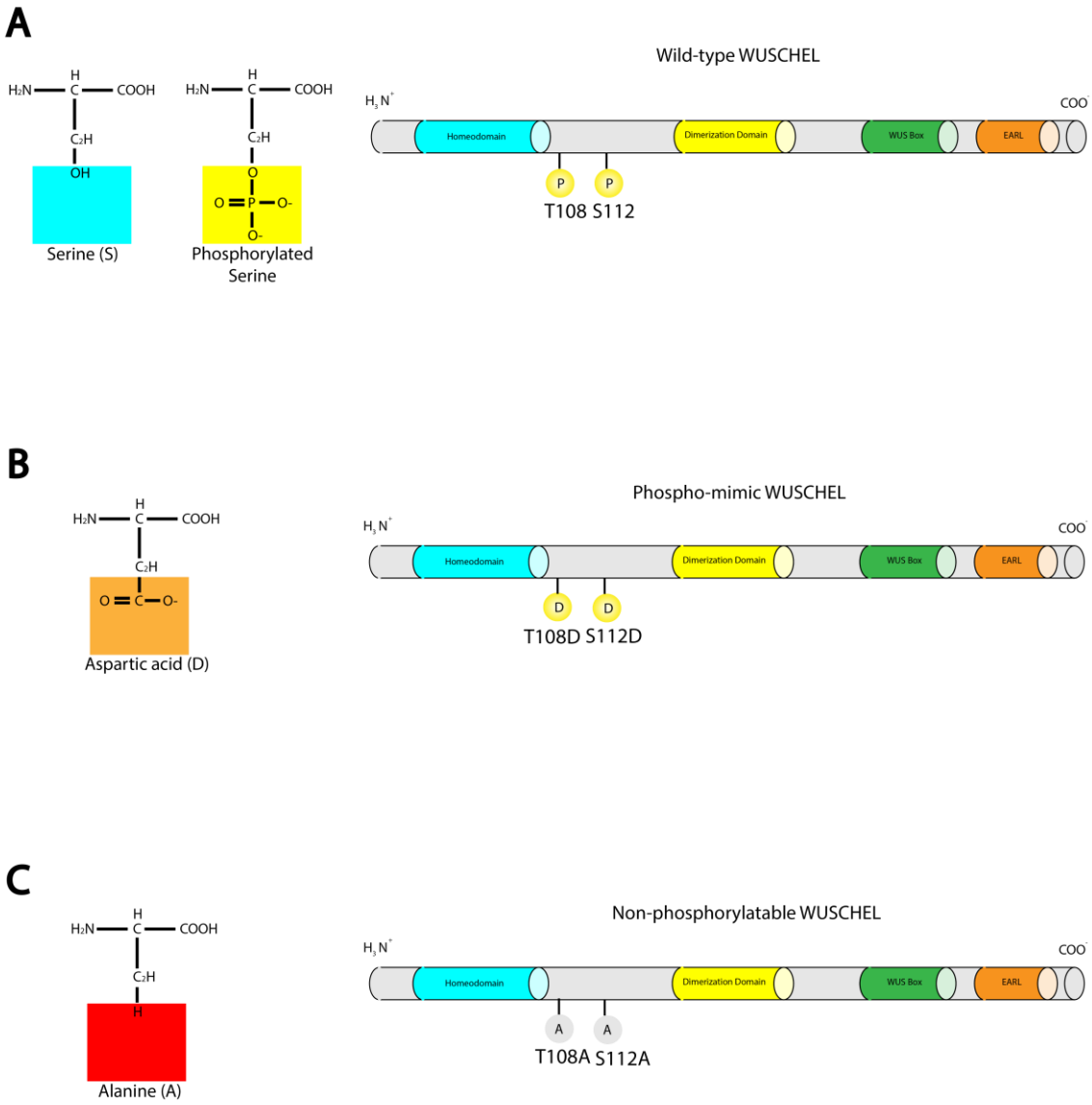
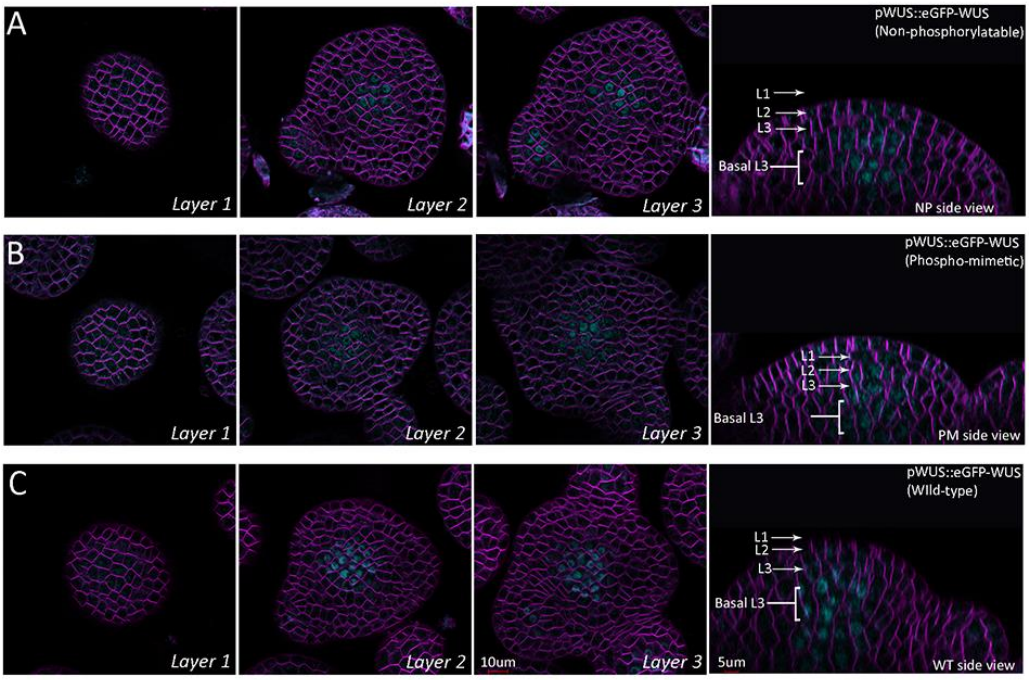


Fig 2.1 Mutations of the N-terminal sites of phosphorylation on the WUSCHEL protein

(A) WUS protein diagram (1aa-209aa) indicating the sites of phosphorylation found at the N-terminal T108 and S112. The two possible phosphorylation states are shown exhibiting how addition of the phosphoryl group to a serine results in an overall negative charge of the residue. (B) Protein diagram of the phospho-mimic (PM) version. Both phosphorylatable residues are converted into aspartic acids (T108D and S112D) which has an overall negative charge comparable to a phosphoryl group. (C) WUS protein diagram of the non-phosphorylatable (NP) version where both phosphorylatable resides are converted into an alanine (T108A and S112A) removing the hydroxyl group needed to accept a phosphoryl group.



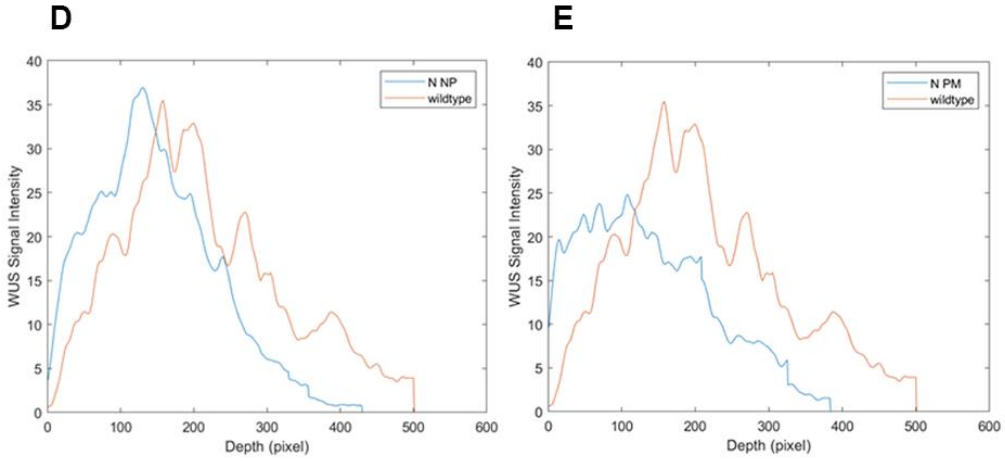


Fig 2.3 WUS N-terminal phosphorylation decreases the nuclear accumulation of the WUS protein
In all images WUS protein is indicated by the cyan fluorescence and the plasmamembrane is indicated by the purple fluoresce (FM4-64). (A) Non-phosphorylatable (NP) version of WUS result in increased nuclear accumulation of the WUS protein. (B) Phospho-mimic (PM) versions result in a reduction of nuclear accumulation which in turn lowers the overall amount of protein compared to the wild-type (C). (D) Relative WUSCHEL signal intensity NP mutants and wildtype. (E) Relative WUSCHEL signal intensity for PM mutants and wildtype. 6 images were analyzed for each mutant background and for wildtype. The central region of the SAM was isolated and the intensity values were average by depth.

Fig 2.3 Disruption of WUS phosphorylation states alters growth rates and developmental patters

(A) Both non-phosphorylatable (NP) and phosphomimic (PM) versions of WUS lead to changes to plant development. NP versions cause result in SAM growth outside of the shoot axis as development of SAMs can be observed on leaf tissues as indicated by the arrows in (B). NP mutant plant also have a disruption of regular leaf pattern and adaxial curling of leaves. PM versions exhibit irregular and relatively enlarged SAMs along the stem of the plant with numerous ectopic underdeveloped flowers. Plant stems were also much greater in thickness and exhibited curling. Plants exhibited alterations to leaf phenotype as seen by the lateral curling of the leaves towards the stem resulting in a “pinwheel” phyllotaxy indicated by the arrows in (B).

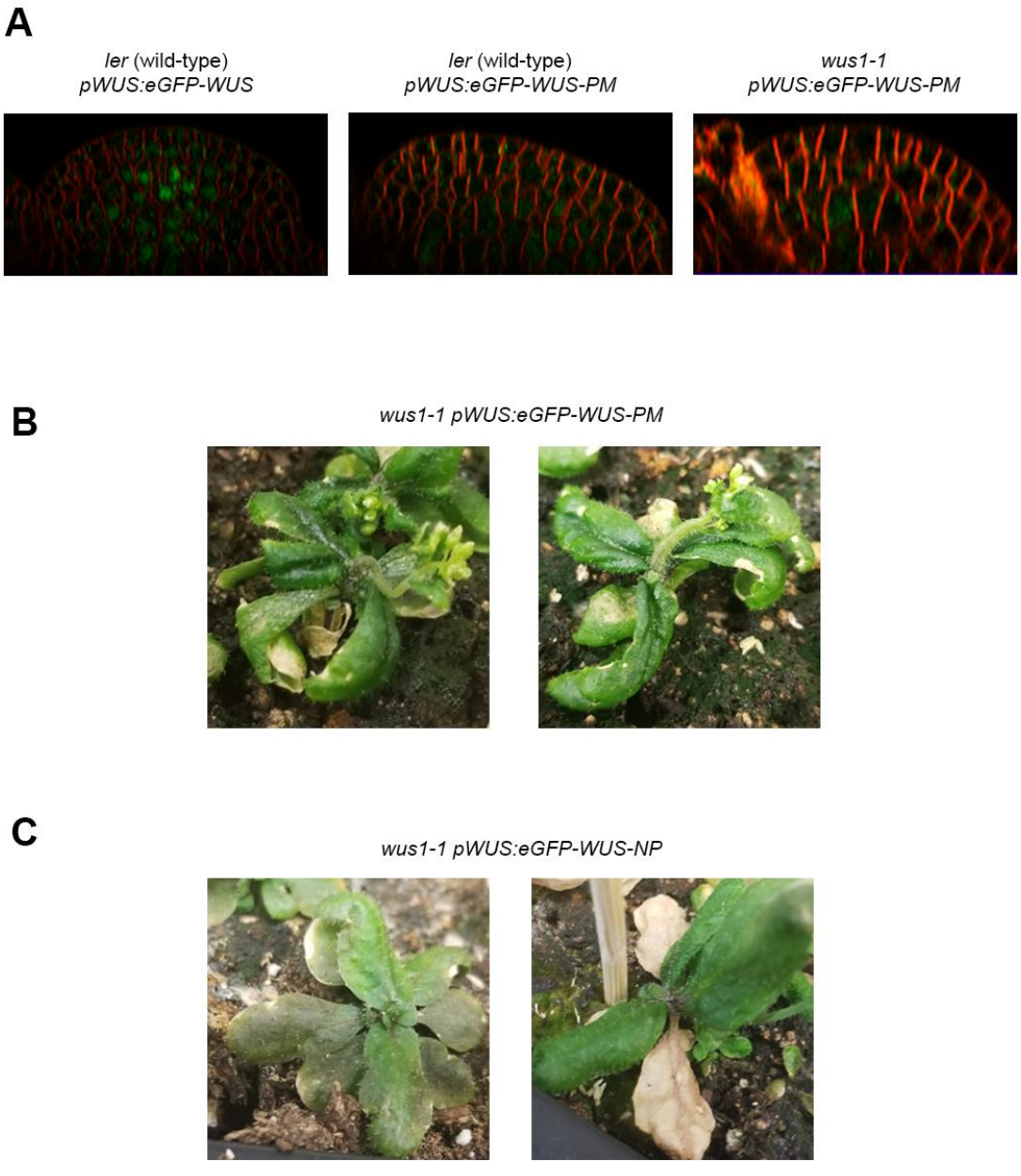


Fig 2.4 Non-phosphorylatable versions of WUS cannot rescue the *wus1-1* null mutant line

(A) Reconstructed side of the SAM with the cell walls stained in red (FM4-64) and WUS protein indicated by green flouresence. Comparisions of WUS and PM version of WUS show a decrease in signal intensity. PM WUS is able to rescue the *wus1-1* null mutant line and maintain a SAM (B) with no change to the WUS PM protein distribution seen in the wild-type background. (C) NP versions of WUS cannot rescure the *wus1-1* phenotype as no SAM is observed in these lines. This was confirmed through phenotypic and genotypic analysis.

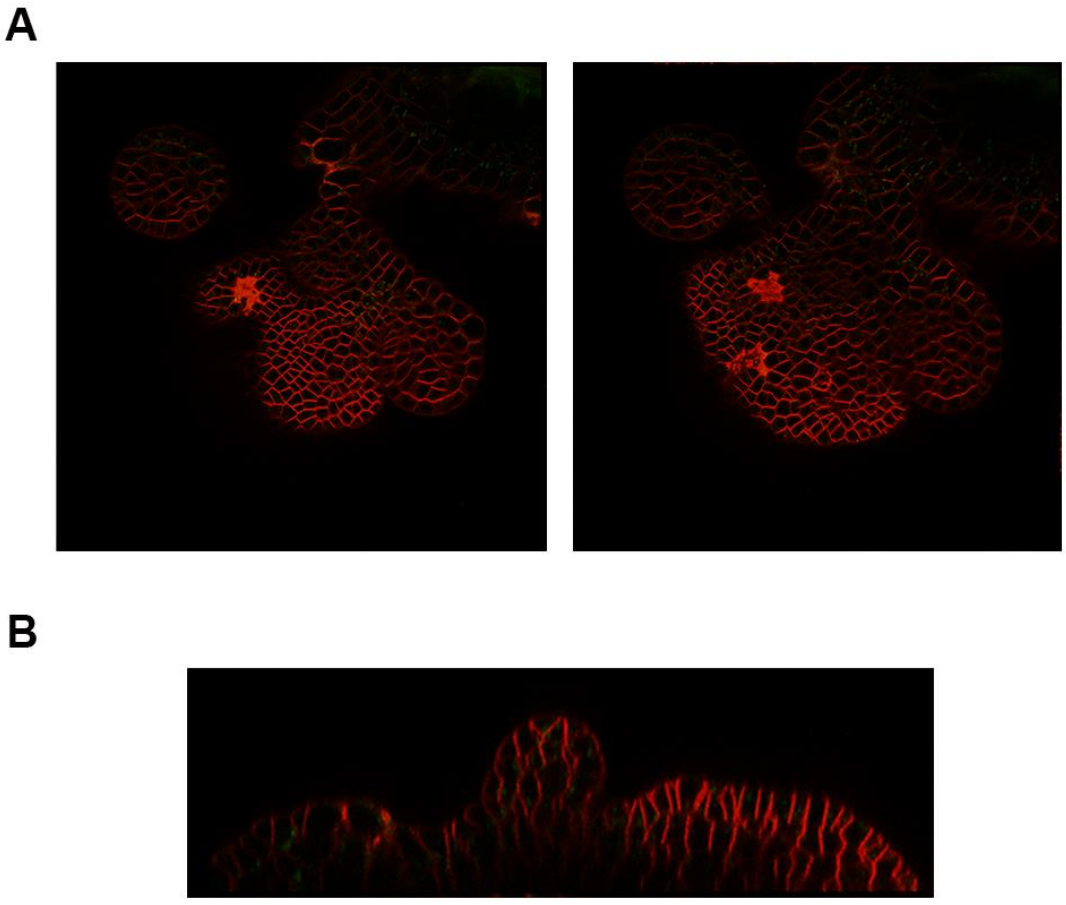


Fig 2.5 Phosphomimic versions of WUS cannot propperly accumulate in the *clv3-2* null mutant line

(A) Reconstructed side of the SAM with the cell walls stained in red (FM4-64) and WUS protein indicated by green flouresence. *pWUS:eGFP-WUS-PM* shows no accumulation in the L1 and L2-L3 of *clv3-2* null mutant lines. This is likely due to the loss of CLV3 signaling and nucelar retentation of WUS associated with it. (B) Reconstructed side view of a *clv3-2* SAM showing no accumulation of *pWUS:eGFP-WUS-PM.*

Chapter 3: Uncovering the Molecular and Genetic Characterization of SNRK1-WUSCHEL Interaction and its effect on SAM Maintenance

Introduction

Identification of functionally relevant WUS PPIs from a high through generated various avenues for studying regulation of the WUS protein gradient (Snipes., 2016). Many of these uncharacterized targets are suggested to or have been previously shown to regulate transcriptional activity, subcellular localization of target, protein stability or protein complex formation through protein-protein interactions or post-translational modifications, which are processes essential to proper protein gradient formation or modulating WUS transcriptional function. One such target identified by this screen is SNF1-related protein kinase KIN11 (Snipes., 2016).

KIN11 is classified as a SNRK1 (SNF1-Related Protein Kinases) protein kinase which are part of an evolutionary conserved family of energy sensor kinases which include the mammalian AMPK (AMP-ACTIVATED PROTEIN KINASE) and the yeast SNF1 (SUCROSE-NON-FERMENTATION1) (Celenza et al. 1986; Hardie et al., 2012; Hardie et al., 1998). Additionally, KIN11 is often associated with KIN10 due to high sequence similarity and function redundancy. The SnRK1 family are known to be key regulators of cellular metabolism that are active during starvation, stress conditions and growth-promoting conditions by completely reprograming transcriptional networks (Wurzinger et al., 2018). KIN10

and KIN11 specifically were found to regulate the expression of 600 genes in response to energy depletion or nutrient signals in protoplasts (Baena-Gonzalez et al., 2007). Additionally, SnRK1 kinases are tasked with regulating daily metabolic transitions associated with day and night cycles and in response to biotic/abiotic environmental stimuli resulting in reduction of ribosomal protein synthesis, adjustments to growth rates and accumulation of protective metabolites or defense compounds. A regulatory role conserved among the SnRK1/AMPK/SNF1 kinase family (Crozet et al., 2014; Cutler et al., 2010; Hrabak et al., 2003; Broeckx et al., 2016). The transcriptional reprogramming associated with SnRK1 kinases is done through direct regulation of transcription factor activity. Previous studies have found that KIN10/KIN11, when active, will activate various transcription factors directly such as SOG1, GBF5 and other bZIP related transcription factors critical for regulating plant growth, development and abiotic stress responses (Wang et al., 2019; Baena-Gonzalez et al., 2007; Hamasaki et al., 2019). Given how broad the regulatory network the SnRK1 kinases operate it opens the possibility for convergence of KIN10 and KIN11 direct regulation onto the WUS-CLV3 negative feedback loop. This is supported by the changes in plant developmental patterns associated with transgenic lines overexpressing KIN10 which result in altered inflorescence architecture and delayed flowering (Baena-Gonzalez et al., 2007). Many of the processes regulated by KIN10 and KIN11 ,via modulation of transcription factor activity, as well as their direct correlation to changes in plant development and identification

as a WUS PPI provide the necessary premise for determining their role in regulating WUS-mediated SAM maintenance.

The following work aims to characterize the effects of SnRK1-WUS interactions and its effect on SAM maintenance. KIN10 and KIN11 docking on the WUS proteins is mapped using the Y2H method while the subcellular localization of their interactions is revealed using BiFC experiments. Lastly, genetic characterization of KIN10 regulation of the WUS-CLV3 feedback loop is done through analysis of changes to various fluorescent reporters in the KIN10 overexpression background.

Results

KIN10/KIN11 Interact with WUS at a region near the C-terminus Including the WUS-Box and EAR-like domains

KIN10/KIN11 are widely known as master regulators of transcriptional networks during energy deprivation conditions and stress (Wurzinger et al., 2018). This function is highly conserved across all eukaryotic taxa as homologs in mammalian (AMPK) and yeast (SNF1) serve a similar purpose, therefore understanding KIN10/KIN11 relevance in WUS-mediated SAM maintenance is important uncovering the mechanisms regulating development and stress response (Celenza et al. 1986; Hardie et al., 2012; Hardie et al., 1998). To do it is important to first map KIN10/KIN11 docking onto WUS. WUS contains many

functional and regulatory domains vital modulating WUS gradient formation and transcriptional function. Starting from the N-terminus there is the homeodomain, which is responsible for DNA binding and WUS transcription factor activity (Mayer et al., 1998, Yadav et al., 2011) (Fig1.2). Next is an additional homodimerization domain needed for dimerization and for regulating WUS mobility (Daum et al., 2014, Rodriguez et al., 2016) (Fig1.2). Following that, near the C-terminus, are the WUS-Box and EAR-Like domains vital for regulating transcriptional function as well as responsible for nuclear retention and nuclear export, respectively (Rodriguez et al., 2016) (Fig1.2). Previous studies have shown that conversions of these hydrophobic residues can influence the subcellular localization of WUS, the mechanisms regulating how these domains are able to modulate their activity is still unknown but phosphorylation by KIN10/KIN11 may play a role as these kinases may possibly docking to these regions.

Conservation of the αβγ across the SNRK1, SNF1 and AMPK is extremely evident. KIN10/KIN11, sharing at 89% sequence similarity, share close to a 50% similarity to their SNF1 and AMPK counter parts with a 60-65% similar seen at the catalytic domain in the α-subunit (Mohannath et al., 2014). This suggests that kinases docking to its target must also share similarities. There is currently no consensus for SNRK1 docking site but looking at AMPK alignments reveal that proper sites have a βΦβ motif, where β represents a basic amino acid residue and Φ represents a hydrophobic amino acid residue (Hardie 2011). Located after

the hydrophobic residue of this motif is a serine or threonine residue which then be phosphorylated by the kinase residue (Hardie 2011). Screening the WUS protein sequence with the AMPK consensus sequence reveals that the WUS-Box and EAR-Like domains contain sites of phosphorylation with similar βΦβ motifs suggesting that KIN10/KIN11 could very well dock to these regions (Fig1.2).

In order to map KIN10/KIN11 docking, WUS deletion and mutations were used as described in (Snipes., 2016). Interactions between these various WUS versions with either KIN10 or KIN11 were determined through Y2H. All interactions were plated on SD-L/W to determine basal growth and on SD-L/W/H/A to test interactions. Plating was done once colonies growth reached an OD_{600} of 0.4, then all subsequent colonies were diluted 10^{-1}x, 10^{-2}x and 10^{-3}x in order to compare the relative strength of interactions. In the negative control interaction with AD empty colonies can only be seen until the 2nd dilution, which is likely due to the initial plating rather than actual colony growth. Testing KIN10 and KIN11 against the AD empty yields the same result as AD and BD empty colonies indicating that the presence of colonies up until the 2nd dilution indicates no interaction. Testing KIN10 and KIN11 against AD full length WUS results in growth until the 4th dilution exhibiting the interaction possible. When KIN10 and KIN11 are tested against AD WUS D1, a deletion of the last 84 amino acids, there are only colonies up until the 2nd serial dilution, comparable to AD empty, indicating no interaction within this region (Fig 3.1). Alternatively, when KIN10 and KIN11 are tested against AD WUS D2, a deletion of the first 208 amino

acids, growth up to the 4th serial dilution can be seen indicating the interaction between this region of WUS and KIN10/KIN11 is as strong as interactions with full length WUS (Fig3.1). Comparative analysis of these interaction levels reveal that KIN10 and KIN11 dock near the C-terminus of WUS in the regions that contain the WUS-Box and EAR-like domains.

Mutations to the WUS-Box and EAR-like domains inhibit KIN10/KIN11 interactions with WUS

To test if the WUS-Box and EAR-like domains and the AMPK consensus sequence mapped to these regions are where KIN10/KIN11 interact, mutations to the hydrophobic residues needed for AMPK docking were mutated as described in (Rodriguez et al., 2016) (Fig1.2). Independently mutating the WUS-Box or EAR-like domains results in no change to the interactions between KIN10 or KIN11 with WUS as growth until the 4th serial dilution can be picked up, similar to that of KIN10/KIN11 interactions with full length WUS (Fig 3.2). When screened against the WUS-Box and EAR-like double mutant, both KIN10 and KIN11 are unable to show growth past the 2nd serial dilution indicating loss of interaction with this region (Fig 3.2). Taken together these results confirm the original screen that showed KIN10/KIN11 are likely to interact with these domains.

WUS Interacts with KIN10 and KIN11 in the Cytosol of *N. benthamiana* Epidermal Cells

Microarray data from the electronic Fluorescent Pictograph (eFP) Browser reveals that both *KIN10* and *KIN11* expression is present in the SAM of *Arabidopsis thaliana* (Winter et al., 2007). Tissue specific data shows that *KIN11* is more highly expressed in the CZ and RM than *KIN10* but both genes are found in these regions that coincide with WUS protein distribution suggesting their interaction may happen *in vivo* (Winter et al., 2007). In order to build confidence in the findings of the Y2H analysis and to demonstrate the presence of this interaction *in planta* given the eFP data, WUS interaction with KIN10/KIN11 was tested utilizing the BiFC method. First, eGFP fusions of WUS, KIN10 and KIN were independently expressed in *N. benthamiana* leaf epidermal cells under the control of the *2xCaMV 35S* promoter (Walter et al., 2004, Kerppola, 2008). This resulted in the nuclear and cytosolic accumulation of WUS, meanwhile KIN10 and KIN11 only accumulated in the cytosol (Fig 3.3). Next, fusions of N-terminal eGFP and C-terminal eGFP with either WUS, KIN10 or KIN11 were generated. Complimentary eGFP truncations fused to WUS or KIN10/KIN11 were then co-expressed and visualized. Interactions with NeGFP-WUS/CeGFP-KIN10, NeGFP-WUS/CeGFP-KIN11, CeGFP-WUS/NeGFP-KIN10 and CeGFP-WUS/NeGFP-KIN11 were all strongly present in the cytosol suggesting the interaction is present only outside of the nucleus (Fig 3.3).

KIN10 Over-expression Lines Result in Changes to Developmental Patterns and Alteration of *pCLV3* and *pWUS* Expression

After KIN10 and KIN11 interactions with WUS were mapped and the interaction was shown to happen *in planta*, characterization of the phenotypic effects of misexpression of KIN10/KIN11 was done in order to understand their role in WUS-mediated SAM maintenance. Previously generated *KIN10* overexpression lines were done by driving expression of the gene with the *UBIQUITIN10* promoter as described in (Baena-Gonzalez et al., 2007). This line was already attributed with altering inflorescence architecture, further analysis reveals this is done by the overproduction of organs seen by increased amount of flower, flower buds and carpels when compared to the *ler* wild-type (Fig 3.5). Additionally, *KIN10* overexpression results in an increased amount of short interspersed axillary meristems and stunted growth (Fig 3.5).

KIN10 overexpression lines were then paired with the fluorescent reporter *pWUS::H2B-YFP* and *pCLV3::H2B-YFP*. Analysis of the changes to *pCLV3::H2B-YFP* when in the wild-type and *KIN10* overexpression background show a reduction of signal intensity at all levels indicating a reduction in *pCLV3* activity (Fig 3.6-3.7). On the other hand *pWUS::H2B-YFP* revealed an increase in signal intensity along with an expansion of the *pWUS* expression domain (Fig 3.6). In some cases ,roughly 30% of all imaged plants, there is even severe fascination of the shoot apical meristem leading to broad meristem tissue

formation as well as *pWUS* activity in the L1 where it is normally not expressed (Fig 3.6).

Discussion

Previous work revealed the SnRK1 protein kinase KIN11 to interact with WUS as a part of a great PPI network (Snipes., 2016). Along with its homolog KIN10, both of these kinase have had extensive work and research revealing their role as master regulators of transcriptional networks for cell energy homeostasis (Wurzinger et al., 2018). One of their most well documented roles is to adjust plant growth rates which is evident by direct regulation of bZIP transcription factors in order to modulate plant growth and development (Wang et al., 2019; Baena-Gonzalez et al., 2007; Hamasaki et al., 2019). Additionally, overexpression of *KIN10* results in changes to plant growth patter as seen by stunted growth as well as alteration to the inflorescence meristem (Baena-Gonzalez et al., 2007) (Fig 3.5). The consensus of all these studies heavily suggest a direct role of KIN10 and KIN11 in the regulation of WUS-CLV3 negative feedback loop. Therefore, this study focused on analysis of KIN10/KIN11-WUS interaction and elucidating the signification of this interaction in stem cell maintenance.

The first objective was to map KIN10 and KIN11 docking onto WUS. Due to the fact that there is no SnRK1 docking consensus at the time of this study, utilizing previously developed AMPK docking consensus sequence worked to

provide the initial identification of putative KIN docking site due to the conservation of the αβγ motifs shared by the SnRK1/SNF1/AMPK family (Hardie., 2011) (Fig 1.2). The initial Y2H screen was able to confirm KIN11 interactions with WUS picked up by the high through put screen but also was able to find that KIN10 similarly interacted with WUS which had been previously unknown (Fig 3.1). This same screen was also able to map interactions between WUS with KIN10/KIN11 to a 84 amino acid region near the C-terminus which included the WUS-Box and EAR-like domains, important regulatory domains that showed sequences similar to the APMK docking sequence surround the phosphor-acceptor amino acid (Fig 3.1). Mutations to the hydrophobic residues of the WUS-Box and EAR-like domains needed for AMPK docking, and thought to similarly be vital to SnRK1 docking, inhibited interactions with KIN10 and KIN11 suggesting that these two domains are where the SnRK1 kinases preferred to dock onto WUS (Fig 3.2). Additionally, the weak growth of colonies testing positive for KIN10 and KIN11 interactions suggests that the KIN-WUS interaction is fairly weak or transient.

BiFC experiments confirm the interaction of WUS with KIN10 and KIN11 but also provide valuable spatial context of the interaction. By co-transfecting into *N. benthamiana* leaf epidermal cells it is possible to understand the *in vivo* localization of this interaction. In both cases, with KIN10 or KIN11, we can observe cytosolic fluorescent signal indicating that the KINs and WUS interact somewhere in the cytoplasm instead of the nucleus (Fig 3.3). Confirming the

interaction between WUS with KIN10 and KIN11 and understanding the spatial context provided a strong basis for further analysis of the KINs regulatory role in shoot stem cell maintenance.

Given that phosphorylation of transcription factors is known regulate transcription factors by modulating DNA binding, cellular localization and stability the fact that the interaction occurs in the cytoplasm suggests that the KINs may play a role in regulating WUS protein levels (Whitmarsh et al., 2000) (Fig 1.2). This can be done by using phosphorylation, via the KINs, to mark WUS to either enhance nuclear accumulation which can work to stabilize the WUS protein, decrease diffusion and increase levels or it can be a mark favoring nuclear export resulting in destabilization of the protein, increased degradation and diffusion. In order to understand this the next objective of this study was to misexpress the KINs and utilize a library of fluorescent reporters to probe how this could be influencing the WUS-CLV3 regulatory mechanism. Analysis of the reporter *pCLV3::H2B-YFP* reveals that in the *KIN10OE* background, *pCLV3* activity is substantially decreased evident by the lower fluorescent levels measured and observed when compared to the wild-type background (Fig 3.6-3.7). On the other hand, *pWUS:H2B-YFP* in the *KIN10OE* background shows expansion of *pWUS* activity evident by the larger expression domain observed (Fig 3.6). Interestingly, in some cases there is even fasciation of the meristem as result of unregulated meristematic tissue production as well as activity of *pWUS* in the L1 layer which is not typically seen (Fig 3.6). This fascinated meristem is similar to that of the

clv3-2 null mutant line but differs from it in that *pWUS* is seen in the L1 (Perales et al., 2016). Although the fasciation can be explained by implying that *pCLV3* may be inactive, the fact that *pWUS* is seen in the L1 can not be so easily explained. As a result, this suggests that changes in *pWUS*, in part, may be independent of the *CLV3* regulatory pathway. Instead the changes to promoter activity and the presence of L1 *pWUS* may be a result of changes to the WUS protein gradient. One proposed mechanism is that the increase of phosphorylation due to the higher levels of KIN10 may be changing the stability, diffusion and/or nuclear accumulation of the WUS protein in some way that is leading to the L1 layer obtaining RM identity and subsequently expressing *pWUS*. In order to fully understand how protein dynamics are being changed under these conditions, observation of the reporter *pWUS::eGFP-WUS* in the *KIN10OE* background will serve to answer these unknown questions. If the proposed scenario is true what we can expect to observe is an increase in WUS proteins nuclear levels across the SAM tissue. In the non-faciated *KIN10OE* SAMs we may observe a higher amount of protein than in the wild-type background but in the faciated *KIN10OE* SAMs we can expect those protein levels to be even higher. This would suggest that phosphorylation by KIN10 works to stabilize the WUS protein and increase protein levels to those found in the RM which inevitably inhibits *pCLV3* leading to the changes observed.

While there are still many questions regarding the role and importance of KIN10 and KIN11 on regulating WUS-mediated SAM maintenance the results in

this study in tandem with all previous work done are beginning to uncover some vital functions of WUS-KIN interaction. Moving forward there are still many experiments and avenues of research to be done regarding WUS-KIN interaction. To start it will be necessary to observe changes to the WUS protein gradient in the *KIN10OE* background as it is the last experiment needed to understand the data previously discussed. Additionally, it will also be important to generate *KIN11OE* lines in order to confirm that similar changes happen as observed with KIN10. Previous studies on KIN10 and KIN11 suggest they have some functional overlap as null mutants in just one of the two kinases does not have any significant phenotypic changes to plant development or stress response therefore it is anticipated that there will be similar observations to the changes in fluorescent reporters (Baena-Gonzalez et al., 2007). These line can be easily generated by utilizing the constructs developed of the BiFC experiments as the *2xCaMV 35S* promoter was used to drive expression of *KIN11* and been used extensively in the field to overexpress genes in the past (Walter et al., 2004, Kerppola, 2008). In contrast to the overexpression data it will also be necessary to observe changes to the WUS-CLV3 regulatory mechanism in the absence of KIN10 and KIN11. Since null mutants of just one of the two kinases yield no changes to developmental patterns and null mutation in both KIN10 and KIN11 leads to embryonic lethality (Baena-Gonzalez et al., 2007). Currently, there are no T-DNA insertion, enhancer or gene trap knockout lines that are able to significantly reduce *KIN10* and *KIN11* expression while avoiding

embryonic lethality, to circumvent this similar RNAi strategies have been utilized to generate *kin10*/*kin11* knockdown lines but have observed issues in developing stable lines presumably because of embryonic lethality (Baena-Gonzalez et al., 2007). We proposed that utilizing an inducible amiRNA system be used to knockdown both *KIN10* and *KIN11* as it would allow for the knockdown of both genes at later stages of plant development circumventing embryonic lethality. Work is already being done to develop such lines by utilizing methods previously described to generate an amiRNA capable of targeting both *KIN10* and *KIN11* (Schwab et al., 2006). Currently, there have been limitations of development of these lines as induction of the knockdown may occur despite there not being a signal to activate the transcription of the amiRNA similarly resulting in embryonic lethality. Therefore, more work must be done in order to overcome this. Previous papers have been able to utilize virus-induced gene silencing (VIGS) to reduce *KIN10* and *KIN11* transcripts with more success than the other methods mentioned (Baena-Gonzalez et al., 2007). Finally, since KIN10 and KIN11 were mapped to the WUS-Box and EAR-like domains investigation of how the phosphorylation state of the phospho-acceptor residues in these domains affects WUS protein gradient formation will shed more light on functional role of these domains. This can be done by similarly creating non-phophoryltable and phosphomimic versions of WUS targeting the WUS-Box and EAR-like domains as done for the N-terminal residues discussed in the previous section. Comparing the *KINOE* lines as well as the *kin10/kin11* knockdown lines with the

WUS-Box and EAR-like phosphomutant versions may yield similar results drawing connections between KIN10/KIN11 and the significance of the phosphorylation states of the WUS-Box and EAR-like domains.

Methods

Generation of Bait and Prey DNA for Y2H Screening

Generation of WUS full length, truncation and mutated construct are described in (Snipes., 2016). Full length KIN10 and KIN11 were amplified and clones into the pGADT7 MCS via NedI and EcoRI sites to generate pGADT7 KIN10 and pGADT7 KIN11.

Y2H Bait/Prey Transformation and Selection

Y2H transformation procedures were adapted from (Geitz et al., 1997). AH109 yeast cultures were grown at 30^{o}C in yeast extract peptone dextrose (YPD) media for 48hrs while been shaken at 200rpm. Yeast was then concentrated by centrifugation at 3,000rpm and a small amount was then resuspended in 1mL of 0.1 M lithium acetate (LiAC). This yeast was pelleted via centrifugation for 30sec, the supernatant was removed, and the pellet was resuspended again in 1mL 0.1M LiAC. The previous step was repeated; however the pellet was resuspended after the addition of the following reagents in order: 240µL of 50% polyethylene glycol (PEG) 3350, 36µL of 1M LiAC, 50µL 2.5uM boiled DNA-salmon sperm, 10µL of pGBKT7 construct (full length WUS,

truncated WUS or mutated WUS) plasmid, 10µL of pGADT7 construct (KIN10 or KIN11) plasmid and 14µL of sterile MQ H2O. After resuspension the transformation mixture was heat shocked in a 42^{o}C water bath for 45mins. Transformed yeast cells were pelleted at 3,000rpm for 1min and supernatant removed. Yeast was suspended in water and plated on synthetic defended (SD)-(W/L) plates and allowed to grow for 2-4d at 30^{o}C.

After 2-4days yeast was then inoculated in 50-10mL of liquid SD-(W/L) and allowed to grow for 5-6 hours at 30^{o}C 200rpm until an OD_{600} of 0.4 was measured. Four 1/100 serial dilutions of the colony was made and 5-6 µL was plated on to SD-(W/L/H/A) and allowed to grow for 3-4 days at 30^{o}C. Growth was then observed and captured.

Bimolecular Fluorescence Complementation in *N. benthamiana*

CDS of the eGFP(AA 1-239), N-terminus of eGFP (AA 1-155, NeGFP) and the C-terminus of eGFP (AA 156-239, CeGFP) were cloned in frame to the N-terminus of WUS, KIN10 and KIN11 to generate fusion proteins under control of the *2xCaMV 35s* promoter. These constructs along with negative controls were transiently syringe infiltrated into 3-4 week old *N. benthamiana* leaves and incubated for 2d in a continuous light growth chamber. Leaf sections were cut and placed onto slides and imaged via the ZEISS 880 upright LSM Confocal microscope. Excitation of eGFP was at 488nm and emission was captured from at 500nm-525nm using a 40x water objective at 1.5x magnification.

KIN10OE Plant transformation and Fluorescent Confocal Imaging

Previously generated *KIN10* overexpression lines were done by driving expression of the gene with the *UBIQUITIN10* promoter as described in (Baena-Gonzalez et al., 2007). The *pCLV3:H2B-YFP* and *pWUS:H2B-YFP* reporters were cross bred into the *KIN10OE* lines and positives were identified by screens checking for fluoresces as described in Chapter 1. *KIN10OE pWUS:eGFP-WUS* was generated via *Agrobacterium*-mediated floral dip transformation and selected on 0.5% MS plates containing 50µg/mL kanamycin in continuous light for 10-12 days.

Confocal images for all plants were obtained as previously described in Chapter 2.

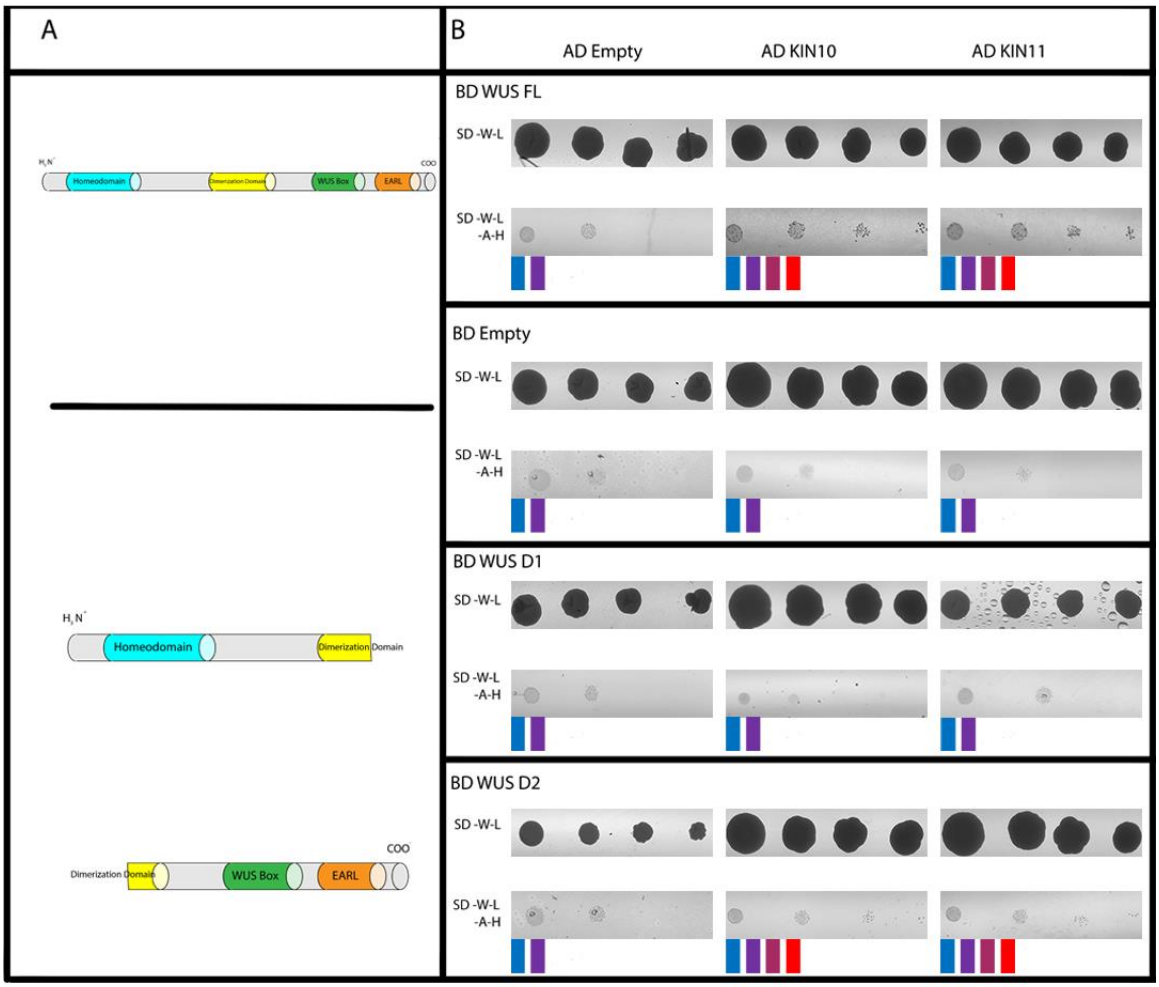


Fig 3.1 Mapping the interation region of WUS and KIN10/KIN11

(A) Each of the WUS truncation constructs diagramed WUS FL (1-292aa), WUS D1 (1-171aa) and WUS D1 (171-292aa). (B) Yeast-two hydrid 10-fold serial dilution analysis of each WUS truncation with KIN10 and KIN11. Colored rectangles indicate strength of the interaction based of the presence of growth, blue indicates the weakest/no interaction while red is the strongest interaction.

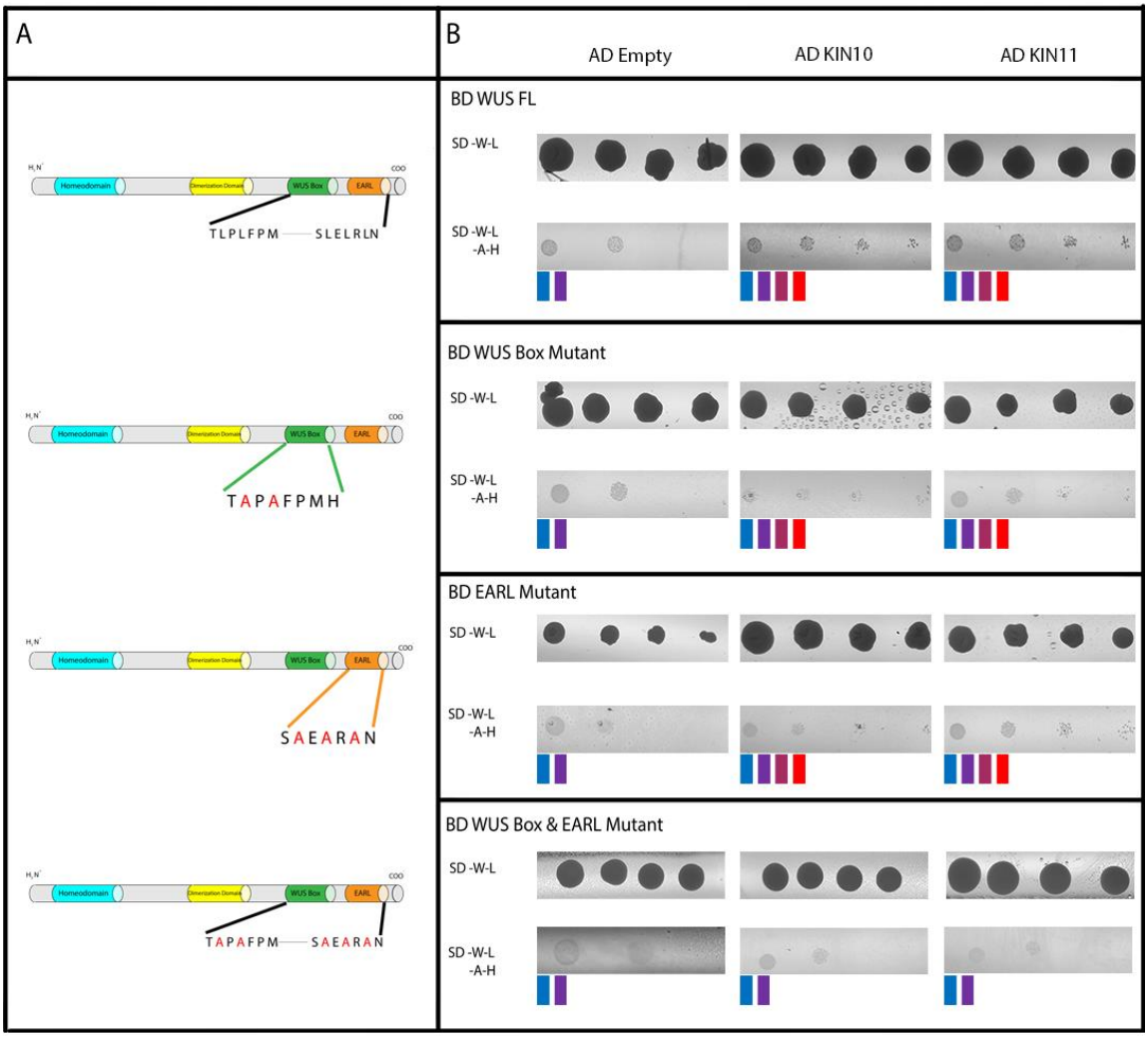


Fig 3.2 Mapping KIN10/KIN11 interactions to the WUS-Box and EAR-like domains

(A) Each of the WUS mutation constructs diagramed, mutations to the hydrophobic residues of the WUS-Box and EAR-like domains were used as described in (Rodriguez et al., 2016). (B) Yeast-two hydrid 10-fold serial dilution analysis of each WUS mutation with KIN10 and KIN11. Colored rectangles indicate strength of the interaction based of the presence of growth, blue indicates the weakest/no interaction while red is the strongest interaction.

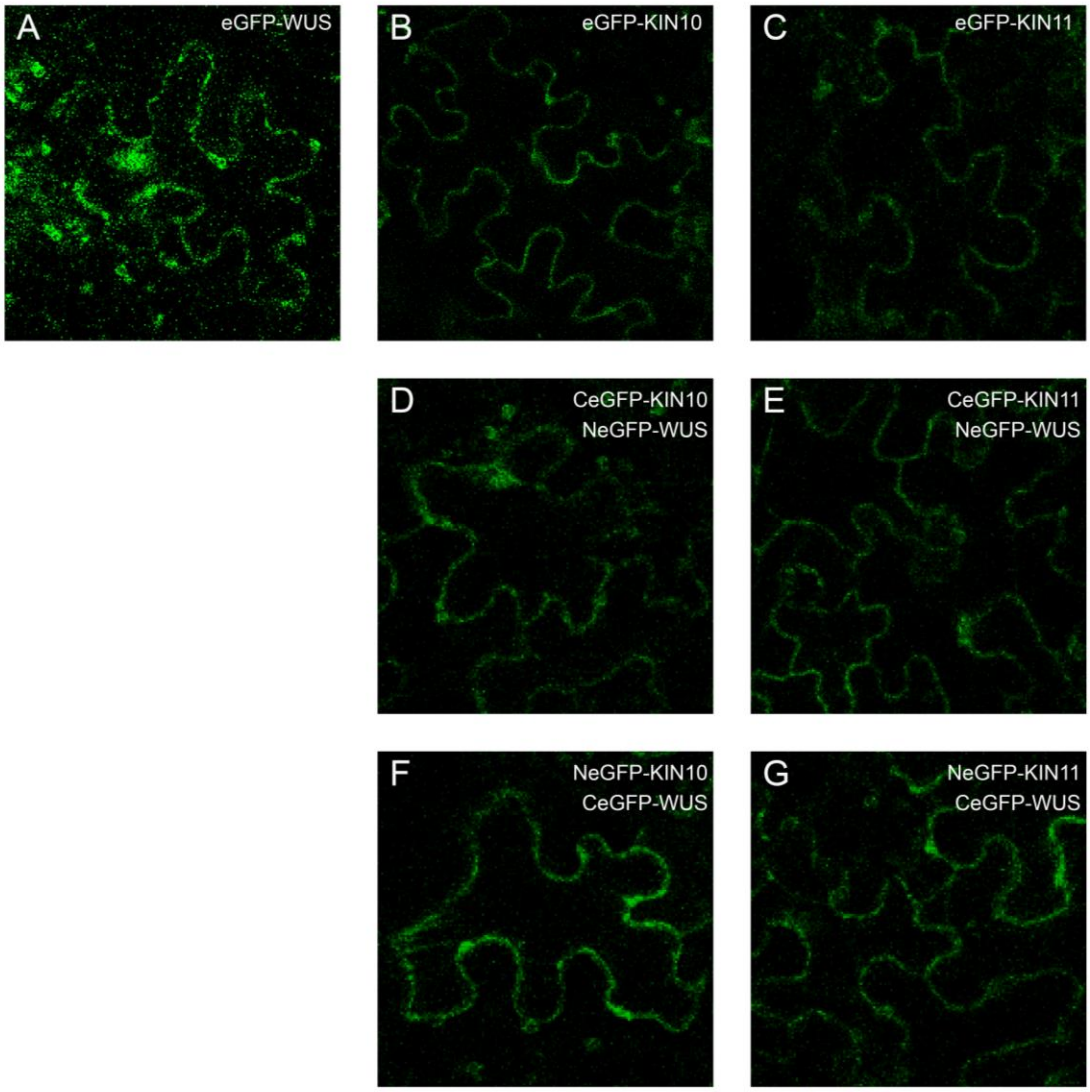


Fig 3.3 BiFC interactions of WUS-KIN10/KIN11

Localization of eGFP-WUS (A), eGFP-KIN10 (B) and eGFP-KIN11 (C) in N. benthamiana epidermal cells. The subcellular accumulation of CeGFP-KIN10/NeGFP-WUS (D) and NeGFP-KIN10/CeGFP-WUS (F) in N. benthamiana epidermial cells shows the interaction occurs in the cytoplasim. CeGFP-KIN11/NeGFP-WUS (E) and NeGFP-KIN11/CeGFP-WUS (G) similarly accumulate in the cytoplasim.

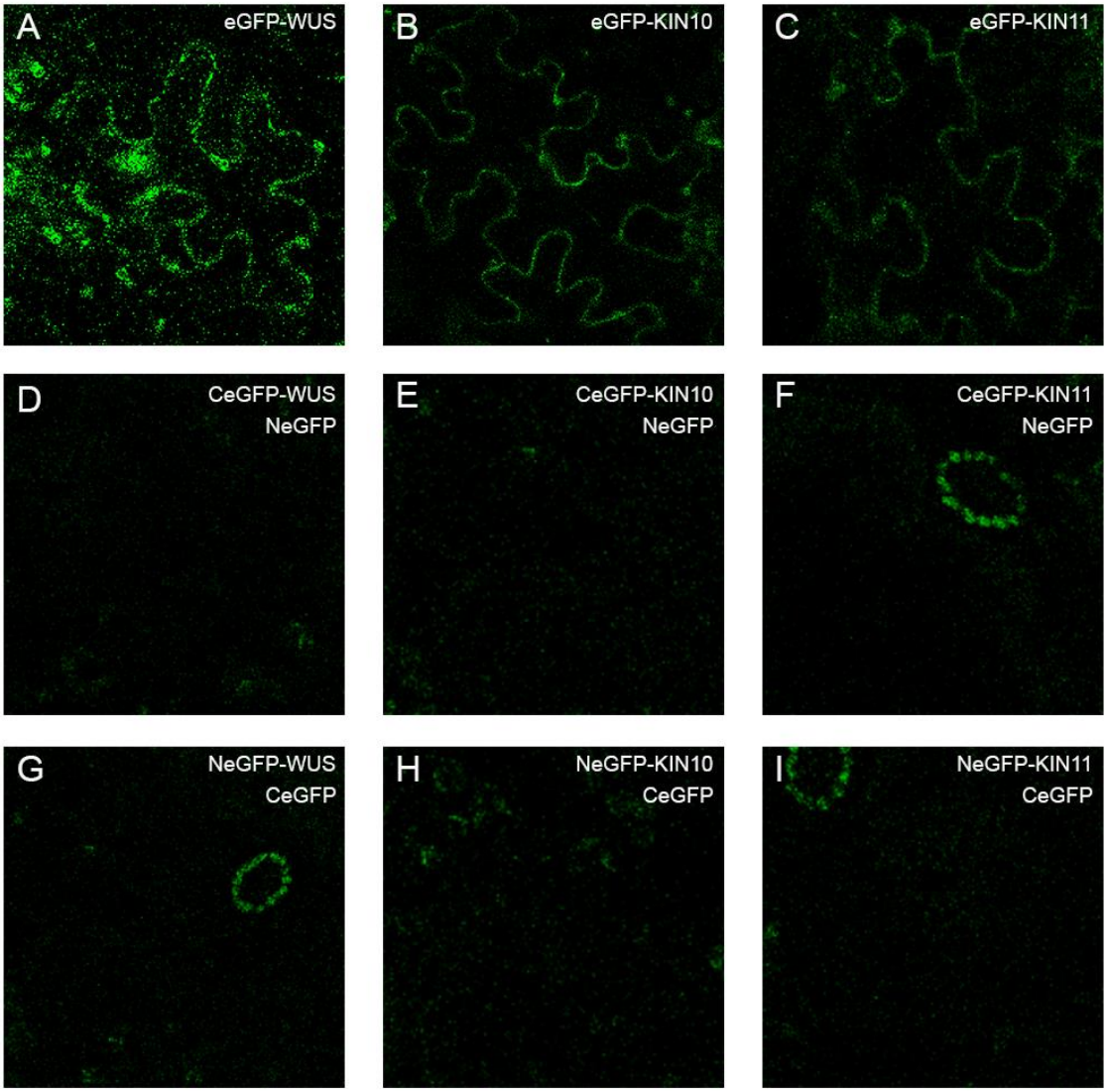


Fig 3.4 BiFC controls for interactions of WUS-KIN10/KIN11

Sub-cellular accumulation of CeGFP-WUS/NeGFP-Blank and NeGFP-WUS/CeGFP-Blank (D&G).
Sub-cellular accumulation of CeGFP-KIN10/NeGFP-Blank and NeGFP-KIN10/CeGFP-Blank (E&H).
Sub-cellular accumulation of CeGFP-KIN11/NeGFP-Blank and NeGFP-KIN11/CeGFP-Blank (F&I).

Fig 3.5 *KIN10OE* Results in an overproliferation of above ground organs and increased axillary meristem production

(A) *KIN10OE* lines produce more above ground organs when compared to wild-type plants as previosly described in (Baena-Gonzalez et al., 2007). (B) Additional analysis reveals the presence of more auxilary meristems branching from the main growing stem indicated by the arrows.

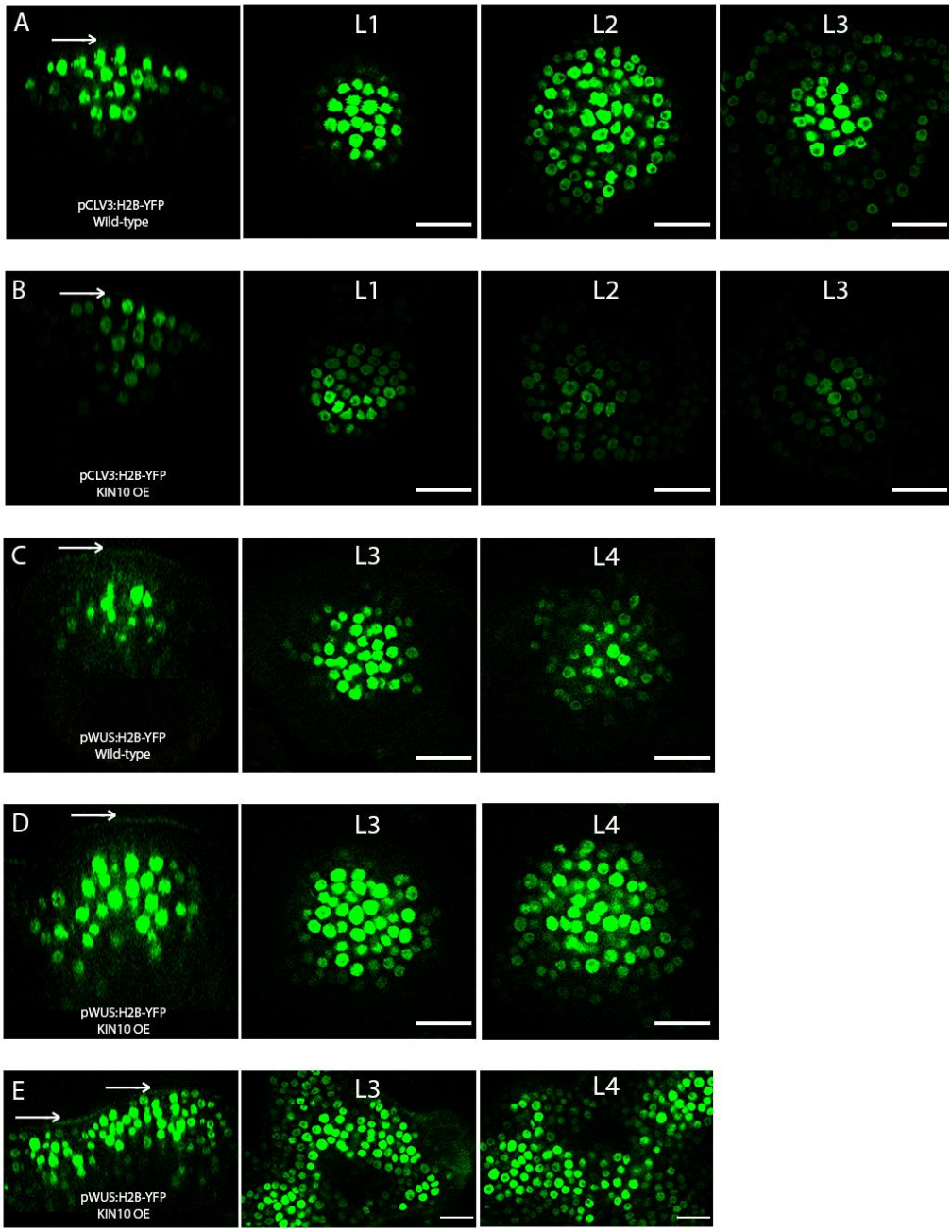
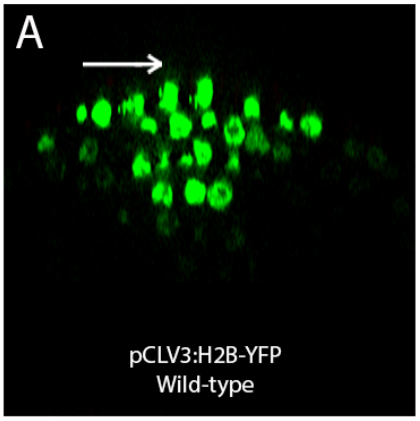
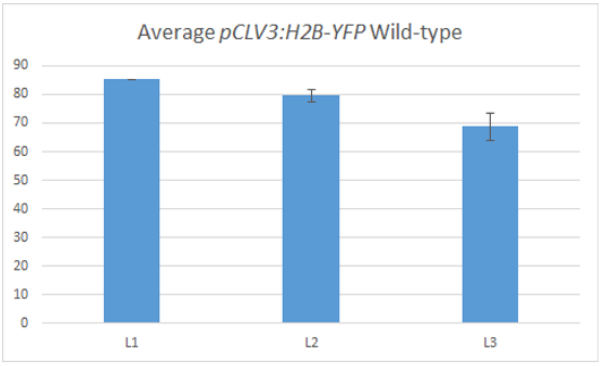
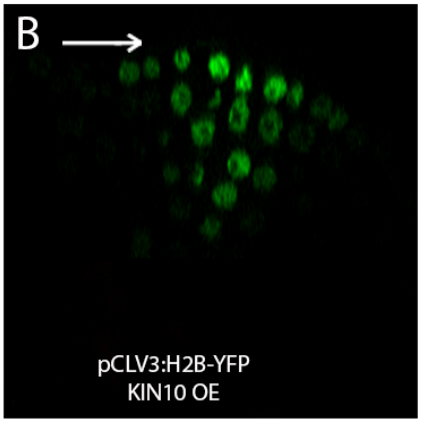


Fig 3.6 *KIN10OE* Results changes SAM architecture, *pCLV3* and *pWUS* expression
(A-B) Reconstructed side-view of a SAM and L1-L3 layers with the flourescent reporter *pCLV3:H2B-YFP* , indicating *CLV3* expression, in green. Arrows indicate the top of the SAM while the while scale bar represents 20µm. (A-B) In the *KIN10OE* background *pCLV3* expression is reduced at all cell layers were it is found. (D-E) The flourescent reporter *pWUS:H2B-YFP* indicating *pWUS* expression shows in increase in singal intensity as well as expression of the *WUS* expression domain when comparing *KIN10OE* and wild-type backgrounds. (E) Aproximately 30% of the plants also exhibited a fasciated mersitem which contains expression of *pWUS* in the L1 layer where it is normally not expressed.







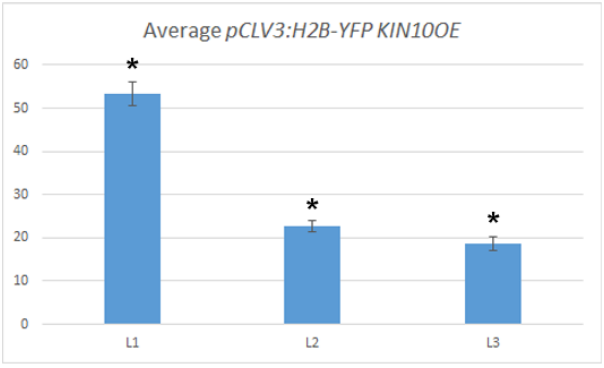


Fig 3.7 *KIN10OE* decreases *pCLV3* expression
Reconstrcuted side-view of wild-type *pCLV3:H2B-YFP* (A) and *KIN10OE pCLV3:H2B-YFP* (B). The relative flourescence of the six most center cells in the L1-L3 layers were measured. Error bars represent standard error. Asterisk indicates a significant difference between the mean of wild-type cell flourescence and *KIN10OE* cell flourescence. (n= 6, p=0.05).

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