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By Steven D. Rowland

Entitled A Lateral Root Defect in the wag1-1/wag2-1 Double Mutant of Arabidopsis

For the degree of Master of Science

Is approved by the final examining committee:

John C. Watson
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A LATERAL ROOT DEFECT IN THE *WAG1-1;WAG2-1* DOUBLE MUTANT OF *ARABIDOPSIS*

A Thesis

Submitted to the Faculty

of

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by

Steven D. Rowland

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This work is dedicated to my parents Steve and Donna Rowland, and to my sister Melissa.

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LIST OF ABBREVIATIONS

ABA	Abcisic Acid
ARG	Altered Response to Gravity
AUX	Auxin Resistance
AXR	Auxin Resistant
IL	Inner Layer
LAX	Like AUX1
LO	Lateral Organ
LR	Lateral Root / Roots
LRP	Lateral Root Primordium / Primordia
OL	Outer Layer
PAT	Polar Auxin Transport
PGM	Phosphoglucomutase
PID	PINOID
PIN	Pin Formed
RSA	Root System Architecture
TIR	Transport Inhibitor Response

ABSTRACT

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The root system architecture of higher plants plays an essential role in the uptake of water and nutrients as well as the production of hormones. These root systems are highly branched with the formation of post-embryonic organs such as lateral roots. The initiation and development of lateral roots has been well defined. WAG1 and WAG2 are protein-serine/threonine kinases from *Arabidopsis* that are closely related to PINOID and suppress root waving. The *wag1;wag2* double mutants exhibit a strong root waving phenotype on vertical hard agar plates only seen in wild-type roots when the seedlings are grown on inclined plates. Here an additional root phenotype in the *wag1;wag2* mutant is reported. The *wag1;wag2* double mutant displays both an increased total number and density of emerged lateral roots (approximately 1.5-fold). An increased LRP density of 1.5-fold over wild-type is observed. To ascertain the role of *WAG1* and *WAG2* in lateral root development we examined promoter activity in the *WAG1*::*GUS* and *WAG2*::*GUS* lines. The *WAG1* promoter showed no detectable activity at any stage of development. The *WAG2* promoter was active in stage IV onward,

however there was no detectable activity in the cell types associated with initiation events. The lateral root density and spatial patterning in wild-type, when grown on inclined hard agar plates, was similar to *wag1;wag2* on vertical plates. Seedlings of both genotypes were treated with hormones such as auxin and MeJA, and inhibitors. Auxin response in *wag1;wag2* was normal with a similar number of LR as the wild-type after treatment. Treatment with MeJA resulted in a similar induction of LRP in both genotypes, however the percent lateral root emergence in *wag1;wag2* was reduced while Col-0 was increased compared to controls. Treatment with the calcium blocker tetracaine resulted in *wag1;wag2* displaying a wild-type level of LR but had no significant effect on wild-type. Genetic analysis of the *wag1;wag2* LR pathway revealed that *WAG1* and *WAG2* are acting in the same pathway as *AUX1*, *AXR1and* PGM1. *pgm1-1* was not previously reported to have a LR defect but showed decreased LR formation here, while *pgm1;wag1;wag2* had a similar LR density to *wag1;wag2*. *TIR7* and *ARG1* were both deduced to operate in separate pathways from *WAG1* and *WAG2*. The data presented here shows that the *wag1;wag2* double mutant has an increased number of LR compared to Col-0. This defect appears to be caused by increased pre-initiation events and seems to be tied to the root waving phenotype. However, the treatment with MeJA revealed a possible role for *WAG1* or *WAG2* in LRP development, potentially under stress conditions. Calcium also seems to play a significant role in the *wag1;wag2* LR phenotype, possibly independent of the root waving phenotype.

INTRODUCTION

Root System Architecture of Plants

The root system architecture (RSA) of plants is essential for proper development. The root system provides many essential components for the plant including anchorage in the soil, finding and uptake of water and nutrients and the production of hormones such as auxin. The root system in plants is highly plastic and can respond to various cues from the external environment in the soil, a crucial ability as the soil does not always contain all the nutrients and water that plants require for proper development. Abiotic factors that contribute to the altering of RSA include water, nitrogen and phosphate availability (44,46,59). When these are not present or present in low amounts the root system will alter its architecture by increasing the amount of branching or increasing root elongation (33,44,46,59). Availability of a carbon source, such as glucose, also alters the RSA. When glucose is readily available, roots will increase branching, growth rate and root hair development, significantly altering their architecture (38). Mechanical stimulation, such as contact and avoidance of a barrier in the soil, can alter RSA significantly, typically resulting in the formation of new lateral roots on specific sides of the primary root, associated with the direction of avoidance (48). Biotic factors

also affect RSA, such as bacteria (infectious or not) and fungi (44). The ways in which the RSA is altered is almost as varied as the number of biotic factors that can affect it.

The plasticity of the RSA is primarily due to post-embryonic de novo organogenesis, or the formation of lateral roots (LR). In *Arabidopsis thaliana*, the root system consists of an embryonically derived primary root and post-embryonic lateral roots. The primary root of Arabidopsis develops in the embryo and emerges from the seed as a developed organ (41). However, unlike mammals that complete organ formation embryonically, plants continue to generate new organs post-embryonically and in the case of the root these are lateral roots. The primary root contains continually dividing cells in a meristem that allows it to grow (41). At later time points additional cells gain the ability to continue to divide and give rise to LR (41). The development of new roots gives the plant the ability to grow in poor soils by allowing it to seek out both nutrient and water supplies not readily available in the local environment (41).

Arabidopsis Root Anatomy

The Arabidopsis root consists of five tissue layers and three distinct zones (12). This simplicity makes the Arabidopsis root highly amenable to study of primary root and LR development. The outer three tissue layers consist of the epidermis, cortex and endodermis and the deep layers are the pericycle and vasculature. The meristematic zone constitutes the distal 250 µm of the root tip (12). This zone can be sub-divided further into the apical meristem and the transition zone (10,60,61), which constitutes the proximal end of the meristem and adjoins the next zone (12). The apical meristem

consists of small cells originating from a group of cells in the quiescent center (12). The quiescent center is surrounded by meristematic initials that continuously divide and allow the root to grow. The apical meristem is above the root cap which contains the columnella cells (12). The transition zone consists of non-differentiated cells that are expanding in size, and is marked by cube-shaped cells.

Proximal to the meristem is the elongation zone (12). This zone is characterized by non-differentiated cells which are elongating anticlinally and constitutes the 750 μm shootward from the meristem (12). Proximal to the elongation zone is the differentiation zone. This zone consists of elongated cells in each layer that mature into their respective tissue types (12). The regulation of the processes occurring in these zones is primarily attributable to the phytohorome auxin. Auxin is a primary regulator of cell division and expansion in the meristem and elongation zone (4,5). Auxin is known to regulate many plant developmental processes (5). Increased levels of auxin such as those found in the meristem result in cell division and the suppression of elongation, however lower levels of auxin results in cell elongation instead of division (4,5). Response to auxin can be modulated by the hormone cytokinin (61). Cytokinin promotes cell elongation through the suppression of auxin signaling and transport and is an essential antagonist to auxin to maintain the correct developmental process in each zone (61).

Auxin can move throughout the root in two primary methods, the first being passive diffusion through cells. However, only protonated auxin can diffuse through cells and only a small percentage of endogenous auxin is in its protonated form at any

given time. The second method is active or polar auxin transport (PAT). PAT is mediated primarily through the AUX and PIN protein families which perform influx and efflux (51). AUX1 and PIN1 located in the vasculature move auxin rootward towards the root tip and the root cap, specifically into the columnella cells (56, 53). From the quiescent center and columnella cells, auxin is moved into the lateral root cap by PIN3 and PIN4 (3). Auxin in the lateral root cap is moved shootward through the epidermal cell layer via the protein PIN2 (1,39). Mutations in PIN2 demonstrated a role for auxin transport in proper gravitropic response, as the roots of PIN2 loss-of-function mutants displayed an agravitropic phenotype (1,39). In the transition zone auxin transported by PIN2 moves inward through the cortex, endodermis and pericycle cell layers into the vasculature where AUX1 and PIN1 again transport it rootward (1,32,39). This cycling of auxin from the root tip to the distal meristem and back to the root tip has been called the auxin fountain system or auxin reflux, and has been shown to be important for LR formation (7).

Other proteins involved in auxin transport include the MDR/PGP/ABCB proteins (51,56). Two important ABCBs are ABCB4 and ABCB19. Loss-of-function mutations of these genes showed an increased and decreased number of LR, respectively (51,56). ABCB19 is a rootward auxin transporter located in the vasculature, much like AUX1 and PIN1 and moves auxin produced in the shoot to the root system (56). Roots with mutations in ABCB19 display a slight agravatropic phenotype similar to but less severe than AUX1 and PIN2 mutations (56).

Auxin reflux plays an important role in many processes in the root including LR formation (34,35). Proper PAT is necessary to develop the correct number and distribution of LR, as well as proper gravitropic response (34). The auxin reflux system as described above relies on the protein transporters primarily of the AUX and PIN families and as described mutating any or several of these proteins results in altered LR numbers and development and altered gravitropism (34,35).

Lateral Root Development

Lateral root development in Arabidopsis occurs in four distinct stages; pre-initiation, initiation, primordia development and emergence. Each stage of development is regulated by auxin and its transport. Pre-initiation occurs in the distal meristem, which is defined as the priming of founder cells (11,27). Auxin is transported through the epidermis via PIN2 and then transported inward to the vasculature in the transition zone. The auxin transporter AUX1 participates in creating an auxin maximum in the protoxylem (11). This auxin maximum spreads out from the xylem poles to the adjacent pericycle cells, which results in the priming of these cells, usually in pairs (11,27). What exactly occurs, such as gene expression, or how one set of pericycle cells are selected versus another, is still unknown (11). At this time no cell division occurs and in fact the primed pericycle cells cannot be distinguished from cells that have not been primed, although founder cells can be visualized through cell lineage marking (27). As the root tip continues to elongate the primed pericycle cells undergo normal cell elongation as the region they are in matures into the elongation zone and then the

differentiation zone (11). Unlike other pericycle cells that in the differentiation zone undergo complete differentiation and cease cell cycle activity, founder cells will re-enter the cell cycle upon proper signaling, which signifies initiation (11).

LR initiation involves a complex set of molecular actions that reset the primed pericycle cells to be able to re-enter the cell cycle and begin dividing. The activation of this process is regulated primarily by auxin (positively) and also by cytokinin (negatively) (8). Shoot derived auxin is transported rootward via the auxin transporter ABCB19 which is required for proper initiation of lateral roots (4,51,56). Auxin transported rootward accumulates in cells adjacent to the primed pericycle cells through the action of PIN2 and AUX1, creating an auxin maximum that triggers the molecular processes of auxin signaling shown in Figure 1 (8,17). Auxin binds to the F-box protein TIR1 of the SCF^{TIR1} complex and derepresses auxin response elements that are blocked by auxin response factors, thus up regulating auxin responsive genes (8). The SCF^{TIR1} complex degrades IAA14/SLR1, which then derepresses AFR7 and ARF19 (Figure 1), leading to the eventual activation of the cell cycle and cell fate respecification (9,18,19,20,42). However, the protein ALF4 is required for cell cycle reactivation through the repression of cyclin B1 and up regulation of CDKB;1. Roots with a knockout of ALF4 develop no lateral roots (9). All of these responses to auxin reactivate the cell cycle only in pericycle cells that had previously been primed and activate the lateral root developmental program (9,18,19,20,42). Additionally an auxin maximum continues to accumulate in the actively dividing cells, which up regulates Like AUX1 (LAX) 3 in the cells of the adjacent tissue layer (53). LAX3 is an auxin influx protein, and, in the case of the initially

dividing pericycle cells, imports auxin into adjacent endodermal cells (53). The influx of auxin again represses Aux/IAA proteins, which in turn negatively regulate LAX3, but most importantly the auxin activates cell wall remodeling (CWR) proteins. CWR proteins begin to break down the cell wall and allowing the developing LR to grow without impedance (53). LAX3 mutants either do not develop LR or have LR that tear through the overlying tissue layers damaging the primary root in the process (53). As the lateral root continues to develop, LAX3 transports auxin into the overlying tissue layer causing the breakdown of the cell walls and allowing for the lateral root to eventually emerge from the primary root without damage.

After the auxin maximum is formed and the pericycle founder cells re-enter the cell cycle, they begin anticlinal division (along the axis of the root) to form a single row of eight to twelve cells (8,36). At this point, initiation of the lateral root is complete and a stage I LRP is formed (36). Figure 1 displays the known molecular process of initiation in LRP founder cells.

The development of LRP has been well defined by Malamy and Benfey (1997) and is characterized by eight anatomical stages and two sub stages. Stage I is defined as a single file of eight to twelve cells that originate from the pericycle founder cells. These cells undergo periclinal division and result in stage II LRP with an outer cell layer (OL) and inner layer (IL) (36). Stage III consists of three cell layers, with the periclinal division of the OL resulting in two OLs (36). Stage IV is formed by the periclinal division of the IL giving rise to a four cell layer LRP with two ILs and two OLs (36). Stage V is sub-divided into Va and Vb; an anticlinal division of the two central cells of OL I and II gives rise to Va

(36). Cells of OL I and II adjacent to the central cells undergo anticlinal division and both IL cells expand to form stage Vb (36). Stage VIa is formed by the periclinal division of all cells in OL II with the exception of the central two creating OL IIa and IIb (36). The central four cells of OL I then undergo periclinal division to form another layer, giving the OL a 4-4-4 cell configuration (36). All the cells in OL I then undergo anticlinal division to give an 8-8-8 cell pattern and form a stage VII LRP (36). From this point the LRP will no longer undergo cell division but will grow by cell expansion throughout emergence and until the meristem becomes active, at which point the mature LR will grow in the same manner as the primary root (36).

The passage through each stage of LRP development is highly regulated, with auxin playing a prominent role (3). The movement of auxin through the LRP is controlled by the PIN proteins, primarily PIN1 through PIN6 (3). In the stage I LRP, PIN3, PIN4 and PIN6 are actively contributing to the auxin maximum at this and the following two stages (3). PIN1 becomes active at stage III at a basal level and at higher levels of activity at stage IV onward (3). PIN2 becomes active much later at stage VI to VII and localizes to the cells that will eventually be the epidermal cell layer (3). PIN6 brings auxin into the LRP from the primary root. Auxin is then transported via PIN1 through central tissues, which will eventually become the vasculature. At the tip of the LRP, both PIN3 and PIN4 are active and are responsible for moving auxin from the tip outward toward what will be the epidermis, where PIN2 moves the auxin out of the LRP back into the primary root (3). At stage I, the auxin maxima is distributed across all cells but localizes more centrally at stage II and continues to be localized to centrally located

cells at all following stages. At stages I-III, the LRP is completely reliant on auxin from the primary root, however at stage IV the LRP begins to produce its own auxin (30). While it still utilizes the auxin from the primary root, it has been shown that LRP excised from the primary root at stage IV or later will continue to develop and produce its own auxin (30).

Malamy and Benfey define the early emerged LR as a stage VIII LRP, and as previously stated the LRP grows through cell expansion rather than cell division at this point (36). At a later time point the meristem will become active and the meristematic initials begin to divide, and the LR will grow via cell division rather than cell expansion (36). At this point the LR develops like the primary root with the same tissue layers and zones.

There are many known LR mutants in Arabidopsis but only a small portion of these mutants are associated with known molecular or cellular processes (45). For those mutations with known actions, many play a role in auxin transport, signaling or response, with the exception of ALF mutants that are required for chromatin remodeling and activation of the cell cycle (7,9,45). Interestingly, many LR mutants reduce or eliminate LR formation, and very few mutations confer an increased number of LR (45). The few mutants that increase LR numbers include *sur1,* sur2 and *arf8,* which are involved in auxin homeostasis, and the chromatin remodeling factor *pickle* (45). Mutants, such as *PIN* and *AUX1* auxin transporters, result in fewer LR with the exception of *abcb4* which confers increased LR pre-initiation and initiation (45). Collectively this shows that many mutations that affect auxin transport, response or homeostasis have

an effect on LR development, indicating auxin as a major contributor to LR development and patterning.

Root Waving

When grown on vertical agar plates, the roots of *Arabidopsis* seedlings grow relatively straight, only meandering slightly off the vertical vector of gravity (21). However, when *Arabidopsis* seedlings are grown on inclined plates (less than 90°) their roots begin a process called root waving, which is the regular sinusoidal movement of the root (21, 54). Thompson and Holbrook (2004) showed that root tip impedance was modulated through normal gravitropic response on inclined plates, resulting in root waving (54). Gravitropism is the re-alignment of the root tip in the direction of the gravity vector, which occurs any time the root tip is angled away from this vector (54). Thompson and Holbrook (2004) were able to show that on inclined plates, seedling roots undergo normal gravitropism, bringing the root tip into more contact with the agar surface. This increased contact generates impedance on the root tip, often causing it to stick to the agar surface, which in turn generated very specific non-tropic bending behind the root tip (54). The bending behind the root tip (and the torsional stress that accompanied it) would cause the root tip to then slip and deflect off a straight vector (54).

After the slippage of the root tip, the root would continue to grow along this new vector until it again underwent gravitropic bending, which redirected the root downward on the plate and against the surface of the agar repeating the process (54).

In this way a sinusoidal wave pattern is generated in seedling roots grown on inclined plates (54). The composition of the growth medium also determined the amount of root tip impedance, as higher agar concentrations (such as 1.5% and up) created greater friction on the root, while lower concentrations of agar allowed the root tip to slide more easily (21,54). Other components of the medium also contribute to the waving pattern of seedling roots. For example, roots will not wave in the absence of sucrose (21,54). The levels of ethylene present also modulate the amount of root waving (21,36,54).

Protein Serine/Threonine Kinases

The AGCVIIIa subfamily of kinases in *Arabidopsis* is a part of the eukaryotic group of regulatory kinases (58). AGCVIII kinases are protein-serine/threonine kinases (2,58). Protein kinases are enzymes that transfer the gamma phosphate of ATP or GTP to a substrate protein (58). This transfer often results in the activation or inactivation of the substrate (58). AGCVIIIa protein kinases are distinguished from other AGC kinases by a conserved DFD motif, and a variable insertion within the catalytic domain (58). Many AGCVIIIa kinases have not been associated with single mutant phenotypes, despite having confirmed insertions within their coding regions, and most likely function redundantly (58). The high conservation between the genes and protein sequences supports this idea (58). Of the Arabidopsis AGCVIIIa kinases, PINOID (*PID*) has been shown to play a positive role in auxin transport by regulating the asymmetrical

localization of membrane proteins involved in PAT (2,58). AGC kinases then can and do play a role in PAT through directing the localization of auxin transport proteins (2,5,49).

Previous researchers in our laboratory investigated protein kinase genes from the garden pea that were regulated by light. Partial cDNA clones were obtained and designated PsPK1-5 (*Pisum sativum* protein kinase 1-5) (62). Of these, the mRNA levels of PsPK3 in 6 day-old etiolated seedlings were found to decline within one hour of constant white light (18,50). A homolog of PsPK3 from *Arabidopsis*, named PK3At1 was cloned (52). Additionally a second homolog in Arabidopsis was found by searching the genome for paralogs of Pk3At1. These were later renamed WAG1 and WAG2, respectively (52). WAG1 and WAG2, like PsPK3, are members of the AGCVIIIa family of protein serine/threonine kinases. They have 69.5% and 68.8% homology with PsPK3, respectively, and are 74% identical to each other with 81% identity in their catalytic domains (Fig. 3).

The *wag1-1* (*wag1)* and *wag2-1* (*wag2)* single mutants contain T-DNA insertions within their coding regions, and are loss-of-function mutations (52). Both *wag1* and *wag2* appear wild-type in most respects except when grown on inclined plates where they show an enhanced root waving phenotype. The *wag1*;*wag2* double mutant shows an even greater enhanced waving phenotype indicating a gene dosage effect and overlap in their function (52). The *wag1;wag2* double mutant roots also wave when grown on vertical agar plates, a phenotype only present in wild-type plants when the plate is inclined to less than 90° (63).

When *wag1;wag2* is grown on inclined plates the waves become even more compressed with shorter wave lengths and larger amplitudes (52), indicating that the gravitropic input, which gives Col-0 its wavy growth pattern on inclined plates, adds to the *wag1;wag2* waving phenotype. The *wag1* and *wag2* single mutants also display a root waving phenotype on vertical plates, however it is much less pronounced than the double mutant. The *wag1* single mutant roots show a compressed wave length when compared to Col-0 grown on inclined plates, while *wag2* showed increased amplitude (52). Along with the 74% identity between *WAG1* and *WAG2*, this suggests that these genes may be functionally redundant. Both *wag1* and *wag2* display a waving phenotype but each modulates that phenotype in a slightly different way. The fact that on inclined plates *wag1;wag2* showed even stronger enhancement of waving may indicate that gravitropism is not responsible for the waving phenotype and only added to the constitutive waving through the normal gravistimulated mechanism explained above. Previously it was shown that *wag1;wag2* did not have altered LR when compared to Col-0 (52). These data were obtained in experiments that measured auxin responsive LR induction in *wag1;wag2,* which required the transfer of seedlings to fresh plates. Preliminary data obtained later with non-transferred seedlings suggested that there was enhanced LR formation in *wag1;wag2*, which provided the impetus for my project.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Col-0, *wag1-1, wag2-1* and *wag1-1;wag2-1* were used previously (52). The triple mutants used were described previously (65). Laboratory seed stocks were grown up and seeds harvested for working stocks. Seeds were sterilized by incubation for 2 minutes in 70% ethanol, followed by incubation for 10 minutes in 25% (v/v) bleach, then washed 5 times with sterile water. The seeds were then imbibed at 4°C in the dark for 72 hours in sterile water. After imbibing, seeds were sown onto 1.5% (w/v) Bacto agar (214010; Becton Dickson) plates containing half-strength MS salts with vitamins (M5519; Sigma-Aldrich) and 1% (w/v) sucrose, with the pH adjusted to 5.6 with sodium hydroxide before autoclaving. The plates were placed vertically in racks under constant cool white fluorescent light of 80 µmol m^{-2} sec^{-1} at 22°C for the times indicated below.

Emerged Lateral Root Quantification

Time Course: Seedlings of Col-0, and *wag1;wag2* were grown for 5 to 10 days and all emerged LR on the primary root were counted on a stereomicroscope. When quantifying LR density, the plates were scanned (HP Scanjet 3970) after LR counts and the root lengths measured using ImageJ (Neurite Tracer) software

(HTTP://RSBWEB.NIH.GOV/IJ/). The number of lateral roots per unit length of primary root was then calculated and referred to as LR density.

Auxin Treatment: Seedlings of Col-0 and *wag1;wag2* were grown to 7 days after sowing and then transferred to fresh media containing either 10^{-6} M NAA (or the solvent control 70% EtOH) and allowed to grow for an additional 3 days. All emerged LR were counted as described above.

Staging of Lateral Root Primordia

To count the total number of LRP at each stage (36), seedlings of Col-0 and *wag1;wag2* were grown from 5 to 7 days after sowing. The seedlings were cleared according to Malamy and Benfey (1997) with the following modification: The incubation time for the first step was increased to 20 minutes. Seedlings were placed in a container with 0.24N HCl in 20% methanol and incubated at 57°C for 20 minutes. This solution was replaced with 7% sodium hydroxide in 60% ethanol and incubated at room temperature for 15 minutes. Seedlings were then rehydrated for 5 minutes each in 40%, 20% and 10% ethanol, and then vacuum infiltrated in 5% ethanol, 25% glycerol for 15 minutes. The seedlings were then mounted in 25% (v/v) glycerol on slides and the LRP counted and their stages recorded on a Nikon Eclipse E800 with DIC optics.

To calculate the density of LRP, slides were scanned (hp Scanjet 3970), and primary root lengths measured using ImageJ (HTTP://RSBWEB.NIH.GOV/IJ/). The density was then calculated as the number of LRP per unit length of primary root.

Germination

Seeds of Col-0 and *wag1;wag2* were sterilized and sown as described above. The number of seeds that germinated (described as those seeds with an emerged radical ≥ half the length of the seed) were counted on a stereomicroscope every 8 hours up to 72 hours. After 72 hours the total number of germinated seeds was normalized to 100% germinated and the percentages for previous time points recalculated according to this number.

Promoter Activity and Lateral Organ Density

To analyze the activity of the *WAG1* and *WAG2* promoters in LRP, seedlings of Col-0 containing *DR5*::*GUS* (65) and Col-0 containing *WAG1*::*GUS* and *WAG2*::*GUS* (52) were grown as described to 7 days after sowing. The seedlings were vacuum infiltrated in GUS stain [50mM sodium phosphate buffer (S-0876; Sigma-Aldrich), 0.5% (v/v) Triton X-100, 10mM Potassium Ferricyanide (P232-500; Fischer Scientific), 10mM Potassium Ferrocyanide (P236-500; Fischer Scientific), 0.5 mg/mL X-Gluc (G1281C1; Gold Biotechnology) (X-Gluc was prepared in dimethylformamide) and brought to volume with sterile water for 2 minutes, incubated at 37°C overnight, and then cleared as described above. Images of the lateral root primordia and primary root tips were acquired with the Nikon Eclipse E800 with DIC optics using a Nikon DXM1200 Digital Camera and the Nikon ACT-1 software. All images were edited in iPhoto.

To facilitate visualization of LRP to calculate total lateral organ density (emerged LR + LRP/cm), seedlings of Col-0 and *wag1;wag2* containing *DR5::GUS* were grown from 3 to 7 days after sowing, stained and cleared as above, and then all lateral organs were counted on the Nikon Eclipse TE200 microscope using phase contrast. The slides were scanned and the root lengths measured as described above.

LR and LRP Density in Zone 1 and Zone 2

Seeds of Col-0 and *wag1;wag2* were sterilized and grown as described above. Seedlings were then cleared as described above, 7 days after sowing, and the LR and LRP in zone 1 and zone 2 (14) were counted. Zone 1 is defined as the LR containing region of the primary root, and zone 2 contains only LRP. The seedlings were imaged and the length of zone 1 and zone 2 determined using ImageJ. LR and LRP density were calculated for each zone.

Lateral Root and Lateral Root Primordia Patterning

Seedlings of Col-0 and *wag1;wag2* were sterilized and grown as described above. The seedlings were then cleared as described 7 days after sowing, mounted on slides in 25% glycerol and each LR and LRP marked with a Sharpie on the coverslip using a Nikon Eclipse TE200 inverted stage microscope. The slides were scanned and the distances of each LR and LRP from the root tip were measured using ImageJ. The inter-LO distance (the average distance between LOs) was calculated from these measurements.

Methyl Jasmonate Treatment

Seedlings of Col-0 and *wag1;wag2* were sterilized and grown for 7 days after sowing either on plates containing 1 μM methyl jasmonate (made from a 1 molar stock in 70% EtOH) or solvent control (70% EtOH) plates. The seedlings were then cleared as described and the LR and LRP counted as described above. Root length was obtained by imaging the slides and measuring in ImageJ and LR and LRP density calculated. Percent emergence was calculated as the percent of LR versus the total LO. Percent LRP per stage was calculated as the LRP at that stage versus total LO.

Calcium Blockers

Seedlings of Col-0 and *wag1;wag2* containing *DR5::GUS* (to aid in visualization of LRP) were sterilized and grown to 7 days after sowing on either 75 μM lanthanum chloride, 75 μM verapamil, 75 μM tetracaine or solvent control (70% EtOH). The seedlings were stained for GUS activity and cleared as described. Total LO were counted and root lengths measured as described above.

RESULTS

Lateral Root and Lateral Root Primordia in *wag1;wag2*

The goal of my project was to determine if the *wag1;wag2* double mutant displayed a LR defect. The first step to investigate this was to measure the number of emerged LR in both Col-0 (wild-type) and the *wag1;wag2* double mutant (Fig. 3). Seedlings were grown on 1.5% (hard) agar with 0.5X MS and 1% sucrose for to the number of days indicated before being scored for total LR. Later time points beyond 5 days were incorporated to investigate if the *wag1;wag2* mutants LR numbers changed over a time course differently than the wild-type. *wag1;wag2* has an increased number of LR as early as day 5 and at each additional time point on both vertical (90°) (Fig. 3A) plates and inclined (45°) (Fig. 3B) plates. The LR number in *wag1;wag2* is approximately 1.5-fold higher than that of wild-type for seedlings grown on vertical plates. Seedlings grown on inclined plates displayed increased LR over vertically-grown seedlings for both wild-type and *wag1;wag2* until day 9, where LR numbers were nearly equal for both genotypes (Fig. 3). At day 10 vertically-grown seedlings of both genotypes had higher LR numbers than their inclined counterparts. Nevertheless, *wag1;wag2* seedlings exhibited a higher number of emerged LR over wild-type at all time points on both vertical and inclined plates.

Whether the increased number of LR over wild-type in *wag1;wag2* seedlings was caused by an actual defect in LR development or indirectly through other processes required further analysis. Therefore I asked if LRP numbers and distribution in *wag1;wag2* were affected (Fig. 4). At day 5, *wag1;wag2* shows an increased number of LRP at almost every stage, however the distribution across stages is similar to that of Col-0. Although the distribution between each stage was similar in the two genotypes, there were an increased number of LRP at each stage in *wag1;wag2* (Fig. 4A). At day 6, LRP numbers and pattern remained relatively unchanged with the exception of stage 5 (Fig. 4B). Col-0 had a similar number of stage 5 LRP at day 6 as was present at day 5, however *wag1;wag2* showed a 1.75-fold increase in the number of stage 5 LRP. At day 7, Col-0 also showed a similar increase in stage 5 LRP (Fig. 4C), although the increase was 3.5-fold. This returned the distribution across stages to a similar state between both genotypes. The increase in stage 5 LRP is not surprising since both stage 4 and stage 5 have been implicated as possible check points or arrest points in LRP development (20), and the increased number of stage 1 and 2 LRP may be responsible for *wag1;wag2* reaching this level earlier.

This discrepancy in stage 5 LRP numbers could also be caused by the possible activity of either *WAG1* or *WAG2* during LRP development. The increased number of LRP and LR of all stages suggests that perhaps *WAG1* or *WAG2* play a significant role in the development of LRP. To investigate this, transgenic lines containing either the *WAG1* or *WAG2* promoter driving *GUS* were used. GUS (β-glucuronidase) is an enzyme that will break down the substrate X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide)

to yield an insoluble dark blue product. In this way the activity of both promoters could be visualized in the LRP at each stage. As a control, a transgenic line was used where *DR5*, an auxin responsive promoter, drives *GUS* expression. *DR5*::*GUS* has been used previously to examine auxin levels in LRP at all stages of development and revealed auxin's dominant role in regulating all stages of LR development (3). Figure 5 shows *WAG1::GUS*, *WAG2::GUS* and *DR5::GUS* images of each LRP stage and the primary root tip. The *WAG1* promoter displayed no detectable activity at any stage of LRP development. In fact, *WAG1::GUS* showed no detectable activity until after emergence, where it showed a staining pattern similar to that found in the primary root tip (data not shown). The *WAG2* promoter was not detectably active in stage 1, 2 or 3, but became active in stage 4 LRP and remained active for all proceeding stages of LRP development. The GUS staining for the *WAG2* promoter was always limited to the cells of the OLs and particularly to the central cells of the OLs, which corresponds with *DR5::GUS* activity at these same stages (Fig. 5). The staining pattern of *DR5::GUS* was similar to that seen in previously published data (3), and the typical pattern in the primary root tip was also observed. In accordance with previously published data (3), about 75% of LRP stained at stage 4 while other stages showed 80% to 100% staining. This has been shown before at stage 4 (3) and is indicative of the change in auxin sources as the LRP begins to produce its own auxin and becomes less reliant on the primary root for auxin (7). The *WAG2* promoter is clearly active only in later stages of LRP development, and neither *WAG1* nor *WAG2* are detectably active at the early stages of LRP development. Therefore, early expression of *WAG1* and *WAG2* does not seem to account for the

increased number of stage 1 and 2 LRP in *wag1;wag2*. This suggests that the *wag1;wag2* mutation causes an increased total number of founder cells during pre-initiation, which then undergo normal development.

To test this possibility, seedlings were grown for 7 days on vertical plates and then root length and total emerged LR were scored. The seedlings were then subsequently cleared according to Malamy and Benfey (1997) and the total number of LRP scored. The density of LR and LRP were calculated from the data (Fig. 6). Root lengths for both genotypes were not significantly different between the two genotypes (Fig. 6A), in agreement with previous data (52). LR density was 1.5-fold higher LR in *wag1;wag2* (Fig. 6B), confirming the previous experiments (Fig. 3.) Interestingly, LRP density was also 1.5-fold higher in *wag1;wag2* compared to Col-0 (Fig. 6C). The magnitude of the increase in LRP density agreed exactly with the LR density, indicating that the increased number of LR was most likely attributable to increased pre-initiation events.

To confirm the results that LRP density is enhanced in *wag1;wag2*, another method was employed. In this case, the LR and LRP densities were calculated in two zones of the primary root. Dubrovsky *et al.* (2007) defined zone 1 of the root as the region containing emerged LR and zone 2 as the region of the root that contained only LRP. Figure 7 shows the densities of both LR and LRP in these zones. Interestingly *wag1;wag2* does not show a higher LR density when only zone 1 is taken into account, but this is primarily because zone 1 is larger in *wag1;wag2* than that found in Col-0 (Fig. 7). However, the LRP densities in zone 1 and zone 2 for *wag1;wag2* were significantly

higher than Col-0 (Fig. 7), potentially indicating an increase in pre-initiation event in *wag1;wag2.* For the analysis shown in Figure 7, founder cells were also included in the count as well as fully initiated LRP. However, all of the data presented, when taken as a whole, suggests that pre-initiation events are affected in *wag1;wag2* resulting in a higher number of LR when compared to Col-0.

Root Waving and Lateral Root Development

The result that LR pre-initiation events are altered in *wag1;wag2* led to the question what leads to this disruption? The primary phenotype of *wag1;wag2* is that its roots wave on vertical plates, a phenotype only seen in Col-0 when grown on inclined plates. To ascertain if the root waving of *wag1;wag2* was responsible for the disturbed pre-initiation seedlings, were grown vertically for 7 days and then cleared (Malamy and Benfey, 2007) to determine the positioning of LR and LRP for both genotypes. Cleared seedlings were mounted on slides in 25% glycerol and each LR and LRP marked under a stereomicroscope. The distance of each LR and LRP from the root tip in each seedling was then measured (Fig. 8). Vertically-grown Col-0 seedlings had a relatively large distance between each lateral organ. Vertical Col-0 seedlings had fewer total LR and LRP than when inclined or in either *wag1;wag2* sample. *wag1;wag2* on vertical plates showed a very regular pattern of lateral organ positioning, consistent with previous work that showed root waving directly affects positioning (10). Inclined Col-0 seedlings, which were also strongly waving, displayed a similar spatial pattern as the vertically-grown *wag1;wag2* seedlings with only slightly larger distances between lateral organs.

Inclined *wag1;wag2* seedlings had even shorter distances between lateral organs (Fig. 8), this is consistent with root waving affecting the spatial pattern since these seedlings wave even more intensely than *wag1;wag2* on vertical plates.

The mean inter-lateral organ (LO) distance was measured for each genotype on both vertical and inclined plates (Fig. 9). The average distance for vertical Col-0 seedlings was nearly twice that of inclined Col-0 seedlings and vertical *wag1;wag2* seedlings. The average inter-LO distance between inclined Col-0 and vertical *wag1;wag2* was not statistically different. These data strongly suggest that the root waving of the *wag1;wag2* double mutant affects the spatial pattern of LR and LRP. De Smet *et al.* (2007) previously showed waving roots have 51% of their LR positioned on the apex of a wave which constitutes only 16% of the roots total length. According to these data *wag1;wag2* seedlings demonstrate a similar alteration in spatial pattern of LR, and look very similar to Col-0 seedlings grown on inclined plates. It could be hypothesized quite reasonably that because of the drastic alteration to LR patterning root waving causes, waving could also be responsible for increased pre-initiation events.

Since the number of LRP in *wag1;wag2* are consistently greater than Col-0 from day 5 onwards, it is possible this difference could be explained by *wag1;wag2* seedlings germinating earlier. While this does not discount the effect root waving has on the LO spatial pattern, earlier germination would give the *wag1;wag2* seedlings an early start on LRP pre-initiation and initiation, effectively setting them ahead. To examine germination, seeds of both genotypes were sown on 1.5% hard agar plates with 0.5X MS and 1% sucrose and scored for seeds that had germinated every 8 hours (Fig. 10).

Neither genotype began to germinate until 24 hours after sowing, and both exhibited 100% germination by 56 hours after sowing. At 32 and 40 hours after sowing there was an increase in the number of *wag1;wag2* seedlings germinated compared to Col-0, however by 56 hours both genotypes reached 100%. The difference in germination at 32 hours, while large, was not significantly different from the wild-type ($p=0.25$), indicating at no time point was *wag1;wag2* germinating at a statistically higher rate than Col-0.

Lateral Root Response to Hormone and Inhibitor Treatments

Previous data reported that there was no statistically significant difference in the number of LR between Col-0 and the *wag1;wag2* double mutant (52). The previous experiments used 7 day-old seedlings of both genotypes, and then transferred them to either solvent control plates or plates that contained 1 μM NAA (naphthalene acetic acid), a synthetic auxin. The seedlings were allowed to grow for an additional three days and then LR were counted. The *wag1;wag2* seedlings on both control and NAA plates showed no significant difference from Col-0 in the inhibition of root elongation or induction of LR. This indicated no difference in the auxin responsiveness between the two genotypes (52). The previous experiment was repeated (Fig. 11). Control and NAA-treated seedlings for both genotypes did not show a significant difference in number of LR. However, both genotypes showed a similar response to the auxin treatment (Fig. 11). This result was directly contrary to previous data here that showed a difference in

LR numbers between the genotypes (Fig. 6). This discrepancy may be caused by the transfer of seedlings from one plate to a fresh plate. The transfer may halt or alter LR development for some period of time, masking the normally apparent LR difference between Col-0 and *wag1;wag2*. If this is the case a count of average LR may not reveal a difference, however LR density may be able to distinguish any differences that are still present.

To ask whether the method of scoring and the transfer itself accounted for the disappearance of genotypic differences, the experiment was repeated with one set of seedlings left un-transferred and the LR density measured (Fig. 12). In the non-transferred seedlings, *wag1;wag2* showed significantly increased LR density compared to Col-0. The mock-treated seedlings showed a similar difference with LR density, although the LR density for both genotypes was smaller. This indicates that transfer of the seedlings alters LR development in both genotypes, although it appears to have affected them similarly. The NAA-treated seedlings did not show a significant difference confirming the previous results (Fig. 12). Therefore, auxin response was similar for both genotypes and with the amount of auxin introduced here the LR difference was most likely masked.

Jasmonate is an important stress hormone in plants and is known to play an essential role in pathogen infection and herbivore attack (64). It also plays a role in regulating development since application of jasmonate inhibits root growth (64). It was recently found that jasmonate has an effect on LR development through the action of

JDL1/ASA1 (64). ASA1 (anthranilate synthase α1) is an important protein in the auxin synthesis pathway (64). Sun *et al.* (2009) found that when Col-0 seedlings are treated with 1 µM methyl jasmonate (MeJA) they produce a greater number of LR and LRP. When the *jdl1/asa1-1* (ASA1 knockout) mutant is treated with MeJA, LR formation is inhibited significantly. This was attributed to MeJA's regulation of auxin synthesis and transport within the root (64). MeJA increases auxin synthesis via ASA1 but independently suppresses the auxin transport through the suppression of PIN1 and PIN2 action. Therefore, in *jdl1/asa1-1* mutants, there was decreased auxin synthesis along with the suppression of PIN1 and PIN2 mediated auxin transport resulting in a net loss of LR and LRP through the loss of auxin maxima (64).

To determine if MeJA and thereby ASA1, PIN1 and PIN2 played a role in the *wag1;wag2* LR phenotype, Col-0 and *wag1;wag2* seedlings were sown on either control plates or plates containing 1 µM MeJA, allowed to grow for 7 days, and then collected and cleared. The number of LRP in stage 1 through 7 and emerged LR were measured. Figure 13 gives the percentage of LRP at each stage for both Col-0 (Fig. 13A) and *wag1;wag2* (Fig. 13B). Col-0 showed the expected response based on Sun *et al.* (2009) with increased percentages of stage 1 and 2 LRP when treated with MeJA, and increased emerged LR. When MeJA was applied, the stage 1 and 2 percentages were increased in Col-0 to the untreated *wag1;wag2* levels (compare Fig. 13A and B). Interestingly, treatment with MeJA had no significant effect on stage 1 and 2 percentages in *wag1;wag2*, but did cause a decrease in the emerged LR percentage (Fig. 13B). Stage 4

was differentially affected in both genotypes, with the percentage of stage 4 LRP in Col-0 decreasing to untreated *wag1;wag2* levels and an increase in *wag1;wag2* (Fig. 13A and B). This suggests that more LRP are spending longer or arresting in stage 4 in *wag1;wag2* in response to MeJA, causing a decrease in the emerged LR percentage. It is interesting to note that stage 4 is also the stage at which the *WAG2* promoter first becomes detectable (Fig. 5).

Figure 14 shows the LR and LRP density as well as the percent emergence in Col-0 and *wag1;wag2* in response to MeJA treatment or on control plates. The LR density increases in both genotypes but *wag1;wag2* LR density only increase 1.8-fold over the control while Col-0 increases 3.3-fold over its control. The LRP density of both genotypes increased equally, with the *wag1;wag2* having a higher LRP density on both control and MeJA containing plates (Figure 14B). The percent emergence (Figure 14C) is similar on control plates but when treated with MeJA the number of LRP emerged in Col-0 increases, while in *wag1;wag2* it decreases, suggesting emergence is where MeJA is exerting its differential effect on the genotypes. While a *jdl1/asa1-1* mutant shows a decrease in total number of LOs, *wag1;wag2* still increases in emerged LR density on MeJA (Fig. 14A). The percent emergence of *wag1;wag2* decreases with MeJA treatment while Col-0 emergence increases, accounting for the lower increase in LR density in *wag1;wag2.* If emergence is the effected process under MeJA treatment and stage 4 is the most affected stage in *wag1;wag2* this poses a potential role for WAG2 in LR development under certain conditions (such as stress conditions), as the *WAG2*

promoter is active at stage 4 (Fig. 5), and this is where the greatest increase of LRP occurs with MeJA treatment.

Both WAG1 and WAG2 contain putative calmodulin binding domains (Watson, personal communication), it was of interest to ascertain whether calcium played a role in LR development in *wag1;wag2*. Calmodulin-like proteins bind calcium directly and interact with other proteins after binding calcium (2). PINOID, the closest relative of both *WAG1* and *WAG2*, is known to interact with TCH3 and PBP1, both calmodulin-like proteins (2). To test if calcium plays an important role in the *wag1;wag2* phenotype, known calcium inhibitors were utilized. Lanthanum Chloride, verapamil and tetracaine are all calcium channel blockers and although they each act differently in blocking calcium movement they each effectively prevent or reduce calcium movement and response.

Figure 15 shows the LO density for each treatment. Both Col-0 and *wag1;wag2* seedlings were sown on mock plates or plates containing 75 µM lanthanum chloride, 75 µM verapamil or 75 µM tetracaine and grown to 7 days after sowing. The mock treatment showed *wag1;wag2* had a greater LO density than Col-0 as expected. However, while Col-0 was not significantly affected by the calcium inhibitor treatments, *wag1;wag2* showed reduced LO densities on each compound. Lanthanum chloride caused only a mild reduction, the result of using a relatively low concentration of the compound. In contrast, both verapamil and tetracaine reduced the *wag1;wag2* LO density to that of Col-0. This suggests that calcium has an important role in the

wag1;wag2 LR phenotype. Based on these data, calcium homeostasis is essential for the increased LO density in *wag1;wag2.*

Genetic Analysis of WAG1 and WAG2 in Lateral Root Development

Previously in our laboratory genetic analysis of the *wag1;wag2* root waving pathway was performed by crossing several characterized mutants into the *wag1;wag2* background, and analyzing root waving in the resulting triple mutant. Those mutations utilized in both root waving analysis and here for analysis of LR development include aux1, axr1 and tir7 (65). Two genes identified to act in root gravitropism *ARG1* and *PGM1* were also utilized (65). AUX1 (Auxin Resistance 1) is an auxin importer which has a known gravitropic defect and reduced LR (23). AXR1 (Auxin Resistant 1) is a part of the SCF^{TIR1} complex and plays a role in auxin response. Seedlings with AXR1 mutations demonstrate a reduced number of LR (30). TIR7 (Transport Inhibitor Response 7) is equivalent to ASA1 and plays an important role in auxin synthesis (60). Interestingly, TIR7 mutants display an increased number of LR in contrast to other auxin mutants that typically have a reduced LR phenotype. PGM1 (Phosphoglucomutase 1) incorporates glucose into starch molecules as well as cleaving glucose from starch. Seedlings with mutations in PGM1 are unable to make starch and have an agravatropic phenotype (52). Mutants in PGM1 had not been previously described as having a LR phenotype. ARG1 (Altered Response to Gravitropism 1) is also an agravitropic mutant with no known LR phenotype, and its exact molecular process is still not fully understood (52).

As was expected, *aux1-7* exhibited a reduced LR density and similarly the *aux1;wag1;wag2* mutant had a reduced LR density not significantly different from the *aux1-7* parent (Fig. 16A). Likewise, *axr1-3* displayed a strong reduction in LR density. The *axr1;wag1;wag2* mutant showed an equivalent LR density reduction to *axr1-3* (Fig. 16B). The similar LR densities of *aux1;wag1;wag2* and *axr1;wag1;wag2* with their single mutant parents reveals that these two genes are both epistatic to *wag1;wag2* with respect to LR development, and potentially are in the same genetic pathway. These data match the root waving analysis as it was found that *AUX1* and *AXR1* were both downstream of *WAG1* and *WAG2* in root waving (65). The last auxin mutant used here, *tir7-1*, had an increased LR density even though this mutation confers reduced auxin availability. In fact, *tir7-1* had a LR density similar to *wag1;wag2*, and the *tir7;wag1;wag2* triple mutant had an even greater increase in LR density (Fig. 16E). As the LR densities of the parental genotypes were similar, it was not possible to determine the nature of genetic interaction was occurring here.

The remaining mutants analyzed, *arg1-42* and *pgm1* are both gravitropic mutants used in the root waving analysis to investigate whether gravitropism played a role in root waving (65). Like *tir7-1*, *arg1-42* (Fig. 16C) had an increased LR density comparable to *wag1;wag2*, and the *arg1;wag1;wag2* triple mutant (65) had a further increase in LR density. Once again, it is not possible to determine the genetic interaction between *WAG1*, *WAG2*, and *ARG1* since the parental phenotypes are so similar to each other. The increased LR density of *ARG1* mutants has not previously been reported, and should be investigated further. *pgm1* had a greatly reduced LR

density, however the *pgm1;wag1;wag2* mutant (65) had a LR density similar to the *wag1;wag2* parent. This suggests that the *wag1;wag2* phenotype is epistatic to the *pgm1* phenotype (Fig. 16D), placing *WAG1*, *WAG2* and *PGM1* in the same LR development pathway. This is in contrast to the root waving phenotype, where *PGM1*, *WAG1* and *WAG2* were found to act in different pathways (65). The reduction in LR density in *pgm1* is interesting as, to our knowledge, it has not been reported previously. The genetic interaction of *WAG1*, *WAG2* and *PGM1* in LR development should be further investigated to understand the specific role of these genes in LR development.

DISCUSSION

Lateral Roots and Lateral Root Primordia in *wag1;wag2*

To investigate whether the *wag1;wag2* double mutant had a defect in LR development, seedlings were grown between 5 to 10 days after sowing, and the average number of LR scored for each day. Additionally, the number of LRP for *wag1;wag2* were scored from 5 to 7 days after sowing to determine if LRP development was affected. *wag1;wag2* had increased number of LR for each time point when compared to Col-0 on both vertical and inclined plates (Fig. 3). This confirms preliminary observations that *wag1;wag2* has a LR defect, but unlike most LR mutants, which typically have reduced or no LR, *wag1;wag2* displays an increased LR number (45). Those mutants that have increased LR with known molecular actions are involved in auxin homeostasis, with the exception of PICKLE, which is involved in chromatin remodeling and activation of the cell cycle (20,34).

The LRP number in *wag1;wag2* also increased (Fig. 4). At 5 days after sowing there were more LRP at every stage in *wag1;wag2* than Col-0. At 6 days after sowing, the number of stage 5 LRP in *wag1;wag2* rose sharply, increasing almost 2-fold compared to 5 day-old seedlings, while Col-0 remained unchanged. A similar increase in stage 5 LRP occurred in Col-0 at 7 days. This could mean that *wag1;wag2* seedlings are chronologically ahead of Col-0 by either starting pre-initiation events earlier, or possibly

germinating earlier. However, neither of these scenarios accounts for the continually higher LRP in wag1;wag2 at all stages. It seems likely that at later days Col-0 would have a similar number of stage 1 and 2 LRP as *wag1;wag2* at an earlier time point, but this is not the case. The number of stage 1 and 2 LRP for both genotypes stays fairly constant across all three days (Fig. 4). These data suggest that *wag1;wag2* seedlings have more LR pre-initiation events than Col-0, which are then developing at normal rates. However, LRP development is so highly regulated at every stage, why does the mutant plant simply not arrest a larger amount to arrive at the "normal" or "necessary" number of emerged LR?

As the number of early stage LRP increased in *wag1;wag2*, it seemed reasonable to suppose that either *WAG1* or *WAG2* play a role early in LRP development. To ascertain whether *WAG1* and *WAG2* exerted this effect during pre-initiation, initiation or during development, transgenic lines of *WAG1::GUS*, *WAG2::GUS* and *DR5::GUS* were used to observe promoter activity in the LRP. The observation of promoter activity would allow us to determine potential areas of activity of WAG1 or WAG2. The *WAG1* promoter showed no detectable activity at any LRP stage, and in fact did not become detectably active until a short time after emergence. The *WAG2* promoter however, was active but not until stage 4 and onward. Stage 4 is an important stage of LRP development, being the stage at which the LRP begins to produce its own auxin and a stage where many LRP arrest their development (7). It is possible that WAG2 plays a role in LRP development or that it is responding to the production of auxin in the LRP. *WAG1* and *WAG2* have been shown to be auxin-responsive (52), but this does not

explain why the *WAG1* promoter is not induced. Nor does it explain why it is only after the LRP begins producing its own auxin that the *WAG2* promoter becomes active. It is also possible that *WAG2* is simply becoming active in the cells that will eventually become the tissue types where *WAG2* would normally be active.

The increased number of LRP at stage 1 and 2 in *wag1;wag2* suggests either increased initiation or pre-initiation events. Since neither *WAG1* nor *WAG2* promoters were found to be detectably active immediately prior to or at stage 1 indicating that the defect may be caused by an increase in pre-initiation events. Measuring LR and LRP densities should clarify this by comparing the two densities. Therefore, 7 day-old seedlings were cleared and counted on a microscope with DIC optics. Col-0 had a LR density of just under 1, while *wag1;wag2* had a LR density of 1.4 (Fig. 6B). The LRP density of *wag1;wag2* was found to be 1.5-fold higher than Col-0, closely matching the increase in LR density (Fig. 6C). Since the increase in LRP density closely matches the higher number of LR, these data are consistent with the idea that *wag1;wag2* has an increased pre-initiation rate, priming 1.5-fold more founder cells than Col-0.

Dubrovsky *et al.* (2009) divided the Arabidopsis root into zone 1, containing emerged LR, and zone 2, containing only LRP, to examine density differences across these zones. For both zone 1 and zone 2, *wag1;wag2* had increased LRP density indicating that it indeed produces more LRP (Fig. 7). In contrast, LR density in zone 1 was similar in both genotypes (Fig. 7). The similarity of LR density in both genotypes using this method is caused by the difference in zone 1 length. While *wag1;wag2* has

only a slightly shorter total root length, it had a longer zone 1 than did Col-0. The LRP density of *wag1;wag2* in zone 1 was higher than in Col-0 (Fig. 7)

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Root Waving and Lateral Root Development

De Smet *et al.* (2007) demonstrated that root waving impacts LR development by changing the LR spatial pattern. When wild-type roots wave on inclined plates, 51% of LR develop on the apex of waves despite these regions accounting for only 16% of the total root length (10). While De Smet *et al.* did not make a direct association between root waving and an increased number of LR, they demonstrated that root waving dramatically impacts the positioning and patterning of LR. To investigate the patterning of LR and LRP in *wag1;wag2*, the LR and LRP distances from the root tip were measured in 7 day-old seedlings. Col-0 on vertical plates revealed the largest distance not only between each LO, but also from the root tip to the first LRP (Fig. 8). The inter-LO distances varied slightly across the length of the root in wild-type. Conversely, *wag1;wag2* on vertical plates had a very regular spacing between each LO, with a shorter distance between each LO. The distance from the root tip to the first LRP was also shorter. This raised the possibility that root waving itself affects LR formation such that vertical *wag1;wag2* seedlings may have a very similar spatial pattern to that of inclined Col-0. On inclined plates the root waving of *wag1;wag2* is increased (not shown) and correspondingly the spacing of LO in these seedlings was even shorter. Interestingly, the distance from the root tip to the first LRP only shortened slightly when *wag1;wag2* was grown on inclined plates (Fig. 8), suggesting this distance may be near

the minimum distance the first LRP can be from the root tip. This distance to the first LRP in Col-0 decreased considerably when grown on inclined plates. The mean inter-LO distance for Col-0 on inclined plates was half that of vertically grown seedlings. *wag1;wag2* on vertical plates had a similar inter-LO distance to that of Col-0 on inclined plates, consistent with the idea that root waving may cause the LR and LRP spatial pattern in *wag1;wag2*.

Root waving represents one possible cause of the increased pre-initiation in *wag1;wag2*, but earlier germination is another possibility for setting up higher numbers of LR and LRP. To investigate if *wag1;wag2* had increased germination, seedlings of both genotypes (Col-0 and *wag1;wag2*) were sown on 1.5% agar plates with 1% sucrose and 0.5X MS, and then scored for germination every eight hours (Fig. 10). Both genotypes began germination 24 hours after sowing, and finished germinating 56 hours after sowing. At 32 hours and 40 hours after sowing, the percent germination in *wag1;wag2* seeds was higher than Col-0, although these differences were not statistically significant. While *wag1;wag2* had have about 20% more germination at 32 hours, this difference quickly decreases, and at 40 hours the difference is only 4-5%. As the majority of *wag1;wag2* seeds germinated at the same rate as Col-0 it would be likely that the percentage of seedlings with earlier LR formation due to germination would not produce a significant difference in LR between the genotypes. Therefore it is unlikely that early germination is the cause of the *wag1;wag2* LR phenotype, although *wag1;wag2* does show slightly enhanced germination.

Lateral Root Response to Hormone and Inhibitor Treatment

The plant hormone auxin is an important regulator of many physiological processes, including LR development. Santner and Watson (2006) compared the induction of LR in response to auxin treatment of *wag1;wag2* and Col-0. The results indicated there was no significant difference in LR numbers between genotypes, as both the control and the treated seedlings had a similar number of LR (52). To investigate why no difference was observed, the experiment was repeated by growing seedlings for 7 days, and then transferring to either control plates or plates containing 1 µM NAA. The seedlings were then allowed to grow for an additional 3 days before the LR were counted. As with the data reported by Santner and Watson (2006), there was no statistically significant difference in LR numbers on either the control plates or the treated plates (Fig. 11). These data contradict the data presented here (Fig. 3), indicating there was a significant difference in LR formation between these genotypes. It is possible that transferring the seedlings to different plates negatively affects LR development, masking the normally apparent increased LR in *wag1;wag2*. Therefore, the experiment was repeated, except a set of non-transferred seedlings were included and the LR density measured for each set. The LR density of *wag1;wag2* that were not transferred had increased LR density. Those seedlings that were transferred to control plates also had a significant difference in LR density. When the average total LR for these plates were calculated there was no significant difference on control plates (data not shown), indicating that the disruption in LR development by transferring the seedlings masked the increased LR formation in *wag1;wag2*. However, using LR density

revealed the difference between Col-0 and *wag1;wag2* LR even when the seedlings were transferred to new plates. Both genotypes, when treated with NAA, showed a similar response with a highly increased LR density, but there was no significant difference between Col-0 and *wag1;wag2*.

Jasmonic acid is another important hormone in plants, and plays a prominent role in pathogen infection and herbivore attack (64). Jasmonic acid is also known to regulate other process in plants including the inhibition of root elongation (64). The mutant *jdl1/asa1-1* is a loss-of-function mutation in ASA1 (Anthranilate Synthase α1), a crucial protein in the auxin biosynthesis pathway (64). When Col-0 seedlings are treated with MeJA they develop significantly more LR; when *jdl1/asa1-1* seedlings are similarly treated they develop few or no LR. To investigate if MeJA would have an effect on *wag1;wag2* LR development, seedlings were grown for 7 days either on control plates or plates containing 1 μM MeJA. The seedlings were cleared, and the number of each stage of LRP and LR were counted. The percentage of total LO for each stage is shown in Figure 13. Treatment with MeJA caused a large increase in stage 1 and 2 LRP in Col-0 (Fig. 13A). This suggests increased pre-initiation in Col-0 when treated with MeJA and results in an increase in the number of emerged LR. Interestingly, MeJA treatment has no effect on stage 1 and 2 percentages in *wag1;wag2,* as they remain relatively unchanged from their untreated levels (Fig. 13B). There is a decrease in the percent of emerged LR of *wag1;wag2* which corresponds to only a modest increase in total number of emerged LR. Col-0 had a 7% increase in emerged LR that is a 2.5-fold increase in the number of emerged LR. The LR density of *wag1;wag2* was higher than

Col-0 on control plates, but when treated with MeJA Col-0 had a higher LR density (Fig. 14A). The LRP density of both genotypes increased by about 2.5-fold, indicating MeJA treatment induced the same number of LRP in both genotypes (Fig. 14B). Since the LRP density increase was equal, but the LR density of Col-0 increased more than *wag1;wag2*, this suggested that the number of LRP emerging is decreased in *wag1;wag2*. Figure 14C shows that MeJA treatment increases the number of emerged LR in Col-0, while suppressing emergence in *wag1;wag2*. This may correlate with the large increase of stage 4 LRP in *wag1;wag2* when treated with MeJA (Fig. 14B), as more LRP may be arresting or pausing in this stage than when untreated. Interestingly, the *WAG2* promoter activity is first detectable at stage 4 (Fig. 5), and may imply a role for *WAG2* under stress conditions, as MeJA is a stress hormone. Without *WAG2* present, LRP halt or arrest at stage 4 more often than they do in Col-0 when treated with MeJA, accounting for the decrease in emergence.

Both *WAG1* and *WAG2* have putative calmodulin binding domains (Watson, personal communication), suggesting that calcium may play a role in the *wag1;wag2* LR phenotype. To investigate this, seedlings were grown on three different calcium inhibitors: lanthanum chloride, verapamil and tetracaine. Each of these displayed some reduction in the LO density of *wag1;wag2*, but had little effect on Col-0 (Fig. 15). Lanthanum chloride did not severely affect *wag1;wag2*, but this may have been caused by the concentration used as 75 µM may be too low of a dose, and therefore the effect seen is only minimal. However, both verapamil and tetracaine reduced the LO density of *wag1;wag2* to that of Col-0 without affecting Col-0, and this suggests an important

role for calcium. PINOID, the closest relative of *WAG1* and *WAG2*, has been shown to interact with TCH3 and PBP1, known calmodulin-like proteins (2). It is not unreasonable to suspect that WAG1 or WAG2 may interact with a calmodulin-like protein, and this could play a role in the change in LO density on calcium inhibitors. However, it is possible that these chemicals have other effects on the plant, and calcium itself plays a part in many biological processes and therefore may be affecting LR development in another way.

Genetic Analysis of WAG1 and WAG2 in Lateral Root Development

Several mutants known to be involved in auxin transport and response, as well as mutants involved in gravitropism, were previously analyzed to determine the placement of *WAG1* and *WAG2* in a root waving pathway (65). To determine if any of these same genes were involved in the LR pathway with *WAG1* and *WAG2*, both parent lines (single mutants and *wag1;wag2*) along with the corresponding triple mutants (65), were grown and scored for LR density.

AUX1 is an auxin transporter and the mutant *aux1-7* displays agravitropic root growth and reduced LR density (23). The triple mutant *aux1;wag1;wag2* (65) had a similar LR density to *aux1-7*, indicating that *aux1* is epistatic to *wag1* and *wag2* (Fig. 16A). *AXR1* is a part of the SCF^{TIR1} complex, which binds to auxin and targets Aux/IAA proteins for degradation, derepressing or repressing many genes involved in auxin response (30). The *axr1-3* mutant has enhanced root elongation and decreased LR density (30). The *axr1;wag1;wag2* (65) mutant also had reduced LR density similar to

axr1-3, indicating that it too is epistatic to *wag1* and *wag2* (Fig. 16B). *ARG1* is involved in root gravitropism, and the *arg1-42* mutant has decreased response to gravitropic stimulation (52). The LR density of *arg1-42* was higher than that of Col-0, but similar to *wag1;wag2* and *arg1;wag1;wag2* (65) showed a further increase in LR density (Fig. 16C). The LR phenotype of *arg1-42* is interesting as it has not been previously reported, however the type of interaction between these genes cannot be determined, since the parental phenotypes are so similar. *PGM1* is phosphoglucomutase 1, and *pgm1* is incapable of creating starch stores (52). Therefore *pgm1* seedlings are unable to sense the direction of gravity, making it agravitropic (52). *pgm1* had a reduced LR density compared to Col-0, however the *pgm1;wag1;wag2* (65) mutant had a LR density similar to *wag1;wag2* (Fig. 16D). This means that *WAG1* and *WAG2* are epistatic to *PGM1* in LR development. This is different than the root waving pathway as *PGM1* was found to be in a separate pathway from *WAG1* and *WAG2* (65). It is unclear at this time how WAG1, WAG2, and PGM1 interact to form LR. However, it is clear from these results (Fig. 16D) that the pgm1 phenotype is masked by the wag1;wag2 phenotype, indicating that these genes act in the same pathway when LR are concerned.

The *tir7-1* mutation reduces the amount of available auxin to the plant (60). The *tir7-1* mutant showed an increased LR density comparable to *wag1;wag2*, and the *tir7;wag1;wag2* mutant had a further increased LR density (Fig. 16E). Like *ARG1* the interaction between *TIR7*, *WAG1* and *WAG2* cannot be determined. Interestingly, *tir7-1* also has a root waving phenotype (65), and has increased LR density like *wag1;wag2.* While it is likely not in the same pathway, it would be interesting to understand how

reduced auxin levels give rise to the root waving, and if the root waving is responsible for the increased LR density.

In conclusion, *wag1;wag2* has been shown to have an increased number of LR, and an increased number of LRP at all time points observed. The *WAG2* promoter has been shown to be active in LRP at stage 4 through emergence, however it is unclear if *WAG2* plays a direct role in LRP development. MeJA treatment suggests that *WAG2* may play a role, as more LRP were found at stage 4 with a corresponding decrease in emergence. This could be conditional and therefore needs further investigation. The *wag1;wag2* mutant has increased pre-initiation, as both LR density and LRP density are 1.5-fold higher than Col-0, with similar emergence between the genotypes. While root waving dictates the patterning and positioning of LR it has not been previously correlated with increased LR. However, here it has been shown that waving roots develop more LR. This suggests that the root waving of *wag1;wag2* is the cause of the increased LO. Without further investigation it is impossible to determine if root waving is the only causative agent, one of several, or not playing a role in the increased LR and LRP numbers.

FUTURE DIRECTIONS

It is clear that *wag1;wag2* has a LR phenotype different than that of Col-0, and that this phenotype may be caused by increased pre-initiation events. The LRP in *wag1;wag2* show the same increase as the LR, compared to Col-0, which is strong evidence that there are more founder cells that undergo normal development at a later time. However it is unclear how the pre-initiation events are increased. The root waving phenotype of *wag1;wag2* modulates LR positioning and thus yields spatial patterns similar to that found in waving Col-0 roots. In order to investigate if root waving is responsible for the LR phenotype it would be necessary to grow the *wag1;wag2* seedlings in some manner that removes root waving as a factor, without modulating other processes within the plant. Phytagel is another common gelling agent used for plant growth, and it does provide a slicker surface which reduces waving in *wag1;wag2*. However, we have found that when seedlings are grown on phytagel there is an inverse relation between free auxin in the root and the percentage of phytagel used (Cheek, Unpublished). Interestingly a lower percentage of phytagel results in higher auxin levels, making this a poor substrate for separating root waving from LR development. Growing *wag1;wag2* in agar also eliminates waving. This method is appealing as it removes waving and seems to have little other effect on the plants, but

seedlings grown in agar develop fewer LR, and this may mask any difference, giving a false result of waving being the determinate factor. The separation of root waving and LR development in *wag1;wag2* is crucial experiment to determine its role.

As was shown here the *WAG2* promoter is active in LRP suggesting that WAG2 is active in LRP, but this needs to be confirmed. The use of *WAG2::WAG2:GUS* or *WAG2::WAG2:GFP* transgenic lines would allow the visualization of WAG2 in the LRP if it is active. MeJA treatment does suggest that *WAG2* may have a role as it reduces emergence with a corresponding increase in the LRP at stage 4 in *wag1;wag2*, which is the first stage the *WAG2* promoter has detectable activity. This may not be a direct link to *WAG2* activity, but this could be further determined by using other hormones that modulate LR and LRP development, such as cytokinin. Cytokinin affects LR development by reducing the number of LR, either through preventing initiation of founder cells, or arresting already developing LRP. Modulation of LRP stage numbers could indicate an action for *WAG2*. ABA, like cytokinin, is known to negatively regulate LR development through modulation of emergence (43), making it a good candidate for observing if *WAG2* plays a role in LRP development. Additionally, the hormones ethylene and gibberellin, both of which affect LR development, are potential treatments (43). *WAG1* promoter activity was not detectable in LRP, however it may be active at a low enough level to not be visible with GUS staining, and therefore GFP-tagged *WAG1* would be useful in determining if there is any activity for both promoter and protein in LRP.

There was no significant difference in the germination rate of *wag1;wag2* and Col-0 shown here, however germination experiments need to be repeated. The current germination assay is done on 1.5% agar, which causes delayed germination and may be exaggerating a small difference. Germination done on ≤ 1% agar should be performed to check if the same difference is present. In the total counts of LR it does appear as if *wag1;wag2* is simply a day ahead, suggesting that perhaps the bulk of seedlings germinated 24 hours or more ahead of Col-0. Although LR density did not bear this out, it is not something that can be ignored, and therefore it is necessary to determine if *wag1;wag2* seedlings are 24 hours ahead overall. Current data does not suggest that *wag1;wag2* seedlings are germinating this far ahead.

The calcium blockers used here reduced the LO density of *wag1;wag2* without greatly modulating Col-0. Tetracaine in particular reduced the LO density of *wag1;wag2* to that of Col-0, indicating that calcium plays an important part in the action of *WAG1* and *WAG2*. As PINOID is the closest relative to *WAG1* and *WAG2*, and it is known to interact with calmodulin-like proteins, it is not unreasonable to suspect that either *WAG1* or *WAG2* may also interact with a calmodulin-like protein. This is compounded when considering the putative calmodulin-binding domains of *WAG1* and *WAG2* (Watson, personal communication). The use of calmodulin antagonists such as W7 would help elucidate whether a calmodulin-like protein is indeed responsible, or if the reduction in LR density is a more general effect of calcium.

Several different genes were identified as participating in the same pathway as *WAG1* and *WAG2* for LR development. *AUX1* and *AXR1* are not surprising, as they both

play a role in auxin transport and response, and auxin is a major director of LR development. The interesting mutant here is *pgm1*, which to my knowledge does not have a previously reported LR phenotype. Here it was shown that *pgm1* has a reduced LR density, however when crossed into the *wag1;wag2* background, the LR phenotype of *pgm1* was completely masked. This indicates that *WAG1* and *WAG2* are acting in the same pathway as *PGM1*. It would be of interest to find other genes in the same pathway as *WAG1* and *WAG2* to obtain a more complete map of exactly where they fit into LR development. The obvious place to start would be other genes involved in auxin transport, response or homeostasis, as these genes play an important role in LR development and would likely be in the same pathway. It would be of interest to cross in knockouts that confer root waving defects, both enhanced or reduced, to compare LR phenotypes to determine the role of root waving in the development of LR.

FIGURES

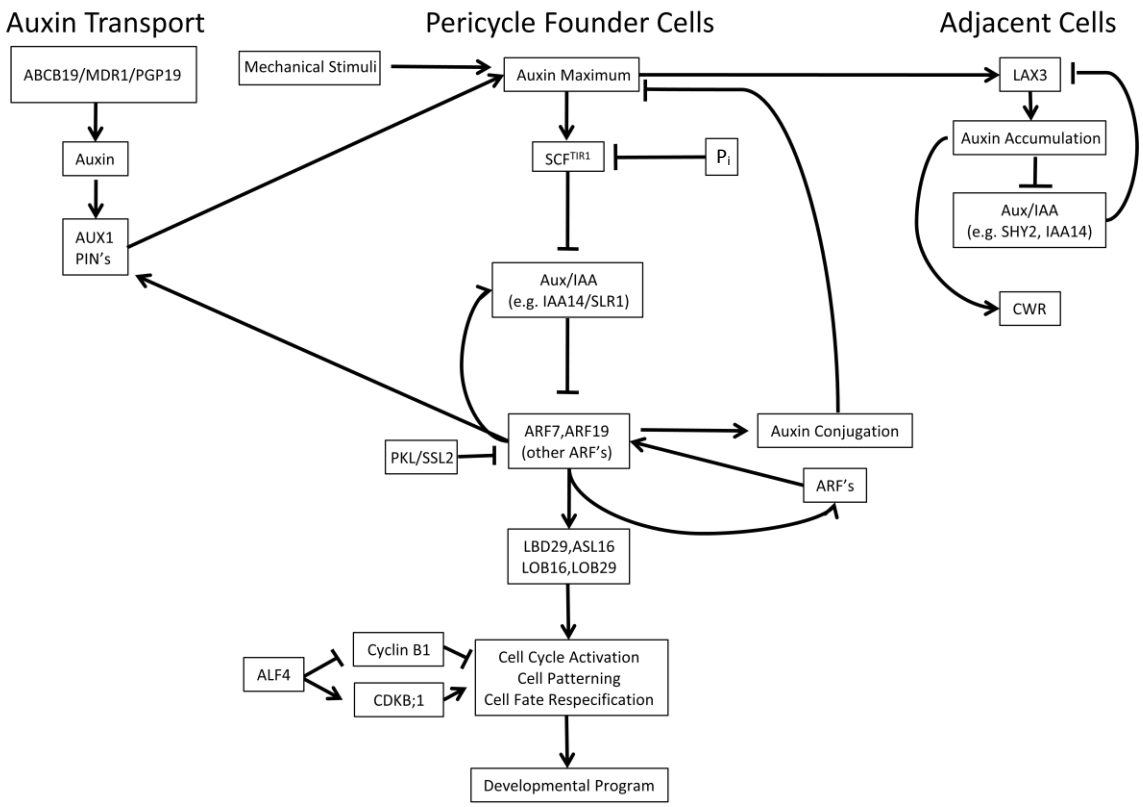

Figure 1. Lateral Root Initiation Pathway. The pathway for LR initiation is shown beginning with shoot-derived auxin transport (left), pericycle founder cell auxin response (middle), and LAX3 auxin transport and activation of CWR proteins (right.) Compiled from recent literature (4,8,9,18,19,20,28,42,51, 53,56.)

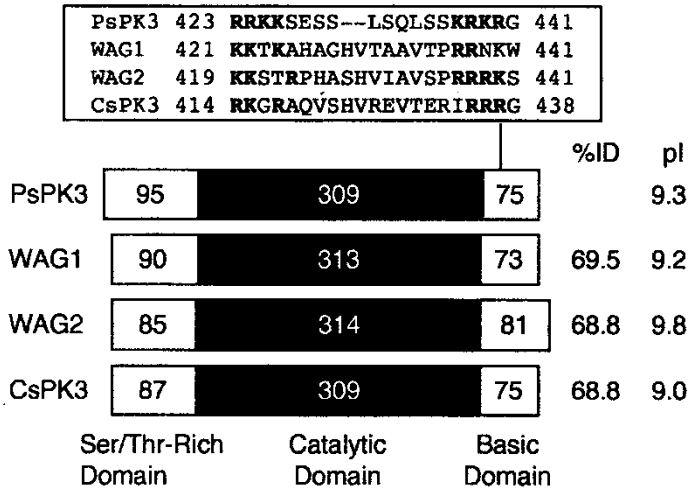

Figure 2. Schematic of the PsPK3-like Proteins. The PsPK3-like genes contain three domains: A serine/threonine rich region at the N-terminus, the catalytic domain and a basic domain at the C-terminus. A putative bipartite nuclear localization sequence is found in the basic domain. The numbers found in each box represent the number of amino acids in each associated region, and the %ID indicates the sequence identity to PsPK3. (Reproduced from 52.)

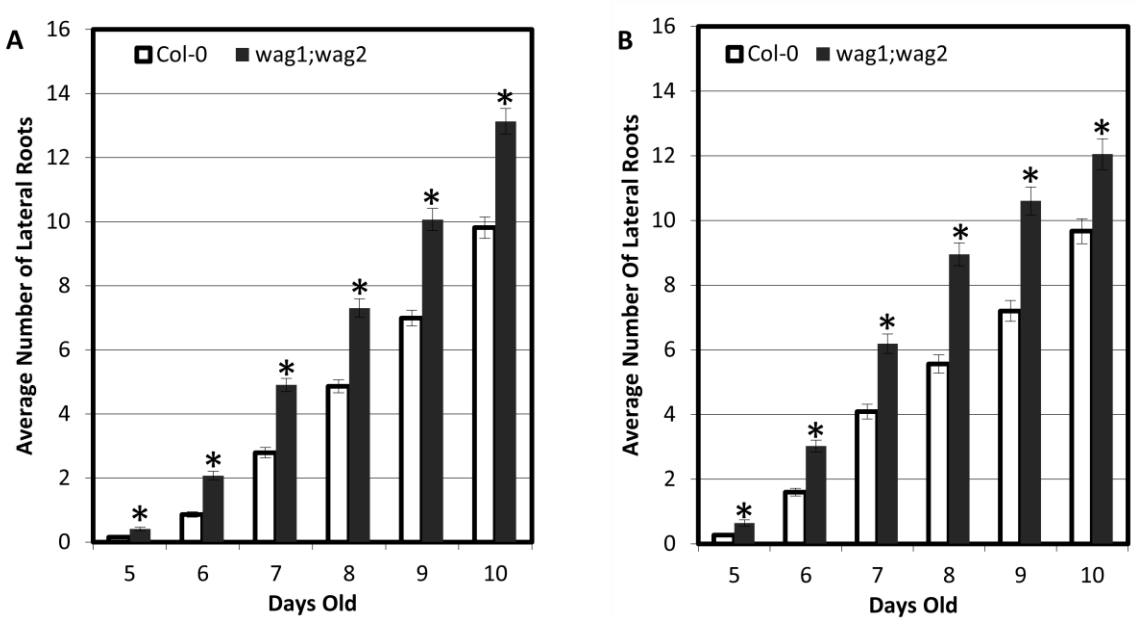

Figure 3. *wag1;wag2* has Increased Lateral Roots. (A) *wag1;wag2* has increased LR formation compared to Col-0 on vertical plates (n≥87, p≤0.001). (B) *wag1;wag2* has increased LR formation compared to Col-0 on inclined plates (n≥82, p<0.001). Seedlings were grown on 0.5X MS, 1% sucrose and 1.5% agar on either vertical or inclined plates, and to the day indicated. Means ± SE are shown. Asterisk indicate significant difference.

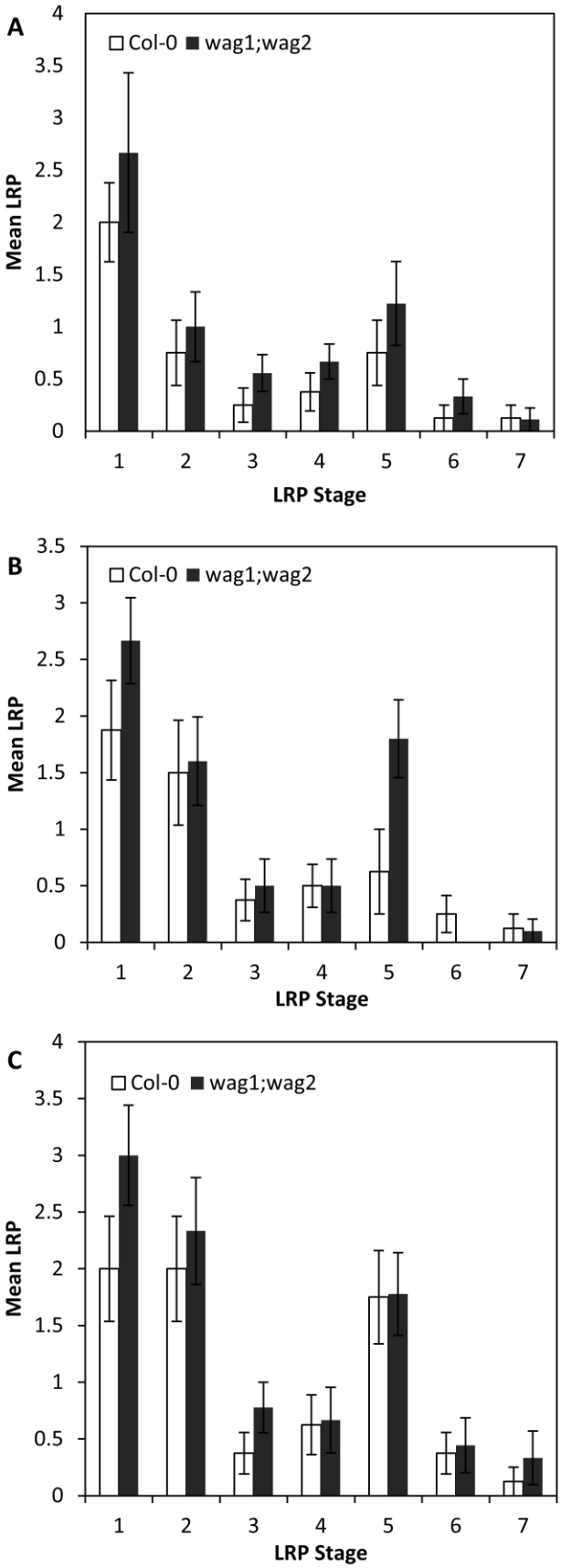

Figure 4. *wag1;wag2* has Increased LRP Formation. LRP stages (A) 5 days after sowing (n=7). (B) 6 days after sowing (n=7). (C) 7 days after sowing (n=7). No significant difference was observed for any individual stage. Seedlings were grown on 0.5X MS, 1% sucrose, 1.5% agar to the days indicated. The seedlings were cleared, and LRP were counted on a microscope with DIC optics. Mean ± SE are shown.

Figure 5. *WAG1* and *WAG2* Promoter Activity. Seedlings were grown for 7 days on 0.5X MS, 1% sucrose and 1.5% agar, , then stained in GUS stain solution and cleared according to Malamy and Benfey (1997). Images were acquired on a Nikon Eclipse E800 with DIC optics, using a Nikon DXM1200 Digital Camera and the Nikon ACT-1 software. *WAG1::GUS* showed no detectable activity at any stage of LRP. *WAG2::GUS* showed GUS staining at stage 4 through emergence. *DR5::GUS* showed staining at all stages.

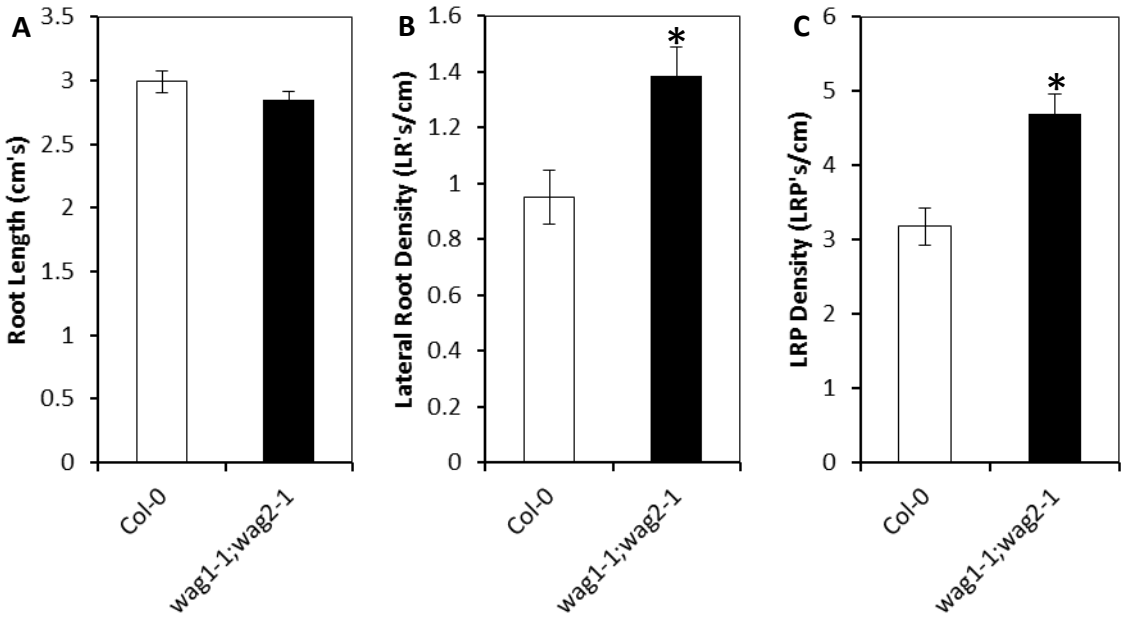

Figure 6. *wag1;wag2* has Increased Lateral Root Formation. (A) *wag1;wag2* shows no significant difference in root length from wild-type (n=78.) (B) *wag1;wag2* shows an increased lateral root density (approximately 1.5-fold) compared to the wild-type (n=78, p=0.002.) (C) *wag1;wag2* shows an increased LRP density (approximately 1.5-fold) compared to the wild-type (n=10, p=0.0006.) Seedlings were grown for 7 days on vertical 0.5X MS, 1% sucrose, 1.5% agar plates. Means ± SE are shown. Asterisks indicate significant difference.

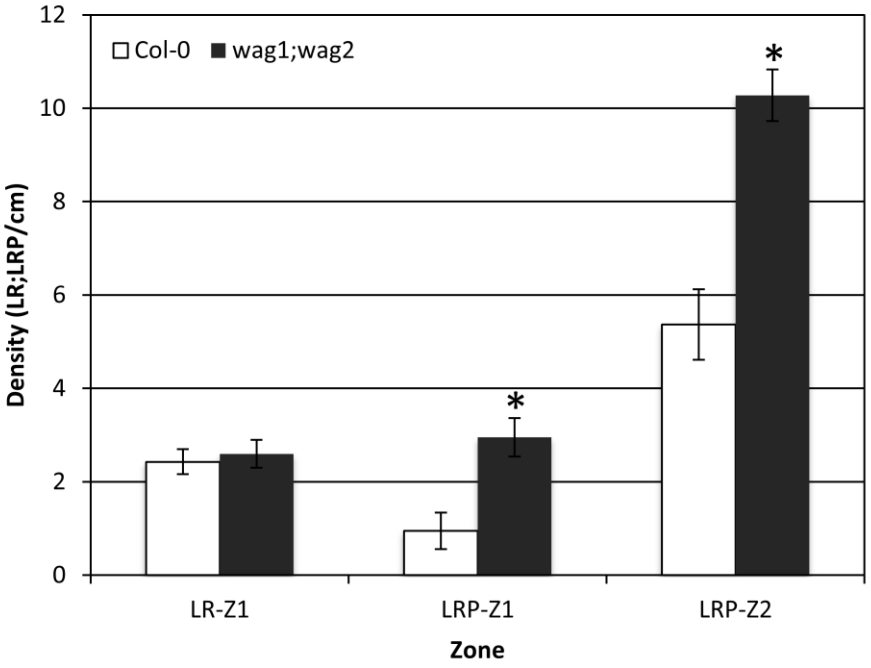

Figure 7. *wag1;wag2* LRP Density is Increased. *wag1;wag2* LRP density in both zone 1 and zone 2 is increased compared to Col-0 (n=7, LRP-Z1: p=0.007, LRP-Z2: p<0.001). Zone 1 is defined as the portion of the root where LR have emerged and zone 2 consists of the root from zone 1 to the root tip (Dubrovsky *et al.*, 2007.) (Zone 1 length of Col-0 = 1 cm; *wag1;wag2* = 1.23 cm.) Seedlings were grown on vertical plates for 7 days, LR were counted on a stereoscope and LRP counted on a DIC microscope. Means ± SE are shown. Asterisks indicate significant difference.

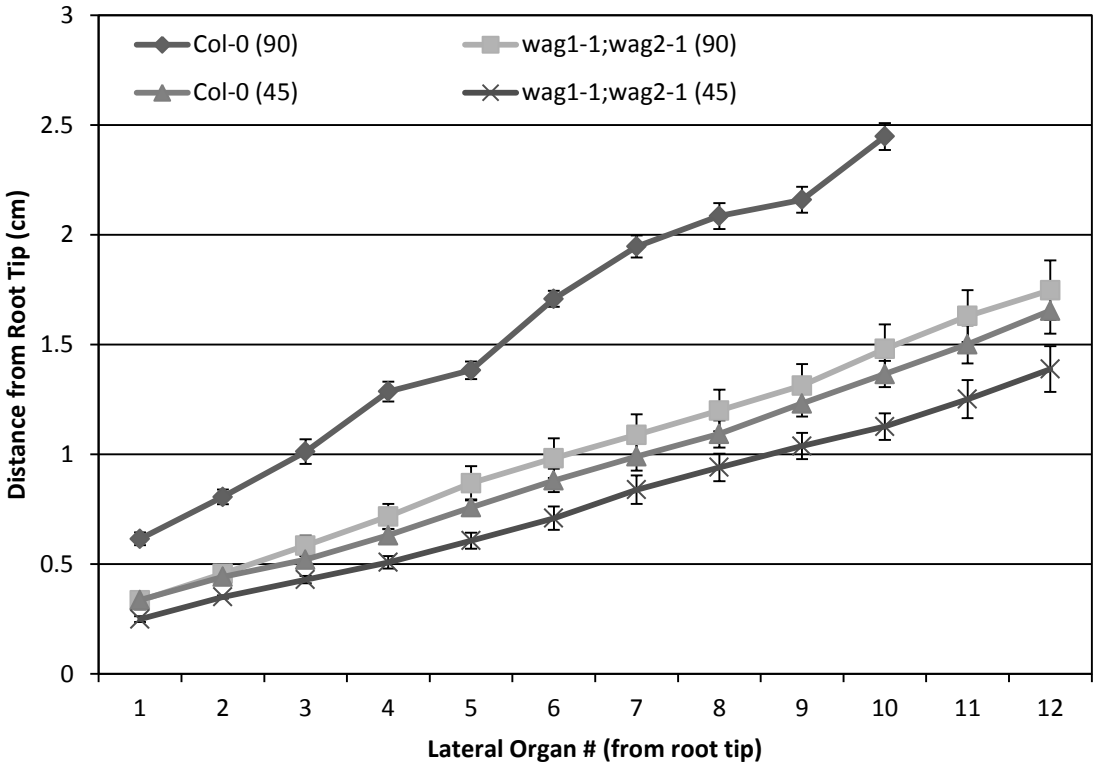

Figure 8. LO Position and Patterning. LO distances from the root tip of 7 day old seedlings were recorded for both vertical (90°) and inclined (45°) plates. Seedlings were cleared (Malamy and Benfey, 1997) and mounted on slides. LOs were marked on the coverslip with a Sharpie on an inverted microscope. Slides were then scanned and distances measured. Col-0 on vertical plates displays a large distance and slightly irregular patterning between LOs, while the inclined Col-0 has a tighter, more regular pattern similar to *wag1;wag2* on vertical plates (n≥11). Mean ± SE are shown.

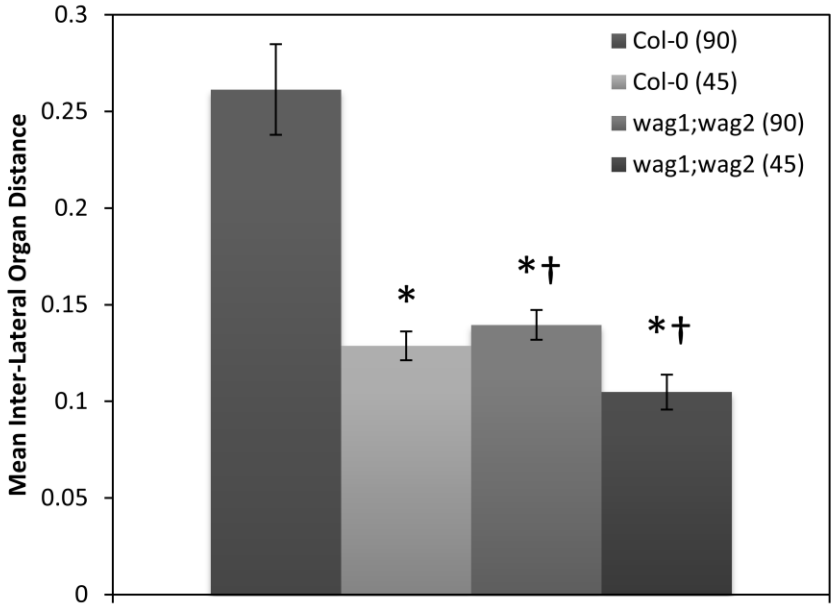

Figure 9. Inter-Lateral Organ Distance. The distance between LOs of 7 day-old seedlings were recorded for both vertical (90°) and inclined (45°) plates. Distances were obtained as in Figure 8. Col-0 vertical displays a large distance between LOs, while the inclined Col-0 has a shorter distance between LOs similar to *wag1;wag2* on vertical plates (n≥11). Mean ± SE are shown. Symbols indicate significant difference.

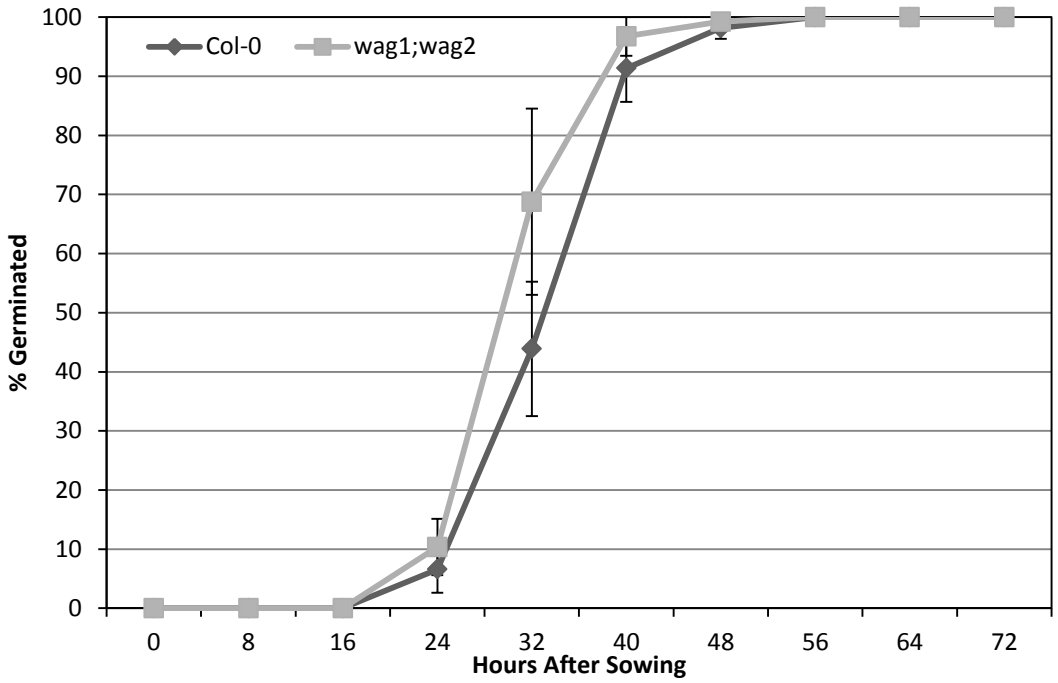

Figure 10. Germination Rate. *wag1;wag2* showed an increase in germination at 32 hours that was not significantly different from wild-type (n=100, p=0.25). At 40 hours there was only a slight increase in *wag1;wag2* germination (p=0.37). Seedlings were sown on 0.5X MS, 1% sucrose and 1.5% agar plates and grown vertically in constant light.

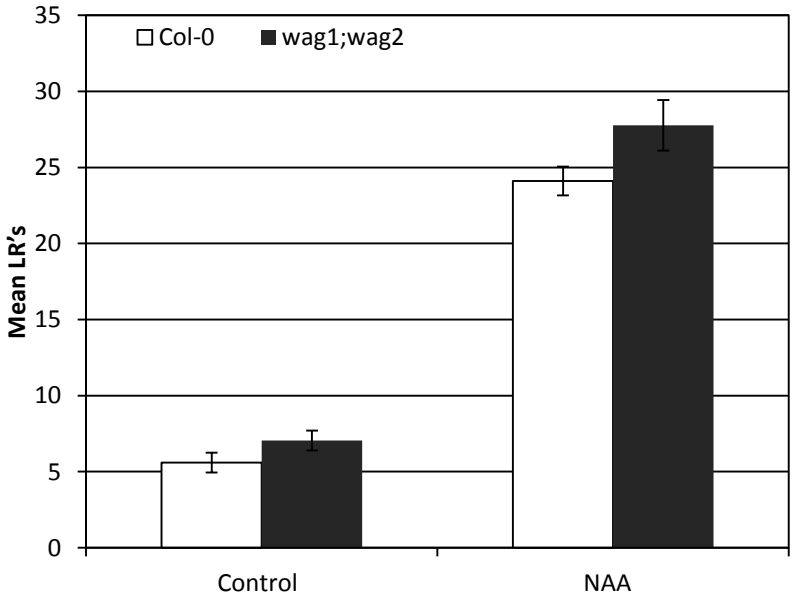

Figure 11. Auxin Response. Auxin induction of LR is similar between Col-0 and *wag1;wag2* (n=17). Seedlings were grown on vertical plates for 7 days after sowing, and then transferred to fresh media containing either the solvent control or 1 µM NAA. They were then allowed to grow an additional 3 days and their LR counted using a stereomicroscope. Means ± SE are shown.

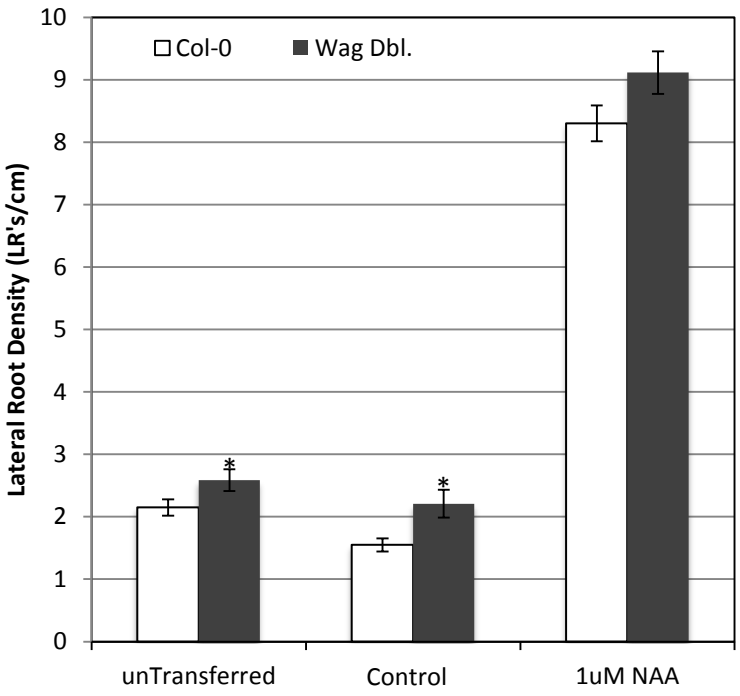

Figure 12. Auxin Induction of LR Formation. Non-transferred *wag1;wag2* seedlings had increased LR density compared to Col-0 (n=12, p=0.05). *wag1;wag2* had higher LR density on mock treatment (n=12, p=0.01). Col-0 and *wag1;wag2* had similar LR densities when treated with NAA (n=12). Seedlings were grown and treated as in Figure 11, except non-transferred seedlings were not moved from their original plates. Means ± SE are shown. Asterisks indicate significant difference.

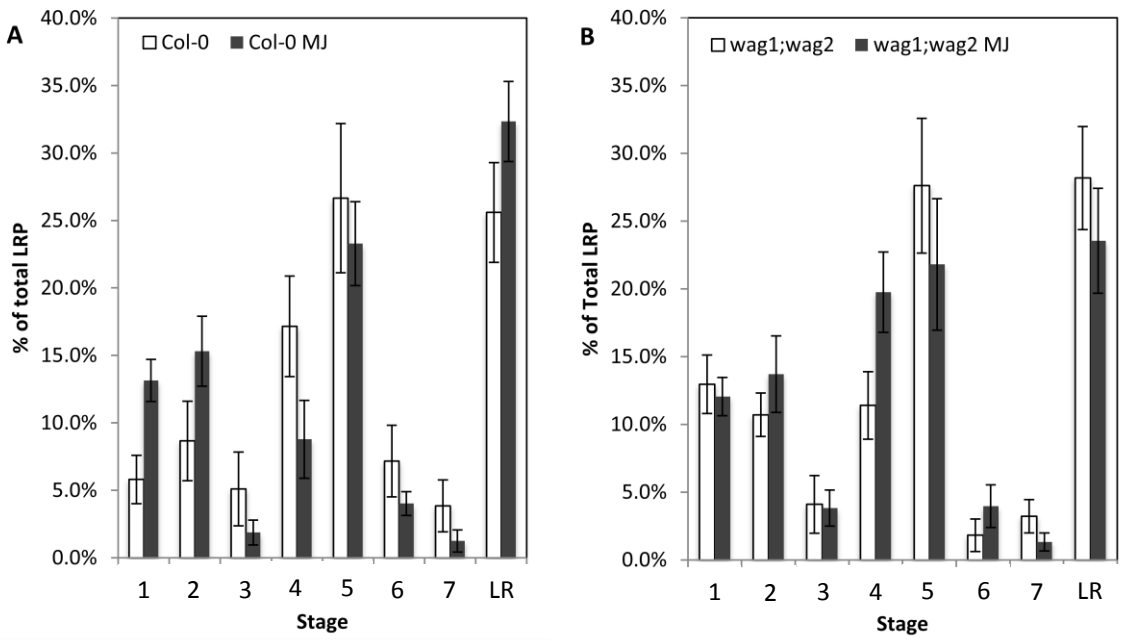

Figure 13. LR and LRP Development with MeJA Treatment. (A) Col-0 on plates containing either solvent control or 1 µM MeJA (n=9). (B) *wag1;wag2* on plates containing either solvent control or 1 µM MeJA (n=9). Seedlings were grown on vertical plates for 7 days after sowing either on solvent control or 1 µM MeJA. They were then cleared, and the LRP and LR counted on a microscope with DIC optics. LRP and LR percentages were calculated for each stage from the total LOs. No statistically significant difference was observed at any individual stage. Means ± SE are shown.

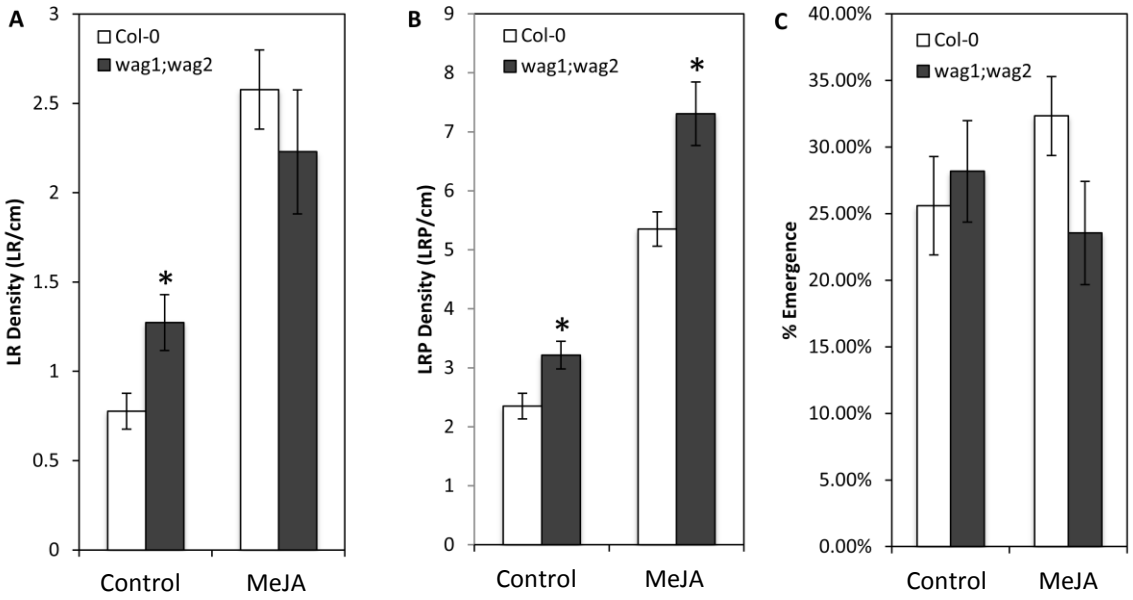

Figure 14. *wag1;wag2* has Decreased Emergence on MeJA. (A) *wag1;wag2* has a lower LR density on MeJA when compared to Col-0 (n=9, Control: p=0.02). (B) Relative LRP density remains unchanged on MeJA (n=9, Control: p=0.01, MeJA: p=0.007). (C) *wag1;wag2* percent emergence is reduced on MeJA treatment (n=9). Seedling were grown and treated as in Figure 13. Means ± SE are shown. Asterisks indicate significant difference.

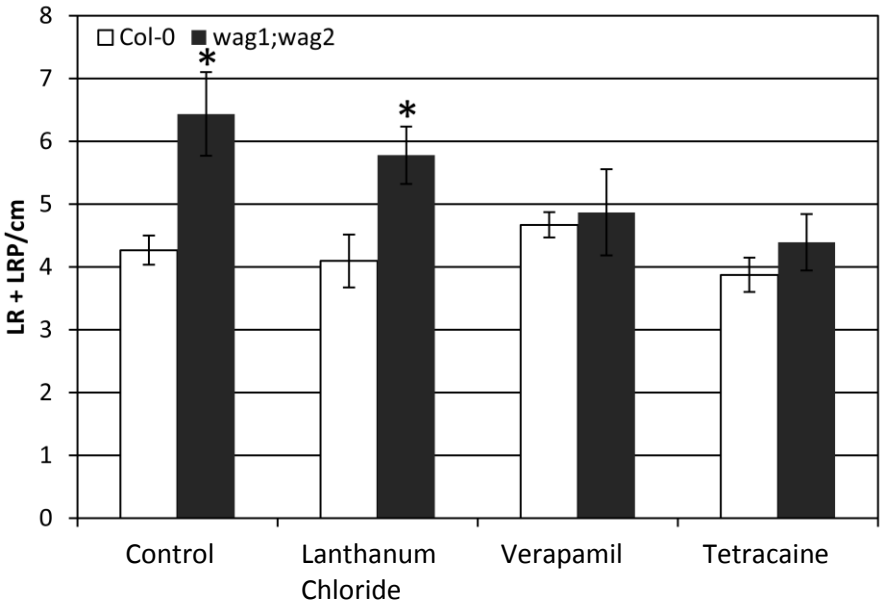

Figure 15. Calcium Inhibitors Reduce *wag1;wag2* LR Formation. *wag1;wag2* has reduced LO density on lanthanum chloride compared to the control. Verapamil and tetracaine reduce the *wag1;wag2* LO density to that of the wild-type. Col-0 showed no significant change on any compound (n=9, Control: p=0.007, lanthanum chloride: p=0.01, verapamil: p=0.78, tetracaine: P=0.33). Means ± SE are shown. Asterisks indicate significant difference.

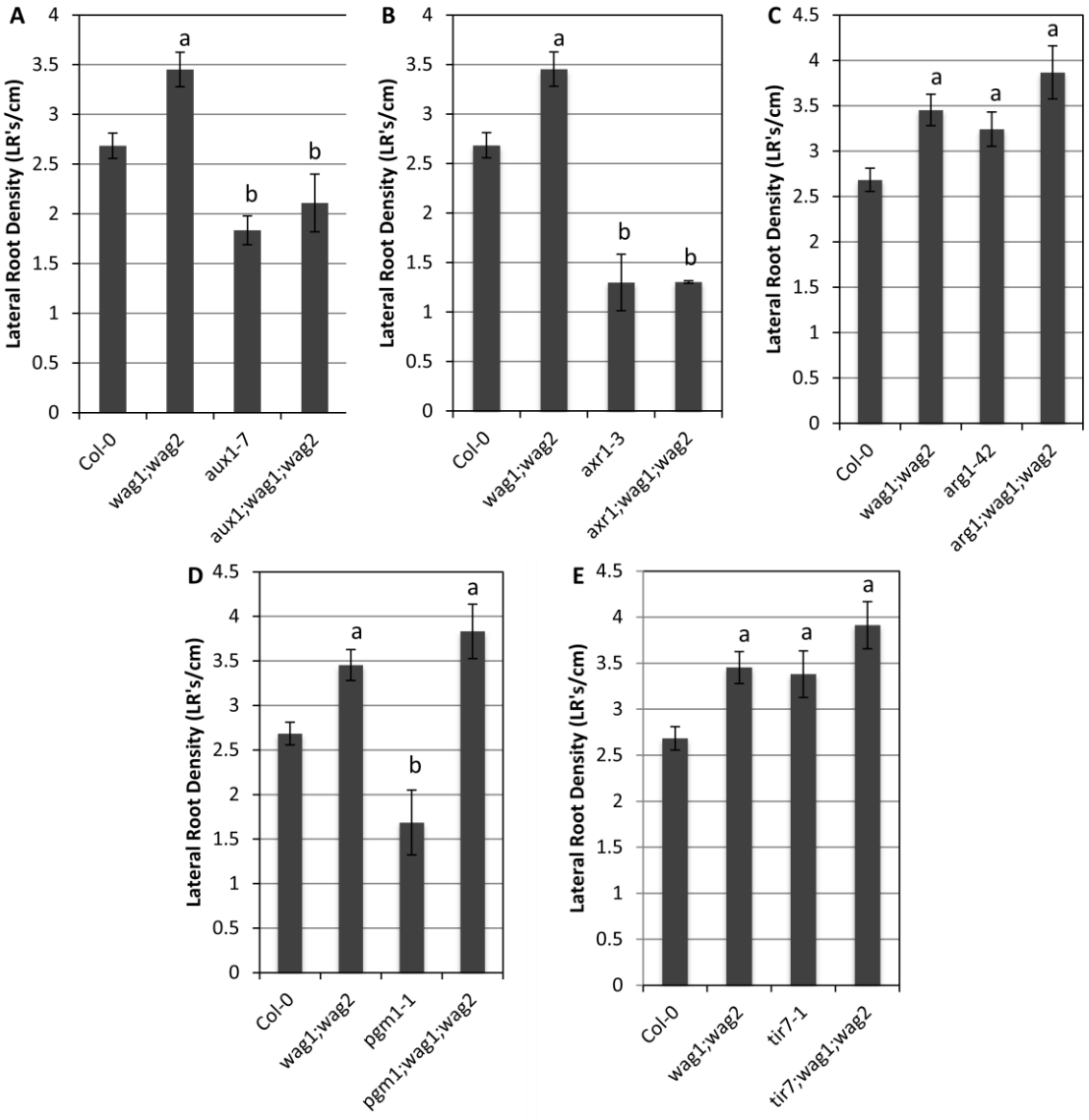

Figure 16. Genetic Analysis of *wag1;wag2* LR Pathway.
(A) *aux1-7* and *aux1-7;wag1;wag2* ($p<0.05$). (B) *axr1-3* and *axr1;wag1;wag2* ($p\leq0.002$). (C) *arg1-42* and *arg1;wag1;wag2* ($p\leq0.02$). (D) *pgm1-1* and *pgm1;wag1;wag2* ($p\leq0.02$). (E) *tir7-1* and *tir7;wag1;wag2* ($p\leq0.025$, $n\geq12$ for all genotypes). Genetic analysis of the *wag1;wag2* LR pathway was performed using single and triple mutants generated for root waving analysis. Seedlings were grown to 7 days old and their LR density calculated. Means ± SE are shown. Different letters signify significant difference.

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APPENDIX

APPENDIX

Introduction

The primary root of *Arabidopsis thaliana* can be subdivided into three distinct zones, each marked by cells of different type and shape (12). The zone encompassed by the root tip is the meristem (12). The meristem can be further sub-divided into two distinct regions, with the most distal portion being the apical meristem, and the more proximal region being the transition zone (12). The transition zone is the region where compact, undifferentiated cells from the apical meristem first undergo elongation, giving rise to cube-shaped cells as they mature into the next zone (12). As the transition zone matures into the elongation zone, the cells undergo elongation, as the name suggests, becoming longer than they are wide; However, these cells remain undifferentiated (12). After elongating, the cells differentiate into specific cell types in the differentiation zone (12).

The size of the apical meristem and transition zone is highly regulated, primarily controlled through the action of auxin and cytokinin (60,61). Auxin induces auxin transporters, such as the PIN family of transporters, thereby increasing the number of cells to which auxin is distributed via shootward transport

from the root tip (61). Increased levels of auxin promote cell division and inhibit cell elongation, so the more cells that receive higher levels of auxin remain compact and do not begin to elongate, which is the mark of the transition zone (60,61). Conversely, cytokinin acts to suppress auxin signaling through the up regulation of SHY2 (SHY2/IAA3), which negatively regulates the expression of auxin transporters, such as the PINs (61). The decrease in auxin reduces the number of cells with sufficient auxin levels to prevent elongation and these begin to elongate, creating the cube-shaped cells of the transition zone (12,60,61). Through the antagonistic action of auxin and cytokinin, the size of the apical meristem and transition zone is maintained (61).

The size of the apical meristem has been defined as the number of cortical cells from the quiescent center to the transition zone (60,61). The number of cortical cells contained in this zone is dependent upon the sufficiently high auxin levels to prevent their elongation as described above. Counting cortical cells is effective in relating not only a relative size of the apical meristem but also, indirectly, the amount of auxin transport and auxin signaling in the meristem. Ioio *et al.* (2008) found that *shy2-31*, a loss of function mutation, had an increased meristem size, with nearly twenty more cortical cells in the apical meristem than the wild-type (61). Conversely, *shy2-2*, a gain-of-function mutation, had a smaller meristem with approximately 10 fewer cortical cells in the meristem (61). From the data obtained by Ioio *et al*. (2008), it was determined that the regulation of the meristem size in Arabidopsis was controlled through a regulatory circuit involving auxin and cytokinin, which converged on *SHY2* (61).

Materials and Methods

Plant Material and Growth Conditions

Col-0 and *wag1;wag2* were used previously (52). Seeds were sterilized by incubation for 2 minutes in 70% ethanol, followed by incubation for 10 minutes in 25% (v/v) bleach, then washed 5 times with sterile water. The seeds were then imbibed at 4°C in the dark for 72 hours in sterile water. After imbibing, seeds were sown onto 1.5% (w/v) Bacto agar (214010; Becton Dickson) plates containing half-strength MS nutrients with vitamins (M5519; Sigma-Aldrich) and 1% (w/v) sucrose, with the pH adjusted to 5.6 with sodium hydroxide. The plates were placed vertically in racks under constant cool white fluorescent light of 80 µmol m^{-2} sec^{-1} at 22°C for the times indicated below.

Quantification of Cortical Cells in the Meristem

Seedlings of Col-0 and *wag1;wag2* were grown from 3 to 9 days as described above. Seedlings were collected and cleared according to Malamy and Benfey (1997) and then mounted on slides in 25% glycerol. Cortical cells were counted and images obtained using a Nikon Eclipse E800 with DIC optics and a Nikon DXM1200 Digital Camera with the Nikon ACT-1 software. The mean number of cortical cells for each genotype at each time point was then calculated.

Results

The *wag1;wag2* mutant has been shown to have increased shootward auxin transport in the meristem (Santner *et al.*, in preparation). Based on the data obtained by Ioio *et al.* (2008), increased auxin transport should give rise to higher auxin levels further from the root tip. With increased auxin transport in the root, I hypothesized that the meristem of *wag1;wag2* would be larger (contain more cortical cells) than Col-0.

To investigate if *wag1;wag2* had an increased meristem size, seedlings were grown vertically on 1.5% agar with 1% sucrose and 0.5X MS. Sets of seedlings were grown to between 3 to 9 days after sowing to observe the change in meristem size. Col-0 does not reach its maximum meristem size until 7 days after sowing, at which point it maintains a constant size (61). Figure A.1A is a representative Col-0 meristem at 7 days after sowing, the point at which it has reached its maximum size. The arrows indicate the first and last cortical cell of the apical meristem. Above the upper arrow is the transition zone consisting of 5 to 6 cortical cells. Figure A.1B shows a representative *wag1;wag2* meristem at 7 days after sowing. As can be seen the number of cortical cells in *wag1;wag2* is greater than Col-0. This increased number of cortical cells is small at 7 days, although statistically significant. Figure A.2 shows the time course of both Col-0 and *wag1;wag2* from 3 to 9 days after sowing. From day 3 to day 6 the meristem of both genotypes was similar, indicating that if there was indeed increased auxin transport in *wag1;wag2* at this time point it was insufficient to create a larger meristem.

Ioio *et al.* (2008), found that *shy2-31* (the loss-of-function mutation) had an increased meristem size 2 days after germination, indicating that a strong induction of auxin signaling through repression of SHY2 would yield a larger meristem early on. This did not happen here, indicating that the increased transport conferred by the *wag1;wag2* mutation was not sufficient to alter the meristem size. At 7 days after sowing, Col-0 reached its maximum meristem size of 32 cortical cells, and maintained this number for both 8 and 9 days old. However, the *wag1;wag2* meristem continued to increase in size beyond 7 days after sowing with 4 more cortical cells on average compared to Col-0 (Fig. A.2).

This increase was maintained in *wag1;wag2* for both 8 and 9 day old seedlings, which indicates that *wag1;wag2* achieves its maximum meristem size at 7 days after sowing as well. The increase in the number of cortical cells is small, especially when compared to *shy2-31*, but was statistically significant for days 7 through 9. This suggests that indeed, the increased level of shootward auxin transport is sufficient for *wag1;wag2* to attain a larger meristem than Col-0 beyond 7 days old. Interestingly, the pattern over the last three days for both genotypes was the same, increasing or decreasing from day to day similarly, suggesting that all the normal meristem maintenance is still active.

Discussion

The interaction of auxin and cytokinin regulates the size of the meristem through activation or suppression of *SHY2* (61). This has been demonstrated with *shy2-31*, a loss-of-function mutation, which has a greatly increased meristem size. *shy2-2*, a gain-of-function mutation, confers a greatly reduced meristem size (61). Here, *wag1;wag2* displayed a normal meristem size for 3 to 6 days after sowing, indicating that during this time the meristem of *wag1;wag2* is developing normally. At 7 days after sowing Col-0 reaches its maximum meristem size, and maintains this number for the remaining days observed. Likewise *wag1;wag2* reached its maximum size at 7 days old, however the number of cortical cells was higher than that seen in Col-0. It is interesting that *wag1;wag2* does not display an increased number of cortical cells in the meristem until 7 days, as increased shootward auxin transport (Santner *et al.*, in preparation) at all time points would suggest a larger meristem at all time points.

The increased number of cortical cells in *wag1;wag2* is small at only 4 more cortical cells over Col-0. The fact that the increase at 7 days is small may give some indication as to why no difference is seen before this. The difference between genotypes is subtle, and during the development of the meristem (as it continues to grow each day) there is no discernable difference because both genotypes are increasing their meristem size over this time through the increase of both auxin transport and auxin response. The *shy2-31* mutant shows an almost immediate increase over wild-type because it has no ability to up-regulate SHY2, and therefore modulate

the action of auxin. SHY2 is presumable fully functional in the *wag1;wag2* background and therefore as the auxin transport and response increases in *wag1;wag2* it is no different than Col-0 at this time.

The difference between the genotypes begins when Col-0 reaches its maximum size and *wag1;wag2* continues to grow in size, most likely caused by the increased shootward auxin transport giving rise to more cortical cells. Because both genotypes increase their meristem over the first several days and the difference observed later is subtle, it can potentially explain why the difference does not became apparent until after Col-0 reaches its maximum size.

Future Directions

It was shown here that *wag1;wag2* has an increased meristem size that is significantly different from that of Col-0. In order to investigate further how the regulation of meristem size is affected in *wag1;wag2*, it would be of interest to treat with both auxin and cytokinin to observe the differential effects these hormones have, if any, on both genotypes. Cytokinin would be the most telling, as it should decrease the meristem size proportional to the concentration applied. If *wag1;wag2* is achieving a larger meristem through increased auxin transport then without a sufficiently large dose of cytokinin, its meristem should remain larger than Col-0. Finding the concentration of cytokinin necessary to reduce meristem size of *wag1;wag2* to that of Col-0 could give some insight into the changes to auxin in the *wag1;wag2* background.

Treatment with auxin should increase the meristem size of both genotypes until a sufficiently high concentration was reached to bring the Col-0 meristem size up to the size of *wag1;wag2*. This approach, similar to treatment with cytokinin to achieve the same end, could give insight into the difference in auxin in *wag1;wag2*. The relatively simply control of meristem size affords the opportunity to explore auxin transport, response and availability in *wag1;wag2.*

Additionally it may be of interest to obtain the *shy2-31* and *shy2-2* mutants and cross them into the *wag1;wag2* background to observe the changes in meristem size. It would be especially interesting to see how the *shy2-2;wag1;wag2* triple mutant meristem size would behave. Since *shy2-2* is a gain-of-function mutation, it would be

expected that the meristem size would decrease, however if the meristem size of *wag1;wag2* decreased to *shy2-2* size would be of particular interest. While SHY2, in the triple mutant, would not be regulated by auxin the increased auxin transport may be enough to counter some of the effects of *shy2-2.* Crossing *shy2-31* into the *wag1;wag2* background would be expected to drastically increase the size of the meristem to larger than either parent, though the possibility of epistasis exists, as these crosses would also reveal if WAG1, WAG2 and SHY2 were in the same pathway.

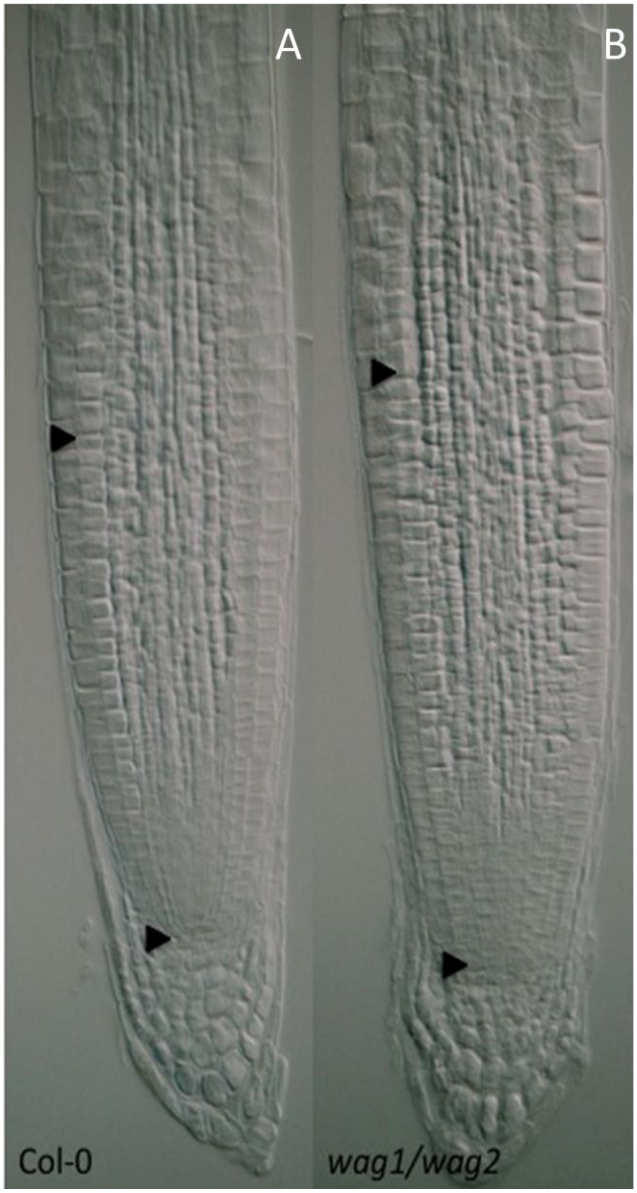

Figure A.1. Increased Meristem Size in *wag1;wag2*.
(A) A representative Col-0 root tip at 7 days with cortical cells of the meristem marked by the black arrows. (B) A representative *wag1;wag2* root tip at 7 days with cortical cells of the meristem marked by the black arrows.

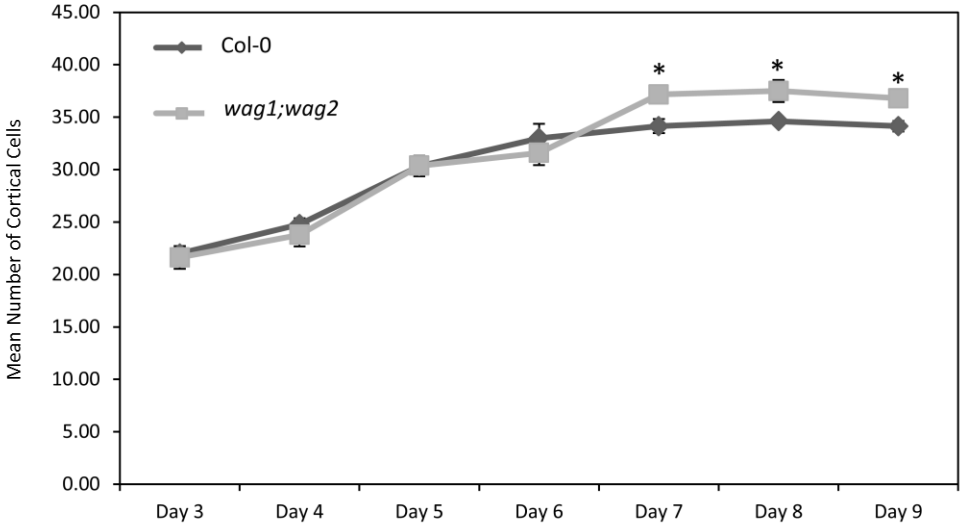

Figure A.2. *wag1;wag2* has an Increased Meristem Size.
At 7 days after sowing the *wag1;wag2* double mutant has a stable but increased meristem size over the wild-type (n=10, p≤0.02). Seedlings were grown to for the number of days indicated, cleared and then mounted on slides. Meristem size was scored as the number of cortical cells between the quiescent center and the transition zone on a microscope with DIC optics. Means ± SE are shown. Asterisks indicate significant difference.