UNDERSTANDING THE FUNCTION OF TANGLED1 IN ARABIDOPSIS THALIANA

By

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Abstract

Owing to the constraints posed by the presence of a cell wall, maintaining proper microtubule dynamics through the behavior of microtubule associated proteins is critical for symmetrical cell division in plant cells. TANGLED1 (TAN1) and AUXIN INDUCED IN ROOT CULTURES9 (AIR9) in Arabidopsis thaliana are microtubule binding proteins present at the division zone during mitosis. Neither single mutant displays a strong phenotype, however the tan1 air9 double mutant produces a strong synthetic phenotype with misplaced cell walls and twisted roots. We studied the response of the single and double mutants to the microtubule altering drugs propyzamide and Taxol. We show that tan1 air9 double mutants are hypersensitive to propyzamide when compared to tan1 and air9 single mutants and did not respond differently to Taxol when compared to air9 single mutants. The roots treated with Taxol and propyzamide also showed an increased twisting phenotype. Hypersensitivity to propyzamide suggested that TAN1 might be required for interphase microtubule organization. To understand where TAN1 acts in dividing and non-dividing cells, we also imaged the native promoter expression of TAN1 in Arabidopsis thaliana roots segregating native promoter driven TAN1-YFP and CFP Tubulin. We demonstrate through confocal microscopy that TAN1 driven by its native promoter is expressed in the meristematic zone of the Arabidopsis root, where cells are rapidly undergoing mitosis, but not the elongation or differentiation zones. Through these studies we demonstrate the importance of TAN1 in potentially stabilizing microtubules during mitosis and expose a potential interphase function.

Acknowledgements

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Glossary of Key Terms

Cell Plate

The precursor to the final cell wall which develops as a free-floating structure in the center of the cell in telophase. It ultimately grows towards the cortex of the cell where it fuses with the cell wall of the mother cell to form two daughter cells.

Phragmoplast

A microtubule structure that directs vesicles to the cell plate during cytokinesis to construct the new cell walls. It spreads from the center of the cell outwards towards the cortex.

Preprophase band

A band of microtubules and microfilaments in the cortex of a plant cell that marks the position of attachment for the new cell wall. It disassembles after the breakdown of the nuclear membrane and formation of the metaphase spindle.

Introduction

Unlike animal cells, plant cells are not able to migrate after their placement in the plant body. As a result, the organization of the plant body is defined through oriented cell divisions in anticlinal or periclinal directions, adding to their current cell file or to a new cell file respectively. A failure to divide asymmetrically or symmetrically in the correct plane, expand in the correct direction, or differentiate into the correct cell type results in improper formation of the plant body (Pickett-Heaps et al. 1999). As a result, division plane orientation, or the control of the placement of the cell wall during cytokinesis, plays a crucial role in plant development.

The cytoskeleton is one of the primary cell components that defines the location of the new cell wall in plant cell division. During late G2 phase, the microtubule and microfilament cytoskeletons form a tight ring around the cortex of the cell called the preprophase band (PPB) (Pickett-Heaps and Northcote 1966). The PPB has been previously shown to correctly predict the future division site, or the new placement of the cell wall at the cortex of the cell (Martinez et al. 2017). However, the PPB disappears during metaphase after the mitotic spindle has formed completely. It is thought that a molecular marker remains at the site of the PPB throughout mitosis to mark the future division plane even when the PPB disassembles (Rasmussen et al. 2013). During the progression to telophase, the microtubules and microfilaments reorganize at the cell plate in a structure called the phragmoplast (Jürgens 2005). These cytoskeletal filaments serve to allow Golgi vesicles enriched in materials for de novo cell wall construction to be delivered to the cell plate (Boruc and Van Damme 2015). The phragmoplast directs cell wall construction from the center of the cell outwards to the cortex of the cell to the position originally marked by the PPB and then disassembles (Rasmussen et al. 2013, Lee and Liu, 2013).

One such molecular marker of the division site is the microtubule-binding protein TANGLED1 (TAN1) protein. *tangled1* mutants were first identified in maize (*Zea* mays) and had misplaced cell walls, a characteristic of division plane defects (Smith et al. 1996). TAN1 was first identified as a division site marker in the *Arabidopsis thaliana* homolog (Walker et al. 2007). When TAN1 was fused to YFP, TAN1 co-localized with the PPB and remained at the site until after the phragmoplast disassembled, even though the PPB disassembled (Walker et al. 2007, Martinez et al. 2017). Furthermore, *tangled1* mutants in maize fail to direct the new cell wall to the region indicated by the PPB, which suggests TAN1 is important in the guidance of the developing cell wall to the cell cortex (Martinez et al. 2017).

Unlike the *tan1* mutant in maize, the Arabidopsis *tan1* mutant shows a weak and inconsistent phenotype in division plane orientation, hindering analysis of the function of the protein in Arabidopsis. However, double mutants lacking both the TAN1 and the AIR9 proteins (*tan1 air9* double mutants) plants show severe division plane defects (Mir et al. 2018). AIR9 is also a microtubule binding protein that localizes to the division site with a weak phenotype in the single mutant (Buschmann et al. 2015). The generation of the *tan1 air9* double mutant allowed for experiments to understand the role of these proteins in maintaining a proper division plane for plant body development.

Materials and Methods

Treatment with Propyzamide and Taxol

Arabidopsis thaliana seeds were sterilized in chlorine gas for 2 hours at room temperature (~21ºC). Seeds were plated on ½ MS (Murashige and Skoog 1962), 0.5 g L^{-1} MES, 0.8% agar, pH 5.7 plates on strips of filter paper and placed at 4ºC for 2 days. Each plate had one of four genotypes in the Landsberg ecotype: wild type, *tangled1* mutant, *air9* mutant, or *tan1 air9* double mutants. After 2 days, plates were moved to a Conviron set at 22ºC with a 16/8-hour light cycle for 4 days to germinate. After 4 days, the sheets of filter paper with germinated seedlings were transferred to new media plates (½ MS, 0.5 g L^{-1} MES, 0.8% agar, 0.05% DMSO, pH 5.7) and one of 5 concentrations of either propyzamide (0, 0.5, 1, 1.5, 3µM) or Taxol (0, 0.2, 0.5, 1, 3µM) and returned to the Conviron for a further 4 days.

Root length was marked immediately after transfer to plates with propyzamide and Taxol to mark the 0 point. Length was marked every 24 hours for a further 4 days. Plates were scanned at 600 dpi with a ruler and TIF files were uploaded to FIJI to measure rate of growth from the length at 4 days after germination (FIJI Is Just ImageJ; http://fiji.sc/). The mean and standard error were calculated for each condition and normalized to 0µM for each day and plotted as percentages. n = 23–45 for each condition. Roots were then imaged by confocal microscopy, outlined below.

Native Promoter TAN1 Screening

A. thaliana roots segregating *air9* mutants, TAN1 fused to YFP expressed from the native promoter (NP TAN1-YFP) and alpha-tubulin fused to CFP (CFP-Tubulin) were screened for TAN1 expression in the meristematic, elongation and differentiation zones of roots. The meristematic zone was imaged just above the root tip, the elongation zone was imaged further up

the root before the emergence of root hairs. The differentiation zone was identified by the presence of root hairs and internal vasculature. All three sections of the roots were imaged in roots expressing NP TAN1-YFP and their negative siblings. If the roots also expressed CFP-Tubulin, the roots were also imaged to see the microtubule structures. Since no differences were noted in expression in 12 plants, the roots were not genotyped for *air9* mutants.

Confocal Imaging

Roots treated with propyzamide and Taxol from root growth experiments were imaged by confocal microscopy at the differentiation zone, which was identified by the presence of root hairs. Images were taken on an inverted Nikon Ti with motorized stage (ASI Piezo) and spinning-disk confocal microscope (Yokogawa W1) run with Micromanager software (micromanager.org) and built by Solamere Technology. Solid-state lasers (Obis) and emission filters (Chroma Technology) were used. To image propidium iodide, a 561 laser with emission filter 620/60 was used. For NP TAN1–YFP, a 514 laser with emission filter 540/30 was used. For CFP–TUBULIN, a 445 laser with emission filter 480/40 was used.

Statistical Analysis

Propyzamide and Taxol

Kolmogorov–Smirnov tests were used to compare *tan1*, *air9* and *tan1 air9* root lengths at each concentration on day 8. Comparisons were compared at $\alpha = 0.05$ to determine statistical significance of differences.

Native Promoter TANGLED1

ANOVA analysis was performed at $\alpha = 0.05$ to compare the difference between the roots expressing TAN1-YFP and the negative siblings at each root zone.

Results

Arabidopsis thaliana roots were treated with the microtubule altering drugs propyzamide and Taxol to assess the role of TAN1 and AIR9 in maintaining proper microtubule dynamics. Propyzamide is a microtubule depolymerizing drug, which favors the creation of tubulin units over microtubules. Taxol is a microtubule stabilizing drug which prevents the depolymerization of microtubules into tubulin units by preventing the hydrolysis of the GTP molecule bound to the tubulin monomers. *tan1* and *air9* mutants responded similarly to the effects of propyzamide at increasing concentrations, while the *tan1air9* double mutant showed hypersensitivity to the drug (Figure 1A). The double mutant showed decreased root growth at 1.5µM, while the two single mutants did not show decreased growth until treatment with 3µM. In contrast, *tan1* showed a different response to Taxol, showing decreased root growth at 0.5 and 1µM when compared to *air9* and *tan1air9* (Figure 1B). Similar trends are seen on days 5-7 post-germination under drug treatment (Figure 2).

These roots were then imaged by confocal microscopy to assess the cell file rotation when treated with microtubule altering drugs. The plants treated with 0, 1 and 3µM Taxol and propyzamide were imaged and cell file rotation was imaged (Figure 1C-H). Treatment with propyzamide or Taxol increased the cell file rotation. However, neither the single nor double mutants showed a differential response when compared to each other.

TANGLED1 has a very clear mitotic function, however it is unclear if TAN1 also possesses an interphase function. To test for the possibility of an interphase function we used confocal microscopy to measure the expression of TAN1 in different regions of the *Arabidopsis* root using a construct of TAN1 fused to YFP controlled by its native promoter. This was conducted in roots segregating the *air9* single mutant phenotype to examine if TAN1 was

differentially expressed in the absence of AIR9. As no difference were noted between any of the samples, the samples were not genotyped for the presence of the *air9* mutation. We measured the arbitrary fluorescence of the expression in the meristematic zone (Figure 3A-B), elongation zone and differentiation zone of 7-day old roots. There was a statistically significant difference between the meristematic zone of roots expressing TAN1-YFP and the meristematic zones of negative siblings (Figure 3C). The fluorescence of TAN1-YFP was below detectable levels in the elongation and differentiation zones and was statistically the same as the negative siblings. Lack of detectable TAN1-YFP fluorescence suggests that it does not play a significant role in elongation or differentiation zones in the root, although it is possible that small but undetectable amounts are present.

Discussion

In unicellular organisms, cell division plays an important role in reproduction, but does not contribute to the organism's development. In contrast, multicellular organisms such as animals and plants depend on cell division to produce the millions of cells that compose each organism. Furthermore, as the different cells are divided into cell types that will organize into tissues, organs and eventually organ systems, regulated and organized cell division is essential. Animals couple the roles of organized cell division with apoptosis and cell migration as the organism develops (Alberts et al. 2015). In contrast, plant development is less reliant on apoptosis, reserving controlled cell death to the formation of very specific organs including the xylem and some complex leaf patterning (Alberts et al. 2015). Furthermore, the plant cell wall prevents cell migration once placed (Alberts et al. 2015). As a result, patterning of the plant body is highly dependent on oriented placement of the new cell wall, suggesting the presence of highly sophisticated organizational systems are present to regulate it.

Plant cells divide in one of two ways: symmetrically, where the resulting daughter cells have identical fates unless acted upon by an external signal, or asymmetrically, where the resulting daughter cells have different fates (Alberts et al. 2015). Asymmetric cell divisions, such as the divisions in the leaves to produce highly specialized stomates for gas exchange or those in the stem cell niche of meristems, require a differential recruitment of transcripts, proteins and organelles while maintaining equal segregation of the genetic material (Pillitteri et al 2016). Symmetric cell division requires an equal allotment of each of those components between the two cells, which produces equal size as well as equivalent components (Besson and Dumais 2011). In this work, we focused on symmetric cell division owing to its prevalence in the epidermal cell layer.

The orientation of the microtubule cytoskeleton, particularly the mitotic spindle and the cortical microtubules arrays, is critical for proper placement of the new cell wall. In both plant and animal cells, microtubule cytoskeletal arrays provide scaffolding to direct the movement of cellular components including the organelles and nuclear genetic information and the mitotic spindle allows for the separation of sister chromatids to evenly divide the genetic material (Alberts et al. 2015). However, while animal cells divide by the formation of a cleavage furrow which is dependent on the reorganization of the actin cytoskeleton for pinching in cytokinesis, plant cells build their new cell walls from the inside out (Alberts et al. 2015). In order to facilitate this construction, a plant cell-specific microtubule structure, the phragmoplast, forms (Jürgens 2005). The phragmoplast is oriented perpendicular to the plane of the cell plate, with the positive ends of the microtubules facing the developing cell wall to facilitate the movement of vesicles containing material to construct the cell wall mediated by positive end-directed motor proteins (Boruc and Van Damme 2015). The phragmoplast stretches outward alongside the developing cell wall, eventually reaching the cell cortex, at which point cytokinesis is complete.

Plants also have mechanisms that seem to mark the position where the new cell wall will fuse with the parental cell wall during cytokinesis, also referred to as the division plane. During late interphase, the cortical microtubules in plant cells form a ring around the cell known as the preprophase band (Pickett-Heaps and Northcote 1966). The preprophase band forms during preprophase in late G2 and remains at the division site until the beginning of metaphase when is dissociates (Rasmussen et al. 2013, Lee and Liu, 2013). In 99.7% of wild type divisions in maize, this marks the location of the future division plane (Martinez et al 2017). Thus, in almost all wild type divisions the developing cell wall will be directed to the site previously marked by the preprophase band during cytokinesis, despite the dissociation of the preprophase band during

metaphase. The placement of the new cell wall in accordance with the preprophase band despite its dissociation early in mitosis suggests the presence of another marker for the division plane.

In order to form structures like the PPB and phragmoplast, microtubules must be highly dynamic; microtubules must be readily built up, rapidly destroyed and rebuilt with the changing needs of the cell as mitosis progresses. When the dynamics of microtubules are altered through the use of microtubule altering drugs, changes to the orientation of the cortical microtubule arrays are stimulated (Baskin et al. 1994). The dynamics of microtubules are controlled endogenously through the activity of microtubule associated proteins (MAPs) (Sedbrook et al. 2008). Extensive literature has been published on MAPs, which interact with microtubules in various ways, including binding at the tips or along the length of the microtubule to prevent growth or promote growth at an end or to stabilize or produce catastrophe along the length (Alberts et al 2015). For example, XMAP215 plays an important role in mitosis in stabilizing microtubules for efficient spindle construction (Alberts et al. 2015). As mentioned above, the division site is marked by a microtubule-based structure, so MAPs serve as a likely candidate for a division site marker. Many division site marking proteins have also been previously described, including MYOSIN VIII and POK1/POK2, which both associate with microtubules (Wu and Bezanilla 2014, Lipka et al. 2014).

In this study, we focus on the division site marker TANGLED1. The original study of the *tangled1* mutants in maize observed the recessive mutation caused by a transposable element that resulted in altered division plane orientation, leading to the characteristic patterning of disorganized cell shape, without altering the plants ability to form most organs, most notably the leaves (Smith et al. 1996). However, the patterning of all epidermal cells, both specialized, as in stomata and trichomes, and unspecialized, are disrupted. In addition, the leaves show a “tangled”

venation network as opposed to parallel networks characteristic of wild type maize plants (Smith et al. 1996). However, the plants still resemble the basic form of a wild type plant, though smaller in stature (Smith et al. 1996). Furthermore, the cells do not display cytokinesis defects, as characterized by the presence of incomplete cell walls or multinucleate cells (Smith et al. 1996). This suggests that the mutant plants are experiencing defects in their ability to orient their divisions that prevents proper elongation in later development rather than an error in placing the new cell wall. This is the result of a phragmoplast guidance defect, where the microtubule structure directing vesicles to the cell plate to build the cell wall is not properly directing the cell wall to the cortical division site marked by the PPB (Cleary and Smith 1998; Martinez et al. 2017).

Later studies furthered identified the study of the missing protein, TANGLED1, the first member of a group of plant-specific proteins necessary for maintaining proper division plane orientation (Rasmussen et al. 2011). TANGLED1 (TAN1) is recruited to the cortical division site, the same location as the preprophase band, before the initiation of prophase. Contrary to the behavior of the preprophase band, TAN1 remains localized at this position until the end of cytokinesis (Rasmussen et al. 2011). Its localization pattern and the resulting phenotype when TAN1 is absent serve as evidence for the hypothesis that TAN1 plays a role in acting as a chemical or physical marker of the division plane to aid in the control of organized cell division.

TAN1 has also been shown to bind to microtubules, allowing it to be added to the growing list of microtubule-associated proteins (Smith et al. 2001). MAPs usually interact with microtubules in discrete categories and function in maintaining the dynamics of the microtubules. The lack of TAN1 also delays the progression through metaphase and telophase owing to slower assembly and disassembly of the phragmoplast, however these were not

connected to the misplacement of the new cell wall (Martinez et al. 2017). The lack of TAN1 also alters the structure of the cytoskeletal arrays, providing further evidence that TAN1 is critical for cytoskeleton organization (Cleary et al. 1998). As a result, we hypothesized that the binding of TAN1 to the microtubules plays a role in either the stabilization, resulting in the building of microtubules, or destabilization, promoting the destruction of microtubules into tubulin subunits.

Most of the work conducted on TAN1, and all of the work previously mentioned, was done in the *tan1* mutant in *Zea mays*. Analogs of the *TAN1* gene in *Arabidopsis thaliana*, which were knocked out to study the gene's function in the model organism, and AtTAN1-YFP was the first recorded positive division site marker in *A. thaliana*, localizing to the same location as the ZmTAN1 protein (Walker et al. 2007). However, the *tan1* single mutant in *A. thaliana* shows a weak and irreproducible phenotype which hampered its usefulness in experiments. However, when a double mutant with AUXIN INDUCED IN ROOT CULTURES9 (AIR9) and TAN1 was generated, a strong division plane orientation defect was observed. AIR9 also binds microtubules and localizes to the cortical division site, and also does not show division plane defects as a single mutant (Buschmann et al. 2006; Buschmann et al. 2015).

The *tan1 air9* double mutant in *A. thaliana* shows a synthetic phenotype that impacts the root growth, division plane orientation and the cell file rotation in the differentiation zone of the root despite neither single mutant showing a phenotype (Mir et al. 2018). Together these phenotypes suggested both a mitotic and interphase function of these proteins (Mir et al. 2018). Furthermore, introducing transgenic expression of TAN1-YFP into the *tan1 air9* double mutant returned the organism to a wild type phenotype, making the double mutant in *A. thaliana* an informative organism to study to elucidate the role of TAN1 in wild type plants (Mir et al. 2018).

The studies conducted measuring the impact of microtubule altering drugs and the expression patterns of TAN1-YFP driven by the native promoter served to aid in identifying the function of TAN1 in vivo.

Proper mitotic progression requires maintenance of very particular microtubule dynamics, thus treatment of plants with drugs that alter the ability of microtubules to build or break apart impacts a cells ability to properly divide in wild type plants. This experiment hoped to see differential responses between the wild type, single mutants and the double mutants to aid in identifying the role that TAN1 played in maintaining the microtubule structures involved during division. These experiments showed that the *tan1 air9* double mutant exhibited reduced root length, an indicator of either a cell's failing to progress through mitosis properly or a disruption in cell expansion Lack of detectable TAN1-YFP fluorescence suggests that it does not play a significant role in elongation or differentiation zones in the root, although it is possible that small but undetectable amounts are present.

, at a lower concentration of the microtubule destabilizing drug, propyzamide, than the wild type and single mutant plants. As the double mutant was hyper-sensitive to the depolymerizing effects of propyzamide, this suggests that the double mutant's microtubule arrays are destabilized, or more prone to breaking apart into tubulin subunits. This suggests a potential stabilizing function of TAN1 in vivo. This was also suggested at the lower concentrations of treatment in relation to the twisting of the roots. Owing to the radial swelling that is common under treatment of microtubule altering drugs the impact was hard to measure at higher concentrations (Baskin et al. 1994).

All studies up until now have been conducted with TAN1-YFP fused to the viral 35S promoter. TAN1 expression has only been recorded in mitotic cells under the constitutive

expression of this promoter (Rasmussen et al. 2011). By fusing the transformed protein to its native promoter, we can learn further information about the localization of the expression of a protein such as in the absence of AIR9. When looking at the expression of native promoter TAN1, in addition to localizing to the microtubule structures of dividing cells, TAN1 localized to the cytoplasm of cells in the meristematic region (Walker et al. 2007, Mir et al. 2018). This finding is consistent with previous ones noting TAN1 expression only in dividing cells. However, this study using the native *TAN1* promoter showed cytoplasmic accumulation which has not been previously observed. Cytoplasmic accumulation in addition to the growth impacts in response to propyzamide suggests a potential interphase function of TAN1. This also demonstrated no differential expression patterns between plants with AIR9 and those without.

With this study, we have elucidated a possible interphase function of TAN1. However, work still needs to be conducted under the native promoter to confirm such a function and to identify the specific role. Further research in identifying the function of TAN1 would include identifying the possibility for interactions or post-secondary modification to the protein. A potential experiment to test for the influence of post-secondary modification would be to change the identity of amino acids in the sequence that could be phosphorylated, namely tyrosine, threonine and serine, into other polar amino acids to maintain their properties for interaction in protein folding without allowing for phosphorylation of the protein. In addition, no work has been done to determine if the impacts on the absence of TAN1 in the distribution of organelles between cells. As the cell is unable to correctly place the new cell wall due to aberrant phragmoplast, it is possible that it is also unable to properly distribute organelles between cells. This has the potential to alter the fates of epidermal cells. Understanding the complete impact of

altering the microtubule dynamics in the *tan1* mutant would aid in understanding the direct role of the protein, but also the downstream impacts of its loss.

Figures

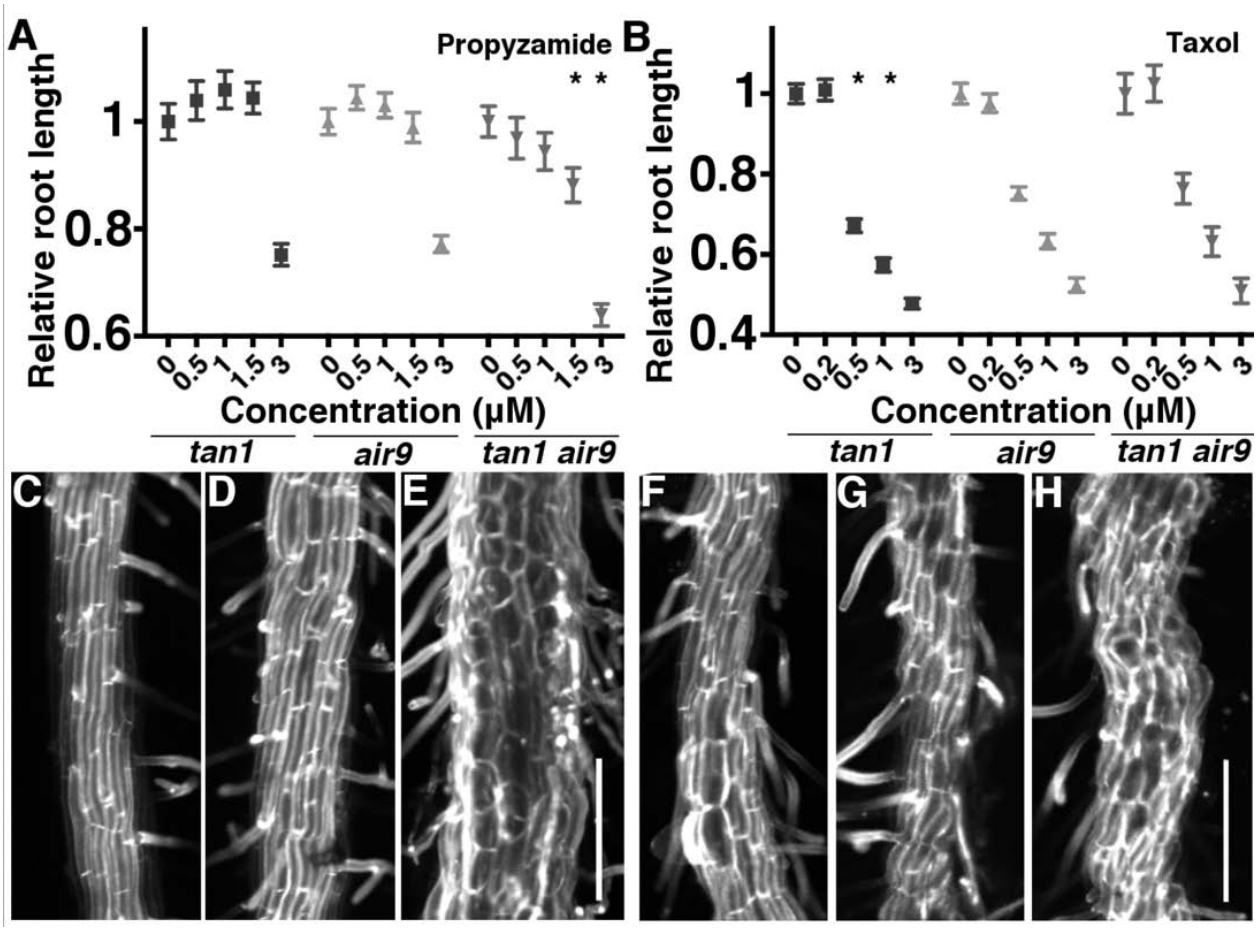

Figure 1. Treatments with Propyzamide and Taxol. **(A-B)** Relative root growth compared to 0μM of 8-day old roots of *tan1, air9* and *tan1air9* grown on different concentrations of (A) propyzamide and (B) Taxol. Asterisks indicate statistically different responses to drug treatments (KS test, $P<0.01$; $n>24$ plants per condition each day). **(C-H)** Maximum projections of 10 (C,D and F-H) and 30 (E) Z-stacks of 8 day old propidium iodide-stained differentiation zone roots of tan1 (C and F), air9 (D and G), and tan1air9 (E and H) treated with 3μM propyzamide (C-E) and 3μM Taxol (F-H). Bars=200μM.

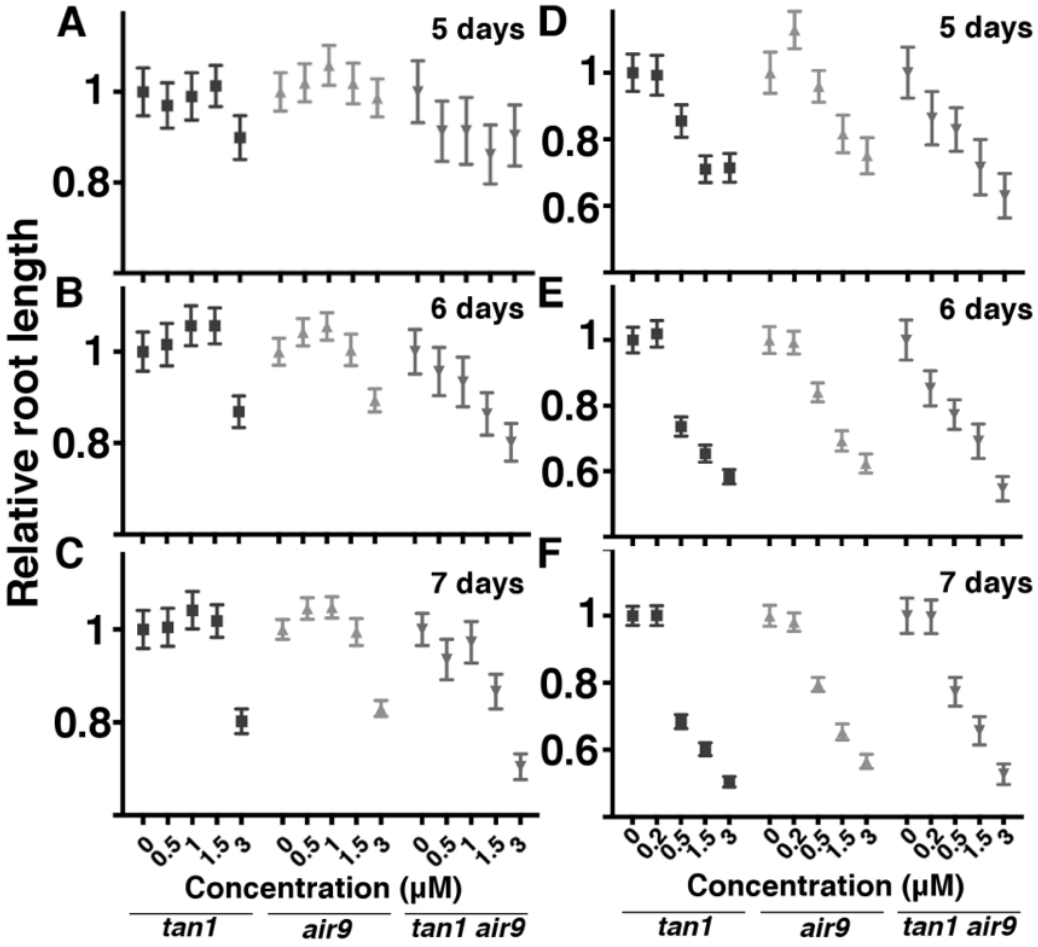

Figure 2. Treatments with Propyzamide and Taxol. **(A-B)** Relative root growth compared to 0µM of 5-, 6-, and 7-day old roots of *tan1, air9* and *tan1air9* grown on different concentrations of (A-C) propyzamide and (D-F) Taxol. N = 23-45 for each genotype and concentration.

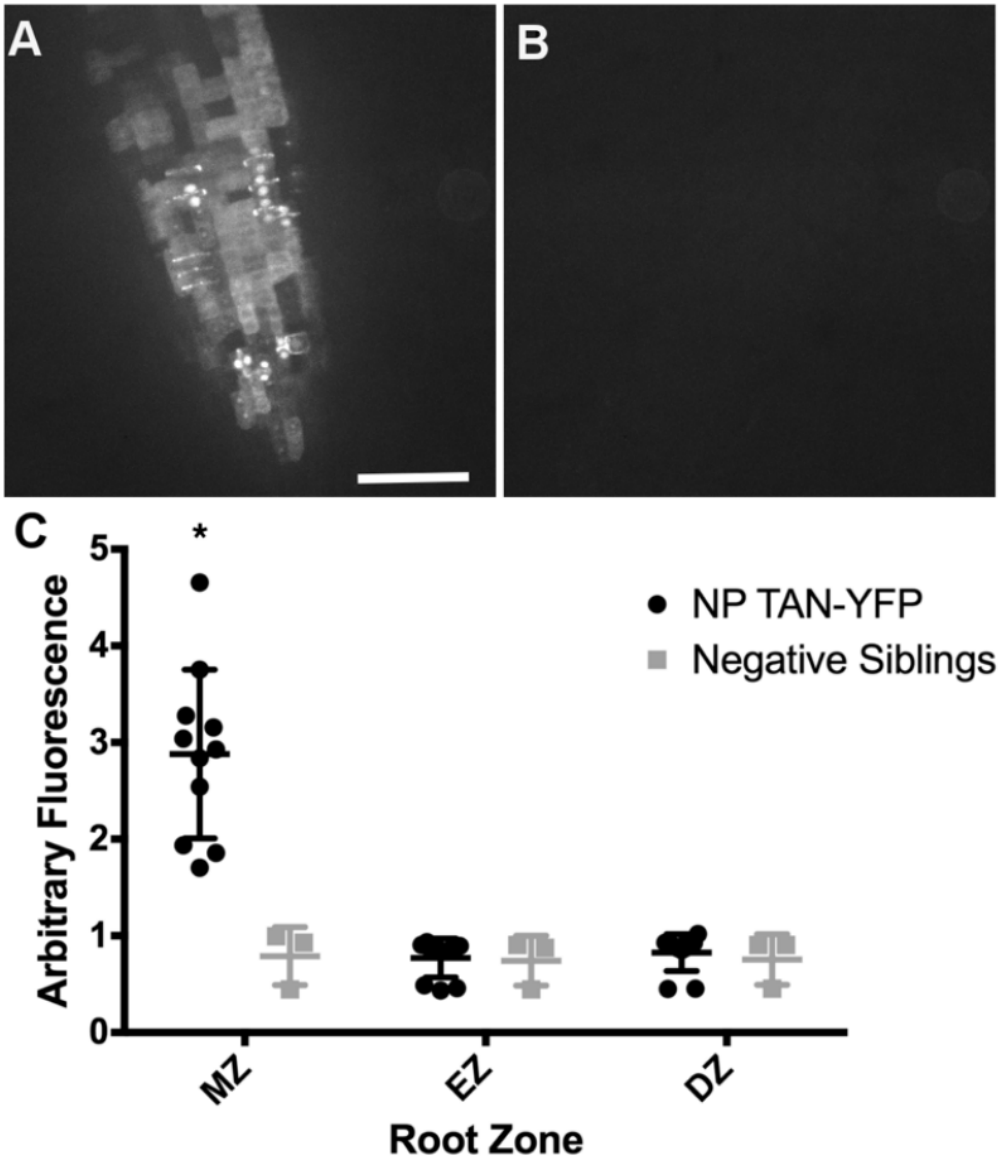

Figure 3. Native Promoter TANGLED1 Screening. **(A-B)** Maximum projections of 20 Z-stacks of 7-day old meristematic zone roots. (A) Representative image of meristematic zone of root expressing Native Promoter TAN1-YFP. (B) Sibling meristematic zone root not expressing TAN1-YFP. Images are the same size and Bar=50µM. **(C)** Quantification of arbitrary fluorescence signal in the meristematic zone (MZ), elongation zone (EZ) and differentiation zone (DZ) in TAN1-YFP (n=12) and negative sibling (n=3) plants. The asterisk above NP TAN1-YFP signal in the MZ indicates a statistically significant difference ($p < 0.001$) compared to all other fluorescence values. No other differences were detected.

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