Optimizing an Inducible PIN Knockout System in *Brachypodium Distachyon*

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Summary

The PIN proteins are a family of transmembrane proteins that transport auxin basipetally through the plant. The polar movement of auxin through the plant has been implicated in a variety of developmental processes, including embryo patterning, lateral organ initiation, and apical dominance (Leyser and Day 2003). Previously we used a reverse-genetics approach to study the effects of *PIN* knockdown on the model grass *Brachypodium distachyon*. We transformed *Brachypodium* to express an artificial microRNA that targeted transcripts of the two *Brachypodium PIN1* orthologs. Transformed plants exhibited reduced apical dominance (Fig 2). Unfortunately *PIN* knockdown transformations produced far fewer transformed events than controls with an artificial microRNA targeting the alcohol dehydrogenase genes. *Brachypodium* transformation involves the infection of embryonic callus with agrobacterium. Because of its role in embryonic patterning, we suspect that strong loss of *PIN* function is lethal in *Brachypodium* embryonic callus. It is therefore desirable to develop an inducible system to study the effects of *PIN* knockdown in adult plants.

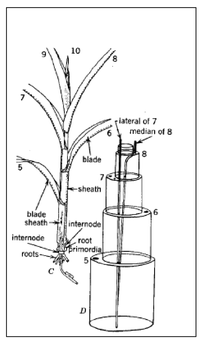
Here, we optimized techniques and treatment conditions that could later be used in an inducible *PIN* knockdown system. In order to observe the kinetics of inducible gene expression, we transformed *Brachypodium* with a construct containing a dexamethazone inducible *GUS* gene (Fig 2). *GUS* catalyzes the production of a blue pigment in the presence of *X-gluc*, making it an easily assayable marker for gene induction. Three transformants expressed *GUS* constitutively in leaf tissue; the rest did not induce at all.

Polar auxin transport plays an important role in phyllotaxy, the regular arrangement of leaves on the stem (Reinhardt 2003). Our goal is to use the inducible system to observe leaf initiation during *PIN* knockdown. It is desirable to know the plastochron length in *Brachypodium* in order to properly time *PIN* knockdown during leaf initiation. We found that *Brachypodium* grown in 20 hours of light have a shorter plastochron (3.79 days/leaf initiation) than those grown in 16 hours of light (7.61 days/leaf initiation) (Fig 5). The slopes were significantly different (one-way ANCOVA, df=1, F=45.07, P<.0001).

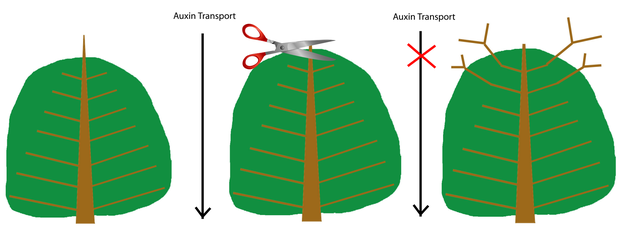
We are also interested in examining *PIN* knockdown during embryo development. Knockdown in embryos will require the embryos to be cultured *in vitro* so their development can be observed without the seed coat obscuring them. Culturing early plant embryos in vitro is challenging because the embryo requires nutrients and hormones from the surrounding ovule. Here we develop a technique that allows some *Brachypodium* embryos that have not yet initiated a meristem to develop to germination *in vitro*. Some early stage embryos appeared to develop normally, however, many embryos developed an abnormally curled scutellum, and the embryos underwent premature germination (Fig 3).

Figure 1: Auxin Transport in *Brachypodium*

Wild-type *Brachypodium* distachyon.

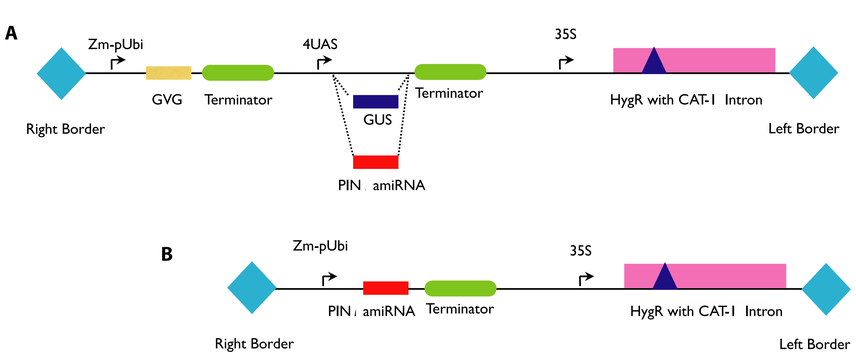


Leaf morphology of the grasses, showing the blade, sheath, node, and internode. Image from Esau (1967).

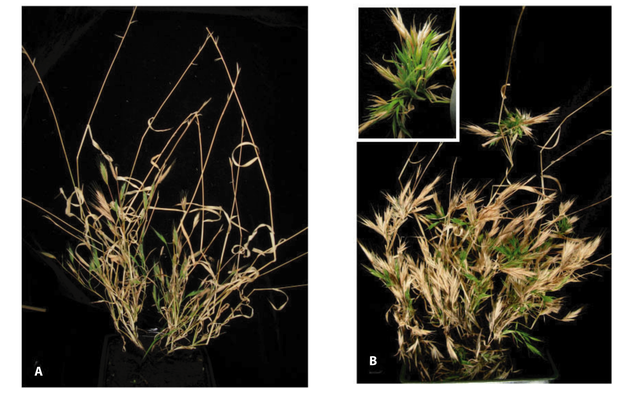


Auxin is a plant hormone involved in many developmental processes including apical dominance, leaf veination, and lateral organ initiation. It is synthesized in new leaves and transported down the plant by PIN proteins in the parenchyma cells. Auxin flux inhibits branching. When the auxin source (the shoot apex) is removed, branching occurs.

Figure 2: PIN amiRNA Knockdown Shows Loss of Apical Dominance

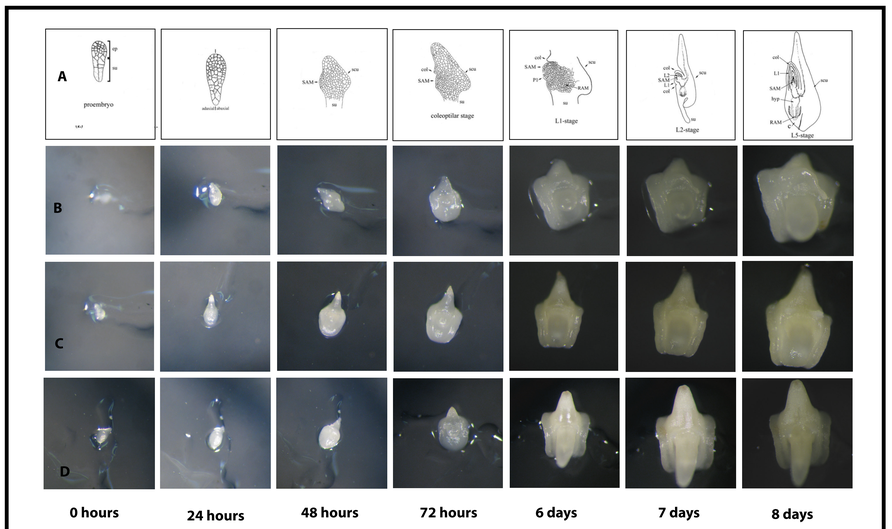


Constructs used to transform *Brachypodium* callus. A: Construct for the dexamethazone-inducible system. The insert contains genes for GVG and hygromicin resistance under constitutive promoters and contains either the gene for a PIN amiRNA or GUS under the control of the 4UAS promoter. B: In this construct, hygromicin resistance and PIN amiRNA are both expressed constitutively.



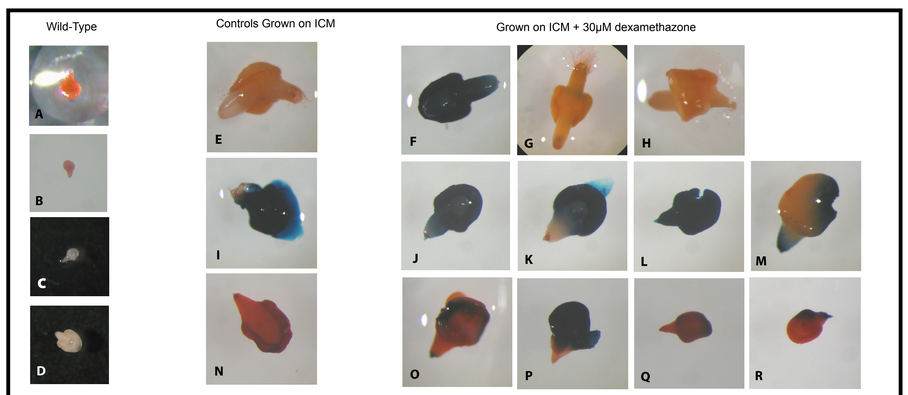
Artificial miRNA knockdown of *PIN*. After callus was transformed, plants were regenerated, transferred to 4° C for two weeks, then to 16 hours of daylight for further growth. A: *Adh* control. B: Plant expressing *PIN* amiRNA and showing loss of apical dominance.

Figure 3: In-Vitro Development of *Brachypodium*



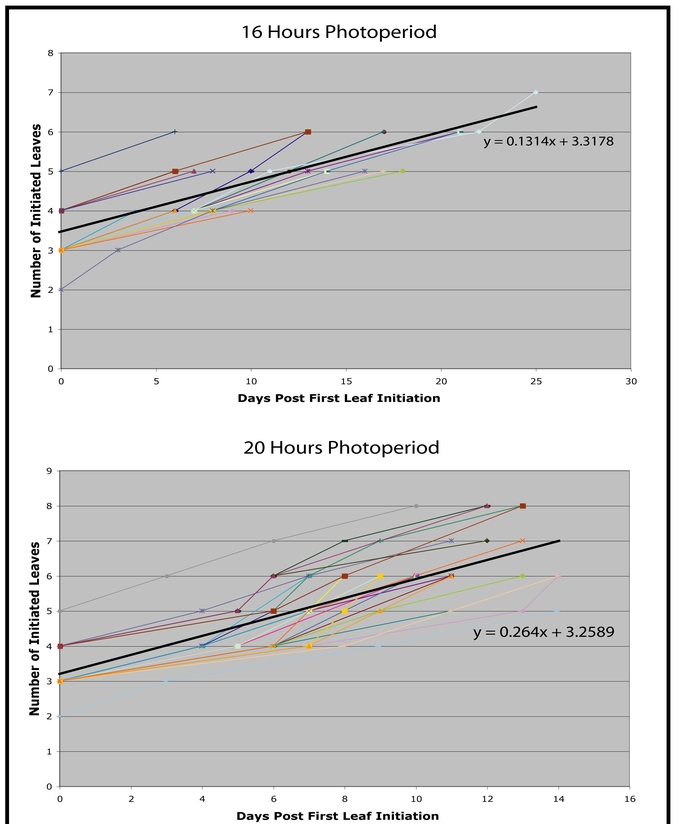
A: Normal development of a grass embryo in the ovule. B-D: *Brachypodium* embryos cultured *in vitro*. Embryos were excised from the ovules under sterile conditions and plated on *in-vitro* culture medium (ICM) as described in Sauer (2004).

Figure 4: *Brachypodium* Embryos Express *GUS* Inducibly



Embryos from plants that were transformed to contain the Dex-Gus gene construct. Embryos were excised from their ovules and grown on ICM containing 30μM dexamethazone. Control embryos were plated on ICM alone. Embryos were stained with a solution containing 10mg/mL X-gluc, 0.1% Triton, 10mM EDTA and 100 mM NaPO4, then fixed in formaldehyde. A-D: Wild-type controls. A and B grown on ICM, C and D grown on ICM + 30μM dexamethazone. E: JJ5602 control. F-H: Induced JJ5602 embryos. I: JJ5621 control. J-M: Induced JJ5621 embryos. Offspring of this transformation event express the *GUS* gene constitutively. N: JJ5605 control. O=R: Induced JJ5605 embryos.

Figure 5: Plastochron Length



Brachypodium leaf initiations under 20 hours and 16 hours of light. Plants were checked daily for new leaf initiations starting at the 3- or 4-leaf stage. We defined an initiation as the first time a new leaf was visible emerging from the sheath of the previous leaf. Each line on the graph represents the growth of one individual; the day of first initiation for each plant was normalized to zero. The inverse slope of the best-fit line is the plastochron (days/leaf initiation). Note the difference in scale on the x-axis. The 20-hour photoperiod plants had a shorter plastochron (3.79 days/leaf initiation) than the 16-hour photoperiod plants (7.61 days/leaf initiation) (one-way ANCOVA, df=1, F=45.07, P<.0001).

Conclusions

We propose supplementing embryo culture media with nutrients and plant hormones that would be provided to the embryo in the ovule to minimize developmental abnormalities. It may be possible to devise media that allows *Brachypodium* embryos to develop normally long enough to observe leaf initiation even if the embryos later germinate prematurely. The limiting factor of dexamethazone induction in adult plants may not be proper insertion of the transgene but getting the dexamethazone into the leaf tissue. In one transformant event, embryos of the plant induced while its leaf tissue did not (Fig 4). We need to devise new ways of overcoming *Brachypodium* leaves' epidermis so the inner tissues are exposed to dexamethazone. Though medium for *Brachypodium* embryo culture and techniques for inducing *GUS* in leaf tissue remain to be optimized, our results will bring a *PIN* inducible knockdown system in adult and embryo *Brachypodium* tissue closer to reality.

References

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Acknowledgments:
Thanks to Devin O' Connor for guidance, advice, and transformed *Brachypodium* plants, the Hake Lab, and the Kolenkow-Reitz fund for making this work possible.