Title: Modification of hypocotyl length and seed size in camelina and canola via manipulation of the AHL gene family.

PI: Michael M. Neff

CoPIs: None

Funding term and duration: 7/1/14 – 6/31/15

Graduate students: David Favero and Reuben Tayengwa are both Ph.D. graduate students contributing to this project. Both receive RAs from other sources of money. They are both in the Molecular Plant Sciences Graduate Program at WSU and working on this gene family in Arabidopsis.

Undergraduate students: Courtney Pierce and Breanna Ervin are both undergraduate students contributing to this project. Both receive support from other sources of money. They are working with Reuben Tayengwa on CRISPR/Cas9-based DNA editing of AHL genes in Arabidopsis. Kim Le is an undergraduate contributing to this project. She receives support from other sources of money. She is working with David Favero on determining how the AHLS interact with hormone and light signaling pathways in Arabidopsis.

Technical Support: Pushpa Koirala (Technician) and Jiwen Qiu (Postdoc) are both working on this gene family in camelina and canola. Both are supported from other sources of money.

Background and justification: In low rainfall, dryland-cropping areas of Eastern Washington, such as the regions around Washtucna, Lind and Dusty, stand establishment can have a major impact on yields of camelina and canola. During dry years, these seeds need to be planted in deep furrows so that the developing seedling has access to soil moisture. In areas with higher rainfall, canola and camelina are often used in rotations where they are planted into wheat stubble left over after harvest to reduce erosion and increase soil quality. One approach to facilitate stand establishment in each of these regions is to develop varieties with larger seeds and longer hypocotyls as seedlings while maintaining normal stature as adults. Unfortunately, few mechanisms have been identified that uncouple adult stature from seedling height.

The Neff lab has identified a group of plant-specific genes that, when mutated in a particular way, increase seed size and seedling height without adversely affecting adult stature. These genes encode AHL (AT-Hook Containing, Nuclear Localized) proteins. When these proteins are over-expressed, the result is seedlings with shorter hypocotyls. When the activity of multiple genes is disrupted, the result is seedlings with taller hypocotyls, demonstrating that these genes control seedling height in a redundant manner (Street et al., 2008). In the Brassica Arabidopsis thaliana, we have identified a unique allele (sob3-6) for one of these genes, SOB3/AHL29, that over-expresses a protein with a disrupted DNA-binding domain and a normal protein/protein interaction domain. In Arabidopsis, this mutation confers normal adult plants that produce larger seeds and seedlings with hypocotyl stems that can be more than twice as long as the wild type.

Objectives: The goal of this project is to enhance camelina and canola seedling emergence when they are planted deeply in low-rainfall dryland-cropping regions (generally less than 12”/year) or in wheat
stubble. This can be achieved by manipulating AHL gene family members to develop varieties that have long hypocotyls as seedlings yet maintain normal growth characteristics as adults.

Methods: This project includes three major sub-aims:
1) Continue characterizing the activity of sob3-6-like mutations in other Arabidopsis AHL genes.
2) Generate transgenic camelina and canola plants over-expressing wild-type and mutant forms of Arabidopsis AHL genes.
3) Identify, clone and characterize AHL gene family members from camelina.

Results and Discussion: During this funding period, the Neff Lab has used a combination of molecular, genetic, biochemical, bioinformatic and biotechnological approaches to understand the role of AHL genes in plant growth and development. Our primary goal has been to characterize AHL genes from Arabidopsis and camelina, including an analysis of the evolution of this gene family (Zhao et al. 2014). The molecular mechanisms underlying the foundation of this work are described in a November 2013 publication from the Neff lab in the Proceedings of the National Academy of Sciences, USA (PNAS) (Zhao et al. 2013).

Related to Sub-aim #1: Generating transgenic Arabidopsis over-expressing AHL genes from complex genomes has been a powerful way to identify those genes with similar function as SOB3/AHL29 and other family members that have been characterized previously in Arabidopsis. For example, as a part of our phylogenetic/evolutionary analysis we over-expressed a dominant-negative AHL gene from soybean (Glycine max) in Arabidopsis and demonstrated a similar long-hypocotyl phenotype to those produced when expressing various dominant-negative sob3 alleles (Fig. 1; Zhao et al. 2014).

Using this phylogenetic/evolutionary analysis, we identified two Arabidopsis AHL genes that were unique as compared to previously characterized AHLs: AtAHL6 and AtAHL20. We over-expressed sob3-6-like mutations for each of these genes in Arabidopsis, and compared them to the over-expression of wild-type forms for these genes. For example, the over-expression of wild-type AtAHL20 leads to plants with delayed flowering time. In comparison, over-expression of a sob3-6-like allele of AtAHL20 leads to the opposite phenotype, with earlier flowering. In contrast, over-expression of wild-type AtAHL6 leads to earlier flowering with necrotic leaves. Surprisingly, over-expression of a sob3-6-like allele of AtAHL6 causes essentially the same phenotype as the wild-type copy of the gene, suggesting that this particular type of mutation does not cause a dominant-negative phenotype in all AHL genes. This finding is part of Reuben Tayengwa’s Ph.D. dissertation as well as a manuscript that will be submitted for peer review in the near future. It is important to note that the sob3-6-like mutation in AtAHL6 represents only one of the three methods we have identified for generating dominant-negative mutations in AHL genes. Thus, it is still possible that one of the other two methods may still create a dominant-negative phenotype for AHL6-like genes.

Reuben Tayengwa, along with two undergraduate researchers, has also been using CRISPR/Cas9-based genome editing to generate loss-of-function and ultimately dominant-negative mutations in AHL genes. This approach uses a transgene-based guide RNA to target specific genes, which are then edited by the Cas9 protein. Once the genome has been successfully edited, the transgene can be crossed out, leading to a non-transgenic product that is currently being considered non-GMO. This approach will be a major area of focus during our next round of funding.

Related to Sub-aim #2: We are also using Arabidopsis transformation as a means for assessing the function of camelina AHLs. For example, we have transformed Arabidopsis with both CsAHL6 and CsAHL20, which are camelina versions of AtAHL6 and AtAHL20. Our T1 CsAHL6 over-expression lines in Arabidopsis confer adult plants with necrotic tissue, a phenotype that is also seen when AtAHL6 is over-expressed in Arabidopsis. In addition, our T1 CsAHL20 over-expression lines in Arabidopsis confer larger
adult plants with delayed flowering, a phenotype that is also seen when AtAHL20 is over-expressed in Arabidopsis. We have also begun expressing camelina AHLs in camelina. For example, we have observed that over-expression of CsAHL6 and CsAHL20 in Camelina confer the same phenotypes described above in Arabidopsis.

As a part of this sub-aim, we have also been attempting to generate transgenic canola using the floral-dip technique. Unfortunately, this approach has not been successful to date. Thus, we are now using a tissue-culture-based approach for generating transgenic canola. This will also be a major area of focus during our next round of funding.

Related to Sub-aim #3: We have been using the camelina genome sequence to identify and clone full-length camelina AHLs. BLAST-search analysis using Arabidopsis genes as a query has led to the identification of homologous camelina sequences for all 29 Arabidopsis AHLs, with most genes having three candidate copies in camelina due to its hexaploid genome. We have cloned versions of the following camelina AHLs: CsAHL29, CsAHL27, CsAHL6, CsAHL19 and CsAHL20. We are currently in the process of cloning six more camelina AHLs. During our next round of funding we will continue to identify and clone AHL genes from camelina. We will also expand our AHL gene cloning to include members from canola.

Impact/Potential Outcomes: We have now shown that expressing at least three different mutant forms of AHL genes leads to larger seeds and taller seedlings in both Arabidopsis and the oilseed crop camelina. Two of these mutant forms encode proteins with a disrupted or deleted DNA-binding domain. The third form encodes an AHL protein with a deleted AHL/transcription-factor-binding domain. Based on our Arabidopsis research, we may be able to double the size of camelina seeds by generating transgenic plants expressing mutant forms of these genes. Even if the total harvestable oil per plant is unchanged, this may lead to an increase in yield/acre by enhancing stand establishment and reducing harvest loss due to blowing out of the combine. During the current funding period, we have begun identifying AHL gene family members in camelina. A key step in this characterization is the demonstration that these and other AHL proteins physically interact with themselves and each other. This observation has led to developing a molecular-genetic method for identifying and cloning AHL family members that are specifically associated with seed and seedling development. This method plays a central role in a USDA/NIFA grant that was recently funded (see below).

Affiliated projects and funding: The characterization of the AHL gene family in Arabidopsis was previously supported by a grant from the Department of Energy. The characterization of the AHL gene family in wheat has been supported by grants from the Washington Grain Commission and the Orville A. Vogel Wheat Research Fund. We used preliminary data from these funds as well as those from the Washington Grain Commission and the Orville A. Vogel Wheat Research Fund as the basis for a $500,000 three-year grant proposal submitted to the USDA/NIFA entitled “Increasing seed size and plant biomass via manipulation of the AHL gene family”. Though the proposal was not funded the first time, the panel ranked the proposal as “high priority”. Based on the progress we made with this project, we resubmitted the proposal to USDA/NIFA, which led to a ranking of “outstanding” and an award of $498,000/three years (start date 12/1/13). Half of this award is for working with wheat, and the other half for camelina.

Publications and Presentations: During the past funding period we published a manuscript describing a phylogenetic/evolutionary analysis of the AHL gene family in plants (Zhao et al. 2014). We are also preparing a manuscript describing the functional analysis of AtAHL6 and AtAHL20. Further, we published an extension bulletin/abstract for the 2014 WSU field days. In addition Dr. Neff spoke with the following groups (>1000 total attendees/participants) about GMOs as well as the AHL gene family and how it can be manipulated to increase seed size and seedling height: 1/15/14, CBCCA Extension Short Course,
Moses Lake WA, ~90 attendees/participants; 2/19/14, Washington Grain Commission Meeting, Pullman WA, ~40 attendees/participants; 2/21/14, Washington Biofuel Cropping Systems Meeting, Pullman WA, ~30 attendees/participants; 2/12/14, Crop Production Services Growers Meeting, Almira WA, ~50 attendees/participants; 3/29/14, Sandhill Crane Festival, Othello WA, ~50 attendees/participants; 4/24/14, University of Massachusetts, Amherst MA, ~50 attendees/participants; 5/14/14, USDA-NIFA Plant Biology/Plant Breeding Project Directors Meeting, Washington DC, ~50 attendees/participants; 7/8/14, Washington State University Extension County Faculty Meeting, Spokane WA, ~100 attendees/participants; 9/9/14, Washington State Master Gardeners Annual Conference, Tacoma WA, ~300 attendees/participants; 11/18/14, Crop Production Services Ag School Winter Conference, Kennewick WA, ~400 attendees/participants; 12/16/14, Washington State University Extension Wheat Academy, Pullman WA, ~60 attendees/participants. We recognize the importance of extension publications for sharing research findings with the stakeholders of Washington State. We now have sufficient material to present a summary of our findings to the general public. We will work with Karen Sowers and other extension specialists on the WOCS team to present this material in a manner that can be understood and appreciated in lay terms.

References:


Tables/Graphs:

Figure 1: Hypocotyl growth of wild-type Arabidopsis (Col-0), Arabidopsis plants over-expressing AtSOB3/AHL29 (SOB3-D) and the dominant-negative PPC domains from AtSOB3/AHL29 (SOB3-PPC-ox) and Gm06g01650 (GmPPC-ox). Plants were grown in 20 µmol/m²/sec of continuous white light. Scale bar = 5 mm (adapted from Zhao et al. 2014 Figure 2c).