

**Washington Grain Commission  
Wheat and Barley Research Final Report**

**Project #:** 13C-3019-3687

**Progress Report Year:**     \_\_3\_\_ of \_\_3\_\_ (*maximum of 3 year funding cycle*)

**Title:** *A Genetic Arsenal for Drought Tolerance, Getting to the Root of the Problem*

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**Executive summary**

Lignin content and accumulation in stems, leaves and roots has been linked with different stress tolerances in crop plants. Lignin confers rigidity to plant cell walls, and increases in response to drought, heavy metals, salinity, and pathogen attack. Therefore, managing overall lignin content, as well as its proportion in the roots versus shoots of crop plants is important for improved stress tolerance. Only a few studies have investigated the role of lignin in grass root systems at present. Reports on maize and wheat showed that lignin content in the root was higher than in the shoot, and that these levels varied depending on genotype. In wheat, lignin concentration was shown to decrease in seedlings and roots when exposed to mineral deficiencies and increase in response to toxic minerals. Given these findings, our preliminary results, there is a need to further investigate the role of lignin in roots. The overall goal of the project is to determine the role of lignin in wheat roots for drought tolerance and disease resistance and to develop a high-throughput method for lignin analysis in wheat roots and straw. We have worked on processing stem and root tissues for overall lignin content using two independent assays as well as sending pulverized stem and root tissues for analysis of aromatics including lignin monomers to the Ralph lab at University of Wisconsin-Madison. We had good success with lignin extraction in stems, but are still working on lignin extraction from root tissues. As such, we are outsourcing the lignin chemistry to a collaborator who works with grasses and lignin in roots and has already developed protocols. We attempted to implement drought studies using the Phenospex drought spotter in the wheat greenhouse, but had technical issues and then had to abandon work due to the COVID-19 pandemic. Therefore, we focused efforts on molecular and *in silico* approaches to understanding two key enzymes that regulate lignin biosynthesis: CAD and COMT.

**Impact**

In addition to stress tolerance, lignin has important implications for the rhizosphere and agricultural soils, particularly since it is a stable component of soil organic matter (SOM). There is evidence that lignin slows down the mineralization of nutrients from crop residues. For example, the ratio of lignin to nitrogen is used as an indicator for litter degradation. Studies have shown that lignin negatively affects short-term nitrogen release from different types of green manures that differ in lignin content and that time is a key factor in the lignin/nitrogen equation. Since SOM contains roughly two-thirds of global terrestrial carbon storage and lignin is an important component of SOM, lignified biomass represents a promising source of sustainable fertilizer, which is a concern for Washington state farmers and globally. Our research has shown the lignin monomer content and not total lignin content in winter wheat stems is important for

residue breakdown and thus management. Long-term our data will shed light on the role of lignin in rhizosphere processes as well—such as soil-borne pathogen management and improving overall plant responses to abiotic stresses like drought, salinity, changes in pH, and cold.

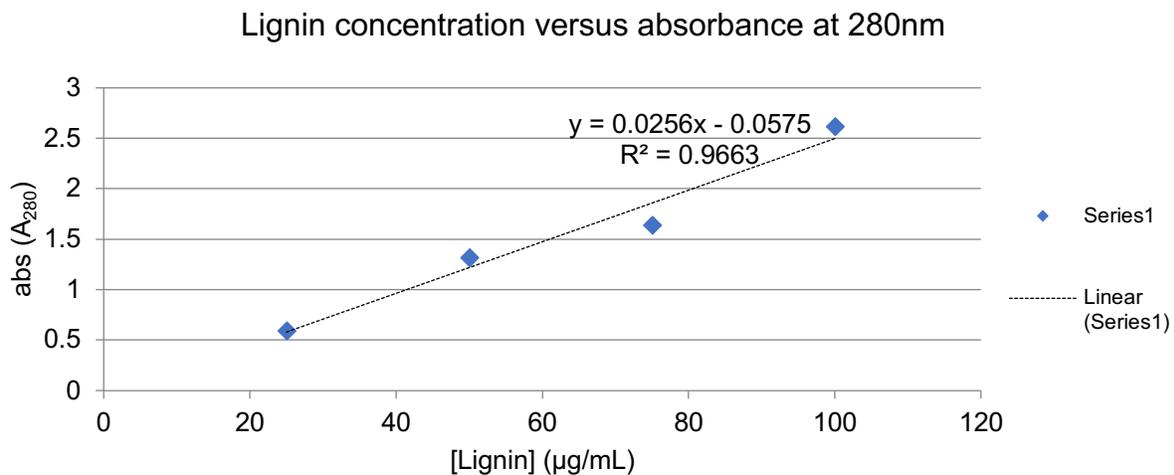
## **Outcomes and Deliverables**

Overall, we were able to confirm initial findings that Louise and AUS28451 roots differed in lignin content. We performed some initial experiments examining *Fusarium* resistance in the backcross Lou/Au populations and attempted to relate that to the total lignin content of the lines. At this point, it is unclear whether the lignin content, monomer ratios or another aromatic compound is contributing to the stress tolerance of AUS28451. To address this, we established a collaboration with Dr. John Ralph's lab at the University of Wisconsin-Madison. He is an expert in lignin biochemistry and is working on running a complete aromatic profile panel on Louise and AUS28451 shoots and roots. While we are awaiting the results, we are eager to determine the differences and hope that these analyses shed light on candidate molecules and breeding targets. We did streamline a total lignin quantification and protocol. Our greenhouse work was stymied in year 3 by the COVID-19 pandemic and therefore will continue into 2021. In year 3, we shifted focus to try to understand the genes that control lignin production in wheat to better understand lignin accumulation in the shoots and roots, particularly in response to biotic and abiotic stress. Below we detail some of our findings from the last three years.

### Lignin quantification protocol

To establish a rapid method to quantify total lignin, wheat stem samples taken at the second internode after grain filling were ground and sequentially washed using a modified in-house cell wall washing protocol adapted from (Fukushima and Hatfield, 2001). The protocol involves washing uniformly ground plant tissue twice with potassium phosphate, three times with Triton X-100, twice with sodium chloride, twice with distilled water, and three times with acetone. Washed samples are then dried in a 60°C oven for 24 hours and weighed again. The acetyl bromide digestion solution includes NaOH, freshly prepared hydroxylamine-HCL, acetic acid, and acetyl bromide. Samples are digested at 70°C for 30 minutes and then spun down. The suspended solution is then tested on a spectrophotometer at 280nm for absorbance compared to an empty digestion solution control.

Ideally, standards should be generated from commercially extracted lignin derived from monocot sources. Instead, standards were generated from commercially extracted lignin derived from a dicot source due to the lack of a commercially available monocot lignin extract. Exactly 50mg of lignin extract was suspended in 10ml of acetyl bromide digestion solution to generate a working stock solution (stored in a sealable glass container). Seven diluted standards were generated, including a blank sample representing the minimum 0ug/ml of lignin, with a dilution series up to 100ug/ml. All standards were then measured at 280nm on an Eppendorf Biophotometer, and plotted on a graph where measured absorbance at 280nm can be equated to a known lignin concentration (Figure 1).



**Figure 1:** A four-point test standard plot derived from absorbance values of lignin solutions resulting from the acetyl bromide digestion protocol. Measuring light absorption relative to known concentrations of lignin generates a regression curve that can be used to estimate the unknown concentrations of test samples.

This protocol has been used to confirm and test lignin concentrations in the Louise and AUS28451 backcross populations but will be repeated to confirm the initial findings.

### Identification of lignin biosynthesis enzymes: CAD and COMT

Lignin is a multifunctional, complex biopolymer that is of critical importance to the overall fitness of terrestrial plants. Evolutionary pressure led aquatic plants to biosynthesize and integrate lignin into their secondary cell walls (SCW), which facilitated the emergence of terrestrial plants that now account for roughly 30% of the total carbon biomass found in the Earth's biosphere. Lignin acts as a mechanical scaffold that enables plants to resist the mechanical forces of gravity and wind. Furthermore, lignification of plant xylary tissue enables plants to resist embolisms caused by the high pressure of upward water transport driven by photosynthesis and evapotranspiration while also providing a hydrophobic surface within xylary tissue that maximizes water retention. Increased lignin deposition has been correlated with drought and salt tolerance. Lastly, evidence suggests lignified tissue provides a protective barrier from both herbivory and pathogen attack.

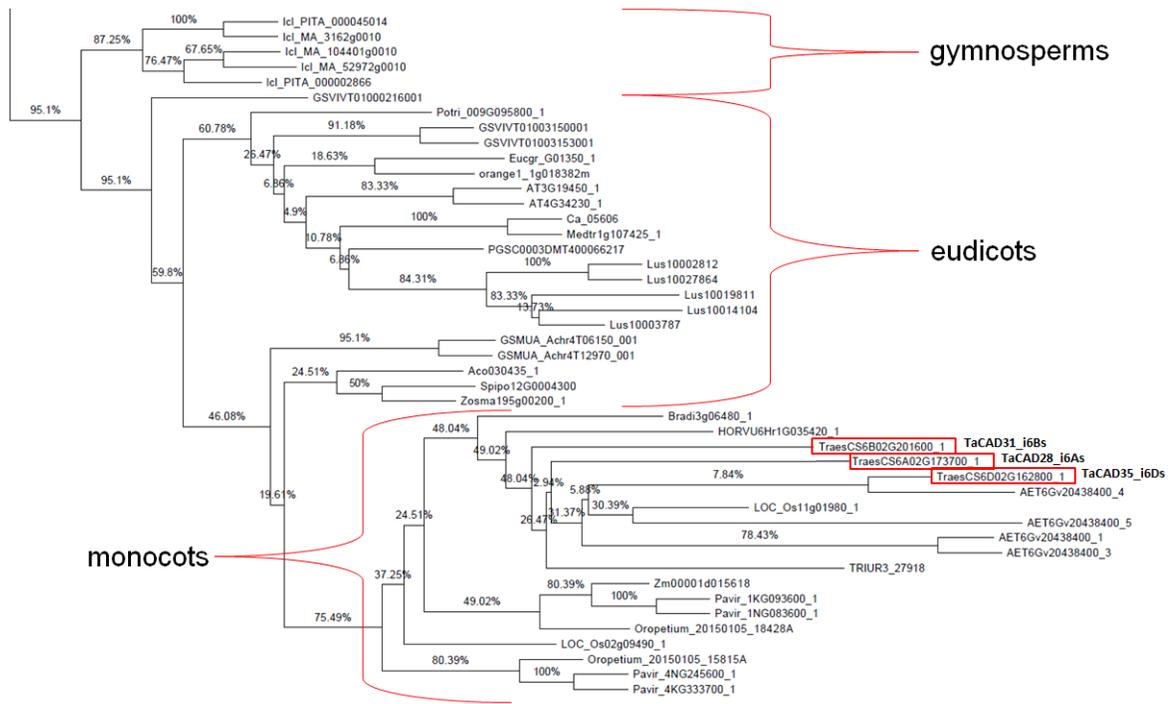
Lignin itself is primarily composed of three aromatic monomers: p-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S). Moreover, there is evidence of additional

monomers that exist but at lower concentrations. Lignin monomer composition is variable and can differ based on species, developmental age, and environment. Lignin biosynthesis begins in the plastids where phosphoenolpyruvate is converted into phenylalanine in the Shikimate pathway. Phenylalanine is shunted into the cytosol, where phenylalanine ammonia-lyase (PAL) commits phenylalanine to the phenylpropanoid synthesis pathway via deamination, generating cinnamic acid. Tyrosine is also deaminated by tyrosine ammonia-lyase (TAL) generating p-coumaric acid, suggesting a redundant substrate intake to the phenylpropanoid pathway. Several cytosolic reactions follow, including the activation of two critical lignin monomer synthesis enzymes: cinnamyl alcohol dehydrogenase (CAD) and caffeic O-methyl transferase (COMT). CAD catalyzes the final step in the biosynthesis of three of the most abundant lignin monomers (S, G, H), which actively converts a phenolic aldehyde into an alcohol group through zinc-mediated hydrolysis of NADPH or NADH. In general, the CAD enzyme belongs to the medium chain dehydrogenase reductase (MDR) family, which is defined by three highly conserved motifs: a structural zinc binding motif, a catalytic zinc-binding motif, and a NADPH/NADH catalytic domain called a Rossmann Fold. COMT has a wide range of substrate affinities. Depending on the organism, COMT participates in multiple steps of phenylpropanoid biosynthesis due to its highly conserved S-adenosyl methionine (SAM) binding domain that acts as a methyl donor in hydroxyl-specific methylation reactions. COMT has been shown to have an affinity for both caffeic acid and hydroxyferulic acid, which becomes ferulic acid and sinapylaldehyde respectively after the fifth hydroxylated position of each ligand is methylated. Moreover, COMT methylates precursors of both coniferyl alcohol and sinapyl alcohol lignin monomers.

The resulting lignin monomers are then quickly transported out of the cell by ATP-binding cassette (ABC) transporters into the apoplast during construction of the SCW. There, lignin monomers are oxidized by laccases and peroxidases, creating lignin radicals that proceed to polymerize via carbon-carbon and ester bonds. Though the polymerization of lignin monomers is highly complex, evidence suggests that the polymer organization is not random, but the mechanisms controlling polymerization have yet to be defined. Both the phenolic chemical basis of the lignin monomers and the carbon-carbon and ester bonds that join the phenolic bases result in tissue that is recalcitrant to degradation. Hydrolysis of the carbon-carbon bonds and ester bonds is energy intensive and generates phenolic free radicals that prove to be highly toxic to the herbivore. Such toxicity and recalcitrant digestibility acts as an innate defense from pathogen attack, and has been observed in several plant species including *Oryza sativa* L., *Populus trichocarpa*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *T. aestivum*, and *Zea mays* L. However, fungal organisms such as *D. citrispora* (white rot) and *P. placenta* (brown rot) have evolved strategies to digest lignified tissue by either reducing the phenolic radicals formed by digestion (white rot), or by digesting the vast quantities of cellulose and hemi-cellulose that surround the lignin polymer (brown rot).

An exhaustive list of all TaCAD protein sequences was generated by BLASTp search of the EnsemblPlants database (version 42 accessed April 2019)

<https://plants.ensembl.org/index.html>], using the protein sequence of the *bona fide* BdCAD1 (Bradi3g06480). The gDNA, cDNA, protein sequences, all physical properties, and location and orientations of 47 unique putative *TaCAD* candidates were organized, given unique names, rooted in the order by which they appear in the *T. aestivum* genome, phylogenetic class, and chromosomal location. CAD enzymes fall into three classes with Class I being the *bona fide* CAD also known as CAD1 in other plant species.



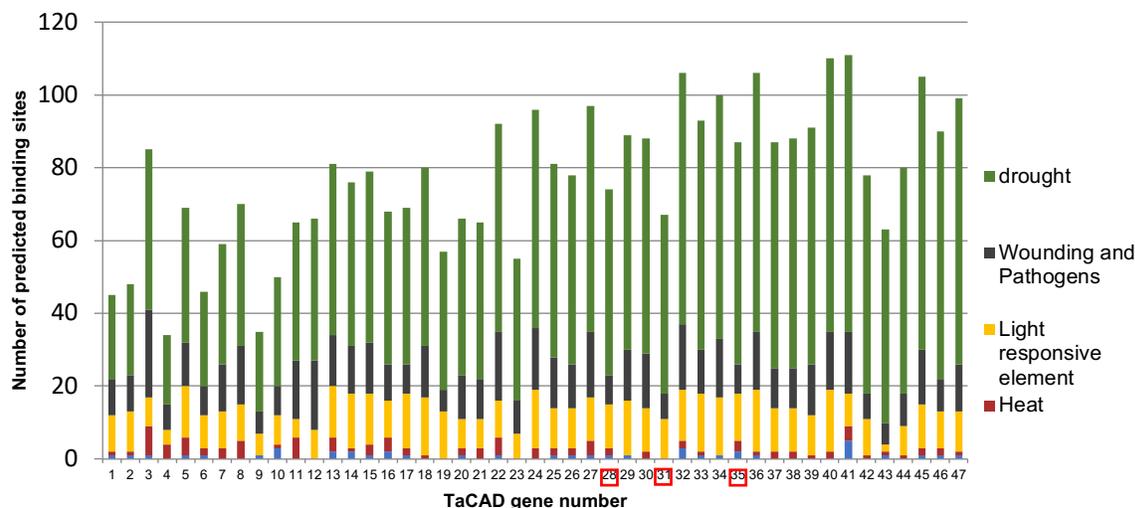
**Figure 2:** Class I CAD subtree. Sequences from gymnosperms, eudicots and monocots segregated into their own subclasses. *T. aestivum* CADs are boxed in red and given common names for simplicity.

The intronic and exonic boundaries of all 47 *TaCAD* genes were resolved by utilizing the Gene Structure Display Server [<http://gsds.cbi.pku.edu.cn/>]. All *TaCAD* protein sequences were aligned relative to *bona fide* CAD sequences *AtCAD4* and *AtCAD5*, *OsCAD2*, *BdCAD1*, and *TaCAD1* using the MUSCLE algorithm provided in the Mega X software suite, where the MDR motifs were identified. Of particular note, *TaCAD1* (Genbank GU563724) corresponds to the EnsemblPlants gene ID *TraesCS6D02G162800* which was renamed *TaCAD35\_i6Ds* (*TaCAD1*). All *TaCAD* protein sequences were then subjected to a phylogenetic analysis to define what classification each putative *TaCAD* falls under and Class I CADs or the *bona fide* CAD proteins are displayed (**Figure 2**). The phylogenetic tree is rooted with CAD sequences from *Aegilops tauschii* (and CAD paralogs), *Anasus comosus*, *Arabidopsis thaliana*, *Asparagus officinalis*, *Brachypodium distachyon*, *Cicer arietinum*, *Hordeum vulgare*, *Linum usitatissimum*, *Musa acuminata*, *Medicago sativa*, *Nicotiana benthamiana*, *Oropetium thomaeum*, *Oryza sativa*, *Panicum virgatum*, *Physcomitrella patens*, *Spirodela polyrhiza*, *Triticum urartu* (and CAD paralogs), *Zea mays*, and *Zostera marina*.

To determine the cues and signaling pathways that govern their expression, a promoter analysis of all *TaCAD* genes was performed. The 5' UTR and 2000bp upstream of the 5' UTR was queried in the PlantCARE promoter element database. The resulting data was collected and compiled into a graph that delineates the number of cis-regulatory elements found in that particular region. We found cis-regulatory elements that are involved in drought responses,

wounding and pathogen response, heat, and light (Figure 3). These data indicate further study of these genes in wheat is warranted.

### Stress-specific cis-element binding sites



**Figure 3:** Number of stress-related transcription factor (TF) binding sites found in TaCAD1-47 cis-regulatory elements. The class I *TaCADs* *TaCAD28\_i6As* and *TaCAD35\_i6Ds* (*TaCAD1*) (highlighted in red) contain cis-regulatory elements for drought, wounding and pathogens, light, heat, and cold. *TaCAD31\_i6Bs* promoter contains TF binding sites for drought, wounding and pathogens, and light, but lacks cis-regulatory elements responsive to heat and cold. All *TaCADs* appear to have cis-regulatory elements responsive to drought, wounding and pathogens, and light exposure.

Recently, TaCOMT-3DL has been found to contribute to resistance of sharp eyespot disease and overall stem mechanical strength. Moreover, the *COMT* gene family in *Z. mays*, *O. sativa*, *B. distachyon* and *Sorghum bicolor* have received attention from research groups interested in reducing stem lignin concentration to increase cellulose bioavailability for biofuel production and livestock forage. We performed preliminary analysis of the recently published *T. aestivum* cv. Chinese Spring genome and found 50 TaCOMT isoforms with a protein sequence similarity of >40% to BdCOMT4 (Bradi3g16530) and an additional 4 TaCOMT paralogues (TaCOMTp) containing the SAM domain. Given the volume of recently acquired preliminary data and the newly available sequenced hexaploid wheat genome of Chinese Spring, it is an opportune time to recharacterize both the *TaCAD* and *TaCOMT* gene families in their entirety. Analysis of TaCAD and TaCOMT will add value to the analysis because such context will give breeders an objective molecular basis to pursue new and potentially improved breeding targets.

### Conclusions

While our progress was hindered by the pandemic and challenges with supply chain issues and ability to physically be in the laboratory, we tried to make the best of the situation and refocused efforts on the molecular nature of lignin biosynthesis. The focus of our next proposal will be to resolve the relationships between lignin, root architecture and biotic and abiotic stress to help breeders select for more resilient root systems.

**WGC project number:** 13C-3019-3687  
**WGC project title:** A Genetic Arsenal for Drought Tolerance, Getting to the Root of the Problem  
**Project PI(s):** Karen A. Sanguinet, Kim Garland-Campbell, Timothy Paulitz  
**Project initiation date:** 7/1/18  
**Project year (X of 3-yr cycle):** year 3 of 3

Objective	Deliverable	Progress	Timeline	Communication
1. Quantification of lignin content in roots and stems	A robust and reliable method to accurately determine lignin content in root samples in a high-throughput manner is the main deliverable of this objective.	The first rounds of lignin extractions were performed with the parental lines: AUS28451 and Louise in 2018 both for total lignin and for lignin monomers. Following optimization of the protocols for root extractions, the Lou/Au BC1F6 populations were analyzed in 2019, but another repetition is needed. We have also identified some additional backcross lines of interest. There were technical issues with quantification of the full aromatic profile so we are now outsourcing that to a collaborator.	The quantification of lignin and optimization of the extraction methods was performed for total lignin. Three independent methods have been used for lignin quantification. We are now performing an entire aromatic profile for AUS28451 and Louise. Due to complications and delays from the COVID pandemic, the samples are to be analyzed by Dr. John Ralph's lab in January 2021.	We developed a robust and high-throughput method for lignin quantification, and are planning a methods protocol for publication in addition to another peer-reviewed publication with the addition of the aromatic profiles generated by the Ralph lab.
2. Assessment of the role of lignin in drought	If lignin in roots is associated with drought, this can then be a desirable trait and selected/screened for in breeding populations.	The parental lines were assessed and protocols established for the greenhouse trials on the BC lines and is being assessed in year 3.	Assessment of the parental lines was completed in 2018 and is being followed by two rounds of greenhouse trials with the backcross lines in year 3 that were delayed until Spring 2021.	The findings and protocols will be reported at field days and in peer-reviewed publications. An abstract was submitted for Lind Field Day in June 2020.
3. Root lignin and soil-borne disease	If lignin in roots is associated with disease resistance as hypothesized, this can then be selected/screened for in breeding populations.	The parental lines have been assessed and protocols established for the greenhouse trial in years 1 and 2 and one round of assessment was performed on the BC population, but that must be repeated.	Assessment of disease resistance was assayed in spring 2019, but needs to be repeated in 2020, but was delayed due to COVID and working remotely for most of the team.	The findings and protocols will be reported at field days and in peer-reviewed publications, in Wheat Life magazine, and via WheatBeat podcasts.