WASHINGTON TURFGRASS SEED COMMISSION
RESEARCH PROPOSAL FOR 2018

New Project Proposal (Yes/No): No **Proposed Duration** (1, 2, or 3 years): 3 years

Project Title: Characterization of vernalization genes and flowering in Kentucky Bluegrass

PI: Michael M. Neff **Co-PI:** None

Organization: Washington State University **Organization:**

Title: Professor, Crop Biotechnology **Title:**

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Cooperators:

Year Initiated: 2017 **Terminating Year**: 2020

Total Project Request: Year 1 $29,912 Year 2 $29,782 Year 3 $ 29,328

Other Funding Sources: *(If no other funding sources are anticipated, type in "None".)*

Agency Name: Brubbaken and Reinbold Monocot Breeding Fund

Amount Requested/Awarded: *(Circle either amount requested or awarded)* *$100,000/yr*

Notes: These funds support our turfgrass breeding program.

Description: (*181 words*)

Objectives: Floral induction is a genetically control event mediated by environmental cues. Kentucky Bluegrass (KBG) requires vernalization and short-day (SD) induction followed by long days (LD) in order to flower and produce seeds. The genes (VRN1, VRN2, VRN3, FDL2) involved in floral induction have been identified and characterized in several species but not in KBG. Herein we propose to:

1) Identify, clone and characterize VRN-related genes in Kentucky Bluegrass.
2) Examine the expression of these genes during vernalization with some varieties that we have identified that do vernalize well compared to ones that do not.
3) Examine the role of specific gibberellin (GA) hormones in inducing flowering with or without vernalization.
4) Examine the role of GAs on expression of VRN-related genes.

Specific Outcomes: At the end of the project, the floral induction of KBG will be genetically characterized. Variants of the main genes will be identified and related to vernalization

requirements. The role of specific GAs on floral induction of KBG will be clarified. In addition, the specific GA's required for accelerating flowering time and seed production will be defined.

Justification and Background: (*391 words*)

Vernalization in KBG and why it should be addressed: KBG is the most widely used cool-season turfgrass in temperate and subarctic climates. KBG requires a double induction for flowering and seed production: one induction at low temperature (vernalization) under SD for several weeks, followed by a second induction at warmer temperatures and long LD (Heide, 1994). The first induction by SD/low temperatures is met by autumn or winter conditions and the second induction under LD typically happens during the spring and summer. Changing climatic conditions with warmer winters could reduce the stimulus for flowering, which would delay flowering time, lower percentage of actual flowers (Heide, 1994) and potentially decrease seed quality and production.

Relationship to other projects: The key floral induction genes (VRN1, VRN2, VRN3, FDL2, CO) have been identified in model and crop species (Trevaskis et al., 2007; Woods et al., 2014; Jokela et al, 2015; Mulki and von Korff, 2016; Woods et al., 2016). However, the specific genetic regulation of the flowering process in KBG has not been described yet, and the genes involved in the vernalization response and flowering process are yet to be identified and cloned. Changes in the floral induction genes could lead to shifting in the time of flowering and variations in vernalization requirements. For example, mutations in the VRN2 gene of winter wheat eliminate the requirement of vernalization (Li et al., 2011). Also, alleles of VRN1 that have high basal levels of VRN1 expression can substitute for vernalization (Trevaskis et al., 2007). In the same way, exogenous application of gibberellins could change the expression of some of the floral induction genes leading to flowering with shorter or no vernalization requirements in LD plants (Mutasa-Göttgens and Hedden, 2009; King, 2012) or to a shorter requirement of the secondary LD induction in species with double-induction requirements (Heide et al., 1998).

Basic approach: We propose to identify, clone and characterize the genes related to flowering induction in KBG. We will investigate the expression levels of the genes in accessions with differential vernalization requirements and the effect of exogenous GAs on gene expression, vernalization and flowering induction. The resulting sequences and variants could be used by breeders as molecular markers and for targeted genetic modification of flowering time. The results of the GA experiments could be used as an agronomic practice for controlling flowering timing and seed production.

Methodology: (*400 words*)

Aim 1- Identify, clone and characterize VRN-related genes in KBG *(month 1 to month 18)*
An RNA-seq approach will be used for the identification and cloning of floral induction genes. Vernalized and not vernalized KBG accessions differing in the vernalization requirements (identified in Dr. Neff's Lab) will be used. Next Generation Sequencing will be used to obtain sequencing data, and *de novo* assembly to obtain contigs. An orthologous search will be performed using related species sequences as a query to identify the homologues in KBG.

In addition, using the sequence information of related species, specific primers for each one of the floral induction genes will be developed. With these primers, the floral genes of KBG will be amplified and sequenced. DNA and cDNA will be used to get information of the genomic

sequence and of the expressed sequences, to obtain the structure of the genes. Once the genes have been identified for one of the KBG varieties, specific primers for those genes will be redesigned and use for amplification and sequencing of the genes of KBG accessions in order to detect specific allelic differences (such as SNPs) which could be responsible for the differential vernalization requirements.

Aim 2- Examine the expression of these genes during vernalization with some varieties that we have identified that do vernalize well (e.g. Jumpstart and an Alaska accession) compared to ones that do not (e.g. Barsweet) ***(month 1 to month 18)*** The RNAseq data will also be used for the differential expression analysis of the floral induction genes. Using the sequence information obtained before, specific fluorescent probes will be designed to further study the expression of the genes by qRT-PCR. Plants with or without the primary and secondary induction of KBG accessions with differential response to vernalization will be used.

Aim 3- Examine the role of GA in inducing flowering with or without vernalization ***(month 18 to month 30)*** Plants of accession with different vernalization requirements will be treated with either GA1 and GA4 (gibberellins related to elongation) or GA5 and GA6 (giberellins related to flowering). The treatments will be applied before, during or after the vernalization treatment. Flowering related variables will then be measured.

Aim 4- Examine the role of GA on expression of VRN-related genes ***(month 30 to month 36)*** RNA of plants under the different GA treatments will be used to determine the differential expression of the floral regulation genes by qRT-PCR.

Anticipated Benefits and Information Transfer: (*100 words maximum)*

The KBG floral induction process will be described. Floral genes and their regulation by vernalization will be identified and characterized. Allelic variants of the genes will be identified in KBG. The potential use of gibberellins for flowering induction and seed production will be assessed. Information on the genes could be used by breeders to identify and produce varieties with differential vernalization requirements and flowering times. GA use could become a regular practice for seed production.

Research results will be transferred by scientific publications, oral presentations to growers and the scientific community. The sequence information will be in publicly available databases.

References:

Heide OM (1994) Control of flowering and reproduction in temperate grasses. New Phytologist 128: 347–362

Heide OM, Blundell C, King RW, Evans LT (1998) Gibberellin substitution for long day secondary induction of flowering in Poa pratensis. PHYSIOLOGIA PLANTARUM 104: 10–16

Jokela V, others (2015) Regulation of flowering and canopy structure in timothy (Phleum pratense L.). UNIVERSITY OF HELSINKI DEPARTMENT OF AGRICULTURAL SCIENCES DOCTORAL PROGRAMME IN PLANT SCIENCES Ph.D.: 51

King RW (2012) Mobile signals in day length-regulated flowering: Gibberellins, flowering locus T, and sucrose. RUSSIAN JOURNAL OF PLANT PHYSIOLOGY 59: 479–490

Li C, Distelfeld A, Comis A, Dubcovsky J (2011) Wheat flowering repressor VRN2 and promoter CO2 compete for interactions with NUCLEAR FACTOR-Y complexes. The Plant Journal 67: 763–773

Mulki MA, von Korff M (2016) CONSTANS Controls Floral Repression by Up-Regulating VERNALIZATION2 (VRN-H2) in Barley. PLANT PHYSIOLOGY 170: 325–337

Mutasa-Göttgens E, Hedden P (2009) Gibberellin as a factor in floral regulatory networks. Journal of Experimental Botany 60: 1979–1989

Trevaskis B, Hemming MN, Dennis ES, Peacock WJ (2007) The molecular basis of vernalization-induced flowering in cereals. Trends in Plant Science 12: 352–357

Woods DP, McKeown MA, Dong Y, Preston, C. J, Amasino RM (2016) Evolution of VRN2/Ghd7-Like Genes in Vernalization-Mediated Repression of Grass Flowering. PLANT PHYSIOLOGY 170: 2124–2135

Woods DP, Ream TS, Amasino RM (2014) Memory of the vernalized state in plants including the model grass Brachypodium distachyon. FRONTIERS IN PLANT SCIENCE 5:99

Budget: *(Indirect or overhead costs are not allowed unless specifically authorized by the Board)*

Budget Item	2018	2019	2020
Salaries[1]	*$17,622.00*	*$18,327*	*$*
Time-Slip	*$5,280*	*$5,491*	*$*
Operations (goods & services)	*$5,194*	*$3,756*	*$*
Travel[2]	*$*	*$*	*$*
Meetings	*$*	*$*	*$*
Other	*$*	*$*	*$*
Equipment[3]	*$*	*$*	*$*
Benefits[4]	*$1,686*	*$1,754*	*$*
Total	***$29,782.00***	***$29,328.00***	***$NA***

Budget Justification:

Salaries and Benefits: This project will support 100% of John Hadish's salary and benefits as a Molecular Plant Sciences Graduate Student. The time slip funds will be used to cover John's summer salary. John has been working on this project since joining the lab in August 2017.

Operations: These funds support the goods and services necessary to complete this project. If additional goods and services funds are necessary, they will be covered by the Brubbaken and Reinbold Monocot Breeding Fund.

WASHINGTON TURFGRASS SEED COMMISSION
PROGRESS REPORT FORMAT FOR 2017 PROJECTS

Project No.: WSU ACCOUNT# 13C-3019-6780

Title: Characterization of vernalization genes and flowering in Kentucky Bluegrass

Personnel: Michael M. Neff Ph.D. (PI) and John Hadish (Graduate Student)

Reporting Period: 7/1/17 – present (4.5 months)

Accomplishments: This project was originally written to support a postdoctoral fellow Dr. Hernan Romero-Angulo, who intended to join the Neff Lab. Due to unforeseen circumstances, Dr. Romero-Angulo decided to not join the lab and work on this project. A new Ph.D. student, John Hadish, joined the Neff lab in August 2017 and began working on this project. In the past three months, John Hadish has cloned fragments of two flowering time genes, VRN1 and VRN3, from the KBG genome. A 275 base-pair sequence fragment of VRN3 was cloned using PCR primers from our collaborator Dr. Ian Burke. A 88 base-pair sequence fragment of VRN1 was cloned using PCR primers that were designed based on genome alignments of known genes from other grass genomes. Using RT-PCR, John has also shown that both are expressed in KBG. He is now working on cloning full-length cDNAs for each of these genes in order to compare their sequences to the varieties Jumpstart and Barsweet as well as the wild Alaskan accession described in our proposal. John will also be able to compare these sequences to known genes from other grasses. In addition, John will continue to attempt cloning other known flowering related genes from the KBG genome.

Results: The VRN3 sequence was compared to other known sequences and was shown to have a 92% identity with the *Lolium perenne* (perennial ryegrass) FT3 gene (an ortoholog of VRN3 in wheat). The sequence was also shown to share high degrees of similarity with orthologous gene sequences previously characterized from *Lolium temulentum*, *L. multiflorum*, *Phleum pratense*, *Festuca pratensis*, *Hordeum vulgare*, *Avena sativa*, and several *Triticum* species. Together, these results indicate that we have successfully cloned VRN3 in KBG. VRN1 was cloned using a primer set designed from a multiple alignment of VNR3/FT1/FT3 genes and corresponding amino acid sequences acquired from genbank. ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align the amino sequences and CEMAsuite (Release 2.0.9) was used to compare the amino acid sequences with corresponding DNA sequences and design primer sets that were conserved across all sequences. The VRN1 was compared to other known sequences and was shown to have a 97% identity with the *Triticum aestivum* VRN-B1 gene and the *Hordeum vulgare* VRN-H1 gene. This is in accordance with past research that indicates there is a high degree of conservation in VRN1. (Shinozuka H, Hand M, Cogan N, Spangenberg G, Forster J (2013) Nucleotide diversity of vernalization and flowering-time-related genes in a germplasm collection of meadow fescue (*Festuca pratensis* Huds. syn. *Lolium pretense* (Huds.) Darbysh.) ECOLOGY AND EVOLUTION 3(13): 4415-4426)

Publications: None at this time.

WASHINGTON TURFGRASS SEED COMMISSION
PROGRESS REPORT FORMAT FOR 2017 PROJECTS

Instructions:
1. Record information for active and pending projects.
2. All current research to which principal investigator(s) and other senior personnel have committed a portion of their time must be listed whether or not salary for the person(s) involved is included in the budgets of the various projects.
3. Provide analogous information for all proposed research which is being considered by, or which will be submitted in the near future to, other possible sponsors.

Name (List PI#1 first)	Supporting Agency and Project #	Total $ Amount	Effective and Expiration Dates	% of Time Committed	Title of Project
	Current:				
Neff, MM	Brubbaken and Reinbold, Inc.	$500,000	11/15/13-11/14/18	5%	"Brubbaken and Reinbold Monocot Breeding Fund"
Neff, MM	USDA-NIFA Foundational Program	$498,000	12/1/13-11/30/17	5%	"Increasing Seed Size and Plant Biomass via Manipulation of the AHL Gene Family"
Neff, MM	Washington State Department of Agriculture Biofuel Cropping Systems Project	$23,000	7/1/17-6/30/18	5%	"Modification of Hypocotyl Length in Camelina and Canola via Manipulation of the AHL Gene Family"
Neff, MM	National Science Foundation	$465,956	7/1/17-6/30/20	5% 5%	"The ATAF2 Transcription Factor, Brassinosteroid Catabolism and Plant Development" "Characterization of vernalization genes

Neff, MM	Washington Turfgrass Seed Commission (This proposal)	$89,022	7/1/17-6/30/20		and flowering in Kentucky Bluegrass”
	Pending: None				