

PROJECT NO: BJKU85, BJKU86

TITLE: Develop Idaho wheat resistant to wireworms by novel RNA interference technology

PERSONNEL: **Daolin Fu (DF)**, **Fangming Xiao (FX)**, Department of Plant Sciences, University of Idaho; **Arash Rashed (AR)**, Department of Entomology, Plant Pathology and Nematology, University of Idaho; **Samuel Hunter (SH)**, Institute for Bioinformatics and Evolutionary Studies (IBEST), University of Idaho

ADDRESS: Daolin Fu, 875 Perimeter Dr., Moscow, ID 83844 (208-885-1542, dlfu@uidaho.edu)

JUSTIFICATION:

Wheat is the second most important cereal crop in the United States. The annual wheat production in the US Pacific Northwest (PNW) is ca. 6.5 million metric tons, and the PNW wheat accounts for a large portion of the US wheat exported abroad. However, many insects feed on cereal plants imposing great threat to wheat production. Wireworms (Coleoptera: Elateridae) are the subterranean larval stage of the click beetle. In recent years, wireworms become prevalent in some cropping systems and pose an increasing economic threat to wheat growers in Idaho. Currently control of wireworms is mainly relying on insecticides which is costly and environmentally harmful. Thus, development of wireworm-resistant germplasm in wheat is urgently needed to safeguard this important Idaho industry.

RNA interference (RNAi) offers a valid tool for insect pest management because it can disrupt insect gene expression when taken up by insect. As one type of RNAi technology, host-induced gene silencing (HIGS) utilizes transgenic plant to express specific double-stranded RNA (dsRNA) that targets vital insect genes. HIGS becomes a promising approach to improve insect resistance. Thus, generation of transgenic wheat lines expressing wireworm-essential dsRNA would be a novel genetic strategy to develop wheat cultivars resistant to wireworms in Idaho, thereby ultimately leading to sustainable wheat production in Idaho.

RNAi strategy relies on short, perfectly matching RNA sequences. One consideration is whether the RNAi works robustly across different populations of a species, but not causing harm to people and non-target organisms. It is important to understand the genetic diversity that exists within the wireworm species, especially when wireworms are present across a large geographic range in PNW.

HYPOTHESIS & OBJECTIVES:

The hypothesis driving this project is that *in planta* expression of the wireworm-essential dsRNA would lead to knockdown of the wireworm gene expression upon feeding, thereby conferring resistance to wireworms, and generation of such transgenic wheat lines would be a novel genetic strategy to develop resistant wheat cultivars. Towards this end, we propose four objectives: 1) understand genetic diversity of predominant wireworm species in Idaho; 2) determine and optimize dsRNA of vital genes that are lethal to wireworms; 3) generate transgenic wheat lines expressing dsRNA that targets vital genes of wireworms; 4) characterize and verify transgenic wheat lines for improved resistance to wireworms.

PROCEDURES:

The main approach for our objectives is to identify dsRNA matching wireworm essential genes by PCR-based molecular cloning and introduce the dsRNA construct into Idaho wheat cultivar(s) via transformation technique to disrupt these genes' expression in the feeding wireworms.

1: Understand genetic diversity of predominant wireworm species in Idaho (SH).

The Idaho Wheat Commission has funded a project to generate RAD sequencing and genomic data for one predominant wireworm species, *Limonijs californicus*. This information will be used to characterize the *L. californicus* and other genetically diverse populations of wireworm so that appropriate testing can be carried out to confirm species specificity and efficacy of the designed RNAi triggers. During development, existing data and samples housed within the IBEST Genomics Resources Core will be leveraged to confirm sequence identity within *L. californicus* populations and to predict whether closely related species may be responsive to RNAi treatment.

2: Determine and optimize dsRNA of vital genes that are lethal to wireworms (FX & AR).

We will target genes essential for wireworm viability to generate the dsRNA constructs. Based on success on other insects (Ulrich et al., 2015), we intend to select genes coding for proteasome, β -actin, and others (Table 1). In total, 10 target genes will be tested. Unique DNA fragments of selected candidate genes will be obtained by PCR and cloned into the RNA *in vitro* expression vectors. Double stranded RNA containing the selected gene fragments will be synthesized using either the commercial service or the *in vitro* expression kits. The effect of dsRNA on wireworm viability will be determined by feeding or injecting dsRNA to wireworms.

3. Generate transgenic wheat expressing dsRNAs that targets vital genes of wireworms (DF).

In *Tribolium castaneum*, Ulrich et al. identified 11 novel and highly efficient RNAi targets, which caused 80-100% mortality to the injected pupae and larvae (Ulrich et al., 2015). Using the *T. castaneum* queries, we have identified eight wireworm homologues (Table 1) in the current wireworm sequence database (IWC wireworm sequencing project). Hopefully, these beetle genes are conserved both in structure and function. We will use these eight genes to generate stable transformation in wheat.

RNAi triggers (200 to 300 bp) will be selected for each target gene. Four gene fragments will be stacked together to generate a 1 to 1.2-kb RNAi trigger. The final RNAi triggers will be cloned into the pANDA vector (Miki and Shimamoto, 2004). RNAi constructs will be used to generate transgenic wheat as described in an established protocol (Lv et al., 2014). For each RNAi construct, at least ten independent T₀ plants will be generated, and they will be advanced to T₁ and higher generations. Positive transgenic plants will be tested for wireworm resistance.

4. Characterize and verify transgenic wheat for improved resistance to wireworms (DF & AR).

Transgenic wheat plants will be verified by PCR using the vector-specific primers. Positive transgenic lines will be further determined for the best expression of the dsRNA. A minimum of 5 transgenic lines are expected to be available for the next stage of this proposal. Once identified, transgenic plants positive for RNAi transcripts will be grown in greenhouse and their interaction

with wireworms will be tested according to previous publications. A minimum of 20 plant replicates, per genetic line, would be available by the spring of 2019. The efficacy of the transgenic lines will be assessed against the sugar beet wireworm, *Limonius californicus*. Evaluations will be conducted at both seed (N = 10) and seedling (N = 10) stages of each of the transgenic wheat lines. Wireworms will be collected from cereal fields in central and eastern Idaho. Wireworms will be maintained in the greenhouse on pure sand, for a 2-week period, prior to the experiments. Transgenic grain seeds will be planted in 1-gallon pots filled with a mix of 30% peatmoss and 70% sand.

Wireworms will be exposed to seed/seedlings (and corresponding non-transgenic controls), for a 3-week period. Then, pots will be inspected to recover wireworms. Wireworms, which were collected alive, will be placed in sand monitored over-time to estimate rates of mortality. Wireworm survivorship will be compared across transgenic treatments and between wireworms exposed to transgenic and non-transgenic plants, using Kaplan-Meier analysis in SPSS. All experiments will be conducted in two time blocks, and time block will be treated as random factor in all of our analyses.

Table 1. *Tribolium castaneum* RNAi targets and their homologues in wireworm

<i>T. castaneum</i> accessions	Gene description	<i>T. castaneum</i> vs. wireworm cDNA identity (%)	<i>T. castaneum</i> vs. wireworm Protein identity (%)
TC002574	Signal recognition particle 54 (srp54k)	78.15	95.47
TC011120	Ras opposite (rop)	73.82	87.48
TC013571	alpha-soluble NSF attachment protein (alpha snap)	73.31	87.33
TC011058	Dynamin-like Protein (2C)	74.25	89.60
TC015321	Serine/threonine-protein phosphatase alpha-2 (6C)	78.51	98.17
TC004425	Heat shock 70 kDa protein cognate 3- like Protein (MC)	76.9	94.50
TC006375	26S proteasome non-ATPase regulatory subunit 6-like Protein	71.66	82.52
TC006679	<i>Tribolium castaneum</i> protein Gawky (GW)	69.79	71.82
Pending	β -Actin	Pending	Pending

DURATION: One year (FY 2019), Objectives 1 - 4

COOPERATION: Daolin Fu (PI), and Co-PIs (Fangming Xiao, Arash Rashed, and Samuel Hunter) will lead the project and work with visiting scholars and/or graduate students on the gene search, expression profiles and data mining. PI and Co-PIs will present research findings at professional conferences, and communicate research progress and results to the Idaho Wheat Commission. Visiting scholars, graduate students and other personnel working on data collection

will reports progress to PI and Co-PIs. The research team will regularly communicate via e-mail, phone, and face-to-face meetings. Wheat wireworm resistance will be measured in facilities accessible to the University of Idaho.

ANTICIPATED BENEFITS/EXPECTED OUTCOMES/INFORMATION TRANSFER:

Thousands of growers and stakeholders will benefit from these efforts. Beneficiaries of this project will be all growers affected or potentially affected by wireworms, as well as the Idaho wheat industry as a whole by providing new genetic material in wheat for resistance to wireworms.

In addition, the generation of wireworm-resistant wheat by transgenic expression the wireworm-essential dsRNA will provide proof-of-concept and help frame future approaches that might be utilized to realize significant gains in wheat production.

At last, the success of the project will be measured by presentation of research results at the annual American Phytopathology Meeting (over 1,500 scientist attendants) and publication of 1-2 research articles on the peer-reviewed journals.

LITERATURE REVIEW:

RNA interference (RNAi) is a post-transcriptional gene-silencing mechanism where exogenous double-stranded (dsRNA) knockdown genes by triggering degradation of target mRNA in cells (Carthew and Sontheimer, 2009). Recently, RNAi-based disruption of gene expression has been developed as a new technology for biological control of some insects, especially those that belong to order Coleoptera (Baum et al., 2007; Tomoyasu et al., 2008), which contains the wireworm species. In particular, dsRNA fed to insects can be taken up by midgut cells and processed into 21-bp small interfering RNAs (siRNAs) by the so-called Dicer complex-mediated cleavage. When the resulting siRNAs encounter the mRNA transcribed from the cognate insect gene, RNA interference will occur and the expression of the insect's gene will be disrupted. If the targeted gene is essential for the insect's basic physiology and viability, such RNA interference might result in the death of insect. Thus, by targeting essential insect genes, dsRNAs can be developed into highly species-specific insecticides.

The overall process of dsRNA-based insect gene-silencing includes delivery of dsRNA, dsRNA uptake and intracellular transport, dsRNA processing to siRNA, binding and digestion of target mRNA. Among these steps, delivery of dsRNA is the most practically challenging in terms of the relevance to crop protection. Originally, RNAi in insect was elucidated by injecting dsRNA directly into the organism of interest. Recently, ingestion of *in vitro* synthesized dsRNA has been shown to be effective in several insect species (San Miguel and Scott, 2016). However, such synthesized dsRNA insecticides are very costly and have limited life-time on the surface of plant leaves. Thus, an *in-planta* expression of dsRNA should be an ideal method to delivery dsRNA into insects fed on crops, especially the field-grown crops. An exciting recent paper has showed that transgenic potato plants, which express the dsRNA targeting the β -actin gene of Colorado potato beetle, exhibit complete resistance to this notorious pest (Zhang et al., 2015). Therefore, expression of dsRNAs targeted against insect genes in transgenic plants is an effective method to protect crops against the insects belonging to the order Coleoptera, including wireworms.

REFERENCES:

- Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O, Johnson S, Plaetinck G, Munyikwa T, Pleau M, Vaughn T, Roberts J** (2007) Control of coleopteran insect pests through RNA interference. *Nat Biotechnol* **25**: 1322-1326
- Carthew RW, Sontheimer EJ** (2009) Origins and Mechanisms of miRNAs and siRNAs. *Cell* **136**: 642-655
- Lv B, Nitcher R, Han X, Wang S, Ni F, Li K, Pearce S, Wu J, Dubcovsky J, Fu D** (2014) Characterization of *FLOWERING LOCUS T1 (FT1)* gene in *Brachypodium* and wheat. *PLoS ONE* **9**: e94171
- Miki D, Shimamoto K** (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant and Cell Physiology* **45**: 490-495
- San Miguel K, Scott JG** (2016) The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest Manag Sci* **72**: 801-809
- Tomoyasu Y, Miller SC, Tomita S, Schoppmeier M, Grossmann D, Bucher G** (2008) Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*. *Genome Biol* **9**: R10
- Ulrich J, Dao VA, Majumdar U, Schmitt-Engel C, Schwirz J, Schultheis D, Ströhlein N, Troelenberg N, Grossmann D, Richter T, Dönitz J, Gerischer L, Leboulle G, Vilcinskas A, Stanke M, Bucher G** (2015) Large scale RNAi screen in *Tribolium* reveals novel target genes for pest control and the proteasome as prime target. *BMC Genomics* **16**: 674
- Zhang J, Khan SA, Hasse C, Ruf S, Heckel DG, Bock R** (2015) Pest control. Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids. *Science* **347**: 991-994

IDAHO WHEAT COMMISSION - BUDGET FORM

Principal Investigator: Daolin Fu

Allocated by Idaho Wheat Commission during FY 2017 \$

Allocated by Idaho Wheat Commission during FY 2018 \$ 48,000

REQUESTED FY2019 SUPPORT:

	Salary (staff, post-docs, etc.)	Temporary Help	Fringe	Travel	OE	Graduate Tuition/Fees	TOTALS
Idaho Wheat Commission	\$ -	\$ 20,000	\$ 1,500	\$ 3,000	\$ 25,000	\$ -	\$ 49,500

TOTAL BUDGET REQUEST FOR FY 2019:

\$ 49,500

BREAKDOWN FOR MULTIPLE SUB-BUDGETS:

	Daolin Fu	Fangming Xiao	Arash Rashed	Samuel Hunter
Salary	\$ -	\$ -	\$ -	\$ -
Temporary Help	\$ 10,000	\$ 10,000	\$ -	\$ -
Fringe Benefits	\$ 750.00	\$ 750.00	\$ -	\$ -
Travel	\$ -	\$ -	\$ 2,000	\$ 1,000
Operating Expenses	\$ 9,000	\$ 9,000	\$ 3,000	\$ 4,000
Graduate Student Tuition/Fees	\$ -	\$ -	\$ -	\$ -
TOTALS	\$ 19,750	\$ 19,750	\$ 5,000	\$ 5,000

Total Sub-budgets \$ 49,500

Explanatory Comments: (see FY2019 RFP for definition)

Fall 2017 Version

ANNUAL REPORT

PROJECT NO: BJKU85, BJKU86

TITLE: Develop Idaho wheat resistant to wireworms by novel RNA interference technology

PERSONNEL: Jiwen Qiu, Yulin Yuan, Fangming Xiao, Daolin Fu, Department of Plant Sciences, University of Idaho

ADDRESS: Daolin Fu, 875 Perimeter Dr., Moscow, ID 83844; 208-885-1542; dlfu@uidaho.edu

ACCOMPLISHMENTS:

Wheat transformation facility: Tissue culture and plant transformation are critical steps of this project. In 2017, we established the Genome Editing and Transformation (GET) lab which allows us to perform biolistic DNA delivery and *Agrobacterium*-mediated DNA transfer. The GET lab will meet our demand on wheat tissue culture and wheat transformation to achieve the Host Induced Gene Silencing (HIGS).

RNAi targets of the wireworm: HIGS is effective for the Colorado potato beetle (Zhang *et al.*, 2015). However, there is no preliminary report on wheat HIGS against the wireworms, the larvae of click beetles (Coleoptera: Family Elateridae). It is essential to target critical wireworm genes. Interestingly, Ulrich *et al.* identified 11 novel and highly efficient RNA interference (RNAi) targets (Table 1) in red flour beetle (*Tribolium castaneum*), which caused 80-100% mortality to the injected pupae and larvae (Ulrich *et al.*, 2015). Hopefully, the beetle genes are conserved both in structure and function.

In collaboration with Dr. Sam Hunter and Dr. Arrash Rashed, we used the *T. castaneum* genes as queries to search the wireworm sequence database (IWC wireworm sequencing project). Many homologues were found in the wireworm genome, but only eight wireworm homologues displayed more than 70% identity to the *T. castaneum* proteins (Table 1). They will be pursued for RNAi constructs using selected fragments of each target (Table 2).

Actin proteins are abundant and highly conserved in all eukaryotic cells. They play essential roles in a variety of cellular processes. HIGS against the β -*Actin* gene is effective on the Colorado potato beetle (Zhang *et al.*, 2015). We will also identify the wireworm β -*Actin* genes and use them for HIGS.

Cloning of the wireworm RNAi triggers: To avoid wheat endogenous targets, we searched the wireworm sequences in the wheat whole genome database and eliminated the homologous regions. The non-homologous regions were selected as RNAi triggers. Accordingly, specific primers were designed to clone these fragments (Table 2). The RNAi triggers (250-350 bp) were amplified by PCR (Fig 1).

Four gene fragments will stacked together to generate a 1 to 1.2-kb RNAi trigger. The final RNAi triggers will be cloned into the pANDA vectors (Miki & Shimamoto, 2004). The RNAi constructs will be transformed into wheat. Positive transgenic plants will be tested for wireworm resistance.

Table 1. *Tribolium castaneum* RNAi targets and their homologues in wireworm

<i>T. castaneum</i> accessions	Gene description	<i>T. castaneum</i> vs. wireworm cDNA identity (%)	<i>T. castaneum</i> vs. wireworm Protein identity (%)
1: TC002003	Cactus (Cact)	60.79	56.77
2: TC002574	Signal recognition particle 54 (srp54k)	78.15	95.47
3: TC011120	Ras opposite (rop)	73.82	87.48
4: TC013571	alpha-soluble NSF attachment protein (alpha snap)	73.31	87.33
5: TC011058	Dynamin-like Protein (2C)	74.25	89.60
6: TC015321	Serine/threonine-protein phosphatase alpha-2 (6C)	78.51	98.17
7: TC008263	Kinesin-like protein KIF11-A (5C)	57.22	51.79
8: TC004425	Heat shock 70 kDa protein cognate 3- like Protein (MC)	76.9	94.50
9: TC006375	26S proteasome non-ATPase regulatory subunit 6-like Protein	71.66	82.52
10: TC006679	<i>Tribolium castaneum</i> protein Gawky (GW)	69.79	71.82
11: TC007999	26S protease regulatory subunit 4-like Protein (MC)	64.1	63.76
12: Pending	β -Actin	Pending	Pending

Table 2. RNAi triggers for wireworm

Trigger ID	<i>T. castaneum</i> accessions	Wireworm sequence ID	Primers for the RNAi trigger (5' → 3')
HIGS1	TC015321	Wireworm_k25_Locus_1327 Transcript_1_1	FP: TCAAACCTTTGGAAGACATTCACA RP: CTCGGCCCCAAAAGTAAAAC
HIGS2	TC002574	Wireworm_k25_Locus_2690 Transcript_1_1	FP: GAAGCCGTTTCGTAAGCAAAC RP: AGCTCTCCGTCATTCATGCT
HIGS3	TC004425	Wireworm_k49_Locus_53 Transcript_3_1	FP: CGTGCTTTGTCTTCCAGTCA RP: GCTTGAACGTGTCACCATA
HIGS4	TC011058	Wireworm_k49_Locus_2081 Transcript_4_1	FP: AGTGGAGACAATCCGCAATC RP: AACAGGTGGTGGAACTGGAG
HIGS5	TC011120	Wireworm_k25_Locus_5101 Transcript_1_1	FP: GCTTGCTGTAAAATGCACGA RP: CACCTGCTGTTCACCTGGGTA
HIGS6	TC013571	Wireworm_k25_Locus_7647 Transcript_1_1	FP: GCATTACGAACAAGCTGCTG RP: GTAGCGTTCCAAGGCATGTT
HIGS7	TC006375	Wireworm_k49 Locus_274_Transcript_1_1	FP: AGAAGGGCTCGAAAAGAACC RP: CTGCGTCTTCAATTGCTTCA
HIGS8	TC006679	Wireworm_k25_Locus_4716 Transcript_3_1	FP: CCAGCAACAATCGAGACTGA RP: CAGGGTTTTCTGTTCAAA

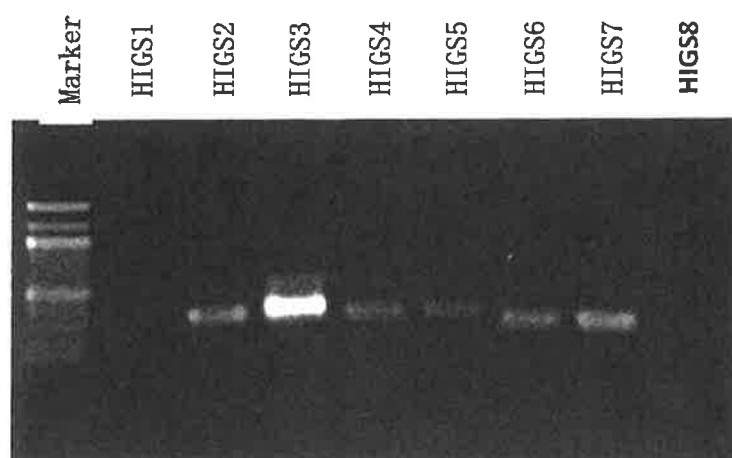


Figure 1 PCR clones of the wireworm RNAi triggers

PROJECTIONS:

In the past six months, we first established a wheat transformation platform. Using the *Tribolium* RNAi target genes, we searched the wireworm database and identified eight close homologues to be used for RNAi. We will generate transgenic wheat that targets to the selected wireworm genes. The selected genes and transgenic wheat will be studied in the second year project, for which we will submit a renewal proposal to the Idaho Wheat Commission.

PUBLICATIONS: NO

REFERENCES:

- Miki, D. and Shimamoto, K. (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant and Cell Physiology*, **45**, 490-495.
- Ulrich, J., Dao, V. A., Majumdar, U., Schmitt-Engel, C., Schwirz, J., Schultheis, D., *et al.* (2015) Large scale RNAi screen in *Tribolium* reveals novel target genes for pest control and the proteasome as prime target. *BMC Genomics*, **16**, 674.
- Zhang, J., Khan, S. A., Hasse, C., Ruf, S., Heckel, D. G. and Bock, R. (2015) Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids. *Science*, **347**, 991-994.

water inputs, and irrigation will be applied based on the spring cereal ET from the AgriMet system (located on the research station) on a weekly basis.

During the growing season, crop physiological measurements will be taken in each plot at stages of booting, heading, anthesis, and grain-filling to explain the impact of water deficiency on crop growth and development. Measurements include normalized difference vegetation index (NDVI) (GreenSeeker, Trimble Inc. Sunnyvale, CA), leaf area index (LAI) (AccuPAR LP-80 unit, Decagon Devices Inc. Pullman, WA), canopy temperature (Apogee infrared radiometer, Apogee Instruments, Inc. Logan, UT), and photosynthetic rate (LI-COR 6400 portable photosynthesis system, LI-COR Inc. Lincoln, NE).

Prior to combining, plant samples will be harvested from each plot and separated into component samples of leaves, stems, and grain heads, and oven dried at 70°C to determine dry biomass. Grain yield components (i.e., grain number per spike, spike numbers in a 3-ft row, and thousand kernel weight, etc.) will also be determined. Water use efficiency (WUE) will be calculated as grain yield divided by seasonal water use (including soil moisture depletion, irrigation, and rainfall) in each cultivar under different irrigation treatments. Grain quality will be evaluated by measuring grain protein, falling number, and in baking tests. Grain yield, yield components, quality, and WUE will be statistically analyzed using ANOVA, and significance of means will be established from multiple comparisons to identify cultivars and irrigation treatments with high yield, quality and WUE.

DURATION: 3 years

COOPERATION:

Katherine O'Brien, University of Idaho Wheat Quality Lab, Aberdeen R&E Center, Aberdeen, ID

ANTICIPATED BENEFITS/EXPECTED OUTCOMES/INFORMATION TRANSFER:

Compared with the 100% ET irrigation treatment, 50-100% ET is expected to maintain grain yield and increase WUE. Cultivars may differ in grain quality under different treatments, and the best deficit irrigation strategy may differ among cultivars and wheat class. Results from this study will provide best strategies for adopting deficit irrigation in spring wheat production in southern Idaho, and recommendations can be provided on reduced irrigation input to maximize economic return in spring wheat production. Results of this research will be communicated to growers and researchers through cereal schools, newsletters, websites, progress reports, conferences, and refereed journal publications.

LITERATURE REVIEW:

Deficit irrigation deliberately imposes drought stress on crops throughout the season, such as irrigating at 75% of recommended crop use rates, maintaining soil water content at 50-60% field capacity, or decreasing irrigation frequency season long or at targeted crop growth stages (Rowland et al., 2012; Du et al., 2010). Deficit irrigation to induce moderate drought stress has been shown to maintain crop yield and quality while improving crop WUE (Du et al., 2010; Masoero et al., 2013; Rowland et al., 2012). For instance, peanuts grown under 75% of recommended irrigation rates throughout the season produced similar kernel yield to those grown under a previously determined full irrigation rate (Rowland et al., 2012). In another study, a 26% reduction in water availability did not affect biomass production, chemical composition, and degradability of forage maize (Masoero et al., 2013). Winter wheat maintained at soil water