Engineering Camelina sativa for Biofuel Production via increasing oil yield and tolerance to abiotic stresses

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ENGINEERING CAMELINA SATIVA FOR BIOFUEL PRODUCTION VIA INCREASING OIL YIELD AND TOLERANCE TO OXIDATIVE/ABIOTIC STRESSES

A Thesis Presented

By

KENNY K. ABLORDEPPEY

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

September 2014

Molecular and Cellular Biology
ENGINEERING CAMELINA SATIVA FOR BIOFUEL PRODUCTION VIA INCREASING OIL YIELD AND TOLERANCE TO OXIDATIVE/ABIOTIC STRESSES

A Thesis presented

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To my family
ACKNOWLEDGMENTS

Special thanks to Dr. Om Parkash, my advisor, whom I am very grateful for providing me with the opportunity to complete this thesis in his lab. I could not have done this without his generous support, and thoughtful guidance during the writing process.

Thanks to all my thesis committee members, Dr. Gross, my undergraduate advisor and Dr. Schnell, my professor for their critical comments and valuable advice during this process.

Hesham Abdullah, Chuanxin Ma, Parul Tomar, Parisa Akbari and all the wonderful undergraduates in Parkash’s lab, thank you for the great experience and excellent atmosphere provided during this research. I could not have achieved all this without your excellent assistance during some of the stressful times.
ABSTRACT

ENGINEERING CAMELINA SATIVA FOR BIOFUEL PRODUCTION VIA INCREASING OIL YIELD AND TOLERANCE TO OXIDATIVE/ABIOTIC STRESSES

SEPTEMBER 2014

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In an effort to engineer *Camelina sativa* for enhanced oil yield and tolerance to abiotic stresses, we have cloned and overexpressed Camelina γ-Glutamyl Cyclotransferase (*GGCT2;1*); a gene involved in oxidative stress tolerance via glutathione homeostasis and Wrinkled 1; a transcription factor that regulates genes involved in fatty acid biosynthesis to increase triacylglycerol (TAG) accumulation in seeds. The *GGCT* gene family in Camelina consists of three genes-*GGCT1, GGCT2;1* and *GGCT2;2*. Camelina *GGCT* genes showed differential expression under oxidative stress caused as a result of exposure to various abiotic stresses. The *GGCT2;1* gene, which showed strong up-regulation, was selected as a candidate gene for further characterization. RT-PCR analysis of overexpressed *GGCT2;1* in leaf tissues from individual T1 seedlings shows increased transcript levels compared to wildtype. T1 plants overexpressing *GGCT2;1* are maturing in the greenhouse and T2 seeds from T1 plants will be used for further analysis for oxidative stress tolerance. The Camelina *WRII* gene showed expression in the early stages of seed development. *WRII* was overexpressed under a seed-specific promoter and
10 independent T1 lines showing DsRed fluorescence were selected. These T1 plants are growing in the greenhouse. In future studies, after characterization of T3 lines of each gene, homozygous T3 lines will be cross pollinated to combine the expression of both genes in single line. These lines will be subject to further analyses for their tolerance to various abiotic stresses and enhanced oil yield. We will compare individual transgenic lines’ total seed weight, total biomass and total oil accumulation per plant with that of wildtype under normal and oxidative stress conditions.
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CHAPTER 1

CHARACTERIZATION OF A γ-GLUTAMYL CYCLOTRANSFERASE (GGCT) GENE IN CAMELINA SATIVA FOR ENHANCED TOLERANCE TO OXIDATIVE STRESS

1.1 Introduction

Oxidative stresses caused as a result of abiotic stresses such as drought, high salt concentration, and heavy metal toxicity have great effect on crops, preventing them from reaching their full yield potential (Boyer, 1982; Edmeades, 2008). There has been intensive research, using genetic approaches such as gene transfer to aid crops become more tolerant to these environmental stresses. Studies from Kasuga et al., (1999) and Jaglo-Ottosen et al., (1998) have shown that overexpression of transcription factors like DEHYDRATION-RESPONSIVE ELEMENT BINDING-1A (DREB1A) and C-BOX BINDING FACTOR-1 (CBF1) led to increased plant (Arabidopsis thaliana) tolerance to cold stress, drought and salt. In rice (Oryza sativa), overexpression of its calcium-dependent protein kinase (CDPK) resulted in tolerance to salt and drought stress (Saijo et al., 2000). It has been reported that the ability of plants to tolerate some of these stresses is associated with the role of glutathione (GSH). GSH is an antioxidant crucial for biotic and abiotic stress management in all living cells. Plants detoxify heavy metals through a Glutathione dependent pathway (Dhankher et al., 2002; Cobbett 2000). Toxic metals such as cadmium (Cd) cause transient depletion of GSH and inhibition of antioxidative enzymes like glutathione reductase (Schutzendubel et al., 2001). According to the same study by Schutzendubel et al., plants with improved capacities for GSH synthesis displayed higher cadmium tolerance. GSH prevents damages to essential cellular
components caused by reactive oxygen species (ROS) such as free radicals and peroxides (Pompella, 2003). GSH acts as a ubiquitous reducing agent in reductive mechanisms involved in protein and DNA synthesis, transport processes, enzyme activity, and metabolism (Oakley et al., 2008). GSH homeostasis in living cell is maintained by the gamma-glutamyl cycle (Figure 1.1), involving GSH synthesis and degradation and recycling of component amino acids.

In *A. thaliana*, the gene of interest, gamma-glutamyl cyclotransferase (GGCT) encoding an enzyme which catalyzes the formation of pyroglutamic acid from dipeptides containing gamma-glutamyl is encoded by a ChaC-like protein with a cation transport regulator-like domain (Paulose et al., 2013). There are three paralogs of this ChaC-like proteins in Arabidopsis (*ChaC1, ChaC2;1* and *ChaC2;2* now referred to as *AtGGCT1, AtGGCT2;1* and *AtGGCT2;2* respectively). Overexpressed lines of *AtGGCT2;1* have been characterized to show high tolerance to arsenic (As) and Cd treatments due to enhanced GSH turnover and homeostasis during heavy metal toxicity by recycling glutamic acid (Paulose et al., 2013).

We have identified a homolog of *AtGGCT2;1* gene in *Camelina sativa* genome with amino acid sequence identity of over 94 percent. *C. sativa*, referred hereafter as Camelina, is a non-food crop that has great potential for biofuel. Also known as gold of pleasure, or false flax, Camelina is a flowering plant belonging to the Brassicaceae family. This annual plant has been proposed to be grown on marginal/waste lands to increase oil yield for the production of biofuel at a lower cost and also as a substitute for petroleum-based fuel. Mature seeds of Camelina contain over 30 wt % of oil. For
Camelina to grow well on marginal land, it has to be able to tolerate oxidative and abiotic stresses such as heavy metal toxicity, drought, high salt concentration and many other stresses present in the environment. However, no effort has been made to engineer Camelina to increase tolerance to the environmental stresses, which could enable this crop to grow on marginal lands.

In Camelina, we have identified three \textit{GGCT} genes (\textit{CsGGCT1}, \textit{CsGGCT2;1} and \textit{CsGGCT2;2}), that are homologous to the \textit{AtGGCT} genes. In this study, we investigated the differential regulation of Camelina gamma-glutamyl cyclotransferase genes under various abiotic and heavy metal stresses. To characterize their function in Camelina, we have isolated and overexpressed a candidate gene \textit{CsGGCT2;1} for enhanced tolerance to oxidative stress caused by heavy metals (As, Cd, Hg, etc), abscisic acid, salt and drought stresses.
Figure 1.1. Gamma-glutamyl cycle in plants for GSH homeostasis and efficient recycling of Glutamate. Adapted from Paulose et al., (2013).
1.2 Materials and Methods

1.2.1 Plant materials and Stress Treatments

Camelina seeds were germinated and grown in vermiculite under greenhouse conditions (16/8 hr light/dark cycles at 25°C). Two weeks old seedlings were transferred to hydroponic solution containing half strength Hoagland modified Basal salt mixture (PhytoTechnology) and allowed to acclimatize for 4-5 days. To initiate stress treatments, old Hoagland solution was replaced with fresh solution supplemented with 25 µM sodium arsenite (AsIII), 40 µM mercury chloride, 75 µM cadmium chloride, 150 mM NaCl (salt stress), 150 mM mannitol (hyperosmotic stress), or 2 mM abscisic acid (hormone stress). Shoot and root tissues were harvested from each treatment at different times (6, 12, 24, or 48 hours after stress induction) and Hoagland medium only control was conducted in parallel. The harvested samples were placed in liquid nitrogen and kept at -80 °C prior to RNA isolation.

1.2.2 RNA Isolation and Differential expression of GGCT genes

Total RNA from root and shoot tissues were extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich), and first strand cDNA was synthesized from total RNA using a Thermo Scientific cDNA kit following the manufactures’ protocol. RNA and cDNA were quantified using NanoDrop spectrophotometry (ThermoScientific, West Palm Beach, FL). Quantitative real-time PCR was performed following the instructions for the Mastercycler ep realplex (Eppendorf) using gene-specific primers with Absolute Blue QPCR SYBR Green Mix (Thermo Fisher Scientific). The primers were designed from gene specific 3’ UTR region of the three GGCT genes sequences (Table 1-1). The
qRT-PCR amplification program was 95 °C for 5 min (1 cycle); 95 °C for 15 seconds, 60 °C for 45 seconds, 72 °C for 1 min (32 cycles); and final extension at 72 °C for 10 min (1 cycle). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All reactions were performed in triplicate, and Actin2 gene primers were used for normalization.

1.2.3 Gene Isolation and Cloning

A Complementary DNA (cDNA) library generated from Camelina wildtype leaves was used to perform PCR with sense and antisense primers (5’ TACGTCGTCGACGTTTAA 3’), (5’ AGATTCGAATCATGATACAAAGACTCTTTGC 3’) designed from Camelina GGCT2;1 (Csa08g024050.1, www.Camelinadb.ca) gene coding sequences. The PCR amplification conditions were: 94°C for 2 min (1 cycle); 94°C for 45 sec, 55°C for 1 min, 72 °C for 45 sec (40 cycles); final extension at 72°C for 10 min (1 cycle). The resulting 654 base pair PCR product was gel purified using Zymoclean Gel DNA Recovery Kit (Zymo research) and ligated into pGem-T easy cloning vector (Promega). The insert was verified by sequencing and then subcloned into the binary vector pCambia Redseed using Sal1 and Xba1 sites for overexpression in plants. The expression of CsGGCT2;1 gene is driven under an enhanced 947 base pair 35S promoter. The 35S promoter was amplified from the pEarlygate 103 plasmid via PCR with primers (5’ TAGCTGGGGCCCGGCGCG 3’), (5’ CGTCGACACTAGTTCCCTCTCCTCAAATGAAA 3’) and after sequence verification, it was subsequently sub-cloned at Apa1 and Sal1 sites of pCambia Redseed vector. The pCambia RedSeed vector contains a DsRed reporter gene and Hygromycin gene for transgenic seed selection using a DsRed filter or hygromycin antibiotics. The resulting plasmid pCambiaRedSeed/35S::CsGGCT2;1
(Figure 1.2) was transformed into plants for driving constitutive expression of the
CsGGCT2;1 gene.
Figure 1.2. pCambia Redseed vector harboring the E35S promoter and the Camelina GGCT2;1 construct.
1.2.4 Plant Transformation

Plasmid pCambiaRedSeed/35S::CsGGCT2;1 was transferred into *Agrobacterium tumefaciens* strain GV3101 using the heat shock method at 37°C for 5 min. Camelina plants in their early flowering stage were transformed with the cells harvested from the Agrobacterium transformation, and suspended in vacuum infiltration medium (2.2 g/L MS, 50 g/L sucrose, 2 mg/L Benzylaminopurine (BAP), pH 5.7, 0.05% (v/v) Silwet L77 (Lehle Seeds, Round Rock, TX, USA)).
1.3 Results

1.3.1 Identification and sequence analysis of Camelina *GGCT* gene family

Amino acid sequences of Arabidopsis GGCT2;1 extracted from the TAIR database was blasted on Camelina database and the highest sequence match was identified and used in this study. Comparison of the amino acid sequences of Arabidopsis and rice *GGCT* genes (Figure 1.3), identified three *GGCT* genes with over 50% match in the Camelina genome (Figure 1.4). Phylogenetic analyses showed close evolutionary ancestry of Camelina *GGCT2;1* with *AtGGCT2;1* as well as with its paralog *AtGGCT2;2*.
Figure 1.3. Multiple sequence alignment of GGCT amino acids from *Arabidopsis thaliana* (AtGGCT1, AtGGCT2;1, AtGGCT2;2), *Camelina sativa* (CsGGCT1, CsGGCT2;1, CsGGCT2;2), and *Oryza sativa* (OsGGCT1, and OsGGCT2). AtGGCT1 (AT1G44790, NM_103560), AtGGCT2;1 (AT5G26220, NM_122523), AtGGCT2;2 (AT4G31290, NM_119278), CsGGCT1 (Csa17g071090), CsGGCT2;1(Csa08g024050), CsGGCT2;2 (Csa12g015850), OsGGCT1 (NP_001052924), OsGGCT2 (NP_001046811)
**Figure 1.4.** Phylogenetic Analysis of GGCT proteins with ChaC-like domains from Arabidopsis, rice and Camelina using CLC genome workbench 7 software with maximum likelihood phylogeny. Accession numbers of sequences; AtGGCT1 (AT1G44790, NM_103560), AtGGCT2;1 (AT5G26220, NM_122523), AtGGCT2;2 (AT4G31290, NM_119278), CsGGCT1 (Csa17g071090), CsGGCT2;1(Csa08g024050), CsGGCT2;2 (Csa12g015850), OsGGCT1 (NP_001052924), OsGGCT2 (NP_001046811)
1.3.2 Differential Expression of Camelina GGCT1, GGCT2;1, and GGCT2;2 relative transcripts

1.3.2.1 Expression of GGCTs in response to heavy metal stress

Camelina seedlings were transferred into hydroponic system containing half strength Hoagland solution supplemented with various heavy metal and abiotic stresses (Figure 1.5). Analysis of GGCT1 relative transcript levels in root and shoot under As(III), Cd and Hg stresses are represented in Figure 1.6. In root samples, the GGCT1 transcript showed a transient expression pattern, level was significantly lower at 12 and 48 hr treatment with As(III), whereas, in shoots the GGCT1 fold levels slightly increased (Figure 1.6A & B). For Cd treatment, GGCT1 transcript levels were higher at 12 and 24 hr in root and almost 3.5-fold higher at 24 hr in shoot tissues (Figure 1.6C & D). For Hg, a gradual increase in GGCT1 fold was observed in root samples compared to control but the shoot samples remained unchanged (Figure 1.6E & F).

For GGCT2;1 in As(III) treated root samples, transcript levels were up-regulated by 16, 6, 8 and 13-folds after 6, 12, 24, 48 hr treatment, respectively (Figure 1.7A). In shoot As(III) treated samples; GGCT2;1 transcript were up-regulated only in the 6 hr sample by 3-folds when compared with control and relative transcript gradually decreased from 12 hr (Figure 1.7B). Cd treated root samples also show an increase in GGCT2;1 relative transcript in the early hours of stress induction, but its transcript level gradually decreased at later treatment times (Figure 1.7C). Shoot samples on the other hand showed a significant down regulation in the transcript levels in 6, 24 and 48 samples (Figure 1.7D). Hg treated samples showed no significant change in GGCT2;1 relative transcript in roots when compared with the no treatment control (Figure 1.7E). However,
GGCT2;1 in Hg treated shoots, similar to that for Cd treatment, showed a significant decrease in relative transcript level in all samples in comparison to control (Figure 1.7F).

For GGCT2;2, a significant increase in transcript level is observed in roots and shoots of AsIII-treated samples when compared with control. The highest fold change levels are observed in the 24 hr root sample with about five-fold increase and 12 hr shoot sample with a two-fold increase (Figure 1.8A & B). In Cd treated samples, all roots and shoot tissue samples except for 48 hr treated shoot showed increased level of GGCT2;2 transcripts. Relative transcript levels increased by more than two-folds in 12 hr and 24 hr root samples as well as in 6 hr, 12 hr and 24 hr shoot samples (Figure 1.8C & D). Similar to As(III) and Cd treatment, under Hg treatment, GGCT2;2 relative transcript levels significantly increased in all roots and some shoot samples (Figure 1.8E & F). Root samples at 12 hr and 48 hr treatment times showed at least two-fold increase in GGCT2;2 relative transcript in comparison to the no treatment control (Figure 1.8E). Overall, in response to heavy metal stress, GGCT2;2 level is significantly up-regulated in both root and shoot samples.

1.3.2.2 Relative transcript levels of GGCTs in Response to Abiotic Stresses

Under abscisic acid stress (ABA), the GGCT1 relative transcript level increased more than two folds in 6 hr treated root tissues but showed no significant change in 12 to 48 hr samples when compared to the no treatment control (Figure 1.9A). In ABA treated shoot samples, the relative transcript levels decreased almost half to that of control samples at all time points. For salt-treated samples, GGCT1 relative transcripts levels had no significant change in fold change in roots but shoot tissues showed significant down regulation in 6, 12 and 24 hr salt treated samples when compared with control samples
(Figure 1.9C & D). In drought stressed samples (mimicked by mannitol treatment), both root and shoot showed a gradual increase in GGCT1 relative transcripts but this increase was less than two fold (Figure 1.9E & F).

For GGCT2;1 fold change levels, roots of ABA treated samples showed more than two-fold increase at all time intervals except for 12 hr. In shoots, transcript was transient, decreasing at 6 and 24 hr and increasing at 48 hr (Figure 1.10A & B). For salt treatment, GGCT2;1 relative transcript in root has no significant change when compared with the no treatment control but shoot samples showed a decrease in relative transcript levels (Figure 1.10C & D). However, in Mannitol treated roots, GGCT2;1 is up-regulated in the 48 hr sample but not at the early time points after stress induction (Figure 1.10E). Shoot samples on the other hand showed decreased level in transcript in all samples when compared with the no treatment control.

The transcript level of GGCT2;2 in ABA treated roots showed decreased levels in 6, 12 and 48 hr samples while there was a slight increase in the 24 hr sample compared to the no treatment control (Figure 1.11A). For shoot ABA treatment, decreased transcript level was observed in 6, and 24 samples compared with controls (Figure 1.11B). Under salt stress, there was almost a two-fold increase in the GGCT2;2 relative transcript in 6 and 48 hr treated roots and the 24 hr treated shoot sample (Figure 1.11C & D). The 12 and 24 hr root samples and 6, 12, and 48 hr shoot samples showed decreased levels compared to control. However, in Mannitol treated roots and shoots, there was a strong increase in GGCT2;2 transcript level in all samples. There was a gradual increase in the relative transcript level from 1.5 fold in 6 hr to almost three-fold in 48 hr root samples compared to control (Figure 1.11E). Mannitol treated shoots also showed a significantly
increased level of \text{GGCT2;2} relative transcripts, ranging from two-fold in 6 hr and fourfold in 24 hr samples (Figure 1.11F).

1.3.3 Cloning and Overexpression of \text{CsGGCT2;1} in Camelina

The Camelina \text{GGCT2;1} gene was identified in the database (camelinadb.ca) after blasting its homolog sequence from the Arabidopsis TAIR database. Amplification of the \text{CsGGCT2;1} gene was performed from leaf cDNA via PCR using gene specific primers. The 654 base pair amplicon after sequence verification was cloned into a pCambia Redseed vector under an enhanced \text{35S} promoter (Figure 1.2). Restriction digestion of the \text{E35S:CsGGCT2;1} construct in pCambia Redseed with the \text{Asc}1 restriction enzyme confirmed the correct size of the gene insert as shown in Figure 1.12.

The \textit{Agrobacterium tumefaciens} GV3101 strain carrying pCambiaRedSeed/35::\text{CsGGCT2;1} construct was grown on large YEP liquid medium and harvested cells were suspended in infiltration medium. Camelina plants with flower buds were dipped into the suspension inside a vacuum chamber. Mature brown pods were harvested 50-60 days after transformation. The pCambia RedSeed vector contains a reporter DsRed gene for transgenic seed selection. Seeds were then screened for red fluorescence using DsRed filter (Figure 1.13C). The transformation efficiency was very low in the first attempt and thus to confirm if the lack of transformants was due to silencing of DsRed fluorescence, \text{T}_0 seeds (mature seeds from transformed plants) were subjected to hygromycin screening. Apart from a DsRed fluorescent selection marker, the pCambiaRedSeed/35S:CsGGCT2;1 vector also has hygromycin resistance gene for plant
selection. All T₀ harvested seeds were also screened on 20 mgL⁻¹ hygromycin antibiotic for seedlings showing resistance. Ten selected independent T₁ DsRed seeds or hygromycin-resistant transgenic seedlings were grown in soil to generate T₂ seeds (Figure 1.13D), which will exhibit a 3:1 (resistance: sensitivity) ratio.

1.3.4 Confirmation of GGCT2;1 transcript level in Transgenic Camelina

Relative transcript of GGCT2;1 was confirmed in the leaf tissues of T₁ seedlings using qRT-PCR. RNA extraction and cDNA synthesis was performed as described above in the “Materials and Methods” section. Of the ten T₁ seedlings growing in the greenhouse, five lines showed over 5 fold increase in GGCT2;1 relative transcript. The highest transcript level was observed in line 10 (Figure 1.14), with over 20-fold increase in its relative transcript level compared to wildtype controls.

Currently, all 10 independent T₁ lines are growing in the soil in the greenhouse and T₂ seeds will be ready for harvest in about 2-3 weeks. T₂ seeds showing a 3:1 segregation ratio for the transgene will be grown further to obtain T₃ homozygous Camelina lines. Independent transgenic lines showing GGCT2;1 relative expression will be analyzed in future work for tolerance to various abiotic and heavy metal stresses.
Figure 1.5. Hydroponic system for Camelina plant stress treatments.
Figure 1.6. RT-PCR Relative Transcript levels of CsGGCT1 in response to heavy metal stress. Fold change analysis from roots and shoots of Arsenite (A, B), Cadmium (C, D), and Mercury (E, F) treated C. sativa plants. Error bars correspond to the standard error of the mean. One-way ANOVA followed by Duncan multiple comparison test (p < 0.01 and p <0.05) was used to determine all differences of statistical significance among treatments.
Figure 1.7. RT-PCR Relative Transcript levels of CsGGCT2;1 in response to heavy metal stresses. Fold change analysis from roots and shoots of Arsenite (A, B), Cadmium (C, D), and Mercury (E, F) treated *C. sativa* plants. Error bars correspond to the standard error of the mean. One-way ANOVA followed by Duncan multiple comparison test (p < 0.01 and p < 0.05) was used to determine all differences of statistical significance among treatments.
Figure 1.8. RT-PCR Relative Transcript levels of CsGGCT2;2 in response to heavy metal stresses. Fold change analysis from roots and shoots of Arsenite (A, B), Cadmium (C, D), and Mercury (E, F) treated C. sativa plants. Error bars correspond to the standard error of the mean. One-way ANOVA followed by Duncan multiple comparison test (p < 0.01 and p <0.05) was used to determine all differences of statistical significance among treatments.
Figure 1.9. RT-PCR Relative Transcript levels of CsGGCT1 in response to various Abiotic stresses. Fold change analysis from roots and shoots of Abscisic acid (A, B), NaCl (C, D), and Mannitol (E, F) treated C. sativa plants. Error bars correspond to the standard error of the mean. One-way ANOVA followed by Duncan multiple comparison test (p < 0.01 and p < 0.05) was used to determine all differences of statistical significance among treatments.
Figure 1.10. RT-PCR Relative Transcript levels of CsGGCT2:1 in response to various Abiotic stresses. Fold change analysis from roots and shoots of Abscisic acid (A, B), NaCl (C, D), and Mannitol (E, F) treated *C. sativa* plants. Error bars correspond to the standard error of the mean. One-way ANOVA followed by Duncan multiple comparison test (p < 0.01 and p <0.05) was used to determine all differences of statistical significance among treatments.
Figure 1.11. RT-PCR Relative Transcript levels of CsgGCT2;2 in response to various Abiotic stresses. Fold change analysis from roots and shoots of Abscisic acid (A, B), NaCl (C, D), and Mannitol (E, F) treated C. sativa plants. Error bars correspond to the standard error of the mean. One-way ANOVA followed by Duncan multiple comparison test (p < 0.01 and p < 0.05) was used to determine all differences of statistical significance among treatments.
Table 1.1. List of primer sequences of Camelina *GGCT* genes used for qPCR.

<table>
<thead>
<tr>
<th>GENE</th>
<th>SEQUENCES (5’-&gt;3’)</th>
</tr>
</thead>
</table>
| 1. CsGGCT2;1 | Forward:  
CTACAGCTACTGGACCATGTG  
Reverse:  
TCACTTCCTCTGGCAAATCG |
| 2. CsGGCT2;2 | Forward:  
CAAGACAAATTGCGACAGCC  
Reverse:  
AACCTTCCTCACCTCGTTTG |
| 3. CsGGCT1  | Forward:  
ACTCAAATTCAGATCCGGCTG  
Reverse:  
AGGCCCTTCAGCTTTAACTATC |
| 4. ACTIN 2  | Forward:  
AAGAGCAGTTCTCTCGGTTGGA  
Reverse:  
TTGATCTTCATGCTGCTTTGGT |
Figure 1.12. Restriction digests of the pCambia Redseed vector harboring our $E_{35S}$::$CsGGCT2;1$ construct.

Lane 1 is the DNA ladder, lane 2 is the undigested construct, lane 3 shows the $E_{35S}$::$CsGGCT2;1$ fragment after digest with Apa1 and Xba1. Lanes 4 and 5 shows the $E_{35S}$ promoter and $CsGGCT2;1$ fragments after being digested with Apa1/Sal1 and Sal1/Xba1 respectively.
Figure 1.13. Camelina transformation and transgenic seed selection.

(A) Flowering Camelina plants ready for transformation. (B) Plants dipped into vacuum infiltration medium containing the E35S:CsGGCT2;1 construct in Agrobacterium tumefaciens strain GV3101. (C) Selection of transgenic seeds using a DsRed filter. (D) Selected T1 transgenic seeds growing to generate T2 seeds with 3:1 resistance to sensitivity ratio.
Figure 1.14. Relative Transcript levels of *GGCT2;1* in T1 seedlings.
1.4 Discussion

A gene encoding gamma glutamyl cyclotransferase in Arabidopsis was recently identified by Paulose et al. (2013). Overexpression of AtGGCT2;1 showed enhanced tolerance of Arabidopsis to As(III) and Cd stress. The additional two GGCT genes have not been characterized in any plant species. In this study, we investigated the differential regulation of transcript levels of GGCT homolog genes from Camelina under As(III), Cd, Hg, ABA, salt and mannitol stresses. The preliminary results show that at least two if not all CsGGCT genes, are differently regulated under these various stresses. Under As(III), Cd and Hg stresses, the expression level of GGCT2;1 and GGCT2;2 increases in the early time points of exposure in roots and shoots, especially the levels of GGCT2;1 in As(III) exposed root with over a 16 fold increase after 6 hr treatment. The transcripts level of GGCT1 under the heavy metal exposure does not show significant change compared to control. GGCT2;1 and GGCT2;2 are paralogs sharing over 72 percent amino acid identity. Expression levels of these paralogs are differentially regulated in some root and shoot tissues under these stresses. Overall, under heavy metal and abiotic stresses, levels of the GGCT2;1 and GGCT2;2 transcripts showed significant change in expression but not for GGCT1. This is the first study of differential expression of Camelina GGCT genes in Camelina plants in response to heavy metal and abiotic stresses.

Since AtGGCT2;1 overexpression has shown enhanced tolerance to heavy metals (Paulose et al., 2013) and also to ABA (Parkash lab, unpublished data), it is expected that overexpression of GGCT2;1 homologs in Camelina will enable this crop to tolerate
oxidative stress caused as a result of heavy metals toxicity and other abiotic stresses. Therefore, to develop transgenic Camelina for enhanced tolerance to oxidative and abiotic stresses, \(CsGGCT2;1\) was overexpressed under a strong constitutive enhanced 35S promoter. Enhanced expression of \(CsGGCT2;1\) was confirmed in independent T1 transgenic lines. These transgenic plants will be subjected to various abiotic stresses for their performance under these stress conditions in future work.

1.5 Conclusion

_Camelina sativa_ is proposed as a dedicated biofuel crop. The genome of this plant is yet to be characterized since the full genome sequence was recently published online. Overexpression of \(GGCT2;1\) in _Arabidopsis_ has been characterized to show enhanced tolerance to As and Cd stress. We have identified homologous genes from Camelina genome. Camelina \(GGCT2;1\) and \(GGCT2;2\) showed strong differential regulation in response to As, Cd, Hg, ABA, NaCl and drought stresses. In addition, \(CsGGCT2;1\) was overexpressed in Camelina to increase its tolerance to several abiotic stresses. Enhanced levels of tolerance to various abiotic and oxidative stresses will enable Camelina production on waste and marginal lands for biofuel production.
1.6 References


Dietz, K., Baier, M., & Krämer, U. (1999). Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. *Heavy metal stress in plants* (pp. 73-97) Springer.


2.1 Introduction

Interest in renewable energy from oilseed crops has increased in recent years. There have been numerous studies on oilseed crops such as soybean, rapeseed, and canola for their oil content properties to be used as petroleum-based fuel substitutes. These oilseed crops also serve as food source for both humans and animals, and thus conflict with use of these crops for biofuels, which can affect the food prices and production. However, an oilseed crop like *Camelina sativa*, mostly used for cosmetic products, culinary oil and animal feed (Sawyer, 2008), has been proposed to be a potential and ideal candidate for the production of biofuel (Rice, 1995, Frohlich et al., 2005). This annual crop grows well in temperate climates. Several studies have reported that compared to most oilseed crops, Camelina has low agricultural inputs, cold-weather tolerance, short growing season (100-110 days), and also is compatible with existing farm equipment (Putnam et al., 1993, Retka-Schill, 2008a and Sawyer, 2008). This annual crop also requires less application of water, pesticides and fertilizers compared to soybean, canola and rapeseed (Budin et al., 1995). Oil from Camelina is high in omega-3 fatty acids, and its chemical composition makes its burning efficiency in engines similar to petroleum-based fuel (Keske et al., 2013). Mature seeds of Camelina contain over 30 percent oil by weight, which is mostly made up of polyunsaturated fatty acids (32.6% linolenic, and 19.6% linoleic acid content) (Moser, 2010).
Increasing the oil accumulation in mature seeds and total per acre oil yield will make Camelina a more attractive biofuel crop. Increase in yield can potentially reduce the land needed for production of Camelina. Pathways to increase oil content and yield in seeds are presently being exploited, mostly by overexpressing genes involved in fatty acid and triacylglycerol biosynthesis (TAGs). In Arabidopsis, canola (*Brassica napus*) and maize (*Zea mays*), increase in oil yield in seeds has been achieved with the overexpression of some transcription factors such as wrinkled 1 (*WRI1*) (Baud et al., 2007; Weselake et al., 2008; Wu et al., 2014; Pouvreau et al., 2011). Wrinkled 1 is a transcription factor that activates target genes involved in fatty acid biosynthesis. In Arabidopsis, WRI1 has been shown to play an important role in oil accumulation in mature seeds. Genes involved in encoding enzymes of late glycolysis, the fatty acid synthesis pathway, and the biotin and lipioc acid biosynthesis pathways have been implicated to be targets of *WRI1* (Baud et al., 2007). WRI1 expressed in plants have high carbohydrate metabolism and efficiently convert sucrose into triacylglycerols. Mutant lines of *wrl1* exhibit 80% reduction in oil content due to inability to incorporate sucrose and glucose into TAGs (Focks and Benning, 1998). In *B. napus*, WRI1 coordinates fatty acid biosynthesis and photosynthesis pathways in developing seeds via directly stimulating expression of GT1 element and/or GCC-box containing genes (Wu et al., 2014).

Pathways have been engineered for biosynthesis of TAG by overexpressing genes such as glycerol-3-phosphate dehydrogenase 1 (*GPD1*), diacylglycerol acyltransferase 1(*DGAT1*), and phosphatidylcholine:diacylglycerol cholinephosphotransferase (*PDCT*) to increase oil accumulation in seeds (Vigeolas et al., 2007, Vanhercke et al., 2013, Hu et
Interestingly, at the late stage of seed development, decline of oil content by 10% has been observed in rapeseed (Kelly et al., 2012), due to breakdown of the final TAG product by lipases. However, suppression of the SUGAR-DEPENDENT1 TAG lipase gene family recovered the oil yield by 8% (Kelly et al., 2012). In a study by van Erp et al., (2014), co-expression of WRl1, DGAT1 and suppression of SDP1 lipase family genes in Arabidopsis resulted in substantial increase in seed oil content and seed mass compared to expression of individual genes separately.

In the recently announced Camelina genome, there are four WRl genes (Table 2-1). Since C. sativa is hexaploid, each gene is represented by three copies. Csa06g028810.1 is the closest homolog of Arabidopsis seed-specific WRl1, involved in fatty acid synthesis in seeds. The Camelina WRl1 gene has not been characterized yet. In this study, Camelina being a model oilseed crop for biofuel production, and with a low-cost of production, we are interested in characterizing the Camelina WRl1 gene and investigating the effect of an overexpressed WRl1 gene on Camelina seed oil content and total oil yield.
Table 2.1. List of Wrinkled genes in *Camelina sativa* based on the nucleotide alignment with Arabidopsis homologous Wrinkled sequences

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>% identity</th>
<th>Homologous in Arabidopsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csa06g028810.1</td>
<td>Integrase-type -DNA-binding super family protein</td>
<td>94%</td>
<td>Wrinkled1</td>
</tr>
<tr>
<td>Csa09g064030.2</td>
<td>Integrase-type -DNA-binding super family protein</td>
<td>92%</td>
<td>Wrinkled1</td>
</tr>
<tr>
<td>Csa04g040400.2</td>
<td>Integrase-type -DNA-binding super family protein</td>
<td>87%</td>
<td>Wrinkled1</td>
</tr>
<tr>
<td>Csa06g047660.2</td>
<td>Integrase-type -DNA-binding super family protein</td>
<td>94%</td>
<td>Wrinkled2</td>
</tr>
<tr>
<td>Csa05g009930.1</td>
<td>Integrase-type -DNA-binding super family protein</td>
<td>94%</td>
<td>Wrinkled2</td>
</tr>
<tr>
<td>Csa04g056170.2</td>
<td>Integrase-type -DNA-binding super family protein</td>
<td>94%</td>
<td>Wrinkled2</td>
</tr>
<tr>
<td>Csa14g020190.1</td>
<td>ARIA-Interacting double AP2 domain protein</td>
<td>90%</td>
<td>Wrinkled3</td>
</tr>
<tr>
<td>Csa03g019870.1</td>
<td>ARIA-Interacting double AP2 domain protein</td>
<td>89%</td>
<td>Wrinkled3</td>
</tr>
<tr>
<td>Csa17g021650.1</td>
<td>ARIA-Interacting double AP2 domain protein</td>
<td>92%</td>
<td>Wrinkled3</td>
</tr>
<tr>
<td>Csa09g093970.1</td>
<td>Integrase-type -DNA-binding super family protein</td>
<td>95%</td>
<td>Wrinkled4</td>
</tr>
<tr>
<td>Csa16g049360.1</td>
<td>Integrase-type -DNA-binding super family protein</td>
<td>95%</td>
<td>Wrinkled4</td>
</tr>
<tr>
<td>Csa07g058830.1</td>
<td>Integrase-type -DNA-binding super family protein</td>
<td>95%</td>
<td>Wrinkled4</td>
</tr>
</tbody>
</table>
2.2 Materials and Methods

2.2.1 Plant Growth, RNA isolation and cDNA Synthesis

Camelina plants were grown under greenhouse conditions (16/8 hr light/dark cycles at 25°C). Flowers of plants were tagged and embryos harvested after 7, 14, 21 and 28 days after flowering (DAF). Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich), and first strand cDNA was synthesized from total RNA using Thermo Scientific cDNA kit following the manufactures’ protocol.

2.2.2 Analysis of Expression of \textit{WRl1} at Seed Developmental Stages by quantitative RT-PCR

In order to study the precise and stage-specific expression of \textit{WRl1} at different seed developmental stage, \textit{WRl1} gene specific primers were used with the Absolute Blue QPCR SYBR Green Mix (Thermo Fisher Scientific), for real time quantitative PCR. The qRT-PCR amplification program was 95 °C for 5 min (1 cycle), 95 °C for 15 s, 60 °C for 45 s, 72 °C for 1 min, (32 cycles); and final extension at 72 °C for 10 min. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All reactions were performed in triplicate. \textit{Actin2} gene primers were used for normalization.

2.2.3 Cloning and Overexpression of \textit{WRl1} in Camelina

A synthetic \textit{WRl1} gene construct consisting of the \textit{Glycine max Oleosin} promoter, \textit{WRl1} coding region and \textit{Oleosin} terminator was used for overexpression in plants. Gene synthesis was done commercially at DNA 2.0. A synthetic gene construct flanked with AscI restriction enzyme sites was cloned into the pCambia Redseed binary vector at its AscI site (Figure 2.1) and transferred into \textit{E.coli DH5}α Electro-competent cells using an
Electroporator 2510 (Eppendorf) at 1730 volts. Cells were supplemented with 1 ml LB miller and incubated at 37°C for 2 hours at 200 rpm. The transformed cells were spread on LB agar plates supplemented with 50 mg/L kanamycin antibiotics and incubated at 37°C overnight. Colonies were randomly picked, purified, and the presence of the gene was confirmed using gel electrophoresis. The construct was subcloned into the Agrobacterium strain GV3101 by heat shocking the reaction for 5 min at 37°C. The cells were spread on YEP medium agar plates supplemented with 50 mg/l Kanamycin, 25 mg/l Gentamycin and 10 mg/l Rifampicin antibiotics after 2 hours of incubation at 30°C, 220 rpm. The plates were incubated at 30°C for 48 hours. Colonies were picked, plasmids were purified, and plasmids were digested with the AscI restriction enzyme to confirm the gene insert.
Figure 2.1. pCambia Redseed harboring CsWR11 expressed under the Oleosin-promoter/terminator cassette.
2.2.4 Plant transformation and Seed selection

The *Agrobacterium* strain harboring the *CsWRl1* construct was inoculated into 5 ml YEP medium supplemented with the above mentioned antibiotics in a test tube and incubated at 30°C overnight at 200 rpm. The overnight culture was transferred into 2 liter flask containing YEP medium with antibiotics and grown again for 48 hours. Cells were harvested by centrifugation of the culture at 4500 rpm and suspending the pellet with vacuum infiltration medium (2.2 g/L MS, 50 g/L sucrose, 2 mg/L Benzylaminopurine (BAP), pH 5.7, 0.05% (v/v) Silwet L77 (Lehle Seeds, Round Rock, TX, USA)). Camelina plants were inoculated with the *Agrobacterium* suspension during their flowering stage as described by Lu, (2008). Mature, transformed seeds were collected and screened using a DsRed filter or half strength MS medium supplemented with 20 mg/l hygromycin as antibiotics to select transgenic seeds for further study.

2.3 Results and Discussion

2.3.1 Sequence Analysis of WRI gene family in Camelina

There are four reported *WRI* genes in Arabidopsis; *WRI1, WRI2, WRI3*, and *WRI4*. However, *WRI1* has been reported to be the only gene to activate fatty acid biosynthesis in seeds for triacylglycerol production whiles the others are required in floral tissues to provide acyl chains for cutin biosynthesis (To et al., 2012). Arabidopsis *WRI1* nucleotide sequences were blasted on the Camelina database and sequences showing highest nucleotide match were further analyzed. We identified three copies of the *WRI1* gene in Camelina and the highest amino acid sequence match with *AtWRI1* was utilized in this study.
Amino acid sequence alignments and the phylogenetic tree of *WRl1* gene in Arabidopsis, Camelina, Brassica and Maize are presented in Figures 2.2 and 2.3 respectively. *CsWRl1* shares over 80% amino acid match with *AtWRl1* and *BnWRl1* but less than 50% match with both maize *WRl1*s. Since Camelina, Arabidopsis and Brassica are from the *Brassicaceae* family, it is not surprising to see such high amino acid sequence match. The phylogenetic tree generated from the sequence alignments shows close evolutionary descent of *WRl1* in these three species.
**Figure 2.2.** Multiple sequence alignment of WR11 amino acids from *Arabidopsis thaliana* (AtWR11), *Camelina sativa* (CsWR11), *Zea mays* (ZmWR11, ZmWR11b) and *Brassica napus* (BnWR11). Accession numbers of sequences: AtWR11 (ATG54320.1), CsWR11 (Csa06g028810.1), BnWR11 (DQ402050), ZmWR11 (NM_001143592.1), and ZmWR11b (NM_001138261.1)
Figure 2.3. Phylogenetic analysis of WRl1 proteins from Arabidopsis, Camelina, Brassica and Maize using the CLC genome workbench 7 with maximum likelihood phylogeny. Accession numbers of sequences; AtWRl1 (ATG54320.1), CsWRl1 (Csa06g028810.1), BnWRl1 (DQ402050), ZmWRl1 (NM_001143592.1), and ZmWRl1b (NM_001138261.1)
2.3.2 Analysis of WRl1 Expression Levels in Developing Seeds

Relative expression levels of WRl1 have been reported in Arabidopsis, showing a high increase of transcript levels in the early stage of seed maturation (Baud et al., 2007), however, information regarding WRl1 expression in Camelina is lacking. We investigated the WRl1 transcript level in Camelina at various seed developmental stages. Developing seeds at stages from 7 to 28 days post anthesis/days after flowering (DPA/DAF), leaves, and flowers were harvested. Real-time quantitative RT-PCR analysis performed on these samples shows a significant increase in WRl1 transcript in 7 DPA samples. However, there is a gradual decrease of transcript from 14 DPA to 28 DPA (Figure 2.4). These findings are consistent with WRl1 transcript expression profiles in Arabidopsis (Baud et al., 2007). In B. napus, time-course expression of genes involved in fatty acid accumulation in developing seeds and chlorophyll content showed the same expression trend as the transcript level of WRl1, indicating that WRl1 functions as a coordinator of both the fatty acid biosynthesis and photosynthesis pathways (Wu et al., 2014).
Figure 2.4. CsWR11 Relative Transcript level in developing seeds.

Transcript analysis of the Wrinkled 1 gene measured by real time qPCR at 7, 14, 21, 28 days post anthesis (DPA). The means are averaged from three replicates. Error bars correspond to the standard error of the mean. One-way ANOVA followed by Duncan multiple comparison test (p < 0.01 and p < 0.05) was used to determine all differences of statistical significance among treatments.
2.3.3 Cloning and Overexpression of WR11 in Camelina

A synthetic gene construct consisting of the *G. max Oleosin* promoter, *WR11* coding region and *G. max Oleosin* terminator was cloned into the pCambia Redseed binary vector at its AscI site (Figure 2.1). For plant transformation, the construct, pCambiaRedSeed/*GmOle-pt::CsWR11 was transformed into *Agrobacterium tumefaciens* strain GV3101. Colonies were selected, purified and restriction digested with AscI to confirm insertion (Figure 2.5). The *Agrobacterium tumefaciens* GV3101 strain carrying our construct was grown on large YEP liquid medium and harvested cells were suspended in infiltration medium. Camelina plants with flower buds were dipped into the *Agrobacterium* suspension inside a vacuum chamber. Mature brown pods were harvested 50-60 days after transformation. Seeds were then screened for red fluorescence using a DsRed filter and for hygromycin resistance as described in Chapter 1. T₀ selected seeds were planted into soil to generate T2 seeds for 3:1 (resistance : sensitivity) ratio.

Ten independent lines of T1 seedlings are growing in soil in the greenhouse and will be ready for harvest in 2-3 weeks (Figure 2.6C). The segregating 3:1 T2 seeds will be grown further to obtain T3 homozygous lines. Our lab has previously overexpressed *GPD1* and *DGAT1* genes (mentioned in the introduction section) involved in TAG accumulation in Camelina seeds. Once characterized for oil content, and yield, the homozygous WR11 lines will be cross-pollinated with the GDP1 and DGAT1 TAG lines to further increase Camelina seed oil yield.
Figure 2.5. Restriction Digest of pCambia Redseed vector harboring *CsWR11* construct.

Lane 1 is the DNA ladder to verify the size of the digested fragments. Lane 2 shows our *GmOleo-pt::CsWR11* fragment (2400 base pairs) digested with AscI and lane 3 also show *GmOleo-pt::CsWR11* fragment (2508 base pairs) digested with BamH1 restriction enzyme.
Figure 2.6. Transgenic Camelina seed selection.

(A) T1 seedling of CsWR11 showing resistance to hygromycin antibiotics, (B) Selected resistant transgenic seedlings grown in soil and placed in a growth chamber, (C) Selected T1 transgenic seedlings growing to generate T2 seeds with 3:1 resistance to sensitivity ratio.
2.4 Conclusion

Many studies exploiting ways to increase oil yield in oilseed crops through genetic approaches have been conducted. Overexpression of transcription factors of genes involved in oil biosynthesis and accumulation are among the many approaches. Engineering Camelina for renewable energy from seed oil looks very promising. Our goal is to increase the seed oil yield in this crop by manipulating genes that can enhance not only the yield but also the quality. We have overexpressed WRl1 gene in Camelina under a seed-specific promoter for driving fatty acid synthesis in developing seeds, which is expected to increase the seed oil yield. RT-PCR analysis of WRl1 expression in early seed developmental stage correlates with the expression of genes involved in glycolysis and fatty acid synthesis. Our lab has already developed Camelina lines with enhanced oil yield by co-expressing yeast GPD1 and Arabidopsis DGAT1 (Parkash Lab, unpublished data). WRl1 transgenic Camelina lines showing higher oil yield potential will be crossed with already existing lines of Camelina expressing GPD1 and DGAT1 for further improving the Camelina oil yield. In the long run, we are proposing to engineer Camelina by combining the expression of GGCT2;1 (Chapter 1) with WRl1, GPD1 and DGAT1 for enhancing the oil yield and tolerance to environmental stresses, enabling its production on marginal/waste lands.
2.5 References


REFERENCES


Dietz, K., Baier, M., & Krämer, U. (1999). Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. *Heavy metal stress in plants (pp. 73-97) Springer.*


