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DEVELOPING PENNYCRESS (*THLASPI ARVENSE*) AS A BIODIESEL
FEEDSTOCK CROP AND PLANT MODEL SYSTEM

MICHAELA G. MCGINN

81 Pages

Thlaspi arvense L. (pennycress) is currently being developed as a profitable oilseed-producing winter annual cover crop with extreme cold tolerance and a rapid life cycle that can be grown on fallow farmland throughout the U.S. Midwest Corn Belt, controlling soil erosion and nutrient runoff while serving as an additional source of income for the American farmer without displacing food crops. The research comprising this dissertation demonstrates that pennycress can serve as a user-friendly model system highly similar to *Arabidopsis thaliana*, and is well-suited for both laboratory and field experimentation, being readily employable in existing growth facilities. After 10 generations of single seed descent, the sequenced diploid genome of a spring-type cultivar which does not require vernalization to flower has been made public, along with a paired homogenous seed lot (Spring32-10) available for research. Plant growth conditions and selectable marker systems for pennycress have been elucidated, and a simple pressurized *Agrobacterium*-mediated floral dip transformation method has been developed. Proof of concept work shows that pennycress has been stably transformed with the diacylglycerol acetyltransferase (*EaDACT*) gene from *Euonymus alatus*, producing low-viscosity acetyltriacylglycerol-containing seed oil suitable as a diesel-engine drop-in fuel. CRISPR-Cas9 constructs were utilized to induce targeted mutations in the pennycress *FATTY ACID ELONGATION1* (*FAEI*) gene, thereby abolishing erucic acid production and generating a seed

oil fatty acid profile that is non-toxic and comparable to canola. CRISPR was also employed to alter putative pennycress glucosinolate target sequences in an attempt to develop pennycress cultivars with reduced sinigrin glucosinolate.

KEYWORDS: Pennycress; Biofuel; Biodiesel; CRISPR-Cas9; Gene editing; Oil engineering

DEVELOPING PENNYCRESS (*THLASPI ARVENSE*) AS A BIODIESEL
FEEDSTOCK CROP AND PLANT MODEL SYSTEM

MICHAELA G. MCGINN

A Dissertation Submitted in Partial
Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences

ILLINOIS STATE UNIVERSITY

2018

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DEVELOPING PENNYCRESS (*THLASPI ARVENSE*) AS A BIODIESEL
FEEDSTOCK CROP AND PLANT MODEL SYSTEM

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ACKNOWLEDGMENTS

Many thank you's to Dr. John Sedbrook and his lab, specifically Brice Jarvis, without whom this work would not have been completed. To Brian, Clayton, Jon, Cara, and the ever resilient McGinns. To my friends and family for doing their level best to keep me sane over the last half decade. Thank you to the bartenders, baristas, counselors, and beautiful freaks for keeping things interesting. Thank you to the 25 Team P undergraduate students who learned with me and kept me grounded. Everything mine is yours.

M.G.M

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CHAPTER I: INTRODUCTION

Cover crops

As the global population continues to grow and arable farmland diminishes and degrades, there is an increasing demand on agriculture to yield sufficient foodstuffs and provide new and readily employable sources of sustainable biofuels and products [1, 2]. Global food security relies in part upon increasing the productivity of existing tillable farmland without exacerbating the negative effects of agriculture on the environment [3, 4]. One method to achieve maximization of current major agricultural systems is the integration of fallow-season crops into existing crop rotations. Fallow-season crops can supplement current production systems without displacing food crops while potentially mitigating some of the environmental effects of modern agriculture [5]. Planting winter cover crops following the fall harvest has been shown to alleviate soil degradation and topsoil erosion while preventing water pollution by scavenging excess nitrogen from the soil and limiting nutrient run-off [6, 7, 8].

Biofuels and products derived from plants have great potential to reduce anthropogenic carbon emissions contributing to global climate change [9]. In the United States alone, the corn/soybean rotation system is currently employed on over 30 million plantable acres of prime farmland [10]. Cover crops can be integrated into this rotation and provide several ecosystem services such as preventing soil erosion, increasing winter biodiversity, decreasing nitrogen run-off, and reducing early spring weed pressure [5, 6, 7, 11]. However, with little economic benefit other than increasing long-term sustainability of the crop rotation system, there has yet to be a wide scale adoption of cover crops [12]. With public opinion shifting to ‘greener pastures’ there is a unique opportunity to develop and implement new ‘cash cover crops’ which provide both the necessary ecosystem services that allow for their integration into current cropping systems while

also supplying a marketable, monetized product.

The research presented throughout this dissertation will outline the improvements made to pennycress, a new fallow-season winter-annual cover crop, which is being developed as a profitable oil-seed for integration into the fallow period of the corn/soybean rotation in the Midwestern United States.

Pennycress Background

Pennycress (*Thlaspi arvense* L., Field Pennycress) is an oilseed-producing plant of the Brassicaceae and is closely related to other agronomically-important mustard family members including rapeseed, canola, and camelina (*Brassica napus*, *Brassica rapa*, and *Camelina sativa*), as well as cabbage, cauliflower, broccoli, and the model plant *Arabidopsis thaliana* [13, 14, 15]. Pennycress was initially native to Eurasia, but ~150 years after its introduction, the plant is considered fully naturalized to the continental U.S. and non-invasive [16].

Pennycress grows throughout temperate North America as a winter or spring annual and possesses a unique combination of attributes allowing for its growth as a winter annual cover crop on farmland that currently lays fallow during the cold months [17, 18]. Those attributes include extreme cold tolerance, over-wintering growth habit, and a short life cycle [13]. Pennycress can be double-cropped between corn and soybeans throughout the U.S. Midwest Corn Belt, planted in standing corn near the time of harvest where it forms a rosette of leaves that overwinter [13, 19].

In early spring, pennycress plants produce multiple white inflorescences with small, self-pollenating flowers from which the characteristic penny-shaped seed pods are formed [16]. Each pod carries 10-14 small, round seeds containing 30%-37% oil by weight in the form of

triacylglycerols (TAGs) [13, 20, 21]. Pennycress seeds are roughly half the size of canola seeds with a 1,000-seed weight of ~1 gram and can be harvested from late May to early June in the Upper Midwest region with the same equipment used to harvest soybeans [13, 21]. Due to the naturally early maturation of pennycress, a full season soybean or other short-season rotation crop can be grown on the same acreage immediately following harvest without affecting the standard crop rotation [22].

In the proposed scenario, pennycress can be grown without displacing currently-grown crops or upsetting established ecosystems [19]. The pennycress growth habit is out of sync with summer crops and the winter plants are easily controlled by tilling or common herbicides, so it is not considered a problem weed [13, 23]. Additionally, pennycress is not considered invasive as it does not readily grow where plants are already established, preferring opportunistic growth in areas where the soil has been disturbed [13].

Pennycress Seed Oil

Each pennycress seed is ~ 34% oil and 19% protein at ~1 mg/seed [13, 20, 21]. Wild pennycress stands can yield an average of 840 liters of oil and 1470 kg press-cake per hectare without genetic engineering or breeding, and could generate sufficient lipid feedstocks to replace ~5% of the petroleum-derived diesel fuel currently used in the United States. [13, 20, 21]. Established oil extraction procedures yield between 600 and 1,200 L of oil/ha for pennycress, which easily outperforms soybean and camelina at 450L and 420-640L respectively [13, 21, 24]. Although pennycress seeds contain high levels of the sulfur-containing glucosinolate sinigrin, the extracted oil is largely depleted of sulfur and phosphorus, which could corrode copper engine parts and damage *catalytic converters* used in emission control systems [21, 25].

Wild type pennycress oil is comprised primarily of C22:1 erucic acid (32.8 wt%), 18:3

linoleic acid (22.4 wt%), and C18:1 oleic acid (11 wt%) with 12.7% of the fatty acids being unsaturated [21]. The conversion of raw pennycress seed oil to fatty acid methyl esters (FAMES) can be accomplished via traditional alkaline-catalyzed methods [26]. The resulting biodiesel has several beneficial characteristics for use in the Midwest such as lower temperatures for cloud point (-10 °C), pour point (-18 °C), and cold filter plugging point (-17 °C) compared to those found for biodiesel made from soybean, camelina, or canola [21, 27]. The main detractor for pennycress biodiesel is a kinematic viscosity of 5.24, which is higher than that of the other biodiesels and is on the borderline of acceptability [21, 28]. This high viscosity is likely due to the high levels of erucic acid [29].

Pennycress Press-Cake

After pennycress seed oil is extracted using a screw press, the crushed seeds and left-over oil remains as a press-cake. This value-added coproduct is 27% protein by weight and energy density due to the residual oil post-pressing [27, 30]. This unprocessed press-cake could be used as a nutrient-rich animal feed. It is hoped pennycress will mirror the adoption and growth of the relatively new crop canola, the press-cake of which is a major commodity and one of the most widely used protein sources in animal feed with over 1 million short tons produced in the U.S. in 2015, valued at ~\$300 million USD [31].

A concern hindering the use of pennycress press-cake as animal feed is its high levels of sinigrin (a pungent glucosinolate) which decreases feed palatability and can be degraded into toxic compounds such as isothiocyanate (AITC) and allyl thocyanate (ATC) by the endogenous enzyme myrosinase [27, 32]. Unprocessed wild type pennycress seed meal cannot currently be used as an animal feed as a result of this high glucosinolate content, but a simple heating step

deactivates myrosinase and renders the meal safe and edible [32, 33, 34]. Without myrosinase heat deactivation, pennycress seed meal can be used as a natural biofumigant [32, 35].

Pennycress Effect on Early Spring Weed Pressure

In the Midwest, early spring weed pressure poses a major threat to soybean production and food security [36]. Currently, the majority of commercially produced soybeans are genetically engineered for glyphosate resistance [37]. However, more than 35 weedy species have recently developed resistance to glyphosate and pose new challenges to soybean farmers [38, 39].

Cover crops such as pennycress can serve as a non-chemical ecological weed management strategy by filling in gaps in cropping systems that would otherwise be susceptible to occupation by weeds [13, 40].

Pennycress can be found growing throughout the Midwestern Corn Belt and is currently managed as a weed, but is easily controlled in farm fields with commonly used herbicides and is not on the federal list of noxious weeds [13, 23]. Pennycress has been planted on more than two thousand acres of prime arable land by farmers in Illinois, Minnesota, and Missouri as part of commercial-scale field trials which resulted in no observed negative effects and minimal or no yield loss of subsequently planted crops [22, 35]. In field trials at two locations in Minnesota during 2011 and 2012, pennycress was shown to significantly reduce the emergence of spring weeds [32, 35, 41, 42]. This ability to suppress weeds was observed when pennycress residues were incorporated or left on the soil surface, suggesting that a portion of the weed suppression effect may be due to biofumigant compounds being released from decomposing pennycress tissue which possesses high levels of sinigrin glucosinolate [32, 35, 41, 42].

Effect of Pennycress on Winter Biodiversity

Finding agricultural crops that can provide plant ground cover, winter forage, and diversify the winter landscape can improve pollinator health and offer foraging resources during periods when few plants are flowering [43, 44]. Two peer reviewed studies investigated the effect of a pennycress double-cropping system on spider and ground beetle diversity and compared it to that of three more common rotations: mustard-corn, green fallow-corn and bare fallow-corn [44, 45]. It was found that the cultivation of pennycress in a double-cropping system has positive effects on spider and ground beetle biodiversity compared with other commonly used cultivation systems [44, 45].

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CHAPTER II: USING PENNYCRESS AS A MODEL/CROP

Abstract

Thlaspi arvense L. (pennycress) is a transformable diploid oilseed plant species being developed as a profitable cover crop and biodiesel feedstock as well as a potential model plant system. Here we outline the user-friendly nature of the pennycress model which is highly similar to *Arabidopsis thaliana*, and is well-suited for both laboratory and field experimentation. Plant growth conditions and selectable marker systems for pennycress have been elucidated, and a simple vacuum pressurized *Agrobacterium*-mediated floral dip transformation protocol has been developed. Several pennycress EMS mutant populations have been generated, and a seed lot of a genetically stable inbred pennycress cultivar (Spring32-10) has been made available for research along with its fully sequenced genome. Adoption of pennycress as a model system will accelerate the oilseed-crop translational research and facilitate the rapid domestication of this new, high-potential crop.

Keywords: Pennycress; Model systems; Mutant population; Genetic transformation; Translational biology; Genetics; Biofuels

Introduction

Pennycress is a high-potential oilseed plant with winter and spring type cultivars that is currently under development as a biofuel feedstock crop. Pennycress is a self-pollinating plant with a relatively small stature and a diploid genome highly similar to model plant and close-cousin *Arabidopsis thaliana* (Arabidopsis), which makes it an excellent candidate as a functional model plant [1, 2]. Adoption of pennycress as a model system could significantly accelerate oilseed-crop translational research and facilitate the rapid domestication of this new, high-potential crop. There is a rich background of basic research on similar model plant species that is readily translatable to pennycress. With modern genetic tools we are poised to rapidly domesticate and engineer novel crop species such as pennycress in order to meet the growing global food and energy demands [3].

Arabidopsis has been used as a model plant for over 40 years and has been the workhorse of intense genetic, biochemical and physiological study. *Arabidopsis* is easy to grow in a laboratory setting due to its modest input requirements, short 8-week lifecycle and diminutive stature. The relatively small, diploid genome is comprised of 5 chromosomes that are extensively mapped and easily modified with binary vectors introduced via *Agrobacterium tumefaciens* (*Agrobacterium*)-mediated floral dipping. However, the *Arabidopsis* model is limited due to its lack of agronomic potential which hinders its ability to address questions specific to crop engineering and field performance. The pennycress model allows us to combine basic and applied research, as experiments can be performed on pennycress that directly test and/or improve agronomic traits. Additionally, the larger cell and organ sizes of pennycress will help facilitate a variety of research including live-cell imaging, biochemical analyses, and omics studies (Figure 1, Figure 2).

To expedite pennycress adoption as both a model system and cash crop, it is necessary to provide the basic toolbox for utilizing pennycress in a lab setting. A model comparable to *Arabidopsis* requires a Columbia-equivalent wild-type germplasm with a reasonably short life cycle and easily employable growth conditions. In order to capitalize on the vast store of information derived from *Arabidopsis* and facilitate genetic research, it is necessary to fully sequence the pennycress genome, elucidate effective transgenic selection protocols, and generate mutant populations available to the research community.

Dorn et al. previously characterized the transcriptome and genome of top-performing winter-type pennycress cultivar MN106 which consists of 539 Mb of DNA organized into 7 chromosomes ($2n=14$) that possess ~86% sequence identity to the *Arabidopsis* genome [2]. Winter-type pennycress cultivars such as MN106 and Elizabeth require a ~21 day/4°C winter cold period called vernalization in order to flower, significantly increasing the generation time and requiring large areas of dedicated cold room space. By definition, spring-type plants do not require this vernalization which allows for more rapid research. A naturally-occurring spring pennycress variant called Spring32 was collected by Dr. Win Phippen of Western Illinois University originating from a natural population of spring-type plants collected in Montana, USA. This variant was provided to us for the purpose of expediting pennycress research.

It is important to generate large mutant populations for model species in order to provide a TILLING (Targeting Induced Local Lesions IN Genomes) platform for forward genetics, functional genomics, introduction of novel allelic variation for breeding, and gene discovery. Ethyl methanesulfonate (EMS) induces random genetic mutations by reacting with guanine in DNA. During DNA replication, polymerases frequently place thymine instead of cytosine

opposite the altered guanine, which can result in mutations at a rate of roughly 5×10^{-4} mutations per gene [4].

Here we present the toolbox for using pennycress in a laboratory setting by outlining growth conditions and a simple genetic transformation protocol, as well as making public a seed lot of 10th generation inbred spring pennycress called Spring32-10 along with its sequenced genome and two large EMS mutant populations. Adoption of pennycress as a model system could significantly accelerate oilseed-crop translational research and facilitate the rapid domestication of this new, high-potential crop. By introducing the pennycress toolbox we are now able to exploit the wealth of information learned from years of basic research in *Arabidopsis*.

Methods

Surface sterilization of pennycress seeds and growth conditions

Pennycress seeds were surface sterilized with a brief rinse with 70% ethanol followed by a 10-minute incubation in a 30% bleach plus 0.01% SDS sterilization solution. The sterilization solution was then removed and the seeds rinsed 3 times with sterile water. For winter-type varieties with primary seed dormancy, seeds were left to soak for 30 minutes in 0.01mM gibberellin 4+7 (PhytoTechnology Laboratories #G358) before plating in order to break dormancy. Gibberellin 4+7 powder was initially dissolved in 95% ethanol then diluted in water to make a 1mM stock solution; the 0.01mM working solution (made from the stock solution) was used within two weeks of being made). Gibberellin treatment was not necessary for spring variety seed germination as it has low primary seed dormancy. The primary dormancy for several winter pennycress cultivars can be broken without GA treatment if left to ripen at room temperature for a few weeks after harvest.

Surface-sterilized pennycress seeds were plated onto 0.8% agar media containing one-half-strength Murishige and Skoog salts, or moistened Whatman 3MM chromatography paper (cat# 3030-6461), in Parafilm-wrapped petri dishes, then immediately placed into a Percival Scientific CU-36L5 incubator (16 hours 4100K fluorescent light $\sim 150\text{-}200\mu\text{E m}^{-2}\text{ s}^{-1}$ /8 hours dark, 22°C). For growth in soil, seedlings were transplanted at a density of 4 plants per 4-inch pot (OBC Northwest Inc.#PPG4) in autoclaved Redi-Earth Plug and Seedling soil mix (or a 50/50 mix of Redi-Earth Plug and Seedling mix and Berger BM 7 bark mix) intermixed with 0.03g/4-inch pot of the insecticide OHP Marathon 1% Granular. When making up the 4-inch pots, a thin layer of wet soil ($\sim 1/4$ inch) was first put in the bottom of the pot, on top of which 1/8 teaspoon of prilled urea (46-0-0; Greenway Biotech, Inc.) was sprinkled before the pot was entirely filled with the wet soil mix. Plants were grown in environment-controlled growth chambers cycling 16 hours light/8 hours dark (light was either 6500K fluorescent or a combination 4100K fluorescent/incandescent lighting, $175\text{-}250\mu\text{E m}^{-2}\text{ s}^{-1}$ light intensity), at 21 or 22°C.

Spring32 whole genome sequencing

Whole-genome sequencing was performed on DNA from 10th-generation inbred seedlings isolated with the DNEasy Plant kit (Quiagen #69106) using Illumina HiSeq 2500 at the University of Minnesota Genomics Center. Paired-end raw reads were processed with the Trimmomatic tool [5] to remove the adaptors and low-quality reads. To identify the nucleotide variants in the Spring32 genome, reads were mapped to the *Thlaspi* v1.0 genome using the Burrows-Wheeler Aligner (BWA) software package. Aligned files were then passed through SAMtools and Picard tools to remove PCR duplicates and assign read groups to the files [2, 6]. Genomic variants in the Spring32 aligned file were identified using HaplotypeCaller in the Broad Institute's Genome Analysis Toolkit (GATK), which is effective in identifying the

INDELs along with the Single Nucleotide Variants (SNVs). Variant files were processed through GATK to select for homozygous variants with the read depth (DP) of ≥ 50 and Quality (QD) of ≥ 20 . Selected variants were then compared to the *Thlaspi* v1 CDS and protein databases to identify the variants in the coding regions. All genome sequencing and analyses were performed by the Marks lab at MNU.

Agrobacterium-mediated transformation of pennycress

To determine if pennycress could be genetically transformed via floral dip, we introduced binary vectors harboring various genes of *in planta* selectable markers (including Hygromycin Phosphotransferase II (*HPTII*) conferring resistance to hygromycin B, Neomycin Phosphotransferase II (*NPTII*) conferring resistance to kanamycin or its analog paromomycin, Bialaphos Resistance (*bar*) conferring resistance to glufosinate, and DsRed which produces a visible red fluorescent protein). Each of these binary vectors was transformed into *Agrobacterium tumefaciens* strain GV3101 using a standard CaCl_2 flash-freeze/thaw transformation method [7].

Cultures of *A. tumefaciens* strain GV3101 containing quality controlled binary vectors with different selectable markers were seeded from glycerol stocks (~200uL inoculated into 50mL Luria Broth (LB) containing 50 $\mu\text{g}/\text{ml}$ Gentamycin, 50 $\mu\text{g}/\text{ml}$ Rifampicin, plus either 50 $\mu\text{g}/\text{ml}$ Kanamycin or 75 $\mu\text{g}/\text{ml}$ Spectinomycin). The 50mL cultures were shaken overnight at 28°C, then added to an additional 200mL LB antibiotic-containing media and incubated overnight in a 28°C shaker. The next morning cultures were centrifuged at 3,500 RPM for 10 minutes and resuspended in 3/4 volume of 5% (w/v) sucrose plus 0.02% (v/v) Silwet L-77.

The fully flowering inflorescences of pennycress plants (Figure 3) were submerged in this *Agrobacterium* solution, then placed under a range of pressure from 0 to ~30 inches mercury

(14.7 psi) vacuum in a 26cm x 25cm x 36.5cm vacuum chamber for 5 to 10 minutes or as otherwise noted, using a diaphragm vacuum pump (60 L/Min pump speed). After dipping, the floral portions of the inflorescences were wrapped in plastic wrap sealed around the stems with twist ties (Figure 3), and the plants placed back into an environmental growth chamber. The plastic wrap covering was removed the following day. Pennycress inflorescences were submerged for durations ranging from 2 to 30 minutes under a range of vacuum pressures.

Ethyl methanesulfonate treatment of pennycress seeds

Forty one grams of surface sterilized pennycress seeds were divided into four 250mL Erlenmeyer flasks holding 10g seed each, with 1g in a separate flask as a control and shaken in distilled water overnight. After 12 hours, the water was removed and 25mLs 0.2% EMS (Sigma #M0880-5G) in 0.1M Sodium Phosphate Buffer (pH 7) was added to each flask (besides the control flask which received only 0.1M Sodium Phosphate Buffer with no EMS). The seeds were shaken in the EMS solution for 18 hours in a fume hood after which the EMS was decanted into a dedicated waste receptacle and 25mLs of dH₂O was added to each flask to shake for 20 minutes. This rinse was decanted into the EMS waste, and the treated seeds were agitated for 20 minutes in 0.1M Sodium Thiosulfate (pH 7.3) in order to deactivate the EMS. After the sodium thiosulfate was decanted into its dedicated waste bottle the seeds were rinse 4x for 15 minutes each with dH₂O. The treated seeds were then dried for 24 hours on filter paper in a chemical flow hood before planting.

Results and Discussion

Growing pennycress in a laboratory setting

Spring32 pennycress grows well in traditional Brassica-species conditions, requiring 16 hours light/8 hours dark under a combination of fluorescent and incandescent lighting (between

175-250 $\mu\text{E m}^{-2} \text{ s}^{-1}$ light intensity), at 21°C and 50% humidity. Light intensity below 175-250 $\mu\text{E m}^{-2} \text{ s}^{-1}$ resulted in spindly plants with elongated hypocotyls and early bolting, while light intensity higher than 300 $\mu\text{E m}^{-2} \text{ s}^{-1}$ stresses the plants and can result in burned leaf edges. The plant growth requirements are so similar to those of *Arabidopsis* that the two plants can be grown side by side in the same growth chamber (Figure 1). Plant generation time can be accelerated by limiting or not applying nitrogen fertilizer, allowing for seed to seed growth in approximately 120 days (results not shown). Spring32 does not require vernalization or gibberellin (GA) treatment and performs consistently well both in laboratory and field settings. After 10 generations of single seed descent, the Spring32-10 germplasm is considered inbred and has been made available for research purposes.

Sequencing Spring32-10

To facilitate genetic research on pennycress and promote its use as a model system, the Spring32-10 genome was sequenced and made publicly available via NCBI by the David Marks lab at the University of Minnesota. The draft captured most of the gene space and identified over 27,000 candidate pennycress orthologues highly similar to *Arabidopsis* genes, including many common crop domestication target sequences. Like other spring-type pennycress cultivars, the Spring32-10 *FLOWERING LOCUS C (FLC)* gene is mutated resulting in the lack of a requirement for vernalization to induce flowering in pennycress [8]. *Arabidopsis* genes and corresponding Spring32-10 pennycress candidate orthologues share about 88% nucleotide sequence identity. These high levels of homology and the diploid nature of the pennycress genome makes translational research between *Arabidopsis* and pennycress straightforward.

Agrobacterium-mediated transformation of pennycress requires floral dip under vacuum

Screening through thousands of T₁-generation seeds arising from dipped plants generated no drug resistant or fluorescent seedlings when no vacuum was applied, signifying that transformation had not occurred. However, transformants among T₁ seeds were identified for plants which had been subjected to a vacuum while submerged in the *Agrobacterium* solution, with the highest transformation efficiency (0.5%) corresponding with the highest vacuum pressure applied (30 inches mercury (Hg), or 14.7 pounds per square inch (psi); Figure 5). We observed no significant differences in transformation efficiencies with vacuum durations longer than 5 minutes (data not shown).

DsRed fluorescence screening using a NightSea dual fluorescent protein flashlight allowed for relatively easy visual identification of transformants at the seedling or adult plant stages (Figure 4, Figure 5). Selection of seedlings transformed with the *HPTII* gene using 40 U/mL hygromycin B worked well, with transformants exhibiting sustained root and shoot growth and lack of cotyledon and leaf yellowing on drug-containing agar media compared to untransformed germinating seedlings (Figure 4, Figure 6). Wild-type pennycress seedlings possess a natural resistance to kanamycin which made it difficult to distinguish transformed seedlings from those untransformed even at high drug concentrations (Figure 6). However, pennycress seedlings exhibited more uniform sensitivity to paromomycin (a kanamycin analog) at a concentration of 100µg/mL, allowing for more accurate identification of seedlings transformed with the *NPTII* gene. *Bar* gene-transformed pennycress seedlings were successfully grown and selected for on agar media containing 18µg/mL glufosinate (119.7µl/1,000mL Finale herbicide) (Figure 6).

Along with Spring32, we used the *Agrobacterium*-mediated floral dip transformation method on four winter-type pennycress cultivars to assess if winter-type cultivars could also be transformed and if there were differences in transformation efficiencies. While all cultivars could be transformed, efficiencies varied from 0.06% to 0.39%, which were all less than the 0.55% efficiency of Spring32 (9 transformants out of 2,300 seeds tested for Elizabeth, 5 out of 1,650 for a proprietary breeding line, 1 out of 1,800 for W12, and 1 out of 1,200 for Beecher, compared to 10 out of 1,824 for Spring32).

EMS mutant populations

Pennycress mutant populations were generated with germplasm from Spring32 and Elizabeth (A registered winter line, Reg.No.GP-36). EMS treated Spring32 pennycress seeds were germinated and grown in an environmental growth chamber at 21°C, 16:8 day/night 50% humidity. Approximately 14 days after planting, plants were thinned and transplanted to a density of 4 plants per 4' pot. The Elizabeth mutant population was field grown at Western Illinois University. In each of the M1 grow outs, the plants displayed characteristic chlorotic leaf sectors that are indicative of a successful mutagenesis (Figure 7). After dry down, ~2000 individual M1 plants were carefully catalogued and harvested. The M2 seeds were surface sterilized, planted, and grown according to the protocols previously described. The resultant plants displayed several unique phenotypes, and the populations have been made available for research purposes (Figure 7).

Conclusions

Pennycress is a new potential model system as well as an emerging cash crop that is as simple to cultivate and engineer as *Arabidopsis*. Experiments can be performed on pennycress that directly test and/or improve agronomic performance, combining basic and applied research.

The compact stature of pennycress allows it to be grown alongside *Arabidopsis* in environmental chambers while its robustness and short life cycle are an asset in field experiments. It is simple and relatively expedient to grow pennycress in a laboratory setting, and the fully sequenced genome can be readily transformed with a pressurized *Agrobacterium*-mediated floral dip. A naturally occurring spring-type pennycress variant was inbred for ten generations of self-pollination and single seed descent in order to produce a homogenous seed lot equivalent to Columbia, and two EMS mutant populations were generated- one in the Spring32 background and one in the Elizabeth background.

Pennycress' amenability to molecular manipulation, ease of cultivation, and the genetic and germplasm resources that including large mutant and wild-type collections and sequenced genomes make pennycress an attractive new model system for both laboratory and field experimentation. With this toolbox we propose pennycress as a new model plant species more closely related to traditional oilseed crops than *Arabidopsis* [1, 2, 9].

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Fig. 1. Pennycress (*Thlaspi arvense*) compared to Arabidopsis (*Arabidopsis thaliana*). In panels A, C, D, and E Arabidopsis is positioned to the left of pennycress. **A.** Plants grown in the same soil and growth conditions. **B.** Pennycress field plots (front left plot is cultivar Spring32). **C.** 7 day-old seedlings grown on vertically-oriented agar medium. **D.** Microscopic images of seedling roots grown on vertically-oriented agar medium. **E.** Silicles of senesced plants. **F.** Seeds of Arabidopsis (top) and pennycress (bottom).

Arabidopsis Root tip

Methylene Blue stain , 40X magnification

Pennycress Root tip

Methylene Blue stain , 40X magnification

Fig. 2. Microscope images comparing the cell sizes (boxes) and nuclei (arrows) in Arabidopsis versus pennycress roots (stained with methylene blue for visualization). Images are shown at the same magnification.

Fig. 3. *Agrobacterium*-mediated floral transformation of pennycress. **A.** Pictured are three racemes of pennycress plants; Dipping of the raceme in the center is likely to produce the highest transformation efficiency. **B** and **C.** Racemes of plants submerged (dipped) in a solution containing *Agrobacterium tumefaciens* (strain GV3101), 5% sucrose, and 0.02% Silwet L-77, within a chamber put under 30 in Hg (14.7 psi) vacuum. **D.** After the vacuum infiltration, the racemes were wrapped in plastic wrap sealed with twist ties, then placed in a growth chamber; **E.** Plastic wrap was removed the following day.

Fig. 4. *Agrobacterium*-mediated floral dip transformation of pennycress. **A.** Transformation efficiencies associated with exposing pennycress racemes submerged in binary-vector-carrying *Agrobacterium* to various vacuum pressures for 5 minutes. **B.** PCR analysis to confirm that red-fluorescing seedlings were transgenic for the DsRed gene. Shown is agarose gel-electrophoresed DsRed gene-derived PCR products, using the following templates: Lane 1: DsRed gene-containing binary vector DNA; 2: Tissue preparation from wild type; 3 through 5: Tissue preparations from four independent DsRed transformants. **C.** A DsRed-fluorescing transgenic pennycress seedling among non-transgenic seedlings on an agar plate, detected using a NightSea fluorescent protein flashlight. **D.** A transgenic pennycress seedling (arrow) carrying the *HPTII* gene and exhibiting resistance to 40 U/mL hygromycin B in an agar medium.

Fig. 5. Visualizing DsRed in Pennycress **A.** Visualization of red fluorescence emanating from transgenic pennycress plants expressing the DsRed protein, using the NightSea fluorescent protein flashlight. **B.** DsRed expressing seedling compared to WT. **C.** 2 week old pennycress rosettes in white light. **D.** Plants from C. viewed with the NightSea flashlight.

Fig. 6. Pennycress seed germination and growth on agar media containing various concentrations of **A.** hygromycin **B.** paromomycin, **C.** kanamycin, and **D.** glufosinate in the herbicide Finale.

Fig. 7. EMS mutagenesis of pennycress. **A.** and **B.** M1 mutant pennycress plants displaying chlorotic leaf sectors indicative of a successful mutagenesis. **C.** Two unique pennycress M2 mutant phenotypes compared to WT

CHAPTER III: GENERATING ZERO ERUCIC ACID PENNYCRESS

Abstract

Pennycress (*Thlaspi arvense*) is an emerging biofuels feedstock crop closely related to Arabidopsis and canola that provides multiple ecosystem services and value-added co-products such as high-protein animal feed. Pennycress possesses a unique combination of attributes including extreme cold tolerance, rapid growth, over-wintering growth habit, and a natural ability to produce copious amounts of seeds high in oil and protein. Pennycress could potentially generate billions of liters of oil annually throughout temperate regions of the world, without displacing food crops or requiring land use changes, e.g. growing double-cropped between corn and soybeans in otherwise vacant fields. Post oil extraction, the pennycress seed meal can be used as a high protein, nutrient-filled animal feed supplement. Work must be done to develop pennycress varieties possessing seed oil and meal compositions better suited for biofuels applications and animal feeds. FATTY ACID ELONGASE1 (FAE1) is an enzyme in the fatty acid biosynthetic pathway that converts C18:1 oleic acid to C22:1 erucic acid. Erucic acid is undesirable for biodiesel production due to its poor cold-flow properties and is restricted in feed/food by the USDA due to possible toxicity. Here we present data and discuss targeted disruption of the *FATTY ACID ELONGATION1* (*FAE1*) gene with the CRISPR-Cas9 genome editing system. We show that pennycress *fae1* mutations genetically mimic mutations that occurred naturally in rapeseed resulting in “zero-erucic” canola oil, thereby making pennycress seed oil less viscous and ideal for conversion to biodiesel. Pennycress *fae1* mutations also result in edible seed castings (meal) suitable for use as animal feed.

Keywords: Pennycress; biofuels; genetics; canola; erucic acid; genetic engineering; edibility; oil

Introduction

Pennycress

Pennycress possesses a unique combination of attributes including extreme cold tolerance, rapid growth, over-wintering growth habit, and a natural ability to produce copious amounts of seeds high in oil and protein [1]. Pennycress could potentially generate billions of liters of oil annually without displacing food crops or requiring land use changes, growing in otherwise vacant fields. Throughout the Midwest, large portions of the rural landscape do not have a living cover from harvest time in the fall until soybean or corn establish in the early summer [2]. As a winter cover, pennycress plants can protect soil from erosion and can scavenge excess nitrogen and phosphorous that would normally be washed into streams and waterways during rain events [3].

Pennycress seeds are ~34% oil by weight and store lipids in the form of triacylglycerols (TAGs) [4]. Post oil extraction, the left over pennycress seed meal can be used as a high protein, nutrient-filled animal feed [1]. While pennycress holds much agronomic promise, the erucic acid naturally accumulated in pennycress seed oil currently renders it too viscous for biodiesel and unsuitable for animal consumption [4, 5, 6, 7, 8]. Work must be done to develop elite pennycress varieties devoid of erucic acid in the seed oil and meal in order to be better suited for diesel engines, as well as making the seed castings (meal) safe and palatable for ruminant farm animals [4, 5, 7, 8].

Erucic acid

Promoting value-added products like animal feed is crucial to the adoption strategy of pennycress for use as a biofuel feedstock [1]. Pennycress seeds are ~34% oil by weight and store lipids in the form of triacylglycerol (TAGs) [4]. TAGs function as energy stores for seed embryos

in Brassicaceae and are composed of three fatty acids of varying carbon chain lengths and degrees of saturation that are ester-linked to a glycerol backbone. Wild-type pennycress seeds naturally store ~33% of their total oil as C22:1 erucic acid, which is viscous and can be troublesome for diesel engines, especially in cold weather [5, 9]. Additionally, the erucic acid left over in pennycress seed castings restrict its use as an animal feed [1].

Rapeseed oil exhibits similar levels of erucic acid and garnered a poor reputation due to the presence of erucic acid, which has a bitter taste and can cause health problems in livestock. [7 8, 10, 11]. Rapeseed oil was subsequently banned as a food product by the FDA in 1956 [12]. Cultivars of rapeseed spontaneously developed a mutation that abolished erucic acid- now called canola, rendering the seed meal and oil safe and palatable for consumption [13]. It is hoped pennycress will mirror the adoption and trajectory of canola (a relatively new crop) the press-cake of which is a major commodity and one of the most widely used protein sources in animal feed with over 1 million short tons produced in the U.S. in 2015, valued at ~\$300 million USD [14].

FATTY ACID ELONGATION I

Fatty acids are commonly classified into four length designations: short chain (SCFA; C2 to C6), medium chain (MCFA; C6 to C12), long chain (LCFA; C13 to C22), and very long chain fatty acids (VLCFA; $\geq$ C22). In Brassicaceae, the FAE1 elongase complex converts 18:1 oleic acid (a lower viscosity, edible fatty acid) to 22:1 erucic acid which exhibits high viscosity poorly suited for biodiesel and is restricted by the USDA for use in feed (15). It has been shown that knockout mutations in the *FATTY ACID ELONGATION 1 (FAE1)* gene that encodes the β -ketoacyl-CoA synthase responsible for the condensation reaction results in the abolishment of

erucic acid in seed oil, and that *FAEI* loss-of-function mutations are what give canola its “Zero Erucic” trait [13, 16, 17, 18, 19].

Methods

Generating CRISPR-Cas9 Vectors

To identify the putative *TaFAEI* gene orthologue in pennycress, we used the *Arabidopsis thaliana AtFAEI* gene as query and searched the *Thlaspi arvense* genome using a pennycress-specific Basic Local Alignment Search Tool (BLAST) program (<http://pennycress.umn.edu/>). We also searched the *Thlaspi arvense* transcriptome shotgun assembly using the National Center for Biotechnology Information (NCBI) nucleotide BLAST online search tool, identifying the putative *TaFAEI* gene and coding sequence (GAKE01018976.1). The *TaFAEI* open reading frame (ORF) was found to be 1,521 nucleotides in length, sharing 87.8% nucleotide sequence identity with the 1,521bp *AtFAEI* ORF (AT4G34520) (Figure 8). The *TaFAEI*-CRISPR-Cas9_Hyg binary vector construct was generated, as described in Fauser et al., 2014 using the vectors pEn-Chimera and pDe-Cas9. The plant selectable marker (*bar*) in the pDe-Cas9 binary vector was replaced with the *Hygromycin phosphotransferase (hpt)* to create a pDe-Cas9_Hyg vector. Bacterial selection used for the binary vector was 75µg/ml Spectinomycin. The following two oligos were annealed to create the 20-mer protospacer specific to the open reading frame of the putative pennycress *TaFAEI* gene:

PennyFAEI_CRISPR_FWD: ATTGTGGCTCTCTACATCGTAACC

PennyFAEI_CRISPR_REV: AAACGGTTACGATGTAGAGAGCCA

Completed constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 using a standard CaCl₂ flash-freeze/thaw transformation method [20].

Agrobacterium-mediated transformation of pennycress

Culture of *Agrobacterium tumefaciens* strain GV3101 containing the *TaFAE1*-CRISPR-Cas9_Hyg plasmid was seeded from a glycerol stock (~200uL inoculated into 50mL Luria Broth (LB) containing 50µg/ml Gentamycin, 50µg/ml Rifampicin, plus 75µg/ml Spectinomycin. The 50mL cultures were shaken overnight at 28°C, then added to an additional 200mL LB antibiotic-containing media and again incubated overnight, then centrifuged at 3,500 RPM for 10 min and resuspended in an equal volume of 5% (w/v) sucrose plus 0.02% (v/v) Silwet L-77. The fully flowering inflorescences of ~1 month old pennycress plants were submerged in this *Agrobacterium* solution, then placed in a vacuum chamber at ~30 inches mercury (14.7 psi) for 10 minutes. After dipping, the floral portions of the inflorescences were wrapped in plastic wrap sealed around the stems with twist ties before being placed back into an environmental growth chamber. The plastic wrap covering was removed the following day.

Surface sterilization of pennycress seeds and growth conditions

Putative *TaFAE1*-CRISPR-Cas9_Hyg transgenic seeds were surface sterilized with a brief rinse with 70% ethanol followed by a 10-minute incubation in a 30% bleach plus 0.01% SDS sterilization solution. The sterilization solution was then removed and the seeds rinsed 3 times with sterile water and plated onto 0.8% agar/one half-strength Murashige and Skoog salts containing 40 U/mL hygromycin B. Seedlings that continued to grow on the antibiotic-containing media were transferred to soil ~8 days after plating. For growth in soil, seedlings were transplanted at a density of 4 plants per 4-inch pot (OBC Northwest Inc.#PPG4) in autoclaved Redi-Earth Plug and Seedling soil mix supplemented with 0.03g/4-inch pot of the insecticide OHP Marathon 1% Granular. Plants were grown in environmental growth chambers

cycling 16 hours light/8 hours dark (light was either 6500K fluorescent or a combination 4100K fluorescent/incandescent lighting, $175\text{-}250\text{ }\mu\text{E m}^{-2}\text{ s}^{-1}$ light intensity), at 21°C

Western blotting

Post hygromycin selection, 100mg of leaf tissue of individual putative CRISPR vector-containing plants were crushed in SDS-PAGE sample buffer and run in 7.5% polyacrylamide gels before being transferred onto a nitrocellulose membrane for western blots. The primary antibody Guide-it Cas9 Polyclonal antibody (Clontech #632607) was used at a 1:1,500 dilution in TBST + 5% milk and α -Rabbit HRP secondary antibody (Thermo #31460) at a 1:5,000 dilution in TBST + 5% milk were used to probe for the Cas9 protein. Membranes were exposed to film after addition of Supersignal chemiluminescent substrate (Thermo #34077). Signal from the *Streptococcus pyogenes* Cas9 protein signal can be detected at 160kDa.

T7 endonuclease

Indels generated by CRISPR were detected using the enzymatic mismatch cleavage (EMC) method following protocols previously described in Pyott et al., 2016. PCR template was extracted from fresh leaf tissue using the Phire Plant Direct PCR Kit (Thermo # F130WH). *FAEI* was amplified from genomic DNA of these putative *faeI* CRISPR-Cas9 plants using:

TaFAEI_OutterF1: ACATGCATGTAAAACGTAACGG

TaFAEI_OutterR1: TGGATTATATCAGGATGTGGCG

Product size: 1,842bp

The PCR master mix was spiked with 0.2uL/reaction wild type DNA to ensure that even in the event of a homozygous edit, a DNA mismatch would occur post DNA hybridization. 10uL of the resultant PCR product was denatured by heating at 99°C for 5 minutes in a thermocycler and hybridized with gradual cooling before being digested with T7 endonuclease I (NEB # M0302S)

for 30 minutes. The digested product was electrophoresed on a 1% agarose gel containing ethidium bromide and visualized with a UV transilluminator to identify samples that partially digested, indicating an *SpCas9* induced indel in *TaFAE1*.

PCR and sequence analysis of putative CRISPR fae1 pennycress

The full ORF of the pennycress *FAE1* gene was amplified from genomic DNA of putative *fae1* CRISPR-Cas9 pennycress plants that showed cleavage in the T7 protocol using Phusion DNA polymerase (NEB #M0530) and primers TaFAE1_OuterF1 and TaFAE1_OuterR1 (sequences listed above). Thermal cycling parameters were as follows: 1-minute initial denaturation at 98°C; 33 cycles of 98°C for 10 seconds, annealing at 55°C for 15 seconds, and a 72°C extension for 2 minutes, followed by a single 5-minute final extension at 72°C. Amplicons were detected by electrophoresis in a 1% agarose gel containing ethidium bromide and visualization with a UV transilluminator. Bands of the expected 1.8kb size were cut from a 1% agarose gel and DNA was extracted with the GeneJET Gel Extraction kit (#K0692) before being sequenced with the primer pennyFAE1_OuterF1. DNA sequences (.abi) were analyzed using either Benchling or Lasergene 13 software to identify/confirm the corresponding mutation.

2.7 Fatty acid analysis of seed oils

Total lipids were extracted from pennycress seeds, and fatty acid methyl esters (FAMES) were generated and analyzed by gas chromatography, as described in Cahoon et al., 2006.

Results

The *TaFAE1*-CRISPR-Cas9_Hyg vector was introduced into pennycress plants using the Agrobacterium-mediated vacuum infiltration method. Initially, transgenic *TaFAE1*-CRISPR-Cas9_Hyg T₁-generation seedlings were screened for the highest *SpCas9* expression by Western blot analysis using an anti-*SpCas9* antibody (Clontech Guide-it™ Cas9 Polyclonal Antibody,

catalog number 632607). However, this approach was abandoned after discovering that Cas9 editing activity did not correlate well with the level of *SpCas9* expression. Instead, we moved to selecting for putative *SpCas9*-induced genome edits in T₂ and T₃-generation seedlings by performing a T7 endonuclease I digestion of PCR products spanning the CRISPR target site before moving on to whole gene sequencing (Figure 9). Of the lines showing heritable mutations, a 4 bp deletion (mutant *fae1-3*), a single A insertion (*fae1-4*), and a single T insertion (*fae1-5*) were chosen for further characterization (Figure 10). Seeds from homozygous *fae1-3*, *fae1-4*, and *fae1-5* plants were analyzed for TAG fatty acid composition, revealing negligible amounts of erucic acid in all three lines in a genetic mimic to the mutation that occurred naturally in rapeseed resulting in “zero-erucic” canola (Table 1, Figure 11, and Figure 12). These plants have no observable phenotype and grow as wild type.

Discussion

Thlaspi arvense L. (pennycress) is being developed as a profitable oilseed-producing winter annual cover crop with extreme cold tolerance and a rapid life cycle that can be double-cropped on fallow farmland throughout the U.S. Midwest Corn Belt, controlling soil erosion and nutrient runoff while serving as an additional source of income for the American farmer without displacing food crops. Post oil extraction, the pennycress seed meal can be used as a high protein, nutrient-filled animal feed supplement. Work must be done to develop pennycress varieties possessing seed oil and meal compositions better suited for biofuels applications and animal feeds. FATTY ACID ELONGASE1 (FAE1) is an enzyme in the fatty acid biosynthetic pathway that converts C18:1 oleic acid to C22:1 erucic acid, which is undesirable for biodiesel production due to its poor cold-flow properties and is restricted in feed/food by the USDA due to possible toxicity. We created a CRISPR-Cas9 binary vector construct named *TaFAE1*-CRISPR-

Cas9_Hyg, utilizing a vector set optimized for use in Arabidopsis [21]. The *TaFAEI*-CRISPR-Cas9_Hyg vector was designed to target edits ~ 216bp downstream of the translational start site of the *TaFAEI* gene. CRISPR-Cas9 editing of *TaFAEI* abolishes erucic acid in pennycress seed triacylglycerols. The Cas9 transgene has been segregated away from the germplasm through a series of back crosses to wild type, resulting in a non-GMO seed lot with no observable deleterious phenotype. This *fae1* mutation is a genetic mimic to the mutation that occurred in toxic rapeseed that resulted in the edible variety known as canola. Pennycress *fae1* mutants accumulate 30% more 18C fatty acids than wild type and no erucic acid- resulting in a lower viscosity, edible seed oil which greatly improves the value of the seed oil both for industrial applications and consumption.

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Seq1(1>1521)	Seq2(1>1521)	Similarity	Gap	Gap	Consensus
FAE1 ORF GAKE01018976	FAE1 ORF AT4G34520.seq	Index	Number	Length	Length
(1>1521)	(1>1521)	87.8	3	6	1524
v10	v20	v30	v40	v50	v60
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^160	^170	^180	^190	^200	^210
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^160	^170	^180	^190	^200	^210
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^9960	^9970	^9980	^9990

Fig. 9. T7 endonuclease assay of segregating *faeI* mutants. Lane 1: 1kb GeneRuler Lane 2: shows a questionable result, 3-8 have only a single undigested band indicating WT *FAEI*, while lanes 8-13 show the WT *FAEI* band at 1.8kb, as well as the T7 digest products at 1.5kb and 300bp, indicating that a DNA mismatch occurred near the targeted PAM ~300bp from the penny*FAEI*_OuterF1.

Fig. 10. DNA sequence chromatograms showing *TaFAE1* coding sequences homozygous for a 4bp deletion in the *Tafae1-3* mutant, an adenine (+A) insertion in the *Tafae1-4* mutant, and a thymine (+T) insertion in the *Tafae1-5* mutant. All three mutations are located at the binding site of the CRISPR-*SpCas9* guide RNA.

Table 1

Table detailing the relative amounts (in mol%) of fatty acids in wild-type pennycress seed oil compared to the three *fae1* pennycress mutants. All three mutants are devoid of 22:1 erucic acid and accumulate ~30% more 18:1 oleic acid.

	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	22:2
WT	3.6	0.4	20.3	21.2	12	0	12	1.4	0	26.8	0.9
Cas+ WT	4.3	0.4	19.3	21	11.1	0	11.2	1	0	31.2	0.3
<i>fae1</i> K/O -4bp	4.3	0.6	52.8	25.2	15.8	0.1	0.8	0.1	0.1	0	0.1
<i>fae1</i> K/O +A	4.5	0.6	43.4	25.8	17.3	0.1	1.7	0.2	0.1	1.1	0.1
<i>fae1</i> K/O +T	3.1	0.5	64.0	16.8	12.7	0.1	1.3	0.0	0.0	0.0	0.0

Fig. 11. The pennycress *Tafae1-3* mutant harbors a 4-bp deletion in the *TaFAE1* coding sequence and contains undetectable amounts of erucic acid (22:1) and greatly reduced amounts of eicosenoic acid (20:1) in seed triacylglycerols. **A.** DNA sequence chromatograms showing the location of the *fae1-3* 4-bp deletion in relation to the CRISPR-SpCas9 protospacer and adjacent PAM site. **B.** Pie charts showing relative amounts of fatty acids in pennycress wild-type, pennycress *Tafae1-3* mutant, and canola seed triacylglycerols.

Fig. 12: Alternate representation of pennycress *fae1* knockout lipid data.

CHAPTER IV: ENGINEERING PENNYCRESS (*THLASPI ARVENSE*) TO PRODUCE REDUCED VISCOSITY SEED OILS FOR ‘DROP-IN’ BIOFUELS

Abstract

The finite nature of traditional fuel sources paired with the negative environmental consequences from their continued use underscore the need for renewable transportation biofuels as global population and energy demand continue to rise. In order to be competitive with fossil fuels, viable biofuel alternatives must be producible on a commercial scale without displacing food crops, provide a net energy gain, and be cost-efficient. Pennycress (*Thlaspi arvense*) is an emerging biofuels feedstock crop that provides multiple ecosystem services and possesses a unique combination of attributes including extreme cold tolerance, rapid growth, over-wintering growth habit, and a natural ability to produce copious amounts of seeds high in oil and protein. Pennycress could potentially generate billions of liters of oil annually throughout temperate regions of the world without displacing traditional crops, growing in what would otherwise be fallow farmland. Pennycress was stably transformed with the *Euonymus alatus* diacylglycerol acetyltransferase (*EaDAcT*) gene to produce a novel lipid with low viscosity. This novel oil shows a reduction in viscosity up to ~30% compared to traditional storage lipids, making them suitable as a biofuel that can be used in a ‘drop-in’ capacity for heavy diesel engines. By successfully expressing a novel lipid biosynthetic gene in pennycress, we demonstrate that pennycress can be used as a platform for the production of unusual lipids, as well as provide a plant-based feedstock of low-viscosity, non-edible seed oils for biofuels applications.

Keywords: Pennycress; Genetics; Drop-in; Diesel; Seed oil; Cover crops; Novel lipids

Introduction

Pennycress as a novel oil feedstock and model

Thlaspi arvense L. (pennycress) is a winter oilseed from the mustard family and is currently under development as a biofuel feedstock crop. Pennycress could be planted between the corn/soybean rotation on millions of hectares of farmland throughout the U.S. Corn Belt, growing in the winter and early spring without displacing food crops [1, 2]. Pennycress seeds are ~34% oil by weight and in the form of triacylglycerols (TAGs), and established oil extraction procedures yield between 600 and 1,200 L of oil/ha for pennycress, easily outperforming soybean and camelina at 450L and 420-640L respectively [1, 3, 4, 5]

Pennycress is a self-pollinating plant with a sequenced diploid genome highly similar to model plant *Arabidopsis thaliana*, which makes it an excellent candidate as a functional model plant [1, 6]. It is simple and relatively expedient to grow pennycress in a laboratory setting, and the fully sequenced genome can be readily transformed with a pressurized *Agrobacterium*-mediated floral dip (McGinn et. al., submitted). With modern genetic tools we are poised to rapidly domesticate and engineer novel crop species such as pennycress in order to meet the growing global energy demands [7]. Adoption of pennycress as a model system could significantly accelerate oilseed-crop translational research and facilitate the rapid domestication of this new, high-potential crop.

Traditional biofuels

Traditionally, biodiesel is derived from a renewable feed stock (i.e. vegetable oil and animal fat) and are most commonly sourced from rapeseed, soybean, palm, and sunflower oils for commercial purposes [8, 9]. However, generating biodiesel from edible oils is highly controversial as using existing crop land to produce fuel feed stocks could endanger food

security and has been projected to increase carbon emissions by up to 50% due to land use changes [10, 11]. Unprocessed seed oil cannot be used as a fuel in existing diesel engines due in large part to its high viscosity [12]. To produce usable biodiesel, the oil or fat is reacted with monohydric alcohol in presence of a catalyst, releasing the mono alkyl esters of long chain fatty acids from their glycerol backbone in a process called transesterification [13]. Aside from the cost of the lipid feedstock itself, the seed oil transesterification reaction represents the highest cost in biodiesel production [14]. This processing step could be eliminated from the seed oil-to-biofuel operation by engineering seed oil with appropriately decreased viscosity.

EaDAcT

The endosperm and embryonic tissues of *Euonymus alatus* (Burning Bush) seeds naturally accumulate high levels of 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs), while the aril around the seed produces long-chain triacylglycerols (lcTAGs) typical of most plants [15]. Burning Bush possesses a specialized diacylglycerol acetyltransferase (*EaDAcT*) which catalyzes the transfer of an acetyl group to the *sn*-3 position of diacylglycerol rather than another long acyl chain, forming an acetyl-TAG in lieu of the conventional lcTAG (Figure 13) [15]. These acetyl-TAGs show a 30% reduction in oil viscosity and improved cold temperature properties compared to lcTAGs, making them suitable as a drop-in biofuel for diesel #4 engines [16]. Additionally, the unique composition of acetyl-TAGs make them valuable as biodegradable lubricants, food emulsifiers, and plasticizers [17].

Methods

Vectors

The pGly*EaDAcT* binary vector (previously described in Durrett et al., 2010) possesses the *Euonymus alatus* diacylglycerol acetyltransferase (*EaDAcT*) gene driven by a seed-specific

Glycine max Glycinin promoter. The construct also contains the *NPTII* gene for 50µg/ml kanamycin selection in bacteria and the *DsRed* gene driven by the Cassava Vein Mosaic Virus (CVMV) promoter for red fluorescent protein visual screening in plants.

Agrobacterium-mediated transformation of pennycress

Culture of *Agrobacterium tumefaciens* strain GV3101 containing the pGlyEaDAcT binary vector was seeded from a glycerol stock (~200uL inoculated into 50mL Luria Broth (LB) containing 50µg/ml Gentamycin, 50µg/ml Rifampicin, plus 50µg/ml Kanamycin. The 50mL cultures were shaken overnight at 28°C, then added to an additional 200mL LB antibiotic-containing media and again incubated overnight, then centrifuged at 3,500 RPM for 10 min and resuspended in an equal volume of 5% (w/v) sucrose plus 0.02% (v/v) Silwet L-77. The fully flowering inflorescences of ~1 month old pennycress plants were submerged in this *Agrobacterium* solution, then placed in a vacuum chamber at ~30 inches mercury (14.7 psi) for 10 minutes. After dipping, the floral portions of the inflorescences were wrapped in plastic wrap sealed around the stems with twist ties before being placed back into an environmental growth chamber. The plastic wrap covering was removed the following day.

Identification of transgenic pennycress plants

Putative pGlyEaDAcT transgenic pennycress seeds were surface sterilized and germinated on either agar media or moist chromatography paper. After ~6 days of growth, seedlings were screened for DsRed expression using a NightSea Dual Fluorescent Protein Flashlight (DFP-GC). To prevent false positives and ensure each transgenic was found, putative DsRed expressing seeds were germinated prior to screening.

PCR analysis of transgenic plants

Genomic DNA was extracted from pennycress tissues using a standard CTAB extraction protocol described in Clarke et al., 2009. PCR was carried out using the following primers to amplify portions of coding sequences from the following genes of interest.

DsRed Forward: CCACAACACCGTGAAGCTGAAGG

DsRed Reverse: GGACTTGAAGTCCACCAGGTAGTGG

Product size: 418bp

EaDAcT Forward: TTCATCAAGGTTTGGGTACAAGC

EaDAcT Reverse: AAGGAATTCAGCTAGGGTTGC

Product size: 528bp

Thermal cycling parameters were as follows: 1-minute initial denaturation at 98°C; 32 cycles of 98°C for 10 seconds, annealing at 55°C for 15 seconds, and a 72°C extension for 1 minute; followed by a single 5-minute final extension at 72°C. Amplicons were detected by electrophoresis in a 1% agarose gel containing ethidium bromide and visualization with a UV transilluminator.

Lipid analysis

To quantify fatty acid composition, total seed lipids were separated using thin layer chromatography on Silica gel 60 plates (Merck) with a 70:30:1 hexane: diethyl-ether: acetic acid (v/v/v) solvent system. Lipids were visualized by spraying with 0.075% 2',7'-dichlorofluorescein in 95% methanol and exposing to UV light. The bands were scraped and directly transmethylated using a base-catalyzed method (PMID: 8727647); the resulting FAMES were analyzed using gas chromatography. All lipid analysis was performed by Sunil Bansal of the Durrett lab at Kansas State.

Results and Discussion

Pennycress was successfully transformed with the pGly*EaD*AcT construct designed to promote seed-specific expression of the specialized *Euonymus alatus* diacylglycerol acetyltransferase (*EaD*AcT) (Figure 14). The heterologous expression of *EaD*AcT in pennycress seeds resulted in the accumulation of acetyl-TAGs in storage lipids as determined by thin layer chromatography and electrospray ionization mass spectrometry (Figure 14, Figure 15). Product ion analysis generated daughter fragments consistent with the loss of an acetate group, confirming the identity of the acetyl-TAGs in the transformed lines (Figure 15). Compared to the lcTAGs still produced in the transgenic pennycress seeds, the *sn*-1 and *sn*-2 positions of the acetyl-TAGs were rarely populated by 22:1 acyl chains, preferring shorter acyl chains (Figure 15). This much lower incorporation of 22:1 in acetyl-TAGs is consistent with observations in other transgenic acetyl-TAGs expressing brassicas which also contain lower levels of very long chain fatty acids (VLCFA). This is likely explained by the *sn*-3 position of acetyl-TAG being occupied by acetate, and therefore unavailable for acylation by VLCFA such as 22:1 that would typically be incorporated there, leading to lower levels of this fatty acid in acetyl-TAGs. [18].

Conclusion

Pennycress is an overwintering oil-seed cover crop that could potentially generate billions of liters of oil annually without displacing food crops or requiring land use changes. Plants typically produce viscous long-chain triacylglycerols (lcTAGs) as the major storage lipid in seeds and therefore must undergo a transesterification reaction before being utilized as biodiesel in order to reduce the oil viscosity to a level that existing engines would find tolerable. This processing step could be eliminated from the seed oil-to-biofuel operation by engineering seed oil with appropriately decreased viscosity. *Euonymus alatus* (Burning bush) seeds naturally

accumulate high levels of 3-acetyl-1,2-diacyl-*sn*-glycerols (acetyl-TAGs) which display a 30% reduction in kinematic viscosity, improved cold temperature properties, and can be utilized as a drop-in biofuel for heavy diesel engines. The seed-specific expression of *EaDAcT* in pennycress resulted in the accumulation of acetyl-TAGs in seed oils. The ability to easily engineer pennycress to produce these useful lipids and then grow high yielding transgenic lines in the field will allow the production of large quantities of this oil for functional testing. Further, pennycress could potentially produce a variety of industrially useful lipids through the expression of the appropriate lipid biosynthetic genes. The ease and speed by which pennycress can be grown in the laboratory and the fact that it can be readily transformed make pennycress an attractive candidate as a new model plant species more closely related to traditional oilseed crops than *Arabidopsis*, as well as a robust platform for the generation of novel lipids without displacing food crops.

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Fig. 13. A specialized diacylglycerol acetyltransferase (*EaDAcT*) from Burning Bush catalyzes the transfer of an acetyl group to the *sn*-3 position of diacylglycerol rather than a long acyl chain, forming a low-viscosity acetyl-TAG in lieu of the conventional lcTAG

Fig. 14. *EaDacT*-expressing transgenic pennycress lines accumulate acetyl-TAG in seeds. **A.** *EaDacT*-expressing transgenic plants detected by the NightSea fluorescent protein flashlight. **B.** PCR analysis to confirm the presence of DsRed gene. Lane +: DsRed gene-containing binary vector DNA; Lane WT: Tissue preparation from wild type; 1 through 3: Tissue preparations from three independent pGly*EaDacT* transformants. **C.** Thin layer chromatography (TLC) analysis showing the presence of acetyl-TAG in transgenic pennycress seed oil. Lanes labeled 1 through 3 contain extracted seed oil from three independent *EaDacT* transgenic lines. WT=wild type seed oil. The last lane contains oil from *Euonymus alatus* (Burning Bush) seeds, whose endosperms contain 95% acetyl-TAG

Fig. 15. Pennycress seeds expressing *EaDAcT* accumulate acetyl-TAGs.

(a) Positive ESI mass spectra of neutral lipids from Pennycress wild-type seed or transgenic T₃ seed expressing *EaDAcT*. Signal peaks possess the m/z value of [M+NH₄]⁺ adduct. For clarity, only the number of acyl carbons and not the number (x) of double bonds in each series of acetyl-TAG molecular species is indicated. (b) ESI-MS² daughter scans of acetyl-TAGs from Pennycress seed expressing *EaDAcT*. Shown are the fragment peaks for acetyl-TAGs with [M+NH₄]⁺ adducts with mass of 676.7. (c) Mean fatty acid composition of acetyl-TAGs and lcTAG fractions from the T₃ seed of 4 independent lines expressing *EaDAcT*. Error bars represent SEM. Asterisks indicate statistical significant (Student's *t*-test, **, P < 0.01; ***, P < 0.01).

CHAPTER V: REDUCING GLUCOSINOLATE IN PENNYCRESS SEED MEAL

Abstract

Pennycress (*Thlaspi arvense*) is a member of the mustard family closely related to Arabidopsis and canola with considerable agronomic and economic potential due to its unique combination of attributes including extreme cold tolerance, rapid growth, over-wintering growth habit, and a natural ability to produce large volumes of seed high in oil and protein. Pennycress could be planted between the corn/soybean rotation on millions of hectares of farmland throughout the U.S. Corn Belt, allowing a single parcel of land to be triple cropped without compromising food production. Post oil extraction, the left over pennycress seed meal could be used as a high protein, nutrient-filled animal feed. A major concern impeding the utilization of pennycress press-cake as animal feed is the high level of sinigrin (a pungent glucosinolate) in the seeds which decreases feed palatability and can be degraded into toxic compounds. Unprocessed pennycress seed meal cannot currently be used in animal feed as a result of this high glucosinolate content. Here we discuss targets and approaches to engineer pennycress varieties that produce edible seed oil and castings suitable for use as a biodiesel feedstock and value-added animal feed.

Keywords: Pennycress; Glucosinolate; Sinigrin; Genetics; Crop domestication; Animal feed

Introduction

Glucosinolates in pennycress

Pennycress seeds are harvested with a standard straight combine in mid-May before soybeans are planted (1). Seed oil extraction via screw press yields between 600 and 1,200 L of oil/ha, easily outperforming soybean and camelina at 450L and 420-640L respectively [2, 3, 4]. After crushing the seeds and recovering the oil the resulting meal is ~27% protein by weight and energy dense due to the residual oil post-pressing, making this value-added coproduct an attractive candidate for use as an animal feed supplement [5, 6].

Promoting value-added products like animal feed is crucial to the adoption strategy for utilization of pennycress as a biofuel feedstock as the meal is projected to make up one third of the crop value (Arvegenix Inc. personal communication). However, pennycress meal has a high level of the anti-nutrient compound sinigrin (allyl-glucosinolate or 2-propenyl-glucosinolate), and without additional treatments, is not competitive with high-value products like the soybean and canola meal commonly used in animal feed [7, 8, 9]. Pennycress requires development to reduce bitter-tasting and potentially toxic compounds in seeds in order to make the left over press cake safe and palatable for livestock.

With the sinigrin glucosinolate reduced to a tolerable level ($< 30\mu\text{mol/g}$), it is hoped that pennycress will mirror the adoption and trajectory of canola (a relatively new crop) the press-cake of which is a major commodity and one of the most widely used protein sources in animal feed with over 1 million short tons produced in the U.S. in 2015, valued at ~\$300 million USD [10]. While unprocessed wild type pennycress seed meal cannot currently be used as feed due to its high glucosinolate content, a simple heating step deactivates myrosinase and renders the meal

safe and edible [7, 11, 12]. Without myrosinase heat deactivation, pennycress seed meal can be used as a natural biofumigant [7, 13].

Glucosinolates and animal feed

Many plants belonging to the mustard family (*Brassicaceae*) including rapeseed and camelina produce high levels of glucosinolates and glucosinolate hydrolysis products, which are proposed to serve as anti-herbivory agents [14]. Glucosinolates are found in all parts of the plant during vegetative development and are transported to the seeds upon senescence [15]. When brassica seeds are processed, the glucosinolates and GSL-hydrolysis breakdown products remaining in the left over seed meal can result in negative effects on animal nutrition [16, 17]. In the presence of water, myrosinase hydrolyzes glucosinolate, forming an aglycone intermediate which is then converted to allyl isothiocyanate (AITC) [14]. This glucosinolate degradation product has genotoxic effects, can cause stomach ulcers, and is toxic to the liver, thymus, and kidneys at high doses in rats [18, 19, 20, 21].

Pennycress possesses a characteristic ‘garlic-like’ odor indicative of glucosinolate, and upon characterization revealed high levels (~100umol/g seed) of a single allelopathic glucosinolate, sinigrin (allyl-glucosinolate or 2-propenyl-glucosinolate) [7]. A major concern impeding the use of pennycress press cake as animal feed is this high concentration of sinigrin glucosinolate which decreases feed palatability and can be hydrolyzed into toxic isothiocyanate (AITC) [9, 22, 7, 23].

Younger animals appear to be more severely affected by diets containing high levels of glucosinolates and exhibit reduced intake and decreased performance when fed glucosinolate-containing seed meal [24]. The thiocyanates produced from the hydrolysis of glucosinolates interfere with iodine uptake leading eventually to hypothyroidism [17]. Farm animals with

hypothyroidism experience growth retardation, reduction in milk and egg production, impaired reproductive activity, and decreased liver and kidney function [17]. Additionally, the garlicky flavor of pennycress press cake can taint the flavor of meat or eggs produced by animals fed glucosinolate containing seed meal [25].

Glucosinolate gene targets

Value-added coproducts like animal feed are crucial to the adoption strategy of pennycress, as the meal represents ~1/3 of the crop value. Due to the potentially deleterious effects that pennycress seed meal feed supplementation could have on livestock, it is imperative that glucosinolate content is reduced in pennycress seeds in order to render the press cake safe and palatable for farm animals [7, 4, 16]. While standard breeding techniques are being employed to reduce glucosinolate, we have yet to find a naturally occurring pennycress variant with significantly lower sinigrin. Glucosinolates are one of the most highly characterized secondary metabolites in *Arabidopsis*, and GWAS (Genome Wide Association Studies) have elucidated several promising gene targets in naturally occurring low-glucosinolate variants of other *Brassica* species [26, 27, 28]. Together, the sequenced pennycress genome and its high sequence similarity to *Arabidopsis* paired with the advent of new tools facilitating targeted genome editing provide a unique opportunity to rapidly alter putative pennycress glucosinolate target genes in a precise manner [29]. The major gene targets for reducing glucosinolate in pennycress seeds are *HAG1*, *CYP83A1*, and *GTR1/GTR2*.

HAG1 (HIGH ALIPHATIC GLUCOSINOLATE) is a transcription factor that functions as a positive regulator of aliphatic (methionine-derived) glucosinolates like sinigrin [30]. Experiments performed in *Arabidopsis* show that knocking down *HAG1* all but abolishes aliphatic glucosinolates in seed oil without a deleterious phenotype [31]. Additionally, genome-

wide association studies in rapeseed have identified deletions in *HAG1* (also referred to as *MYB28*) as the orthologue associated with the low seed glucosinolate phenotype of canola, and is an attractive target for glucosinolate knockout in pennycress (32).

CYP83A1 (*CYTOCHROME P450 MONOOXYGENASE*) is involved in the final steps in the methionine-derived glucosinolate biosynthesis pathway and is responsible for the oxidation of the aliphatic oximes and their conjugation with a sulfur donor [25]. In Arabidopsis, *REDUCED EPIDERMAL FLUORESCENCE 2* (*ref2*) mutants were found to harbor a disruption of *CYP83A1* and showed reduced levels of all aliphatic glucosinolates and no detrimental phenotype when compared to Columbia wild type [33].

Previous work in Arabidopsis identified two redundant glucosinolate transporter proteins, *GTR1/2* (*GLUCOSINOLATE TRANSPORT RECEPTOR1* and 2), which are responsible for translocating glucosinolates from the vegetative tissues into the seeds [34]. The Arabidopsis *gtr1/gtr2* double mutant showed negligible levels of glucosinolates in seed, and it is hypothesized that loss-of-function mutations in the pennycress GTR-like genes will similarly reduce seed glucosinolate levels.

In field trials at two locations in Minnesota during 2011 and 2012, pennycress was shown to significantly reduce the emergence of spring weeds [7, 35, 36, 37]. This ability to suppress weeds was observed when pennycress residues were incorporated or left on the soil surface, suggesting that a portion of the weed suppression effect may be due to biofumigant compounds released from decomposing pennycress tissues which possesses high levels of sinigrin glucosinolate [7, 35, 36]. This weed-suppression benefit would likely be lost if glucosinolate were entirely abolished (as in the cases of *HAG1* and *CYP83A1*), so despite the

30% seed yield loss seen in Arabidopsis, it is worth pursuing *gtr1/2* double mutants in pennycress [33].

Methods

Ethyl methanesulfonate mutagenesis

Forty one grams of sterilized pennycress seeds were agitated in distilled water overnight. After the water was removed, 25mLs of 0.2% EMS in 0.1M sodium phosphate buffer (pH 7) was added to each of the four 250 mL Erlenmeyer flasks containing 10g seed each. 1g of control seeds in a separate flask received phosphate buffer with no EMS. The flasks were shaken in a fume hood for 18 hours. The EMS solution was decanted into an EMS-specific waste bottle, and 25mL of dH₂O was added to each flask and agitated for 20 minutes. This rinse was also decanted into the EMS-specific waste bottle. The seeds were then washed for 20 minutes in 0.1M sodium thiosulfate (pH 7.3) in order to deactivate the EMS before the sodium thiosulfate solution was decanted into the EMS waste bottle. The seeds were rinsed 4 times with dH₂O for 15 minutes each and germinated directly in autoclaved Reddiearth soil at a density of approximately 10 seeds per 4-inch pot.

CRISPR-Cas9 vector construction

The CRISPR-Cas9_Hyg binary vectors were generated as described in Fauser et al., 2014 using the vectors pEn-Chimera and pDe-Cas9. The plant selectable marker (*Bar* gene) in the pDe-Cas9 binary vectors were replaced with the Hygromycin phosphotransferase (*hpt*) gene to create a pDe-Cas9_Hyg iteration. Bacterial selection used for the binary vector was 75µg/ml Spectinomycin. The following paired oligos were annealed to create the 20-mer protospacer specific to the open reading frame of the putative pennycress target genes:

PennyHAG1_*sp*CRISPR_FWD: ATTGTCAAGAAAGCCGTGTTGTGT

PennyHAG1_*sp*CRISPR_REV: AAACACACAACACGGCTTTCTTGA

PennyCYP83A1_*sa*CRISPR_FWD: ATTGCCAATCTTATCATACAAGAT

PennyCYP83A1_*sa*CRISPR_REV: AAACATCTTGTATGATAAGATTGG

PennyGTR1/2_*sa*CRISPR_FWD: ATTGGAAGAAGTGAAGTGCATTGT

PennyGTR1/2_*sa*CRISPR_REV: AAACACAATGCACTTCACTTCTTC

Due to the high sequence similarity, it is possible to target both putative pennycress *GTR* genes with a single protospacer. The pDe-*Sp*Cas9_Hyg, pDe-*Sa*Cas9_Hyg containing the *Streptococcus pyogenes* Cas9 (*Sp*Cas9) and the *Staphylococcus aureus* Cas9 (*Sa*Cas9) cassettes with the corresponding sequence-specific protospacers were transformed into *Agrobacterium tumefaciens* strain GV3101 using a freeze/thaw method described in Holsters et. al., 1978.

Agrobacterium-mediated floral dip

Culture of *Agrobacterium tumefaciens* strain GV3101 containing the CRISPR-Cas9_Hyg plasmids were seeded from glycerol stocks (~200uL inoculated into 50mL Luria Broth (LB) containing 50µg/ml Gentamycin, 50µg/ml Rifampicin, plus 75µg/ml Spectinomycin). The 50mL cultures were shaken overnight at 28°C, then added to an additional 200mL LB antibiotic-containing media and again incubated overnight, then centrifuged at 3,500 RPM for 10 min and resuspended in an equal volume of 5% (w/v) sucrose plus 0.02% (v/v) Silwet L-77. The fully flowering inflorescences of ~1 month old pennycress plants were submerged in this *Agrobacterium* solution, then placed in a vacuum chamber at ~30 inches of mercury (14.7 psi) for 10 minutes. After dipping, the floral portions were wrapped in plastic wrap sealed around the stems with a twist tie before being placed back into an environmental growth chamber. The plastic wrap covering was removed the following day.

Screening putative transgenic pennycress seeds and growth conditions

Pennycress seeds were surface sterilized with a brief rinse with 70% ethanol followed by a 10-minute incubation in a 30% bleach plus 0.01% SDS sterilization solution. The sterilization solution was then removed and the seeds rinsed 3 times with sterile water. Surface-sterilized pennycress seeds were plated onto 0.8% agar media containing one-half-strength Murishige and Skoog salts, or moistened Whatman 3MM chromatography paper (cat# 3030-6461), in Parafilm-wrapped petri dishes, then placed into a Percival Scientific CU-36L5 incubator (16 hours 4100K fluorescent light $\sim 150\text{-}200\ \mu\text{E m}^{-2}\text{ s}^{-1}$ /8 hours dark, 22° C). Surviving hygromycin-resistant T1-generation seedlings were transplanted into autoclaved Reddiearth soil mix and grown in an environmental growth chamber set to 16-hour days/8-hour nights at 21°C and 50% humidity.

Identifying CRISPR-induced mutations with T7 endonuclease I

Indels generated by CRISPR were detected using the enzymatic mismatch cleavage (EMC) method following protocols previously described in Pyott et al., 2016. PCR template was extracted from fresh leaf tissue using the Phire Plant Direct PCR Kit (Thermo # F130WH). The genes of interest were amplified from genomic DNA of their respective putative CRISPR-Cas9 expressing plants using:

TaHAG1_OuterF1: 5' AGACCCAAGAGCGTTTCTCG 3'

TaHAG1_OuterR1: 5' TCACACAAACCCTAAACCTGGG 3'

product 1,724bp

TaCYP83A1_OuterF1: 5' AGCTGTGTGCTGTTTATGTACC 3'

TaCYP83A1_OuterR1: 5' TTTTGGCTCGGCTATCTAGC 3'

product 1,840bp

TaGTR1_OuterF1: 5' ACATGTCTCTATCCTCCACAGC 3'

TaGTR1_OuterR1: 5' CTTCTTTTAGTGGTTCGTGACG 3'

product 3kb

TaGTR2_OuterF1: 5' TACCACTTTGTCTAGATTCCGG 3'

TaGTR2_OuterR1: 5' GATTACTTGGTTTCTCTATGGGC 3'

product 2,672bp

The PCR master mix was spiked with 0.2uL/reaction wild type DNA to ensure that even in the event of a homozygous edit, a DNA mismatch would occur post DNA hybridization. 10µl of the resultant PCR product was denatured by heating at 99°C for 5 minutes in a thermocycler and hybridized with gradual cooling before being digested with T7 endonuclease I (NEB # M0302S) for 30 minutes. The digested product was electrophoresed on a 1% agarose gel containing ethidium bromide and visualized with a UV transilluminator to identify samples that partially digested, indicating a Cas9 induced indel in the gene of interest.

PCR and sequence analysis of putative mutant pennycress

The full ORFs of pennycress genes of interest were amplified from genomic DNA of putative CRISPR-Cas9 expressing pennycress plants that showed cleavage in the T7 protocol using Phusion DNA polymerase (NEB #M0530) and the outer primers listed above. Thermal cycling parameters were as follows: 1-minute initial denaturation at 98°C; 33 cycles of 98°C for 10 seconds, annealing at 55°C for 15 seconds, and a 72°C extension for 2.5 minutes, followed by a single 5-minute final extension at 72°C. Amplicons were detected by electrophoresis in a 1% agarose gel containing ethidium bromide and visualization with a UV transilluminator. Bands of the expected sizes were cut from a 1% agarose gel and DNA was extracted with the GeneJET Gel Extraction kit (#K0692) before being sequenced with the OuterF1 primer of each respective

gene of interest. Sequences (.abi) were analyzed using either Benchling or Lasergene 13 software to identify/confirm the corresponding mutation.

NIRS analysis

Pennycress seeds were scanned using a Metrohm NIRS XDS MultiVial Analyzer to assess the amount of sinigrin as previously described [38]. Intact seed samples were placed in the NIRS sample holder (3cm diameter round cell) until it was three-fourths full, and their Near infrared (NIR) spectroscopic analysis was compared to the range of sinigrin in corresponding wild type seeds in order to determine their approximate sinigrin content. These analyses captured information related to the approximate levels of total glucosinolate and were used to identify low sinigrin candidates. Seeds showing a predicted sinigrin reduction were used in a wet lab analysis to confirm or determine the sinigrin amount with better accuracy.

Glucosinolate quantitation

Pennycress lines that displayed sinigrin content significantly below population average in the NIR scan were further analyzed along with their controls using a method adapted from Kliebenstein et.al, 2001. This purification technique follows the basic sephadex/sulfatase Arabidopsis protocol previously described [39]. These analyses were performed by Joe Lyons of Arvegenix Inc.

Results and Discussion

EMS Mutants

Two pennycress EMS mutant lines (I87113 and I87207) accumulated seed glucosinolate at or below the 30umol/g edibility threshold (Figure 16), as indicated first by NIRS analysis where sinigrin peaks were significantly lower than the corresponding ranges found in control parental seeds (Data not shown) and validated by wet chemistry. Unfortunately, both lines display severe,

semi-sterile phenotypes and will likely not be included among the candidates for the breeding program due to their reduced vigor.

CRISPR-Cas9 Mutants

Two unique pennycress *hag1* CRISPR-Cas9 mutants were generated (-G and +A) 17bp from ATG start, each resulting in a premature stop codon (Figure 17). However, when subjected to NIRS and wet chemistry, the mutants showed WT accumulation of seed glucosinolate (data not shown). It is hypothesized that transcription machinery may have exploited an alternate reading frame resulting in the last $\frac{3}{4}$ of the gene being transcribed and translated as usual. This region contains highly active domains that may be sufficient for glucosinolate biosynthesis (Figure 2).

Two unique Cas9-induced mutations in the ORF of pennycress *CYP83A1* (+T and +A) 206bp from ATG start result in premature stop codons (Figure 18). A range of deleterious phenotypes were seen in homozygous pennycress *cyp83a1* mutants. After the plants have senesced and the seeds are harvested, NIRS and wet bench analysis will be performed, and we hypothesize that the more extreme phenotypes will correlate with lower levels of sinigrin glucosinolate.

A flexible protospacer was designed to target the ORF of pennycress *GTR1* and *GTR2* at the same time without any predicted off-target effects. Sequence analysis of the T1's revealed Cas9 activity in *GTR2*, but none in *GTR1* (Figure 19). We hypothesize that because the flexible protospacer was designed with a preference for *GTR2*, *GTR1* editing will be seen in the next generation when the nuclease has had more opportunities to generate targeted double stranded breaks.

Conclusions

Pennycress is a high-potential oilseed crop that could provide billions of liters of oil as well as value-added products such as animal feed without displacing traditional food crops. After oil is pressed from pennycress seed, the remaining press cake still has value as a high protein animal feed. A major concern impeding the utilization of pennycress press cake as an animal feed is the high level of sinigrin (a pungent glucosinolate) in the seeds which decreases feed palatability and can be degraded into toxic compounds that result in negative effects on animal nutrition. Using the fully sequenced diploid genome of pennycress and its high level of sequence similarity to *Arabidopsis*, we are able to target putative pennycress genes involved in sinigrin biosynthesis and/or transport in a precise manner. Pennycress EMS mutants with low seed glucosinolate display severe phenotypes. Pennycress *cyp83a1* mutants also show a range of deleterious phenotypes. Pennycress *hag1* mutants possessed seed glucosinolate comparable to wild type, but this may be due to an alternate transcription start site that renders a partially functional gene product sufficient for glucosinolate biosynthesis. The *GTR1/2* CRISPR-Cas9 mutants are early in development and require two more generations of self-pollination and glucosinolate analysis. A theme of low sinigrin and deleterious phenotype has been noted, and future work will focus on alternative means to develop safe and palatable pennycress seed meal.

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Fig. 16. Seed glucosinolate content in promising pennycress EMS mutants. **A.** Bar graph of seed glucosinolate content (average of 3 biological reps) in wild type spring pennycress (102umols/g sinigrin), the I87207 mutant (25umols/g sinigrin), the I87113 mutant (30umols/g sinigrin) compared to canola (15.3umols/g glucosinolate). **B.** Pennycress WT compared to I87207 and I8113 low sinigrin mutants, all 5 weeks old. Scale bar represents 5cm. **C D** and **E** show the associated floral phenotypes of each

Fig. 17. CRISPR-Cas9 induced *hag1* pennycress mutants. **Left:** Two unique, heritable homozygous mutations in the ORF of pennycress *HAG1* (-G and +A) 17bp from ATG start, each resulting in premature stop codons. **Right:** Expected pennycress *HAG1* protein products: WT, Mutant, and Mutant in an alternate reading frame.

Fig. 18. CRISPR-Cas9 induced *cyp83a1* pennycress mutants. **A.** A simplified representation of the glucosinolate biosynthetic pathway and associated genes. **B.** Two unique, heritable homozygous mutations in the ORF of pennycress *CYP83A1* (+T and +A) 206bp from ATG start, each resulting in premature stop codons. **C.** A range of deleterious phenotypes seen in homozygous pennycress *cyp83a1* mutants (in order: WT, +T, +A, +T, +A) and their ‘crinkled leaf’ phenotype.

Fig. 19. CRISPR-Cas9 induced mutations in pennycress *GTR*-Like genes. **Left:** Sequencing of hygromycin resistant T1 plants showing heterozygous mutations in *GTR2*. **Above:** Transgenic plants grow as WT. Scale bar represents 5cm.