Implementation of Synthetic Biology for Industrial and Next-Generation Biofuel Crop Improvement

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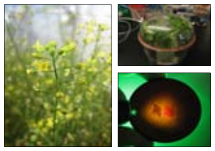
Overview

Meeting the challenge of feeding and fueling the world's population will require new strategies for rapid crop improvement that target advanced biofuel and industrial traits and also agronomic traits. Conventional plant breeding and biotechnology approaches for crop improvement are making important inroads into meeting these challenges, but these approaches typically target only one or a small number of traits at a time. In contrast, the emerging discipline of synthetic biology offers tools for making step changes in crop improvement by enabling integration of many trait genes into the host crop genome in a single genetic transformation event. We have constructed ten modules with different trait genes using GoldenBraid technology to generate improved sustainability and biofuel and industrial quality traits and to evaluate the upper limit of transgene numbers that can be introduced into camelina from one construct. These trait targets include genes for heat tolerance, seed size, oil content, oil quality, and protein quality. These gene modules were used to assemble constructs of six, eight, ten, or twelve transcriptional units, and the resulting constructs were transformed into camelina. Improved oil content and oil and protein quality traits that have been achieved to date, including high monounsaturated fatty acid-containing oils, will be presented. The same gene assembly strategy is also being applied to sorghum to express eleven genes for improved nitrogen-use efficiency, cold tolerance, lignin content, vegetative oil.

Camelina



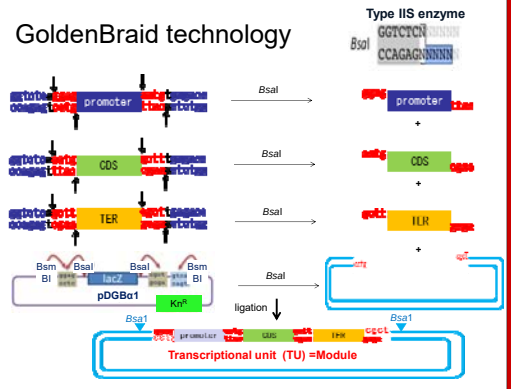
Pros:
*Seeds are oil-rich (30-40 wt%)
*Relatively short growing season (100-120 days)
*Easy to genetically engineer by Agrobacterium transformation

Cons:
*Heat sensitive
*Seed oil quantity and quality not optimal
*Seed protein quality limits profitability
*Small seeds

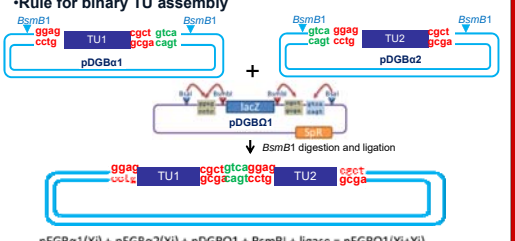
Targets for Camelina Improvement

•**Seed size** (1 gene): Arabidopsis G protein γ3 (AGG3)
•**Heat tolerance** (2 genes): Arabidopsis thermotolerance genes (*AtPARK13, AtHsfA1a*) expressed under control of heat shock promoter AtHSP18.2
•**Oil quantity** (2 gene): Arabidopsis wrinkled 1 transcription factor (*AtWRI1*), *Neurospora crassa* DGAT2 (*TaDGAT2*) **Oil quality** (5 genes): High oleic--RNAi suppression of *FAD2*, *FAD3*, *FAE1* genes;Palmitoleic—Com25 16:0-ACP desaturase, *C. elegans FAT5* 16:0-CoA desaturase
•**Protein meal quality** (2 genes): suppression of 2S seed storage proteins, insect resilin (industrial protein)

GoldenBraid technology



•**Rule for binary TU assembly**



pEGBα1(Xi) + pEGBα2(Xj) + pDGBΩ1 + BsmBI + ligase = pEGBΩ1(Xi+Xj)
pEGBα1(Xi) + pEGBα2(Xj) + pDGBΩ2 + BsmBI + ligase = pEGBΩ2(Xi+Xj)
pEGBΩ1 (Xi) + pEGBΩ2(Xj) + pDGBα1 + BsaI + ligase = pEGBα1(Xi+Xj)
pEGBΩ1 (Xi) + pEGBΩ2(Xj) + pDGBα2 + BsaI + ligase =pEGBα2(Xi+Xj)

<Reference > Sarrion-Perdigones *et al.* (2013) GoldenBraid 2.0: a comprehensive DNA assembly framework for plant synthetic biology. Plant physiology, 162, 1618-1631.

Goal

(1) To develop crop-based synthetic biology tools and techniques.
①Generation of an optimized, minimal binary vector for transgene expression.
②Develop expertise for gene silencing.
③Develop expertise for multi-gene assembly.
④Create a repository for biofuel trait gene modules to promote standardized synthetic biology use in crop improvement.
(2) To implement synthetic biology tools and techniques for rapid improvement of agronomic and biofuel traits in sorghum and camelina, including the introduction of high-value co-products.
①Assemble and express linked trait genes for improved cold tolerance, nitrogen use efficiency, lignin content, and vegetative oil in sorghum.
②Assemble and express linked trait genes for improved heat tolerance, increased seed size, and enhanced seed oil biofuel performance in camelina.
③Assess efficacy of multi-gene expression in sorghum and camelina by genotypic and phenotypic evaluation of transformed plant material.
④Systematically evaluate the transformation efficiency in camelina of expression vectors with increasing numbers of assembled trait genes.

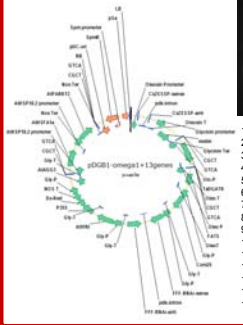
List of 10 gene modules for Camelina transformation (12 gene targets)

promoter	gene	terminator	vector
Oleosin	Cs2SSSP-RNAi	Oleosin	pDGB1-α1
Glycinin	Resilin	Glycinin	pDGB1- α2
Oleosin	TaDGATII	Oleosin	pDGB1- α1, pDGB1- Ω2
Oleosin	FAT5	Oleosin	pDGB1- α1
Glycinin	Com25	Glycinin	pDGB1- α2
Glycinin	CsFAD2+CsFAE1+CsFAD3-RNAi	Glycinin	pDGB1- Ω2
Glycinin	AtAGG3	Glycinin	pDGB1- α1, pDGB1- Ω2
Glycinin	AtWRI	Glycinin	pDGB1- α1
AtHSP18.2	AtHSFA1a	NOS	pDGB1- α1
AtHSP18.2	AtPARK13	NOS	pDGB1- α2

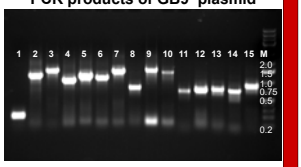
The list of gene assembly for Camelina transformation.

Gene assemly	vector
AtHsfA1a+AtPARK13	pDGB1-Ω1, pDGB1-Ω2
AtHsfA1a+AtPARK13+AtAGG3	pDGB1- α1
AtHsfA1a+AtPARK13+AtAGG3+Ds-red	pDGB1- Ω1 (GB3)
AtWRI+DsRed	pDGB1-Ω1 (GB1)
AtWRI+DsRed+AtAGG3	pDGB1- α2 (GB2)
AtWRI+DsRed+AtAGG3+TaDGAT2	pDGB1-Ω1 (GB7), pDGB1-Ω2
Com25+Fat5	pDGB1-Ω1
Com25+Fat5+CsFDA2+CsFAE1+CsFAD3-RNAi	pDGB1- α1
Com25+Fat5+CsFDA2+CsFAE1+CsFAD3-RNAi+DsRed	pDGB1-Ω1(GB5)
Com25+Fat5+CsFDA2+CsFAE1+CsFAD3-RNAi+AtWRI+DsRed+AtAGG3	pDGB1-Ω1(GB4)
Com25+Fat5+CsFDA2+CsFAE1+CsFAD3-RNAi+AtWRI+DsRed+AtAGG3+AtHsfA1a+AtPARK13	pDGB1- α2 (GB8)
Cs2SSSP-RNAi+resilin	pDGB1-Ω1, pDGB1-Ω2
Cs2SSSP-RNAi+resilin+AtAGG3	pDGB1- α1
Cs2SSSP-RNAi+resilin+AtAGG3+DsRed	pDGB1-Ω1 (GB6), pDGB1-Ω2
Cs2SSSP-RNAi+resilin+ TaDGAT2	pDGB1- α1
Com25+Fat5+CsFAD2+CsFAE1+CsFAD3-RNAi+AtWRI+DsRed+AtAGG3+AtHsfA1a+AtPARK13 +Cs2SSSP-RNAi+resilin+ TaDGAT2	pDGB1-Ω1 (GB9)

Map of GB9 (12 genes +Ds-red) binary plasmid including 12 genes

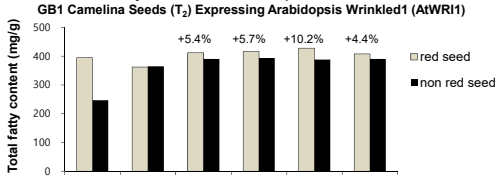


PCR products of GB9 plasmid



1. CsSSP-RNAi
2. Resilin
3. TaDGATII
4. FAT5 (1,002 bp)
5. Com25 (1,191 bp)
6. CsFAD2+CsFAE1+CsFAD3-RNAi (1,105 bp)
7. AtWRI (1,317 bp)
8. AtAGG3 (756 bp)
9. AtHSFA1a (1,488 bp)
10. AtPARK13 (1,287 bp)
11. Ds-Red (685 bp)
12. AtHSP18.2-promoter (720 bp)
13. CsFAD2+CsFAE1+CsFAD3-RNAi (sense) (750 bp)
14. CsFAD2+CsFAE1+CsFAD3-RNAi (antisense)
15. TaDGATII –Fp2 + oleosin-terminator

Total Fatty Acid Content and Composition are Altered in GB1 Camelina Seeds (T_2) Expressing Arabidopsis Wrinkled1 (AtWRI1)



Increased total fatty acid content and increase in linoleic acid (18:2, not shown) was detected in camelina seeds expressing AtWRI1.

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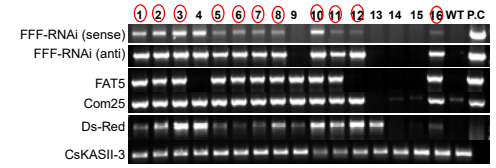
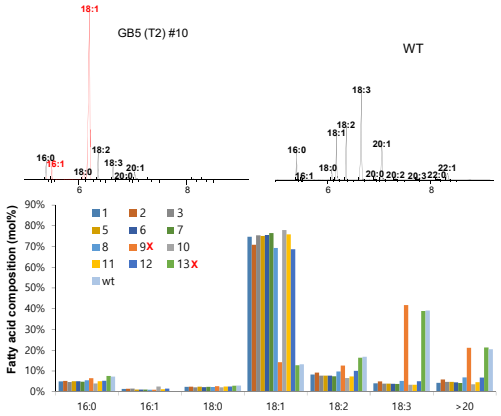
Characterization of GB5 transgenic Camelina (T_1) - gDNA PCR



➢GB5:Com25+Fat5+CsFDA2+CsFAE1+CsFAD3-RNAi+DsRed/pDGB1-Ω1 (13,850 bp)

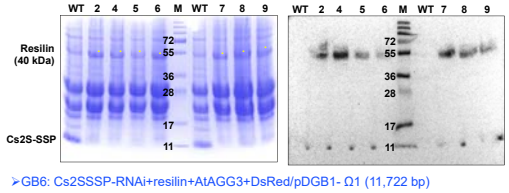
CsKASII-3 was used for confirmation of gDNA condition. Most of T1 plants contain all 5 genes.

Production of a High Monounsaturated Camelina Oil: Fatty acid analysis of FAD2+FAE1+FAD3-RNAi, 16:0-ACP Desaturase, and 16:0-CoA Desaturase transgenic camelina (T_2 seeds)



Increased 18:1Δ9/Δ11 (78%) and 16:1Δ9 (2.5%) content and decreased content of all other fatty acids were detected in seeds of GB5 camelina lines.

Production of the Industrial Protein Resilin and Knockout of 2S Seed Storage Proteins in Camelina Seeds: Protein analysis of GB6 transgenic camelina (T_2 seeds)



➢GB6: Cs2SSSP-RNAi+resilin+AtAGG3+DsRed/pDGB1- Ω1 (11,722 bp)

SDS gel image of total protein (A) and western blot image (B) in GB6 camelina seeds. Resilin antibody was used for the Western blot.

Sorghum

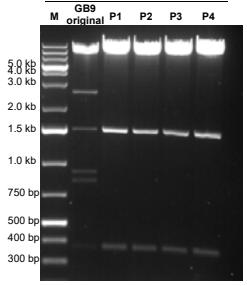
Pros:
*C4 photosynthesis
*Rich in lignocellulose and sugars for biofuels
*Wealth of available genomic resources

Cons:
*Cold sensitive
*Requires extensive nitrogen fertilization
*Lignocellulose & sugars may not be sufficient for profitability

Targets for Sorghum Improvement: Nitrogen-use efficiency (NUE), cold tolerance, lignin content, vegetative oil

•NUE (4 genes): maize *Dof1* transcription factor, rice glutamine synthase (*OsGS1*), rice glutamate synthase (*OsGOGAT*), barley alanine aminotransferase (*HvALAT*)
•Cold tolerance (2 genes): Arabidopsis sphingolipid acyl-desaturase (*ADS2*), Arabidopsis long-chain base D8 desaturase (*SLD1*)
•Lignin (2 gene): maize phenylalanine ammonia lyase (*ZmPAL*), Sorghum *myb* transcription factor
•Vegetative oil (3 genes): oat wrinkled 1 transcription factor (*AsWRI1*), *Neurospora crassa* DGAT2 (*NcDGAT2*), Arabidopsis oleosin (*AtOLE*)

Challenge



➢In some cases, rearrangement of large binary vectors occurred in Agrobacterium.
➢RNAi part of large binaries was particularly unstable in Agrobacterium.
➢It might be caused by repetitive using of same promoter, structural and size problem of RNAi construction, or Agrobacterium strain.
➢Replacement of promoter, reducing size of RNAi, and replacement Agrobacterium strain are ongoing to address this challenge.

Digestion of re-transformed plasmid from Agrobacterium to *E.coli*